Optimization of production of chlamydospores of the nematode-trapping fungus Duddingtonia flagrans *in solid culture media*

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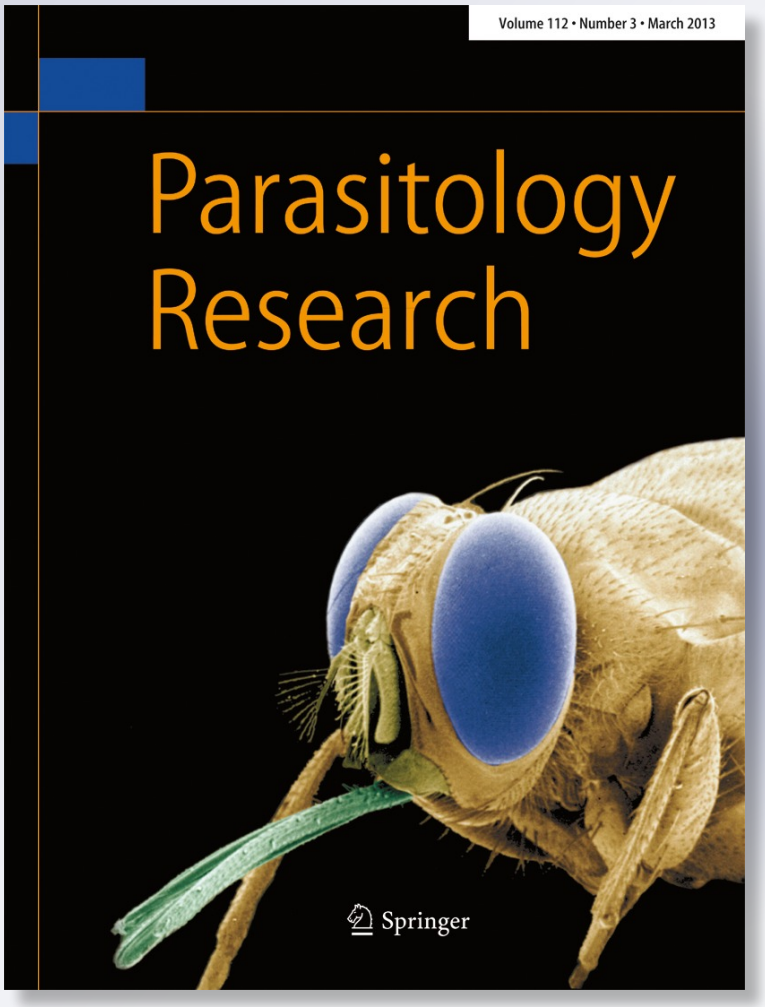

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ORIGINAL PAPER

Optimization of production of chlamydospores of the nematode-trapping fungus *Duddingtonia flagrans* in solid culture media

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Abstract The large-scale production of nematophagous fungi as agents of biological control is one of the main challenges to be commercially used. In order to improve growth of microorganism in a culture medium, the addition of growth inducer is common. At the moment, the action of their addition in the mycelia growth and sporulation rate of nematophagous fungi is not known. The purpose of this trial was to evaluate the sporulation rate of *Duddingtonia flagrans* by adding two growth inducers, *meso*-inositol and Tween 80, both at 0.5 % in a traditional culture medium Sabouraud glucose agar (SGA) and also in a traditional culture medium enriched with wheat flour and milk powder. From a traditional sterile culture of *D. flagrans*, four groups were made: SGA; Sabouraud glucose agar–*meso*-inositol 0.5 %; Sabouraud glucose agar–Tween 80 0.5 %; and Sabouraud glucose agar-enriched (SGA-E). These media were placed at a constant temperature of 27 °C for 4 weeks. Following this, chlamydospores were gently rinsed off with sterile water and counted using a Neubauer haematocytometer to estimate the number of chlamydospores per millilitre of water. The addition of *meso*-inositol 0.5 % to SGA promoted a significant increase ($p<0.05$) in chlamydospore production obtaining an average of 51,715,000 chlamydospores per Petri dish. The highest chlamydospore concentration was observed in the SGA-E in comparison with SGA ($p<0.01$) obtaining an average of 208,760,000 chlamydospores. The aim of this study was to obtain basic knowledge regarding the effect of enriched culture medium and growth-inducing *meso*-inositol and Tween 80 on mycelial growth and production of chlamydospores.

