Volume 60

2014

An NRC Research Press Journal

Une revue de NRC Research Press

www.nrcresearchpress.com

Canadian Journal of Microbiology

Revue canadienne de microbiologie

NRC Research Press

NRC Research Press

ARTICLE

A simple method for the detection of *Leptolegnia chapmanii* from infected *Aedes aegypti* larvae

Renan N. Leles, Cláudia C. López Lastra, Juan J. García, Éverton K.K. Fernandes, and Christian Luz

Abstract: Significant progress in developing *Leptolegnia chapmanii* as a biological control agent against mosquitoes will be accelerated by improved and simpler methods to detect and to isolate this virulent and rapidly lethal watermold from field-collected mosquito larvae. To date, however, this oomycete has remained understudied and little used. This study presents a simplified method to detect *Leptolegnia* in infected *Aedes aegypti* larvae. The development of *L. chapmanii* inside mosquitoes is easily monitored when pathogen-treated larvae are quasi-immobilized for an initial 48 h in the water film on plates of water agar amended with antibiotic (chloramphenicol, 0.5–1 g/L) and fungicide (thiabendazole, 4–8 g/L) and then transferred to a larger volume of water for an additional 48 h. Surprisingly, chloramphenicol stimulated oosporogenesis by *L. chapmanii*. The method permits processing of large numbers of *A. aegypti* and other culicid larvae and is useful for both obtaining new strains and also monitoring the efficacy of *L. chapmanii* during field tests.

Key words: watermold, Straminipila, Chromista, Peronosporomycetes, Saprolegniales, mosquitoes.

Résumé : Des méthodes de détection et d'isolation de *Leptolegnia chapmanii* améliorées et simplifiées, à partir de larves de moustiques prélevées sur le terrain, pourraient fortement accélérer l'élaboration d'agents de lutte biologique s'attaquant aux moustiques et s'appuyant sur ce oomycète virulent et rapidement mortel. Or, à ce jour, cet oomycète demeure mal étudié et sous utilisé. La présente étude illustre une méthode simplifiée permettant la détection de *Leptolegnia* dans des larves de *Aedes aegypti*. Le développement de *L. chapmanii* à l'intérieur des moustiques peut être facilement observé en continu lorsque les larves traitées avec le pathogène sont presque immobilisées pendant une durée initiale de 48 h dans la pellicule d'eau de géloses d'agar aqueux auxquelles on a ajouté un antibiotique (chloramphénicol à 0,5–1 g/L) et un fongicide (thiabendazole à 4–8 g/L), pour ensuite être transférées dans un plus grand volume d'eau pour un autre 48 h. Fait surprenant, le chloramphénicol stimule l'oosporogénèse chez *L. chapmanii*. La méthode permet d'examiner un grand nombre de *A. aegypti* et d'autre larves de culicidés et facilite l'identification de nouvelles souches et la surveillance de l'efficacité de *L. chapmanii* lors d'essais sur le terrain. [Traduit par la Rédaction]

Mots-clés : oomycète, Straminipila, Chromista, peronosporomycète, Saprolegniales, moustiques.

Introduction

The increasing interest in entomopathogenic fungi and the development of mycoinsecticides for biological control of important mosquito vectors stimulate the search for powerful entomopathogens. *Leptolegnia chapmanii* (Peronosporomycetes: Saprolegniales) is an oomycete watermold that specifically infects and quickly kills culicid mosquito larvae. Despite their traditional treatment as fungi, oomycetes such as *Leptolegnia* are now excluded from the true fungi because they have biflagellate zoospores on which one of the flagella is of the tinsel-type; all organisms forming this type of flagellum are now classified in the highly diverse kingdom Straminipila (Chromista) (Dick 2001).

As with most other entomopathogenic fungi, *L. chapmanii* usually infects by its zoospores encysting on and then penetrating through the host cuticle (Zattau and McInnis 1987; Pelizza et al. 2008). Additionally, however, ingestion of encysted zoospores of *L. chapmanii* can result also in larval infections through the gut (Zattau and McInnis 1987; Pelizza et al. 2008). By either route of infection, encysted zoospores produce germ tubes that penetrate to the coelomic cavity to establish fatal infections of larvae (Zattau and McInnis 1987). Larval death is followed by hyphal growth through the cuticle into the surrounding water with the subsequent formation of terminal zoosporangia. New anteriorly biflagellate primary zoospores are released into the water, encyst on some substrate, and then germinate to release bean-shaped, laterally biflagellate secondary zoospores that can infect other susceptible larvae (Zattau and McInnis 1987). Any sexually produced oospores of *L. chapmanii* resist environmental conditions for vegetative growth, but mature oospores are rarely observed for this pathogen because oosporogenesis most often terminates (for unknown reasons) without completing the formation of fertilized oospores (Zattau and McInnis 1987; Pelizza et al. 2010).

