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# Interspecific competition between the nematode-trapping fungus, *Duddingtonia flagrans*, and selected microorganisms and the effect of spore concentration on the efficacy of nematode trapping

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## Abstract

The fungus, *Duddingtonia flagrans*, is able to trap and kill free-living nematode larvae of the cattle parasite *Cooperia oncophora* when chlamydospores are mixed in cattle faeces. Isolates of *Bacillus subtilis* (two isolates), *Pseudomonas* spp. (three isolates) and single isolates of the fungal genera *Alternaria*, *Cladosporium*, *Fusarium*, *Trichoderma* and *Verticillium* were isolated from cattle faeces and shown to reduce *D. flagrans* growth on agar plates. When these isolates were added to cattle faeces containing *D. flagrans* and nematode larvae of *C. oncophora*, developing from eggs, none of the isolates reduced nematode mortality attributed to *D. flagrans*. Similarly, the coprophilic fungus *Pilobolus kleinii*, which cannot be cultivated on agar, also failed to suppress the ability of *D. flagrans* to trap and kill developing larvae of *C. oncophora*. Increasing chlamydospore doses of *D. flagrans* in faecal cultures resulted in higher nematode mortality. Thus, no evidence of interspecific or intraspecific competition was observed. The consequences of these findings are discussed.

## Introduction

This paper presents results from the last of three series of basic experiments on biotic and abiotic factors influencing the predacious efficiency of the nematode-trapping fungus *Duddingtonia flagrans* (Cooke, 1969), which is characterized by the production of numerous thick-walled spores (chlamydospores). Earlier experiments have been published by Grønvold *et al.* (1996, 1999), using the strain 'CI3' while the present study concerns the strain 'Trol A'.

*Duddingtonia flagrans* is able to trap nematodes in adhesive traps, which are induced after physical contact with small migrating nematodes (Grønvold *et al.*, 1996). After feeding chlamydospores to domestic animals, viable spores are excreted in their faeces together with eggs of gastrointestinal nematode parasites, e.g. *Cooperia oncophora* of cattle. Danish experiments have shown that *D. flagrans* chlamydospores fed to domesticated animals survive the alimentary tract in sufficient numbers to reduce the number of parasitic nematode larvae developing from eggs in faeces, and to prevent clinical disease

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(Grønvold *et al.*, 1993; Wolstrup *et al.*, 1994; Larsen *et al.*, 1995, 1996; Nansen *et al.*, 1995, 1996; Githigia *et al.*, 1997).

Faeces contain, or are invaded by, a range of microorganisms, such as bacteria and fungi. To be effective in controlling gastrointestinal parasitic nematodes developing in faeces, *D. flagrans* must be able to compete with these potentially antagonistic microorganisms, at least until a substantial number of nematode larvae have been trapped. Antagonistic interactions are a common phenomenon, e.g. it is difficult to establish a fungus in soil in which it does not normally occur, the so-called fungistasis. However, removal of the competing organisms by sterilization often allows a new fungus to establish without difficulty.

This paper describes the ability of *D. flagrans* to compete with selected numbers of bacteria and fungi isolated from faeces (interspecific competition) and also investigates the effect of chlamydospore concentration on the nematode mortality (intraspecific competition) in cattle faeces.

## Materials and methods

### *Experimental animals*

A group of housed calves infected with a monoculture of a Danish strain of *Cooperia oncophora* Railliet 1898 were reared under conditions that did not allow transmission of parasites. The calves produced a pure culture of *C. oncophora* eggs in their faeces. These faeces were sampled for the series of experiments described below.

### *Nematode-trapping fungus*

*Duddingtonia flagrans* Cooke 1969 (strain 'Trol A'), used for the experiment, was produced on millet seed as described by Grønvold *et al.* (1993). However, compared to the original description, barley grains were changed with millet grains, as fungal growth medium. The cultures were produced by Chr. Hansen A/S, Hørsholm, Denmark, and chlamydospore suspensions were made by washing the substrate in tap water.

### *Isolation and selection of antagonistic microorganisms on agar*

From different cow pats, 20 bacterial strains and 14 fungal strains were isolated on tryptic soya agar (TSA), and malt agar (MA), respectively. Selection was performed by allowing these 34 microorganisms to grow together with *D. flagrans* on agar.

