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INTRACELLULAR SYMBIOTES OF THE ASTER LEAFHOPPER, *MACROSTELES FASCIFRONS* (STÅL)[1]

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ABSTRACT

Liang-Yung Wei and Marion A. Brooks (1978). *Intracellular Symbiotes of the Aster Leafhopper*, Macrosteles fascifrons (*Stal*). Bull. Inst. Zool., Academia Sinica, **17**(1): 61-66. The aster leafhopper, *Macrosteles fascifrons*, possesses intracellular microorganisms in specialized organs, the mycetomes. The mycetomes are a pair of organs located laterally in the first and second abdominal segments, surrounded by fat body. The mycetomes can be recognized in advanced embryos (8-10 days old). The mycetomal symbiotes are irregular, cylindrical structures, whose taxonomic position has not yet been determined. They seem to be very sensitive to the ionic ratio of saline solution.

Intracellular symbiotes are found in members of Homoptera, Hemiptera, Coleoptera, Orthoptera, Mallophaga, Anoplura, and a few Diptera and Hymenoptera (Brooks, 1963). Buchener (1965) categorized the homopteran insect intracellular symbiotes by letters of the alphabet. He described in the leafhopper *a*-, *H*-, *t*-, and companion-symbiotes present in certain combinations.

The aster leafhopper, *M. fascifrons*, has two types of mycetomal symbiotes that, according to the nomenclature of Müller (1949), are called "*a*-symbiote" located in the *a*-organ and "*t*-symbiote" located in the *t*-organ. The symbiotes are transmitted from one generation to the next by transovarial infection, the typical way of transmission of leafhopper symbiotes. The symbiotic relationship between the leafhopper and its symbiotes was said by Buchner (1965) to be of a mutualistic rather than parasitic nature. Presumably, symbiotic microorganisms are beneficial to their host (Musgrave, 1964).

MATERIAL AND METHODS

Aster leafhopper were reared on a chemically defined diet developed by Hou and Brooks (1975) in small vessels in a bench-top chamber that maintained high humidity. The relative humidity was 68-75%, the temperature 22±3°C, and the photoperiod 16 L/8 D.

1. Dissection:

Carbon dioxide anesthetized insects were placed ventral side down on a slide in a drop

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of saline solution. With two small needles mounted in match sticks, the abdominal and thoracic sternites were teasted apart exposing the viscera. Various organs, including mycetomes, guts, testes, ovaries, salivary glands, and malpighian tubules, which were suspected of harboring microorganisms, were extirpated, smeared, and stained with Gram's stain. For examining mycetomal symbiotes under phase contrast microscopy, the mycetomes were dissected and crushed in three kinds of saline solutions (Table 1), and in 45% acetic acid (Mitsuhashi and Kono, 1975).

TABLE 1
Composition of Saline Solutions,gm/l

Tyrode's; Na/K=32.6, mOsm=278, freezing pt.=−0.52°C	
NaCl	8.00
KCl	0.20
$CaCl_2$	0.20
$MgCl_2 \cdot 6H_2O$	0.10
$NaH_2PO_4 \cdot H_2O$	0.05
$NaHCO_3$	1.00
Glucose	1.00
Ringer's Drosophila; Na/K=16.1, mOsm=238, freezing pt.=−0.44°C	
NaCl	7.50
KCl	0.35
$CaCl_2$	0.21
**Kurtti's SIM*; Na/K=0.24, mOsm=405, freezing pt.=−0.75°C	
NaCl	2.340
KCl	4.475
KH_2PO_4	0.082
K_2HPO_4	3.380
Sucrose	68.460

* Unpublished data from the insect-microbiology laboratory at the University of Minnesota

2. Histology:

After removal of legs or wings or both, three incisions were made through the integument of the thorax and abdomen. But for embryos, since the yolk material is very soft, the eggs were incubated in fixing solution for one or two days before they were pricked. Adults, nymphs, and embryos (8–10 days old) were fixed in one of the following (Table 2).

i. Bouin's solution at room temperature for at least 24 hours;

ii. Carnoy's solution at room temperature overnight;

iii. Duboscq-Brasil solution (alcoholic Bouin's) at 37°C for 2 days.

