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In vitro rearing of *Anagrus breviphragma* (Hymenoptera: Mymaridae), an egg parasitoid of *Cicadella viridis* (Hemiptera: Cicadellidae), from second instar larva to adult on diets without insect components

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Abstract. We describe here the in vitro rearing of *Anagrus breviphragma* Soyka, an egg parasitoid of *Cicadella viridis* (Linnaeus), from the second instar larva to the adult stage. Three media, containing mainly either a commercial tissue culture medium (IPL-41), skimmed milk or veal homogenate, were tested. Larval development occurred on all the diets but few larvae reached the pupal stage on the diets containing skimmed milk or veal homogenate. On the tissue culture medium, pharate adults, albeit malformed, developed. Supplementing the tissue culture medium-based diet with chicken egg yolk alone, or combined with yeast extract, further improved parasitoid development. The addition of both egg yolk and yeast extract resulted in twelve out of twenty larvae reaching the adult stage, of which only four females emerged.

INTRODUCTION

Rearing entomophagous insects on an artificial diet may provide a good opportunity to reduce the costs associated with the production of biocontrol agents. Moreover, in vitro rearing may facilitate experimental studies on the biology, physiology and behaviour of parasitoids and predators (Grenier et al., 1994). Complete or partial development on artificial diets, with or without insect components, is reported for many oophagous parasitoids, including several species of the genus *Trichogramma* (Thompson, 1999), *Anastatus japonicus* Ashmead (Liu et al., 1988) and *Edovum puttleri* Grissell (Hu et al., 1998). These results stimulate research on the in vitro culture of other oophagous species with the aim of improving the rearing technique and enriching our understanding of their biology.

Anagrus breviphragma Soyka, an oophagous parasitoid widely distributed in the holarctic and neotropical regions, is an important antagonist of *Cicadella viridis* (Linnaeus) and other leafhoppers and planthoppers. Depending on the size of the host egg, it can be solitary or gregarious. It has two larval instars and a pupal stage (Moratorio & Chiappini, 1995). It has never been used for biological control, unlike other *Anagrus* species (Triapitsyn & Beardsley, 2000). The availability of a suitable artificial medium may open possibilities for simplifying its production so as to make easier its possible use as a biocontrol agent.

As an idiobiontic, polyphagous and facultative gregarious parasitoid, *A. breviphragma* may be a good candidate for in vitro rearing, because its development is not synchronised with that of its host, it has a high tolerance of variation in diet composition (Grenier et al., 1994) and can be cultured in groups instead of individually (Mellini & Campadelli, 1995).

This paper describes the results obtained by rearing second instar larvae of *A. breviphragma* on artificial media devoid of insect components.

MATERIAL AND METHODS

Carex riparia Curtis leaves bearing eggs of *C. viridis*, healthy and parasitised by *A. breviphragma*, were collected in winter beside the river Po in the province of Piacenza (Italy), wrapped in wet paper towelling, placed in closed plastic bags and kept at 4°C until required (Moratorio & Chiappini, 1995). When needed, the parasitised eggs were transferred to 20±1°C until parasitoid emerged. The second instar larvae for the experiments were removed from *C. viridis* eggs, which had been exposed to mated *A. breviphragma* females 5 days earlier (Moratorio & Chiappini, 1995).

The research was performed in two steps. First, diets based on media developed for other parasitoids were tested. Second, we compared diets made after the results of the first trial. The media utilised are reported in Table 1. Diets MEYS (containing liquid skimmed milk, chicken egg yolk, yeast extract and saccharose) and HEYS (containing veal homogenate, chicken egg yolk, yeast extract and saccharose) were respectively based on those successfully employed by Mellini & Campadelli (1995) and Dindo et al. (1999) for the tachinid parasitoid *Exorista larvarum* (Linnaeus). Diet IF included IPL-41, a commercially available insect cell culture medium and foetal bovine serum, two ingredients of the medium developed by Digilio (1999) for *Aphidius ervi* Haliday. Diets MEYS and HEYS were prepared by the procedures described by Farneti et al. (1998) and Dindo et al. (1999). Diet IF was prepared by mixing IPL-41, foetal bovine serum and gentamicin solution in a beaker. The agar-water suspension was prepared separately and added to the beaker's content as described by Farneti et al. (1998).

The other four diets tested (Table 1) were all based on diet IF, supplemented with one or two common ingredients used in media developed for several parasitoids (Bratti, 1990), in order to evaluate their effectiveness. Diet IFE was supplemented with chicken egg yolk in the same proportion, not considering gentamicin and agar (20% w/w), as in the media developed for *Trichogramma galloi* Zucchi and *Trichogramma pretiosum* Riley by Consoli & Parra (1997). The egg yolk was treated and added

TABLE 1. Diets used for rearing *Anagrus breviphragma* in the first and second trials.

