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ORIGINAL PAPER

Nematodes of *Rhynchophorus palmarum*, L. (Coleoptera: Curculionidae), vector of the Red Ring disease in coconut plantations from the north of the Rio de Janeiro State

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Abstract *Rhynchophorus palmarum*, the palm weevil, has been reported as a pest of palms and sugarcane plants. The Red Ring Disease is an infectious plant disease caused by nematodes. The etiological agent, *Bursaphelencus* (*Rhadinaphelencus*) *cocophilus* (Nematoda: *Aphelenchoididae*), completes its life cycle within 9 to 10 days inside the palm tree. The main symptom is a permanent wilting of the plant aerial parts. Previous studies stated that *B. cocophilus* cohabits with other nematodes the gut of *R. palmarum*. The aim of this study is to identify nematodes collected from palm weevil found in coconut plantations from the north of the Rio de Janeiro State. Light (LM) and scanning electron microscopy (SEM) analyses were carried on samples of infected *R. palmarum* and fragments and fresh juice of infected plants with *B. cocophilus*. Observations of *R. palmarum* fecal material made by LM and SEM showed three species cohabiting these samples, being also present in fresh juice and fragments of infected coconut tree: *B. cocophilus, Teratorhabditis palmarum* (Nematoda: Rhabditidae) and *Diplogasteritus* sp (Nematoda: Diplogasteridae). These findings confirm previous studies, which related that *R. palmarum* own a varied nematode fauna. Nematodes associated to *B. cocophilus* probably could be co-participates of the etiology of the Red Ring disease.

Keywords *Bursaphelencus cocophilus* · Coconut palm · Palm weevil · Plant parasite nematodes · Red Ring disease · *Rhynchophorus palmarum*

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Introduction

Rhynchophorus palmarum, L. (Coleoptera: Curculionidae) occurs on 35 plant species of 12 different families but is found predominantly on *Arecaceae* (Esser and Meredith 1987; Griffith 1987; Wattanapongsiri 1966; Jaffé and Sánchez 1990; Sánchez and Cerda 1993). It has only been reported as a pest on palms and sugarcane (Arango and Rizo 1977; Restrepo et al. 1982). In other plants, *R. palmarum* for example, it has been reported as a commensal, feeding on ripe fruits but not causing economic damage. Their main hosts are *Cocos nucifera*, *Elaeis guineensis*, *Euterpe edulis*, *Metroxylon sagu*, *Phoenix canariensis*, *Phoenix dactylifera*, and *Saccharum officinarum* (OEPP/EPPO 2005).

As reported by Wattanapongsiri (1966), the genus *Rhynchophorus* has an extensive worldwide distribution but is mostly concentrated in the tropics (Caribbean, Central and South Americas: Argentina, Bolivia, Colombia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Surinam, Uruguay and Venezuela). In Brazil, its distribution occurs from the North to the Southeast region (Ferreira et al. 1998).

Bain and Fedon (1951) determined that *R. palmarum* is the most important vector of the nematode *Bursaphelenchus* (*Rhadinaphelenchus*) *cocophilus* (Cobb 1919) Baujard 1989 (Nematoda:Tylenchida; Baujard 1989), which causes the Red Ring disease of coconut trees and other plants of the Arecaceae (Palmae) family (Agrios 2005; Griffith 1987; Lucas 1998).

Plant parasitic nematodes (PPN) are a threat to important crops worldwide. Intensive trade has made many PPN, which were once localized in specific areas of the world, cosmopolitan. A global survey indicated that the annual yield losses due to parasitic nematodes are approximately 12% in agricultural productivity (Sasser and Freckman 1987). The annual worldwide losses caused by nematodes on the life-sustaining crops, which include all grains and legumes, banana, cassava, coconut, potato, sugar beet, sugarcane, sweet potato, and yam, are estimated to be about 11% (Agrios 2005). Besides causing direct damage to plants, some of the PPN are vectors of troublesome plant viruses (Zuckerman and Rohde 1981). Billions of dollars are spent annually on soil fumigants and other nematicides. However, the current trend is to minimize the use of these environmentally unsafe toxicants (Jasmer et al. 2003).

