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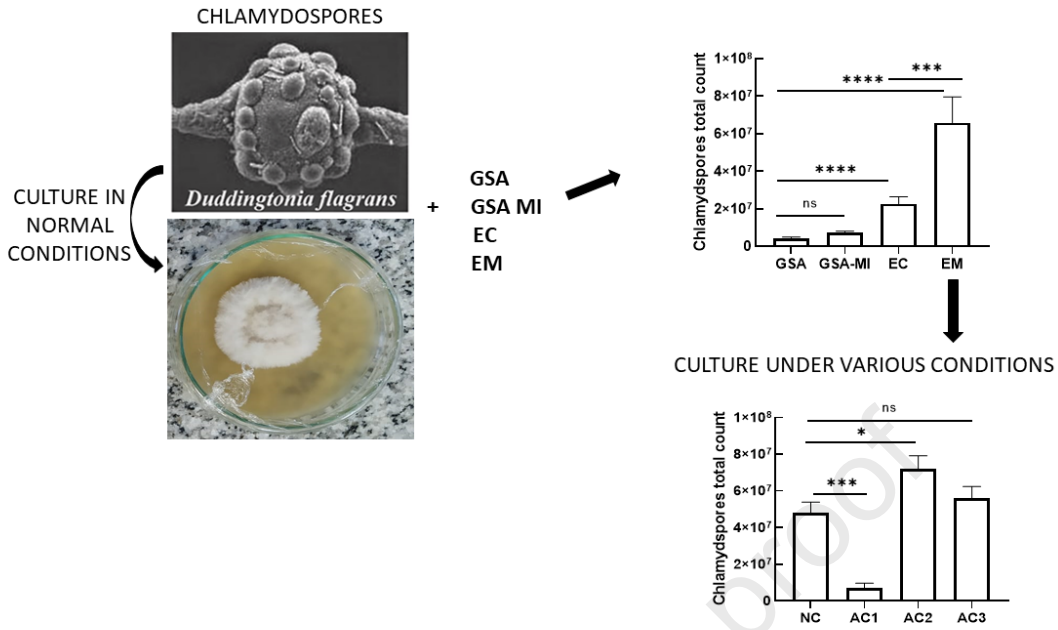

Enhancing Chlamydospore Production in *Duddingtonia flagrans* on solid substrate: The Impact of Mannitol and Varied Cultivation Conditions

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Abstract

Duddingtonia flagrans is a nematophagous fungus which has shown promising results as a non-chemical parasitic control tool. The fungus disrupts the parasite's life cycle by trapping larvae in the environment through the networks generated from chlamydospores, thus preventing the reinfection of animals. One barrier to the development of a commercial product using this tool is the need to increase chlamydospore production in the laboratory for its administration to livestock. The purpose of this study was to evaluate the addition of mannitol to an enriched culture medium and the effect of adverse cultivation conditions on chlamydospore production. *D. flagrans* was cultivated on Petri dishes with corn agar for 4 weeks at 27°C and 70% relative humidity (RH). Four groups were then formed, all with Sabouraud agar as a base, to which different growth inducers were added: GSA (glucose Sabouraud agar), GSA-MI (glucose Sabouraud agar + meso inositol), GSA-E (enriched glucose Sabouraud agar), and AE-M (enriched agar + mannitol). After 4 weeks, chlamydospores were recovered by washing the surface of each plate with distilled water and then quantified. The medium that yielded the highest amount of chlamydospores was subjected to different cultivation conditions: NC (normal conditions): 70% RH and 27°C, AC (adverse conditions) 1: 20% RH and 40°C, CA2: 60% RH and 27°C, and CA3: 55% RH and 24°C. It was determined that mannitol increases chlamydospore production ($65x10^6$

chlamydospores/plate), and when reducing humidity by 10% under cultivation conditions it resulted in an approximately 10% increase in chlamydospore production compared to the control group. These results suggest that the addition of polyols, as well as its cultivation under certain environmental conditions, can improve chlamydospore production on a laboratory scale.

Keywords: *Duddingtonia flagrans* – culture media – mannitol – temperature – humidity

1. Introduction

D. flagrans is one of the most studied species for the biological control of gastrointestinal nematodes. Its high degree of development, ease of laboratory production and ability to pass through the gastrointestinal tract of animals are characteristics of interest for the use of this fungus in production animals (Ojeda Robertos *et al.*, 2009).

