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NEMATODA

SCIENTIFIC NOTE

Culturing *Bursaphelenchus cocophilus in vitro* and *in vivo*

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HIGHLIGHTS

- Culturing of the nematode in coconut seedlings was marginally successful.
- Culturing of the nematode on several fungi endophytic to coconut was not successful.

ABSTRACT: Red ring disease (RRD) is of particular importance in many African oil palms- and coconut-producing regions in Central and South America and the Caribbean. Its causal agent, the nematode *Bursaphelenchus cocophilus* (Cobb) Baujard, causes extensive damage to tissues in the plant trunk that typically leads to plant death within months. Nearly 100 years after its first report RRD remains understudied largely because the nematode cannot be cultured *in vivo* or *in vitro*, what hinders sustained research efforts on basic and applied aspects of the pathosystem. To overcome this problem we attempted *in vivo* culturing in coconut seedlings, paying attention to aspects that had been overlooked in previous trials. We also attempted *in vitro* culturing on several fungi endophytic to healthy and RRD-affected coconut trees. In the two *in vivo* assays performed we were able to recover hundreds of nematodes from the seedlings up to 60 days after inoculation, but the nematodes seemed unable to sustain parasitism in most seedlings. No nematode was recovered from the endophytic fungal cultures in neither of the two assays performed. Hence *B. cocophilus* continues to stand as the only obligatory plant-parasitic species of the speciose genus *Bursaphelenchus* and a somewhat intractable plant-parasitic nematode to study on.

Keywords: red ring disease, nematode culturing, obligatory plant-parasitic species.

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INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is an important crop in most tropical countries. In addition to a range of uses and by-products it offers, the crop sustains millions of direct and indirect job positions and it provides revenue for medium- and small-holders worldwide[1].

Among the several pests and diseases that affect coconut, the red ring disease (RRD) – which also affects several other palms – is of particular importance in countries in Central and South America and the Caribbean region[2, 3]. Red ring disease is caused by the nematode *Bursaphelenchus cocophilus* (Cobb) Baujard, which is vectored primarily by a coconut pest, the red palm weevil *Rhynchophorus palmarum* L. (Curculionidae: Coleoptera). The nematode colonizes primarily the coconut trunk, where it causes extensive damage to tissues. The reddish to brownish ring that may be seen transversally in the trunk – a sign of the disease - results from oxidation of phenolic compounds produced by parenchima cells, presumably in response to nematode parasitism. Oclusion of xylem and phloem elements through tylose

accumulation hinders water and nutrient translocation[4]. The coconut leaves wilt, develop chlorosis and necrosis, and the plant dies within 2-4 months, although older plants may last longer.

Red ring may arguably be called a neglected disease because its importance to many coconut-producing regions has not been matched by sustained basic and applied research efforts. Management of RRD has been centered on the use of traps to capture the nematode´s vector, an effective method if properly conducted[5]. Notwithstanding there has been no sustained studies on new management options; plant-nematode interactions at biochemical, cell and tissue levels; or sources of and mechanisms involved in coconut resistance to the nematode or tolerance to RRD. The slow advance in understanding basic and applied aspects of *B. cocophilus* and RRD is due at least partially to the difficulty in culturing the nematode *in vivo* and *in vitro*. Hence, long term research programs can only be conducted near endemic areas, and coconut trees must be felled for one to obtain fresh nematodes because they don´t remain active for long outside its host.

Goberdhan (1962) (cited by Giblin-Davis et al.[6]) was the first to attempt to cultivate *B. cocophilus in vitro*, but no nematode survived in the fungus *Alternaria alternata* (Fr.) Keissl. Gibblin-Davis et al.[6] attempted to cultivate the nematode in excised stalks of sugarcane (*Saccharum officinarium* L.); excised leaf stalks of coconut and cabbage palmetto (*Sabal palmetto* Walter); monoxenic cultures of sugarcane callus; oligidic culture media; and in cultures of the fungi *Monilinia fructicola* (Winter) Honey and *Botrytis cinerea* Pers. No nematodes were recovered from the fungal cultures, sugarcane callus or leaf stalks of sugarcane and cabbage palmetto. Some nematodes survived – but did not reproduce – in some of the oligidic media tested. About half of the coconut leaf stalks were seen infected by the nematode three weeks after inoculation, when the authors ended the assay.

