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**Thielaviopsis paradoxa and cultivable mycobiota associated with
carapace of Rhynchophorus palmarum L. (Coleoptera: Curculionidae) in
the state of Alagoas, Brazil**

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Bacharelado em Farmácia.

Orientador: Prof. Dr. Valter Alvino
da Silva.

Maceió/AL



DECLARAÇÃO

Declaramos para os devidos fins que **JOÃO LUCAS LETTIERI ELIZIARIO**, matrícula **19210990**, teve seu Trabalho de Conclusão de Curso (Artigo): "THIELAVIOPSIS PARADOXA AND CULTIVABLE MYCOBIOTA ASSOCIATED WITH CARAPACE OF RHYNCHOPHORUS PALMARUM L. (COLEOPTERA: CURCULIONIDAE) IN THE STATE OF ALAGOAS, BRAZIL." avaliado e aprovado com nota 10,0 (dez) pela Banca Examinadora, listada abaixo, em 25/10/2024.

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Original Article

Thielaviopsis paradoxa and cultivable mycobiota associated with carapace of *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) in the state of Alagoas, Brazil

Thielaviopsis paradoxa e micobiota cultivável associada à carapaça de *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) no estado de Alagoas, Brasil

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Abstract

Rhynchophorus palmarum (Coleoptera: Curculionidae) is a significant agricultural pest in palm plantations across tropical America, playing a critical role as a vector of the fungus *Thielaviopsis paradoxa*, which is the causative agent of stem bleeding disease in coconut palms. This disease has raised concerns due to its rapid spread and subsequent reduction in coconut production in northeastern Brazil. Additionally, this insect can establish mutualistic interactions with various fungi, including saprophytic, phytopathogenic, and entomopathogenic fungi, underscoring the importance of identifying its external mycobiota. The aim of this study was to assess the presence of *T. paradoxa* in the digestive tract and identify the cultivable mycobiota associated with the carapace of *R. palmarum*. To achieve this, a mycological study was conducted by culturing the external surface and digestive tract of field-caught adult insects (10 males and 10 females) on potato dextrose agar (PDA) in Maceió, Alagoas, Brazil. Fungal identification was performed by correlating microscopic features with the macroscopic characteristics of the obtained colonies. The results showed that *T. paradoxa* was detected in 15.0% of carapace isolates but was not found in the insects' intestinal tract. Additionally, nine fungal genera frequently associated with saprophytic or phytopathogenic behaviors were identified on the carapace. Eight of these genera belong to the Ascomycota phylum, while one is classified in the Basidiomycota phylum. The ubiquitous presence of *Paecilomyces* spp. and the occurrence of *Trichosporon* spp. in 95% of the assessed insects stand out. Furthermore, other potentially phytopathogenic fungi such as *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp., as well as fungi with entomopathogenic potential like *Paecilomyces* spp., *Trichoderma* spp., *Metarhizium* spp., and *Beauveria bassiana*, were detected. These findings enhance the understanding of the complex interactions between *R. palmarum* and its fungal hosts, providing insights for integrated pest management strategies.

Keywords: *R. palmarum*, coconut, *T. paradoxa*, coconut stem bleeding disease, *Paecilomyces* spp.