Understanding the physiology of nematophagous fungi (NF) is necessary to comprehend their predatory capacity, as well as their response to different nutritional sources. Different nutrients can regulate the sporulation of fungi. The concentration of these nutrients in the culture media determines the quality and quantity of the fungus,encourages sporulation and prevents mycelial growth (Krishna, 2005).

Implementing biological control requires increasing chlamydospore production in the laboratory. Stimulating the formation of these resistance structures on a massive scale is possible by adding growth and sporulation inducers to the culture media (Sagués *et al.*, 2013), although there are also environmental signals that influence chlamydospore formation and are often species-specific (Lin and Heitman, 2005).

Diverse authors have reported the addition of different components to traditional culture media in order to promote the development of this fungus. Silva *et al.* (2016), evaluated chlamydospore production using by-products from the industry and different amounts of initial inoculum. Sagués *et al.* (2013), evaluated the addition of meso-inositol and Tween 80. Cuadrado Osorio *et al.* (2021), found sodium acetate and ammonium sulfate to be enhancing elements in the production of resistance structures. Santurio *et al.* (2009), focused on a biphasic production system. Other authors have evaluated the physical conditions of chlamydospore production, such as Blair and Biddle (2020), who evaluated fungal

dehydration as a strategy to increase spore production by subjecting the fungus to periods of slow and rapid drying.

Mannitol functions as a metabolic precursor, participating in various biochemical pathways which can provide energy to the cell (Goh *et al.*, 2009; Son *et al.*, 2012), in addition to regulating osmotic balance and interacting with reactive oxygen species in cell membranes (Meena *et al.*, 2015).

The aim of this study was twofold: first, to assess the efficacy of mannitol as an inducer of fungal development; and second, to investigate various cultivation conditions to determine if unfavorable temperature and humidity conditions could serve as stress factors leading to chlamydospore formation.

2. Materials and methods

2.1 Fungal material

A local isolate of *Duddingtonia flagrans* 03/99 (Saumell *et al.*, 2015) was used. The fungus was grown on enriched Sabouraud agar cultures at 25 ºC, composed of 20 g of corn agar per liter of distilled water, which was sterilized in an autoclave at 121 ºC/17 minutes. Fresh fungal chlamydospores were recovered from enriched Sabouraud agar cultures that had been incubated for 28 days at 27ºC and were kept at 4 ºC until used. Each inoculum consisted of squares of agar measuring 1,2 X 1,2 mm cut from the colony and placed upside down of Petri dishes containing each agar.

2.2 Experimental design

One-factor-at-a-time (OFAT) method was applied to evaluate the different nutrients and the various temperature and humidity conditions. This design depended on studying one factor while the other variables were constant. Four culture media were evaluated:

(1) Glucose Sabouraud Agar (GSA): prepared by dissolving 65 g of Britania Sabouraud Dextrose in 1 liter of distilled water;

(2) Glucose Sabouraud Agar with the addition of meso-inositol (GSA-MI): the base culture was prepared by dissolving 65 g of Britania Sabouraud Dextrose in 1 liter of distilled water, with the addition of 0,5% of meso-inositol;

(3) enriched culture medium (EC): composed of 65 g of Britania Sabouraud Dextrose in 1 liter of distilled water, with the addition of 0,5% of meso-inositol, wheat flour (composition per 100 g: energy: 339 kcal; fat: 1.87 g; protein: 13.70 g; carbohydrates: 72.57 g; fiber: 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 IU; vitamin B1 (Thiamine): 0.4 mg; vitamin B2 (Riboflavin): 0.215 mg; vitamin B3 (Pyridoxine): 0.341 mg; vitamin E: 1.23 mg; folic acid: 44 µg) and whole milk powder (composition per 26 g: energy value: 61 kcal; carbohydrates: 4.8 mg; proteins: 3.3 mg; saturated fats: 1.9 g; trans fats: 0.0 g; dietary fiber: 0.0 g; sodium: 46 mg; calcium: 113 mg; vitamin A: 64 µg; vitamin D: 1.0µg);

(4) enriched medium with the addition of 2% mannitol (EM).

