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Evaluation of Histological Techniques for the Detection of Fungal Infections Caused by *Leptolegnia chapmanii* (*Oomycetes*: *Saprolegniales*) in *Aedes aegypti* (*Diptera*: *Culicidae*) Larvae

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ABSTRACT. We evaluated which of the fixatives and stains most frequently used for observation of insect tissues were the most appropriate for histopathological visualization of entomopathogenic fungal infections with *Leptolegnia chapmanii* in larvae of *Aedes aegypti*. The best contrast between the host tissues and the fungal structures was obtained when using a combination of Carnoy fixative with Grocott staining contrasted with light green. Masson trichromic stain combined with 10 % formaldehyde–phosphate buffer also provided satisfactory results – a good contrast and clearly distinguishable host tissues and fungal structures.

References about histological techniques for insect tissues are scarce; they focus particularly on the evaluation of the most accurate methods to observe infections caused by entomopathogenic fungi (McCauley *et al*. 1968; López Lastra 1988; Allen *et al*. 1990). Some data were reported on the way of action of entomopathogenic fungi (Vey and Fargues 1977; Nnakumusana 1986; Lord and Fukuda 1988; Weiser *et al*. 2003).

The goal of our study was to evaluate the best fixatives and stains to be used in histopathological sections of mosquitoes infected with the fungus *Leptolegnia chapmanii*, comparing host larval tissues treated with the fungus with control (nontreated) insect tissues.

MATERIAL AND METHODS

We used the fungus *L. chapmanii* SEYMOUR (*Oomycetes*: *Saprolegniales*) isolated from *Aedes albifasciatus* (MACQUART) (*Diptera*: *Culicidae*) larvae (López Lastra *et al*. 1999). The fungal isolate was deposited at the CEPAVE (*Centro de Estudios Parasitológicos y de Vectores*, La Plata, Argentina) fungal culture collection, and in the *Entomopathogenic Fungal Culture Collection at the US Department of Agriculture* (USDA)–*Agricultural Research Service* (ARS) (ARSEF; Ithaca, USA) registered under the access no. CEP 010 and ARSEF 5499, respectively. The fungal culture was maintained in routine Emerson YPSS agar medium (*Difco*) at 22 °C for 7 d. Inoculum was prepared by culturing mycelial cubes in agar medium (5 mm each). In order to induce zoosporulation, mycelial squares containing hyphae were placed on 90 × 15 mm Petri dishes with 20 mL sterile distilled water for 3 d in darkness at 22 °C. The presence of zoospores was confirmed by observation in an optical microscope; they were quantified in a hemocytometer (Neubauer chamber), revealing a concentration of 10^5 zoospores per mL. One mL of this zoospore suspension was added to 99 mL distilled water in a 250-mL plastic container. After 1, 2 and 3 d, three treated larvae and three controls were taken out and fixed in either Carnoy, Bouin or 10 % formaldehyde–phosphate buffer for 1 d. Afterwards, they were embedded in Paraplast® and sliced into 6 μm sections by a manual microtome. These sections were stained using 4 different colorations: hematoxylin–eosin, Masson trichromic (with light green) stain, periodic acid–Schiff reaction (PAS)–hematoxylin and Grocott methenamine silver stain (with light green) (Ferron *et al*. 1966); 3 replicates of each stain were performed. All microscopic slides with histological sections were deposited at the CEPAVE collection, under access numbers 1876a, 1876b, 1876c, 1878a, 1879a, 1879b, 1879c and 1885a. Slide preparations were observed in the CH 30 optical *Olympus* microscope; images were

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recorded with the SC 35 (Type 12) *Olympus* camera fitted to the microscope, using a 35 mm *Ilford* black-and-white film (ISO 400).