Specimens were dehydrated and cleared by washing in 70% ethanol for 24 hours; then in a solution containing (V/V) 55% *n*-butanol, 40.5% ethanol and 4.5% H_2O for about 24 hours; next in a solution of 75% *n*-butanol, 22.5% ethanol and 2.5% H_2O for about 20 hours; and finally in three changes of absolute *n*-butanol for 2–3 days. Materials were infiltrated with Tissuemat, M. P. 58°C and blocked in Tissuemat, M. P. 61°C. Sections were cut at 5 μm, dried at 45°C on a warm plate, then passed from xylene and a series of ethanol solutions. Delafield's hematoxylin, modified Goodpasture's and Mallory's triple stains (Table 3) were tried. A modification of Mallory's triple stain was found to be the most suitable for the differentiating the

TABLE 2
The Formulae of Fixing Solutions

Bouin's Fixative	
1.2% aqueous picric acid	75 ml
40.0% formaldehyde	25 ml
glacial acetic acid	5 ml
Carnoy's Fixative	
absolute ethanol	60 ml
chloroform	30 ml
glacial acetic acid	10 ml
Duboscq-Brasil Fixative	
80.0% ethanol	150 ml
40.0% formaldehyde	60 ml
glacial acetic acid	15 ml
picric acid	1 gm

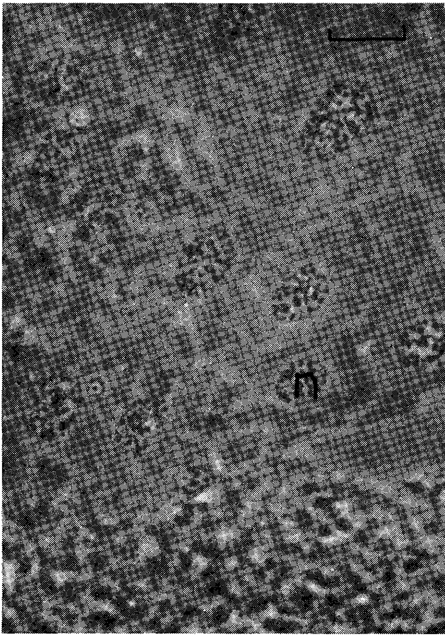

Fig. 1

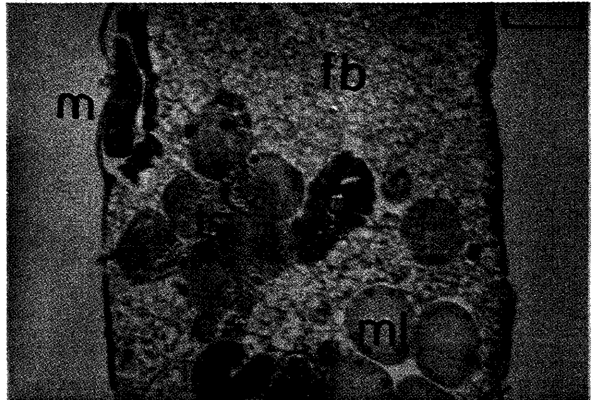

Fig. 2

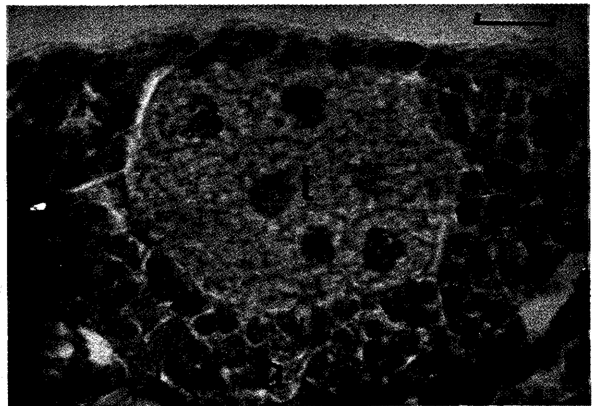

Fig. 3

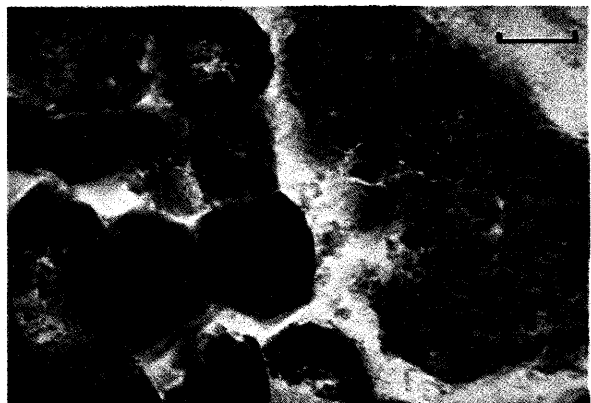

Fig. 4

Fig. 1. Photomicrograph of a mycetome freshly dissected in Drosophila Ringer's solution, showing globular mycetocytes with large nucli. Phase contrast. Bar = 3.96 μm.