All nematode–plant interactions involve cellular modifications of feeding cells and surrounding tissue (Hussey 1989). Plants respond to the presence of PPN by employing various generic defense strategies (Veech 1981) that include the release of naturally occurring nematicides, phytoalexins, and other compounds (Gommers and Bakker 1988), systemic induction of pathogenesis-related proteins (Hammond-Kosack et al. 1989), production of protease inhibitors (Brenner et al. 1998), and reactive oxygen (Waetzig et al. 1999). In addition to these chemical barriers, plants reinforce the cell wall by lignification, suberization, and callose deposition at the site of stylet penetration (Hussey et al. 1992). Plants have evolved a defense system against PPN that is mediated by a set of disease resistance genes (Dangl and Jones 2001).

The Red Ring disease was named on basis of the characteristic red band always present in the stem of affected palms (Franco 1964; Ferreira et al. 1998). Red Ring external symptoms are confined to the leaves, which turn yellow from the tip of the leaflets to the basis of the raquis and, then, turn brown. Leaf symptoms start on the lowest leaves and progress upward. The internal symptom is a circular anthocyanin deposition at cortex tissue of affected organs. This ring is usually red in tall cultivars but may be browner in dwarf and hybrid cultivars. The internal symptom is fully developed before external symptoms become visible (Franco 1964; Griffith 1987).

Gerber and Giblin-Davis (1990a, b) showed the association of *B. cocophilus, Teratorhabditis palmarum* and other nematode species with the palm weevils, *R. palmarum*, and *R. cruentatus* from many countries [Trinidad and Tobago, Ecuador, Colombia and USA (Florida)]. Those palm weevils could be internally infested by other nematodes that cohabit the gut with the Red Ring nematode.

Scanning Electron Microscopy has been used extensively as a valuable investigative tool in taxonomic and structural studies of nematodes (Gibbons 1986, 2002). Its higher resolution power has enabled to achieve surface architecture details not perceived by classical works that utilized only light microscopy (LM) analysis (Halton 2004). The purpose of this study is to identify the nematodes present in palm weevils collected at coconut plantations from the north of the Rio de Janeiro State using SEM as a powerful tool to provide new information concerning the nematodes surface.

Material and methods

Plant material

The study was done near Conceição de Macabu municipality (22°05′07″ S–41°52′06″ W) on the northern of Rio de Janeiro State, SE, Brazil. Experiments were carried out on three commercial farms during 2003 and 2004. There were approximately 44.300 coconut trees (*Cocos nucifera* L. ecotype Green Dwarf) divided in ten plantation areas. Symptomatic palms for Red Ring disease occurred randomly and all of them (n=8) were collected. Fresh juices were observed under differential interferential contrast microscopy to confirm field diagnosis.

Insects

Insect traps to *R. palmarum* were located at the periphery of the ten coconut plantation areas. They were divided in two groups. Trap type A (n=10), white plastic 100-l bucket presenting a 2.5-cm aperture and containing 4 kg of sugarcane 25 cm long tablets in 2003 and 4 kg of sugarcane 25 cm long tablets plus adult male of *R. palmarum* in 2004. Trap type B (n=10), transparent plastic 6-l recycled pet bottle for soft drink (a low cost trap) presenting a 2.2-cm aperture and containing 250 g of sugarcane 10 cm long tablets in 2003 and 250 g of sugarcane 10 cm long tablets plus adult male of *R. palmarum* in 2004. Pheromone and insecticides were not used. Collections were made once every 14 days. Two empty traps (type A and type B) were used as control for each collecting point. Insects were maintained at laboratory conditions (room temperature± 25°C, 80% humidity) in aired plastic boxes (20×15 cm) feed with sugar cane previously washed with 2% hypochlorite and distilled water.