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Introduction

New alternatives for the control of gastrointestinal nematodes affecting livestock production worldwide have been previously studied. Amongst them, biological control is one of the most promising strategies by its ecological sustainability characteristics. Between all natural antagonists studied till now, the nematode-trapping fungus *Duddingtonia flagrans* has the best possibilities of controlling gastrointestinal nematodes in livestock. This microfungus has the advantage of being able to pass through the gastrointestinal tract of the animal host without losing its predatory capabilities due to the anatomical characteristic of its resting spores: the chlamydospores (Larsen et al. 1992, 1999; Grønvold et al. 1993). Because of this, the administration of *D. flagrans* to animals incorporated into their feed has been considered favourably by some researchers (Waller et al. 2001). Sagüés et al. (2012) testing in vitro and in vivo native strain (03/99) from Argentina demonstrated the faculty of reproduction into the laboratory and the predatory efficacy on infective larvae of parasites in the environment. The in vitro tests characterized the daily radial growth, different growth temperatures and the predatory ability of *D. flagrans* in different culture medium (Sagüés et al. 2012). Their behaviour in the environment was reflecting through a performance test in

sheep when the chlamydospores were incorporated into an energy block (Sagüés et al. 2011). The absence of large-scale production methods for these organisms is a major drawback for the effective use of them in biological control. The mass production of fungal biocontrol agents is still one of the major constraints for their large-scale use and commercialization (Santurio et al. 2009). There is little information about the culture media used to maximize the production of spores and even less concerning nematophagous fungi.

The most common commercial culture medium used to promote the growth of nematophagous fungi are Sabouraud glucose agar, corn meal agar, faecal agar and water agar (Waller et al. 2001). The nematophagous fungi culture can be performed in the laboratory in a liquid culture medium based in corn meal agar (CMA) (Lohmann and Sikora 1989), potato dextrose (Lohmann and Sikora 1989) and V8 juice (Gardner et al. 2000). Also, it can be grown in solid media such as CMA (Nordbring-Hertz 1968; Fernández et al. 1999), Sabouraud dextrose (Gardner et al. 2000) and faecal agar (Fernández et al. 1999). Another fungi culture alternative may be in sterile grains such as corn, birdseed, wheat, sorghum (Jobin et al. 2008), millet and barley (Waller et al. 2001).

In order to improve the growth of microorganism, it is common to add a traditional culture medium growth inductor. Until now, the effect of adding inductors in the mycelia growth and sporulation rate of nematophagous fungi is unknown. The *meso*-inositol is a growth inductor and an essential nutrient factor for certain microorganisms. Besides, in some higher animals, it has not been associated with a metabolic function which completely explains its role (Lewin 1965). Eastcott (1928) reported that inositol is required for normal growth of yeast. Schopfer et al. (1962) studied the effects of inositol deficiency in *Schizosaccharomyces pornbe* and observed marked changes in cellular morphology, concluding that inositol has a role as a regulator in cytogenesis and morphogenesis. Another growth inductor is Tween 80, a polyoxyethylene sorbitan monooleate, non-ionic surfactant that contains a mono-unsaturated fatty acid (oleate). Tween 80 has been extensively employed as a vehicle for the addition of water-insoluble compounds. Tween 80 is known to interfere with the permeability of cell membranes and enhances the nutritional input from the surroundings to the cell body (Scardovi 1981). The addition of Tween 80 to a culture medium containing the fungus thermophilic *Thermomyces lanuginosus* increased growth capacity and the biomass and the production of extracellular enzymes (Arnesen et al. 1998). The traditional culture medium can be enriched by substances to increase the mycelium production and sporulation. Michel-Acheves et al. (2008) emphasize the importance of finding a medium to increase the production of spores of fungi in an economic way and it could be used for a commercial or industrial purpose.

The wide-scale development of *D. flagrans* chlamydospore production is a challenge to overcome, if biological control is to become a real alternative in the fight against gastrointestinal nematodes. Until now, there is no information in the literature about the ability of *D. flagrans* to produce chlamydospores in different solid culture media. In this work, *D. flagrans* sporulation in commercial and enriched solid culture medium at two constant temperatures (24 and 27 °C) was evaluated, with the objective of finding the best combination of culture medium and temperature.

Material and methods

Fungal material

The strain used in this work was *D. flagrans* 03/99 that was previously selected from six strains tested, isolated from bovine faecal matter decaying in Tandil, Buenos Aires Province, Argentina (Saumell and Fuse, personal communication). The isolate was cultivated in CMA at 25 °C and kept frozen until used.

Temperature regimes

To evaluate the best combination of culture medium and temperature for chlamydospore production, they were incubated in Sabouraud glucose agar *meso*-inositol (SGA-MI) and Sabouraud glucose agar-enriched (SGA-E) at 24 and 27 °C in a climatic chamber (VWR Scientific Products, model 2005, low temperature incubator).

