In vitro assessment of the influence of nutrition, temperature and larval density on trapping of the infective larvae of Heligmosomoides polygyrus by Arthrobotrys oligospora, Duddingtonia flagrans and Monacrosporium megalosporum

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SUMMARY

The influence of nutrient level, temperature and larval density on the trapping of Heligmosomoides polygyrus L$_3$ by the nematophagous fungi Arthrobotrys oligospora, Duddingtonia flagrans and Monacrosporium megalosporum were investigated by quantification of trapped nematodes. All 3 factors were found to have a significant effect on the number of larvae trapped by A. oligospora and M. megalosporum. Decreased nutrient concentrations resulted in increased trapping for these 2 fungi, but nutrient availability was not found to have a significant effect on trapping by D. flagrans. The 3 fungi were found to have similar responses to temperature, with peak trapping occurring at or near the optimum growth temperatures. Nematode trapping was found to be density dependent for all 3 fungi, with increased percentage trapping at increased larval densities. Comparison in a single experiment of the relative importance of these factors to each fungus showed that nutrient level was the main factor influencing trapping by A. oligospora, whereas D. flagrans and M. megalosporum were more dependent on larval density.

Key words: Heligmosomoides polygyrus, nematophagous, nutrition, temperature, density dependence.

INTRODUCTION

Currently, the control of nematode infections of humans and animals is based mainly on the regular administration of anthelmintic drugs. However, long-term chemotherapy can lead to the evolution of resistance in nematodes, as has happened widely in response to the intensive use of the anthelmintic benzimidazoles (Jackson, 1993; Waller, 1990). Alternative control strategies are therefore urgently required.

Nematophagous fungi constitute a diverse group of fungi which feed on nematodes in the soil. They have been used, with varying degrees of success, as biological control agents for a variety of plant parasitic nematodes (Cayrol & Frankowski, 1979; Kerry, 1980), human parasitic nematodes (Soprano, 1958) and their potential for controlling animal parasitic nematodes is currently being evaluated (Grønvold et al. 1989; Waller, 1993; Larsen et al. 1995).

To be useful as a biological control agent, candidate micro-organisms must operate over a range of environmental conditions, since fastidious agents would have restricted activity in the field. Ideally, implementation of such agents requires evaluation of the factors which are likely to influence optimal performance, ahead of field trials. In the case of nematophagous fungi, cultures grown on agar plates provide a model system for obtaining preliminary information on the factors which affect fungal viability and the extent of trapping of target nematode species.

The effect of nutrient levels on the predacity of A. oligospora has been investigated with various free-living and plant-parasitic nematodes as the target species (Olthof & Estey, 1966; Nordbring-Hertz, 1968; Scholler & Rubner, 1994), but relatively little is known about the influence of other abiotic and biotic conditions, such as temperature and larval density, on the formation of traps and the subsequent capture and infection of nematodes. Moreover it is not clear whether those factors which have already been suggested to be important in the case of A. oligospora are also applicable to other nematophagous fungi.

In the current study, we tested the hypothesis that the predacity of A. oligospora is highly dependent on nutrient status, and measured trapping of the infective L$_3$ stages of H. polygyrus over a range of temperatures and at varying nematode density, such
as might be encountered in the field. In comparative assays D. flagrans and M. megalosporum were tested and the relative importance of each of these 3 variables was evaluated for each of the fungal species, in order to identify the principal determinants of predacity for each species of fungus.

**MATERIALS AND METHODS**

**Fungi**

*Arthrobotrys oligospora*, *Duddingtonia flagrans*, and *Monacrosporium megalosporum*, supplied by Professor B. Kerry of IACR-Rothamsted, were used throughout the experiments. All 3 fungi are predator nematophagous fungi, capturing nematodes by means of 3D adhesive networks. They were cultured on corn meal agar (CMA, Oxoid) in 9 cm Petri dishes at 25°C. Pieces cut from the colony margin of actively growing stock cultures were used to inoculate cultures for trapping experiments.

**Nematodes**

*Heligmosomoides polygyrus* is a gastrointestinal trichostrongyloid nematode of mice, which is commonly used as a model for nematodes of veterinary and medical importance. The parasite was maintained by passage through outbred CFLP mice. The infective (L$_3$) stages were obtained by incubating a slurry of infected mouse faeces, charcoal and distilled water on moist filter paper for 7 days at 25°C (Behnke & Wakelin, 1977). At the end of this period the larvae were collected from the back of the filter paper by washing in distilled water.

