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Modification of the Oligidic Medium for Axenic Culture of *Aphelenchoides rutgersi*

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Abstract: Aphelenchoides rutgersi was axenically cultured in modified Soytone, yeast extract, lyophilized chick embryo extract medium (3% ST:2% YE:20% CEE-L, w/v:w/v:v/v). Earlier formulations used 10% CEE, v/v, before the manufacturer changed the preparation. After reestablishing *A. rutgersi* in medium that permitted continuous subcultivation and reproduction, a second medium was tested that contained 0.5% sucrose and 0.5% Lipid Concentrate. The commercially available Lipid Concentrate made it possible to incorporate nonaqueous soluble chemicals into the medium. In addition, 0.1% Fast Green #3 was added to both media to visually demonstrate active ingestion of nutriment.

Key words: Aphelenchoides rutgersi, axenic medium, culture, nematode, oligidic medium.

Successful axenic cultivation in aqueous, oligidic medium of a stylet-bearing nematode later identifed as *Aphelenchoides rutgersi* Hooper & Myers, 1971 (2) was first reported by Myers (3,5,7). Since the first report of axenic cultivation, *A. rutgersi* has been cultured in partially defined (meridic) medium, and its requirements for amino acids, carbohydrates, nucleic acids, and some vitamins have been studied (1,4,6,8,10,12).

Recently, research on the cultivation of *A. rutgersi* was resumed. However, modifications to the original culture medium were necessary because the aqueous chick embryo extract (CEE) used in the previously developed medium was unavailable, and the preparation of lyophilized CEE (CEE-L) had been modified by the manufacturer. Other basic problems also existed in the formulation of media that could be used for the cultivation of stylet-bearing nematodes. Low solubility or insolubility of lipids, certain vitamins, cholesterol, and similar molecules limited their availability to stylet-bearing nematodes feeding in aqueous media.

Ingestion of nutriment (feeding) can only be assumed by subsequent growth, development, or reproduction of cultured nematodes, but when these parameters are absent, ingestion of nutriment cannot be confirmed and suitability of media cannot be verified. This problem has been partially alleviated by the addition of dye such as Amaranth to demonstrate feeding and ingestion visually (13).

The objectives of this research were to re-establish *A. rutgersi* in axenic culture, to develop an oligidic medium in which lipid soluble nutrients could be dissolved, and to select a dye that would provide a visual indication of the ingestion of nutriment.

MATERIALS AND METHODS

Nematode preparation: Aphelenchoides rutgersi was reared on *Botrytis cinerea* Pers. growing on potato dextrose agar (PDA). A 2 cm^2 piece of PDA and fungus, which also included nematodes, was placed in the center of a petri dish (10 cm-d) containing 1.5% water agar (WA). After several hours, when the nematodes had migrated throughout the layer of WA, they were extracted from strips of WA in an autoclaved Baermann funnel apparatus. The apparatus consisted of a wire screen that was covered with tissue paper and inserted in a test tube (2 × 10 cm). The tube contained deionized water above the level of the tissue paper and the apparatus was closed with a metal cap to maintain sterility. Nematodes that migrated from the WA strips and through the tissue paper were used for inoculum. The number of nema-

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todes was adjusted in concentration by dilution with sterile water. Nematodes inoculated into salt solution controls (SS) were counted to estimate the initial number of nematodes inoculated into the various media.

Medium preparation: Sterile plastic, screw-cap tubes (13 × 100 mm) were inoculated either with 0.5 ml of media or SS containing adult females and juvenile nematodes. Five replications of media and the SS control were usually used but when dyes were incorporated, 10 replications were used. Tubes were slanted to provide greater surface area for oxygen diffusion and reduced depth of nutriment. SS mixed at 2× strength contained 240 mM NaCl, 40 mM KCl, 8 mM $MgCl_2$, and 8 mM $CaCl_2$.

