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SHORT COMMUNICATIONS

Screening for a culture medium yielding optimal colony growth, zoospore yield and infectivity of different isolates of *Leptolegnia chapmanii* (Straminipila: Peronosporomycetes)

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Abstract *Leptolegnia chapmanii* is an aquatic fungus that has demonstrated marked pathogenicity towards the larvae of a number of mosquito species with little or no effect on non-target insects. The aim of this work was to determine the best culture medium for colony growth, and production and infectivity of zoospores among different isolates of *L. chapmanii*. No significant differences (P >0.01) were observed for the media $FORT_{(agar)}$, PYG, YPSS, and PYG_{SF} in terms of colony diameter of the different isolates evaluated. In these culture media, all *L. chapmanii* isolates had the highest growth (45 mm^2) after 7 days. The nine isolates tested produced the greatest number of zoospores in $FORT_{(agar)}$ medium 3 (1×10^6 zoospores/ml). Medium YPSS exhibited significant differences (P <0.0001) compared to other culture media with respect to maintenance of virulence and infectivity of the isolates, producing 100% larval mortality after 48 h of inoculation. Thus, for the cultivation and maintenance of *L. chapmanii* isolates, the medium of choice among those tested is $FORT_{(agar)}$, as it is both inexpensive to prepare and ensures optimal colony development, extremely high zoospore production, and excellent maintenance of fungal infectivity.

Keywords *Leptolegnia chapmanii* · Isolate · Culture medium · *Aedes aegypti*

Introduction

Culicidae are vectors of serious illnesses in humans—e.g., malaria, filiariasis, yellow fever, and diseases caused by other arboviruses. Dengue and dengue hemorrhagic fever are considered the most severe and widely disseminated viral diseases transmitted by mosquitoes (Pan American Health Organization 2009). *Aedes aegypti* L. (Diptera: Culicidae) plays a crucial role in the transmission of these infections (Jansen and Beebe 2010). The global use of insecticides for mosquito-vector control in recent decades has caused environmental pollution of aqueous ecosystems and has resulted in insecticide resistance in many mosquito species. Thus the burgeoning interest in alternative nonchemical strategies over the last decades is hardly surprising. The use of biological control agents such as bacteria, protozoa, nematodes, and fungi in order to control mosquito populations has been explored and developed (Federici et al. 2007).

Although a number of oomycete watermolds are parasitic on plants and animals, only *Lagenidium giganteum* (Couch) has been widely recognized to have a significant impact as a natural control agent for mosquitoes (Umphlett and Huang 1972; World Health Organization 1979). Seymour (1976)

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reported the isolation of a watermold from a parasitized mosquito larva that was tentatively identified as a *Leptolegnia* species, but no infection experiments from the infected larvae were attempted at that time. *Leptolegnia chapmanii* Seymour was subsequently isolated from the larvae of several mosquito species (McInnis and Zattau 1982; Seymour 1984; Lord and Fukuda 1988; Fukuda et al. 1997; López Lastra et al. 1999).

Leptolegnia chapmanii is an aquatic fungus[1] that has demonstrated marked pathogenicity toward the larvae of a number of mosquito species, with little or no effect on nontarget insects (McInnis and Zattau 1982; McInnis and Schimmel 1985; López Lastra et al. 2004).

The high pathogenicity and virulence of these fungal isolates, coupled with their specificity for members of the Culicidae family and their ability to be cultivated on artificial media make them good candidates for biological agents in the control of mosquito larvae. The aim of this work was to determine the optimal culture medium for the colony growth and zoospore production and infectivity of the different *L. chapmanii* isolates.

