Artificial Media and the Control of Microorganisms in the Culture of Tephritid Larvae (Diptera:Tephritidae)

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The results of initial trials using a blended[2] Hubbard squash, *Cucurbita maxima* var., diet for culturing the larvae of the oriental fruit fly, *Dacus dorsalis* Hendel, were very erratic; at times a good yield of mature larvae was obtained, while at other times little or no development occurred. These inconsistent results were probably caused by certain microorganisms invading the food, since no effort was made to sterilize equipment or to protect the medium from airborne contamination.

Experiments using blended squash under aseptic conditions demonstrated that this medium alone was apparently nutritionally deficient Under the same sterile conditions good larval development was obtained when the blended squash was supplemented with certain amounts of dried brewers' yeast. These results suggested that certain microorganisms, perhaps certain airborne yeasts, in controlled amounts, were beneficial but that other yeasts, molds and bacteria were detrimental.

It was considered impractical to carry on a mass-culture program under aseptic conditions, so a search was made for other means of controlling microorganisms.

The first step was to formulate a synthetic diet that would inhibit the growth of undesirable microorganisms without changing the basal nutrients required for good larval development. The basis of this work was a synthetic medium developed by Beck *et al.* (1949) for rearing the larvae of the corn borer, *Pyrausta nubilalis* (Hübner) under aseptic conditions. It was found that in order to adapt this medium to the peculiar requirements of the fruit fly larvae, certain physical and nutritional changes had to be made. In order to learn these requirements, the larvae had to be reared aseptically and fed known nutrients.[3] The optimal concentrations of many of the nutritional factors were determined.

The larvae of the oriental fruit fly, *Dacus dorsalis* Hendel, the melon fly, *D. cucurbitae* Coquillett and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) were all reared under aseptic conditions on synthetic media. These tephritid larvae were also reared on synthetic media exposed to airborne contaminants.

Materials and Methods.—The materials and methods used will be discussed under the subsequent sub-topics.

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[2]A commercial high speed blender designed for home use was employed.

[3]Since dried brewers' yeast was used as the B group vitamin source, individual vitamins other than choline were not varied. The protein source was dried brewers' yeast and casein in some cases.

Separation and Sterilization of Eggs

The eggs of *D. dorsalis* and *C. capitata* were deposited in sections of orange rind which had been sealed to a glass slide with paraffin and placed in the cages with the gravid flies. This technique of obtaining eggs was developed by the earlier workers on the Mediterranean fruit fly when it was present in Florida. The eggs were washed from the rind section into water. Only well separated eggs were pipetted and placed in a 20 milliliter centrifuge tube containing mercuric bichloride solution (1:1000). The tube of eggs was shaken from time to time during a ten minute sterilization period, after which time the eggs were rinsed three times with sterile distilled water. The disinfected eggs were then pipetted onto a piece of thin, white cloth in a sterilized petri dish. Sterile water was added to keep the egg pad moist. The eggs were held for about 24 hours, in which time most of the eggs hatched. The larvae were used within a few hours after hatching.

Preparation of the Artificial Media

The agar agar was placed in a flask with water and brought to a boiling point. The remaining ingredients were added and the mixture boiled for a few minutes. In each test diet, only one factor was varied. The approximate pH of the media was 5.5. Nitrazine paper was used to determine the pH of the media. Five milliliters of medium were placed in 12 x 125 mm. test tubes, which were plugged with cotton and the upper portions of the tubes covered with paper. The tubes were autoclaved at fifteen pounds pressure for fifteen minutes, then slanted and shaken just before the agar set to insure uniform distribution of nutrients in the medium.

Larval Culture

Five larvae, hatched from surface sterilized eggs, were placed on the artificial medium slant by a sterilized dropper. The slant was examined every day. At the end of six days, only the full grown larvae were counted and weighed. The larval response to a medium was calculated in terms of the weights and the per cent of larvae maturing (herein referred to as recovery).

Sterility Test

At the end of the larval growth period, sterility tests of each tube of medium were run. The tests were made by inoculating a loopful of "digested" medium into brewers' fluid thioglycolate medium and tryptone or nutrient broth. No growth of microorganisms in the broth indicated that the larvae developed under aseptic conditions. Contaminated cultures were discarded. Only 1.4 per cent of the culture became contaminated.

Experimental Design

Twenty-one different diets were tested in triplicate. This entire series was again replicated, and the data shown in Tables 2, 3, and 5 represent the averages from both series.

The diet composition shown in Table 1 was used as the control, or standard diet, in these tests. Unless otherwise idicated, only a single ingredient was varied in concentration from that indicated in the standard diet. The results in larval weights and larval recovery shown opposite each ingredient in all the tables are based upon rearing tests where each listed ingredient was in each case used at the listed concentration as a part of a diet which was identical with the standard diet except in the quantity used of that particular ingredient.

