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Rearing of *Exorista larvarum* (Diptera Tachinidae): simplification of the *in vitro* technique

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Abstract

The tachinid *Exorista larvarum* (L.) was developed *in vitro* from egg to adult using absorbent cotton in replacement of more expensive agar as a physical support of the artificial medium. Puparial yields and adult emergence did not differ significantly between agar-containing and cotton absorbed diets. The heaviest puparia (mg 66-69 for males and 59-60 for females) were obtained when 15 mg cotton were used to absorb 0.4 ml diet. With this ratio of absorbent mass to volume of absorbed liquid, puparial yields reached as high as 61% and over 92% of puparia emerged as adults. Subsequently a comparison was made of *in vitro* rearing procedure conducted in or out of a laminar flow hood with a cotton absorbed diet devoid of antifungal agents. When the procedure was conducted out of a laminar flow hood no significant increase of mould contamination or decrease of adult yields occurred. The weights of male puparia were significantly lighter than the weights of puparia reared in a laminar flow hood. Both male and female puparia obtained out of a laminar flow hood however weighed more than those previously cultured *in vivo* from monoparasitized *G. mellonella* larvae.

Key words: *In vitro* rearing, parasitoids, *Exorista larvarum*, Tachinidae, diet physical substrate, mould contamination.

Introduction

The tachinid *Exorista larvarum* (L.), a polyphagous gregarious larval parasitoid of *Lymantria dispar* (L.) and other lepidopterous defoliators, has been cultured *in vitro* on various insect material-free artificial diets based on crude ingredients and containing agar as a physical support. Adult yields approached those commonly obtained in the factitious host *Galleria mellonella* (L.) and puparial weights were comparable or even higher in the parasitoids produced on artificial media than in the *in vivo*-reared ones (Mellini and Campadelli, 1995, 1996; Dindo *et al.*, 1999). There was no substantial difference between *in vitro*- and *in vivo*-reared flies as to female longevity and performance against natural or factitious hosts under laboratory conditions. Other quality parameters (including efficiency in parasitizing *L. dispar* in the field and total aminoacid larval body content) were however lower in artificially than in naturally reared parasitoids (Dindo *et al*., 2002a, b).

The purpose of this study was to simplify the *in vitro* rearing of *E. larvarum* in order to facilitate its production on artificial diets, and to evaluate the quality of the *in vitro*-grown flies. Quality control is a major concern of programmes aimed at rearing entomophages *in vitro* (Thompson, 1999; Grenier and De Clercq, 2003).

Physical support is very important for successful *in vitro* rearing of *E. larvarum*, because its larvae form primary integumental respiratory funnels and breathe atmospheric oxygen and reject CO_2 from the beginning of their development (Mellini *et al.*, 1993). Mellini *et al.* (1993) first suggested the possibility to use absorbent cotton in lieu of agar as a diet support for this tachinid. Adult yields and puparial weights were however lower on cotton -absorbed than on agar-based diets, possibly because of inadequate ratio of absorbent mass to volume of absorbed liquid (50 mg cotton to 1 ml diet). Because cotton is far more economical than agar, and cotton-supported media are easier to prepare than agar-based diets, in the first experiment attempts were made to culture *E. larvarum* on diet-soaked cotton balls.

A critical factor for the *in vitro* rearing of parasitoids is the control of contamination by bacteria and fungi. Bacteria can be easily controlled by integrating diets with antibiotics, but it may be more difficult to control fungi, which can rapidly spread on the media from an initial contamination point by mycelia and spores. Unfortunately parasitoids are generally very sensitive to antifungal agents (Grenier and Liu, 1990). As for other parasitoids, also for *E. larvarum*, the *in vitro* rearing procedure has always been performed in a laminar flow hood. Under these conditions the use of fungicides in the diet has never proved to be necessary (Mellini and Campadelli, 1995; Dindo *et al.*, 1999). The possibility of conducting the procedure out of a laminar flow hood could however make the artificial culture of *E. larvarum* simpler and more economical. Therefore in the second experiment a comparison was made of *in vitro* rearing procedure conducted in or out of a laminar flow hood with a diet devoid of antifungal agents.