The experiment, concerning the 20 bacterial isolates was performed by placing *D. flagrans* and the single selected bacterial strains on the same potato dextrose agar (PDA) in Petri dishes (diameter: 8.5 cm). A square (5 × 5 mm) of corn meal agar (CMA) was cut from a *D. flagrans* stock fungal culture and placed in the centre of the PDA-dishes. At the same time the bacteria were inoculated on to the same dish in two streaks (on a line through the centre of the Petri dish) starting from near the edge of the fungal inoculum and following the line to the rim of the Petri dish on both sides of the inoculum. For each bacterial isolate five Petri dishes were prepared. In case of inhibition due to bacteria, fungal growth was depressed in the vicinity of the bacterial streaks.

The 14 'other fungi', were tested for antagonistic properties on MA in the following way. Each isolated fungus was tested against *D. flagrans* by placing 3 × 3 mm blocks, cut from CMA cultures of *D. flagrans*, at the periphery of a Petri dish (diameter 8.5 cm) diametrically opposite to 3 × 3 mm MA blocks cut from cultures of the 'other fungus'. Two Petri dishes were set up for each fungus. The influence of possible competing fungi on *D. flagrans* was estimated after inspection under the microscope. In case of a positive effect from the isolated microorganisms, the growth of *D. flagrans* was inhibited in the vicinity of the competing microorganisms.

Five bacterial strains and five fungal strains were selected as competitors to *D. flagrans* on agar and further investigated in cattle faeces. The very common coprophilic fungus, *Pilobolus kleinii*, cannot be grown on agar, but was tested in cattle faeces only.

### *Interspecific competition with bacteria in faeces*

The five antagonistic bacteria selected were two strains of *Bacillus subtilis* Cohn 1872 (a and b; Gram positive rods) and three strains of *Pseudomonas* spp. (c, d and e; Gram negative rods) (table 1). These five isolates were tested in faecal cultures in the laboratory. Each culture consisted of a mixture of 10 g cattle faeces containing on average a concentration of 967 eggs per gram (EPG) of *C. oncophora* + 3 g vermiculite + 8 ml of tap water with or without (controls) one of the bacterial isolates. Except for the negative controls (with *C. oncophora* eggs only), all faecal cultures were given a dose of *D. flagrans* chlamydospores at a concentration of 25000 chlamydospores per gram of faeces. Except for the negative and positive controls (with *D. flagrans* chlamydospores only), all test cultures also contained bacteria of the five isolated strains at $8 \times 10^7$ or $2 \times (8 \times 10)^7$ CFU per gram of faeces; CFU is colony forming units (cells). The mixtures were carefully blended and placed in humidity chambers, as described by Henriksen & Korsholm (1983). The faecal cultures were cultivated for 2 weeks at room temperature (20–22°C) and 95–100% RH. At the end of the 2-week period the cultures were extracted using a Baermann apparatus and surviving infective *C. oncophora* larvae were isolated and counted. Thus the test was indirect and measured the trapping efficiency of *D. flagrans* against the free-living larvae of *C. oncophora*. Three replicates were prepared for all cultures.

### *Interspecific competition with* Pilobolus *and 'other fungi' in faeces*

The following tests, concerning fungi, were also indirect.

#### *Pilobolus*

*Pilobolus kleinii* Tode 1784 spores, harvested from cattle faeces, were used in increasing concentrations together with *D. flagrans* chlamydospores. The experiment was performed in plastic flowerpots (upper diameter: 10 cm; lower diameter: 7 cm; height: 7 cm). One hundred grams

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Table 1. The number of infective *Cooperia oncophora* larvae ($L_3$) harvested from faecal cultures, after different combinations of *Duddingtonia flagrans* chlamydospores and bacterial cells from five isolates, i.e. two *Bacillus subtilis* (a, b) and three *Pseudomonas* spp. (c, d, e) isolates, were added.

