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In vitro assessment of the influence of nutrition and temperature on growing rates of five *Duddingtonia flagrans* isolates, their insecticidal properties and ability to impair *Heligmosomoides polygyrus* motility

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Abstract

Diverse effects of two temperature regimes (20 and 30 °C) on the growing rates of five *Duddingtonia flagrans* isolates (MUCL 28429, CBS 143.83, CBS 561.92, CBS 565.50, and CBS 583.91) propagated on two liquid (MM, LB) and four solid substrates (CMA, SAB, SAB-GM, and SAB-HP) were observed. All *D. flagrans* isolates were able to produce chlamydospores but not on all substrates. None of the isolates produced trapping nets and conidia under applied growing conditions. *D. flagrans* isolates showed moderate insecticidal properties against *Galleria mellonella* larvae with mortality rates below 20%. Preincubation (18 h) of *Heligmosomoides polygyrus* infective (L_3) larvae in fungal homogenates highly impaired in vitro spontaneous motility of nematodes. This may indicate the potential of *D. flagrans* bioactive substance(s) for use as biocontrol agents of parasitic nematodes.

Index Descriptors and Abbreviations: BALB/c mice; *Duddingtonia flagrans*; *Heligmosomoides polygyrus*; *Galleria mellonella*; Chlamydospores; Motility; Insecticidal properties; Nematocidal activity; CMA, corn meal agar; SAB, Sabouraud medium; SAB-GM, SAB supplemented with *Galleria mellonella* larval homogenate; SAB-HP, SAB supplemented with *Heligmosomoides polygyrus* larval homogenate; LB, Luria broth; MM, minimal medium; PBS, phosphate-buffered solution; BSA, bovine serum albumin; SEM, standard error of the mean

1. Introduction

The potential of using soil fungi to prevent nematodosis caused by parasites with free-living larval stages is well documented. In this respect, *Duddingtonia flagrans*, a net-trapping, nematode-destroying fungus, appears to be the most promising candidate (Larsen, 2000). In field trials *D. flagrans* reduces the number of infective larvae in faeces from ruminants fed fungal spores (Larsen et al., 1997). Spores of *D. flagrans* survive passage through the gastro-intestinal tract of animals and grow in the faeces to attack parasite larvae (Faedo et al., 1997). Viability of *D. flagrans* spores is preserved during prolonged exposure to elevated temperature in air or under an atmosphere of the major ruminal gases and remains stable in tabletted form for several months (Waller et al., 2001).

Duddingtonia flagrans traps migrating nematodes in three-dimensional adhesive nets. As with many other nematode-trapping fungi, *D. flagrans* only produces traps when it is induced to do so, e.g., by physical contact with nematodes. The fungus may produce two types of spores: thin-walled conidia on the top of conidiophores and thick-walled intercalar chlamydospores. The manner in which *D. flagrans* attracts, captures, and penetrates nematodes has not yet been investigated, but it may be comparable to the mechanisms found in other nematode-trapping fungal species. Nematodes are attracted by predacious fungi which release into the

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immediate surroundings several unstable attractants luring nematodes into the traps (Balan et al., 1976; Field and Webster, 1977). The capture of nematodes is thought to be mediated by the recognition of carbohydrate residues on the surface of nematodes by lectins on the surface of traps (Balogh et al., 2003). Capture of nematodes is followed by cuticle penetration by invasive fungal hyphae and death of the nematode. Fungal enzymes, especially extracellular proteases, play a crucial role in this process (Ahman et al., 2002). Soon after penetration, the movement of captured nematode becomes inhibited due to a secreted nematotoxin (not identified yet) that either paralyses or kills the nematode (Gronvold et al., 1993a).