Coconut trees, 4-12 year-old, can be inoculated with *B. cocophilus* with ease, leading to RRD and plant death within weeks. However this is not a useful *in vivo* cultivation method because it is costly and requires considerable field area. Malaguti[7] and van Hoof & Seinhorst[8] attempted to reproduce RRD through inoculation of young African oil palms (*Elaeis guineenses* Jacq.). A marginal success was achieved by the former.

Fenwick & Mohammed[9] reported that coconut seedlings became infected by the nematode when the seednuts fell and remained on the ground amongst coconut trees affected by RRD. Remarkably, no RRD symptoms were observed in those young plants. Following this observation the authors inoculated seednuts and 8-12 month-old coconut seedlings with 4,000 nematodes. *B. cocophilus* was recovered from seednuts and seedlings occasionally, leading to the conclusion that nematode reproduction did occur, but the population did not last long. Some seedlings presented wilting and tissue discoloration and necrosis, but not always associated with the presence of nematodes. Blair[10] inoculated eggs of *B. cocophilus* in the mesocarp of immature coconuts; ten days later the author saw juveniles and adults, but he did not provide information whether the infection lasted beyond the 13th day of observation. Better results were reported by Blair & Darling[4] who reported thousands of live nematodes in green nuts three weeks after inoculation. Collectively these results indicate that nearly 100 years after its original description *B. cocophilus* remains the only plant-parasitic nematode that cannot be cultivated for proper investigation.

In *B. xylophilus* (Steiner & Buhrer) Nickle – the only other *Bursaphelenchus* species accepted as plant-parasitic – the ancestral mycophagy occurs on fungi that colonize the plant host, primarily *Pinus* spp. The recently evolved plant-parasitism prompts a hypersentive reaction by the host, which damages its own tissues, alters its physiology and leads to plant death[3].

The tri-trophic interaction nematode-coconut tree-weevil may also be a recent evolutionary phenomenon[3]. Therefore, we hypothesize that *B. cocophilus* could have an ancestral mycophagy on coconut-associated fungi and that only fruit-bearing coconut trees respond to nematode parasitism through a strong hypersensitive reaction, which is perceived as RRD.

Therefore, in attempting to cultivate *B. cocophilus in vitro* we turned to several fungi endophytic to healthy and RRD-affected coconut trees. To attempt to cultivate the nematode *in vivo* we turned to inoculation of coconut seedlings, on the assumption that the nematode could establish infection and colonization without mounting a strong defensive reaction that characterizes RRD, as observed in coconut seedlings[9].

MATERIAL AND METHODS

Origin of isolate and nematode extraction

Trunk pieces of *B. cocophilus*-infected coconut trees 'Yellow Dwarf' were obtained from a plantation located in the municipality of São Mateus, State of Espírito Santo, Brazil (18°43'51"N, 40°03"12"S). For nematode extraction trunk fragments were placed over paper tissue in trays filled with tap

water for 48 hours, at room temperature. The nematodes that passed through the paper tissue were collected and passed through 60 and 500 mesh screens. The resulting nematode suspension was used immediately in the assays.

Nematode "in vivo" culturing

Coconut seedlings (1.5 year-old) were removed from a sandy germination bed and individually transplanted to 20 L capacity plastic pots filled with riverbed sand, to which 20 g of single superphosphate and 20 g of potassium chlorate were added.

Thirty days later a 0.5 cm deep hole was punctured in the seedlings at one of three distinct positions (treatments): at the base of the seedling or at 3 or 6 cm high. One millilitre of nematode suspension – calibrated to contain 1,000 nematodes – was injected into the hole with an automatic pipette (Figure 1), and a piece of tape was used to seal the hole. Non inoculated seedlings, which had tap water injected into the hole, served as controls. Twenty replicates (seedlings) were prepared for each treatment, for a total of 80 seedlings. The seedlings were arranged in the greenhouse in four entirely randomized blocks.