Twelve plates of each culture medium were inoculated and placed in an incubator at 27 ºC and 70% relative humidity (RH). Weekly controls were performed to check contamination. Each plate was directly observed under a microscope to determine the presence of mycelial growth, spores, chlamydospores, and/or three-dimensional networks during the incubation period (4 weeks). Before harvesting the chlamydospores, they were moistened with distilled water and gently scrapped of the agar surface using a stainless steel scraper. The fungal mass obtained was strained through a nylon mesh to break up the mycelia and release the individual chlamydospores. These were collected in glass beakers to which distilled water was added up to 40 mL. A 10 µl sample from this solution was further diluted in distilled water up to 1 mL, from which a second 10 µl aliquot was taken in order to count the chlamydospores under a light microscope.

2.3 Cultivation conditions

Four cultivation conditions were evaluated using medium supplemented with mannitol because it yielded the highest production of chlamydospores One group was maintained at

27ºC and a relative humidity of 70% (NC). A climatic chamber (WTB Binder Labortechnik GmbH WTBbinder) was used for the other conditions, which were: 40ºC and 20% RH (AC1); 27ºC and 60% RH (AC2); and 24ºC and 55% RH (AC3).

2.4 Evaluation of the predatory capacity of *D. flagrans*

The nematophagous capacity of the fungus was evaluated through fecal cultures. The cultures were performed using the modified technique of Roberts and O'Sullivan described by Niec (1968). Ten grams of fecal matter from naturally parasitized calves, without prior anthelmintic treatment, were mixed with styrofoam. The value of the egg per gram (epg) was 550 and the infective larvae presence was represented by *Cooperia* spp., *Haemonchus* spp., *Ostertagia* spp and *Oesophagostomum* spp. According to the dosage levels evaluated by Bilotto *et al.* (2012) and Zegbi *et al.* (2021), 11000 chlamydospores/g of fecal matter were added, and this culture was left for 15 days in a climatic chamber (24ºC and 64-65% RH). To quantify the number of L3 in the fecal matter, the Baermann method (1917) was used. The fecal matter from each coproculture was wrapped in gauze in a small package and submerged in warm distilled water inside a plastic container for 24 hours. Afterwards, the containers and the supernatant were removed, while the remaining content was transferred and decanted into glass tubes. Once the supernatant was discarded from the glass tubes, the remaining 10% of each Baermann device was placed in a small glass receptacle with the addition of some lugol drops and observed under an optical microscope (40X and 100X). Ten coprocultures were performed for each tested cultivation condition and the development of infective larvae was evaluated after 15 days to determine if the addition of mannitol or adverse cultivation conditions had an effect on the predatory activity of the fungus. The percentage of L3 recovery was calculated using the following formula:

$$\text{L3 recovered (\%)} = \frac{L3\ average\ per\ gram}{\text{average epg}} X\ 100$$

2.5 Statistical analysis

Data were expressed as average ± SEM. Data normality was assayed using Brown-Forsythe and Bartlett´s test. Kruskall Wallis along with Dunn Multiple Comparison or One-way

ANOVA along with Dunnett´s Multiple Comparison post hoc test was used to compare differences between experimental groups. *P* values less than 0.05 were considered statistically significant. Data were analyzed by the GraphPadPrism 8.0.2 software (GraphPad Software Inc.).

3. Results

3.1 Culture media

The lowest chlamydospore production was observed with the GSA culture medium, followed by GSA-MI, GSA-E, and AE-M. The group supplemented with mannitol showed statistically significant differences compared to the medium reported in the literature as the solid culture medium with the highest production yield. Overall, all the evaluated culture media resulted in higher chlamydospore production compared to the traditional fungal culture medium (Fig. 1). The average chlamydospore production for the GSA, GSA-MI, GSA-E, and AE-M media was 4.4 X 106 (±1.7 X 106), 7.5 X 106 (±1.9 X 106), 2.2 X 107 (±1.7 X 107), and 6.5 X 107 (±3.9 X 107), respectively.

3.2 Predatory capacity of *D. flagrans*

Despite variations in the culture media employed, the nematophagous efficacy of the organism remained consistently robust, as evidenced by a significant 99% reduction in larval development observed across all experimental conditions (Fig. 2 and 4). This pronounced decrease underscored the potent parasitic control capabilities of the nematophagous agent.