Resumo

Rhynchophorus palmarum (Coleoptera: Curculionidae) é uma praga agrícola de plantações de palmeiras na América tropical, apresentando grande importância por ser vetor do fungo *Thielaviopsis paradoxa*, agente causal da doença de sangramento do tronco (resinose) em coqueiros, que tem causado grande preocupação devido à sua rápida disseminação e consequente redução na produção de coco no nordeste do Brasil. Além disso, este inseto pode apresentar interações mutualísticas com diferentes fungos, incluindo fungos saprofíticos, fitopatogênicos e entomopatogênicos, destacando a importância de identificar sua micobiota externa. O objetivo deste estudo foi verificar a presença de *T. paradoxa* no trato digestivo e identificar a micobiota cultivável associada à carapaça de *R. palmarum*. Para isso, um estudo micológico foi realizado através de cultivo em ágar batata dextrose (APD) da parte externa e do trato digestivo de insetos adultos capturados em campo (10 machos e 10 fêmeas) na cidade de Maceió, Alagoas, Brasil. A identificação dos fungos foi realizada associando os aspectos microscópicos às características macroscópicas das colônias obtidas. Os resultados revelaram que *T. paradoxa* foi detectado em 15,0% dos isolados da carapaça, mas não foi encontrado no trato intestinal dos insetos. Além disso, foram identificados na carapaça nove gêneros fúngicos frequentemente associados a comportamentos saprófitos ou fitopatogênicos. Oito desses gêneros pertencem ao filo Ascomycota, enquanto um é classificado no filo Basidiomycota. Destaca-se a presença ubíqua de *Paecilomyces* spp. e a ocorrência de *Trichosporon* spp. em 95% dos insetos avaliados. Adicionalmente, outros fungos potencialmente fitopatogênicos, como *Penicillium* spp., *Fusarium* spp. e *Aspergillus* spp., e fungos com potencial entomopatogênico, como *Paecilomyces* spp., *Trichoderma* spp., *Metarhizium* spp. e *Beauveria bassiana*, foram detectados. Essas descobertas ampliam a compreensão das complexas interações entre *R. palmarum* e seus fungos hospedeiros, fornecendo informações para estratégias de manejo integrado de pragas.

Palavras-chave: *R. palmarum*, coco, *T. paradoxa*, resinose do coqueiro, *Paecilomyces* spp.

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1. Introduction

Rhynchophorus palmarum Linnaeus, 1764 (Coleoptera: Curculionidae), commonly known as the South American palm borer or coconut eye borer, is an important pest in palm plantations throughout tropical America, including Venezuela, Mexico, Brazil, and the Caribbean region (Löhr et al., 2015; Batalha et al., 2020; Hoddle et al., 2020; Calumby et al., 2022a).

This insect has a broad host range, impacting 35 plant species from 12 different families, with a preference for Arecaceae (EPPO, 2007; Araujo-Dalbon et al., 2021). It primarily infests *Cocos nucifera* L. (coconut tree), *Elaeis guineensis* Jacq. (oil palm), *Euterpe edulis* Mart. (juçara palm), *Metroxylon sagu* Rottb. (sago palm), *Phoenix canariensis* Chaub. (Canary Island date palm), *Phoenix dactylifera* L. (date palm), and *Saccharum officinarum* L. (sugar cane) (Plata-Rueda et al., 2016; Martínez et al., 2019; Hoddle et al., 2020). Additionally, adult insects are attracted to and feed on other plants such as *Carica papaya* L. (papaya tree), *Ananas comosus* (L.) Merr. (pineapple tree), and *Musa paradisiaca* L. (banana tree), leading to losses and damage in these plantations (Ferreira et al., 2014).

Among the insects that damage coconut trees, *R. palmarum* demands increased attention and monitoring due to its potential economic, ecological, and agricultural significance. These beetles have remarkable olfactory abilities, enabling them to locate host plants over significant distances (Hoddle et al., 2020). When they feed on the internal plant tissue, they create galleries that damage the plant, rendering it vulnerable to diseases and other pests. In severe cases, they can destroy the meristem, leading to the death of the plant (Ferreira et al., 2014). The impact of such damage extends beyond agriculture, affecting regional economies, ecosystems, and biodiversity. Coconut trees are critical to local economies and food security, and their loss can disrupt ecosystems and jeopardize regional livelihoods. Therefore, understanding and mitigating the threat posed by *R. palmarum* is essential not only for agriculture but also for regional sustainability.

In addition to the aforementioned impacts, some studies have proposed that *R. palmarum* may serve as a potential vector for the fungus *Thielaviopsis paradoxa* (De Seynes) Höhn (1904), an asexual form of the ascomycete *Ceratocystis paradoxa* (Dade) C. Moreau (1952), responsible for coconut stem bleeding (resinosis) (Carvalho et al., 2013). In the northeastern region of Brazil, this fungal disease has become a significant concern among producers and research institutions due to its rapid spread and subsequent decline in coconut tree production (Carvalho et al., 2011). Furthermore, the fungus can survive in decomposed crop residues and endure in the soil for extended periods by forming resilient structures known as chlamydospores, posing challenges for its eradication (Nascimento et al., 2020).

