Aseptic rearing of the aster leafhopper, *Macrosteles fascifrons* (Stål), on a chemically defined diet

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Summary. The normal symbiotic aster leafhoppers, *Macrosteles fascifrons,* were reared under aseptic conditions on the holidic diet through 5 successive generations with normal rates of development, survival and reproduction. Thus the artificial diet is completely adequate for normal insects and dependence on gut organisms is not part of the physiology of this species.

Leafhoppers have been known for long as vectors in the transmission of plant viruses. That makes aseptic rearing of leafhoppers an important project, because the aseptic insects provide the most suitable materials for leafhopper tissue culture, considered to be a useful tool for the study of plant viruses[2,3]. Also, for designing a chemically defined diet, aseptic rearing technique can solve the problem of contamination by microorganisms and their concomitant metabolic reactions.

Materials and methods. Aster leafhoppers, *M. fascifrons,* were reared on a chemically defined diet modified slight from that of Hou and Brooks[4,5] (table 1) 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 16L/8D.

All the metal instruments were sterilized by dipping them in 95% ethanol and evaporating them to dryness over a gas flame. All glassware was sterilized in a 200 °C oven for 2 h. All filter paper and cotton was sterilized in an autoclave (16 lbs, 121 °C) for 15 min. The Parafilm M was cut into suitable pieces and stored in 70% ethanol until it was stretched, by holding only the edges of it. The diet was sterilized by filtering it through a Millipore disposable filter (0.45 µm). Eggs were collected 8–10 days after oviposition and surface-sterilized by immersion for 35 min in 0.025% sodium hypochlorite ($NaClO \cdot 5\ H_2O$), 5–10 sec in 70% ethanol, and 4 changes in sterile distilled water. The eggs were transferred aseptically to a 15 mm diameter watch glass (i.e. the egg chamber) containing sterile wet cotton covered with filter paper to keep the humidity high. The diet chamber was put above the egg chamber and the whole system was covered with a sterile beaker. When the nymphs hatched, the egg chamber was replaced with a water chamber (a glass bottle 13 mm in diameter and 50 mm in height). Between the diet chamber and the water chamber there was a sterile disc of filter paper which was picked in many places to prevent the nymphs from falling into the water.

Adult females laid eggs into the diet sachets. The eggs were transferred with a sterile pipette from the diet chamber to the egg chamber. This technique was used for continuous rearing of successive generations.

Results and discussion. At the conclusion of this experiment, aster leafhoppers reared under aseptic conditions have completed 5 generations. Their development, survival and oviposition are similar to that of control insects (table 2). Eggs, nymphs, adults and diets were tested in the aseptic system and found to be sterile. The tests were as follows:

1. The sterilization tests of eggs, nymphs and adults. All the eggs tested had been either surface-sterilized (designated as 'S') or laid in a colony under aseptic conditions (designated as 'A'). Nymphs and adults had been reared under aseptic conditions without any treatment.

Small tubes of sterile media, either nutrient broth or brain-heart infusion were allowed to stand for 1 week in the laboratory, to confirm sterility, before they were inoculated. There were 3 replicates of 5 tubes, each of which received one of the following:

1. 4 eggs (S and A), nymphs and adults not crushed. 2. 20–30 eggs (S and A), 5 nymphs and adults homogenized. 3. 10 eggs (S and A), 5 nymphs and adults placed in the broth and then crushed with a sterile glass rod. 4. 10 unsterilized eggs, used as a check of the sterilizing effect of immersion in sodium hypochlorite. 5. Nothing added, to check further on the sterility of the broth.

Surface sterilization of the eggs was assumed to have been accomplished if the broth remained clear for 48 h at 37 °C. For further testing, loopfuls from each tube were streaked on the surface of 3 media: nutrient agar, brain-heart infusion agar and tryptone glucose extract agar in petri plates, and these were incubated at 37 °C for 48 h. All the tubes containing surface-sterilized eggs or laid by aseptic colonies, whether crushed or not, remained clear. The

Table 1. Composition of diet, mg/100 ml

L-Alanine	20
L-Arginine HCl	80
L-Asparagine	60
L-Aspartic acid	20
L-Cysteine (free base)	10
L-Cystine	1
r-Amino butyric acid	4
L-Glutamic acid	40
L-Glutamine (commercial)	120
Glycine	4
L-Histidine (free base)	16
DL-Homoserine	160
L-Isoleucine (allo-free)	40
L-Leucine	40
L-Lysine HCl	40
L-Methionine	20
L-Phenylalanine	20
L-Proline	20
L-Serine	20
L-Threonine (allo-free)	40
L-Tryptophane	20
L-Tyrosine	4
L-Valine	40
Biotin	0.05
Calcium pantothenate	2.50
Choline chloride	25.00
Folic acid	0.25
i-Inositol (meso)	25.00
Nicotinic acid	5.00
Pyridoxine HCl	1.25
Riboflavin	0.25
Thiamine HCl	1.25
$CuCl_2 \cdot 2\ H_2O$	0.051
$FeCl_3 \cdot 6\ H_2O$	0.534
$MnCl_2 \cdot 4\ H_2O$	0.100
$CaCl_2$	0.444
Zn-sequestrene	0.160
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	10.00
$MgSO_4 \cdot 7\ H_2O$	242.00
KH_2PO_4	250.00
Sucrose	5,000.00
Ascorbic acid	100.00
Cholesterol S.C.W.	5.00
Lecithin (vegetable)	5.00
Tween 80	10.00