Table 1 Comparison between the measurements and ratios obtained from ten *B. cocophilus* adults collected from juice and plant fragments in coconut commercial plantations from the Conceicão de Macabu municipality, in the north of the Rio de Janeiro State, Brazil, and the original description made by Cobb 1919 in Franco 1964

B. cocophilus	Male		Female	
	Present study	Cobb 1919; Franco 1964	Present study	Cobb 1919; Franco 1964
L (mm)	0.98 (0.86–1.15)	1.02 (0.84–1.16)	1.03 (0.95–1.03)	1.05 (0.97–1.18)
A	118.0 (98.0–150.0)	120.0 (100.0–179.0)	85.0 (70.0–90.0)	87.0 (78.0–96.0)
B	6.4	6.5	8.5	8.7
C	26.0 (24.0–33.0)	28.0 (24.0–35.0)	10.5 (10.5–14.6)	11.6 (9.5–13.2)
T (%)	57.0 (48.0–65.0)	59.0 (50.0–68.0)	–	–
V (%)	–	–	67.0 (65.0–70.0)	66.0 (64.0–68.0)

L Total length, *A* ratio total length/wide, *B* ratio total length/esophagus length, *C* ratio total length/tail length, *T* (%) total length/testis extremity, *V* (%) total length/vulva position

Nematodes

Nematodes were collected from insect fecal material, fresh plant juice, and plant fragments. Samples were washed three times in a saline solution at room temperature or 4°C and fixed by 2 h at room temperature or overnight at 4°C in 2.5% glutaraldehyde, with 4% freshly prepared formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2.

Light microscopy

For LM, extracts, fragments, and insect fecal material previously fixed were washed in 0.1 M cacodylate buffer, pH 7.2, distilled water and mounted in lactophenol. Slides were observed in Axioplan Zeiss microscope. Images were digitized with a CCD ZVS Zeiss camera (Germany) controlled by image analysis system AnalySIS® (Soft Imaging System, Zeiss, Germany). Morphometry was carried out through this system. The measurements are given in micrometers unless stated differently.

Scanning electron microscopy

For SEM, extracts, fragments, and insect fecal material previously fixed were washed in 0.1 M sodium cacodylate buffer, pH 7.2. Post-fixation was carried out with 1.0% Osmium tetroxide in the same buffer for 2 h. They were dehydrated in ethanol series, dried by CO_2 critical point in a Balzer's apparatus (CPD 030), covered by sputtering with 20 nm of gold in a Balzer's apparatus (SCD 050), and observed under SEM at 25 kV in a Zeiss DSM 962.

Results

Observations made by LM (morphometry) and SEM revealed that three species of nematodes (Tables 1, 2, and 3) were present in insect fecal material: L_3 of *B. cocophilus* (Fig. 1a), *Teratorhabditis palmarum* (Nematoda: Rhabditidae) Gerber and Giblin-Davis 1990 (Fig. 2a–f) and *Diplogasteritus* sp (Nematoda: Diplogasteridae) Paramonov 1952 (Fig. 3a–d). In juice and plant fragments were

Table 2 Comparison between the measurements and ratios obtained from five *T. palmarum* adult male and eight females collected from fecal material of *R. palmarum*, juice and fragments of infected plants in coconut commercial plantations from the Conceicão de Macabu municipality, in the north of the Rio de Janeiro State, Brazil, and the original description made by Gerber and Giblin-Davis 1990a, b

T. palmarum	Male		Female	
	Present study	Gerber and Giblin-Davis 1990a, b	Present study	Gerber and Giblin-Davis 1990a, b
L	780.0 (710.0–805.0)	830.0 (723.0–1093.0)	1050.0 (970.0–1415.0)	1216.0 (948.0–1397.0)
A	16.2 (15.2–21.0)	16.6(15.6–23.3)	17.5 (15.5–25.2)	20.2 (17.6–23.1)
B	4.8 (4.2–5.6)	4.3 (3.2–5.7)	6.5 (4.0–7.5)	5.1 (3.6–6.0)
C	28.7 (26.3–39.8)	24.4 (19.5–33.3)	47.5 (37.5–80.0)	43.4 (26.5–61.1)
spicule (μm)	52.0 (48.0–60.0)	56.0 (51.0–67.0)	–	–
V (%)	–	–	92.0 (89.2–98.0)	95.0 (92.8–96.0)