Lyophilized CEE (CEE-L, #620-5115AD GIBCO/BRL, Grand Island, NY) was used in place of the unavailable aqueous or lyophilized CEE. Initial attempts failed to establish *A. rutgersi* in the previously successful medium containing 3% Soytone plus 2% yeast extract supplemented with the substituted 10% CEE-L (9). Dry weight of just three samples (bottles) of CEE-L ranged from 0.26 to 0.31 g. The CEE-L was tested at concentrations of 10, 15, 20, and 25% in media containing Soytone (ST, #0436-01 Difco Laboratories, Detroit, MI), Select yeast extract (YE, #M00393B, GIBCO/BRL), and antibiotic solution consisting of 10,000 µg/ml Penicillin G (sodium), 10,000 µg/ml Streptomycin sulfate, and 20 µg/ml Amphotericin B dissolved in 0.85% NaCl solution (#600-5240PE GIBCO/BRL). Constituent concentrations and preparation of C-1 medium are listed in Table 1. Chick embryo extract, ultrafiltrate (CEE-U, #620-6460AD GIBCO/BRL) was examined as a possible substitute for 20% CEE-L in C-1. During commercial preparation of CEE-U, molecules larger than 60 A, including the reddish-colored hemoglobin, were removed. CEE-U was tested with and without 10 µg/ml of hemoglobin (#0136-02-5 Difco Laboratories, Detroit, MI).

Lipid Concentrate (LC, #680-1900AG GIBCO/BRL) was incorporated into C-1 and tested at 0.01%, 0.05%, 0.1%, 0.5%, and 1% (v/v). Aqueous LC contained 1,000 mg/L cod liver oil, 450 mg/L cholesterol, 100,000 mg/L Pluronic F-68, 200 mg/L D-α-tocopherol acetate, and 2,500 mg/L Tween 80. The 0.5% LC contained a final concentration of 2.25 µg cholesterol/ml of C-2 (Table 1). In addition to 0.5% LC, 0.5% sucrose, w/v, (S, #4702-01, J. T. Baker, Phillipsburg, NJ) was also included in C-2 (Table 1).

Stock cultures of *A. rutgersi* were maintained at 20–22C in 30 ml tissue culture flasks (Cat. No. 10-126-9, Fisher Scientific Co., Springfield, NJ) containing 5 to 10 ml of C-1 medium, and these were subcultured every 1–½ to 2 months.

TABLE 1. Media used for rearing *Aphelenchoides rutgersi* axenically. Diet was prepared in the order of occurrence of ingredients to make a final volume of 10 ml.†

Diet ingredient	C-1 (ml)	C-2 (ml)
10% yeast extract + 15% Soytone, aqueous solution, w/v‡	2.0	2.0
Water	4.0	3.0
Antibiotics§	1.0	1.0
Chick embryo extract-lyophilized. Rehydrate with 10 ml water	2.0	2.0
10% lipid concentrate, aqueous solution, v/v		0.5
10% sucrose, aqueous solution, w/v		0.5
1% Fast Green, aqueous solution, w/v‖		
Nematodes in water or media	1.0	1.0

† Autoclave 5× yeast extract–Soytone solution, sucrose, water, and dye solutions at 121 C for 15 minutes before mixing ingredients.

‡ Adjust to pH 7.0 with 0.1N KOH before autoclaving.

§ Adjust volume of water as necessary if antibiotics or dye solution is deleted or added to medium.

‖ Fast Green #3 may be added to media to produce a 0.1% final concentration to visually demonstrate feeding.

Staining procedures: The following food colorings were tested at a concentration of 0.25% (w/v) to verify media ingestion: FD&C Amaranth Red #2 (CI 16185), FD&C Brilliant Blue #1 (CI 42090), FD&C Fast Green #3 (CI 42053), FD&C Erythosine Red #3 (CI 45430), FD&C Allura Red AC #40 (CI 16035), and Citrus Red #2 (CI 12156). Dyes were added to SS as controls, and each treatment was replicated ten times. Nematodes were removed from the dye solutions after 1, 7, and 14 days by washing them with water on a 25-μm-pore (500 mesh) screen, and any coloration of cuticle, intestinal contents, or total body was observed by brightfield microscopy at ×100 and ×400 magnification. Nematode numbers were determined after 28 days. In addition, Fast Green #3 was tested at concentrations of 0, 0.0001, 0.001, 0.01, 0.1, 0.15, 0.20, and 0.25% (w/v) in both SS and in C-2. In other experiments, 0.1% Fast Green #3 was incorporated into both C-1 and C-2 to demonstrate nutriment ingestion.