Coconut stem bleeding represents one of the most devastating diseases affecting coconut crops, with significant lethality and destructive potential (Ferraz et al., 2020). The disease is characterized by the emergence of a reddish-brown liquid that seeps through cracks in the trunk, subsequently darkening to a reddish or blackish hue upon drying. It leads to the rotting of internal tissue, a reduction

in leaf emergence, stunted growth of young leaves, thinning of the trunk near the canopy, and the presence of brittle, brownish-yellow foliage (Carvalho et al., 2011).

In addition to its role as a vector of *T. paradoxa*, insects such as *R. palmarum* can engage in mutualistic interactions with various groups of fungi. These interactions encompass fungi that serve as a food source (Ambrosia fungi), fungi that can act as primary pathogens, opportunistic pathogens, or secondary colonizers, and occasional interactions with fungi that may be transported by the insects, regardless of their potential as phytopathogens (Ramos et al., 2015). Additionally, these arthropods can become infected by entomopathogenic fungi, which are commonly used as biological control agents for insect pests (Pedrini, 2018), emphasizing the importance of identifying the mycobiota associated with *R. palmarum*.

Considering the factors mentioned above, the main objective of this study was to detect *T. paradoxa* in the digestive tract and profile the cultivable mycobiota found on the carapace of *R. palmarum*. The research focused on evaluating the prevalence of infected insects in plantations in Alagoas, Brazil, and confirming the potential role of *R. palmarum* as a vector of this important phytopathogenic agent. Additionally, the study aimed to uncover the presence of other fungal genera that constitute the cultivable mycobiota associated with this significant insect pest.

2. Material and Methods

2.1. Sampling of *R. palmarum* adult insects

Adult *R. palmarum* insects were collected during the spring season in the metropolitan region of Maceió, Alagoas, Brazil, using traps baited with the aggregation pheromone Rincoforol® and sugarcane pieces, as described by Duarte et al. (2003). The captured insects were promptly transported to the laboratory for analysis and placed in plastic cages with a volume of 30 cm³. They were provided with sugarcane as their food source and placed in a climate-controlled room at room temperature, with a relative humidity of 60 ± 10%, for up to one week to prevent alterations in the mycobiota.

2.2. Isolation of *T. paradoxa* and other fungi from the carapace of *R. palmarum*

The methodology was based on the study conducted by Carvalho et al. (2011). To ascertain the presence of *T. paradoxa* and isolate the fungi that constitute the cultivable mycobiota associated with the carapace of *R. palmarum*, each of the 20 adult insects was individually rubbed onto Petri plates containing Potato Dextrose Agar (PDA), with one insect per plate.

To assess the presence of *T. paradoxa* in the insect's digestive tract, the intestines were extracted using the method described in Calumby et al. (2022a). In brief, the insects had their elytra and membranous wings removed and were subsequently surface-disinfected by immersing them in 2% sodium hypochlorite for 30 seconds, followed by three rinses in sterilized distilled water. Next, the intestine of each insect was carefully separated from the

carcass using fine dissecting tweezers and needles, and then sectioned into three segments. Each segment was placed in a Petri dish containing PDA supplemented with chloramphenicol (50 mg/L). The plates were incubated at 28°C in a microbiological incubator for up to seven days and examined every 24 hours during this period.

2.3. Fungi identification

Filamentous fungi were identified by observing and comparing the macroscopic and microscopic characteristics of the colonies with descriptions available in the specialized literature for fungal genera. Macroscopic characteristics such as colony color, texture, size, and pigmentation were observed on Sabouraud Dextrose Agar (SDA), as described by Hoog et al. (2000), Lacaz et al. (2002), Sidrim and Rocha (2010) and Zaitz et al. (2010). Microscopic features were observed by creating microcultures on Lactrimel agar using the Ridell technique (Calumby et al., 2019) and examining them under a bright-field microscope with 10X and 40X objectives. Yeasts were identified through a morphological analysis of colonies on ASD and microscopic analysis using the microculture technique on Corn Meal Agar. The results were correlated with assimilation and fermentation tests of carbon and nitrogen sources (Zaitz et al., 2010; Calumby et al., 2022b).

