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INFLUENCE OF FIXATION OF THE BLACKFLY *SIMULIUM VITTATUM* ON MORPHOLOGICAL CHARACTERS OF THE TRICHOMYCETE *SMITTIUM CULISETAE*

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ABSTRACT – The present study investigates the symbiotic trichomycete fungus *Smittium culisetae* (Harpellaceae: Legeriomycetaceae) in larvae of the blackfly *Simulium vittatum* Zetterstedt (Diptera: Simuliidae). Trichomycetes show some degree of host preference, but many species have been found inhabiting more than one host species. They therefore cannot be identified solely on the basis of the host species. Accordingly, morphological characters such as thallus and trichospore measurements are critical in species identification. Trichomycetes are typically identified from freshly dissected hosts, but the ability to identify fungi from preserved host material would provide a wealth of ecological and biogeographical information. The intent of this study, therefore, was to examine the possibility of alteration of selected morphological characters in *S.. culisetae* from blackfly hosts fixed in 70% ethanol.

KEY WORDS: Harpellales, Simuliidae, fixation, symbiosis, trichomycete

INTRODUCTION

The present study investigates the trichomycete *Smittium culisetae* Lichtwardt (Harpellaceae: Legeriomycetaceae) in larvae of the blackfly *Simulium vittatum* Zetterstedt (Diptera: Simuliidae). Trichomycetes (Zygomycota) are a cosmopolitan class of filamentous fungi that are found living as obligate commensals in the digestive tract of various marine, freshwater, and terrestrial arthropods (LICHTWARDT, 1986, 1996). In this association, arthropods are typically referred to as the "host" and trichomycetes as the "symbiont" (MCCREADIE ET AL., 2005).

The arthropod hosts become infected with *Smittium* upon ingestion of trichospores (basipetal, monosporous sporangia) (LICHTWARDT, 1996). Following sporangiospore extrusion, young thallus attach to the larval hindgut cuticle, grow, and produce new trichospores (LICHTWARDT, 1986), often as early as 22 hours after attachment (WILLIAMS AND LICHTWARDT, 1972). Upon maturation, trichospores are shed into the gut lumen and expelled out of the larvae through the host's anus.

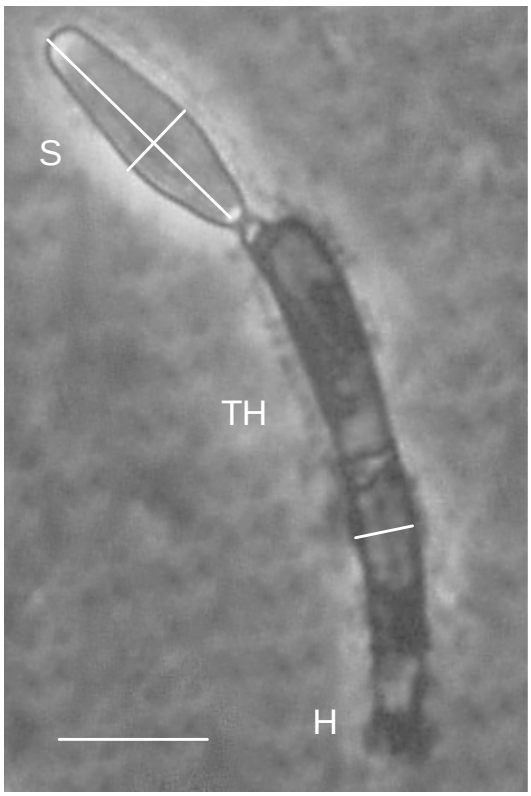

Fig. 1. *Smittium culisetae* attached by the holdfast (H) to the hindgut of the blackfly host fixed in 75% ethanol. Spore (S) length and width were measured, as well as thallus (TH) width. Scale bar ~ 10 µm.