Materials and methods

The isolates of *L. chapmanii* used in this work were provided by the Entomopathogenic Fungal Culture Collection of Department of Agriculture United States, Ithaca, NY (ARSEF). Five isolates were used: LPSc #1051 (ARSEF 2562, isolated from *Mansonia titillians* Walkers in Ohio, 1988)., LPSc #1052 (ARSEF 2680, isolated from *M. titillians* in Florida, 1989), LPSc #1054 (ARSEF 2681, isolated from *Culex pipiens quinquefasciatus* Say in South Carolina, 1982), LPSc#1055 (ARSEF 2682, isolated from *Culex* sp. in Levi County, Florida, 1987) and LPSc #1056(ARSEF 5499, isolated from *Aedes albifasciatus* Macquart in Melchor Romero, Argentina, 1996).

The isolates were deposited in the mycological collection of the Institute Spegazzini (LPS culture collection), La Plata (Buenos Aires Province), Argentina. All isolates were maintained on water agar.

Mosquito larvae

The larvae of *Aedes aegypti* used in this study were obtained from colonies maintained following standard mosquito-rearing techniques (Gerberg et al. 1994).

[1] We recognize that all taxa of oomycete watermolds are now classified as belonging to the kingdom Chromista (= Straminipila) and have been formally excluded from the true fungi. For the sake of convenience, however, we continue to refer to oomycetes in this paper in the historically broad sense as fungi.

Culture media

Nine culture media were tested to assess which was the most appropriate for colony growth, zoospore production and infectivity of the five extant isolates of *L. chapmanii* (Table 1).

Colony growth

To evaluate the optimal medium for the colony growth of the *L. chapmanii* isolates, 5-mm circles of mycelia were removed with a sterile punch from the different fungal isolates and placed in the center of Petri dishes, with each vessel containing a different culture medium. Fungal growth was then assessed by measuring colony diameters after 7 days at 25±1°C. Five replicates were performed for each isolation and medium used.

The data were transformed by means of a log (x+0.5) function and analyzed by the Bifactorial-Anova Test. The parameters were culture media and *L. chapmanii* isolates, and the variable evaluated was the colony diameter. When the parameters of the interaction (culture medium and isolates) were statistically significant, the influence of each culture medium on a given isolate was evaluated by the ANOVA test, followed by the Duncan test for post-hoc comparison.

Zoospore production

To assess zoospore production for the five isolates of *L. chapmanii* after growth in the nine different culture media, eight cubes of mycelium-containing agar (1 cm^3) were removed with a sterile punch. The cubes were then placed in a sterile 250-ml Erlenmeyer flask containing 100 ml sterile distilled water and incubated with constant shaking for 48 h at 25°C. Each fungal strain was tested with all nine culture media in triplicate.

After the incubation period, the mycelium from each cube of agar was separated with tweezers, returned to the flask, and mixed by magnetic stirring for 20 min. Ten tubes, each containing 10 ml suspension, were centrifuged at 78 *g* (1,000 rpm) for 15 min, the supernatant removed, and the pellets collected and pooled. The combined pellet fraction was then recentrifuged under the same conditions to give a final pellet of about 2 ml per replicate, or 6 ml for the combined triplicate samples.

The number of zoospores per combined sample was counted in a Neubauer hemocytometer and the counts expressed as mean values.

The number of zoospores produced by the five isolates in each of the nine culture media was analyzed statistically as described above.

Table 1 Chemical composition of the nine culture media in which the infective capacity, zoospore production, and colony development of the five *Leptolegnia champanii* isolates were evaluated

Component	Culture medium								
	$CGPSC_1$	$CGPSC_2$	FORT $_{(agar)}$	PYG	YPSS	PYGSF	RGNSF	CMDP	CMDP $_{(modf.)}$
Peptone				1.2 g/l		1.2 g/l		10 g/l	5 g/l
Yeast extract				1.2 g/l	4 g/l	1.2 g/l			
Glucose	12 g/l	12 g/l		3 g/l		3 g/l		5 g/l	10 g/l
Cornmeal								40 g/l	40 g/l
K_2HPO_4	10 mM	10 mM			1 g/l				
$MgSO_4$					0.5 g/l				
Starch					15 g/l				
Casamino acids	20 g/l	10 g/l							
NH_4Cl	0.26 g/l	0.26 g/l							
L-Methionine	50 ppm	50 ppm							
$CaCl_2$ $2H_2O$	1 g/l	1 g/l							
Fortisip®			100 ml						
Sunflower oil						10 ml	10 ml		
Groundnut cake							2.5 g/l		
pH	7	7	7	7	7	7	7	7	7
Agar	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l
Distilled water	1,000 ml	1,000 ml	900 ml	1,000 ml	1,000 ml	990 ml	990 ml	1,000 ml	1,000 ml