Nematodes were counted after 14 and 28 days by inverted brightfield microscopy (×40), using the top half of 6 cm-d tissue culture dishes (#3009, Falcon Plastic, Los Angeles, CA 90045) on which parallel lines were scribed 1 cm apart. Media were diluted with water to reduce nematode numbers between 50 and 400/dish, and the nematodes occupying 50% of the dish surface area were counted. Usually, only total numbers of nematodes consisting of both parthenogenetic females and juveniles were counted; but in some experiments the numbers of living and dead nematodes, eggs, juveniles, and adults were also counted. Nematodes were tallied as dead when no movement was detected and their bodies were in a linear or relaxed condition.

Data analysis: Data were analyzed using various SAS programs (11). Means and standard deviations were calculated. The GLM procedure for ANOVA, and Duncan's multiple-range test were selected to test means ($P \leq 0.05$).

Results

The numbers of *A. rutgersi* per tube after 28 days in aqueous 3% ST + 2% YE medium containing either 15% CEE-L (5,610 ± 527) or 20% CEE-L (5,330 ± 1,170) were greater than the numbers initially inoculated as represented by the SS control (188 ± 36, $P \leq 0.05$). Little reproduction occurred in media compounded with 5% CEE-L (790 ± 43) or 10% CEE-L (652 ± 194).

Over 10 serial subcultures were completed in medium C-1 without loss of fecundity. Egg counts were generally between 3,000 to 6,000 in C-1 or C-2 at 28 days, and these cultures usually contained about 15% adult females. Seven-day-old cultures sometimes contained two to three times as many females as 28-day-old cultures. Up to 50,000 eggs accumulated after 1 to 2 months in cultures initially inoculated with 1,000 or more nematodes/ml into media ≥1 cm deep. Although egg hatch was retarded, maturation to egg-producing adult females continued. Egg hatch was not retarded in 28-day-old cultures when medium was reduced to 5 mm deep, initial inoculum restricted to under 500 nematodes/tube, and tubes were slanted. However, final numbers of all stages were affected by the number of parthenogenetic females initially inoculated into tubes. When stock cultures were kept for over 2 months, egg hatching was also retarded. Hatching was not stimulated by decreasing the medium to 5 mm depth to increase gaseous diffusion. Rapid hatching of eggs occurred when the medium was diluted 2× with water.

No increase in total mean numbers of *A. rutgersi* over the SS control (244 ± 64 nematodes/tube) was found 28 days after inoculation, when 20% CEE-U (319 ± 86 nematodes/tube) was used instead of 20% CEE-L in medium C-1, or when 20% CEE-U with 10 μg/ml of hemoglobin (340 ± 45 nematodes/tube) was substituted for CEE-L ($P \leq 0.05$). Culture medium prepared with 20% CEE-L contained 2,536 ±

817 *A. rutgersi*/tube and was significantly greater than the SS controls ($P \leq 0.05$). CEE-U could not be substituted for CEE-L.

There was no difference in the total number of nematodes at 21 days within the range of 0.01% (2,428 ± 41 nematodes/tube) to 1% LC (2,587 ± 378 nematodes/tube), when incorporated into diet C-1, as compared with C-1 without LC (2,853 ± 596 nematodes/tube). In two of these experiments, nematodes reared in C-2 produced greater numbers of progeny (Table 2).

After 24 hours incubation in C-1 medium containing 0.25% Amaranth Red #2, a faint pink color was noted in the intestinal lumen of some *A. rutgersi*. However, color did not intensify in the intestinal lumen even after 28 days, and the intestinal contents remained unstained. Some loss of intestinal contents was noted at 7 days, but the loss of granular materials from the intestine was severe by 28 days. Granules remained only in a thin layer lining the lumen of the intestine. Loss of intestinal contents coincided with reduced reproduction (Table 3, Expt. 1). Only an occasional nematode ingested a small amount of 0.25% dye when incubated in SS containing any of these dyes. Citrus Red #2 reacted with the C-2 medium and a precipitate formed (Table 3, Expt. 2). Color intensity of Amaranth Red #2 and Allura Red #40 in the intestinal lumen was less than optimal. Both Fast Green #3 and Brilliant Blue #1 were more easily observed in the intestinal lumen than the other dyes, and their effect on nematode reproduction was less, especially when dyes were incorporated in C-2. Very slight cuticular coloring was noted when nematodes were incubated in Brilliant Blue #1.

