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Development of the mosquito pathogen *Leptolegnia chapmanii* (Straminipila: Peronosporomycetes) on an inexpensive culture medium based on sunflower seed

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An inexpensive culture medium based on sunflower seed extract (SSE) for production of *L. chapmanii* was developed. Vegetative growth on solid and liquid SSE was compared with two culture media used routinely (PYG and Emerson YPss). Results indicate that the oomycete is able to grow on SSE medium, producing more zoospores at a faster rate as well as inducing higher mortality rates in *Ae. aegypti* larvae.

Keywords: Mosquito control, diseases-borne vector, entomopathogen, Straminipila, Oomycete culture.

Leptolegnia chapmanii (Seymour) (Straminipila: Peronosporomycetes) is an aquatic pathogen of mosquito larvae (McInnis & Schimmel, 1985; López Lastra et al., 2004). Its virulence and pathogenicity against *Aedes aegypti* larvae has been studied (McInnis & Zattau, 1982; López Lastra et al., 2004). It persists enabling it to reduce mosquito population for weeks after a single application (Rueda et al., 2015). It grows readily on culture media as PYG and Emerson YPss (Pelizza et al., 2011). However, the use of such culture media for its mass production could be expensive. A culture medium based on sunflower extract (SSE) was reported as an alternative for culture of the

entomopathogen *Lagenidium giganteum* (Couch) (Guzman & Axtell, 1986). The mass production of zoospores from *L. giganteum* maintained on *in vitro* culture media by its immersion in water was used by Jaronski & Axtell, (1983) and a medium-scale production of *L. giganteum* and its application in laboratory and field was reported by Kerwin et al., (1994).

Consequently we developed and evaluated a culture medium based on sunflower seeds as an inexpensive alternative for the mass production of *L. chapmanii.* SSE medium was prepared following the protocol proposed by Jaronski & Axtell (1984). To prepare one liter of medium, ten grams of sunflower seeds were blended with 100 ml distilled water for one minute, filtered through 2-4 layers of cheesecloth and the residue was blended again with 100 ml of distilled water for one minute. Suspensions were filtered, mixed and the final volume was increased with distilled water to one liter. The PYG (Peptone 1.25 g, Yeast extract 1.25 g, Glucose 3 g, distilled water 1000 ml) and E. YPss (Yeast extract 1 g, soluble Starch 20 g, K_2HPO_4 1g, $MgSO_4.7H_2O$ 0.5 g, distilled water 1000 ml) culture media were routinely prepared. Media were solidified adding 15 g of agar per liter of culture medium. Both liquid and solid culture media were sterilized by autoclaving at 120 ºC and 20 PSI for 25 minutes.

Growth and development of *L. chapmanii* in solid and liquid SSE culture media was compared with traditional media PYG and E. YPss, used for its culture. Zoospore production and their pathogenicity against *A. aegypti* larvae were also evaluated. An Argentinean isolate of *L. chapmanii* (CEP 010) from the Fungal Entomological Collection of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE) in La Plata, Buenos Aires, Argentina was used. *Aedes aegypti* larval populations (L2/L3) from a colony established at CEPAVE were used in pathogenicity bioassays

Petri dishes (100 mm diam.) with solid media were used to evaluate radial growth of *L. chapmanii*. Treatment medium dishes were inoculated by placing 1cm^2 plug from PYG solid medium with *L. chapmanii.* The treatments were maintained at 25 °C in an incubator and the radial growth was assessed after 48, 72 and 96 h. Each treatment had four replicate dishes and the assay was repeated three times. The formation and release of zoospores was induced by adding five pieces (1cm^2) of each solid medium treatment into 100 ml distilled water. Zoospore concentrations were determined after 48 h and their viability and pathogenicity evaluated through bioassay against *A. aegypti* larvae (n=25). Larval mortalities were recorded after 48 h.

L. chapmanii growth in liquid media was evaluated by initial inoculating of 200 ml of each liquid medium with a 1 cm^2 plug from cultures grown on of the PYG solid medium, incubating for 7 days at 25ºC and 0.18 g (180 rpm) in an orbital shaker, then transferred to 400 ml of each respective culture medium for an additional 7 days of culture under the same laboratory conditions. After the 14-day culture period, biomass from each culture medium was filtered and fresh weight recorded. Cost of production based on biomass obtained in different culture media was estimated and compared. For zoospore formation and release, 10 g of biomass produced in liquid cultures were added to 200 ml sterile distilled water in 500 ml Erlenmeyer flasks on and shaken on an orbital shaker at 0.18 g (180 rpm). Production of zoospores was determined daily for 4 days. Their viability and virulence were confirmed by pathogenicity tests against *A. aegypti* larvae (n= 10) using 10 ml of the zoospore suspensions in plastic containers

(50 ml). Larval mortality was recorded after 48 h. Each treatment had three replicate containers and one negative control with distilled water. Assays were repeated three times at different dates.

In all cases, the concentration of zoospores was determined using a Neubauer chamber. Results were analyzed by ANOVA and the *pos hoc* test of Student Newman Keuls (SNK). Larval mortalities were transformed to arsine square-roots for statistical analysis. The SPSS statistics software from IBM was for the analyses.

Leptolegnia chapmanii developed on solid culture media with different growth rates. Higher production of zoospores as well as mortalities against *Ae. aegypti* larval population were obtained with *L. chapmanii* from the alternative SSE medium (Table 1A). It grew also in liquid culture media developing a great number of small mycelial masses in SSE medium compared with a single large biomasses formed in PYG and E. YPss. Higher production of zoospores (Fig 1) and host mortality (Table 1 B) was obtained with *L. chapmanii* from SSE medium. Nulls mortalities were registered on negative controls.