Infectivity tests

In order to determine which of the nine culture media was the optimal for maintaining the infectivity of zoospores produced by the five isolates of *L. chapmanii*, 25 healthy fourth-instar larvae of *Ae. aegypti* were exposed to 1 ml zoospore suspension in 46-cm^2 sterile plastic containers containing 150 ml sterile distilled water.

Three replicates were tested along with a control sample treated in the same way as above but without the addition of the fungal inoculum. Larval mortality, confirmed by phase-contrast microscopy at 40X, was recorded after 48 h.

Larval mortality produced by each of the five *L. chapmanii* isolates after cultivation in each of nine media were analyzed by the General Linear Model through the GENMOD Procedures of Sas. The model function applied was: $\eta = \mu + \alpha_i + \delta_k + (\alpha\delta)_{ik}$, where μ is general mean, α_i the effect of the culture media (CGPS;$_1$, $CGPSC_2$, $FORT_{agar}$, PYG, YPSS, PYG_{SF}, RGNSF, CMDP, and $CMDP_{(modif)}$) to nine; δ_k the effect of the isolates (1051, 1052, 1054, 1055, and 1056); and $(\alpha\delta)_{ik}$the interaction between α_i and δ_k

Larval mortality of the five *L. chapmanii* isolates against mosquito larvae in the nine culture media was compared by the Linear- Contrast test.

Results

Colony growth

ANOVA test indicated significant differences with the two parameters varied: F=256.9, df=8, P <0.0001 for the media; F=31.7, df=4, P <0.0001 for the isolates; while the interaction between them was also significant: F=12.6, df=32, P <0.0001. No significant differences (P >0.01) were observed for the media $FORT_{agar}$, PYG, YPSS, and PYG_{SF} with respect to the colony diameters of the different isolates evaluated. In these culture media, all five *L. chapmanii* isolates had equally high growth (45 mm^2) after 7 days. Colony growth to 45 mm^2 after 7 days was also recorded in the following isolate-media combinations: LPSc #1055 and medium CMDP, LPSc #1056 and media $CGPSC_1$ and CMDP, and either LPSc #1051 or LPSc #1052 and the media $CGPSC_2$ and $CMDP_{(modif)}$. In contrast, growth of the five *L. chapmanii* isolates was limited in RGNSF medium (Fig. 1).

Production of zoospores

ANOVA test showed significant differences with the two parameters varied: F=31.56, df=8, P <0.0001 for the culture media; F=7.36, df=4, P<0.0001 for the isolates;