Leptolegnia chapmanii can be cultured easily in liquid or on solid media, and the zoospores released from *Aedes aegypti* larvae and maintained in water at 25 °C remained infective to new larvae for more than 50 days (Pelizza et al. 2008, 2011). After contact with zoospores from dead larvae, healthy larvae were infected by exposures as short as only 2 min, and began to die within 24 h (Scholte et al. 2004; Pelizza et al. 2008). Infection of new, healthy larvae depended on the number of zoospores produced on dead larvae and was inversely proportional to the area of the water surface (Pelizza et al. 2007*a*). Younger *A. aegypti* and *Culex pipiens* instars were more susceptible to *L. chapmanii* than older instars, and the optimal water temperature for infection by *L. chapmanii*

Received 27 November 2012. Revision received 25 April 2013. Accepted 28 April 2013.

R.N. Leles. Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, C.P. 131, 74001-970 Goiânia, GO, Brazil; Centro de Estudios Parasitológicos y de Vectores, Universidade Nacional de la Plata, La Plata, Buenos Aires, Argentina.
C.C. López Lastra and J.J. García. Centro de Estudios Parasitológicos y de Vectores, Universidade Nacional de la Plata, La Plata, Buenos Aires, Argentina.
É.K.K. Fernandes and C. Luz. Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, C.P. 131, 74001-970 Goiânia, GO, Brazil.
Corresponding author: Christian Luz (e-mail: wchrisluz@hotmail.com).

was around 25 °C with a permissive temperature range of 10 to 35 °C for both mosquito species (Pelizza et al. 2007*b*). pH values of 4–10 and salinity of the water did not interfere with infectivity of *L. chapmanii* in *A. aegypti* larvae, but the susceptibility of *C. pipiens* to infection was decreased at pH values lower or higher than 7 and was inversely proportional to the level of salinity (Pelizza et al. 2007*b*).

Mosquito-pathogenic species of *Leptolegnia* other than *L. chapmanii* have received only limited attention (Scholte et al. 2004), and little is known about their natural occurrences. *Leptolegnia chapmanii* was first recorded in the USA in 1971 on a larva of *Ochlerotatus triseriatus*. To date, there are only few reports about natural isolation of *L. chapmanii*, *Aphanomyces* sp., or *Lagenidium giganteum* (Peronosporomycetes: Lagenidiales) from mosquitoes in the USA, and only a single record of *L. chapmanii* in Argentina (Seymour 1984; Fukuda et al. 1997; López Lastra et al. 1999; Scholte et al. 2004). The exceptionally high virulence of *L. chapmanii* (based on the rapid onset of mortality of exposed larvae) makes this pathogen an unusually attractive candidate for biological control of important culicid vectors. There has been no selective medium to enhance the likelihood of detecting *Leptolegnia* species from infected larvae. During infection and after host death, entomopathogens have to overcome saprobic microorganisms, such as fungi and bacteria, that might slow down or inhibit sporulation or that overgrow the desirable pathogen and, thereby, hamper the detection and later isolation of this pathogen (Tsao 1970; Roy and Pell 2000; Wraight et al. 2007). Growth of entomopathogens can be restrained by fungivorous arthropods, especially mites (Yoder et al. 2009; R.N. Leles and C. Luz, unpublished data). Other inhospitable conditions for fungal recycling, such as absence of liquid water, unfavorable humidities or temperatures, and excessive sunlight, affect post-mortem development and impede the isolation of pathogens from the host (Luz and Fargues 1998; Arthurs and Thomas 2001).