First trial		Diet		
Component		MEYS[a]	HEYS[b]	IF
Liquid skimmed milk (M) (g)		16.3		
Veal homogenate (H) (g)			13	
Chicken egg yolk (E)(g)		2.8	1.9	
IPL-41 Insect Medium (I) (g)[c]				14.7
Yeast extract (Y) (g)		1.4	0.7	
Foetal bovine serum (F) (g)[c]				2
Saccharose (S) (g)		0.4	0.4	
Distilled water (g)			1.9	
Gentamicin (10 mg^{-1} solution) (ml g^{-1} diet)[c]		0.06	0.06	0.06
Agar-water suspension (6% of agar) (ml g^{-1} diet)		0.2	0.2	0.2
	ph	6	6	7.4
	Osmotic pressure (mOsm/Kg)[c]	567	691	240

Second trial		Diet			
Component		IFE	IFM	IFY	IFEY
IPL-41 Insect Medium (I) (g)[c]		14.7	14.7	14.7	14.7
Foetal bovine serum (F) (g)[c]		2	2	2	2
Powdered skimmed milk (M) (g)			4.2		
Chicken egg yolk (E)(g)		4.2			4.2
Yeast extract (Y) (g)[c]				1.2	1.2
Gentamicin (10 mg^{-1} solution) (ml g^{-1} diet)[c]		0.06	0.06	0.06	0.06
Agar-water suspension (6% of agar) (ml g^{-1} diet)		0.2	0.2	0.2	0.2
	ph	7.1	7.4	6.2	6.4
	Osmotic pressure (mOsm/Kg)[d]	251	634	535	488

[a]Diet composition according to the formulation of Mellini & Campadelli (1995).
[b]Diet composition according to the formulation of Dindo et al. (1999).
[c]Sigma Chemical Co.
[d]Measured using a cryoscopic osmometer (Dic, Japan).

to the media using the procedure described by Farneti et al. (1998). In diet IFM, chicken egg yolk was replaced with an equal amount of powdered skimmed milk, which was dissolved in the IPL-41 and bovine serum mixture, before adding gentamicin and agar. We used powdered milk in order to reduce the total water content of the diet. Diet IFY was supplemented with yeast extract in the same proportion, not considering gentamicin and agar (6.6% w/w), as was diet MEYS. The yeast extract was added to the media using the procedure described by Farneti et al. (1998). Diet IFEY included chicken egg yolk and yeast extract in the same amount as in diets IFE and IFY.

Each medium was placed in the wells of a 24-well plastic rearing plate (Nunclon, Denmark) (0.4 ml per well). The plates were sealed with Parafilm, wrapped in tinfoil and stored at –18°C until required. *C. viridis* eggs, parasitised 5 days earlier, were disinfected for 2 min in 1.1% sodium hypochlorite, rinsed with sterile physiological saline solution and dissected on a concave slide. Second instar parasitoid larvae were placed onto a diet using a sterile brush (1 larva per plate well), after which the plates were sealed with Parafilm and maintained at 20±1°C, 75% RH and 16L : 8D photoperiod. Instruments and glassware were sterilised by autoclaving for 20 min at 120°C and pressure of 1 bar or by immersion for 1 h in 1.1% sodium hypochlorite. All operations were performed in a laminar flow hood.

Each diet was tested on 24 larvae. The parasitoid development on a diet was followed daily by visual inspection under a microscope and a score for degree of development was attributed.

Each score corresponds to the age in days of the larva inside a host at a specific degree of development characterised by a particular morphology and behaviour (Moratorio & Chiappini, 1995), as indicated below:

5 – white larva (newly-moulted second instar),
8 – orange-coloured larva with white spots,
10 – immobile larva with apical transparent spots behind the mandibles (prepupa),
12 – recognisable ommatidia (pupa),
18 – dark eyes and visible valvae in females (pharate adult).

In both trials about 15 parasitised *C. viridis* eggs were maintained in the same environmental conditions as the rearing plates, in order to verify the correspondence of each score to the matched developmental time.

The results were evaluated in terms of maximum reached score, number of pupae obtained and parasitoid development rate (= days necessary to reach a certain score from score 5 in vitro / days necessary to reach the same score from score 5 in vivo).

A one-way ANOVA was performed on the maximum scores on each of the diet. The data were transformed by y = exp (y) (first trial) or y = Log y (second trial). The development rates were analysed by one-way ANOVA (first trial) or Student t-test (second trial). The data for the number of pharate adults were analysed by 2×2 contingency tables (STATISTICA for WINDOWS, 1994).