Growth time and incubation time

The production of chlamydospores in a solid culture medium was evaluated at the second, third and fourth weeks with the purpose to determine the higher production in the least time.

Substrates used

Four substrates were used for the determination of the chlamydospore production: Sabouraud glucose agar (SGA), SGA-MI 0.5 %, Sabouraud glucose agar–Tween 80 0.5 % (SGA-TW8 0.5 %) and SGA-E. The SGA was prepared as follows: Sabouraud dextrosa britania (65 g) and distilled water (1 L) were autoclaved at 121 °C for 20 min. The SGA-MI 0.5 % was prepared as follows: Sabouraud dextrosa britania (65 g) and distilled water (1 L) were added to 5 g *meso*-inositol ($C_6H_{12}O_6$ 180.16 g/mol) (Anedra, *meso*-inositol 180.16 g/mol, USA). The SGA-TW8 0.5 % was prepared as follows: Sabouraud dextrosa britania (65 g), distilled water (1 L) and 5 g of Tween 80 (Biopack, Tween

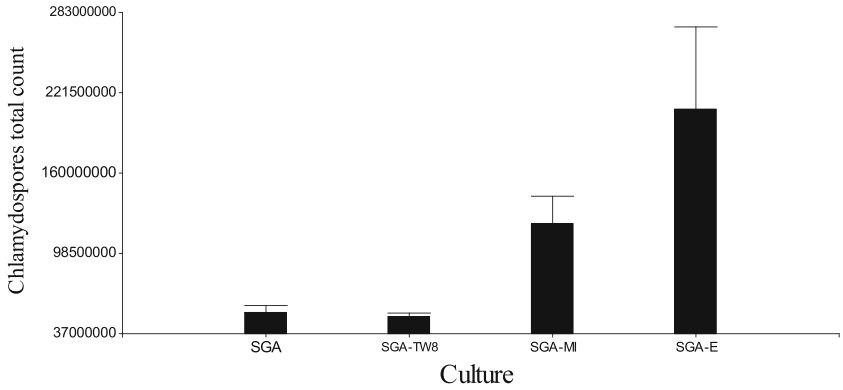

Fig. 1 The average numbers of chlamydospores of *Duddingtonia flagrans* growth in Sabouraud glucose agar (*SGA*), Sabouraud glucose agar *meso*-inositol 0.5 % (*SGA-MI 0.5 %*), Sabouraud glucose agar–Tween 80 0.5 % (*SGA-TW8 0.5 %*) and Sabouraud glucose agar-enriched (*SGA-E*)

80, 1 L=1.08 kg) were added. The SGA-E was prepared as follows: Sabouraud dextrosa britania (65 g) and distilled water (1 L) were added to 5 g of *meso*-inositol (Anedra, *meso*-inositol 180.16 g/mol, USA), wheat flour (composition per 100 g: energy, 339 kcal; grease, 1.87 g; protein, 13.70 g; carbohydrate, 72.57 g; fibre, 12.2 g; potassium, 405 mg; phosphorus, 346 mg; iron, 4.64 mg; sodium, 5 mg; magnesium, 138 mg; calcium, 34 mg; copper, 0.38 mg; zinc, 2.93 mg; manganese, 3.79 mg; vitamin C, 0 mg; vitamin A, 0 UI; vitamin B_1 (thiamine), 0.4 mg; vitamin B_2 (riboflavin), 0.215 mg; vitamin B_3 (pyridoxine), 0.341 mg; vitamin E, 1.23 mg; folic acid, 44 mcg) and whole milk powder La Serenísima® (composition per 26 g: energy, 61 kcal; carbohydrate, 4.8 mg; protein, 3.3 mg; saturated fat, 1.9 g; trans fat, 0.0 g; fibre, 0.0 g; sodium, 46 mg; calcium, 113 mg; vitamin A, 64 mcg; vitamin D, 1.0 mcg). The Petri dish preparation with different culture media was sealed with film and stored in frozen until used.

Experimental design

The trial was carried out in the Parasitology Laboratory, Faculty of Veterinary Sciences, UNCPBA, located in Tandil, Buenos Aires Province, Argentina. The isolate of *D. flagrans* 03/99 was grown in CMA for 2 weeks until it was used to inoculate Petri dishes (8.5 cm diameter). Each inoculum consisted of small squares of agar measuring approximately 5×5 mm, which was cut from the colony margin and placed upside down at the edge of Petri dishes containing either agar. Ten replicates for each solid medium were incubated for 4 weeks at 27 °C in a climatic chamber (VWR Scientific Products, model 2005, low temperature incubator). After 4 weeks of incubation, the chlamydospores on the agar plates were gently rinsed off with sterile water and the concentration of spores per millilitre of water was calculated using a haemocytometer.