L Total length, *A* ratio total length/wide, *B* ratio total length/esophagus length, *C* ratio total length/tail length, spicule length, *V* (%) total length/vulva position

Table 3 Comparison between the measurements and ratios obtained from five *Diplogasteritus sp* adult male and eight females collected from *R. palmarum* fecal material and of juice and fragments of infected plants in coconut commercial plantations from the Conceicão de Macabu municipality, in the north of the Rio de Janeiro State, Brazil, and the original description made by Paramonov 1952

Diplogasteritus sp	Male		Female	
	Present study	Paramonov 1952	Present study	Paramonov 1952
L	550.0 (547.0–702.5)	630.0	690.0 (633.5–1010.0)	1000.5
A	20.9 (18.7–21.2)	20.0	27.5 (24.0–33.1)	24.0
B	4.8 (4.6–5.0)	4.9	5.5 (5.3–6.1)	5.1
C	3.7 (3.3–4.1)	3.9	5.1 (4.7–5.8)	5.9
spicule (μm)	17.5 (16.3–21.2)	18.0 (15.0- 20.5)	–	–
V (%)	–	–	466.2 (350.2–507.6)	511.0

L Total length, *A* ratio total length/wide, *B* ratio total length/esophagus length, *C* ratio total length/tail length, spicule length, *V* (*%*) total length/vulva position

observed all species cited above; however, only adult forms of *B. cocophilus* were observed (Fig. 1b).

By SEM, the cephalic end of *B. cocophilus* (Table 1) was slightly flat with a simple oral opening surrounded by a labial disc and most externally by six lips (Fig. 1c and d). The anterior region of both *B. cocophilus* sex genders were not offset by a constriction but were narrower than the body, presenting a modified pattern of cuticular ornamentation (Fig. 1c). In the cephalic end, two pairs of inner papillae were present. Two amphid apertures were observed