Table 1. **Composition of standard diet.**[4]

Substance	Grams per 100 ml. distilled water
Flaked agar agar	1.1
Glucose	4.9
Casein	1.5
Cholesterol	0.175
Wheat germ oil	0.175
Salt mixture (U.S.P.XIII)[5]	0.35
Brewers' yeast	1.75
Choline chloride	0.07

[4]pH 5.5

[5]Nutritional Biochemicals salt mixture No. 2.

Results.—The experiment outlined in Table 2 was conducted at a mean temperature of 80° F., fluctuating from 72° to 87° F. The relative humidity averaged about 62 per cent, with a range of from 40 to 80 per cent.

Table 2. **Effect of various nutrients on *D. dorsalis* larval growth and recovery under aseptic conditions.**

Diet	Varied ingredient in otherwise standard diet	Per cent	Aver. larval wt. in mg.	Aver. % larval recovery
1.	Brewers' yeast	0.0	All died	0.0
2.	Brewers' yeast	2	21.4	76.6
3.	Brewers' yeast	4	19.9	66.7
4.	pH 4.5		21.0	85.0
5.	Cholesterol Wheat germ oil	0.0 0.53	20.8	73.3
6.	Cholesterol	0.35	20.7	85.0
7.	Cholesterol Wheat germ oil	0.35 0.0	21.0	90.0
8.	Standard diet[6]		20.4	88.3

[6]Composition of standard diet shown in Table 1.

The data shown in Table 2 indicate that the 1.75 per cent level of brewers' yeast which was present in the standard diet stimulated the best recovery of *D. dorsalis* of any of the levels of yeast used. When brewers' yeast was omitted entirely from the diet none of the larvae matured. Increasing the concentration of brewers' yeast above 1.75 per cent appeared to be somewhat detrimental to larval recovery. The omission of both cholesterol and wheat germ oil from the diet (not tabulated) resulted in poor growth. The standard amounts of cholesterol and wheat germ oil (1.75 per cent of each), or increasing the cholesterol alone (diet 6), did not produce the degree of recovery produced by the 0.35 per cent level of cholesterol without the wheat germ oil (diet 7).

Since cholesterol is rather expensive for rearing large numbers of larvae, lanolin was tried as a substitute. The larvae reared on diets containing from 0.05 to 0.25 per cent lanolin did not develop normally. Lanolin (hydrous wool fat) is an ester of cholesterol and apparently is not easily hydrolyzed.

Another series of experiments was conducted varying the remaining nutritional factors not treated in the foregoing tests. This series of experiments (Table 3) was carried out at room temperatures fluctuating between 74° to 86° F., and the relative humidity varied from 46 to 80 per cent. The mean temperature and humidity were 80°F. and 63.5 per cent, respectively.

Table 3.—**Effect of various nutrients on *D. dorsalis* larval growth and recovery under aseptic conditions.**

Diet	Varied ingredients in otherwise standard diet	Per cent	Aver. larval wt. in mg.	Aver. % larval recovery
1.	Glucose	2	21.7	36.6
2.	Glucose	6	20.3	56.6
3.	Sucrose Glucose	2 0	20.7	63.3
4.	Sucrose Glucose	4 0	21.7	100.0
5.	Salt Mixture (U.S.P. XIII)	0	18.7	66.6
6.	Salt mixture (U.S.P. XIII)	0.6	19.8	73.3
7.	Choline chloride	0	18.4	76.6
8.	Choline chloride	0.2	20.5	66.6
9.	Peptone Casein	2 0	21.7	13.3
10.	Casein	0	20.5	49.8
11.	Casein Brewers' yeast	0 3.25	20.0	80.0
12.	Casein	3	20.5	60.0
13.	Standard diet[7]		19.9	80.0

[7]Standard diet formula shown in Table 1.

The data shown in Table 3 indicate the best levels for the following: sucrose 4.0 per cent; salt mixture 0.35 per cent; choline chloride 0.07 per cent; brewers' yeast 3.25 per cent.

The diet containing 2.0 per cent sucrose apparently did not furnish sufficient carbohydrate as indicated by the excellent larval recovery from a 4.0 per cent sucrose diet. The best larval recovery from a glucose source was obtained at a concentration of 4.9 per cent (standard diet). The larval recovery from the 2.0 per cent sucrose diet (diet 3) was nearly double that of the 2.0 per cent glucose diet (diet 1). This possibly indicates that the fructose molecule of sucrose is an important factor. This is shown also when comparing the glucose control diet to the 4.0 per cent sucrose diet. However, under insectary conditions where aseptic techniques were not exercised, it was found that 4.9 per cent glucose concentration gave better results than did the 4.0 per cent sucrose concentration. It appears that using sucrose under these conditions permits vigorous and detrimental fermentation.