A culture medium amended with antibiotics and fungicides that specifically favors the development of a wanted entomopathogen while inhibiting undesired contaminants provides an important tool for a more selective detection. Various semiselective culture media are well known for isolations of specific entomopathogenic fungi or groups. Many of these media, however, are amended with dodine, a fungicide that is now difficult to obtain and that presents acute toxicity issues (Chase et al. 1986; Liu et al. 1993; Keller et al. 2003; Hughes et al. 2004; Luz et al. 2004; Kegley et al. 2010). Rocha and Luz (2009) tested the inhibition of saprobic fungi by several fungicides and suggested the broad usefulness of thiabendazole at 4 mg/L for semiselective culture media. Other dodine-free semiselective media to isolate *Culicinomyces clavisporus* (Panter and Frances 2003), *Beauveria* spp., and *Metarhizium* spp. (Luz et al. 2007; Fernandes et al. 2010) are known, but no specific medium favoring growth by *L. chapmanii* has been described. This pathogen attacks only aquatic stages and, unless it is causing epizootic events, is more difficult to detect than most common terrestrial entomopathogenic fungi.

Current studies in our laboratory focus on techniques to detect entomopathogenic fungi directly on field-collected culicid larvae, using captured larvae as a specific substrate (R.N. Leles and C. Luz, unpublished data). The objective of the present study was to provide a simple method for a more effective detection of and, consequently, isolation of *L. chapmanii* from infected mosquito larvae.

Materials and methods

Preparation of water agar media

A very soft water agar (WA; 10 g agar/L) medium was prepared as the basal and control medium to be amended with chloramphenicol (Oficinal, Brazil) at 50, 100, 300, 500, or 1000 mg/L (WA+ca); thiabendazole (Nortec Química, Brazil) at 8 mg/L (WA+tb); crystal violet (hexamethyl pararosaniline chloride, Cromato Produtos Químicos, Brazil) at 10 mg/L (WA+cv); deltamethrin (k-othrine CE 25, Bayer CropScience, Brazil) at 50 mg/L (WA+dm); or a combination of thiabendazole (8 mg/L) and chloramphenicol (500 mg/L) (WA+tb+ca). For WA without additives or added with crystal violet or deltamethrin, distilled water was heated to 50 °C at 500 r/min on a magnetic stirrer before adding agar and either additive; the medium was then agitated until any additives were completely dissolved. Thiabendazole and chloramphenicol were dissolved separately in 1 mL aliquots of absolute ethanol before being added to the hot water agar prepared as noted above. The pH of all media was adjusted to 7 before being autoclaved and dispensed into sterile Petri dishes (35 mm × 15 mm).

Origin and culture of the watermold and production of infected larvae

Leptolegnia chapmanii ARSEF 5499 was originally isolated in 1996 from a fourth instar *Aedes albifasciatus* larva (L4) in the city of La Plata, Argentina (López Lastra et al. 1999). The strain was cultivated on Emerson's YPSS agar, and its virulence maintained by successive passage through laboratory-reared L4 *A. aegypti*. This strain, when inoculated in L4 larva, produces about 10^5 zoospores per infected larva, 48 h after inoculation and exposure at 25 °C (Pelizza et al. 2008). Fifteen dead L4 larva were transferred to a plastic cup (260 cm^3) with 100 mL of sterile distilled water. Then 50 healthy *A. aegypti* L4 larva, obtained from a colony maintained at Centro de Estudios Parasitológicos y de Vectores (CEPAVE, La Plata, Argentina), were added to the cup and exposed to infection by zoospores (1.5×10^4 zoospores/mL) for 6 h.

Evaluation of the effect of additives on the development of *L. chapmanii*

Four living *A. aegypti* L4 larva infected with *L. chapmanii* as mentioned were carefully set on WA and incubated at 25 °C, 12 h photophase, and saturated humidity for 48 h. The development of *L. chapmanii* on larvae was examined with a phase contrast microscope. Larvae were then transferred individually with 2 mL of distilled water into wells on a sterile plastic multiwell cell culture plate (Cellstar, Greiner Bio-One) and maintained for 48 h at ambient room temperature and humidity to allow the development of asexual and sexual structures. To verify the viability of zoospores, larvae were then removed from the well, and a healthy L4 exposed to this water. Infection and mortality of these larvae with *L. chapmanii* were assessed microscopically at 48 h incubation. Control groups were assessed with healthy L4 maintained on WA media with or without additives and the same posterior processing as described.

Analysis of results

Four independent repetitions with 4 L4 were made for all tests, and for each test a new culture of *L. chapmanii* was used. Percent mortalities were arcsine-square root transformed and then analyzed using analysis of variance (ANOVA) and the Student–Newman–Keuls multiple range test for comparison of means. Means were considered to be statistically different at $P < 0.05$.