TABLE 2. Development of *Anagrus breviphragma* on artificial diets. Maximum scores and development rates [= days from score 5 (2nd instar larva) to 10 (prepupa) or 12 (pupa) in vivo / days from score 5 to 10 or 12 in vitro].

First trial		
Diet	Maximum score	Development rate (from score 5 to 10)
MEYS	12.0±3.1 a	1.53±0.5 a
HEYS	11.7±3.1 a	1.56±0.4 a
IF	17.4±1.8 b	0.97±0.2 b
F	29.15	19.39
df	2, 59	2, 51
P	<0.0001	<0.0001
Second trial		
Diet	Maximum score	Development rate (from score 5 to 12)
IFM	6.91±1.10 a	
IFY	7.13±1.79 a	
IFE	13.47±3.15 b	0.99±0.2 a
IFEY	15.60±3.02 b	0.92±0.1 a
F (df)	95.25 (3, 86)	
t (df)		1,81 (41)
P	<0.0001	0.08

Means in a column followed by the same letter are not significantly different (P<0.05 Tukey-Kramer multiple range test).

RESULTS

The maximum scores and parasitoid development rates are given in Table 2. In the first trial the latter were calculated from second instar larva to prepupa (score 10) as few parasitoids reached the pupal stage (score 12) on the MEYS and HEYS diets. The difference between the numbers of pharate adults obtained on diets IF (= 19) and MEYS (= 3) or HEYS (= 3) was significant (Yates corrected chi-square value = 18.9, df = 1, P = 0.00001). On diet IF almost all parasitoids reached score 18 (pharate adult) but they were deformed and none emerged. Moreover, on this diet the parasitoid developed faster than in the egg of its host.

On diet IFM, 7 out of 24 larvae reached score 8 (orange-coloured larva with white spots) and eventually died. On diet IFY, 6 out of 24 larvae reached the prepupal stage (score 10). There was no significant difference between the numbers of pupae (score 12) obtained on the two diets supplemented with egg yolk, whether alone (diet IFE = 19 pupae) or combined with yeast extract (diet IFEY = 20 pupae). (Yates corrected chi-square value = 0.04, df = 1, P = 0.83). On diet IFE 10 pupae developed to pharate adult stage (score 18) but none emerged. On diet IFEY 12 pupae reached score 18. Of these four females emerged and appeared normal. They were washed and placed, together with in vivo-reared males, on healthy *C. viridis* eggs, but neither mating nor oviposition were observed.

DISCUSSION

The diets tested in the first trial were inadequate for rearing *A. breviphragma*, but, as almost all the larvae reached the pharate adult stage when reared on the IF-diet (cf. Table 2), the latter was selected as the basis for the four media assessed in the second trial.

As found by Consoli & Parra (1997) for *T. pretiosum*, powdered skimmed milk proved to be detrimental for the in vitro culture of *A. breviphragma*. The poor development of the mymarid on diet IFM may have been due to the high osmotic pressure of this diet, rather than the milk itself. In fact dietary osmolarity is an important factor for many endoparasitoids, particularly for those with a thin larval integument (Grenier et al., 1994).

Egg yolk is widely utilised in artificial media for oophagous parasitoids (Grenier et al., 1995; Consoli & Parra, 1997). This ingredient provides a high concentration of well-emulsified lipids, important for both parasitoid and predator development (Grenier et al., 1994). The addition of egg yolk also improved the diet for *A. breviphragma*, as on the medium integrated with this component alone (diet IFE) the ten parasitoids that developed to adulthood were not malformed, though did not emerge. The lack of deformities may have been due to the fatty acids in this diet, which are known to affect the development of other hymenopterous parasitoids (Thompson, 1981; Yazgan, 1981).

Yeast extract is a commonly utilised nutrient source (proteins, amino acids, vitamins and water-soluble growth factors) in insect diets and a key component in artificial media for parasitoids, including oophagous species (Hu et al., 1998). The fact that *A. breviphragma* only reached the prepupal stage on diet IFY, may have been due to the high osmolarity of this diet. This hypothesis is supported by the observation that the parasitoid developed to adult emergence on diet IFEY, which has a lower osmotic pressure. The role of yeast extract in improving the in vitro development of *A. breviphragma*, as well as the tolerance of this parasitoid to high osmolarity deserves further investigation.

Until now the best results were obtained by rearing oophagous parasitoids on diets including insect haemolymph (Thompson, 1999). The deletion of insect material from artificial diets remains a major goal in order to rear parasitoids by eliminating the necessity of production of hosts (Grenier et al., 1994). This work represents the first step in the in vitro rearing of *A. breviphragma* on diets devoid of insect components.

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