Blackfly larvae infected with members of the Harpellales have been found in streams of North America (LABEYRIE ET AL., 1996), South America (LICHTWARDT AND ARENAS, 1996), Europe (TAYLOR ET AL., 1996), China and Far Eastern Russia (ADLER ET AL., 1996), Japan (LICHTWARDT ET AL., 1987), New Zealand (WILLIAMS AND LICHTWARDT, 1990), Australia (LICHTWARDT AND WILLIAMS, 1990, 1992), Thailand (TAKAOKA AND ADLER, 1997) and the Galapagos Islands (NELDER ET AL., 2004). Although many freshwater trichomycetes have been reported from only a single host species, many are found infecting several to many host species (LICHTWARDT, 1986). Hence, trichomycetes cannot be identified solely on the basis of the host species. Accordingly, morphological characters such as thallus and trichospore measurements (Fig. 1) are very critical in species identification.

Due to the low axenic culturability of trichomycetes, identification of these fungi has typically been based on material from freshly dissected hosts (LICHTWARDT, 1986). However, dissection of fresh hosts precludes the examination of a large number of specimens. On the other hand, a wealth of preserved material exists in museum specimens. The most common method of host fixation is in 70% ethanol (LICHTWARDT, 1986). But fixatives such as alcohol tend to harden host tissue, making it difficult to dissect; in addition, trichomycete morphology may be altered as a result of fixation.

ADLER ET AL. (1996) and BEARD ET AL. (2003) reported the detection of *Harpella melusinae* Leger and Duboscq from ethanol-fixed material, but did not detect Legeriomycetaceae in the same material. Recently, hindgut trichomycetes were found to be readily detectable: i) in ethanol-fixed blackfly larvae from the Galapagos Islands (NELDER ET AL., 2004); ii) in acetic ethanol and ethanol-fixed blackflies from Armenia (NELDER ET AL., 2005) and iii) in 15-20-year-old acetic ethanol-fixed material from Newfoundland blackflies (NELDER, unpublished data). Since morphology is heavily used in the identification of trichomycetes, the possibility that linear characteristics of trichomycetes change during fixation of the host requires attention. Changes in the morphology of *S. culisetae* Lichtwardt during ethanol fixation of the blackfly host *S. vittatum* Zetterstedt were therefore investigated in this study.

MATERIALS AND METHODS

Maintenance of inoculum. Culturing, trichospore harvesting, and dosage calculations followed the protocols of BEARD (2002) and MCCREADIE AND BEARD (2003), with a brief outline presented here. Stock cultures were maintained on plates of 3.7g/L (1/10) Brain Heart Infusion agar (Difco 0037-15-0) at 23–25 °C, with monthly transfers to fresh plates. Thallus subcultures were transferred to new plates 10 days prior to the start of any experiment. Plates were covered with a sterile water overlay to induce trichospore production after six days. Trichospores were harvested by filtering the overlay through glass-wool four days later. The resulting trichospore suspension was centrifuged at 900g for 10 min and the trichospore 'plug' rinsed once in water. Trichospore concentration in the resulting suspension was determined using a counting slide (Hemacytometer, improved Neubauer scale). A dosage suspension of 4000 trichospores per mL of host rearing water (MCCREADIE AND BEARD, 2003) was then added to the treatment containers (see below).

Host maintenance. The blackfly host used in all experiments were obtained from a colony housed at the University of Georgia (Athens, GA, USA). This colony is known to be free of trichomycetes, nematodes, and microsporidians (ADLER, unpublished data). All experiments were conducted in five-shelf Precival® incubators held under a 16 / 8 h light / dark schedule. The rearing system was described in detail by MCCREADIE AND BEARD (2003), and only the pertinent details are presented here. Each experimental incubator was supplied with air by an external Sweetwater® 5.5 CFM pump. The experimental rearing containers each consisted of a 12-cm (diameter) x 11 cm (height) round polypropylene plastic container fitted with a screw-top. Air tubing (0.4 cm) was inserted into the screw-top via a stiff tubing collar and fitted with an AS1 Sweetwater® air-stone to create an aeration current. Air supplied to the rearing water creates currents, simulating a running-water environment.

