IN VIVO AND IN VITRO CULTURE OF THE RED RING NEMATODE, *RHADINAPHELENCHUS COCOPHILUS*[1]

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Accepted:
24.VII.1989
Aceptado:

ABSTRACT

Giblin-Davis, R. M., K. Gerber, and R. Griffith. 1989. In vivo and in vitro culture of the red ring nematode, *Rhadinaphelenchus cocophilus*. Nematrópica 19:135–142.

Inoculations of 100 persistent juveniles (JIII) of the red ring nematode, *Rhadinaphelenchus cocophilus*, into coconut leaf stalks produced red ring infestations in 50–60% of the stalks after 3–4 weeks. Monoxenic cultures of the fungi, *Monilinia fructicola* and *Botrytis cinerea* and undifferentiated sugarcane leaf spindle callus were inoculated with ca. 100 surface-sterilized juveniles (JIII) of *R. cocophilus*. At 1 week, no nematode survival was observed on either fungus. Molting was observed in one culture of sugarcane callus after 4 weeks. Several oligidic media were tested for *R. cocophilus* survival and development. Red ring nematodes survived longer than 70 days in autoclaved red ring stem tissue infusion water (RRW) that was unsupplemented or was supplemented with 5.00 g D-glucose/500 ml RRW, 3.85 g lactose/500 ml RRW, or 10.00 g Bacto-lactose broth + 5.00 g D-glucose/500 ml RRW (R + LB + G). A single adult female, four J4's, and nine JIII-J4 intermolts were recovered after 70 days in the R + LB + G medium and these nematodes were infective to a healthy coconut leaf stalk.

Key words: coconut palm, *Cocos nucifera*, culture, red ring nematode, red ring disease, *Rhadinaphelenchus cocophilus*, Trinidad.

RESUMEN

Giblin-Davis, R. M., K. Gerber y R. Griffith. 1989. Cultivos in vivo e in vitro del nematodo del anillo rojo, *Rhadinaphelenchus cocophilus*. Nematrópica 19:135–142.

Las inoculaciones de 100 larvas persistentes (JIII) del nematodo del anillo rojo, *Rhadinaphelenchus cocophilus*, en petíolos de hojas de cocotero tuvieron éxito en un 50–60% de los petíolos mostrando infestaciones de anillo rojo a las 3–4 semanas. Se inocularon también cultivos monoxénicos de los hongos *Monilia fructicola* y *Botrytis cinerea* y de callos de floema de caña de azúcar con ca. 100 juveniles (JIII) de *R. cocophilus* esterilizadas en

[1]This research was supported in part by U.S. Department of Agriculture Special Grant in Tropical and Subtropical Agriculture; 86-CRSR-2-2841, to R. M. G-D. Contribution from Florida Agricultural Experiment Stations; Journal Series No. 9845.

sus exteriores. Después de 1 semana no hubo supervivientes en los cultivos con hongos aúnque se observó muda de cutícula en uno de los cultivos de callo a las 4 semanas. Se probaron varios medios oligídicos para observar el desarrollo y supervivencia de *R. cocophilus*. Los nematodos supervivieron por más de 70 días en una infusión acuosa de tejido con anillo rojo esterilizado al autoclave sin suplemento alguno o suplementado con (g/500 ml): 5 g D-glucosa, 3.85 g lactosa, 10 g de caldo Bacto-lactosa + 5 g D-glucosa (R + LB + G). Se observaron una hembra adulta, cuatro J4's, y nueve JIII-J4's en entremuda después de 70 días en el cultivo R + LB + G todos los cuales resultaron infectivos en un petíolo de hoja de cocotero.

Palabras claves: cocotero, *Cocos nucifera*, cultivos, mal del anillo rojo, nematodo del anillo rojo, *Rhadinaphelenchus cocophilus*, Trinidad.

INTRODUCTION

The red ring nematode, *Rhadinaphelenchus cocophilus* (Cobb) Goodey, is the causal agent of the lethal red ring disease and chronic "Little Leaf" disease of coconut (*Cocos nucifera* L.) oil palm (*Elaeis guineensis* Jacquin) and other palm species in the Neotropics (4,9). *Rhadinaphelenchus cocophilus* does not survive long when separated from its palm or weevil (*Rhynchophorus palmarum* L.) hosts (9) and nematodes usually are procured for study from naturally or artificially infected 3–10-year-old palms. The task of obtaining and maintaining the nematode in this way is expensive and time consuming and makes geographical and host-resistance studies difficult. Immature coconuts have been inoculated successfully with R. *cocophilus* (1,2) and will sustain limited growth and reproduction of the nematode for several weeks (2).