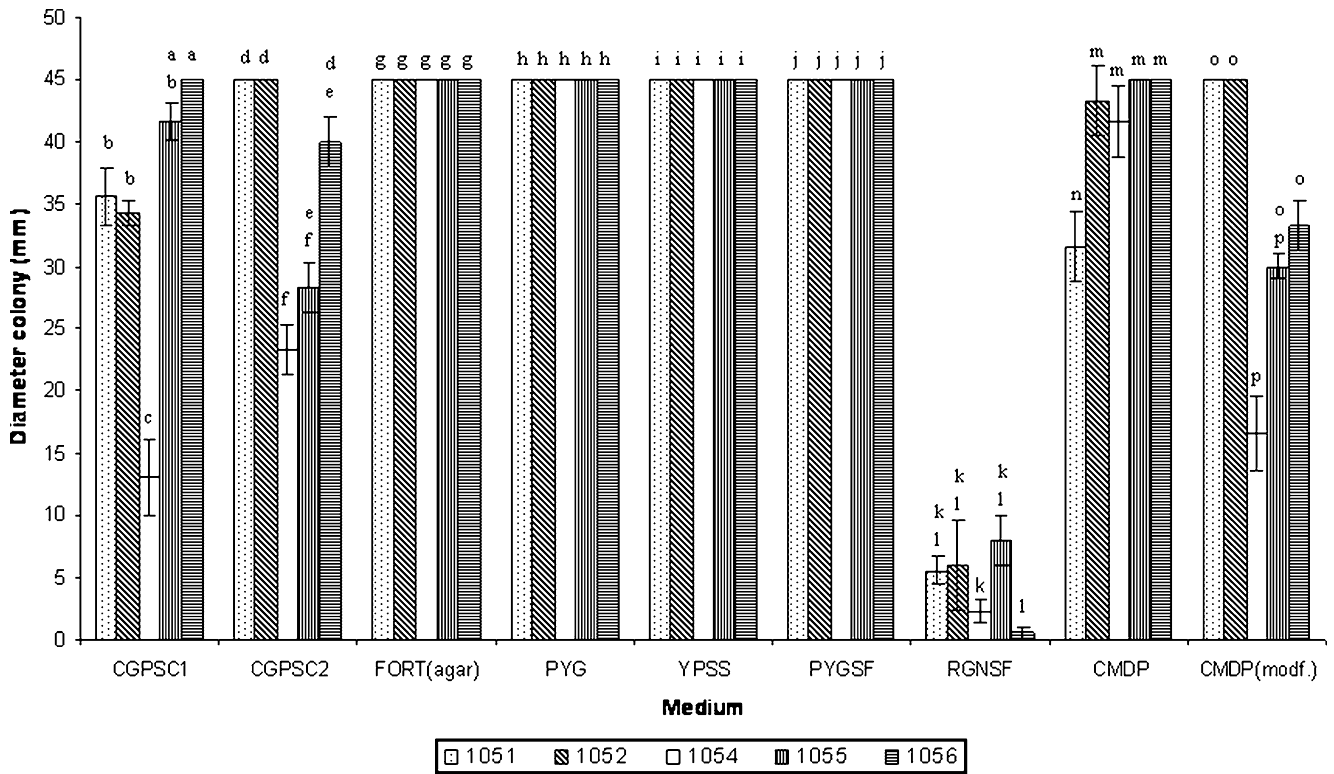

Fig. 1 Colony growth (mm^2) of *Leptolegnia chapmanii* isolates assessed in nine culture media after 7 days. Columns with different letters indicate statistical significant differences ($P<0.01$) for Duncan's test results between the five isolates by each one of the nine culture media