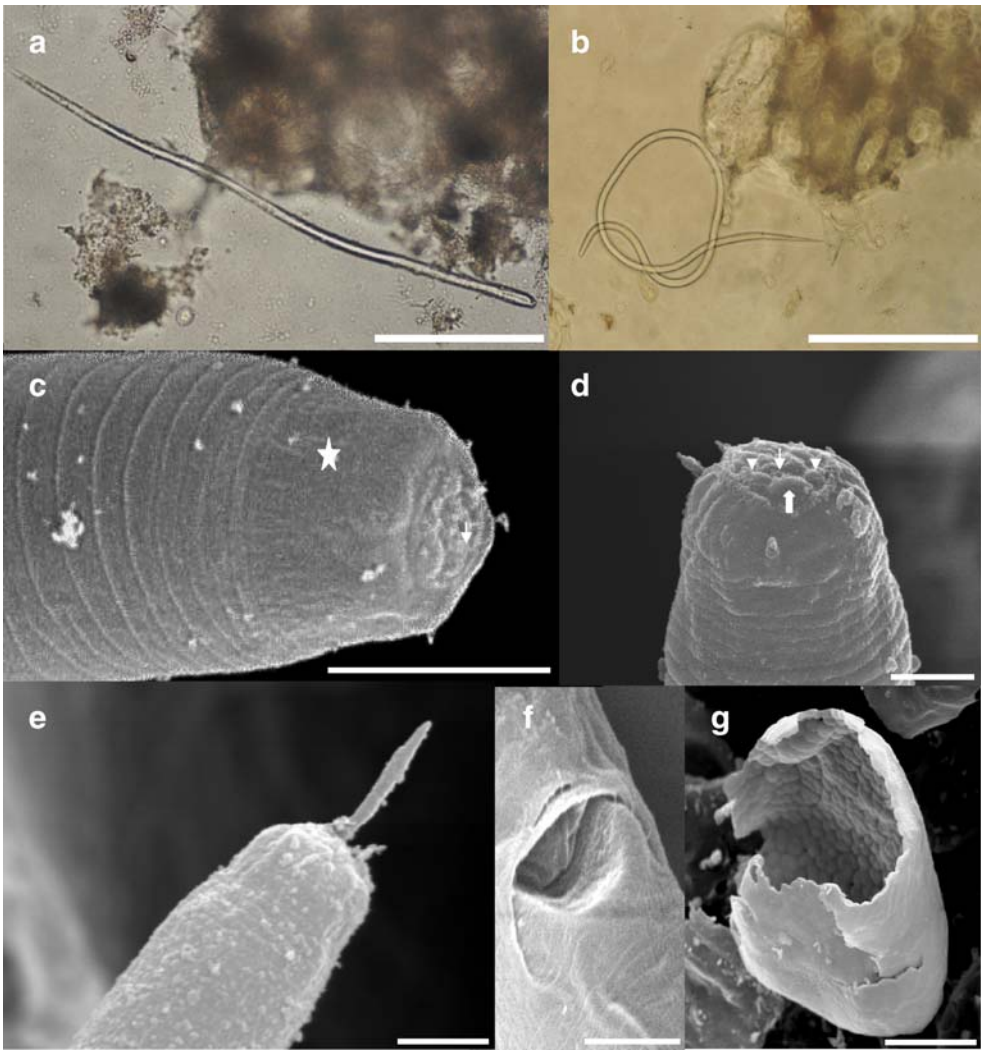

Fig. 1 (**a–f**) Light and scanning electron microscopies of *Bursaphelencus (Rhadinaphelencus) cocophilus* (**a**) Light microscopy (LM) of L_3 in fecal material from *Rhynchophorus palmarum*, collected from Conceição de Macabu, in the north region of the Rio de Janeiro State. *Bar* 150 μm. (**b**) LM of adult in tissue fragment and juice of an infected plant. *Bar* 200 μm. (**c**, **d**) SEM views of cephalic end of *B. cocophilus*, which was slightly flat and had a simple oral opening surrounded by a labial disc and most externally by six lips. The cuticle in this region presented a modified ornamentation pattern (*star*). The cephalic end presented two pairs of inner papillae (*arrowheads*) and two amphids apertures (*slender arrow*) in a slightly lateral position, near to outer papillae (*large arrow*). *Bars* 5 μm. (**e**) SEM view of anterior region of adult *B. cocophilus*, showing the conical form spears. *Bar* 10 μm: (**f**) SEM of a female of *B. cocophilus*: vulva presenting a thin anterior lip and a thicker posterior one surrounded and covered by a modified cuticular pattern, ventral view. *Bar* 2.5 μm. (**g**) SEM of an egg which was elliptical and presented a smooth outer and a rough inner surface. *Bar* 20 μm