**Experimental procedure**

Predacity of the fungi was assessed under a standard experimental system which consisted of approximately 0.5 ml of CMA spread thinly in 5.5 cm diameter Petri dishes. The fungi were allowed to grow until the colony margin reached the edge of the Petri dishes and then infected with approximately 400 L$_3$ *H. polygyrus*. The dishes were incubated at 25°C for the period of time required. Control Petri dishes, uninoculated by fungi, were set up at the same time. Untrapped larvae were recovered by washing the plates 4 times in 1 ml aliquots of distilled water. They were quantified by allowing the larvae to settle, reducing the total volume to 1 ml, then counting the number of larvae present in 5 aliquots of 50 µl, and multiplying to give the total number present. Total number of larvae recovered was compared with the number of larvae originally added to each dish, and a value for percentage recovery was calculated. Microscopical examination of the plates confirmed the cause of the reduction to be the predacious action of the fungi. This standard system was manipulated to investigate the effect of different factors on trapping.

**Effect of nutrient level**

The effect of nutrients on nematode capture was examined by the preparation of dilutions of CMA. Undiluted (100%) CMA was prepared according to manufacturer’s instructions. Dilutions of 75, 50, 25 and 10% were prepared by replacement of cornmeal with the appropriate quantity of agar. Tap Water Agar (TWA) was used to represent 0% CMA and was prepared by dissolving 20 g agar (Sigma) in 1 litre of tap water and autoclaving for 15 min. At least 5 replicate standard experimental dishes were then prepared for each fungus, for each concentration of CMA. Petri dishes containing each concentration of CMA were also left uninoculated with fungus to act as controls for the recovery of nematodes. The effect of nutrient levels on the predacity of *A. oligospora* was investigated separately from its effect on *D. flagrans* and *M. megalosporum*.

**Effect of temperature**

Radial growth rates were determined by inoculating 3 replicate Petri dishes centrally with a 5 mm diameter disc of each fungus, taken from the growing margin of stock cultures, and recording the colony margin each day. Petri dishes were incubated at a range of temperatures from 7 to 37°C. Five replicate standard experimental Petri dishes, along with equivalent uninoculated control dishes, were incubated at a range of temperatures from 4 to 33°C, to investigate the effect of temperature on predacity of the fungi.

**Effect of larval density**

Suspensions of larvae containing from 50 to 3200 L$_3$ in 0.1 ml of distilled water were prepared and used to inoculate at least 5 standard experimental dishes. The effect of larval density on the predacity of *A. oligospora* was investigated separately from its effect on *D. flagrans* and *M. megalosporum*.

**Statistical analyses**

Differences in normalized data associated with the experimental and control plates were tested using ANOVA. In experiments where 1 fungus was tested against a control, 5% was used as the level of significance. In those cases where the results for 2 fungi were analysed separately against 1 set of controls, the level of significance was corrected to 2.5%, and for 3 fungi analysed separately against 1 set of controls, it was corrected to 1.7%. Data were analysed using the Minitab statistical package.

**RESULTS**

**Effect of nutrient level**

The percentage reduction in the number of larvae
Fig. 1. Mean percentage reduction in the number of larvae recovered from nematophagous fungus-inoculated experimental plates relative to the controls after (i) 1, (ii) 4 and (iii) 7 days, at different dilutions of CMA.
Fig. 2. (A) Growth rate and (B) percentage reduction in larvae recovered from nematophagous fungus-inoculated experimental plates relative to the controls, after 7 days at different temperatures.

Fig. 3. Percentage reduction in larval recovery from nematophagous fungus-inoculated experimental plates relative to the controls, at different larval densities after 4 days.

recovered from fungal inoculated Petri dishes relative to control dishes, at different concentrations of CMA, at different time intervals is shown in Fig. 1. Statistical analysis of the data from the *A. oligospora* dishes showed a significant interaction between fungal treatment and the concentration of CMA ($F_{1.295} = 309.94$ (fungal treatment), $F_{2.294} = 99.10$ (time), $F_{3.291} = 8.43$ (% CMA), $F_{2.294} = 34.62$ (fungal treatment x time), $F_{3.291} = 15.57$ (fungal treatment x % CMA), $P < 0.001$), indicating that nu-
Factors influencing nematode trapping

Table 1. Environmental conditions created for each group for comparison of the relative influence of nutrient status, temperature and fungal density on trapping by *Arthrobotrys oligospora*, *Duddington flagrans* and *Monacrosporium megalosporum*

| Group | Nutrient status | Temperature (°C) | Larval density |
|-------|-----------------|-----------------|---------------|
|       | TWA CMA         | 16 25           | 100 1000      |
| 1     |                 |                 |               |
| 2     |                 |                 |               |
| 3     |                 |                 |               |
| 4     |                 |                 |               |
| 5     |                 |                 |               |
| 6     |                 |                 |               |
| 7     |                 |                 |               |
| 8     |                 |                 |               |

Effect of temperature

The growth of the 3 fungi over a range of temperatures is shown in Fig. 2A. Peak growth rate was found to occur between 20 and 25 °C for *A. oligospora*, between 25 and 33 °C for *D. flagrans* and between 25 and 28 °C for *M. megalosporum*.