TABLE 3. Mean numbers of *Aphelenchoides rutgersi* after 28 days in diets C-1 or C-2, and C-1 or C-2 with 0.25% dyes incorporated.

Incubation liquid	Experiment 1	Experiment 2
C-1	4131 a†	
C-1 + Amaranth Red #2	1489 b	
C-2 + Allura Red #40		3360 a
C-2		2821 b
C-2 + Fast Green #3		2766 b
C-2 + Brilliant Blue #1		2730 b
C-2 + Amaranth Red #2		1818 c
C-2 + Citrus Red #2‡		
Salt solution control§	192 c	231 d

† Mean numbers of nematodes in columns (10 replications) followed by a different letter are significant at $P \leq 0.05$.
‡ Because Citrus Red #2 reacted with C-2, precipitate prevented accurate nematode counts.
§ Mean number of nematodes in the salt solution control is the initial number of nematodes inoculated into culture medium.

Reproduction was reduced in cultures (C-1) containing ≥0.1% Fast Green #3. The SS control averaged 111 ± 25 *A. rutgersi*/tube, 28 days after inoculation, whereas C-1 yielded 2,788 ± 565 nematodes/tube. Cultures containing 0.1% Fast Green #3 contained significantly fewer nematodes (1,586 ± 357 nematodes/tube), whereas the numbers of nematodes in cultures containing ≤0.01% Fast Green #3 were not significantly different from those found in C-1 ($P \leq 0.05$). The color inten-

TABLE 2. Mean total numbers of *Aphelenchoides rutgersi* after 28 days in diets C-1 and C-2, and in modified C-1 and C-2.

Incubation liquid	Experiment 1	Experiment 2	Experiment 3†
C-2	9491 a‡	8196 a	6174 ab
C-1 + 0.5% Lipid Concentrate	5039 b	6496 bc	5720 ab
C-1 + 0.5% Sucrose	4160 bc	7276 ab	5512 b
C-1	3781 c	6584 bc	6858 a
C-1 + 0.1% Fast Green		6184 c	1972 d
C-2 + 0.1% Fast Green		5052 d	3152 c
Salt solution control§	325 d	315 e	191 e

† The 5× yeast extract–Soytone solution mixture and the 1% Fast Green #3 were autoclaved three additional times.
‡ Mean numbers in columns (five replications) followed by a different letter are significant at $P \leq 0.05$.
§ Mean number of nematodes in salt solution control is the initial number of nematodes inoculated into culture medium.

sity of Fast Green #3, while visually detectable at 0.1%, was below optimum at 0.01% in the intestinal lumen of fourth-stage juveniles and adults. The increased magnification needed to detect color in the intestinal lumen, and the small size of other juveniles, caused unreliable color observations.

Cuticular bubbles, especially near the anus, and misshapen tails sometimes developed as females matured in 5× strength ST-PY autoclaved several times. Repeated autoclaving of Fast Green #3, before its incorporation into nutriment, had an adverse effect on nematode reproduction (Table 2, Expt. 3).

Discussion

Axenic culturing of *A. rutgersi,* in oligidic media as described in this study, refers to serial subcultivation in media made from crude, undefined tissue extracts. Reproduction from egg to egg occurred within a normal period of time (9). Prior to being described as *A. rutgersi* Hooper & Myers, 1971, this nematode was mistakenly identified as *A. dactylocercus* or *A. sacchari* (2). *Aphelenchoides cibolensis, A. dactylocercus, A. sacchari,* and *Aphelenchus avenae* were also cultured successfully in oligidic and meridic media (Myers, unpubl.).