3. Results and Discussion

Isolations conducted from the carapace of adult *R. palmarum* insects revealed a mycelial growth of *T. paradoxa* in 15.0% of the samples, which corresponds to three infected insects out of the total of 20 analyzed (two males and one female). However, *T. paradoxa* was not detected in the intestinal tract of the examined insects, as indicated in Table 1. The findings of this study contrast with those reported by Carvalho et al. (2011), who isolated *T. paradoxa* from the carapace (99.6%) and digestive tract (77.5%) of *R. palmarum* captured in the municipality of Neópolis, SE, Brazil.

The relatively low isolation rate of *T. paradoxa* observed in this study can be attributed to the limited occurrence of coconut stem bleeding in the Maceió, Alagoas region.

Table 1. Infection rates of *T. paradoxa* in the carapace and gut of adult *R. palmarum* insects in Maceió, Alagoas, Brazil.

Colonization by <i>T. paradoxa</i>	Males	Females	Total
Carapace			
Infected	2	1	3 (15.0%)
No infected	8	9	17 (85.0%)
Total	10	10	20 (100.0%)
Gut			
Infected	0	0	0 (0.0%)
No infected	10	10	20 (100.0%)
Total	10	10	20 (100.0%)

Carvalho et al. (2011) describe that *R. palmarum* is not a natural host of this fungus but acquires it while feeding on contaminated plantations. According to Nascimento et al. (2020), *T. paradoxa* is a pathogen that primarily infects through wounds and natural fissures, and its spread is facilitated by insect vectors like *R. palmarum*, as well as contaminated soil and tools.

While *R. palmarum* is not considered a natural host of *T. paradoxa*, there is evidence suggesting the possibility of the fungus completing its life cycle within the insect (Nascimento et al., 2020). During feeding on infected plantations, *R. palmarum* can acquire *T. paradoxa* through natural wounds or fissures, as well as externally dispersed on its carapace through spores. As a result, when feeding on healthy plants, *R. palmarum* can serve as a vector in transmitting the infection. Furthermore, the dispersion of *T. paradoxa* can occur through mechanisms such as rain, wind, injuries, and tools used in harvesting, cultural practices, or eradication of diseased plants. These additional mechanisms contribute to the spread of the fungus in the environment, expanding its potential impact (Álvarez et al., 2012).

Macroscopically, *T. paradoxa* exhibited a velvety texture with delicate and aerial mycelium, demonstrating rapid growth and covering nearly the entire surface of the Petri dish within 48 hours of incubation. Over the course of 3 days, the coloration underwent variation, initially displaying white mycelium and gradually transitioning to black by the third day (Figure 1A). Microscopically, the presence of primary conidia, referred to as endoconidia, was observed. These conidia were aseptate, hyaline, and rectangular in shape. Additionally, aseptate secondary conidia were identified, characterized by their brownish hue and oblong to oval morphology (Figure 1B). Furthermore, aleurioconidia were present, exhibiting a spherical and smooth structure with thick, brown walls, as previously described by Warwick and Passos (2009).

In addition to *T. paradoxa*, several other fungi were identified on the carapace of *R. palmarum*, as shown in Figure 2. *Paecilomyces* spp. was isolated from all evaluated insects, followed by *Trichosporon* spp., which was present in 19 insects (95.0%). *Penicillium* spp. (45.0%), *Fusarium* spp. (25.0%), *Aspergillus* spp. (25.0%), *Trichoderma* spp. (20.0%), *Acremonium* spp. (15.0%), *Metarhizium* spp. (5.0%), and *Beauveria bassiana* (5.0%) were also identified. In Figure 3, the macroscopic and microscopic characteristics of the fungi identified on the exoskeleton of *R. palmarum* are presented. These features are essential for accurate identification of these fungi through detailed morphological analysis.

In this study, most of the identified genera are widely recognized in the literature as saprophytes or potential agents of plant diseases, notable for their broad distribution across various ecosystems and their remarkable adaptability to a variety of substrates and ecological niches (Ramos et al., 2015). These genera predominantly belong to the phylum Ascomycota, with a total of eight identified genera, and one genus belongs to the phylum Basidiomycota (*Trichosporon* spp.), including five in the order Hypocreales, three in the order Eurotiales, and one in the order Trichosporonales.