| Microorganisms added to the faecal cultures | Number of *Cooperia oncophora* $L_3$ larvae per g faeces | |
|---|---|---|
| Negative controls (no fungi added) | 553 (142) | |
| Positive controls (+ 25000 D.f. CHL per g) | 1 (0.3) | |
| | Bacterial concentrations | |
| + 25000 D.f. CHL per g + bacteria | $8 \times 10^7$ CFU per g | $2 \times (8 \times 10^7$ CFU per g) |
| *Bacillus subtilis* (a) | 2 (0.5) | 2 (1.2) |
| *Bacillus subtilis* (b) | 1 (0.4) | 2 (1.5) |
| *Pseudomonas sp.* (c) | 2 (1.1) | 2 (0.7) |
| *Pseudomonas sp.* (d) | 1 (0.3) | 2 (1.3) |
| *Pseudomonas sp.* (e) | 2 (0.6) | 3 (1.2) |

The S.D. is shown in parentheses (n = 3). CFU, colony forming units (cells); D.f., *Duddingtonia flagrans*; CHL, D.f. chlamydospores.

of cattle faeces (with a surface area of approximately 45 $cm^2$), containing on average a concentration of 1050 *C. oncophora* EPG, was placed in each flowerpot. Transparent Petri dishes (diameter: 8.5 cm) were used as lids. Each portion of faeces had been mixed with 8 ml of water containing different concentrations of *D. flagrans* chlamydospores and *P. kleinii* spores before placing in the flowerpots (table 2). The flowerpots were placed in the laboratory at 20–22°C, 90–95% RH and 500–1000 lux in the daytime. After cultivation for 25 days, 100 g faecal portions were extracted and infective larvae enumerated. *Pilobolus* fungi discharge their sporangia daily during a 1- to 2-week period. Each sporangium, containing approximately 7000 spores, is discharged towards the light and, in this case, adhered to the underside of the Petri dish lids, allowing the production and diameter of sporangia to be determined. Three replicates were prepared for the different types of cultures.

*Other fungi*

*Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., *Trichoderma* sp. and *Verticillium* sp. were added to cattle faecal cultures. Each culture comprised 10 g of cattle faeces, containing on average a concentration of 4129 EPG of *C. oncophora* + 3 g of vermiculite + 8 ml of tap water containing a known amount of spores of *D. flagrans* and 'other fungi' (table 3). The mixtures were carefully homogenized and placed in humidity chambers (95–100% RH) as described by Henriksen and Korsholm (1983). Cultures were kept at 20–22°C for 4 weeks when the cultures were harvested. Three replicates were prepared for each different type of culture.

*Effect of increasing chlamydospore concentrations on nematode trapping in faeces*

Cultures were prepared as described previously in the section 'other fungi', containing on average

Table 2. The total number of infective *Cooperia oncophora* larvae ($L_3$) harvested from the surface (45 $cm^2$) of the 100 g portions of faeces, when increasing numbers of *Pilobolus kleinii* spores in combination with *Duddingtonia flagrans* chlamydospores were added. Reduction (red. %) in $L_3$ larval numbers harvested in relation to negative controls are also shown.

| Admixture of fungal spores per g faeces | | Total harvest of *C. oncophora* infective larvae from 100 g faeces | | Harvest of *P. kleinii* sporangia per 45 $cm^2$ (100 g faeces) | |
|---|---|---|---|---|---|
| (D.f.) | (P.k.) | $L_3$ (S.D.) | red. (%) | No. | Sporangial diam. ($\mu$m) |
| Negative controls | | | | | |
| 0 | 0 | 823 (154) | – | 4867 | 373 |
| Positive controls | | | | | |
| 25000 | 0 | 146 (35) | 82% | 5200 | 333 |
| Addition of *Pilobolus* | | | | | |
| 0 | 500 | 596* | – | 4000 | 320 |
| 25000 | 500 | 199 (158) | 76% | 5183 | 380 |
| 25000 | 50000 | 153 (72) | 81% | 2967 | 162 |

The S.D. is shown in parentheses (n = 3). D.f., *Duddingtonia flagrans*; P.k., *Pilobolus kleinii*.
* Only one sample.