Predacity of the fungi at different temperatures is illustrated in Fig. 2B. Trapping of all 3 fungi was found to be maximal between 25 and 28 °C. The interaction between fungal treatment and temperature indicated that there were significant differences between the extent of trapping at different temperatures for all 3 fungi (*A. oligospora*: $F_{1,192} = 194.82$ (fungal treatment), $F_{6,123} = 5.43$ (temperature), $F_{6,123} = 9.83$ (interaction), $P < 0.001$; *D. flagrans*: $F_{1,129} = 427.02$ (fungal treatment), $F_{6,134} = 13.69$ (temperature), $F_{6,134} = 25.08$ (interaction), $P < 0.001$; *M. megalosporum*: $F_{1,137} = 559.71$ (fungal treatment), $F_{6,132} = 21.49$ (temperature), $F_{6,132} = 38.63$ (interaction), $P < 0.001$). It is also worth noting that effective trapping occurred across the whole range of temperatures supporting saprophytic growth.

Effect of larval density

Figure 3 shows that increased percentage nematode trapping occurred at increased larval densities. The significant interaction between fungal treatment and larval density indicated that effective trapping was highly dependent on larval density (*A. oligospora*: $F_{1,83} = 91.94$ (fungal treatment), $F_{6,78} = 11.66$ (larval density), $F_{6,78} = 8.21$ (interaction), $P < 0.001$; *D. flagrans*: $F_{1,64} = 130.45$ (fungal treatment), $F_{6,59} = 45.83$ (interaction), $P < 0.001$; *M. megalosporum*: $F_{1,58} = 101.39$ (fungal treatment), $F_{6,55} = 29.05$ (interaction), $P < 0.001$).

The percentage reductions at different concentrations of nutrient status, indicating that loss of nematodes had occurred independently of nutrient concentration ($F_{1,192} = 805.21$ (fungal treatment), $F_{6,183} = 298.95$ (time), $F_{6,183} = 7.69$ (% CMA), $F_{6,183} = 160.06$ (fungal treatment × time), $P < 0.001$, $F_{5,188} = 1.18$ (fungal treatment × % CMA), $P = 0.32$).

Figure 4. Mean larval recovery from control and nematophagous fungus-inoculated experimental plates, after 7 days at conditions either optimal (low nutrients, high temperature, high larval density) or suboptimal (high nutrients, low temperature, low larval density) for nematode capture.

![Graph showing the effect of fungal treatment, temperature, and larval density on nematode capture](Image)

Nutrient concentration influenced the extent of trapping, with increased trapping as the concentration of CMA fell. Over time this difference between the percentage reductions at different concentrations of CMA became less pronounced, presumably as the nutrients in the richer media were depleted and the fungus compensated by greater trapping. This pattern was repeated for *M. megalosporum* ($F_{1,192} = 270.13$ (fungal treatment), $F_{2,192} = 166.24$ (time), $F_{9,188} = 5.35$ (% CMA), $F_{2,192} = 59.81$ (fungal treatment × time), $P < 0.001$, $F_{5,188} = 3.28$ (fungal treatment × % CMA), $P = 0.007$). For *D. flagrans*, although there was a significant overall effect of the nutrient status of the fungus, there was no significant interaction between the fungal treatment and nutrient status, indicating that loss of nematodes had occurred independently of nutrient concentration ($F_{1,192} = 805.21$ (fungal treatment), $F_{6,183} = 298.95$ (time), $F_{6,183} = 7.69$ (% CMA), $F_{6,183} = 160.06$ (fungal treatment × time), $P < 0.001$, $F_{5,188} = 1.18$ (fungal treatment × % CMA), $P = 0.32$).
The different levels of each factor were then selected for each factor affecting nematode trapping. From the results of the previous experiments two factors were considered to include the conditions most optimal for trapping (low nutrients, low temperature, high larval density) and Group 5 the least optimal for trapping (high nutrients, low temperature, low larval density). The percentage reductions in larvae from these two groups were significantly different (interaction), $F_{4,60} = 10.73$ (larval density), $F_{4,60} = 8.79$ (interaction), $P < 0.001$.