Attempts to culture *R. cocophilus* on the fungus, *Alternaria alternata* (Fr.:Fr.) Keissl. grown on corn meal agar and coconut extract-sucrose agar were unsuccessful and few nematodes were recovered alive 24 hours after inoculation (8). Attempts to culture *R. cocophilus* in autoclaved coconut water and in autoclaved healthy coconut stem tissue infusion were not successful; nematodes survived less than 7 days (8). Unfortunately, all of these studies were done with nematode inocula that was not surface-sterilized. Our study was conducted to test the ability of *R. cocophilus* to survive, develop, and reproduce when inoculated into excised leaf stalks of coconut, cabbage palmetto (*Sabal palmetto* Walter), and sugarcane stalks (*Saccharum officinarium* L.), monoxenic cultures of undifferentiated sugarcane callus, two species of fungi, and several oligidic culture media.

MATERIALS AND METHODS

Red ring diseased stem tissue from coconut palms (3–5 years old) from the Cocal plantation in Manzanilla, Trinidad, was collected and harvested for persistent-stage juveniles (JIII) of *R. cocophilus* as described by Giblin-Davis et al. (7).

Leaf stalk culture: In Experiment 1, 10 young leaf stalks (1.0–1.5 m long) from undiseased coconut palms (ca. 3–5 years old) from Manzanilla, Trinidad, were trimmed of the leaf, a plug (1 cm deep) taken with a #4 corkborer 25–30 cm from the basal end of the petiole, and 100 JIII of *R. cocophilus* were added to the hole. The wound was plugged with the original piece of tissue, wrapped several times with Parafilm® (American Can Co. Greenwich, CT 06830, U.S.A.) and the petioles placed upright in a bucket of tapwater in the laboratory. Two control petioles were treated as above but not inoculated. Experiment 2 was a repeat of Experiment 1, except that the cut ends of the leaf stalks were sealed with Parafilm® and were laid horizontally on a table. Laboratory temperature was about 28 C during these experiments. In Experiment 1, leaf stalks were harvested 3 weeks after inoculation by cutting three 5-cm-long pieces, centered from the inoculation wound. Each piece of stalk was weighed, chopped up, and extracted separately on Baermann funnels. Extraction of nematodes was not attempted from the rest of the leaf stalk except for those stalks in which no nematodes were found. Experiment 2 was terminated 4 weeks after inoculation as for Experiment 1. The procedure for Experiments 1 and 2 was repeated with young leaf stalks from two cabbage palmettos growing in the Royal Botanical Garden in Port-of-Spain, Trinidad. Sugarcane stalks (35–40 cm long) were tested using the same methods as for Experiment 2.

Surface sterilization of nematodes: Harvested nematode juveniles were surface sterilized by transferring them into a 0.1% thimerosal (w/v) (sodium ethylmercurithiosalicylate) solution for 15 minutes followed by a 15 minute rinse in autoclaved distilled water in a 30-ml sterile capped centrifuge tube. The sterilization procedure was repeated three times. In most of the experiments, the nematodes were incubated in an antibiotic solution (6 mg/L penicillin-G, 1 mg/L streptomycin sulfate, 1 mg/L chlortetracycline HCl, and 13 mg/L kanamycin sulfate) for 1 hour before thimerosal treatment. Surface-sterilized nematodes were pipetted aseptically into a 150 ml glass bottle for dilution to appropriate inoculation densities. When counting nematodes for inoculation, the surface-sterilized inoculum was cooled in a refrigerator (ca. 5 C) for 20 minutes to alleviate the problem of swarming in which tight knots of nematodes formed at the bottom of suspensions at room temperature (28 C). Approximately 100 JIII of *R. cocophilus* in 0.1 ml of water (range = 75–150) were used for each axenic or monoxenic culture. Duplicate nutrient agar and glycerol-supplemented potato dextrose agar plates (GPDA) (5) and the test media were used to indicate failures in the sterility of the inoculum.

