THREE METHODS FOR THE *IN VITRO* CULTURE OF *TRICHOGRAMMA PRETIOSUM* RILEY

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ABSTRACT

Three methods, which are more efficient than previously reported techniques, are described for *in vitro* production of *Trichogramma pretiosum* Riley in numbers ranging from a few to thousands. Several antibiotics with concentrations as high as 0.5% were not toxic to the parasitoids, reduced microbial growth, and permitted the successful rearing to the pupal stage of *T. pretiosum* on artificial diet composed of insect hemolymph, egg yolk, and milk. The likelihood that *Trichogramma* can be mass produced efficiently *in vitro* has been significantly increased, but it is essential that the cost and nutritional quality of the artificial diet be significantly improved.

Key Words: *Trichogramma*, parasitoids, rearing, *in vitro*, artificial diets, mass production, growth inhibitors, antibiotics, fungicides.

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INTRODUCTION

One of the the obstacles to the large-scale *in vitro* laboratory production of *Trichogramma* spp. for use in augmentation programs is that rearing containers and methods do not allow the efficient production of these insects on artificial diets. Various containers and techniques have been used for rearing *Trichogramma in vitro* on a small scale. Hoffman et al. (1975) used a small piece of filter paper moistened with artificial diet in a petri dish. The hanging drop technique in depression slides and polyethylene coated artificial eggs was used by Guan et al. (1978) and the Hubei Research Group (1979). However, these methods are not satisfactory for large scale production because they allow only a relatively few *Trichogramma* larvae to be reared in the same container and excessive amounts of diet and labor are required. Microbial contamination also is a problem. Because completely sterile techniques are time consuming and expensive, chemical control of microbial contamination is used for most insect artificial diets. There are no published data concerning the toxicity of widely used microbial inhibitors to developing *Trichogramma*. In this paper we describe new methods and modifications of existing methods which demonstrate the feasibility of rearing large numbers of *Trichogramma in vitro*. With modifications, these methods could be adapted for large-scale production.

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METHODS AND MATERIALS

Stock cultures of *T. pretiosum* were maintained on *Sitotroga cerealella* Olivier eggs at 27°C and 80% RH as described by Morrison et al. (1978); newly emerged adults were used in all tests.

The diet (HEM) was composed of heat-treated insect "hemolymph" (50%), fresh egg yolk (25%), 15 g dried milk suspension/100 ml water (25%, V:V), and 0.15% gentamycin. Hemolymph was obtained from mature *Manduca sexta* (L.) larvae (5th instar) by immersing them in hot water (60 - 62°C) for 10 min, cutting a proleg, and gently squeezing each larva. To kill micro-organisms the milk suspension was stirred and boiled for 15 min in a beaker covered with aluminum foil. Fresh chicken egg yolk was collected using a sterile syringe. All operations were performed in a laminar flow hood. The diet was stored at -14°C.

Trichogramma eggs were obtained using diet HEM (for the microtiter plate and multiple drop methods) or an autoclaved ovipositional stimulant solution (OSB, 0.62% KCl - 0.60% $MgSO_4 \cdot 7H_2O$) (Nettles et al. 1982) for the Petri dish method. The diet or salt solution was encapsulated inside wax spheres (Hagen and Tassan 1965) by placing a thin layer of a mixture of paraffin and petroleum jelly (3:1) on the surface of the ovipositional solution in a glass vial held at 58 - 64°C. The tip of a Pasteur capillary pipette was immersed in the liquid, removed, and touched to the inner surface of a polystyrene petri dish. The resulting wax spheres had a diameter of about 2.0 - 2.5 mm and were exposed to 30 - 50 *T. pretiosum* adults/sphere for 4 - 5 hrs. During exposure the dishes containing the wax spheres were held at 27°C and 70% RH, and rotated at a rate of one rpm to evenly distribute the *T. pretiosum* over the artificial eggs. We could collect about 100 - 200 *T. pretiosum* eggs/wax sphere in 4 - 5 hrs.

After oviposition, the surfaces of the wax spheres were cleaned by filling the petri dish with 0.1% solution of liquid household dishwashing detergent for 1 min and rinsing with cool tap water until all *T. pretiosum* adults and debris were removed. The *T. pretiosum* eggs were then transferred to a container with a glass micropipette and incubated at 27°C, 85 - 95% RH inside a glass desiccator with NaOH solutions used to control humidity.

