In Vitro Rearing of an Endoparasitic Fly, *Exorista sorbillans* (Diptera: Tachinidae)

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In order to establish an *in vitro* rearing method for an endoparasitic fly, *Exorista sorbillans*, various liquid media originally formulated for parasitoid rearing or for insect cell cultures were tested. About 50% of parasitoid larvae molted to the 2nd instar in the basic medium, an insect cell culture medium, MGM450, when it was fortified with 10% *Bombyx mori* hemolymph and 10% fetal bovine serum, whereas fewer or no larvae did so in the parasitoid rearing media formulated by other workers. Qualitative and quantitative differences were observed in hemolymph amino acids between parasitized and unparasitized 7-d-old 5th instar larvae of the host, *B. mori*. Heat-treated hemolymph of the host alone enabled the parasitoid larvae to develop to the pupal stage. Among the hemolymph samples taken from host larvae during the first 4 d of the 5th instar, the sample obtained from 4-d-old 5th instar larvae proved to be the best medium, and it sustained the development of larvae to the pupal stage. When such hemolymph was mixed with a modified melon fly diet or with wheat bran, some individuals of *E. sorbillans* completed development to the adult stage.

Key words: *Exorista sorbillans*, endoparasitic fly, *in vitro* rearing

INTRODUCTION

In vitro rearing of insect parasitoids is expected to contribute to investigations of insect physiology and to mass production of parasitoids for biological control (THOMPSON, 1986; NETTLES, 1990). Generally, artificial rearing of endoparasitic flies is very difficult. This is probably related to the fact that the maggots are normally exposed to drastic changes in environment, because the internal conditions of the host change with their growth. Furthermore, they respire mainly through the pair of spiracles at the end of the abdomen. Therefore, care must be taken to prevent maggots from drowning in *in vitro* rearing.

HOUSE (1954) reported rearing larvae of the sarcophagid fly, *Pseudosarcophaga* (= *Agria*) *affinis*, to the adult stage in a holidic medium. GRENIER et al. (1978) was the first to report rearing larvae of a tachinid fly, *Lixophaga diatraeae*, to the adult stage, and then NETTLES et al. (1980) and FANTI and BRATTI (1991) succeeded in rearing of two additional tachinid species, *Eucelatoria bryani* and *Pseudogonia rufifrons*, respectively.

Exorista sorbillans is an endoparasitic fly, and parasitizes to larvae of many lepidopteran insects including *Bombyx mandarina*, *B. mori* and some bagworm moths. MITSUHASHI and OSHIKI (1993) reported for the first time that *E. sorbillans* could be reared to pupae in a meridic liquid medium. In the present study, by testing various modifications of rearing conditions, development from the egg to the adult stage was achieved.

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MATERIALS AND METHODS

Insect. Adults of *E. sorbillans* were collected in a mulberry field in Fuchu, Tokyo and Tsukui, Kanagawa Prefecture, Japan from May to September, 1993. Insects were supplied with powdered sugar and water absorbed in cotton. Larvae of the host, *B. mori* (J122 × C115), were reared on an artificial diet of Silkmate 2S' (Nihon Nohsan Kogyo, Tokyo, Japan). The parasitized hosts were reared at 25°C under a 16L-8D photoperiod.

In vitro rearing. Parasitoid eggs were removed from the body-surface of *B. mori* larvae within 2 d after deposition. The eggs were washed with water, surface-sterilized by submersion in 70% ethanol for 5 min, and rinsed with sterilized distilled water before being placed in media. Larvae that hatched in the media were individually transferred to a well in a 24-well tissue culture plate (Corning, New Jersey, U.S.A.) containing one drop of the medium (200 µl) and incubated at 25°C under a 16L-8D photoperiod. In order to examine the effect of agarose on parasitoid development, various amounts of agarose (Ultra low gelling temperature agarose, Sea Prep, FMC) were added with distilled water and heated at 110°C for 15 min. After cooling down to 40°C, the agarose solutions were mixed with MGM450 medium (MITSUHASHI and INOUE, 1988), and then fortified with 10% *B. mori* hemolymph (Bmh) and 10% fetal bovine serum (FBS). To obtain the maggots used in rearing experiments from the 2nd instar, host larvae were surface-sterilized for 5 min in 70% ethanol (ethanol was changed once) 3–4 d after parasitization, after which 2nd instar maggots were removed from the larvae by dissection. The maggots were washed 4–5 times in CARLSON's solution (CARLSON, 1946) and transferred individually to a drop of medium in a 24-well plate.