At 30, 60, 90 and 120 days after inoculation a block was randomly chosen and all seedlings were submitted to evaluation. For each treatment, the seedlings had their leaves, stem and seednut dissected and inspected for tissue lesions. The tissues were cut in small pieces and submitted to nematode extraction as described before. The number of nematodes recovered from each seedling was assessed 48 hours later. The assay was repeated once.

Nematode "in vitro" culturing

Trunk fragments were collected from 50 healthy and five RRD-affected coconut trees from several plantations in the municipalities of Campos dos Goytacazes and Quissamã (State of Rio de Janeiro) and São Mateus. To collect the fragments, an alcohol-disinfested drilling bit was mounted on an electrical drill and inserted about 10 cm deep into the trunk. In 20 trees the holes were punctured at about 1 m high, while in 35 trees the holes were made at about 3.5 m high. The fragments were disinfested in aqueous alcohol solution (70%) for 1 minute, followed by aqueous sodium hypochlorite solution (1%) for 2 minutes. After double rinsing in sterile, distilled water, the fragments were transferred to sterilized Petri dishes with solid potato-dextrose-agar (PDA) medium. The resulting fungal cultures were sub cultured from single spores and identified to genus level based on taxonomic keys[11-13].

For each of the nine endophytic fungi isolated (see Results and Discussion) a colonized agar plug was transferred to 10 Petri plates with PDA medium. Five days later, 100 *B. cocophilus* were transferred to

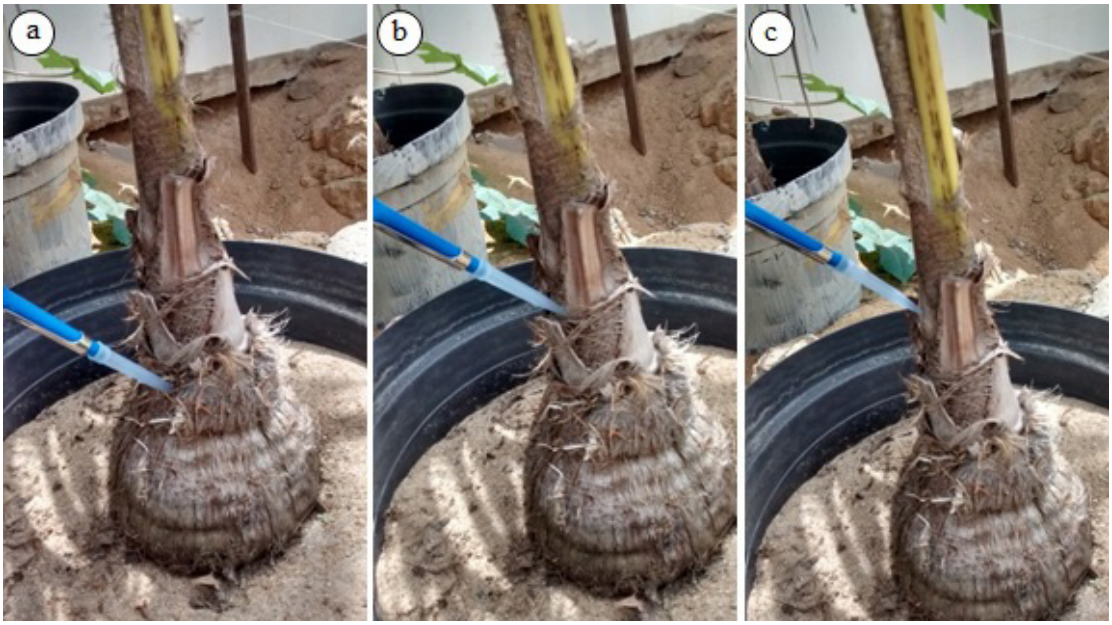

Figure 1. Inoculation of *Bursaphelenchus cocophilus* in 0.5 cm deep holes punctured at three distinct positions: a) at the base of the seedling; b) at 3 cm high; c) at 6 cm high.

each Petri plate. The plates were incubated in growth chamber at 25 °C in the dark. Twenty days later the entire content of each Petri dish was cut into small pieces and processed for nematode extraction as described before. Three aliquots (1 mL) of each nematode suspension were transferred to Peter`s slides for nematode counting under a stereo-microscope. The assay was repeated once.