Even though the production of zoospore by *L. chapmanii* had been described without sterol enrichment of culture medium (Pelizza et al., 2007), the present results suggest that *L. chapmanii* could be an auxotrophic organism that develops vegetatively on poor sterol media, but requires it for initiation of the reproductive cycles (sexual / asexual) as described for *L. giganteum* (Domnas et al., 1977; Kerwin & Washino, 1983). An increased production of zoospores by the enrichment of culture medium with sunflower oil has also been demonstrated for *L. giganteum* (Balaraman & Hoti, 1986; Maldonado-Blanco et al., 2011). Sunflower seeds are a great source of phytosterols (Merah et al., 2012). Higher mortalities of *Ae. aegypti* larvae were related with higher zoospore concentrations (Pelizza, 2007).

We conclude that *L. chapmanii* was able to grow on SSE solid and liquid medium. Based on local prices of compounds required to prepare the culture media used, to produce 1 kg of *L. chapmanii* biomass in PYG would have a cost of $US10, $7 with and $3 with SFE liquid culture media This relatively inexpensive medium providing the nutritional requirements for the vegetative and reproductive development of the pathogen.

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Table 1. A) Assays with *L. chapmanii* developed on solid culture media. B) Assays with *L. chapmanii* increased in liquid culture media.

A.

Medium	Radial Growth (mm) * 48 h	72 h	96 h	Zoospore s.ml^{-1}	(%) Mortality of *Ae. aegypti* larvae**
PYGE	18 ± 0.6^{a}	31 ± 1.0^{a}	40 ± 1.6^{a}	583 ± 193	53^{b}
YPss	18 ± 0.2^{a}	30 ± 0.5^{a}	41 ± 0.9^{a}	1250 ± 329	67^{b}
SSE	10 ± 0.5^{b}	17 ± 0.7^{b}	26 ± 0.7^{b}	2083 ± 668	86^{a}

B.

Fresh mass (g)	(%) Mortality of *Ae. aegypti* larvae ◊ 24 h^{ł}	48 h^{ł}	72 h^{ł}	96 h^{ł}
48.5	94 ± 4.0^{a}	99 ± 1.0^{a}	100 ^{a}	100 ^{a}
93.5	0^{b}	31 ± 16^{b}	10 ± 6^{c}	5 ± 3^{c}
62.4	0^{b}	25 ± 13^{b}	57 ± 16^{b}	93 ± 7^{b}

ł Time after zoosporogenesis induction by immersion of *L. chapmanii* in water.

Note: Letters grouped the treatments without statistical differences.

* $F_{2,33}$(48h) = 98, $F_{2,33}$(72h)= 97 and $F_{2,33}$(96h)= 57. In all cases P <0.001

** $F_{2,51}$=6.6, P< 0.05

◊ $F_{2,24}$(24h) = 331, $F_{2,24}$(48h)= 17, $F_{2,24}$(72H)= 59 and $F_{2,24}$(96h)= 192. In all cases P <0.001

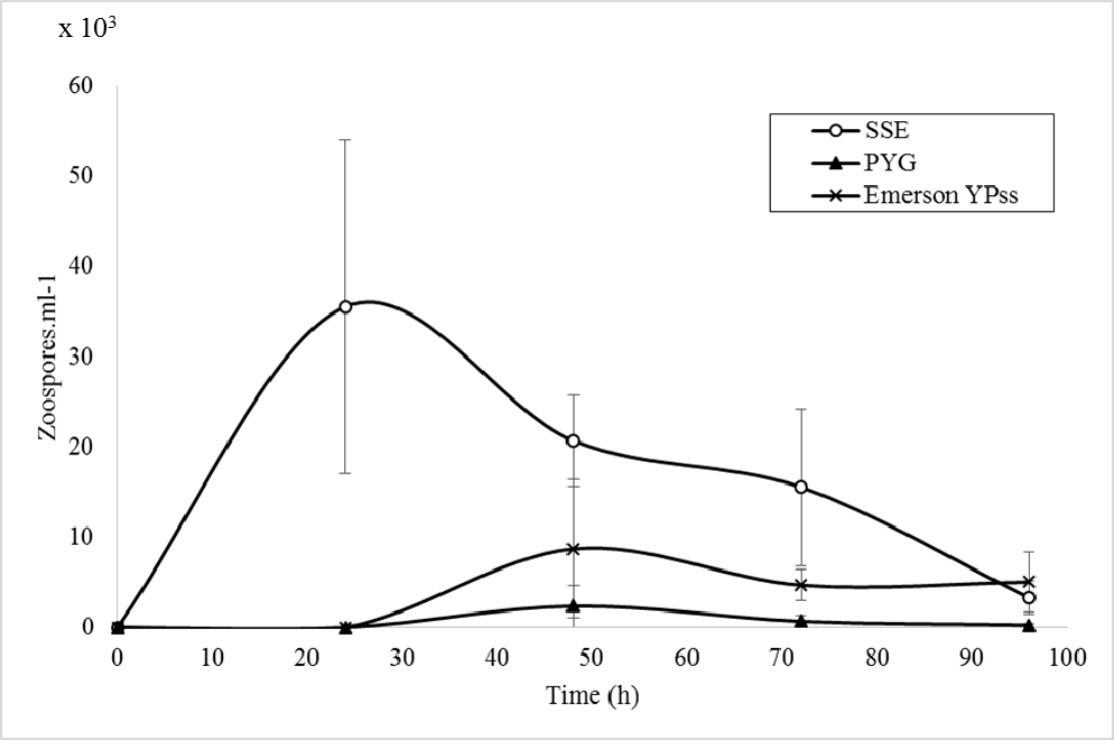

Figure 1. Assays with biomass samples from liquid culture media. Production of zoospores by *Leptolegnia chapmanii* along time.