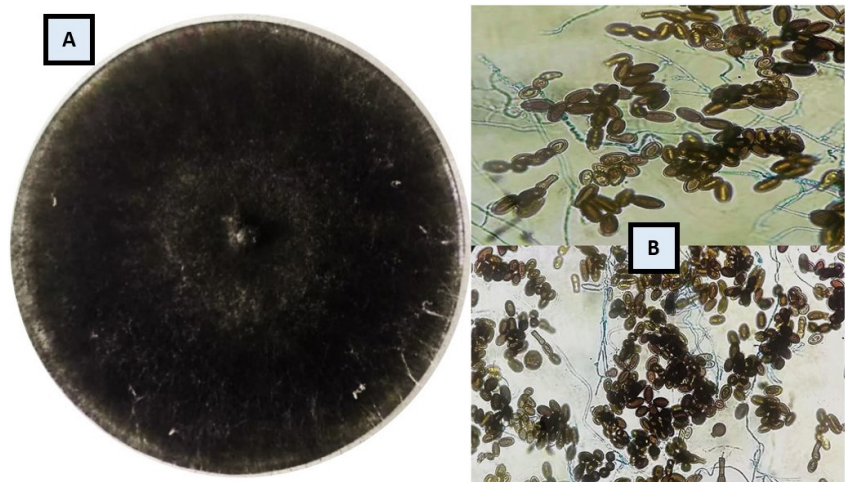


Figure 1. *Thielaviopsis paradoxa*. (A) Macroscopic characteristics on PDA agar after 72 hours of incubation; (B) Microscopic features on a slide stained with lactophenol cotton blue.

There is a scarcity of available studies on fungi associated with insects of the genus *Rhynchophorus*, particularly concerning the species *R. palmarum*. However, some studies have been conducted with *R. ferrugineus*. Ramos et al. (2015) identified 59 species of fungi associated with various structures of *R. ferrugineus*, including the rostrum/antennae, elytra, legs, and samples of internal organs, in Portugal. The majority of the species found in the aforementioned study are known to be saprophytic or phytopathogenic. Additionally, the presence of *Nalanthamala vermoeseni* and *T. paradoxa*, important agents causing diseases in palm trees, as well as fungi from the genera *Metarhizium* and *Paecilomyces*, known to play a relevant role as entomopathogens, was observed.

In another study conducted by Wahizatul et al. (2013), seven fungal genera (*Acremonium*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Geotrichum*, *Penicillium*, and *Trichoderma*) were identified in adults of *R. ferrugineus* captured in Thailand. It is important to highlight that many of these genera were also found in our study with *R. palmarum*, reinforcing the similarity in fungal composition associated with these insect species.

Insects serve as transient and nutrient-rich environments. The ability of various microorganisms to colonize the habitats of these animals involves several characteristics, including mechanisms to evade or modulate the host's immune system and metabolic adaptations to utilize the host's resources. Fungi associated with insects encompass both pathogens, whose survival is linked to the host's vulnerability, and beneficial forms that contribute nutrients, offer protection, and participate in metabolic processes that enhance the host's performance (Wong et al., 2015).

The insect's exoskeleton, composed primarily of chitin and keratin, provides a favorable environment for the colonization of various fungi that utilize these nutrients for their growth (Tetreau and Wang, 2019). While many of the fungi isolated from *R. palmarum* can be considered symbiotic or commensal to insects and plants, certain genera can be pathogenic to specific plants, either through direct action or by producing mycotoxins. This is the case

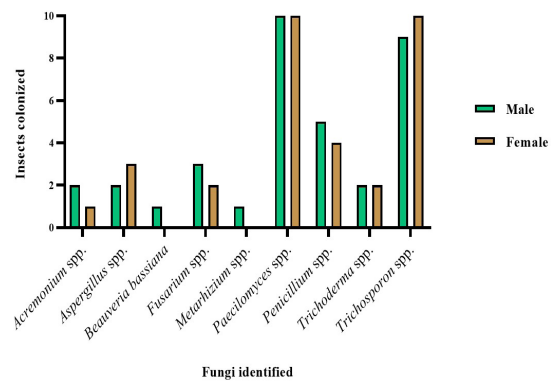


Figure 2. Cultivable mycobiota identified on the exoskeleton of adult *R. palmarum* insects captured in Maceió, Alagoas, Brazil.

with *Fusarium* spp., *Penicillium* spp., and *Aspergillus* spp. (Habschied et al., 2021).