Statistical analysis

The statistics were performed with a Kruskal–Wallis test (nonparametric ANOVA) to analyse the differences between amounts of chlamydospores in different solid medium cultures. Analyses were made with Instat Statistical Software 2008. To analyse the chlamydospores weekly count to different temperatures (24 and 27 °C), an ANOVA test was performed using Infostat 1.0v.

Results

Chlamydospore production in the culture medium

The solid culture medium that generated more chlamydospores was SGA-E, being the only one which differs from the rest of the culture medium tested ($p<0.01$). The medium that followed in quantity of chlamydospore production was SGA-MI 0.5 %, and there were no differences between SGA vs. SGA-TW8 (Fig. 1 and Table 1).

The statistical test showed significant differences when the totality of the culture media was compared between them ($p<0.0001$). When the traditional culture medium was compared with the rest of media using Dunnett's comparison, significant differences were observed (Table 1).

Temperature regimes and incubation time

The studies performed showed that both culture media (SGA-E and SGA-MI 0.5 %) gave a higher production of chlamydospores at 27 °C (Fig. 2). The SGA-E at 27 °C was the best medium that generated the highest production of chlamydospores. At 24 °C, the culture medium presents a much slower production of chlamydospores when compared with 27 °C (Fig. 3).

Table 1 Statistical analysis of numbers of chlamydospores of *Duddingtonia flagrans* grown in SGA, SGA-MI 0.5 %, SGA-TW8 0.5 % and SGA-E media

Comparison of culture medium	*p* value
SGA vs. SGA-E	<0.01
SGA vs. SGA-MI	<0.05
SGA vs. SGA-TW8	>0.05

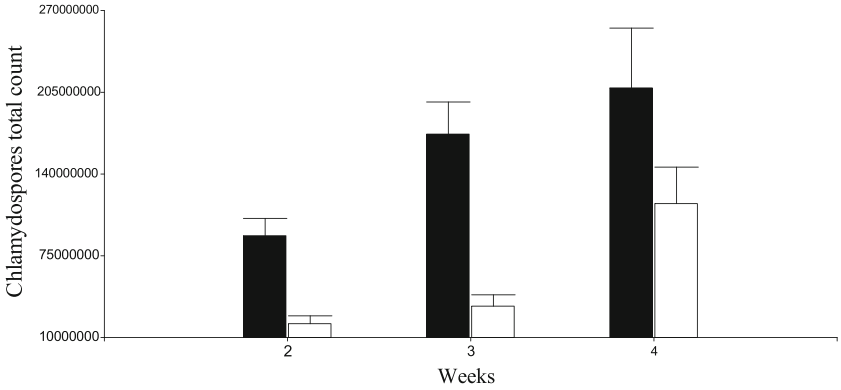

Fig. 2 Weekly chlamydospore count in the culture media SGA-E and SGA-MI at 27 °C. The *black column* is the medium SGA-E and the *white column* is the medium SGA-MI at 27 °C

Growth and sporulation of chlamydospores were increased through the incubation weeks, having the maximum production (quantity and maturity) at the fourth week (Fig. 2). Routinely, the predatory capability was evaluated weekly, for each medium, being observed as tridimensional network formations (unpublished data). There was a interaction in the chlamydospore production in SGA-E and SGA-MI 0.5 % medium and growth temperature in every evaluated week, 2 weeks ($p<0.0001$), 3 weeks ($p<0.0001$) and 4 weeks ($p=0.0016$).