RESULTS AND DISCUSSION

To succeed in culturing *B. cocophilus in vivo* we paid attention to aspects that may have been overlooked in previous attempts. The inoculation hole was punctured at different positions to provide the nematodes with entry points to distinct plant tissues underneath; and a relatively low inoculum was used to minimize defence reactions by the seedlings and inter-specimen competition.

We were able to recover hundreds of nematodes from the seedlings up to 60 days after inoculation (Table 1), but the nematodes seemed unable to sustain parasitism in most seedlings. The seedlings that sustained nematodes presented no red ring in the stem. Leaf chlorosis and tissue necrosis were observed in several seedlings, but not always associated with the presence of nematodes.

Apparently at least two novel approaches should be pursued in new attempts to cultivate *B. cocophilus in vivo*: **i)** histological examination of seedlings several days after inoculation, so that nematode migratory and feeding behaviours, as well as host response, can be understood, and **ii)** use of juveniles extracted from the insect vector – not from coconut trunks – as inoculum for coconut seedlings. If the tri-trophic interaction nematode-coconut tree-weevil is a recent evolutionary event[3], an unknown factor present in the insect could facilitate plant parasitism once the *dauer* juveniles leave the insect and enter the coconut seedlings.

The collection and incubation of trunk fragments obtained from tens of coconut trees resulted in the isolation of several endophytic fungi (Table 2). No connection seemed to exist between the incidence of the different fungi and **i)** the position in the trunk where the drilling occurred; **ii)** the status of the coconut tree – either healthy or affected by RRD; or the geographic distribution of the sampling sites, which were up to 500 km apart.

The assumption that an ancestral mycophagy of *B. cocophilus* on fungi endophytic to coconut trees could favour the cultivation of the nematode *in vitro* seems not to hold true. No nematode was recovered from the fungus cultures in neither of the two assays performed. Hence *B. cocophilus* continues to stand as the only obligatory plant-parasitic species of the genus.

As early as 1965, the difficulty of working with *B. cocophilus* because it could not be cultivated nor lasted long out of its host had been highlighted[10]. Despite the importance of RRD to cultivation of

Table 1. Recovery of *Bursaphelenchus cocophilus* from coconut seedlings following injection of 1,000 specimens into 0.5 cm-deep holes punctured in one of three different positions.

Treatments	Average of nematodes recovered/seedling; rate of seedlings positive for nematode	
	30 days after inoculation	60 days after inoculation
Assay 1		
1*	1,151; 4/5	850; 2/5
2	0; -	0; -
3	280; 1/5	0; -
Non Inoculated control	0; -	0; -
Assay 2		
1	0; -	722; 2/5
2	0; -	0; -
3	0; -	0; -
Non Inoculated control	0; -	0; -

*Treatments: 1: hole punctured at the base of the seedling; 2: at 3 cm high; 3: at 6 cm high.

Table 2. Fungus endophytic to coconut trees used for culturing *B. cocophilus*.

Genera	Deposit code*
Trichoderma sp.	CM/Uenf 407
Phialophora sp.	CM/Uenf 408
Penicillium sp.	CM/Uenf 409
Gliocladium sp.	CM/Uenf 410
Rhizoctonia sp.	CM/Uenf 411
Nigrospora sp.	CM/Uenf 412
Hyalodendron sp.	CM/Uenf 413
Fusarium sp.	CM/Uenf 414
Aspergillus sp.	CM/Uenf 415

*Deposits made at the mycological collection of the Departament of Entomology and Plant Pathology at Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Brazil.

coconut and African oil palm in South and Central Americas and the Caribbean, *B. cocophilus* remains a somewhat intractable nematode to study on. Culturing this nematode remains a challenge certainly worth pursuing.

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