Results

Living larvae exposed to *L. chapmanii* zoospores and then removed from zoospore suspensions to WA typically started dying on the WA surface within 24 h regardless of any additive tested except that WA+dm induced total mortality within a 3–5 min after exposure to the medium, whether or not they were previously exposed to infective zoospores. For the other additives, mortalities after a 48 h exposure of larvae on WA media varied from 100% (WA+ca100, WA+ca300, and WA+tb+ca) to 58.3% (WA+cv) (Table 1). High numbers of uninfected control larvae survived a 48 h exposure on WA media without additives (100%) or WA+cv or WA+tb (100%), WA+tb+ca (93.8% ± 6.2%), or WA+ca1000 (87.5% ± 12.5%). With the exception of deltamethrin, other additives had

Table 1. Infectivity of *Leptolegnia chapmanii* zoospores against *Aedes aegypti* larvae (L4) and subsequent development of reproductive structures for extended periods after 2 days of initial incubation on amended water agar.

		Phase 1: Post-treatment incubation for additional 48 h*			Phase 2: Extended treatment in same water after replacing exposed L4 with healthy L4†		
Additive	mg/L	Mortality (%)	Zoosporangia (%)	Zoosporangia and oogonia (%)	Mortality (%)	Zoosporangia (%)	Zoosporangia and oogonia (%)
Chloramphenicol	1000	87.5±10.8	87.5±10.8	87.5±10.8	81.2±10.4	81.2±10.4	25.0±0.0
	500	87.5±6.2	87.5±6.2	50.0±22.8	87.5±6.3	87.5±6.3	12.5±7.2
	300	100.0	100.0	33.3±27.2	100.0	100.0	16.6±13.6
	100	100.0	91.6±6.8	0.0	91.7±6.8	91.7±6.8	16.6±13.6
	50	91.6±6.7	88.3±9.5	0.0	66.7±13.6	66.7±13.6	8.3±6.8
Thiabendazole	8	75.0±8.9	75.0±8.9	6.2±2.0	87.5±12.5	87.5±12.5	16.6±13.6
Thiabendazole + chloramphenicol	8+500‡	100.0	100.0	50.0±19.8	93.7±5.5	93.7±5.5	0.0
Crystal violet	10	58.3±18.0	0.0	0.0	16.6±6.9	0.0	0.0
Deltamethrin	50	100.0	0.0	0.0	100.0	0.0	0.0
None	—	100.0	100.0	0.0	93.7±5.4	93.7±5.4	0.0

*Four L4 per treatment (with 4 repetitions) were exposed to 1.5×10^3 zoospores/mL for 6 h, placed on amended water agar for 2 days at 25 °C, then moved to individual wells with 2 mL of sterile, uninoculated water at 25 °C, 12 h photophase, and saturated humidity for 48 h.

†Infection and development after replacing initially exposed larvae in these wells with new, healthy L4 larvae and incubated under the same conditions for another 48 h.

‡Eight mg/L thiabendazole plus 500 mg/L chloramphenicol.

Fig. 1. *Leptolegnia chapmanii* on *Aedes aegypti* fourth instar larva on water agar medium. (A) Zoosporangia filled with zoospores (arrow 1) or empty (arrow 2), and free zoospores (12 μm diameter, arrow 3). (B) Zoosporangia releasing zoospores (arrow 4). Bars = 24 μm.