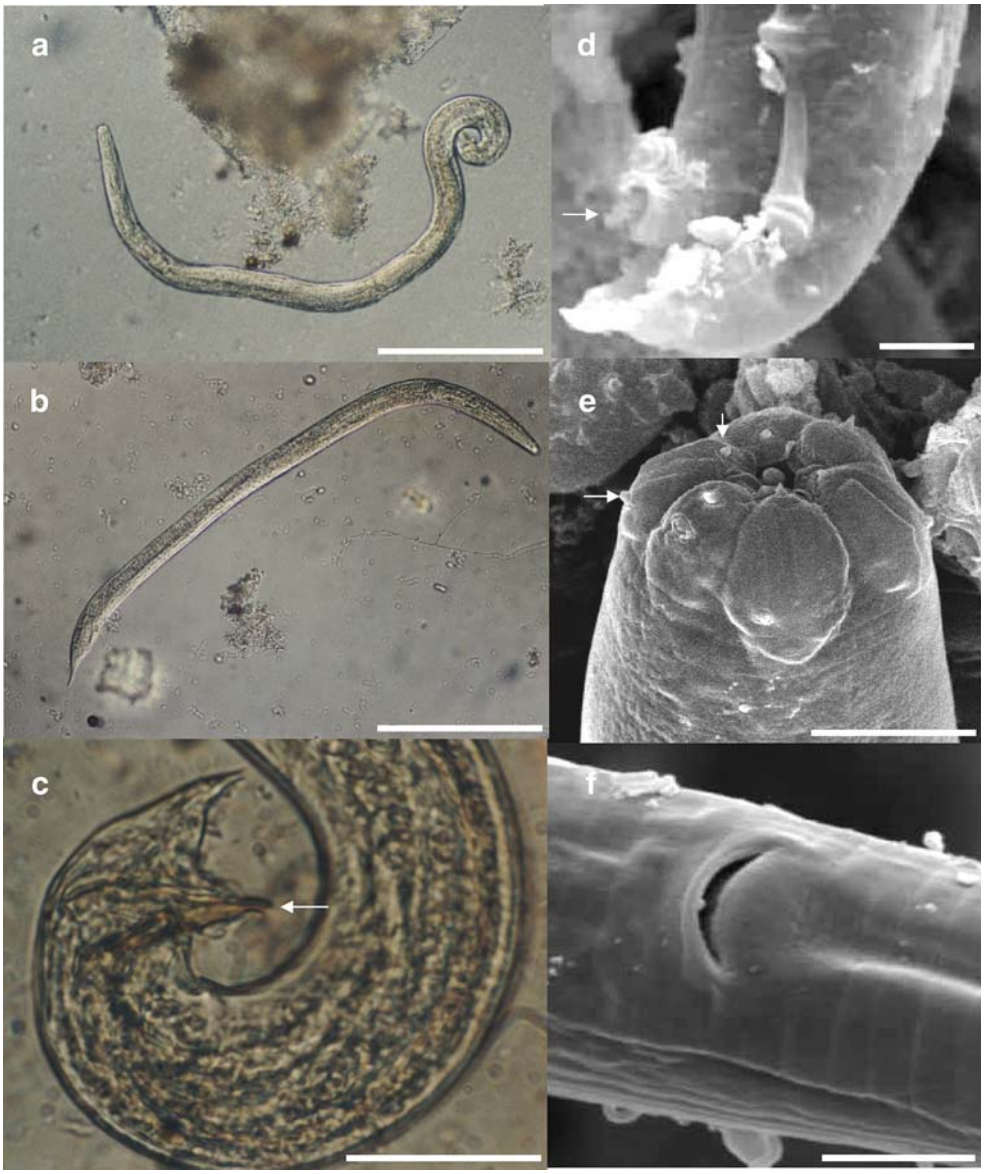

Fig. 2 (**a**–**f**) Light and scanning electron microscopies of the *Teratorhabditis palmarum* (**a**, **b**) LM of male (**a**) and female (**b**) of *T. palmarum.* Note the rounded, spicate, and with sharply pointed spine female tail (**b**). *Bars* (**a**) 150 and (**b**) 300 μm. (**c**, **d**) LM and SEM views of male posterior region showing spicules, which were completely fused at distal ends and connected dorsally for a half of the spicule length (*arrows*). *Bars* (**c**) 20 and (**d**) 10 μm. (**e**) SEM view of cephalic end presenting six lips of uniform shape, each one with a papillae pair (*arrows*). *Bar* 5 μm. (**f**) SEM view of anus region of female. *Bar* 10 μm

in a slightly lateral position near to papillae (Fig. 1d). Cuticular striations near the anterior region of *B. cocophilus* males and females were 0.18–0.27 μm wide in lateral view (Fig. 1c). Spears presented conical form and sizes varying from 12.0 to 15.0 μm (Fig. 1e).

B. cocophilus females presented a vulval flap localized from 650.0 to 700.0 μm from the cephalic end, which was crescent-shaped posteriorly in ventral view (Fig. 1f). The posterior lip of the vulva was heavily sclerotized and thicker (Fig. 1f).

By SEM, the eggs were elliptical, presenting a rough inner and a smooth outer surface (Fig. 1g). Their measures were approximately 60 μm long and 40 μm wide.