Fig. 2. Part of a longitudinal section of adult male abdomen, showing mycetome (m), testes (ts), gut (g), fat body (fb), and malpighian tubules (ml). Bar = 36.8 μm.

Fig. 3. Longitudinal section of 8-10 day old embryo, showing *a*-symbiotes and *t*-symbiotes. Bar = 3.9 μm.

Fig. 4. Part of a section of an adult mycetome, showing *a*-symbiotes and *t*-symbiotes. Bar = 3.9 μm.

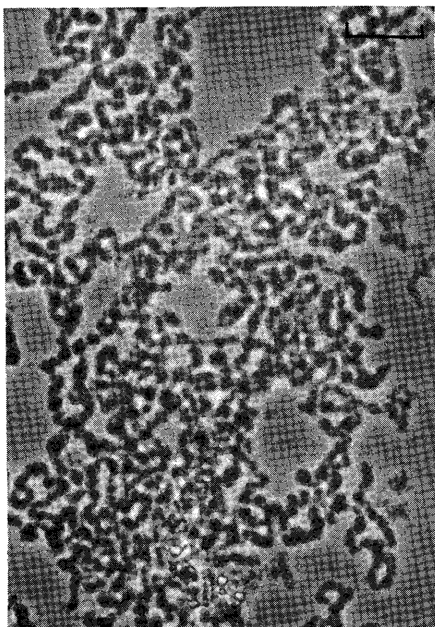

Fig. 5

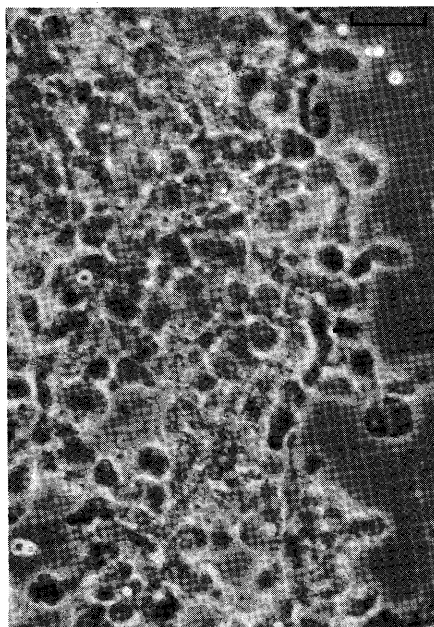

Fig. 6

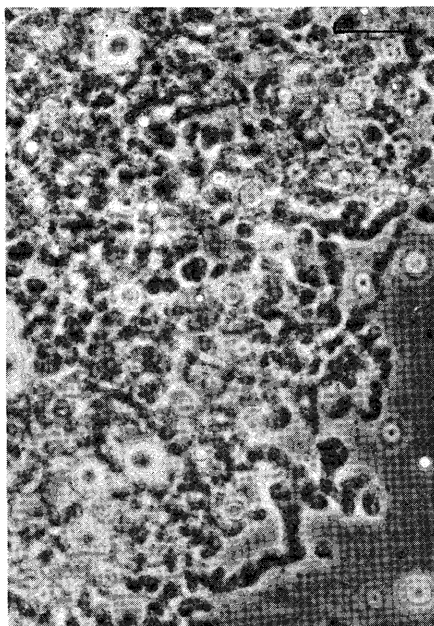

Fig. 7

Fig. 5. Photomicrograph of *a*-symbiotes from an adult mycetome. The mycetome was fixed in 45% acetic acid and crushed. Phase contrast. Bar = 3.96 μm.

Fig. 6. Photomicrograph of *a*-symbiotes from an adult. The mycetome dissected and crushed in Drosophila Ringer's solution. Phase contrast. Bar = 3.96 μm.

Fig. 7. Photomicrographs of symbiotes from an adult. The mycetome was dissected and crushed in Kurtti's SIM solution. Phase contrast. Bar = 3.96 μm.

TABLE 3
The Formulae of Staining Solutions

Delafield's Hematoxylin Stain	
heamtoxylin	6.4 gm
ammonium alum	60.0 gm
ethanol	40.0 ml
methanol	160.0 ml
glycerol	160.0 ml
water	640.0 ml
Goodpasture's Stain	
basic fuchsin	0.5 gm
carbolic acid, crystals	1.0 gm
anilin oil (freshly distilled)	0.5 ml
30.0% ethanol	100.0 ml
Mallory's Triple Stain	
i. acid fuchsin	0.1 gm
water	100.0 ml
ii. aniline blue	0.5 gm
orange G	2.0 gm
phosphomolybdic acid	1.0 gm
water	100.0 ml

mycetomal symbiotes, and the procedure was the following: From distilled H_2O, the sections were stained for 15 seconds in 0.1% acid Fuchsin solution, (the time being especially critical), followed by 80 minutes in the second solution. Immediately after staining, the slides were differentiated in three changes of 95% ethanol for a total of 3 minutes. This was followed by 2 minutes in absolute ethanol, then 50% xylene-absolute ethanol, and finally xylene. Excess xylene was drained off and the sections were cover-slipped with Apochromount (Health and Science Division, Aloe Scientific).