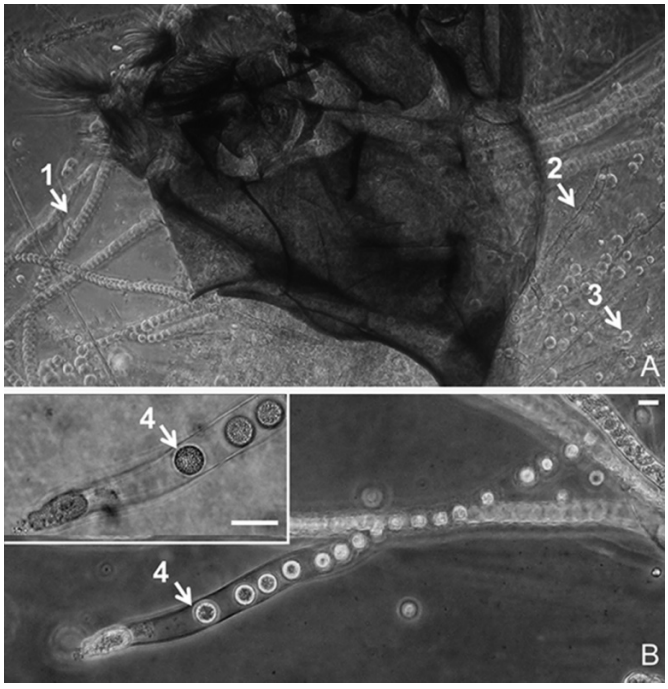
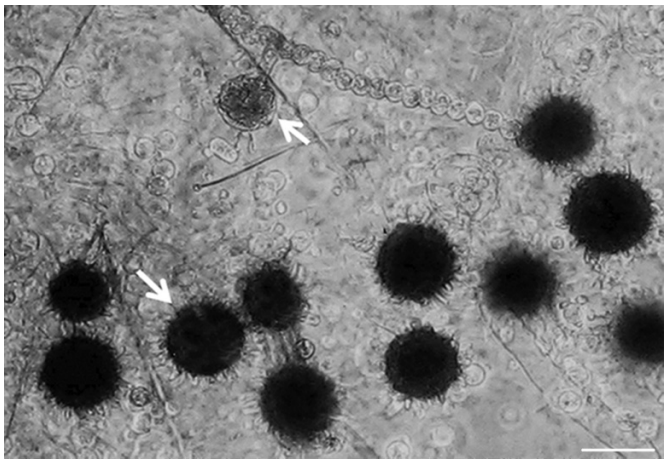


Fig. 2. *Leptolegnia chapmanii* on *Aedes aegypti* fourth instar larva on water agar medium amended with chloramphenicol (1 g/L). Oogonia (40 μm diameter, arrow). Bar = 40 μm.



no effect on survival of uninfected larvae on WA ($F_{[7,16]} = 0.89$; $P = 0.54$).

A few hours after larval death, mycelium and zoosporangia were observed emerging from larvae that were previously treated with *L. chapmanii* and still exposed on unamended WA or, after a somewhat longer time, on larvae placed on WA+tb. No outgrowth or sporulation from infected larvae was observed on WA including any other additives, although mycelium, zoosporangia, and eventually, oogonia were formed after dead larvae were transferred to liquid water in a well plate. Zoosporangia were either filled with zoospores or already empty after the release of zoospores (Fig. 1A). Figure 1B and video (see supplementary material)[1] show a zoosporangium releasing zoospores. Zoosporangia were produced in larvae exposed to WA without additives, but no oogonia were formed. Zoosporangia emerged on dead larvae exposed to WA+ca regardless of the concentrations tested (0.05–1 g/L), and the percentage of larvae with oogonia after 48 h exposure increased proportionally with the concentration of chloramphenicol. Notably, the pathogen in every infected larva placed on WA+ca1000 formed oogonia (Fig. 2). No oogonia were formed in larvae on WA+ca ≤100 mg/L, and in only 6.2% of larvae on WA+tb. On WA+tb8+ca500, all larvae were found with zoosporangia but only 50% had zoosporangia and oogonia (Table 1). No mycelium, zoosporangia, or oogonia were formed in larvae on WA+cv or WA+dm (Table 1). All oogonia found with chloramphenicol-amended media were abortive in their development.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2012-0703.

When healthy new L4 were added to zoospore suspensions in wells after the removal of previously tested larvae, mortality rates of ≥66.7% were obtained in wells that had contained larvae exposed on WA+ca (chloramphenicol: 50–1000 mg/L), WA+tb, WA+ca+tb, and without additives. Only 16.6% mortality was obtained from larvae added to zoospore suspensions from larvae initially exposed on WA+cv at the same moment. Whereas zoosporangia and zoospores developed on all dead larvae within a 48 h period after exposure of healthy larvae, oogonia were apparent on only 0%–25% of these secondarily exposed dead larvae (Table 1). All control larvae survived at least for 48 h regardless of the additive or combination tested except on WA+dm, on which 100% mortality was observed within minutes.

Discussion

Several culture media are excellent for sustaining the vegetative growth of *L. chapmanii* (Pelizza et al. 2011), but the studies reported here focus on facilitating the detection of this entomopathogen. This is the first description of a simple method to detect *L. chapmanii* from living, infected culicid larvae. The technique provides favorable conditions for this pathogen to develop on a supposedly infected living larva that is more or less effectively immobilized on a water agar without nutrients but amended with additives that eventually inhibit growth by numerous unwanted bacterial and fungal contaminants. The method uses the host insect as a selective substrate and permits individual processing of large numbers of larvae even under field conditions.