T. palmarum male (Table 2), as seen by LM (Fig. 2a), presented the posterior region slightly dorsoventrally curved. By LM and SEM, bursas have shown crenated margins. The ten bursal rays were variable in width and shape (not shown) and spicules were completely fused at distal ends and connected dorsally for a half of the spicule length (Fig. 2c and d). The female vulval aperture was located in posterior region near the anus, and the distance between them was slightly greater then the tail length (not shown). The tail was rounded, spicate, and with sharply pointed spine (Fig. 2b). The cephalic end of both sex genres observed by SEM presented six lips of uniform shape, each one with a papillae pair. The lip margins were strongly cuticularized (Fig. 2e). The female anus was terminal and crescent-shaped posteriorly in ventral view, presenting a sclerotized anterior lip (Fig. 2f).

Diplogasteritus sp (Table 3) en face view presented three lips little sclerotized, each one with a papillae (Fig. 3a). Amphid apertures were not observed. The vulva was median, and its protuberant lips had a modified pattern of cuticular arrangement (Fig. 3b). The tail was long (143–176 μm) and narrow (Fig. 3c). Anal aperture presented a

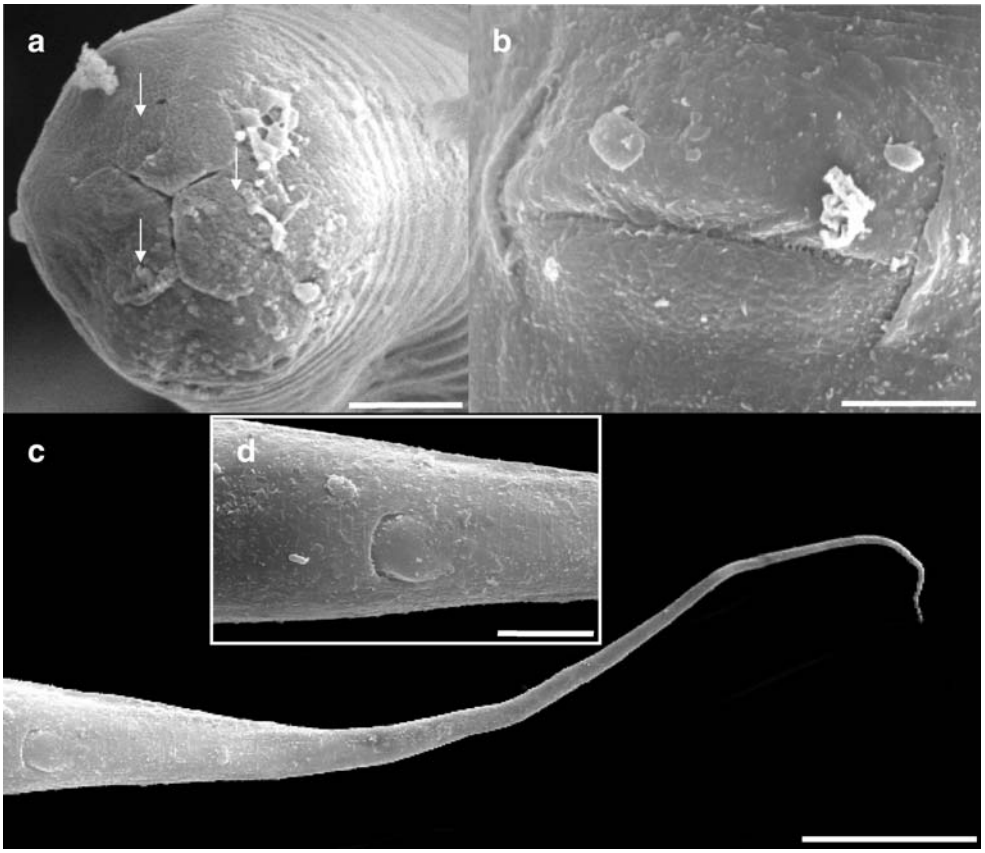

Fig. 3 (**a–d**) SEM of the *Diplogasteritus* sp (**a**) SEM view of cephalic end presenting three little sclerotized lips, each one with one papilla (*arrows*). *Bar* 5 μm. (**b**) SEM of a female showing vulva with protuberant lips which have a modified pattern of cuticle arrangement. *Bar* 5 μm. (**c**, **d**) SEM views of female posterior region showing the long and narrow tail (**c**). Anal aperture (**d**) had a slit-like form and presents a sclerotized and striated anterior lip. The posterior lip was smooth. *Bars* (**c**) 50 and (**d**) 10 μm

slit-like form and a sclerotized and striated anterior lip. The posterior lip was smooth (Fig. 3d).