The nematophagous fungi can live saprophytically as well as predatorily. The transition from a saprophytic to a predacious phase of growth could be brought about either by addition of living nematodes and certain other soil animals, or by addition of small peptides (Nordbring-Hertz, 1977). Trap formation can be regarded as a symptom of nutrient deficiency, while formation of chlamydospores is in much cases a symptom of unfavourable growing conditions (Scholler and Rubner, 1994). The induction of nets in nematode-trapping fungi is influenced by temperature, number of larvae, atmosphere, light, and growing media composition (Fernandez et al., 1999; Friman, 1996; Gronvold et al., 1993b, 1996; Morgan et al., 1997; Waller et al., 1994). Growing conditions, temperature and substrates, strongly influence mycelial growth rates and attained biomass (Fernandez et al., 1999; Gronvold et al., 1996; Morgan et al., 1997).

In the current study, we measured the growing rates of five *D. flagrans* isolates propagated at two temperature regimes on two liquid and four solid media, to find growing conditions stimulating luxuriant fungal growth. Heavy production of mycelia could be important for the future attempts to isolate bioactive fungal compounds impairing nematode motility and/or exerting nematocidal effect. The experiments showed, for the first time, that a nematophagous fungus, *D. flagrans*, produces metabolites that impair nematode motility and demonstrates moderate insecticidal properties.

2. Materials and methods

2.1. Fungi

Five isolates of *D. flagrans*: MUCL 28429, CBS 143.83, CBS 561.92, CBS 565.50, and CBS 583.91 were used throughout the experiments. They were cultured in darkness at 20 and 30 °C on four solid media: (1) CMA, corn meal agar (Merck), (2) SAB, Sabouraud prepared according to Benke (1957), (3) SAB-GM, Sabouraud supplemented with *Galleria mellonella* larval homogenate (10% w/v final concentration of insect homogenate which corresponds to circa 85 mg of insect proteins/ml), (4) SAB-HP, Sabouraud supplemented with *Heligmosomoides polygyrus* larval homogenate (final concentration of nematode homogenate: 0.1 mg of nematode proteins/ml), and in two liquid media: (5) LB, Luria broth and (6) MM, minimal medium. Both liquid media were prepared after Lech and Brent (1991).

For solid media, standard sterile 9 cm petri dishes were used, while fungal propagation in liquid media (30 ml) was performed with the use of sterile 50 ml cell culture tubes (Corning). Agar blocks 3×3 mm cut from the colony margin of actively growing stock cultures (propagated on SAB medium) were used to inoculate experimental cultures both liquid and solid. For each inoculation only one agar block was used. Petri dishes were inoculated centrally. The colony margin was recorded during three consecutive weeks in 3-days intervals. Each experimental variant was performed in 4–6 independent replications. Three-weeks-old colonies were used for insect mortality tests and as a source of mycelia (material was harvested by scraping mycelia from fungal cultures).

Liquid cultures were performed without shaking. Three weeks after inoculation, mycelia were collected from liquid cultures by means of filtration through nitrocellulose filters (0.22 μm), lyophilized, and weighed. Each experimental variant was performed in 4–5 independent replications.

For the nematode motility tests mycelia were collected from colonies propagated in conditions stimulating growth (LB, SAB-GM, and SAB-HP at 30 °C), ultra-sonicated in sterile PBS, pH 6.8 (Sigma), and stored at −20 °C before use (up to 4 weeks). Protein concentration in homogenates was determined according to the method of Bradford (1976) as well as spectrophotometrically (Clausen, 1972) using a Beckman DU-68 spectrophotometer.

Mycelia of all fungal isolates cultured in conditions described above were observed under phase contrast microscope (Zeiss Axiophot) and inspected for the presence of conidia, chlamydospores and trap structures. Examinations were performed on the 1- and 3-week-old colonies, i.e., at the time of maximal production of nets and chlamydospores, respectively (Gronvold et al., 1996). Due to the rank growth of fungi on rich substrates counting of chlamydospores was impracticable.

2.2. 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 BALB/c mice. Quarterly 10–20 BALB/c males were orally infected with 100–150 *H. polygyrus* larvae. Three weeks later, infective (L_3) stages were obtained by incubating a slurry of infected mouse faeces and distilled