Of the salt concentrations used, the control diet, which contained 0.35 per cent salt mixture, stimulated the best larval development, while increasing the salt content to 0.6 per cent, or omitting it altogether was inferior. However, the diet was not entirely devoid of salts in the latter diet for brewers' yeast contains minerals.

The most effective concentration of choline chloride was found to be 0.07 per cent. Although the diets were not supplemented with choline, a sufficient amount was present, evidently being provided through the use of brewers' yeast. A diet containing 3.25 per cent brewers' yeast and no casein (diet 11) stimulated nearly the same degree of larval recovery as did the control diet (diet 13) which contained 1.5 per cent casein and 1.75 per cent dried brewers' yeast. Nearly half the weight of brewers' yeast is protein. Consequently, increasing the yeast concentration in a diet compensated for the omission of casein as a protein source. A diet without casein and containing only 1.75 per cent brewers' yeast only gave 50 per cent larval recovery. Increasing the concentration of casein to the 3.0 per cent level was detrimental to larval development. The use of peptone as a source of protein supported good growth but permitted poor larval survival.

A new, improved diet was formulated, and was the product of the foregoing experiments in that the proved levels of each ingredient were used. The formula of the improved diet is shown in Table 4.

Table 4.—**Composition of an improved purified diet for rearing *D. dorsalis* larvae under aseptic conditions.**

Substance	Grams per 100 ml. distilled water
Flaked agar agar	1.1
Sucrose	4.0
Cholesterol	0.35
Salt mixture (U.S.P. XIII)	0.35
Brewers' yeast	3.25
Choline chloride	0.07

The results obtained from comparing the improved diet (Table 4) with that of the original standard diet (Table 1) are shown in Table 5. Also, two other media having the same composition as the standard diet but differing only in pH were tested. These data are also shown in Table 5. The above experiment was subject to room temperatures fluctuating between 74° to 85°F., and the humidities varied from 48 to 80 per cent. The mean temperatures and humidities were 80.5 and 62 per cent respectively.

Table 5.—**Effect of different pH reactions and the new, improved diet upon the development of *D. dorsalis* larvae, aseptically reared.**

Diet	pH	Aver. larval wt. in mg.	Aver. % larval recovery
Improved[8] with pH	5.5	24.34	100.0
Standard[9] with pH	5.5	23.32	96.6
Standard with pH	7.0	23.1	90.0
Standard with pH	8.0	23.0	96.6

[8]Composition of Improved diet shown in Table 4.
[9]Composition of Standard diet shown in Table 1.

The data shown in Table 5 indicate that the improved diet stimulated better larval growth and recovery than was obtained from the standard diet, although the differences are so small as to suggest need for further testing.

The *D. dorsalis* larvae apparently can tolerate a wide range of acidity and alkalinity under aseptic conditions, for it has been shown that they developed equally well in media of pH 4.5, 5.5, 7.0 and 8.0. However under insectary conditions where aseptic techniques were not followed any media with a pH higher than 5.5 permitted poor larval growth as a result of heavy bacterial contamination. Thus it was possible to mass-culture the larvae under normal laboratory conditions in the presence of bacteria by adjusting the acid reaction to pH 4.5.

Larval Population Density supported by an Artificial Medium

The following experiment was designed to determine the number of *D. dorsalis* larvae that one gram of synthetic medium would support. The medium employed was the improved diet shown in Table 4. Five milliliters of this medium were placed in test tubes and slanted. The eggs were sterilized as already described. Triplicate tubes were used for each larval density tested. The results obtained are shown in Table 6.

Table 6.—**Population density of aseptically reared *D. dorsalis* larvae supported by one gram of artificial medium.**[10]

No. of larvae per gram of medium[10]	Larvae per tube	Aver. No. of larvae recovered	Per cent recovery	Aver. wt. of mature larva in mg.
3	15	13	86.6	17.9
4	20	17	85.0	16.7
5	25	23	92.0	16.6
6	30	24	81.6	14.6
7	35	32	91.4	14.5
8	40	32	80.0	15.0
9	45	41	92.2	13.9
10	50	44	88.0	12.4

[10]Composition of medium shown in Table 4.

The data shown in Table 6 show that as the number of larvae per gram of medium increased the larval weight decreased. The number of larvae maturing remained fairly constant. The optimal range appears to be between three to eight larvae per gram of medium. Apparently one gram of medium can actually support more than ten larvae, but the exact number was not determined.