Discussion

Our observations based in LM, morphology and morphometry, and SEM revealed that three species of nematodes were presented in insect fecal material: L_3 of *B. cocophilus*, all stages of *T. palmarum*, and *Diplogasteritus* sp. In juice and plant fragments, all these species were present; however, only adult forms of *B. cocophilus* were observed. These observations agreed with Gerber and Giblin-Davis (1990a, b) who verified all these species infecting *R. palmarum*. Griffith (1987) stated that *B.cocophilus*, adult and young forms, were present at *R. palmarum* digestive tract. Nevertheless, this author emphasized that some specimens of *R. palmarum* had the ability to keep only *B. cocophilus* young forms in their digestive tract, reducing the potential of parasite infection. Gerber and Giblin-Davis (1990a) did not observe an existing correlation between the size of the insect, the stage, and the amount of parasite nematodes.

Our observations of cephalic ends of *B. cocophilus* agree with those from Giblin-Davis et al. (1989). In this study, we showed the conical spears of *B. cocophilus*. Phytonematodes present spears for vegetal cell penetration, which is fundamental in plant–pathogen relationships (Agrios 2005). *B. cocophilus* females presented a posterior crescent-shaped vulval flap in ventral view. Both lips of the vulva were heavily sclerotized. These observations differ from those from Giblin-Davis et al. (1989), who observed only a posterior vulval lip heavily sclerotized.

Previous studies (Gerber and Giblin-Davis 1990a, b) showed the association between *B. cocophilus* and other nematode species with the palm weevil, *R. palmarum*. In our samples, *T. palmarum* was associated with *R. palmarum*, and this nematode was also present in juice and fragments of infected plants. It is highly probable that *T. palmarum* performed an opportunistic parasitism feeding on bacteria, yeasts, or other fungi that grows in infected plants. We are in accordance with those authors, who affirm that *R. palmarum* biology is not affected by *T. palmarum*. However, we believe that the presence of *T. palmarum* in infected plants should increase the Red Ring disease symptoms. With regard to our *T. palmarum* SEM observations, we added the en face view to the original description. Gerber and Giblin-Davis (1990b) described the cephalic end, based on LM, presenting six lips of uniform shape, each with one papilla and a short seta. Our observations revealed that cephalic end had six lips, each one with a pair of papillae.

Diplogasteritus sp is considered a free-living nematode (Paramonov 1952). There are no reports that show these

nematodes associated with the Red Ring disease. Notwithstanding, Gerber and Giblin-Davis (1990a) associated these nematodes with the vector *R. palmarum* as a routine inhabitant of the insect digestive tract. In our samples of infected vegetable material, we observed the presence of these nematodes. Probably, the presence of this nematode on infected plants could be related to the insect's habit to defecate inside of galleries it has done in the plant. It is known that *R. palmarum* is a poliphagous insect (Arango and Rizo 1977) and *Diplogasteritus* sp is a free-living nematode (Paramonov 1952). A relationship between secondary infections caused by the presence of this nematode associated with the Red Ring disease could be pertinent. By SEM, we observed the en face view (Fig. 3a), and the fine structure of cuticular striations at the vulva region revealed a modified cuticular pattern (Fig. 3b). In addition to it, we observed the long tail, a strongly feature of this genus, presenting an anal aperture with a slit-like form. The cuticular ornamentation of this region has not been described yet.

These findings confirm previous studies which relate that *R. palmarum* presents a varied nematode fauna. Nematodes associated with *B. cocophilus* could probably be co-participates of the etiology of Red Ring disease.

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