Of particular note, despite the substantial decrease in larval numbers, our investigation successfully identified recovered larvae, attributed to the genera *Cooperia* spp., *Ostertagia* spp., *Haemonchus* spp and *Oesophagostomum* spp. The L3 were not detected in groups AC2 and AC3, suggesting that the predation efficiency of the fungus in these groups was 100% (Table 1 and 2).

Table 1. Percentage of parasitic genera present following the nematophagous activity of *D. flagrans* after 15 days of cultivation, using chlamydospores originating from the ASG, ASG MI, EC and EM groups.

Culture media with additives					
	Control (%)	**GSA (%)**	**GSA MI (%)**	**EM (%)**	**AS (%)**
***Cooperia* spp.**	50,4	44	68	47	7
***Ostertagia* spp.**	24,6	16	18	20	57
***Haemonchus* spp.**	24,4	40	14	33	36
***Oesophagostomum* spp.**	6				

Table 2. Percentage of parasitic genera present following the nematophagous activity of *D. flagrans* after 15 days of cultivation, using chlamydospores originating from culture media exposed to various conditions.

Culture media under various conditions			
	Control (%)	**EM (%)**	**CA1 (%)**
***Cooperia* spp.**	36,5	57,1	91
***Ostertagia* spp.**	35,5	28,5	4,5
***Haemonchus* spp.**	22	14,4	4,5
***Oesophagostomum* spp.**	6		

3.3 Cultivation conditions

The condition of maximum temperature (40°C) and minimum relative humidity (20%) resulted in the lowest chlamydospore production among the evaluated conditions, while reducing the relative humidity by 10% (60%) in the culture resulted in a higher chlamydospore production. When the temperature was lowered by three degrees (24°C) and the humidity was reduced by 15%, an increase in chlamydospore production per plate was observed. However, the results were not statistically significant compared to the conditions used routinely. The average chlamydospore production for the NC, AC1, AC2, and AC3 media was 4.8 X 106 (±1.6 X 106), 6.8 X 106 (±6.6 X 106), 7.1 X 107 (±2.3 X 107), and 5.6 X 107 (±1.7 X 107), respectively.

4. Discussion

188 The culture medium with mannitol showed significantly higher chlamydospore production
189 per plate compared to the control group ($p<0.001$). Moreover, when compared to two
190 enriched media, GSA-MI and EC, the mannitol-supplemented medium exhibited increased
191 production of these structures. Although the culture medium supplemented with mannitol
192 exhibited the highest rate of chlamydospore production, the GSA-MI and EC media showed
193 an even higher production compared to the commercial culture medium. Specifically, in the
194 case of EC medium, this disparity was statistically significant.(Fig. 1). Although the
195 experimental design did not include the assessment of interactions among the different
196 components of the culture media, a positive stimulus between these components can be
197 inferred, leading to increased chlamydospore production. Meso inositol stimulates both the
198 cytogenesis and morphogenesis of the fungus (Coscarelli and Pramer, 1962), while the
199 addition of whole milk increased chlamydospore production possibly by the addition of
200 proteins, carbohydrates, and vitamins (Blackburn and Hayes, 1963, Sagués *et al.*, 2013).

201 Mannitol serves as a readily available carbon source crucial for fungal growth and
202 dissemination. Its addition to the culture medium was based on observations indicating
203 increased activity of mannitol dehydrogenase enzyme during both sexual and asexual
204 developmental stages (Trail *et al.*, 2002; Min *et al.*, 2010). Chlamydospores, a pivotal phase
205 of fungal reproduction, are formed during this developmental phase (Sagués *et al.*, 2013).
206 This suggests a probable involvement of mannitol in the fungus morphogenesis (Ruijter *et*
207 *al.*, 2003). Moreover, studies on other fungi like *Gibberella zeae* have demonstrated that the
208 introduction of mannitol prompts the transformation of conidia into structures resembling
209 chlamydospores (Son *et al.*, 2012). These morphological changes in chlamydospores could
210 be associated with increased stress resistance, indicating that some fungal systems develop
211 resistance by accumulating mannitol. The high concentrations of mannitol in the conidia of
212 different *Aspergillus* species support its role in spore survival. These results are consistent
213 with those obtained in the present study, where the addition of 2% mannitol to the culture
214 medium resulted in a statistically significant increase ($p<0.001$) in chlamydospore production
215 after 4 weeks of incubation, compared to the traditional culture medium. These findings are
216 supported by experiments that have shown that mannitol is a requirement for completing the
217 life cycle of Ascomycetes and Basidiomycetes, as evidenced by an increase in mannitol

dehydrogenase and fructose-6-phosphate dehydrogenase enzymes during the fruiting of different fungi (Aguilar Osorio *et al.*, 2009; Patel and Williamson, 2016).