Rearing *D. dorsalis* Larvae in synthetic Media exposed to airborne Contaminants

Since it was impractical to mass-culture the tephritid larvae under aseptic conditions, various artificial media were tested, utilizing facts which were learned from the experiments with aseptic media. Marucci and Clancy (1950) found that the agar base medium known as the Texas *Drosophila* formula and a yeast-agar-water medium supported larval development of *D. dorsalis, D. cucurbitae* and *C. capitata*. The control of detrimental microorganisms was apparently obtained by relying on larval density. The density found necessary to prevent a surface scum was from 50 to 100 larvae per Petri dish.

Since it was rather difficult to depend on a definite number of larvae hatching, because of some variability in egg fertility, it was necessary to develop a medium that would support a wide variation of larval population densities. The only limit necessary to observe would be that of overpopulating a tray of medium, which would result in smaller sized flies. Consequently, a medium was desired that would support a lower density than the actual medium potential in the event of poor egg hatch and in spite of being exposed to microorganisms.

The formula of the purified medium adopted for mass-culturing *D. dorsalis* larvae exposed to potential airborne contamination is shown in Table 7.

Table 7.—Composition of a purified diet utilized in the mass-culture of *D. dorsalis* larvae in the presence of microorganisms.

Substance	Grams per 100 ml. of water
Agar	1.3
"Butoben"[11]	0.12
Glucose	4.9
Wheat germ oil	0.175
Cholesterol	0.175
Salt mixture (U.S.P. XIII)	0.35
Brewers' yeast	3.0
Choline chloride	0.07

[11]N-butylparahydroxybenzoate—Merck.

The preparation of the medium outlined in Table 7 involved boiling the mixture for several minutes to dissolve or suspend the ingredients, allowing it to cool and adjusting the reaction to approximately pH 4.5 with 0.1 N hydrochloric acid. After the agar had set, the medium was blended in a high speed blender to obtain a pulpy consistency. The medium was stored in a refrigerator and used when needed.

The cost of this synthetic medium for mass-culture of *D. dorsalis* was nearly prohibitive even though an average of 5000 mature larvae were obtained, in eight days, from 7000 eggs placed in 800 milliliters of medium. However, the knowledge gained from developing the above diets made possible the final practical medium. This proved to be a blended vegetable medium which supplied the bulk of the expensive nutritive factors, lipids primarily, fortified with certain nutrients that were lacking. Brewers' yeast was used to provide the deficient nutritional factors. Since agar was not used, the preparation of the medium was simplified. From the foregoing results and those of previous workers dealing with other insects, it was found that "Butoben" would inhibit the molds and yeasts. The bacterial growth was controlled by altering the pH to 4.5 by adding a certain amount of hydrochloric acid.

Larval Culture of *Ceratitis capitata*

The standard synthetic medium (Table 1) was used for culturing *C. capitata* larvae under aseptic conditions using proved methods and techniques as in *D. dorsalis* experiments. In the following experiment only the level of the brewers' yeast was varied. A pumpkin medium supplemented with two per cent dried brewers' yeast was also tested. The various yeast concentrations tested and the results obtained are shown in Table 8.

Table 8.—**Effect of different brewers' yeast concentrations in various artificial media upon the larval development of *C. capitata* under aseptic conditions.**

Medium	Per cent brewers' yeast	Aver. larval wt. in mg.	Aver. % larval recovery
Standard[12]	1.75	14.53	86.6
Standard	2.0	12.66	80.0
Standard	4.0	13.85	46.6
Pumpkin[13]	2.0	13.3	66.6

[12]Composition of standard medium shown in Table 1.
[13]Pumpkin pulp was blended. A variety of *Cucurbita pepo* was used.

The data shown in Table 8 indicate that 1.75 per cent brewers' yeast level in a synthetic medium permitted growth of larger larvae and a greater number to mature.

Larval Culture of *Dacus cucurbitae*

A single experiment was conducted to determine whether the melon fly larvae would develop in a synthetic medium under aseptic conditions. Blended pumpkin, *C. pepo* var., and the standard purified medium (Table 1) were tested. The same techniques and methods were used as in the previous experiments.

The larval development was better in the standard medium than that obtained from the blended pumpkin medium supplemented with yeast. The larval growth was variable when melon fly larvae were reared in the pumpkin medium, as indicated by the number of undeveloped larvae which resulted. However, in the artificial medium (Table 1), the larval development was good and the sizes of the larvae were more uniform.

Summary

A method was found whereby *Dacus dorsalis, D. cucurbitae* and *Ceratitis capitata* larvae could be reared in synthetic media under aseptic conditions. Major nutritional factors were varied to ascertain "optimal" levels of each factor. The synthetic diets led to the development of an economical artificial medium[14] which was used in the mass-culture of three species of fruit fly larvae without requiring expensive aseptic techniques or the use of purified diets.

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[14]This medium will be described in a future paper by G. L. Finney.