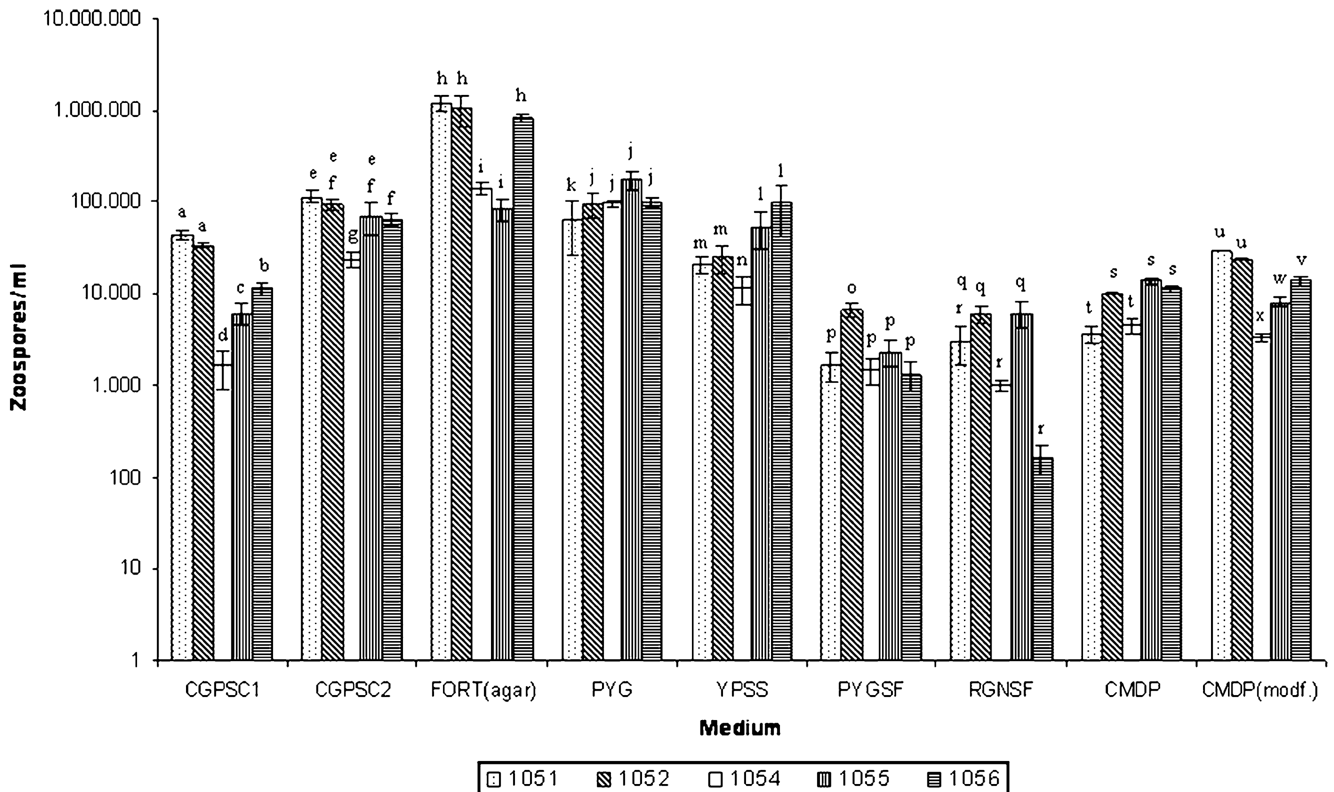

Fig. 2 Zoospore production of *L. chapmanii* isolates in nine different culture media. Columns with different letters indicate statistically significant differences ($P<0.01$) for Duncan's test results between the five isolates by each one of the nine culture media

while the interaction between them was likewise significant: $F=2.84$; $df=32$; $P<0.0001$.

The isolates LPSc #1051, LPSc #1052, LPSc #1054 and LPSc #1056 produced the greatest number of zoospores in $FOTR_{agar}$ medium at 1 x 10^6 ml^{-1}. In contrast, the isolate LPSc #1055 registered the maximum number of zoospores in PYG medium at only 1.7×10^5 ml^{-1} (Fig. 2).

Infectivity tests

With respect to infectivity, culture in YPSS medium evinced significant differences ($P<0.0001$) as compared to the other culture media. Zoospores produced by the five isolates were able to maintain their virulence and infectivity in this medium even 48 h after inoculation, and were capable of producing larval mortality of 100% at that time (Fig. 3). Similarly, zoospores of isolates LPSc #1051 and LPSc #1052 cultured in $FORT_{agar}$ medium produced 100% mortality, while those from isolate LPSc #1056 gave 80 % mortality after 48 h. $CGPSC_a$ medium yielded mortalities that ranged from a low of 4±2.8 % for isolate LPSc #1054 up to 62.6±2.5 % for the isolate LPSc #1051. PYG medium produced mortalities ranging from 14.6±1.1% to 45.3±3.2 % for different isolates of the fungus (Fig. 3).