For mass rearing, 1.2 ml of sterile heat-treated (60°C, 15 min) *B. mori* hemolymph and 1.0 g of sterilized supporting material (either toilet paper, cotton, modified melon fly diet, wheat bran, banana or apple) were mixed in a 35 mm sterile culture dish (Beckton-Dickinson, New York, U.S.A.). The modified melon fly diet consisted of 15.3 g wheat bran, 3.3 g dry yeast (Ebiose, Asahi Breweries, Tokyo, Japan), 3.3 g soy bean protein, 6.6 g sugar, 2.6 g toilet paper and 81.6 ml distilled water, and the pH was adjusted to 6.4 with 1 M KOH. Fifteen to 20 eggs obtained as described above were incubated in each medium.

Medium. The following media were tested: 100% heat-treated *B. mori* hemolymph (Bmh), MGM450 and MM (MITSUHASHI and MARAMOROSCH, 1964) insect cell culture media, NETTLES' (NETTLES et al., 1980) and GUERRA's (GUERRA et al., 1993) media for parasitoid rearing. NETTLES' medium was modified as follows: riboflavin and cholesterol were

Table 1. Larval development of *E. sorbillans* in 100% heat-treated hemolymph from 5th instar larvae of *B. mori* at different ages

Donor age (d)	<i>N</i>	No. of 2nd instars obtained (%)	No. of 3rd instars obtained (%)	No. of pupae obtained (%)	Pupal weight (mg)
0	20	16 (80)	2 (10)	0 (0)	—
1	15	11 (73)	4 (27)	0 (0)	—
2	20	17 (85)	0 (0)	0 (0)	—
3	12	11 (92)	1 (8)	0 (0)	—
4	28	26 (93)	11 (39)	1 (4)	14

reduced to 1/10 and 1/100 of the original dosages, respectively. MnCl_2 was replaced by the same molar of $\text{Mn}(\text{CH}_3\text{CO}_2)_2$, and $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and agar were eliminated. Ten percent FBS and Bmh were added to all media.

Analysis of free amino acids in host hemolymph. Host hemolymph was collected in a chilled 1.5 ml plastic tube by cutting the prolegs of *B. mori* 5th instar larvae. Three-fold 4% sulfosalicylic acid was added to the hemolymph, and the mixture was centrifuged at $1,500 \times g$ for 5 min. The supernatant was diluted to 1/12.5 with 0.1 M HCl and was filtrated through 0.45 μm Millex Hv filter (Millipore, Bedford, U.S.A.). The filtrate (200 μl) was analyzed with a MLC-203 high speed amino acid analyzer (Atto, Tokyo, Japan).

RESULTS

1. Development in host hemolymph

Parasitoid eggs were incubated in hemolymph collected from 0 to 4-d-old 5th instar larvae of *B. mori*. Hemolymph from 5 and 6-d-old larvae was not tested because it coagulated after heat treatment. Most parasitoid eggs hatched and attained the 2nd instar (Table 1). Relatively few maggots molted to the 3rd instar except in the hemolymph taken from 4-d-old 5th instar larvae of *B. mori*. In this sample, one pupa was also obtained. No adults emerged in any of the hemolymphs tested.

2. Development in various artificial media

Several media for insect cell cultures and parasitoid rearing were tested for their ability to support the growth of *E. sorbillans*. MGM450 medium appeared to be the best among all the media tested (Table 2). In this medium, 50% of the larvae reached the 2nd instar. It was, thus, employed as the basic medium (BM) in the following two experiments.