PROCEDURES AND RESULTS

Microtiter plate rearing

Microtiter plates or tissue culture wells containing 96 flat bottom wells (6.4 mm diameter) were used as *in vitro* rearing containers. Each well contained 7.5 - 10.0 μl of diet, developing larvae could usually be seen with a dissecting microscope about 20 hours after oviposition. The insects grew rapidly and an average of 60 *T. pretiosum* larvae could be reared in each cell. About 66.7 - 78.6% of the larvae developed to pupae, but more than 60% were malformed with oversized abdomens or the pupae were unsegmented. Only 6.5% of the larvae became adults (Table 1). The development from the egg to the adult took 10 - 12 days which was 2 - 4 days longer than those reared on host eggs.

Multiple drop rearing

Plastic petri dishes (5 cm diameter) also were used as *in vitro* rearing containers. Each dish held 5 drops (ca. 15 μl each). The covered petri dish was placed inside

Table 1. Results of rearing *Tricohgramma pretiosum* using three *in vitro* rearing methods.

Method	Ovipositional stimulant-diet*	Number of replicates	No. of larvae	% Yield (range) Pupae	% Yield (range) Adults	Developmental Time-egg to Adult
Microtiter plate	HEM-HEM	4	2,220	73.0(66.7-78.6)	6.5(4.8-7.9)	10-12
Multiple drop	HEM-HEM	3	6,346	60.6(57.6-65.2)	3.2(2.2-4.0)	9-12
Petri dish	OSB-HEM	5	24,472	74.2(38.4-90.9)	2.1(1.4-3.6)	10-13

* HEM-ovipositional stimulant or diet composed of hemolymph (50%), egg yolk (25%), and milk (25%); OSB-ovipositional stimulant composed of KCl(0.60%), $MgSO_4 \cdot 7H_20$(0.62%).

a glass desiccator for incubation at 27°C, 95% RH. After 5 - 6 days when the *Trichogramma* had developed to the prepual stage, the top or bottom of the petri dish holding the developing *T. pretiosum* was placed inside a larger petri dish (10 cm diameter). It was sealed with a piece of masking tape and was placed inside a desiccator at 27°C, 85% RH. The secretion of liquid before adult emergence interfered with the development of other *T. pretiosum*. Therefore, the lower humidity was necessary for higher yields of adults. We reared an average of about 90 larvae per drop. The pupation and adult emergence percentages were 60.6 and 3.2%, respectively (Table 1). The development time from egg to adult was 9 - 12 days. More than 60% of the larvae usually grew excessively and failed to form normal pupae or to become adults.

Petri dish rearing

A top or bottom of a glass petri dish (5 cm diameter) was used as the container. About 5000 - 6000 *T. pretiosum* eggs were transferred from the wax spheres containing KCl - $MgSO_4$ ovipositional solution to a petri dish. One to two minutes after being placed in the container, the *T. pretiosum* eggs settled to the bottom and became weakly attached to the surface of the glass dish. The oviposition stimulant solution was removed gently with a slow speed pump, and we then immediately added about 1.2 ml of HEM diet per dish. The petri dish was held horizontally inside a glass desiccator so that the diet was spread evenly over the bottom of the petri dish. The dishes were incubated at 27°C and 95% RH. Figure 1 shows some of the *T. pretiosum* larvae reared in a petri dish; most of the liquid diet medium has been ingested.

Usually the diet was exhausted when 3500 - 5000 larvae grew to the mature larval stage on the fourth or fifth day. Excessive diet or free liquid remaining on the surface of the prepupae inhibited pupation and reduced yields. Therefore, when there was excessive diet, we transferred the insects with sterile distilled water or a dilute amphotericin B solution (12.5 μg/ml) to a piece of fine plastic cloth (0.22 mm diameter openings) which we used as a sieve. The excess liquid was removed by placing sterile absorbent tissue paper below the cloth sieve. Then the cloth and parasitoids were placed in another sterile petri dish and they were incubated at 27°C, 80 - 85% RH. Amphotericin B was used because prepupae and