Relative importance of factors

From the results of the previous experiments two levels, one optimal and one suboptimal, were selected for each factor affecting nematode trapping. The different levels of each factor were then combined into different groups (Table 1) to determine the relative importance of each factor for each fungus. Group 4 was considered to include the conditions most optimal for trapping (low nutrients, high temperature, high larval density) and Group 5 the least optimal for trapping (high nutrients, low temperature, low larval density). The percentage reductions in larvae from these two groups were compared directly by ANOVA. The data from the entire experiment were analysed using a 4-way ANOVA, to determine the relative importance of the different factors for each fungus. Figure 4 shows the mean number of untrapped larvae recovered from control and experimental cultures in Group 4 and Group 5. After 7 days under optimal conditions, there was a 91, 71 and 86% reduction in the number of larvae recovered from experimental plates treated with A. oligospora, D. flagrans and M. megalosporum respectively, whereas there were 0, 13 and 0% reductions under suboptimal conditions. The significant interactions between fungal treatment and environmental conditions showed these results to be significantly different ($A. \text{ oligospora}: F_{1,38} = 82.94$ (fungal treatment), $F_{1,38} = 63.90$ (conditions), $F_{1,38} = 84.97$ (interaction), $P < 0.001$; $D. \text{ flagrans}: F_{1,38} = 19.88$ (fungal treatment), $P < 0.001$, $F_{1,38} = 4.00$ (conditions), $P = 0.05$, $F_{1,38} = 8.20$ (interaction), $P < 0.001$; $M. \text{ megalosporum}: F_{1,38} = 34.99$ (fungal treatment), $F_{1,38} = 18.02$ (conditions), $F_{1,38} = 37.99$ (interaction), $P < 0.001$). The F values calculated for each factor for the entire experiment by 4-way ANOVA are shown in Table 2. From examination of the F values for each fungus, it is clear that the nutrient level was the major factor influencing the predacity of $A. \text{ oligospora}$, whereas larval density appeared to be more important to $D. \text{ flagrans}$ and $M. \text{ megalosporum}$, as reflected in the interactions between fungal treatment and the other factors.

**DISCUSSION**

This study shows that nutrient level, temperature and larval density can each affect the predacious activity of predator nematophagous fungi, when other factors are kept constant, but the relative importance of these factors varies between the different fungal species. The work of Blackburn & Hayes (1966) on the saprophytic phase of $A. \text{ oligospora}$ and $A. \text{ robusta}$ showed that predator fungi have simple nutrient requirements, suggesting no benefit from their ability to catch nematodes other than the provision of an alternative source of nutrients. A limited supply of nutrients has been shown to govern the shift from saprophytism to parasitism in several studies (Eren & Pramer, 1965; Balan & Lechevalier, 1972; Nordbring-Hertz, 1968, 1973, 1977; Saxena, Dayal & Mukerji, 1987). If the agar substrate contains a sufficient concentration of carbon and nitrogen the fungus does not form traps and lives as a saprophyte (Scholler & Rubner, 1994). The results reported here support these findings as reduced concentrations of CMA induced a higher degree of predation by $A. \text{ oligospora}$ and $M. \text{ megalosporum}$. The current results, however, do not support the finding of Saxena *et al.* (1987) and Nordbring-Hertz (1973) who found comparatively poor trap induction on water agar for *Arthrobotrys* species, attributing this to a lack of growth factors and certain essential ions in this medium. Oltlfof & Estey (1966) also suggested that predation would not occur if available carbon sources had been completely exhausted. Our study showed a high level of predation on TWA within 24 h. However, the investigations carried out by Saxena *et al.* (1987) and Nordbring-Hertz (1973) examined trap formation

**Table 2.** F and P values calculated by ANOVA for the effect of different factors on the number of larvae recovered from experimental and control Petri dishes, and the interaction of fungal treatment with these factors

| Factor                | Arthrobotrys oligospora | Duddington flagrans | Monacrosporium megalosporum |
|-----------------------|-------------------------|---------------------|-----------------------------|
|                       | F           | P            | F            | P            | F            | P            |
| Fungal treatment      | 135.10      | < 0.001*    | 54.35        | < 0.001*    | 77.67        | < 0.001*    |
| Nutrients             | 90.71       | < 0.001*    | 1.45         | 0.230       | 18.92        | < 0.001*    |
| Temperature           | 1.11        | 0.294       | 0.00         | 0.989       | 9.11         | 0.003*      |
| Larval density        | 0.34        | 0.561       | 5.00         | 0.027       | 0.39         | 0.531       |
| Fung. × Nutrients     | 55.78       | < 0.001*    | 0.60         | 0.440       | 4.70         | 0.032       |
| Fung. × Temp.         | 0.50        | 0.480       | 0.04         | 0.837       | 0.21         | 0.645       |
| Fung. × L.D.          | 3.69        | 0.057       | 11.97        | < 0.001*    | 18.90        | < 0.001*    |