Discussion

Studies focused on optimizing chlamydospore production in solid culture media showed that it is possible to make a mass production in the laboratory, when the medium is enriched with nutrients needed for growth and sporulation of *D. flagrans*. In our assay, the inclusion of whole milk and wheat flour, low-cost ingredients as a nutrients to the traditional culture medium containing *meso*-inositol 0.5 %, showed increased production of *D. flagrans* chlamydospores at the fourth week at 27 °C. Due to the addition of protein, carbohydrate, vitamins and minerals, the production of chlamydospores was increased. Similarly, the addition of K_2HPO_4, KCL, $MgSO_4$, H_20, yeast extract and Fe^{3+} in a culture medium based on corn meal agar at 20 °C enhanced substantially the capture of nematodes by the fungus *Arthrobotrys oligospora* (Nordbring-Hetz 1968). The search for the optimization of a fungal culture should be based on knowledge of the growth nutrients needed as well as their biology. Coscarelli and Pramer (1962), evaluating the nutrition and growth of *Arthrobotrys conoides*, observed that the addition of biotina (B_7 or B_8) and thiamine (B_1) (belonging to vitamin B complex) into an inorganic culture medium composed of $NaNO_3$, 0.37 %; $KHPO_4$, 0.1; KCL, 0.05; $MgSO_4$, 7; H_2O, 0.05; $FeSO_4$, 7; and H_2O, 0.001 increased significantly the mycelium production. The above data are in accordance with the results of the present assay where a significant rise of chlamydospores in the enriched medium was observed because the milk added contains thiamine, riboflavin and pyridoxine, vitamins of the B complex. The essential vitamins for development of *A. conoides* in medium supplemented with zinc were biotin and thiamine. When only thiamine was supplied, growth of the fungus was less than one half that obtained in the presence of both vitamins. In the absence of biotin and thiamine, *A. conoides* failed to develop (Coscarelli and Pramer 1962). In the data obtained in the present experiment, there was no possibility of comparison with other studies because of the absence of information about nematophagous fungus production in the laboratory.

Among the growth inductors we studied, *meso*-inositol was superior when compared with Tween 80. *Meso*-inositol stimulates the cytogenesis and morphogenesis of the fungus, promoting the development of chlamydospores. Blackburn

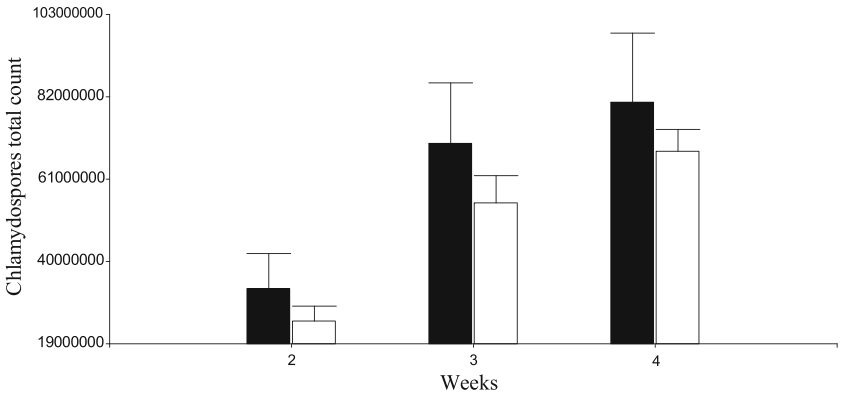

Fig. 3 Weekly chlamydospore count in the culture media SGA-E and SGA-MI at 24 °C. The *black column* is the medium SGA-E and the *white column* is the medium SGA-MI at 24 °C

and Hayes (1963) observed that the incorporation of *meso*-inositol in solid culture medium with agar or silica gel provided a better sporulation in the following fungi: *A. oligospora*, *Arthrobotrys robusta*, *Dactylella doedycoides* and *Dactylaria candida*, when compared with medium without *meso*-inositol. By adding 50 mg/L of *meso*-inositol, they observed the improving of the nematophagous fungus sporulation in two culture media of corn meal agar and sucrose–nitrate–salt solution. In our experiment, the addition of 0.5 % of *meso*-inositol to the traditional culture medium enhanced the sporulation of the fungus.

Otherwise, the addition of Tween 80 as a growth inductor provided less production of chlamydospores, when compared with *meso*-inositol and enriched medium. However, Tween 80 could be used when a production of mycelium was the target. Arnesen et al. (1998) demonstrated that the addition of Tween 80 improved the mycelial growth of thermophilus fungus *T. lanuginosus* and also increased 2.7 times the production of extracellular enzyme α-amylase. That could be useful in nematophagous fungus which can form with loss of its predatory capability, such as *A. conoides* (Etchepare et al. 2004), through the gastrointestinal tract in the mycelium.

The growth temperature was an essential factor to the fungus development and also for the posterior production of chlamydospores. Coscarelli and Pramer (1962) observed that *A. conoides* fungus growing at 28 °C was optimal when compared with 20 °C. Pelloile (1991) observed that *D. flagrans* fungus growth was faster when incubated at 30 °C. This study is in accordance with the present assay where the fungus growth was the best at 27 °C.

To conclude, the present study achieved an increase in the chlamydospore production of the nematophagous fungus *D. flagrans*. The enriched medium showed the most amounts of chlamydospores generated in the fourth week of incubation. Consequently, this medium can be used to produce chlamydospores of *D. flagrans* on a large scale. Future studies will seek to further optimize proposed culture media and their usefulness for the production of other species of nematophagous fungi in veterinary or agronomic use attractive for biological control of nematode parasites in the environment.

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