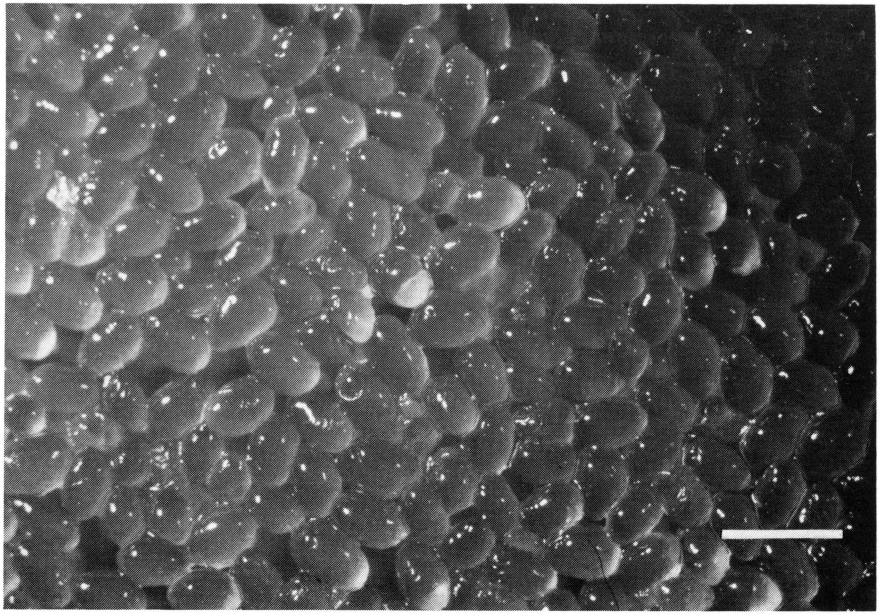

Fig. 1. *Trichogramma* larvae reared in a petri dish, most of liquid diet ingested. Bar = 1.0 mm.

pupae of *Trichogramma* often were attacked by fungi. When microbial contamination (usually *Aspergillus* spp.) appeared, it quickly spread and killed the parasitoids. The experimental results were frequently not uniform. Pupation averaged 74.2% and ranged from 38.4 - 90.9%. A mean of only 2.1% became adults. The development time from egg to adult was 10 - 13 days.

Effects of Antibiotics

Trichogramma pretiosum eggs did not hatch when the artificial diet contained either sorbic acid (0.5 - 2%) or methyl-p-hydroxy-benzoate (0.1 - 0.4%). However, *T. pretiosum* developed when the diet contained as much as 0.5% kanamycin sulfate, 0.5% streptomycin sulfate, 0.15% gentamycin, and 30 μg/ml of amphotericin B. Even at these high levels of inhibitors, usually about 10 - 20% and sometimes even more of the containers became contaminated with microorganisms. The main source of contamination probably is the material that the *T. pretiosum* adults deposit on the surface of the wax eggs. This is the reason for carefully cleaning the surfaces of the wax eggs and removing all of the ovipositing females with a dilute solution of liquid household dishwashing detergent and water before collecting the *T. pretiosum* eggs.

DISCUSSION

Although our yields of *T. pretiosum* pupae were good for all 3 rearing methods (60.6 - 74.2%), mean adult yields were poor and ranged from 2.1 - 6.5% depending

on the rearing method used. This problem was solved by the addition of *M. sexta* egg liquid (Xie et al. 1986) to diet HEM. Because we obtained yields of *T. pretiosum* adults as high as 75.9%, our low adult yields were a result of a deficiency in the HEM diet rather than in our laboratory protocol. Consequently, the methods reported here are suitable for satisfactory production of both pupae and adults.

Comparisons of rearing techniques demonstrate the advantages, significance, and suitable applications of our methods. The depression slide hanging drop technique has been preferred for *in vitro Trichogramma* research. However, this method is very time consuming and provides poor control of humidity or aeration (Liu et al. 1979). Reduced aeration during larval development is probably why our yields of pupae were very low with this hanging drop technique (data not shown). The microtiter plate technique is more convenient and efficient. It allows for better humidity and aeration control which produces higher pupal yields than does the use of the depression slides. While the microtiter plates are suitable for screening artificial diets, neither the hanging drop nor the microtiter plate method is suitable for mass production. However, the multiple drop and the petri dish methods may be suitable for mass production because they provide good control of aeration, humidity, and the quantity of diet per parasitoid, and they allow space to be used efficiently. To conserve space, *Trichogramma* larvae should be reared together in large numbers in the same container; we have reared thousands of mature larvae and pupae of *T. pretiosum* in single containers with these techniques.

The size of the petri dish had no apparent detrimental effect on the growth and development of *T. pretiosum* larvae. When the size of the petri dish was increased to a diameter of 10 cm, we obtained as many as 10,000 larvae per dish. The use of this basic rearing concept with a suitable artificial diet should lead to markedly increased *in vitro* production of *Trichogramma.* However, the larger containers tended to become contaminated more frequently with microorganisms, usually *Aspergillus,* which led to the large variability in pupal yields (Table 1). Thus it was essential that we use microbial growth inhibitors.

The most promising technique for mass production of *Trichogramma* spp. is the multiple drop method using petri dishes or other similar containers. Because the space surrounding each drop of medium strongly inhibited the spread of microbial contamination from drop to drop, the multiple drop procedure is much less susceptible to the spread of microorganisms than is the petri dish method containing hundreds or thousands of parasitoids in the same volume of diet. Although we used small petri dishes, larger vessels undoubtedly would be satisfactory and a variety of simple, inexpensive, autoclavable, and reusable containers could be used. It would not be difficult to develop an automated procedure for quickly dispensing multiple drops of diet containing *Trichogramma* eggs into relatively large containers. The production of 2.1% adults when the ovipositional stimulant was OSB is significant because this is the first time that a species of *Trichogramma* has been reared to the adult from eggs collected using a salt solution as an ovipositional stimulant. An improved diet has increased adult yields to as high as 47% (Xie et al. 1986) when eggs were collected in OSB. Because microbial contamination is a major problem in the *in vitro* rearing of *Trichogramma,* the use of OSB and washing eggs with inexpensive salt solution may allow microorganisms to be reduced to low levels. It also may allow the use of lower concentrations of expensive antibiotics.