The impact of mannitol addition on chlamydospore production has shown variability in different experiments. Cuadrado Osorio *et al.* (2021), examined mannitol supplementation in a solid culture medium, where polyols like glycerol or mannitol can serve as reserve sources during adverse conditions. In their study, no water stress was observed. However, Gardner *et al.* (2000), revealed an increase in chlamydospore numbers upon transferring the culture from a liquid to a solid medium with glycerol. It is plausible that the stress induced by transitioning from a liquid to a solid medium triggered higher production of resistant structures. This discrepancy suggests that the impact of mannitol on chlamydospore production may vary depending on the specific experimental conditions, like the concentration and the nature of the stressors imposed on the fungal cultures. Further research on the various concentrations of this component is needed to optimize the current medium even more.

Mannitol is crucial for the safety of spores against cellular damage that occurs under conditions of high temperature, drying or increasing stress (Meena *et al.*, 2015). It has been reported that polyols such as glycerol and mannitol could be used as a reserve source when cells face adverse conditions. Several fungi also increase stress tolerance by accumulating mannitol (Meena *et al.*, 2015). For this reason, in this work it was decided to expose the fungus to different culture conditions in order to elucidate whether the stress response produces a greater number of chlamydospores in cultures with the presence or absence of mannitol in the medium. The optimal formation temperature of these structures was compared based on the experiments carried out by Sagués *et al.* (2013), where it was determined that the greatest growth of the fungus was at 27ºC. This temperature is within the range of those found by other authors for the growth and formation of *D. flagrans* traps (Youssar *et al.*, 2019 and Vieira *et al.*, 2020).

The literature on the stimulation of chlamydospore production through modifications in the physical cultivation conditions of this fungus is scarce. Blair and Biddle (2020) evaluated the improvement in *D. flagrans* chlamydospore production through dehydration. The fungus was

247 divided into three groups: fast drying at 38ºC and 37% RH for 48 h, slow drying at 24ºC and
248 55% RH for 10 days or kept at 30ºC and sealed with parafilm to avoid loss of RH as a control.
249 As in the present work, the results demonstrate the greater production of chlamydospores
250 when modifying the culture conditions (Fig. 3). By exposing the fungus to 27ºC and 60%
251 RH, the greatest number of chlamydospores was obtained with statistically significant results
252 compared to the control ($p<0.05$), and they maintained their viability, an expected result
253 since the spores survive that same temperature range when passing through the
254 gastrointestinal tract of animals (Ojeda Robertos *et al.*, 2009). By exposing the fungus to
255 extreme desiccation conditions, a decrease in chlamydospore production was observed
256 ($p<0.001$). These results agreed with the ones obtained by Cuadrado Osorio *et al.* (2020) and
257 Blair and Biddle (2020) where the number of chlamydospores per plate decreased
258 considerably at 35ºC. Insufficient substrate humidity can lead to various effects, including
259 decreased air diffusion coefficient within the substrate, resulting in delayed or halted fungal
260 metabolism. Additionally, it can activate enzymes causing metabolic imbalances and affect
261 membrane permeability for nutrient exchange (Gervais and Molín, 2003).The percentage of
262 larval recovery in the coprocultures with the addition of *D. flagrans* was lower compared to
263 the control group (without addition of fungus), thus demonstrating that the conditions
264 evaluated do not affect the predatory capacity of the fungus (Fig. 2 and 4). Tables 1 and 2
265 present the percentages of various parasitic genera recovered from coprocultures. It was
266 observed that genera of parasitic nematodes in domestic animals, whose larvae exhibit high
267 mobility, promote a greater production of three-dimensional networks compared to those
268 larvae with slower movements (Nansen *et al.*, 1988). Consequently, the presence of different
269 parasitic genera in the fecal matter used in this study induced the formation of traps and the
270 capture of parasites. While the capture percentage was notably high, it did not reach 100%.
271 This indicates that although the nematophagous activity effectively impedes larval
272 development, it does not completely eliminate the presence of parasitic nematodes. Such
273 findings play a crucial role in unraveling the complexities of parasitic control mechanisms
274 and hold implications for the formulation of future strategies targeted at managing parasitic
275 infections and agricultural settings.
276 The production of chlamydospores in the laboratory can be optimized by adding different
277 components and modifications to the culture medium. The search for different inducers must