RESULTS AND DISCUSSION

Hematoxylin–eosin stain combined with fixation in 10 % formaldehyde buffer allowed the distinction of insect host tissues (Fig. 1A) from fungal structures (hyphae and/or zoospores) (Fig. 1B–D); it was visible up to 3 d after infection, although by this time the host tissues in infected insects were mostly lyzed and completely invaded by fungal hyphae. Therefore, the optimum observation time was considered to be 1 d. However, this stain was not optimal for obtaining the desired differential contrast.

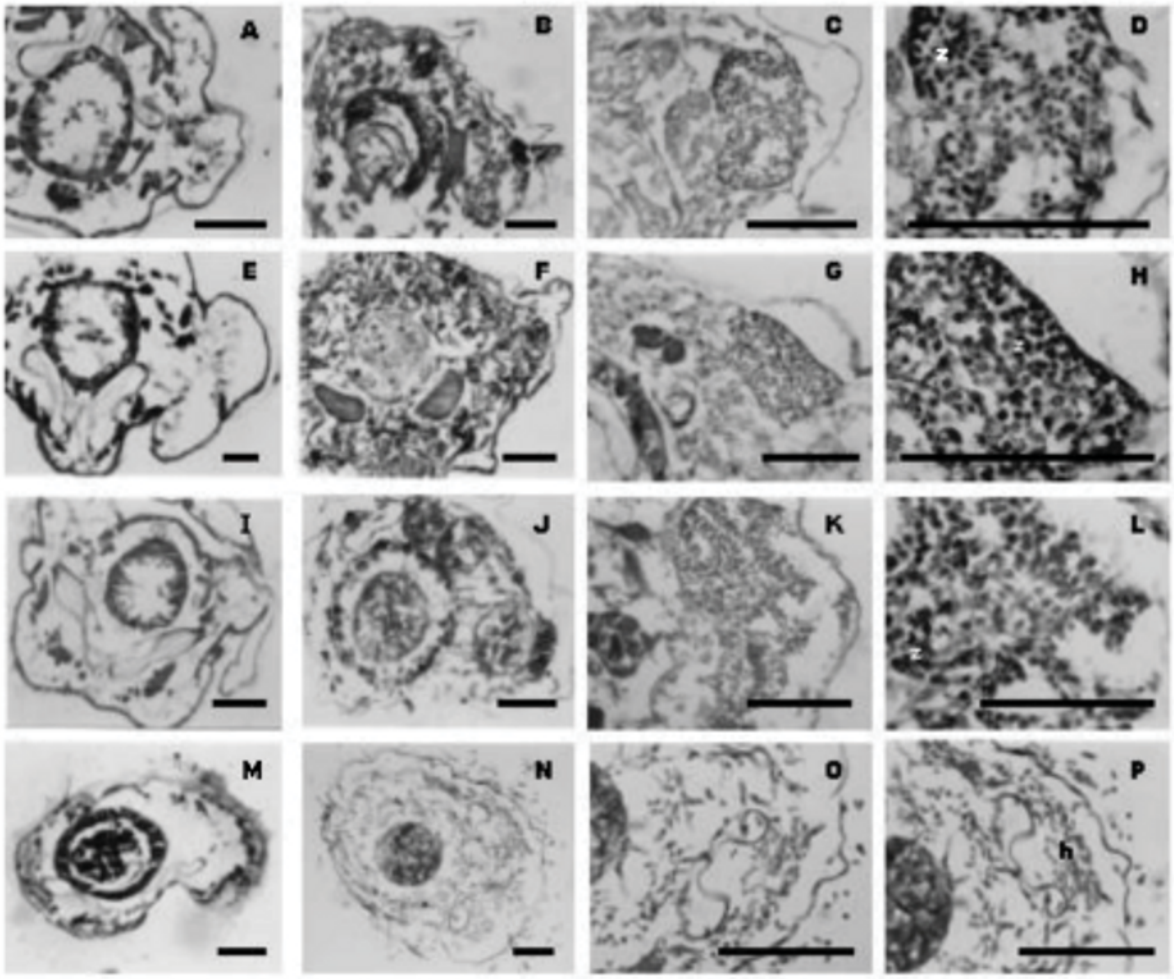

Fig. 1. **A**: Transverse section of *Aedes aegypti* larvae control fixed in 10 % formaldehyde–phosphate buffer and stained with hematoxylin–eosin. **B**: Transverse section of *A. aegypti* larvae infected with *Leptolegnia chapmanii*. **C**, **D**: Detail of transverse section of *A. aegypti* larvae (treated) showing *L. chapmanii* zoospores (z). **E**: Transverse section of *A. aegypti* larvae (control) fixed in 10 % formaldehyde–phosphate buffer and stained with Masson trichromic. **F**: Transversal section of *A. aegypti* larvae infected with *L. chapmanii*. **G**, **H**: Detail of transverse section of *A. aegypti* larvae (treated) showing *L. chapmanii* zoospores. **I**: Transverse section of *A. aegypti* larvae (control) fixed in 10 % formaldehyde–phosphate buffer and stained with periodic acid–Schiff reaction and hematoxylin. **J**: Transverse section of *A. aegypti* larvae infected with *L. chapmanii*. **K**, **L**: Detail of transverse section of *A. aegypti* larvae (treated) showing *L. chapmanii* zoospores. **M**: Transverse section of *A. aegypti* larvae (control) fixed in Carnoy and stained with Grocott. **N**: Transverse section of *A. aegypti* larvae infected with *L. chapmanii*. **O**, **P**: Detail of transverse section of *A. aegypti* larvae (treated) showing *L. chapmanii* hyphae (h). *Scale bars* correspond to 100 μm.

When using Masson trichromic staining with light green, good contrast was achieved in 1 d with 10 % phosphate buffer as fixative (Fig. 1E–H). Nevertheless, when Carnoy fixative was applied, sufficient contrast and coloration intensity were also observed.