3. Analysis of free amino acids in host hemolymph

Table 3 shows the composition of free amino acids in the hemolymph of parasitized and unparasitized 7-d-old 5th instar larvae of *B. mori*. The amino acid pool of parasitized hosts was smaller than that of unparasitized ones, whereas the amounts of certain amino acids, such as cystine, glutamic acid, methionine, tyrosine and valine, were greater in the former (Table 3). Modification of MGM450 medium to allow its amino acid composition to simulate that of parasitized or unparasitized larval hemolymph did not improve the development of parasitoid larvae, and no pupae were obtained (Table 4).

Table 2. Larval development of *E. sorbillans* in various media

Media ^a	N	No. of 2nd instars obtained (%)
MGM450	16	8 (50)
MM	17	1 (6)
M. NETTLES SF	16	0 (0)
M. NETTLES	16	1 (6)
3/4 diluted-M. NETTLES	16	5 (31)
GUERRA SF	16	0 (0)
GUERRA	16	0 (0)

^a All media were fortified with 10% FBS and 10% Bmh except those marked SF (serum free). M. NETTLES, modified NETTLES medium.

Table 3. Composition of free amino acids in the hemolymph of parasitized and unparasitized host larvae, and basic MGM450 medium (mean mg/100 ml)^a

Amino acids	Parasitized hemolymph ^b	Unparasitized hemolymph	MGM450
α -Aminobutyric acid	29.5	37.2	0.0
λ -Aminobutyric acid	3.3	10.5	0.0
α -Alanine	16.8	29.6	26.3
β -Alanine	— ^c	—	16.7
Arginine	31.6	46.2	48.3
Asparagine	69.5	83.0	29.3
Aspartic acid	0.5	0.5	29.3
Cystathionine	12.7	15.6	0.0
Cystine	3.6	—	2.1
Glutamic acid	4.3	—	50.0
Glutamine	190.2	244.0	50.0
Glycine	27.4	89.4	54.2
Histidine	253.8	281.7	208.3
Isoleucine	15.3	16.7	4.2
Leucine	16.0	23.0	6.3
Lysine	56.2	57.0	41.9
Methionine	11.2	5.1	4.2
3-Methyl-histidine	1.1	0.9	0.0
Phenylalanine	11.7	18.0	12.5
Phosphoserine	1.0	1.0	0.0
Proline	18.1	21.6	29.2
Serine	80.9	87.9	91.7
Threonine	14.7	24.4	14.6
Tryptophan	—	—	8.3
Tyrosine	43.6	28.6	4.2
Valine	19.9	10.1	8.3
Total	932.9	1132.0	739.9

^a All host larvae were 7-d-old 5th instar females. Analyses were made in triplicate.^b Host larvae were parasitized by 4 or 5 maggots 4 or 5 d before hemolymph was drawn.^c Below detectable level.Table 4. Effect of amino acid composition of BM^a on *E. sorbillans* development

Amino acid composition ^b	N	No. of 2nd instars obtained (%)	No. of 3rd instars obtained (%)
Parasitized	31	14 (45)	3 (10)
Unparasitized	8	3 (38)	0 (0)
BM (control)	21	12 (57)	3 (14)

^a MGM450 with 10% FBS and 10% Bmh.^b For amino acid composition of parasitized and unparasitized host larvae, refer to Table 3.

4. Effects of supporting material on development

Various amounts of agarose dissolved in distilled water after heating or pieces of toilet

Table 5. Effects of the addition of agarose or toilet paper to BM^a on the development of 2nd instar larvae of *E. sorbillans*

Supporting materials ^b	N	No. of 3rd instars obtained (%)	No. of pupae obtained (%)	Mean pupal weight (mg)
Grown in BM during the 1st instar				
2.0% Agarose	20	13 (65)	4 (20)	13
1.5	12	6 (50)	0 (0)	—
1.0	12	7 (58)	1 (8)	12
0.5	20	8 (40)	0 (0)	—
0 (control)	24	6 (25)	0 (0)	—
Grown in host during the 1st instar				
Toilet paper	15	13 (87)	1 (7)	10
None (control)	14	10 (71)	1 (7)	9

^a MGM450 with 10% FBS and 10% Bmh.^b The amount of BM was 200 μ l per culture except in the case where toilet paper was used as the supporting material (500 μ l BM + 600 mg toilet paper).Table 6. Effect of addition of various kinds of supporting material to *B. mori* hemolymph (Bmh) on *E. sorbillans* development^a