Fusarium spp. are known to cause numerous diseases in economically important plant species. However, they are also associated with the production of beneficial compounds that inhibit the growth of phytopathogens affecting agricultural crops (Husaini et al., 2018). Some of the agricultural plants of significance that can be affected by *Fusarium* include pineapple (Silva et al., 2020), maize (Torovic, 2018), soybean (Schollenberger et al., 2007), and wheat (Orlando et al., 2019). As for *Penicillium* and *Aspergillus*, certain species within these genera are known to produce toxins and hold economic importance in the cereal industry (Alshannaq and Yu, 2017).

Fungi that may potentially serve as important entomopathogenic agents have also been isolated from the external surfaces of *R. palmarum*, such as *Beauveria bassiana*, *Metarhizium* spp., *Paecilomyces* spp., and *Trichoderma* spp. Although many insects successfully inhabit hazardous environments exposed to diverse microbial communities,

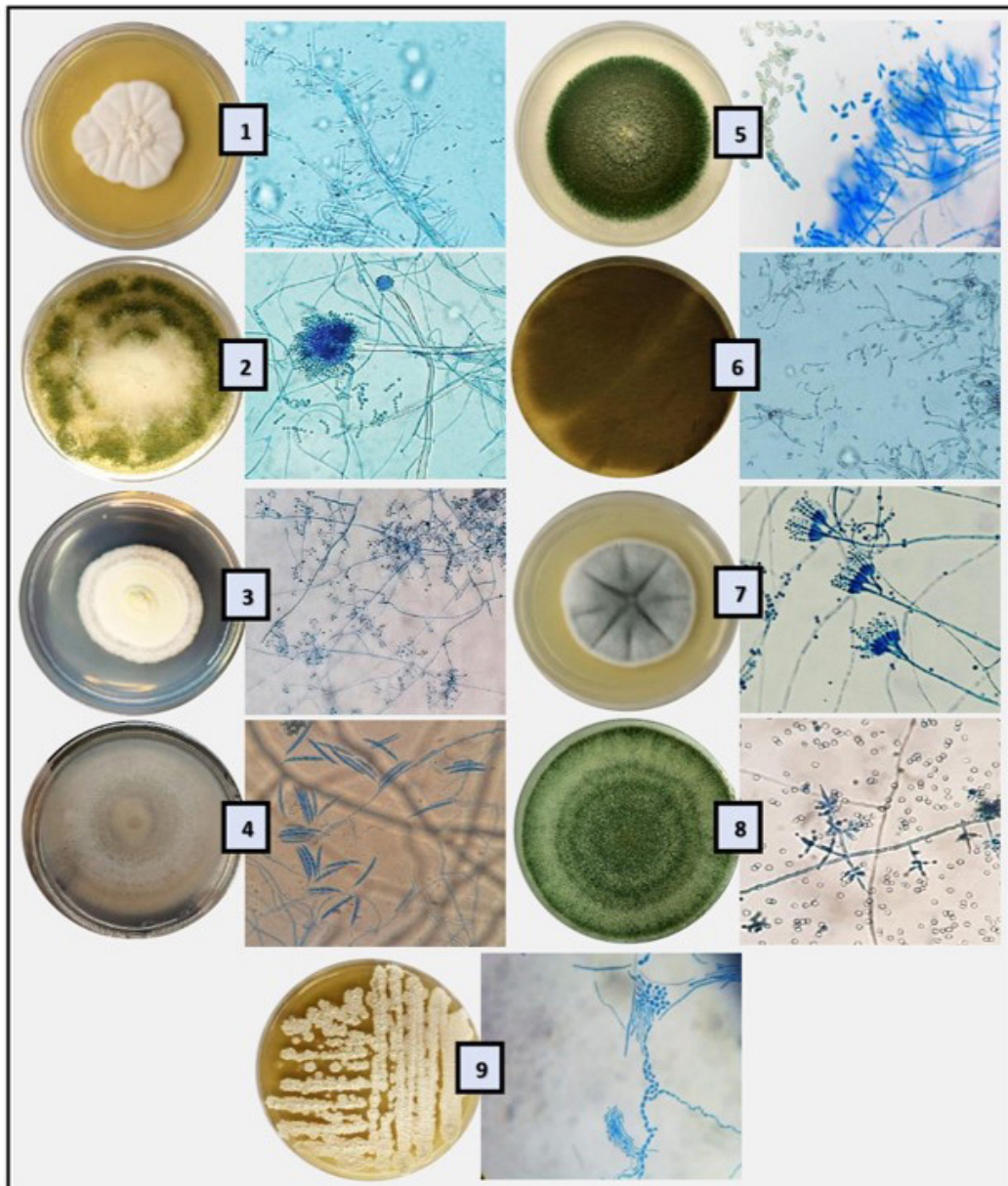


Figure 3. Macroscopic and microscopic features of the fungi identified on the carapace of *R. palmarum*, cultured on Petri dishes containing SDA at 28 °C. Slides stained with lactophenol cotton blue. Samples: (1) *Acremonium* spp.; (2) *Aspergillus* spp.; (3) *Beauveria bassiana*; (4) *Fusarium* spp.; (5) *Metarhizium* spp.; (6) *Paecilomyces* spp.; (7) *Penicillium* spp.; (8) *Trichoderma* spp.; (9) *Trichosporon* spp.

they can also be susceptible to colonization and/or lethality by specialized pathogens (Butt et al., 2016).

Entomopathogenic fungi are organisms that have evolved to exploit insects, and they include a broad spectrum of morphologically, phylogenetically, and ecologically diverse fungal species (Araújo and Hughes, 2016). These fungi have emerged as ecologically sound alternatives to chemical insecticides in biocontrol programs targeting agricultural pests and disease vectors (Zhao et al., 2016).

Paecilomyces spp. was the most abundant fungus on the carapace, being present in all evaluated insects, apparently without causing damage. This suggests that the

species colonizing it may potentially have a commensal relationship. However, further investigations involving genetic sequencing of the isolates and pathogenicity assays are necessary to validate this hypothesis. Among the fungi identified in *R. palmarum*, the genera *Metarhizium* and *Beauveria* stand out as potential agents in insect pathogenesis. These fungi have received significant attention from researchers in the field of agricultural pest control (Jaber and Enkerli, 2016; León-Martínez et al., 2019).