All experiments were conducted at 22 ºC with a 16 / 8 h light / dark schedule. One hour before the start of each experiment, six sets of 30 *S. vittatum* larvae were transferred from rearing

containers into treatment containers with 500 mL of aged tap-water. Blackfly larvae were then dosed with *Sm. culisetae* trichospores (4000 trichospores per mL of host rearing water**)** and fed a daily diet of 3 mL of fish food slurry per container. Forty-eight hours post inoculation, 10 larvae from each of three containers were removed from the inoculated water and dissected immediately. These larvae were used as the control group. An additional 30 larvae (three sets of 10) were fixed in 70% ethanol for 10 days and then dissected. Thallus width, trichospore length, and trichospore width (Fig. 1) were measured using an ocular micrometer. This experiment was repeated three times and each experiment analyzed as a one-way anova, with each measured character as the response variable.

RESULTS

Since each experiment considered three measurements and each experiment was repeated three times, there were a total of nine outcomes (Table 1). Each character showed a significant effect of fixation only once (Table 1): otherwise, the results were somewhat inconsistent (Table 2). This inconsistency is further compounded because significant results were not found in the same

Table 1. Anova of fixation (70% ethanol) effects on selected linear characteristics of *Smittium culisetae* in blackfly hosts. Characters included trichospore length, trichospore width, and thallus width.

		Mean effects ± SE		Anova	
	Characters	Ethanol	Fresh	F	p
Experiment 1					
	Trichospore length	16.9 ± 0.57	15.8 ± 0.72	1.64	0.205
	Trichospore width	4.7 ± 0.15	4.0 ± 0.20	8.15	0.006
	Thallus width	5.0 ± 0.22	4.1 ± 0.29	5.90	0.018
Experiment 2					
	Trichospore length	16.5 ± 0.54	14.6 ± 0.67	4.75	0.034
	Trichospore width	4.9 ± 0.20	5.0 ± 0.24	0.12	0.733
	Thallus width	5.6 ± 0.24	4.9 ± 0.30	3.67	0.061
Experiment 3					
	Trichospore length	16.5 ± 0.85	16.4 ± 0.65	0.00	0.958
	Trichospore width	5.2 ± 0.32	4.4 ± 0.24	3.27	0.078
	Thallus width	5.5 ± 0.38	4.9 ± 0.28	1.82	0.186

Table 2. Results of the anova of linear measurements of *Smittium culisetae* from blackfly larvae.

	Spore length	Spore width	Thallus width
Experiment 1	NS[1]	S	S
Experiment 2	S	NS	NS
Experiment 3	NS	NS	NS

[1]NS = Non-significant (at $p > 0.05$), S = Significant (at $p < 0.05$).

experiment, i.e., spore length was significantly affected by fixation in experiment 2, whereas trichospore width and thallus width showed significant effects only in experiment 1 (Table 2).

Although results were inconsistent with regard to the morphology of *S. culisetae* between fixed and fresh specimens, actual differences in the measurements were minimal. For example, trichospores in ethanol shrank only slightly compared to fresh material, with fixed trichospores 0.6 to 13 % shorter than freshly dissected trichospores. Similar results were observed for trichospore width and thallus width.

DISCUSSION

This results suggest that the morphology of *S. culisetae* is somewhat altered when the blackfly host is fixed in 75% ethanol. However, the trend was not consistent throughout all experiments, with two non-significant results and one significant result per morphological character measured. We feel that the morphology of *S. culisetae* within fixed blackfly hosts is not altered enough to preclude their use. However, one should be cautious when identifying fixed specimens where measurements are at the extreme lower end of reported values, since minimal shrinkage might make such measurements unreliable. To fully understand the implications of fixation, this experiment should be repeated using different hosts and other cultures of trichomycetes. The effect of fixation over a prolonged period of time also should be evaluated.

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