In both experiments the artificial diet described by Mellini and Campadelli (1995) was used. This diet was selected because it is the more efficient, economical and easier to prepare among the media till now developed for *E. larvarum*.

Materials and Methods

A laboratory colony of *E. larvarum* was maintained on the factitious host *Galleria mellonella* by the methods described by Mellini *et al*. (1993). The artificial medium utilised throughout the trial consisted of skimmed milk (30 ml), chicken egg yolk (5.5 ml), yeast extract (Sigma Chemical Co.) (2.7 g), sucrose (0.8 g), Gentamicin (10 mg^{-1} solution) (Sigma Chemical Co.) 0.01ml/ml diet

(Mellini and Campadelli, 1995). The rearing containers were 24-well plastic plates. Each well had a diameter of 17 mm, a height of 18 mm and a capacity of 3.3 ml. *E. larvarum* eggs were collected from superparasitized *G. mellonella* larvae and transferred onto the diet by the method described by Bratti and Coulibaly (1995) (1 egg per well). In both experiments after placing the parasitoid eggs, the plates were sealed with Parafilm and kept in darkness at 26±1°C and 70% RH throughout the trial except when they were removed for daily inspections. The diet was basically prepared as described by Farneti *et al.* (1998), except for the differences indicated below.

Comparison of agar-based medium versus liquid diet on cotton.

This experiment was mainly aimed at investigating the possibility of replacing agar with absorbent cotton as a diet physical substrate. The effects of different diet amounts on parasitoid development were also tested. Six treatments were compared. In treatment (A) 0.4 ml medium supplemented with 1.2% agar were placed in each well, similarly to the standard *in vitro* rearing procedure of *E. larvarum*. The diet thickness was of about 3.5 mm. In treatment (B) the diet was also supplemented with 1.2% agar, but the diet amount per well was 0.3 ml. The diet thickness was of about 3 mm. In treatments (C) and (D) 0.4 ml of liquid diet were pipetted onto small balls of absorbent cotton which weighed 15 mg. The thickness of the diet-soaked cotton layers was of about 2.5 mm. In treatments (E) and (F) 0.3 ml of liquid diet were pipetted onto balls of absorbent cotton which weighed 10 mg. The thickness of the diet-soaked cotton layers was of about 2 mm. Cotton balls weighing less than 10 mg were not tested because they were insufficient to cover the well bottom. In treatments (C) and (E) each ball was separately placed in a 1.5 ml-capacity plastic micro-tube for autoclaving, whereas in every replicate of treatments (D) and (F) all balls were wrapped in tinfoil and then placed in the autoclave. After autoclaving the cotton balls were placed singly into the wells of a rearing plate using forceps. The diet was then pipetted onto the balls.

Cotton, instruments and glassware were autoclaved for 20 min at 120°C. All operations, including daily inspections were conducted in a laminar flow hood.

Twelve replicates were performed, each consisting of 6 eggs per treatment.

Evaluation of *in vitro* rearing procedure conducted in or out of a laminar flow hood.

Two treatments (I and II) were compared. In both 0.4 ml liquid diet were pipetted onto 15 mg-cotton balls which were placed in 1.5 ml-capacity micro-tubes for autoclaving as in treatment (c) of the previous experiment. Cotton, instruments and glassware were autoclaved for 20 min at 120°C. All operations were conducted in clean room conditions in (treatment I) or out of (treatment II) a laminar flow hood. These operations included diet preparation, laying of cotton balls and diet in the plate wells, egg collection and transfer onto the medium, daily inspections and collection of the newly-formed puparia.

Five replicates were performed each consisting of 24 eggs per treatment.

Result evaluation and statistical analysis.

The results were evaluated in terms of percentages of hatched eggs, puparia and adults, of male and female puparial weights 24 h after formation (in mg) and of development times from egg to adult (in days). The percentages were based on the number of eggs originally placed on the media for hatched eggs and puparia, and of puparia for adults. In the second experiment the percentage of wells containing diet contaminated by moulds was also determined. This percentage was calculated on the total number of wells containing diet per replicate (= 24).