These studies made clear that most control larvae (that were not previously infected with *L. chapmanii*) survived for at least 48 h in the aqueous film on 1% WA. Chloramphenicol (50–1000 mg/L), thiabendazole (8 mg/L), combined chloramphenicol (0.5 g/L) and thiabendazole (8 mg/L), and also crystal violet (10 mg/L) had no toxic effect on *A. aegypti* larvae (L4) at the concentrations tested. Because deltamethrin (50 mg/L WA) was highly toxic to larvae, this and other insecticides that quickly kill larvae must be avoided for detecting or isolating pathogens from these larvae. The survival of larvae on WA permitted the development of *L. chapmanii* in the larvae without their needing to be swimming freely in water. The reduced mobility of larvae in the superficial water film on WA increases the likelihood of detecting this pathogen in larvae, even at low infection rates.

Most *Leptolegnia*-exposed L4 died from the infection within 48 h of incubation in the water film on WA and produced zoosporangia and zoospores when transferred to free water. Interestingly, the formation of oogonia (albeit without subsequent production of oosporangia or oospores) was stimulated and accelerated by increasing concentrations of chloramphenicol. Chloramphenicol is a broad-spectrum thermoresistant bactericide that is commonly used in semiselective media for entomopathogenic fungi (Veen and Ferron 1966; Tsao 1970; Fernandes et al. 2010; Rangel et al. 2010). While the factors triggering sexual reproduction by *L. chapmanii* are not yet clearly understood (Zattau and McInnis 1987; Pelizza et al. 2010), Pelizza et al. (2010) reported the postmortem initiation of oogonial development at 25 °C after only 5 days of incubation of infected *A. aegypti* larvae. In any case, the formation of sexual structures is not necessary for successful identification or isolation of this pathogen from culicid larvae or for its effective use as a biological control agent.

Although crystal violet is often used in selective media for both its bactericidal activity and intense coloring that helps the visualization of growing pathogens (Chase et al. 1986), this amendment completely inhibited the development of *L. chapmanii* and can not be used for the detection of this pathogen.

One percent WA seems to be most useful to facilitate field collections and for immobilization of large numbers of mosquito larvae without killing them while favoring the development of *L. chapmanii*. After 24–48 h of incubation on WA, any larvae remaining alive in the water film are probably not infected with *L. chapmanii* and can be excluded from further processing. Dead individuals, on the other hand, can be easily transferred from the agar to liquid water to permit formation of zoosporangia and, possibly, to initiate oosporogenesis. Mycelium and reproductive structures are then either directly transferred to an appropriate culture medium, such as Emerson's YPSS agar medium (Pelizza et al. 2011), for further examination or used as inoculum against healthy laboratory-reared larvae for in vivo propagation of this pathogen.

Based on the findings of this study, WA amended with both chloramphenicol (up to 1 g/L) and (or) thiabendazole (4–8 mg/L), depending on the types and titers of bacterial and fungal contaminants in the material being collected, seems to fit best for the recovery from natural sites of new strains of *Leptolegnia* attacking aquatic stages of culicid mosquitoes and also to monitor the effectiveness of field trials with *L. chapmanii*.

Improving the chance to detect such little-studied oomycetes as *L. chapmanii* and *Lagenidium giganteum* that have a high potential for the control of vectors such as mosquitoes and blackflies may depend on using such an initial survey technique as that discussed here. Numerous individual, field-collected larvae can be surveyed rapidly if they are semi-immobilized at first on a contaminant-suppressive medium, thus making the identification of single infected larvae dramatically faster and increasing the chance of isolating any pathogen. To wait for infected larvae to die and to sporulate is slower and tends to interfere with the isolation and identification of pathogens owing to the increasingly problematic presence of bacterial and fungal contaminants.

It is reasonable to assume that the initial semi-immobilization of living larvae on contaminant-suppressive agar surfaces should be a widely applicable strategy to enhance the detection and isolation of the full range of oomycete and other flagellate fungal pathogens from the aquatic stages of a diverse range of vector dipterans. The low cost and technical simplicity of such an approach should advance the studies of these pathogens in all regions of the world where there is a pressing need for more environmentally friendly means than chemical pesticide applications to control these insect vectors and the diseases they transmit.