Callus tissue culture: Culture of *R. cocophilus* was attempted on undifferentiated leaf spindle callus of sugarcane. Leaf spindle tissue from cv. 65–357 sugarcane was agitated in 95% ethanol for 20 seconds, rinsed

in sterile distilled water, and placed on a modified Murashige and Skoog medium for callus induction in sugarcane (10). The medium was modified by omitting coconut water and using the vitamin ratios as described for Gamborg's B-5 medium. Sixteen plates (100 × 15 mm) of 4-month-old callus were inoculated aseptically with *R. cocophilus*. Plates were inspected weekly for 4 weeks and the nematodes extracted from the callus using a Baermann funnel, and examined for survival, development, and reproduction.

Culture on fungi: Two species of fungi, *Monilinia fructicola* (Wint.) Honey and *Botrytis cinerea* Pers. *ex* Fr., both good hosts for several species of *Bursaphelenchus* (5), which is in the same family as *Rhadinaphelenchus*, were tested as hosts for *R. cocophilus*. Sixteen culture plates (100 × 15 mm) of each fungus were cultured on GPDA for 1 month at 27 C prior to inoculation with surface-sterilized *R. cocophilus*. Four plates of each fungus were harvested separately each week for 4 weeks and nematodes were extracted on a Baermann funnel and examined and counted. All monoxenic and axenic cultures were kept in an incubator at 27 ± 2 C.

Culture using oligidic media: Several oligidic media were tested for their ability to sustain *R. cocophilus* under axenic conditions. Test media (10 ml) were pipetted aseptically into sterile 30-ml plastic culture flasks (Corning Glass Works, Corning, NY 14831, U.S.A.). Each culture was counted as a replicate, and at least five cultures were used for assessing the performance of a medium. Cultures were examined regularly for numbers of motile *R. cocophilus* and at termination, surviving nematodes were examined with the light microscope. The test media were prepared as follows: WATER = autoclaved deionized water; HC = healthy coconut petiolar tissue extract (petiolar tissue was chopped up and soaked in tap water overnight [100 g fresh tissue/L]). The following day the fluid was decanted through a 38-μm-pore sieve and Whatman #1 filter paper and autoclaved for 30 minutes at 160 C and 7 kg/cm^2); R = red ring diseased stem tissue extract (red ring stem tissue treated as for HC); R + LB = 500 ml of R prior to autoclaving + 10.00 g Bacto-lactose broth (Difco Laboratories, Detroit, MI, U.S.A.), autoclaved as above; R + G = 500 ml of R prior to autoclaving + 5.00 g D-glucose, then autoclaved as above; R + L = 500 ml of R + 3.85 g Bacto-lactose (the L fraction of Bacto-lactose broth), then autoclaved as above; R + BP = 500 ml of R prior to autoclaving + 3.85 g Bacto-peptone (the BP fraction of Bacto-lactose broth), then autoclaved as above; R + LB + G = 500 ml of R prior to autoclaving + 10.00 g Bacto-lactose broth + 5.00 g D-glucose, then autoclaved as above; FSC = filter sterilized water from two green immature coconuts (6–7 months old) was filtered through a Whatman #1 filter and then twice aseptically on separate prefilters over 0.2 μm Gelman GA-82 membrane filters.

RESULTS AND DISCUSSION

Leaf stalk culture: Attempts to culture *R. cocophilus* on leaf stalks of *S. palmetto* and stems of sugarcane were unsuccessful. The leaf stalk tissue from *S. palmetto* was hard and dry compared with the succulent tissue present in stems of sugarcane and leaf stalks of coconut. In Experiment 1, 60% of the inoculated coconut leaf stalks were infected with nematodes 3 weeks after inoculation. Seventy-four percent of the harvested nematodes were juveniles, 22% were females, and 4% were males (female:male sex ratio = 6:1). There were 1 814 ± 2 441 nematodes per infected leaf stalk (range = 263–6 300) or 13 ± 5 nematodes/g of harvested tissue (range = 2–45). The nematodes were distributed evenly in the three sections of leaf stalk examined. In Experiment 2, 50% of the inoculated coconut leaf stalks were infected with nematodes 4 weeks after inoculation. There were 537 ± 770 *R. cocophilus* per infected leaf stalk (range = 47–1 896), or 1 ± 2 nematodes/g harvested tissue (range = 0–5). In both experiments, infected palm petioles turned yellow 3 weeks after inoculation, whereas control petioles remained green. In cross section, the infected fronds were reddish in color compared with no discoloration in control fronds. The control coconut petioles for in vivo culture of *R. cocophilus* were negative for nematodes in both experiments.