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Table 3. The number of infective *Cooperia oncophora* larvae ($L_3$) harvested from faecal cultures, after high numbers of spores of 'other fungi' together with *Duddingtonia flagrans* chlamydospores were added.

| Fungi | Spore concentration of 'other fungi' (spores per g faeces) | Number of infective *Cooperia oncophora* larvae ($L_3$ per g) |
|---|---|---|
| Negative controls (no fungi added) | | 6230 (1194) |
| Positive controls (25000 D.f. CHL per g) | | 4 (1.2) |
| 25000 D.f. CHL per g + other fungi | | |
| *Alternaria* sp. | $7 \times 10^5$ | 1 (0.4) |
| *Cladosporium* sp. | $1 \times 10^6$ | 6 (5.8) |
| *Fusarium* sp. | $26 \times 10^6$ | 20 (5.6) |
| *Verticillium* sp. | $16 \times 10^6$ | 11 (13.0) |
| *Trichoderma* sp. | $8 \times 10^6$ | 2 (0.9) |

The S.D. is shown in parentheses (n = 3). D.f., *Duddingtonia flagrans*; CHL, D.f. chlamydospores.

a concentration of 1050 EPG of *C. oncophora* and an increasing concentration of *D. flagrans* chlamydospores. Controls only contained pure tap water. Cultures were maintained as previously described and harvested after 2 weeks. Three replicate cultures were prepared for each fungal isolate.

## Results

### *Addition of* D. flagrans *to faeces*

In all faecal cultures, with or without admixture of selected microorganisms, addition of 25000 *D. flagrans* chlamydospores per gram resulted in over 75% reduction in the number of infective *C. oncophora* larvae, relative to negative control cultures, where the nematode-trapping fungus was absent (tables 1, 2, 3 and fig. 1).

### *Interspecific competition with bacteria in faeces*

When compared with the positive control (see table 1), faecal cultures containing *Pseudomonas* sp. (isolate e) significantly reduced the trapping ability of *D. flagrans* when added at a concentration of $2 \times (8 \times 10^7)$ CFU per gram of faeces (Mann-Whitney U test; $P = 0.05$ (Siegel, 1956)). However, the reduction was very small and *D. flagrans* still reduced the number of nematodes emerging from the faeces by over 99% (table 1). The two strains of *Bacillus subtilis* (a and b) and the two other strains of *Pseudomonas* sp. (c and d) did not reduce the trapping effectiveness of *D. flagrans* significantly at either of the two concentrations tested, and reduction in nematodes in all treatments exceeded 99% (table 1). Thus, despite the bacteria being antagonistic on agar they did not prevent the nematode-trapping ability of *D. flagrans* in cattle faeces, even at very high spore concentrations.

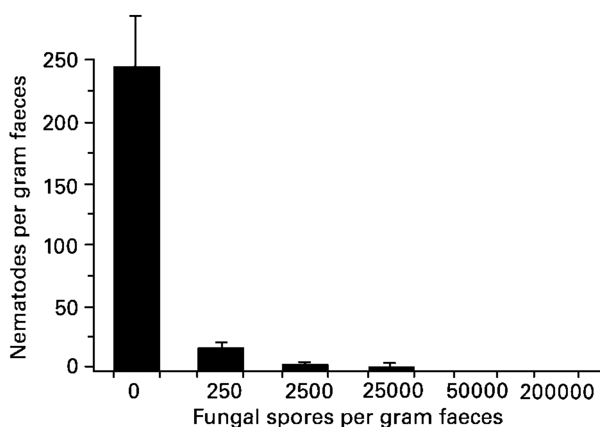


Fig. 1. Number of *Cooperia oncophora* infective nematode larvae harvested per gram of faeces from cultures containing increasing concentrations of *Duddingtonia flagrans* chlamydospores. + S.D. is indicated on top of each bar (n = 3).

### *Interspecific competition with* Pilobolus *and 'other fungi' in faeces*

*Pilobolus*

When compared with the positive control, the addition of *P. kleinii* spores to cattle faeces did not reduce the trapping efficacy of *D. flagrans* significantly (table 2). No effect was observed even at 50000 spores of *P. kleinii* per gram of faeces, with 81% of the *C. oncophora* larvae being killed. However, both the production and diameter of *P. kleinii* sporangia were reduced at this high *Pilobolus* spore concentration level (table 2), indicating a crowding effect.