Zoospores of the different isolates obtained from the culture media $CGPSC_1$, PYG_{SF}, RGNSF, CMDP, and $CMDP_{(modif)}$, however, produced mortalities below 10% on the test larvae at 48 h (Fig. 3). Interestingly, none of the isolates cultured on $CMDP_{(modif)}$ media produced any mosquito larval mortality (Fig. 3). The generalized linear model showed that the culture media and the isolates have a significant effect on the ability of *L. chapmanii* zoospores to infect *Ae. aegypti* larvae (Culture media: $df=8$; $X^2=$ 1,406.5; $P<0.0001$. Isolates: $df=4$; $X^2=53.01$; $P<0.0001$. Isolates*culture media: $df=32$; $X^2=121.97$; $P<0.0001$). The linear-contrast results are summarized in Fig. 3

Discussion

We investigated the effect of the chemical composition of different culture media on the colony growth of five *L. chapmanii* isolates and the production and infectivity of their zoospores. This work constitutes the first investigation

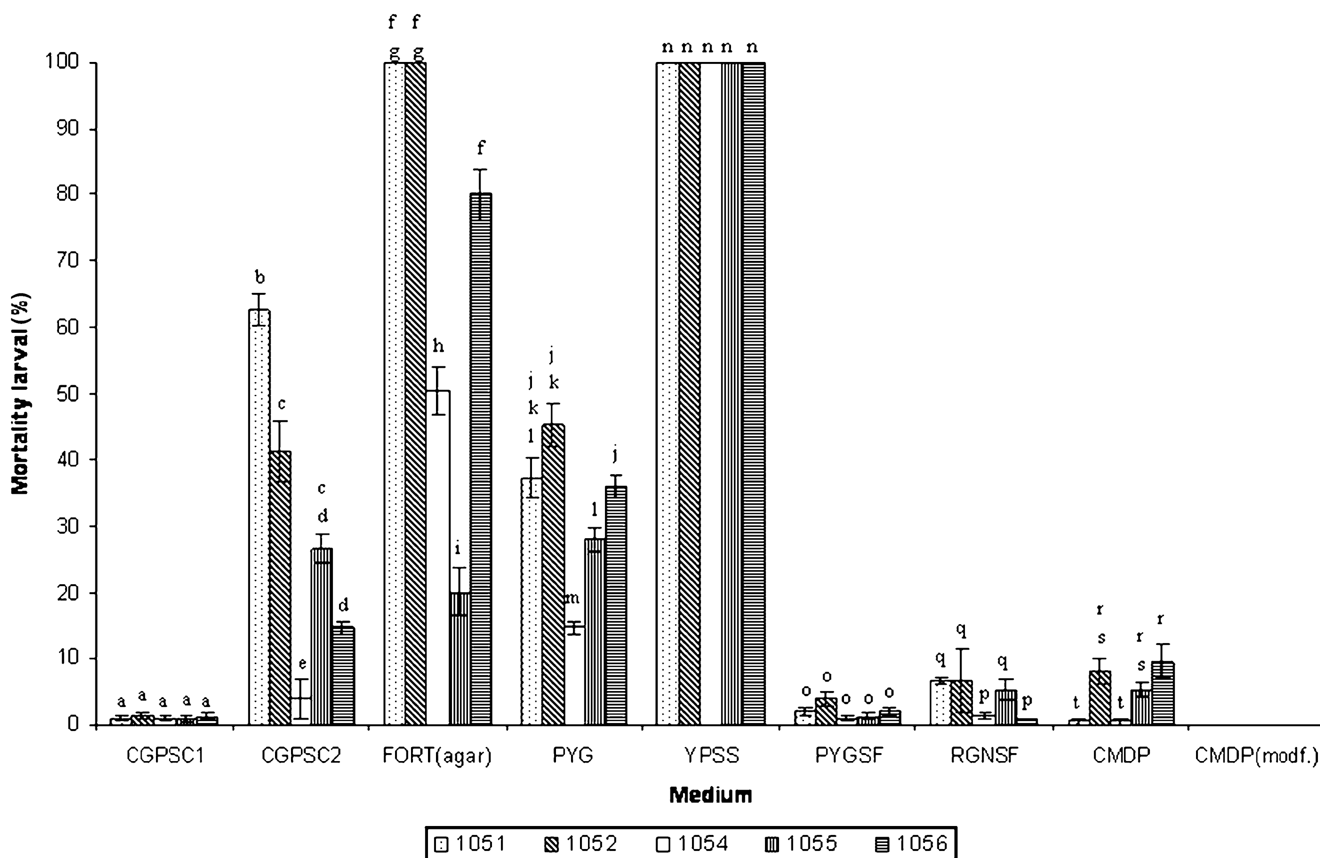

Fig. 3 *Aedes aegypti* larval mortality (%) with zoospores from the five *L. chapmanii* isolates after previous growth in nine different culture media. Columns with different letters indicate statistical significant differences ($P<0.01$) for Linear-Contrast test results between the five isolates by each one of the nine culture media

of this nature on the five known *L. chapmanii* isolates in the world.

The retention of a high level of pathogenicity and a maximum capacity to produce infective spores are primary considerations when culturing entomopathogenic fungi (Shah and Pell 2003). Several studies have been published on the effects of culture in artificial media or serial passage through host insects on the resulting vigor of such fungi (Humber 1997). For some species, e.g., *Verticillium lecanii* (Hall 1980) and *Culicinomyces clavisporus* (Sweeney 1981), no loss of vigor appeared to be associated with prolonged culture in vitro. For others—*Metarhizium anisopliae* (Fargues and Robert 1983) and *Entomophaga maimaiga* (Hajek et al. 1990)—a decline in virulence developed after in vitro culture, but the strength of infectivity was regained after serial passage through the appropriate hosts.

In the present study, the majority of the culture media supported colony growth to give the maximum possible final diameter (45 mm^2) at the end of 7 days. The exception was RGN_{SF} medium, in which colony growth was quite poor for all the *L. chapmanii* isolates. These results differ from those of Hoti and Balaraman (1990), who observed that this medium supported good colony growth as well as zoospore production and infectivity of the fungus *L. giganteum*.

