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Sterile Culture of *Rotylenchulus reniformis* on Tomato Root with Gellan Gum as a Supporting Medium[1]

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Abstract: Rotylenchulus reniformis was repeatedly propagated in sterile excised tomato roots growing on modified White's medium with gellan gum as the support. Gellan gum provided an optically clear support medium that could be liquified by adding 5 mM disodium ethylenediaminetetraacetate (EDTA) to facilitate nematode extraction. Liquefaction of the gellan-gum medium by EDTA allowed efficient recovery of eggs and vermiform stages of *R. reniformis*. Extraction efficiency was quantified with *Radopholus similis* as a test organism. The efficiency of extracting *R. similis* from the gellan gum did not vary with the concentrations of EDTA tested.

Key words: culture, disodium ethylenediaminetetraacetate (EDTA), gellan gum, *Lycopersicon esculentum, Rotylenchulus reniformis,* sterile culture, technique, tissue culture, tomato.

The gnotobiotic culture of plant-parasitic nematodes on root explants is an effective means for studying the biology of host–parasite interactions (4,5,7). The use of root-explant cultures is often hindered by difficulties in observing nematodes within roots because support media are opaque. Additionally, recovering nematodes after the roots grow into the support medium is difficult (2).

Ko and Van Gundy (2) demonstrated that pluronic F127 polyol could be used as a semisolid support medium for culturing plant-parasitic nematodes in excised root tissue. Polyol is optically clear and is a liquid at low temperatures, allowing the recovery of nematodes from the medium; however, polyol is toxic to some nematodes and other organisms (2).

Gelrite (Kelco Division of Merck and Company, San Diego, CA), a gellan gum agar substitute, is optically clear, free of contaminants, and a highly suitable medium for the culture of root explants. Preliminary work in our lab demonstrated that root cultures of tomato and cucumber grew better in Gelrite than in Difco bacto-agar (unpubl. data). We have had excellent results using a modified White's nutrient medium (8) to which we add iron sulfate chelated with disodium ethylenediaminetetraacetate (EDTA).

MATERIALS AND METHODS

Rotylenchulus reniformis Linford & Oliveira was successfully cultured on excised roots of tomato, *Lycopersicon esculentum* Mill. cv. Tropic. Seeds were soaked for 1 minute in 95% ethanol, transferred to 1% sodium hypochlorite, and placed in vacuum for 10 minutes. Seeds were picked directly from the solution and placed on 2% water agar petri plates to germinate at 25 C. When tomato roots were 1–2 cm long, they were excised, placed on 15-cm-d petri plates of White's medium, and allowed to grow for 1–2 days before inoculation with reniform nematodes.

Rotylenchulus reniformis was extracted from soil using sugar-flotation centrifugation. Several hundred males and juveniles were hand picked and placed in filter-sterilized 1% streptomycin sulfate. Using a modified column technique (3) for surface sterilization, the nematodes were pipetted into chromatography columns (12 mmd) half full of 60–80-mesh glass beads as a substrate for nematode movement. The beads facilitated the removal of debris adhering to the nematode cuticle as the nematodes moved to the lower portion of the column. After 24 hours at 25 C, the nematodes were collected in a sterile microcentrifuge tube. Thereafter, all manipulations were performed in a transfer hood using sterile techniques. The nematodes

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were centrifuged at 5,000 rpm for 1 minute in a microcentrifuge, the supernatant was removed with a pasteur pipet, and the nematodes were rinsed twice in sterile tap water to remove residual antibiotic. Sterile water was added to the nematode pellet to achieve a final concentration of approximately 50 nematodes per drop from a pasteur pipet. One or two drops of nematode suspension were placed on an excised tomato root tip using a pasteur pipet. Cultures were maintained by transferring blocks of gellan gum (2–3 cm^2) from established, contaminant-free cultures to new plates containing fresh root explants.

The culture-plate contents were placed on Baermann funnels in an attempt to collect vermiform *R. reniformis* from the cultures. Culture contents were comminuted in water using a blender, and half the resulting slurry was passed through a 149-μm-pore sieve (100-mesh) nested over a 20-μm-pore sieve (635-mesh) in an attempt to recover eggs. The remaining slurry was subject to centrifugation to pellet eggs for collection. We tested the ability of EDTA to liquify the Gelrite medium by comminution of the *R. reniformis* cultures in the presence of 5 mM EDTA using a blender and sieving the resulting liquid to collect eggs and vermiform nematodes.

The influence of EDTA concentration on the efficiency of extracting eggs and vermiform stages from Gelrite cultures was assessed with the burrowing nematode, *Radopholus similis* (Cobb), as a test organism. Eggs and vermiform stages were collected from carrot disk cultures (1,6), suspended in warm (ca. 45 C) Gelrite and White's medium at a concentration of 223 eggs and 485 vermiform stages per millilitre, and poured into petri plates. After 24 hours the contents of one petri plate were placed in a 400-ml beaker. Water and EDTA were added to achieve concentrations of 5, 10, 15, 20, or 25 mM EDTA in a final volume of 150 ml and the resulting slurry was placed in a blender for 30 seconds at the lowest speed. Eggs and nematodes were collected by pouring the blended solution through a 149-μm-pore sieve (100-mesh) nested over a 20-μm-pore sieve (635-mesh). Although a few pieces of solid media remained after blending, liquid passed through the sieves freely. Since some nematodes and eggs pass through a 20-μm-pore sieve (635-mesh), the rinse water from each replicate was screened twice to minimize loss. The contents of the sieve were rinsed into a beaker and brought to a volume of 65 ml and the number of eggs and nematodes in 1 ml was counted.

Results and Discussion

Rotylenchulus reniformis penetration and development were variable from plate to plate, but the inoculations were generally successful, with adult females visible in the roots and viable egg masses produced. By 60 days, population increase was rapid. Optimal quantities of *R. reniformis* were harvested within 50–60 days after block inoculation transfers; however, cultures could be maintained for several months.

Although recovery efficiency was low, males and juveniles were collected from Baermann funnels. Attempts to recover eggs by comminution in water with a blender were not successful because the Gelrite medium remained viscous and did not pass through sieves. The eggs could not be separated from the medium by centrifugation.

We found that we could successfully comminute the culture contents in a blender by adding 5 mM EDTA to the blending solution. The EDTA chelated magnesium ions in the Gelrite medium, breaking the bonds responsible for the gel matrix (G. Veeder, pers. comm.). This resulted in a liquid suspension that easily passed through sieves.

The concentration of EDTA did not affect extraction as similar numbers of vermiform stages of *R. similis* (437 ± 41, 417 ± 37, 426 ± 34, 422 ± 60, 413 ± 65 [means ± SE of six replicates]) and eggs (202 ± 27, 228 ± 26, 236 ± 20, 234 ± 31, 216 ± 23) were recovered from all concentrations of EDTA (5, 10, 15, 20, 25 mM). ANOVA performed on these data indicated no effect of varying EDTA con-

centration (F = 0.22 and 0.73 for vermiform stages and eggs, respectively).

Although no quantitative data on the application of this technique to *R. reniformis* cultures are presented here, we have routinely used this method with *R. reniformis* and obtained high recoveries of viable eggs and vermiform stages. *Rotylenchulus reniformis* and *R. similis* remained active and exhibited normal movement after exposure to EDTA. The effect of EDTA on nematode infectivity was not determined.

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