*Other fungi*

A small but significant reduction (Mann-Whitney U test; $P = 0.05$ (Siegel, 1956)) in the trapping ability of *D. flagrans* was observed in cultures with *Fusarium* sp., when compared with the positive control (see table 3). However, over 99% of the nematodes were still killed in the presence of this isolate. The remaining fungi had no significant effect on the trapping ability of *D. flagrans*, even when added to faeces at between $7 \times 10^5$ and $16 \times 10^6$ spores per gram of faeces (table 3). It is unclear why the average EPG value of 4129, at the start of the experiment, was lower than the average number of 6230 infective larvae that could be harvested from the negative control cultures at the end of the experimental period. However, this problem cannot alter the conclusion.

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*Effect of increasing chlamydospore concentration on nematode trapping in faeces*

The effectiveness of *D. flagrans* in trapping nematodes was illustrated by the reduction of 93% obtained with a spore concentration of 250 chlamydospores per gram of faeces (fig. 1). Increasing the chlamydospore concentration increased nematode mortality to 99%. This strongly indicates that there is no severe intraspecific competition between the *D. flagrans* chlamydospores, when increasing their concentration.

## Discussion

In the present work the primary selection for antagonists was performed to find isolates that were able to reduce the growth of *D. flagrans* on agar. Secondly, positive candidates were indirectly tested in cattle faeces to elucidate any reduction in the trapping ability of *D. flagrans* in the presence of high concentrations of the primary selected competitors. Surprisingly, the five antagonistic bacteria isolated on agar (two *Bacillus subtilis* and three *Pseudomonas* spp. isolates) were not able to depress the nematode-trapping capacity of *D. flagrans* in cattle faeces.

In relation to coprophilic fungi, *P. kleinii* and other *Pilobolus* species are relevant because they are common in faeces and they are also involved in the transmission of the cattle lungworm *Dictyocaulus viviparus* Bloch 1782. Jørgensen *et al.* (1982) have demonstrated that infective *D. viviparus* larvae may be spread from faeces to herbage, clinging to the airborne sporangia of *Pilobolus* spp. In this way, grazing calves are exposed to infective lungworm larvae. As spore concentration in faeces increased, both sporangial production and sporangial size were reduced, but *P. kleinii* did not interfere with the trapping capacity of *D. flagrans* against *C. oncophora*.

As in the case with bacteria, ‘other fungi’ belonging to the genera *Alternaria Cladosporium*, *Fusarium*, *Trichoderma* and *Verticillium* were not able to depress the nematode-trapping capability of *D. flagrans*, in cattle faeces, as judged by its ability to depress the parasitic larval population of *C. oncophora*.

Increasing the chlamydospore concentration of *D. flagrans* increased its nematode-killing activity. Thus, there were no indications of intraspecific competition. When cattle are orally dosed with *D. flagrans* chlamydospores, dose levels are calculated to end up with 25000 chlamydospores per gram of excreted faeces. Reaching this dose level normally results in more than 75% reduction in parasite larval number in faeces. However, it should be noted that a dose of only 250 chlamydospores per gram in faecal culture is enough for a 93% reduction, when chlamydospores are placed directly into faecal cultures, which was the case in this experiment. This strongly indicates that possibly more than 90% of the chlamydospores are killed during passage through the alimentary tract of cattle. But part of the explanation for the success in faecal cultures may also be the optimal conditions used in the laboratory cultures. If, however, the competitive ability of *D. flagrans* is dependent on chlamydospore concentrations in faeces, future experiments may elucidate reduced biological control by *D. flagrans* at lower chlamydospore dosing rates caused by competition only.

Using a constant concentration of 25000 chalmydospores per gram of faeces, it may be concluded that *D. flagrans* is a good competitor in cattle faeces, which may be due to the high organic content in faeces, which may temporarily switch off competition at this concentration (Mankau, 1962). If competition in cattle faeces is significant, *D. flagrans* obviously is able to be active in a period before competition becomes severe.

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