The original liquid or lyophilized CEE, no longer available, had been prepared from 10-day-old embryos homogenized in Gey's balanced salt solution, with 100U/ml streptomycin sulfate added. The CEE-L currently available was prepared in a similar manner, except that 7- to 9-day-old embryos were used before lyophilization. CEE-L was reconstituted with 10 ml of sterile water, but dry weight/bottle of lyophilized CEE varied by over 15%. Originally 10% CEE, either liquid or lyophilized, supported normal growth and reproduction of *A. rutgersi, A. sacchari, A. cibolensis,* and *A. avenae,* but currently 15% to 20% concentrations of CEE-L is required to culture *A. rutgersi.* The quality of commercial batches of CEE also was not constant, as indicated by varying reproductive rates. Neither C-1 nor C-2 supported reproduction of *A. avenae,* collected from diseased *Phlox subulata.* Failure of *A. rutgersi* to reproduce on either the old CEE-U (#646) or the new CEE-U (#620-6460AD) could result from nutritional deficiencies or toxins formed during commercial preparation of these extracts. In earlier experiments, *A. rutgersi, A. sacchari, A. cibolensis, A. dactylocercus,* and *A. avenae* had high rates of reproduction on meridic medium, M-12 (4), supplemented with 20% chick embryo extract.

Tsai and Van Gundy (13) reported that *Pratylenchus scribneri* required many days of feeding for 0.25% Amaranth Red #2 to move the full length of the intestine. My results with 0.25% Amaranth Red #2 incorporated into both C-1 and C-2 verified media ingestion within 24 hours, but Amaranth Red #2 was difficult to visually distinguish in *A. rutgersi.* Amaranth Red #2 is broken down and decolorized within the intestines of some mammals (D. Frick, Warner-Jenkinson Co., pers. comm.), and this may occur within *A. rutgersi.* Therefore, Fast Green #3 proved a useful substitute because it is a readily available histological stain, is a common food dye, and had less effect on the reproduction of *A. rutgersi* than Amaranth Red #2. The incorporation of Fast Green #3 into nematode nutriment such as C-1 or C-2 should help distinguish the unsuitability of nutriment from a failure to ingest nutriment.

Tsai and Van Gundy (13) also indicated that ST, YE, CEE, and the original medium (old formulation) compounded for *Aphelenchoides* spp. lacked a feeding stimulus for *P. scribneri.* Because *A. rutgersi* obviously feeds on both C-1 and C-2, it may not require an external stimulus to trigger feeding, or a feeding trigger may be present in these extracts that is not recognized by *P. scribneri.* Perhaps other stylet-bearing nematodes require stimuli to recognize the presence of otherwise suitable nutriment, and if such stimuli were added to C-1 or C-2, other nematodes could be cultured. More rapid intake of Amaranth Red #2 occurred when *P. scribneri* were

incubated in solutions containing sucrose, dextrose, methionine, corn root filtrate, and Gamborg's B5 (13). Thus, physical contact with solid substrate may be unnecessary. Because sucrose induced a feeding response in *P. scribneri* (13) and sometimes promoted increased reproduction of *A. rutgersi,* it was included in C-2 (Table 1).

Successful cultivation depends on the nematode's capability for recognizing and actively ingesting sufficient quantities of nutriment. Some passive ingestion apparently occurs when nematodes ingest the surrounding fluids to restore osmotic balance or bulk in their intestine. *Aphelenchoides rutgersi* reared in C-1 containing 0.1% Fast Green #3 were observed actively feeding. Such feeding was characterized by stylet movement, lengthy periods of pulsation of the median bulb, and the accumulation of dye within the intestine of the nematodes. Active feeding should promote sustained growth, maturation, and egg production. The LC in the C-2 diet caused a problem with microscopic observations of nematodes. Light was diffused so that internal structures and body outline of nematodes appeared unfocused, but total counts could still be made.

Culture media for nematodes should be chemically, nutritionally, and environmentally stable. Cuticular bubbles and retarded reproduction sometimes occurred, and these phenomena were associated with repeatedly autoclaving 5× strength ST-YE and Fast Green #3, respectively. When media ingredients were freshly prepared, these phenomena did not occur. They are probably caused by heat modification of nutrients or the physical instability of ingredients. Waste products secreted and excreted by nematodes will also modify the suitability of media, but many of these problems may be overcome by replacing media as necessary, or by shorter time intervals between serial subcultivation of stock cultures.

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