continue to achieve a scale-up in the production of the fungus and a commercial formulation of the same to include it in integrated parasitic management programs.

5. Conclusions

The supplementation of mannitol in the enriched culture medium resulted in asignificant increase in the production of chlamydospores after a 4 week incubation period compared to the control group. Altering the culture conditions, albeit not drastically, contributed to heightened formation of these structures when conditions are deviated from the optimal. However, under more extreme conditions, a decrease in their formation was observed. This observation should be considered a limiting factor in the design of industrial production for this species when utilized as a parasitic control agent.

This study substantiates the significant role of 2% mannitol in facilitating the formation of resilient structures in the nematophagous fungus *D. flagrans*. Additionally, it demonstrates mannitol´s role in preserving the viability of chlamydospores when confronted with 27ºC and 60% RH culture conditions. The continuous advancement in chlamydospore production techniques not only promotes the sustainability of production but also contributes to preserving environmental health by reducing dependence on more invasive and less sustainable parasitic control methods.

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7. Conflict of interest

The authors declare no competing interests.

No potential conflict of interest was reported by the author (s).

8. Ethical approval

Not applicable.

9. Consent to participate

Not applicable.

10. Data availability

The data generated during and/or analyzed during the current research are available from the author (Vet. Milagros Junco) on reasonable request.

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12. Contributions

MJ: Conceptualization, Investigation, Methodology, Writing – original draft. **LI:** Investigation, Methodology, Validation. **GB, MF, FS**: Investigation, Methodology, Writing – review & editing. **ER, SZ, IG**: Supervision, Writing – review & editing. **CS, ASF**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing

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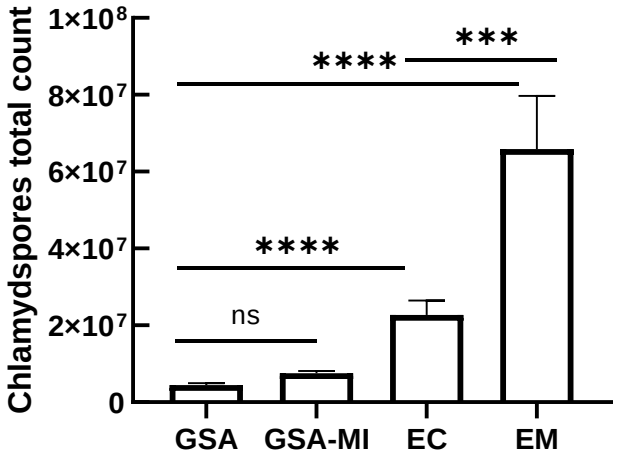
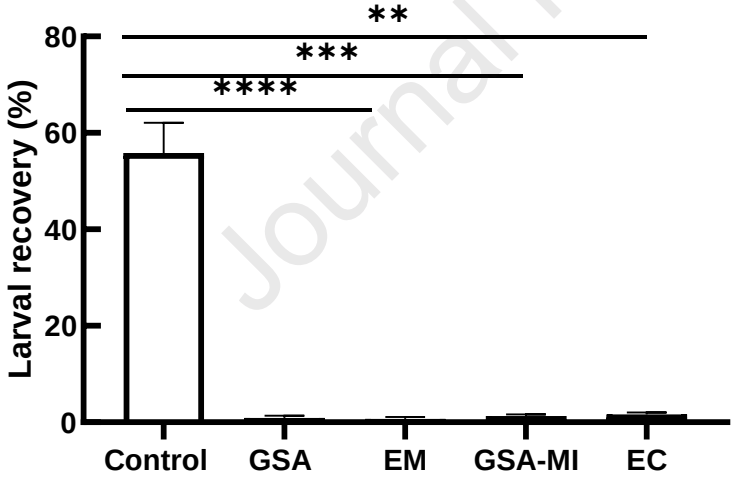
Figures