According to Nolan (1983), $CGPSC_1$ medium is optimum for the vegetative growth of *Leptolegnia* sp. This finding does not agree with these present results since, in our hands, colony growth of *L. chapmanii* isolates LPSc #1051, LPSc #1052, and LPSc #1054 proved not to be optimal on this medium.

Our studies indicate that the best culture medium for the production of zoospores with four of the five *L. chapmanii* isolates studied is $FPRT_{agar}$, as it produced a higher number of zoospores (1×10^6 zoospore/ml) than even the quantity observed previously on fourth-instar larvae of *Ae. aegypti* (9.6×10^4 zoospore/ml) 48 h after death (Pelizza et al. 2008). This finding indicates that $FORT_{agar}$ medium is highly suitable for the in vitro production of zoospores with all known isolates of this species of *Leptolegnia*. Another medium that supported a high level of zoospore production by the isolate LPSc #1055 was PYG. These results disagree with those reported by Lord and Roberts (1986), who concluded that this medium caused a loss of the capacity of *L. giganteum* to produce infective zoospores for *Ae. aegypti* larvae. When assessing the infectivity of zoospores of different isolates of *L. chapmanii* for *Ae. aegypti*, we observed that YPSS medium to be the best medium for maintaining virulence with the five different *L. chapmanii* isolates; these produced between 10.000 and 100.000 zoospores/ml, always causing 100% larval mortality according to the results obtained by Pelizza et al. (2007), who also observed 100% larval mortality when 2.8×10^5 zoospores/ml were assayed. On the other hand, the $FORT_{agar}$ medium also maintained quite respectable zoospore infectivity in four of those five, giving larval mortalities of above 50%.

In accordance with the main objective of this work—to find an optimal culture medium for maintaining different strains of *L. chapmanii* isolated in Ohio, South Carolina, Florida, and Argentina—we conclude that, of the all culture media tested in these experiments, the one that can be recommended for the maintenance of those various isolates is $FORT_{agar}$. This formulation is not only low cost to prepare but also allows optimal colony growth, a high level of zoospore production, and excellent preservation of larval infectivity.

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