Table 2. Development of leafhopper under aseptic condition

	Normal condition*	Aseptic condition**				
	Control	1st generation	2nd generation	3rd generation	4th generation	5th generation
Mean nymphal periods (days)	23.3	23.6	23.2	23.4	23.3	23.8
Means survival 30 days (%)	74.8	74.9	74.6	75.0	74.3	74.6
Adult emergence (%)	75.4	75.2	75.0	75.1	75.6	75.4

*Mean value of 10 replicates; 10–18 insects per replicate. **Mean value of 5 replicates; 10–15 insects per replicate.

streak plates derived from these tubes produced no growth. Contaminants isolated from eggs that had not been surface-sterilized were Gram-negative rods, Gram-positive cocci and some fungi.

2. The sterilization tests of old diets. Tests were made on 3 types of old diets as follows:

a) Diet which had been fed to insects for more than 2 days. b) The first diet, but subsequently incubated at room temperature for 3 days. c) The first diet that contained moulted skins.

The diets were all streaked on the 3 kinds of agar plates and incubated at 37 °C for 48 h. All the plates remained clear and without growth. This demonstrates that under aseptic conditions, the insects, and even their droppings and exuviae, always keep clean.

One problem encountered was the drowning of the newly hatched nymphs in the condensed water in the egg chamber. The aseptic conditions necessitated a closed system, so the relative humidity was almost 100% and the water often condensed on the edge of the egg chamber. Some of the newly hatched nymphs drowned before they could jump to the diet. But once on the diet membrane, the insects stayed there most of the time, even during the moulting process. In an undisturbed condition, the insects grew stronger and high humidity was no longer a problem. Actually, high humidity is very favorable for survival. Mitsuhashi[6] stated that for survival of very small nymphs, above 80% relative humidity; the higher the humidity the better the survival.

From results described as above, we found no indication that the normal leafhoppers have gut microorganisms. Also this is supported by Dubroscky[7] based on her histological observations.

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Slime from thermotactic plasmodium of *Physarum polycephalum*

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Summary. Thermotactic plasmodium of *Physarum polycephalum* leaves behind its external layer of slime as it migrates. This rapid tactic movement provides an easy means to collect slime for mechanistic studies.

The plasmodium of *P. polycephalum,* an acellular slime mold, is an attractive system for phenomenological and behavioral studies of nonmuscle motility. The organism migrates by a net flow of cytoplasm through incessant shuttle streaming[1]. With speed reaching 1350 microns/sec at its maximum, the cytoplasmic flow of this organism has been recognized to be the highest in all motile systems[2]. The direction of migration can be random or dictated by external stimuli including light, heat, electricity and chemicals[3–8]. Although much information exists indicating actin and myosin as major components of the machinery of the shuttle streaming, very little is known about how the mechanism may be controlled[9–11]. In the cellular slime mold, *Dictyostelium discoideum,* however, it has been suggested that a gradient in the thickness of the surface sheath plays a role in the control of directional motion as well as in morphologensis[12]. Similar suggestions that slime may indeed possess certain physiological activities instead of a merely inert secretion have been reported in *Physarum* as well as *Dictyostelium* species[13–16]. In addition to slime, Anderson observed that a migrating plasmodium has a potassium gradient[17]. His assays indicated that a higher potassium content was found in the anterior region of the plasmodium. There was no attempt reported to identify the exact location of this ion, hence leaving a possibility that the potassium ion may actually be left in the slime fraction of the anterior region of the migrating plasmodium. Even though the chemical components of the slime have been preliminarily investigated by Simon and Henney, Jr[18], their samples were obtained from microplasmodia grown in liquid culture with proper agitation, and were separated from other cellular components by ethanol precipitation. There is no evidence that the microplasmodia grown in such an environment were exhibiting oriented migration. Whether slime is involved, or some other component (such as potassium ion) is involved in controlling directional movement similar to that found in *Dictyostelium,* requires a chemical study of slime collected from a 'migrating' plasmodium. We report here such a simple separation method.

Essentially the plasmodium was handled as reported in a previous publication[4]. The plasmodium of *P. polycephalum* M_3C was grown by spreading a suspension of microplasmodia onto a 25 mm millipore filter laid on the surface of agar supplemented with semi-defined growth medium with hemin and citrate[19]. The filters, when covered with freshly grown plasmodia at log phase, were lifted and excess