In the first experiment the male and female development times were analysed by Kruskall-Wallis non parametric test. Means were compared using a non parametric multiple comparison test when a significant difference ($P < 0.05$) occurred. The other parameters were analysed by one-way analysis of variance. In the first experiment means were compared using the LSD test where a significant difference occurred. The percentage values were transformed for the analysis using an arcsine transformation (Mosteller and Youtz, 1961). Statistical tests were done with STATISTICA for WINDOWS (1994).

Results

Comparison of agar-based medium versus liquid diet on cotton.

Differences for the percentages of hatched eggs, puparia and adults and for male development times were not significant among treatments. Male and female puparial weights were significantly higher when 0.4 ml diet were pipetted onto 15 mg cotton (treatments C and D) compared to the agar-based media (treatments A and B). When 10 mg cotton were used to absorb 0.3 ml diet (treatment E and F) puparial weights were not significantly different from those obtained from the other cotton-absorbed or agar-based diets. The female development times were significantly longer on agar-based than on cotton-absorbed diets. There were not significant differences between cotton autoclaved in micro-tubes (treatments C and E) and in tinfoil (treatments D and F) in any parameter but female development times. The latter were however of about 24 days for all the cotton-treatments (table 1).

Evaluation of *in vitro* rearing procedure conducted in or out of a laminar flow hood.

There were no significant differences between treatments in percentages of plate wells containing fungus. The percentages of hatched eggs, puparia and adults, the female weights and the male and female development times were not significantly influenced by the *in vitro* rearing procedure (in or out of a laminar flow hood). The male puparia reared under aseptic conditions were significantly heavier than were those reared out of a laminar flow hood (table 2). The latter were however comparable in size to those obtained *in vivo* from monoparasitized *G. mellonella* larvae by Dindo *et al.* (1999).

Table 1. Developmental parameters (%), male and female puparial weights (mg), and development times from egg to adult (days) of *Exorista larvarum* reared on agar-based artificial diet and liquid diet on cotton. Number of replicates given in parentheses above the means (±SD). Means in a column followed by the same letter are not significantly different (P<0.05) (nonparametric multiple comparisons [male and female development times]; LSD multiple range test [other parameters]).

Treatment		Parameters						
	Diet per well	Hatched eggs[1]	Puparia[1]	Adults[2]	Male weights	Female weights	Male times	Female times
Agar-based (1.2%) medium								
(A)	0.4 ml	(n=12) 75±15.1a	(n=12) 63.9±17.2a	(n=12) 78.5±23.7a	(n=10) 57.7±7.6a	(n=11) 51.8±5.2abf	(n=10) 24.1±0.7a	(n=11) 24.9±0.8a
(B)	0.3 ml	(n=12) 73.6±19.4a	(n=12) 61.2±20.5a	(n=12) 93.9±14.3a	(n=10) 55.1±5a	(n=11) 49.1±5.4ac	(n=10) 23.8±0.4a	(n=11) 25.3±0.4b
Liquid diet on cotton								
(C) 15 mg cotton autoclaved in a plastic micro-tube	0.4 ml	(n=12) 76.4±21.8a	(n=12) 55.6±24a	(n=12) 92.1±12.3a	(n=12) 65.9±11.7bc	(n=7) 59.7±8.6de	(n=12) 23.7±0.6a	(n=7) 23.8±0.6c
(D) 15 mg cotton autoclaved in tinfoil	0.4 ml	(n=12) 83.4±12.3a	(n=12) 61.1±19.3a	(n=12) 92.8±13.5a	(n=10) 69±10.4b	(n=8) 59.1±9bd	(n=10) 23.6±0.8a	(n=8) 24.3±1.1d
(E) 10 mg cotton autoclaved in a plastic micro-tube	0.3 ml	(n=12) 77.8±16.4a	(n=12) 44.5±28.8a	(n=11) 74.2±38.7a	(n=6) 58.3±6.6ac	(n=7) 50.3±10.9fc	(n=6) 23.5±0.8a	(n=7) 24.8±1.2d
(F) 10 mg cotton autoclaved in tinfoil	0.3 ml	(n=12) 75±27.1a	(n=12) 50±26.6a	(n=12) 93.7±15.5a	(n=10) 56.1±9.5a	(n=7) 53.1±10.8bce	(n=10) 24. ±1.5a	(n=7) 23.9±1.4c
F (df)		0.29 (5,66)	1.09 (5,66)	1.33 (5,65)	4.07 (5,52)	2.53 (5,45)		
H (N)							8.51(58)	15.7 (51)
P		0.92	0.37	0.26	0.003*	0.04*	0.13	0.008*