Young leaf stalks from coconut palms (3–7 years old) may be appropriate for use in laboratory maintenance and comparison of geographical or host isolates of *R. cocophilus*. In addition, use of the leaf stalk for screening of different varieties of coconut palms for resistance against *R. cocophilus* would be more appropriate than use of immature coconuts, because coconuts are not normally infected with red ring nematodes and may give misleading results. Not all leaf stalks in our study became infected with nematodes, which suggests that variability in the inoculum, or physiology and/or age of the leaf stalk may be important. Also, inoculation densities may need to be increased. One drawback to this technique is that wounds made during the removal of leaf stalks must be sealed to prevent subsequent attack by *R. palmarum*.

Surface sterilization of nematodes: The mass surface-sterilization technique used in this study was only marginally acceptable, even with the 1 hour preincubation in antibiotics. Failures in obtaining surface-sterilized nematodes were common (20–100% contamination rate), and increased the difficulty and time entailed to evaluate cultures. The mass surface-sterilization technique of Giblin and Platzer (6) was attempted without success; *R. cocophilus* did not migrate through the agar.

Callus tissue culture: At the end of 4 weeks, only one of the seven uncontaminated plates of inoculated sugarcane callus yielded live *R.*

cocophilus. The nine nematodes that were recovered were molting from the third to the fourth stage.

Culture on fungi: Rhadinaphelenchus cocophilus survival, development, or reproduction was not observed in any of the inoculated *M. fructicola* or *B. cinerea* cultures.

Culture using oligidic media: The survival of *R. cocophilus* in oligidic media is presented in Fig. 1. *Rhadinaphelenchus cocophilus* survived longer than 40 days in 20% of the containers (n = 5) of sterile water, but were dead before 14 days in the HC extract (n = 10) (Fig. 1A). When LB (n = 9) or the BP fraction of LB was added to R medium (n = 6), survival was very poor at 28 days postinoculation (Fig. 1A and 1B). Conversely, the R extract alone (n = 7), R plus the sugars, D-glucose (n = 7) or lactose (n = 8), and R plus Bacto-lactose broth and D-glucose (n = 15) allowed for survival of *R. cocophilus* in > 80% of cultures after 50 days (Fig. 1A) with > 10% of the inoculated nematodes remaining motile. Examination of the surviving nematodes from the R + LB + G medium 70 days after inoculation revealed nine juveniles in the process of molting, four J4s, and one young adult female. One undiseased coconut petiole from the field was inoculated as described previously with 10 *R. cocophilus* juveniles that survived 70 days in R + LB + G medium versus one control petiole. Three weeks after inoculation, the inoculated petiole yielded a total of 4 950 nematodes or 11 *R. cocophilus*/g of tissue compared with no nematodes from the control petiole.

These data suggest that soluble materials extracted from tissue harvested from the red ring area of a red ring diseased coconut stem after autoclaving are able to support the survival of juveniles of *R. cocophilus* better than sterile water or autoclaved extract of healthy coconut petiolar tissue. The addition of D-glucose, lactose, or Bacto-lactose and D-glucose broth did not substantially improve the performance of the red ring extract. Under these conditions, the BP fraction of LB and LB alone appeared to be antagonistic to survival of *R. cocophilus*. That one female of *R. cocophilus* was produced in the R + LB + G medium is encouraging because it suggests that it may be possible to culture the nematode axenically in an oligidic medium.

Filter-sterilized endosperm from immature coconuts (FSC) (n = 15) was not appreciably better than the HC medium for survival of *R. cocophilus*. Bolla and Jordan (3) reported that *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle could be cultured axenically on 4% soy-peptone, 1% yeast extract, 50 µg/ml cholesterol, and 50 µg/ml bovine