Trichogramma are known to be gregarious *in vitro* and the present study demonstrates that *T. pretiosum* are gregarious *in vitro*. We did not directly observe cannibalism among *T. pretiosum* larvae. We did observe that presumably dead eggs disappeared after *Trichogramma* larvae begin to develop. For example, in the tissue culture plate method, when all the eggs in the well were dead, the eggs remained intact in the medium for at least a week. Thus the eggs that disappeared probably were digested by the developing *T. pretiosum* larvae. This process probably is similar to the preoral ingestion of the host egg described by Hawlitzky and Boulay (1982). We confirmed that extra-corporeal digestion occurred by adding pieces of collagen to the medium. Collagen was digested and the liquid was ingested by the *T. pretiosum* larvae. We occasionally observed that newly emerged *T. pretiosum* adults inserted their ovipositors into slowly developing pupae and larvae; this was not a major reason for the low yields of adults on diet HEM.

With the suitable rearing procedures described here and the improved diet described by Xie et al. (1986) *T. pretiosum* can be reared to the pupal and adult stages in large numbers and with high yields. *Trichogramma* are good choices for augmentation programs because they theoretically can be reared *in vitro* in very large numbers. Inexpensive and effective oviposition stimulants are available (Nettles et al. 1982, 1983; Wu and Qin 1982), and cannabalism or fighting does not decrease yields as it does for at least several other species of important egg parasitoids. Mass production now is dependent mainly on the development of improved and inexpensive artificial diets.

LITERATURE CITED

Guan, X. C., Z. X. Wu, T. Y. Wu, and H. Feng. 1978. Studies on rearing *Trichogramma dendrolimi* Matsumura in vitro. Acta Entomol. Sinica 21: 122-26.

Hagen, K. S., and R. L. Tassan. 1965. A method of providing artificial diets to *Chrysopa* larvae. J. Econ. Entomol. 58: 999-1000.

Hawlitzky, N., and C. Boulay. 1982. Regimes alimentaires et developpement chez *Trichogramma maidis* Pintureau et Voegele (Hym. Trichogrammatidae) dans l'oeuf d' *Anagasta kuehniella* Zeller (Lep. Pyralidae). Les Trichogrammes, Les Colloques de l'INRA. pp. 101-6.

Hoffman, J. D., C. M. Ignoffo, and W. A. Dickerson. 1975. *In vitro* rearing of the endoparasitic wasp *Trichogramma pretiosum*. Ann. Entomol. Soc. Am. 68: 335-6.

Hubei Research Group. 1979. Studies on the artificial host egg of the endoparasitoid wasp *Trichogramma*. Acta. Entomol. Sinica 22: 301-9.

Liu, W. H., Z. N. Xie, G. F. Xiao, Y. F. Zhou, D. H. Ouyang, and L. Y. Li. 1979. Rearing of the *Trichogramma dendrolimi* in artificial diets. Acta Phytophylacia Sinica 6: 17-25.

Morrison, R. K., S. L. Jones, and J. D. Lopez. 1978. A unified system for the production and preparation of *Trichogramma pretiosum* for field release. Southwest. Entomol. 3: 62-8.

Nettles, Jr., W. C., R. K. Morrison, Z. N. Xie, D. Ball, C. A. Shenkir, and S. B. Vinson. 1982. Synergistic action of potassium chloride and magnesium sulfate on parasitoid wasp oviposition. Science (Washington). 218: 164-6.

Nettles, Jr., W. C., R. K. Morrison, Z. N. Xie, D. Ball, C. A. Shenkir, and S. B. Vinson. 1983. Effect of cations, anions and salt concentrations on oviposition by *Trichogramma pretiosum in wax eggs. Entomol. Exp. et Appl. 33:283-9.*

Wu, Z. X., and J. Qin. 1982. Ovipositional response of Trichogramma dendrolimi to the chemical content of artificial eggs. Acta Entomol. Sinica. 25: 363-72.

Xie, Z. N., W. C. Nettles, Jr., R. K. Morrison, K. Irie, and S. B. Vinson. 1986. The effect of ovipositional stimulants and diets on the growth and development of *Trichogramma pretiosum* Riley *in vitro*. Ent. Exp. Appl. (in press).