[1] Percentages based on eggs placed on the media
[2] Percentages based on puparia

Table 2. Comparison of *in vitro* culture of *Exorista larvarum* conducted in and out of a laminar flow hood: percentages of plate wells containing fungus, developmental parameters (%), male and female puparial weights (mg) and development times from egg to adult (days). Means (±SD). Number of replicates = 5. Means in a column followed by the same letter are not significantly different (P<0.05) (one-way analysis of variance).

In vitro rearing procedure	Parameters							
	Plate wells containing fungus[1]	Hatched eggs[2]	Puparia[2]	Adults[3]	Male weights	Female weights	Male times	Female times
In a laminar flow hood	3.7±5.1a	82±14.6a	43.8±17a	88.5±12.7a	70±5.9a	62.8±7.4a	24.8±1.2a	25.8±1.3a
Out of a laminar flow hood	4.1±2.9a	79.3±10.6a	47.8±12.8a	89.6±11.5a	56.6±2.3b	57.4±6.5a	24.1±1.9a	24.1±1.2a
F (1,8)	0.3	0.1	0.2	0.02	22.4	1.6	0.5	0.2
P	0.63	0.75	0.66	0.89	0.001*	0.25	0.5	0.69

[1] Percentages based on the total number of wells containing medium per treatment (= 24)
[2] Percentages based on eggs placed on the media
[3] Percentages based on puparia

In both experiments the parasitoid adults obtained in all treatments mated and the females laid eggs on *G. mellonella* larvae, producing a second generation *in vivo*.

Discussion

The results of the first experiment showed that replacing agar with absorbent cotton did not significantly affect the development of *E. larvarum* on artificial diets and, in particular, puparial yields and adult emergence. Puparial yields were slightly higher (but the difference was not significant) when 15 mg of cotton with 0.4 ml diet instead of 10 mg of cotton with 0.3 ml diet were used. The puparia were heavier when 15 mg cotton were used to absorb 0.4 ml diet, but in all treatments the weights were higher than those of the puparia obtained *in vivo* from monoparasitized *G. mellonella* larvae by Dindo *et al.* (1999). A correlation was often found between size and individual fitness and, in particular, with female fecundity for both *in vivo*- and *in vitro*-reared parasitoids (Reitz and Adler, 1995; Rojas, 1996). For *E. larvarum*, however, puparial weight may not be considered as the only criterion for estimating either the efficiency of an *in vitro* rearing procedure (including the optimal quantity of media per insect) or the quality of artificially reared *E. larvarum* flies. In fact, the puparial weight of *E. larvarum* females was significantly higher when reared on veal homogenate-based artificial diet compared with *G. mellonella*, but the number of eggs laid throughout female lifespan was only slightly, and not significantly, higher in individuals reared *in vitro* than *in vivo* (Dindo *et al*., 1999). Moreover female flies reared on the same skimmed milk-based diet employed in the present study, and emerged from puparia weighing 30-40 mg, had a significantly lower fecundity than those cultured *in vivo* and emerged from puparia of similar size (Dindo *et al*., 2002). As emphasised by Grenier and De Clercq (2003) other factors (i.e. the composition of proteins and lipids in parasitoid body) may influence fecundity, as the relationship between this parameter and body size is not always clear, especially for *in vitro*-reared entomophages. Further study is needed to ascertain the correlation between puparial weight and quality control parameters of *in vitro*- and *in vivo*- reared *E. larvarum*, and thus determine to what extent it is convenient to produce puparia with high weight.