Trichoderma spp. is another important microorganism utilized as a biocontrol agent. This fungus plays a crucial ecological role in the decomposition and mineralization

of plant residues, thereby enhancing nutrient availability for plants. It exhibits rapid growth and is considered an excellent natural biofungicide, effectively suppressing the proliferation of other fungi in agricultural crops (Ferreira and Musumeci, 2021; Nascimento et al., 2022).

In this study, we also observed a high percentage of colonization by *Trichosporon* spp. on the exoskeleton of the evaluated insects. The genus *Trichosporon* is widely distributed in natural environments, particularly in tropical and temperate regions, and it is found in various sources, including soil, decomposing wood, air, bodies of water, cheeses, as well as in the feces of birds, bats, pigeons, and cattle (Colombo et al., 2011). The presence of *Trichosporon* spp. on the carapace of *R. palmarum* is consistent with the study conducted by Górz and Boroń (2016), in which the species *Trichosporon lactis* was isolated as an epizotic colonizer on the exoskeletons of beetles of the genus *Onthophagus* in Poland. This finding highlights the remarkable affinity of species within this genus in establishing a symbiotic relationship with insects, suggesting a crucial role in the ecology of the mycobiota associated with the carapace of *R. palmarum*.

4. Conclusion

In conclusion, this study confirms the presence of *T. paradoxa* on the carapace of *R. palmarum*, highlighting its potential role as a vector of Coconut stem bleeding (resinosis). Furthermore, we identified a diverse mycobiota on the external surface, which includes potentially phytopathogenic fungi such as *Penicillium* spp., *Fusarium* spp., *Aspergillus* spp., and potentially entomopathogenic fungi such as *Paecilomyces* spp., *Trichoderma* spp., *Metarhizium* spp., and *Beauveria bassiana*.

These findings offer valuable insights for the development of integrated pest management strategies and underscore the ongoing importance of monitoring the mycobiota associated with *R. palmarum*. A more profound comprehension of the interactions between the insect and host fungi is crucial for the effective implementation of control measures. Furthermore, it opens up a promising field for future studies on the microbiome associated with this insect, with the aim of exploring the potential of the diverse fungi found as potential regulators of *R. palmarum* populations under natural conditions.

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