RESULTS AND DISCUSSION

A: Basic Internal Anatomy

Preliminary examinations of the internal anatomy were done in order to become familiar with the relative positions of various organs with respect to one another. The survey was also made the hope of finding those organs which might harbor microorganisms other than symbiotes.

No microorganisms were found in a smear of the gut and malpighian tubules. Furthermore, contaminating microorganisms were not found in ovary, testis and salivary gland smears.

1. The mycetomes:

(a) General observation—The mycetomes are surrounded by fat body, in the first and second abdominal segments, adjacent to the hypodermis. When observed with a phase contrast microscope, mycetomes were found to be composed of globular mycetocytes (Fig. 1). In adults, mycetomes were slightly bigger in females than in males. An average adult (2.75 mm long) usually had a mycetome 260 μm long. Smears of these organs yield no reaction to Gram's stain.

(b) Histological studies—The mycetomes gave a characteristic staining reaction and were easily recognized. Each mycetome consisted of two components—the part lying nearest the hypodermis being the so-called *t*-organ. The *t*-organ appears to be made up of distinct cells, the mycetocytes, each with a large nucleus. The *t*-organ is enclosed by the *a*-organ, which appears to be a large syncytium (Fig. 2). The mycetome, easily recognizable in full-term embryos (8–10 days old) (Fig. 3), persists throughout life.

2. Mycetomal Symbiotes:

(a) Sectioned material—It was found that Dubscq-Brasil fixing fluid followed by Mallory's triple stain was the best for differentiating the mycetomal symbiotes. All of the color photomicrographs were made of material prepared in this way. Delafield's hematoxylin-erythrosin and modified Goodpasture's-picric acid did not differentiate the symbiotes from the cytoplasm very well. In sections, *a*-symbiotes were seen as intertwining, irregular, cylindrical structures in the cytoplasm; they stained a homogeneous blue color. The *t*-symbiotes were somewhat smaller, globular bodies in the cytoplasm, and

they stained a reddish-brown color (Fig. 4). Thus the *a*- and *t*- symbiotes of the aster leafhopper resemble those of the leafhopper *Helochara communis* described by Chang and Musgrave (1972). Based on the similarity of both peripheral envelopes and cytoplasmic extensions, Chang and Musgrave suggested that the *a*-symbiotes have been derived from *t*-symbiotes. If this is true, then according to our observation, there is at least some chemical change in the *a*-symbiotes since the two symbiotes show different reactions to Malloy's triple stain. One must still consider the alternative hypothesis, that the *a*- and *t*- symbiotes belong to different strains or species of microorganisms.

(b) Smear preparations—When the mycetomes were fixed in 45% acetic acid, crushed and examined under phase constrast optics, the symbiotes look like twisted cylinders (Fig. 5) comparable to the symbiotes of the green rice leafhopper described by Mitsuhashi and Kono (1975). If the mycetomes were crushed in Drosophila Ringer saline solution, again the symbiotes looked like twisted cylinders at first (Fig. 6) but they began to disintegrate in about 20 minutes. After that they looked like oil droplets, an effect observed by Mitsuhashi and Kono when they used Tyrode's saline solution. But if Kurtti's SIM solution was used, the symbiotes did not lyse so quickly, their morphological integrity being preserved for up to 40 minutes (Fig. 7). In comparing the formulae of the three saline solutions, it is obvious that they differ greatly in Na^+ content and in Na^+/K^+ ratios. It seems reasonable that, being intracellular organisms, the symbiotes would require a high potassium concentration.

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翠蘭浮塵子細胞內共生生物之研究

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翠蘭浮塵子 (*Macrosteles fascifrons*) 具有細胞內共生微生物，其位於一種特殊的器官，懷菌體 (Mycetome) 中，比器官是成對的，位於第一、二腹節側面脂肪體中。於 8～10 日老熟的胚胎內即可辨認懷菌體的存在，生於其內的共生生物 (Symbiote) 是不規則的圓柱體構造，分類地位尚未被確定，其似乎對於生理食鹽水中的離子比率敏感度很高。