*Significance. Cut off level for significance is $P < 0.017$. Temp., temperature; L.D., larval density.
rather than quantifying trapped nematodes. One possibility is that initial nematode capture on water agar is not by the formation of traps, but by another mechanism such as undifferentiated adhesive hyphae, shown to occur in fungi which produce adhesive networks (Barron, 1979; Jansson & Nordbring-Hertz, 1981; Den Belder & Jansen, 1994). The use of unmodified adhesive hyphae for catching nematodes may be more widespread than previously thought (Gray, 1987).

Our results support the finding that there is no direct relationship between predacious activity and the proliferation of mycelium (Nordbring-Hertz, 1968), since the sparse hyphal growth on the lower nutrient media supported the highest levels of predacious activity.

With regard to the effect of temperature, although predacious activity occurred at temperatures at which saprophytic growth did not, provided mycelium was already present, optima for both growth and predacious activity were found to be approximately the same. Feder (1963) suggested that there is little correlation between increased fungal growth rates and nematode capturing efficiency. The results of Duddington (1957), Cooke (1968) and Monoson (1968) indicated that peak predacious activity occurs at a temperature several degrees lower than that of peak growth (between 15 and 20 °C). Our results differ from these earlier studies but are more in accordance with the findings of Virat & Peloille (1977) on trapping of Haemonchus contortus by A. oligospora. They showed increased trapping at higher temperatures, but did not report on the peak growth rate of the strain used. Furthermore, our observations are also in accordance with the results of Den Belder & Jansen (1994), Soprunov (1958) and Grønvold (1989) who found that development of adhesive networks by A. oligospora was significantly slower or inhibited at lower temperatures. Density-dependent parasitism occurs when the probability of a host being parasitized increases with increasing host density (Jaffee et al., 1994), an important process in the regulation of pest populations (Stirling, 1988). Previous investigations into the effect of larval density on predacity have not given a clear indication of whether density-dependent predation occurs in the nematophagous fungus–nematode system. Soprunov (1958) found increased numbers of larvae trapped at higher nematode densities, for a mixture of Arthrobotrys species and Grønvold (1989) found increased trap induction at higher nematode densities (up to concentrations of 1000 larvae/cm²) for A. oligospora. Other investigators (Gray, 1985; Jaffee, Tedford & Muldoon, 1993; Jaffee et al., 1994) have found, however, that density dependence only occurs in the more obligate parasitic fungi, while the facultative predator fungi, such as those using adhesive networks, were reported to be independent of nematode density. However, findings from the latter two studies may have been confounded by the nematode used (Jaffee & Muldoon, 1995). Virat & Peloille (1977) investigated predacity of A. oligospora at different larval densities, at different temperatures. They found predacity to be density dependent at 15 °C, but density independent at 10 °C and 22 °C. The results from our study indicate that, all other factors being constant, all 3 fungi show density-dependent predation, with an increase occurring as larval densities rise, perhaps up to a limit, as found by Grønvold (1989).

Investigation into the relative importance of these factors showed that environmental conditions are of major importance in determining the level of predacity induced. A delicate balance exists between hyphal development and trap formation and this balance is regulated by environmental conditions (Scholler & Rubner, 1994). Our study has shown that it is possible, using the same fungus and nematode species, but altering environmental factors, to create conditions where no trapping occurs at all and where trapping is maximized. It was also possible to ascertain which factor was the most important in determining the level of predacity for each fungus. It appears that for A. oligospora, nutrient status of the fungus is the most important factor. Hence when nutrients are limited, this fungus traps nematodes. Conversely D. flagrans and M. megalosporum appeared to be more influenced by larval density, indicating that if nematodes are in plentiful supply (perhaps between certain threshold densities), these fungi increase trapping rates. These findings are relevant in the choice of fungi as biological control agents, since different fungi are likely to perform better under different environmental conditions. They also underline the fact that use of the same trapping mechanism does not necessarily mean that identical factors are important for each fungus. Trap formation is a complex response to the individual needs of each fungus rather than simply a response to exogenous stimuli (Cooke, 1977). Each fungus will have its own set of environmental conditions under which it performs efficiently as a biological control agent in the field. It remains to be determined whether these are the same conditions as those identified through laboratory based experiments.

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