Supporting material ^b	N	No. of 2nd instars obtained (%)	No. of 3rd instars obtained (%)	No. of pupae obtained (%)	Mean pupal weight (mg)	No. of adults emerged (%)
Modified melon						
fly diet	23	19 (83)	13 (57)	5 (22)	22	1 (4)
Wheat bran	13	8 (62)	5 (38)	1 (8)	41	1 (8)
Banana	18	0 (0)	—	—	—	—
Apple	17	0 (0)	—	—	—	—
Cotton	14	8 (57)	5 (36)	3 (21)	18	0 (0)
Toilet paper	12	9 (75)	4 (33)	2 (17)	27	0 (0)

^a 15–20 eggs/medium.^b Each supporting material, cut or grated, was mixed with Bmh, adjusted to bring the pH to 6.4 with 1 M KOH, and then autoclaved at 110°C for 15 min.

paper were added to the basic medium to examine how these modifications influenced the development of parasitoids. An addition of 1 or 2% agarose to the basic medium improved the development of maggots when compared to the control (χ^2 test ; $p < 0.05$ for 1% agarose, $p < 0.01$ for 2% agarose) (Table 5). However, no adults were obtained by either treatment. Toilet paper did not improve the development markedly.

A modified melon fly diet was prepared by mixing wheat bran, banana and apple with *B. mori* hemolymph (4-d-old 5th instar) to test whether these additives could improve the development of parasitoids. In this test, 15–20 parasitoid eggs were inoculated in a dish. The results summarized in Table 6, indicate that the melon fly diet or wheat bran added to *B. mori* hemolymph allowed the parasitoid to develop from the 1st instar to the adult stage. Banana and apple were found to be rather detrimental to the maggots.

DISCUSSION

E. sorbillans does not molt to the 2nd instar until its host reaches the 3rd d of the 5th instar, and then it begins to develop rapidly. According to SAKURAI (1984), hemolymph of *B. mori* larvae before the 3rd of the 5th instar has a relatively high juvenile hormone (JH) titer. The maggot is able to develop beyond the 2nd instar in an isolated abdomen of a 4th instar host, but molting to the 2nd instar is suppressed by application of JH to the isolated abdomen. It is therefore considered that the 1st molt of the maggot is induced by the disappearance of JH in the host body (OSHIKI and NAKAZAWA, 1987). In the present study, the maggot developed to the 3rd instar in heat-treated hemolymph collected from 0 or 1-d-old 5th instar larvae of the host. This result may be due to the disappearance of JH activity in the hemolymph.

Tachinid maggots grow in host hemolymph during the 1st instar and obtain oxygen through their integument by gaseous diffusion. Upon reaching the 2nd instar, they become attached to the host tracheal system for respiration or use a hole made on the host cuticle at the time of penetration into the host body cavity (THOMPSON, 1986; MITSUHASHI and OSHIKI, 1993). In *in vitro* rearing of tachinids, therefore, it is thought to be important to ensure the respiration of maggots by keeping them in contact with air. This was also emphasized by YAGI et al. (personal communication) who reared *Exorista japonica* *in vitro*. Agarose, agar, cotton and other materials have been used to support maggots in liquid media (GRENIER et al., 1978; NETTLES et al., 1980; BRATTI and NETTLES, 1992).

In this study, the modified melon fly diet and wheat bran were found to be suitable substrates for the growth of *E. sorbillans* maggots. By adding these substrates to *B. mori* hemolymph, we succeeded in rearing *E. sorbillans* from the egg to the adult stage. This parasitoid is said to require a pupal weight of more than 30 mg for successful adult emergence (OSHIKI et al., 1989). Some pupae in the present study met this criterion. The two female adults obtained were within the range of body sizes normally observed in nature. The present success may be attributed to the following: (1) the addition of modified melon fly diet or wheat bran to the liquid medium enabled maggot spiracles to be exposed to air; (2) wheat bran might contain some important nutrient; (3) mass rearing might have some effect, or (4) a combination of these possibilities. Further analysis is required to elucidate the reasons for this successful rearing.

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