In all treatments the male and female development times were 7-8 days longer than usually observed *in vivo* at the same temperature and the differences among treatments were small. The times were consistent with those observed on artificial diets in previous studies (Mellini and Campadelli, 1995; Dindo *et al.*, 1999) and probably resulted from the large amounts of food available for the *in vitro*-reared parasitoids. The immature development of parasitoids is often longer under artificial conditions than in natural hosts, but contrary to here, the parasitoids grown *in vitro* have usually a lower weight than *in vivo* (Grenier and De Clercq, 2003). Female times were slightly, but significantly, longer on agar-based diets than on liquid diets on cotton. Moreover in female times there were significant differences among cotton-supported media. The relationship between this parameter and diet substrates was however not clear.

Thompson (1975) successfully used cotton-supported artificial diets for the hymenopteran parasitoid *Exeristes roborator* (F.). Subsequently, for the tachinid *Eucelatoria bryani* Sabrosky Bratti and Nettles (1992) showed that cotton, used a support for a liquid medium, gave yields of puparia and adults equal to those obtained on agar-based diet. The weights of puparia from the cotton-supported medium were however about 16% lighter than were those of puparia from the agar-based medium. The inferior weights possibly resulted from partially inadequate rearing container size and/or ratio of absorbent cotton mass to volume of absorbed liquid. In fact, each rearing cell had a capacity of 0.4 ml (about one eighth of that of wells employed in the present study) and contained either 40 or 45 mg cotton balls which were used to absorb 200 µl (=0.2 ml) diet. As emphasised by Grenier *et al.* (1994) the ratio of container size to diet volume may be critical for gas exchange between larvae and surrounding air, diet and parasitoid larvae and, ultimately, for parasitoid growth and development. The *in vitro* development of *E. larvarum* was found to be considerably affected by rearing container volume, as at equal amount of agar-based diet the yields of puparia were significantly lower in 0.4 than in 3.3 ml-capacity rearing wells (the ones used in the present study), and indeed in the smaller wells no adult was found to emerge (Baronio *et al.*, 2002).

No substantial difference was found between the two methods to autoclave cotton for the parameters considered, but placing each ball in a plastic micro-tube was less time-consuming and thus more recommendable.

Based on the results of the first experiment, in the second, 15 mg of cotton were used to absorb 0.4 ml of liquid diet, and cotton balls were autoclaved in plastic micro-tubes. Although antifungal agents have not been added, the *in vitro* rearing procedure conducted out of a laminar flow hood had no apparent effect on the occurrence of mould contamination, because the percentage of wells containing fungus was very similar in the two treatments. The puparial yields, adult emergence and development times did not differ significantly between procedures conducted in or out of a laminar flow hood. The puparia were heavier when the rearing was performed in rather than out of a laminar flow hood, but only for males the difference was significant. The puparia obtained by the procedure conducted out of a laminar flow hood however weighed more than those obtained *in vivo* from monoparasitized *G. mellonella* larvae by Dindo *et al.* (1999). More research is needed to compare the quality of the flies reared *in vitro* in or out of a laminar flow hood. For the parameters considered in the present study, however, it seems to be possible to conduct the *in vitro* rearing of *E. larvarum* out of a laminar flow hood, at least when the procedure is performed in clean room conditions. This could lead to significant savings in production costs in case of large-scale *in vitro* culture of *E. larvarum*. Should the rearing

be performed in highly contaminated air, the use of fungicides in the diet may however be necessary. Grenier and Liu (1990) showed that when used at proper doses some antifungal agents, including nystatine, can control moulds without preventing *Trichogramma* spp. to develop normally *in vitro* even when lot of fungi are present in the air of the laboratory. Further study is needed to investigate the use of fungicides in artificial media for *E. larvarum* in contaminated conditions.

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