Acknowledgements

We thank Evangelina Muttis and Manuel Enrique Rueda Páramo for technical assistance and Richard A. Humber (United States Department of Agriculture, Agricultural Research Service, Ithaca, New York) for the critical review of the manuscript. This research was funded by the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil, PPCP 016/2011), the National Council of Scientific and Technological Development (CNPq, Brazil, 473141/2010-8), and the State of Goiás Research Support Foundation (FAPEG grant 201010267000250).

References

Arthurs, S., and Thomas, M.B. 2001. Effects of temperature and relative humidity on sporulation of *Metarhizium anisopliae* var. *acridum* in mycosed cadavers on *Schistocerca gregaria*. J. Invertebr. Pathol. **78**(2): 59–65. doi:10.1006/jipa.2001.5050. PMID:11812107.

Chase, A.R., Osborne, L.S., and Ferguson, V.M. 1986. Selective isolation of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium. Fla. Entomol. **69**(2): 285–292. doi:10.2307/3494930.

Dick, M.W. 2001. Straminipilous fungi: systematics of the Peronosporomycetes: including accounts of the marine straminipilous protists, the plasmodiophorids and similar organisms. 1st ed. Springer–Verlag, New York.

Fernandes, É.K.K., Keyser, C.A., Rangel, D.E.N., Foster, R.N., and Roberts, D.W. 2010. CTC medium: a novel dodine-free selective medium for isolating entomopathogenic fungi, especially *Metarhizium acridum*, from soil. Biol. Control, **54**(3): 197–205. doi:10.1016/j.biocontrol.2010.05.009.

Fukuda, T., Willis, O.R., and Bernard, D.R. 1997. Parasites of the Asian tiger mosquito and other container inhabiting mosquitoes (Diptera: Culicidae) in northcentral Florida. J. Med. Entomol. **84**(2): 226-233. PMID:9103767.

Hughes, W.O.H., Thomsen, L., Eilenberg, J., and Boomsma, J.J. 2004. Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*. J. Invertebr. Pathol. **85**(1): 46–53. doi:10.1016/j.jip.2003.12.005. PMID:149922860.

Kegley, S.E., Hill, B.R., Orme, S., and Choi, A.H. 2010. PAN pesticides database [online]. Available from http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC33273 [accessed 25 November 2012].

Keller, S., Kessler, P., and Schweizer, C. 2003. Distribution of insect pathogenic soil fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metarhizium anisopliae*. Biocontrol, **48**(3): 307–319. doi:10.1023/A:1023646207455.

Liu, Z.Y., Milner, R.J., McRae, C.F., and Lutton, G.G. 1993. The use of dodine in selective media for the isolation of *Metarhizium* spp. from soil. J. Invertebr. Pathol. **62**(3): 248–251. doi:10.1006/jipa.1993.1107.

López Lastra, C.C., Steciow, M.M., and García, J.J. 1999. Registro más austral del hongo *Leptolegnia chapmanii* (Oomycetes: Saprolegniales) como patógeno de larvas de mosquitos (Diptera: Culicidae). Rev. Iberoam. Micol. **16**: 143–145. PMID:18473561.

Luz, C., and Fargues, J. 1998. Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. J. Invertebr. Pathol. **72**(2): 97–103. doi:10.1006/jipa.1998.4774. PMID:9709008.

Luz, C., Rocha, L.F.N., Nery, G.V., Magalhães, B.P., and Tigano, M.S. 2004. Activity of oil-formulated *Beauveria bassiana* against *Triatoma sordida* in peridomestic areas in Central Brazil. Mem. Inst. Oswaldo Cruz, **99**(2): 211–218. doi:10.1590/S0074-02762004000200017. PMID:15250478.

Luz, C., Netto, M.C.B., and Rocha, L.F.N. 2007. In vitro susceptibility to fungicides by invertebrate-pathogenic and saprobic fungi. Mycopathologia, **164**(1): 39–47. doi:10.1007/s11046-007-9020-0. PMID:17574540.

Panter, C., and Frances, S.P. 2003. A more selective medium for *Culicinomyces clavisporus*. J. Invertebr. Pathol. **82**(3): 198–200. doi:10.1016/S0022-2011(03)00017-X. PMID:12676556.