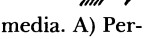
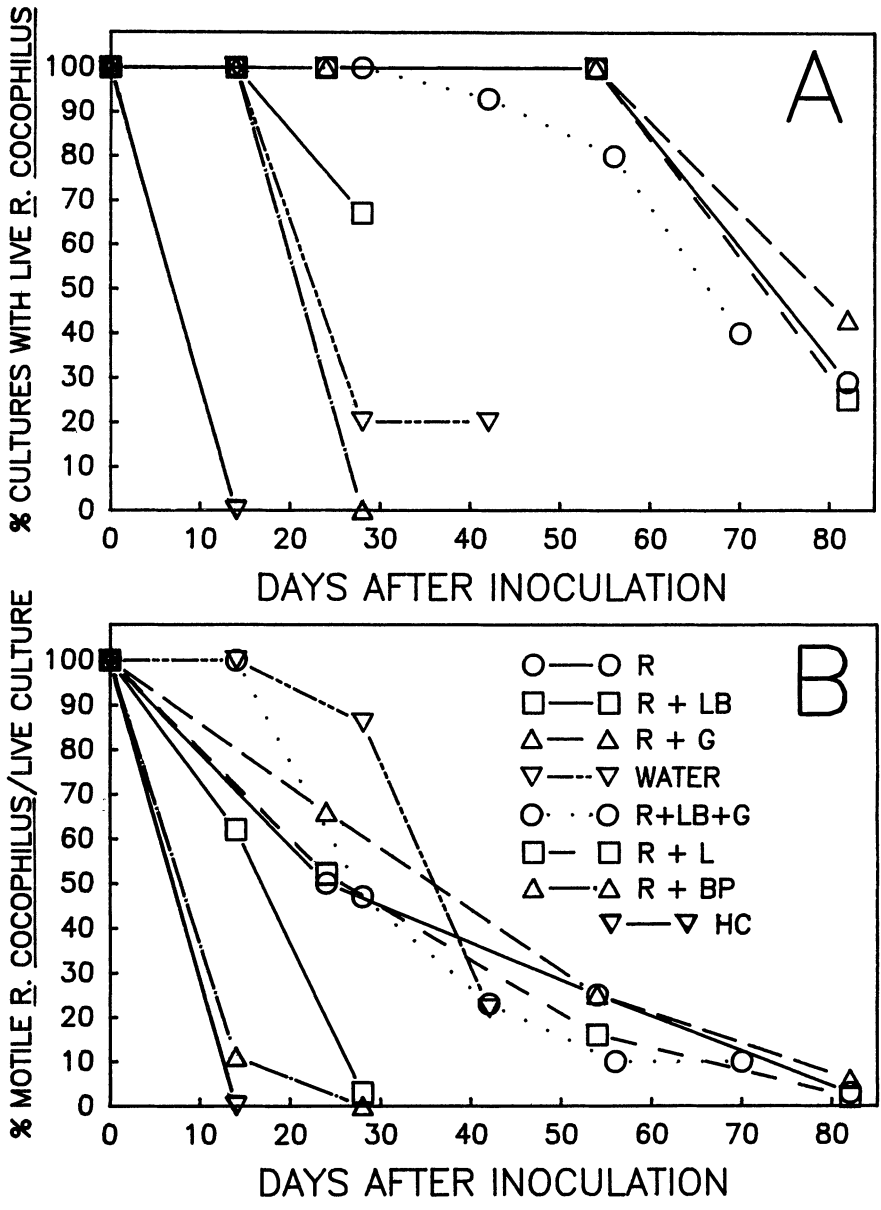


Fig. 1. Survival of surface-sterilized *Rhadinaphelenchus cocophilus* in oligidic media. A) Percentage of cultures with living *R. cocophilus* relative to time after inoculation. B) Percentage of motile *R. cocophilus* per cultures which sustained any living red ring nematodes; Symbols



indicate time after inoculation when cultures were examined; WATER = autoclaved deionized water; HC = autoclaved healthy coconut petiolar tissue extract; R = autoclaved red ring diseased stem tissue extract; R + LB = 500 ml of R + 10.00 g Bacto-lactose broth; R + G = 500 ml of R + 5.00 g D-glucose; R + L = 500 ml of R + 3.85 g Bacto-lactose (the L fraction of Bacto-lactose broth); R + BP = 500 ml of R + 3.85 g Bacto-peptone (the BP fraction of Bacto-lactose broth); R + LB + G = 500 ml of R + 10.00 g Bacto-lactose broth + 5.00 g D-glucose.

hemoglobin. We attempted to culture *R. cocophilus* with this medium, but because of contamination problems, the results were difficult to interpret. We observed good survival of nematodes 21 days after inoculation in two cultures which were not contaminated. This suggests that further work with this medium is warranted.

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Received for publication:
23.III.1989
Recibido para publicar:

ACKNOWLEDGEMENTS

We thank the staff of the Coconut Research Division and the Ministry of Food Production, Marine Exploitation, Forestry, and the Environment of Trinidad for their cooperation in this project and the Royal Botanical Garden, Port-of-Spain, Trinidad for providing cabbage palmetto leaf stalks. In addition, we thank Dr. R. P. Esser, Florida Department of Agriculture and Consumer Services, and Dr. G. C. Smart Jr., University of Florida, IFAS, for critical review of the manuscript.