PAS and PAS plus hematoxylin stain was not so intense nor differentiating when compared with the other stains tested. Formaldehyde–phosphate buffer (10 %) proved to be the best fixative for PAS, and the best results were seen after 1 d (Figs 1I–1L). According to Goettel (1988), PAS stain for mosquito larvae infected with *Tolypocladium cylindrosporum* yielded sufficient results for histological sections; however, we have not been able to replicate these results. Nnakumusana (1986) reported that mosquito larvae invaded by *Leptolegnia* sp., using 5 % formaldehyde–0.95 % NaCl solution as fixative and stained with hematoxylin–eosin for larval transverse sections showed fungal zoospores attached and encysted on the cuticle, and in the gut epithelium, and hyphal penetration into a fat tissue starting 1 d and completed 2 d after fungal invasion/infection.

As to entomopathogenic fungi producing fungal toxins the action of tolypines and cyclosporines was reported by Weiser and Maťha (1988*a*,*b*) and Maťha *et al*. (1992). Using transmission electron microscopy they showed histological and cellular alterations caused by the entomopathogenic fungus *Tolypocladium* sp. in mosquito larvae which demonstrated that the affected tissues were midgut, connective and muscle with mithochondrial and nuclear disintegration and vacuolization of cells. We could not observe the same symptoms because *L. chapmanii* does not produce any toxins.

Grocott stain with light green undoubtedly provided the best results for control and fungal treated (Fig. 1M–P) samples; the best, it was combined with Carnoy fixative since when using phosphate buffer as fixative, the host tissues were observed to be softer and disintegrated. Fungal hyphae were grey to black in contrast to the reddish coloration of host muscle whereas digestive epithelium and epidermis turned brown. We consider this staining to be the best among all those tested since by using it a sharp contrast was achieved. Similarly to our data, Ferron *et al*. (1966) reported good results with Grocott staining for *Melolontha melolontha* (L.) (*Coleoptera*: *Scarabeidae*) infected with the fungus *Beauveria tenella* (SACC.) MACLEOD.

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