Pelizza, S.A., López Lastra, C.C., Becnel, J.J., Bisaro, V., and García, J.J. 2007*a*. Biotic and abiotic factors affecting *Leptolegnia chapmanii* infection in *Aedes aegypti*. J. Am. Mosq. Control Assoc. **23**(2): 177–181. doi:10.2987/8756-971X(2007)23[177:BAAFAL]2.0.CO;2. PMID:17847851.

Pelizza, S.A., López Lastra, C.C., Becnel, J.J., Bisaro, V., and García, J.J. 2007*b*. Effects of temperature, pH and salinity on the infection of *Leptolegnia chapmanii* Seymour (Peronosporomycetes) in mosquito larvae. J. Invertebr. Pathol. **96**(2): 133–137. doi:10.1016/j.jip.2007.04.005. PMID:17521667.

Pelizza, S.A., López Lastra, C.C., Becnel, J.J., Humber, R.A., and García, J.J. 2008. Further research on the production, longevity and infectivity of the zoopores of *Leptolegnia chapmanii* Seymour (Oomycota: Peronosporomycetes). J. Invertebr. Pathol. **98**(3): 314–319. doi:10.1016/j.jip.2008.04.006. PMID:18511067.

Pelizza, S.A., Scorsetti, A.C., López Lastra, C.C., and García, J.J. 2010. Production of oogonia and oospores of *Leptolegnia chapmanii* Seymour (Straminipila: Peronosporomycetes) in *Aedes aegypti* (L.) larvae at different temperatures. Mycopathologia, **169**(1): 71–74. doi:10.1007/s11046-009-9224-6. PMID:19603285.

Pelizza, S.A., Cabello, M.N., Tranchida, M.C., Scorsetti, A.C., and Bisaro, V. 2011. Screening for a culture medium yielding optimal colony growth, zoospore yield and infectivity of different isolates of *Leptolegnia chapmanii*. Ann. Microbiol. **61**(4): 991–997. doi:10.1007/s13213-011-0232-7.

Rangel, D.E.N., Dettenmaier, S.J., Fernandes, É.K.K., and Roberts, D.W. 2010. Susceptibility of *Metarhizium* spp. and other entomopathogenic fungi to dodine-based selective media. Biocontrol Sci. Techn. **20**(4): 375–389. doi:10.1080/09583150903518370.

Rocha, L.F.N., and Luz, C. 2009. Utility of six fungicides for selective isolation of *Evlachovaea* sp. and *Tolypocladium cylindrosporum*. Mycopathologia, **167**(60): 341–350. doi:10.1007/s11046-009-9186-8. PMID:19205922.

Roy, H.E., and Pell, J.K. 2000. Interactions between entomopathogenic fungi and other natural enemies: implications for biological control. Biocontrol Sci. Techn. **10**(6): 737–752. doi:10.1080/09583150020011708.

Scholte, E., Knols, B.G.J., Samson, R.B., and Takken, W. 2004. Entomopathogenic fungi for mosquito control. A review. J. Insect Sci. **4**: 19–43. PMID:15861235.

Seymour, R.L. 1984. *Leptolegnia chapmanii*, an oomycete pathogen of mosquito larvae. Mycologia, **76**(4): 670–674. doi:10.2307/3793224.

Tsao, P.H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. **8**: 157–186. doi:10.1146/annurev.py.08.090170.001105.

Veen, K.H., and Ferron, P. 1966. A selective medium for the isolation of *Beauveria tenella* and of *Metarhizium anisopliae*. J. Invertebr. Pathol. **8**: 268–269. doi:10.1016/0022-2011(66)90145-5. PMID:5949419.

Wraight, S.P., Inglis G.D., and Goettel, M.S. 2007. Fungi. *In* Field manual of techniques in invertebrate pathology. *Edited by* L.A. Lacey and H.K. Kaya. Springer, Netherlands. pp. 223–249.

Yoder, J.A., Chambers, M.J., Condon, M.R., Benoit, J.B., and Zettler, L.W. 2009. Regulation of the external mycoflora of the giant Madagascar hissing-cockroach, *Gromphadorhina portentosa*, by its mite associate, *Gromphadorholaelaps schaeferi*, and its implications on human health. Symbiosis, **47**(2): 93–98. doi:10.1007/BF03182292.

Zattau, W.C., and McInnis, T., Jr. 1987. Life cycle of infection of *Leptolegnia chapmanii* (Oomycetes) parasiting *Aedes aegypti*. J. Invertebr. Pathol. **50**(2): 134–145. doi:10.1016/0022-2011(87)90113-3. PMID:3624894.