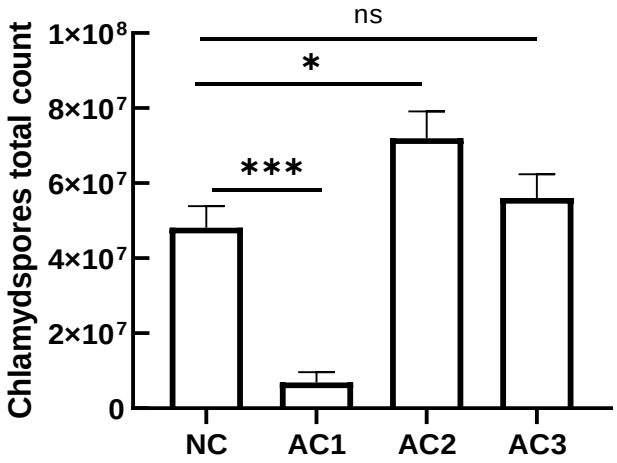
414

415 **Figure 1.** The average numbers of chlamydospores of *D. flagrans* growth in: ASG (Glucose
416 Sabouraud Agar), ASG-MI (Glucose Sabouraud Agar + Meso Inositol), EC (enriched culture
417 medium) y EM (enriched culture medium + Mannitol). Ns: non-significant. ***p<0.001,
418 ****p<0.0001.



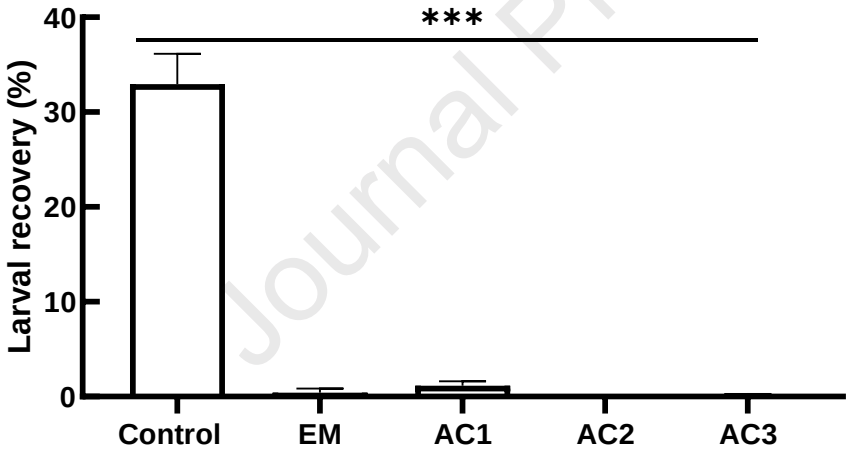
419

420 **Figure 2.** Larval recovery as described in Materials and Methods. Control (without *D.*
421 *flagrans*). GSA (Glucose Sabouraud Agar), GSA-MI (Glucose Sabouraud Agar + Meso
422 Inositol), EC (enriched culture medium) y EM (enriched culture medium + Manitol). Ns:
423 non-significant. **p<0.01, ***p<0.001, ****p<0.0001.



424

425 **Figure 3.** The average numbers of chlamydospores of *D. flagrans* growth in EM under
426 different culture conditions. NC (EM 27 C, 70% HR), AC1 (EM 40 C, 20% HR), AC2 (EM
427 27 C, 60% HR) y AC3 (EM 24 C, 55% HR). *p<0.05, ***p<0.001.



428

429 **Figure 4.** Larval recovery as described in Materials and Methods. Control (without *D.*
430 *flagrans*). EM (27 C, 70% HR), AC1 (EM 40 C, 20% HR), AC2 (EM 27 C, 60% HR) y AC3
431 (EM 24 C, 55% HR). ***p<0.001.

HIGHLIGHTS

- The production of chlamydospores in *Duddigntonia flagrans* is enhanceable by adding mannitol to the culture medium.
- Adverse conditions in the cultivation of *Duddigntonia flagr*ans stimulate chlamydospore production.
- *Duddingtonia flagrans* retains its nematophagous capacity following the addition of mannitol or the assessed adverse culture conditions.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: