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The Association Between Basidiomycete Fungi and Mealybugs Pseudococcidae Affects Coffee Plants

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Abstract: Some Pseudococcidae species interact with *Coffea arabica*'s roots and are associated with basidiomycete fungi. The fungal mycelium envelops the roots, which hinders their water and nutrient absorption. Combined with the feeding activity of the insects, this results in chlorosis, defoliation, and even plant death. Despite the significance of these interactions, they remain under-studied. To investigate the relationship between sporocarps found at the base of coffee trees, the cysts covering their roots, and the mealybug insects within them, samples of these three organisms—sporocarps, cysts, and mealybugs—were collected from 27 coffee plants across three farms in the departments of Norte de Santander and Quindío, Colombia. Fungi and cysts were identified by sequencing a nuclear gene region of the 28S large ribosomal subunit (28S rDNA) using the primers LSU200-F and LSU481-R. Fungal identification was further confirmed through classical taxonomy. Mealybugs were identified by sequencing a region of the mitochondrial gene Cytochrome C oxidase subunit I (COI) with CIF-CIR primers, corroborated through classical taxonomy. This study identified four fungal species associated with four species of *Pseudococcidae*. The fungus *Phlebopus beniensis* was associated with the mealybugs *Pseudococcus elisae*, *Dysmicoccus neobrevipes*, *D. brevipes*, and *Pseudococcus nr. sociabilis*. *Phlebopus portentosus* was linked to *D. neobrevipes*, while *Xerophorus olivascens* and *Boletinellus rompelii* were associated with other *Pseudococcidae* species. Additionally, the fungus *Pseudolaccaria pachyphylla* was found in coffee plants harboring mealybugs. These findings confirm the existence of specific associations between fungal species and mealybug insects that affect coffee plants.

Keywords: biotrophic; coffee; mealybugs; mutualism; roots; sporocarps

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1. Introduction

Mealybugs (Hemiptera: Coccomorpha: Coccoidea) are pests of the *Coffea* species (Rubiaceae) found in different coffee-producing countries, and they can affect both the roots and the aerial parts of the plants [1]. In the case of those that attack the roots, some species, mostly Pseudococcidae Cockerell, 1905, are associated with basidiomycete fungi. Their mycelium covers the affected plants' primary and secondary roots and forms corky structures as a cyst that protects the mealybugs that live and develop inside. The corky layer of the mycelium prevents the absorption of water and nutrients and, in combination with the feeding of the insects, generates chlorosis, defoliation, and even plant death [1,2]. Pseudococcidae groups approximately 258 genera and 2034 species around the world, and 74 species are reported in coffee crops [3,4]; of these, 25 are present in Colombia in the roots *Coffea arabica* L. (Rubiaceae), including the genera *Chorizococcus* McKenzie, 1960,

Distichlicoccus Ferris, 1950, *Dysmicoccus* Ferris, 1950, *Paraputo* Laing, 1929, *Phenacoccus* Cockerell, 1893, *Planococcus* Ferris, 1950, *Pseudococcus* Westwood, 1840, and *Spilococcus* Ferris, 1950 [3,5,6].

In Colombia in recent years, the populations of different mealybug species present on coffee roots have increased, causing economic losses. Furthermore, their hypogeous behavior makes their management difficult and expensive; consequently, some producers prefer to abandon coffee farming [2].

For coffee crops in Colombia, there is information on three species of Pseudococcidae that are associated with basidiomycete fungi: *Dysmicoccus texensis* (Tinsley, 1900), *Pseudococcus elisae* Borchsenius, 1947, and *Pseudococcus jackbeardsleyi* Gimpel & Miller, 1996 [2], while in China, Fang et al. [7] and Zhang et al. [8] reported an association between the fungus *Phlebopus portentosus* (Berk. & Broome) Boedijn and the insects *Dysmicoccus neobrevipes* Beardsley, 1959, *Formicococcus polysperes* Williams, 2004, and *Planococcus minor* (Maskell, 1897) (Pseudococcidae).

According to Zhang et al. [8], some of the fungi that are associated with mealybugs, mainly Pseudococcidae, belong to the Boletinellaceae family of the order Boletales, which comprises two genera, *Phlebopus* (R. Heim) Singer (1936) and *Boletinellus* Murrill (1909). Fungi species of both genera can be saprotrophic but have a mutually beneficial biotrophic association between insects and fungi [8,9]. The fungus, the insect (mealybug), and the plant form a unique tripartite nutritional relationship, in which the roots of the plants are wholly or partially covered by fungal fruiting bodies that produce cysts, where the walls of the cavity are formed by hyphae of fungi instead of plant tissues with mealybugs inside [10]. In this association, the role of the insects is of great importance, since the sugary excretions produced by mealybugs are rich in sugars, amino acids, niacinamide, proteins, minerals, and vitamin B [11] and can promote mycelium growth [8]. Likewise, Fang et al. [7] indicate that mealybugs can be entirely protected by the fungus through the cyst but cannot obtain any nutrients. To feed, the mealybug inside the cysts penetrates the stylet through the mycelial layer and extracts sap from the host plant's roots. Cysts from fungi and insects can have parasitic effects on plants, and the plants can die when the cysts densely cover the entire root system; additionally, both fungi and mealybug insects take their nutrients [12].

The tripartite relationship has also been documented in *Phlebopus portentosus* present in Australia, Brazil, Costa Rica, China, India, Mexico, New Zealand, Sri Lanka, the Republic of Sierra Leone, the Lao People's Democratic Republic, Thailand, and South Africa [13–18]. The mycelium of this fungus has been found in the roots of 37 species of plants from 21 families. However, in only 21 species, including *C. arabica*, were 13 species related to the fungus found. Among them, 11 belong to Pseudococcidae, while the others belong to Monophlebidae and Eriococcidae [8].

Other species of fungi also form tripartite associations with Coccomorpha and plants, such as *Phlebopus tropicus* (Rick) Heinem. & Rammeloo (Boletinellaceae), which forms cysts around the roots of *Citrus* sp. (Rutaceae), in which colonies of *Pseudococcus comstocki* (Kuwana, 1902) are enveloped by the fungus [19]. *Boletus dimocarpicola* M. Zang & Sittigul (Boletaceae) forms cysts with *Paraputo banzigeri* Williams, 2004 on *Dimocarpus longan* Lour. (Sapindaceae) [7,12,20]. Later, Mei et al. [9] reported *Phlebopus roseus* M. Yang, C.-Y. Liu & Y. Wang forming cysts with *Coccus hesperidum* Linnaeus, 1758 (Coccidae) on *Eriobotrya japonica* (Thunb.) Lindl trees (Rosaceae).

In an agricultural context, interactions between fungi, herbivores, and plants can affect crop production. However, underground interactions are challenging to observe and are often overlooked, so they have been under-studied [21]. Therefore, this research aimed to describe the association between sporocarps in the base of coffee trees, the cysts that

cover their roots, and the mealybug insects inside. The damage caused by the association between fungi and mealybugs in coffee is also described.

2. Materials and Methods

2.1. Data Collection

Sporocarps, cysts, and mealybugs were collected from *Coffea arabica* plants in three coffee farms in Norte de Santander and Quindío, Colombia (Table 1, Figure 1). The farms were selected based on the information recorded by Gil et al. [2]. In this work, the authors identified and recorded farms with mealybugs and sporocarps on the roots of coffee plants in eight coffee-growing departments, including Norte de Santander and Quindío-Colombia.

Table 1. Collection sites of sporocarps, cysts, and mealybugs associated with the roots of *Coffea arabica*.

Department	Municipality	Farm	Altitude	Georeference		Soil pH	Organic Matter	Nitrogen Content
Quindío	Quimbaya	La Esperanza	1251	4°34′42″ N	75°47′44″ O	5.4	4.7	0.21
Quindío	Montenegro	El Agrado	1275	4°31′4″ N	75°48′30″ O	4.6	4.7	0.20
Norte de Santander	Lourdes	El Panteón	1398	7°56′54″ N	72°49′51″ O	4.6	3.9	0.18

Figure 1. Study sites located in Colombia coffee-growing region. https://earth.google.com, (accesseed on 10 January 2025).

On La Esperanza farm, a plot of 18-month-old coffee plants of the Castillo® variety was selected, which had a planting density of 3463 trees. Over 50% of the plants were chlorotic or dead, and some planting sites had been lost. The remaining plants, despite being infested with mealybugs, were still productive. The diagnostic evaluation found that 100% of the plants had mealybugs, some of which were associated with basidiomycete fungi. The infestation was so severe that the basidiocarps of the fungus were visible on the base of the stem above the ground.

On the El Agrado farm, a plot of three-year-old coffee plants of the Cenicafé1 variety was selected, which had a planting density of 8333 plants and a total area of 0.94 hectares. During the initial diagnosis, trees were found that presented symptoms of yellowing in the

aerial part, along with several species of Pseudococcidae in the roots. In some plants, cysts were observed in the primary and secondary roots as sporocarps at the base of the tree.

On the Panteón farm, a plot of four-year-old coffee plants of the Castillo® variety was selected, which had a planting density of 6700 plants. A total of 60% of the trees showed chlorosis symptoms in the aerial part and sporocarps at the base of the trees; other trees were dead.

The samples, i.e., sporocarps on the base of coffee trees, the cysts covering the roots, and the mealybug species inside it, were collected between September and November 2021 and March and May 2022, the rainy periods of those years.

2.2. Collection and Identification of Sporocarps

On each farm (Table 1), one plot was visited. In each of them, a census was carried out to determine plants with chlorosis or plants that showed some symptom in the aerial part, which was selected and marked with colored tape; subsequently, the base of the stem was examined in these plants, and for those where sporocarps (with a pileus between 3.0 and 13.0 cm) were observed, all those present in a horizontal radius of 25 cm from the base of the stem above ground were collected; subsequently, they were wrapped in damp paper towels and labeled Ziploc bags and transported in Styrofoam coolers with cooling gels to the laboratory of the entomology department of the National Coffee Research Center—Cenicafé, Manizales, Colombia. The samples were cleaned in the laboratory with distilled water and ultrapure water. Furthermore, the macroscopic and microscopic characteristics were described following the terminology proposed by [22–24]. The observations, measurements, and photographs were carried out with a Nikon SMZ1500 stereomicroscope (Nikon Instruments Inc., Melville, NY, USA), with a SIGHD DS-5M digital camera and the NIS-Elements software version 6.10. Identification by classical taxonomy was carried out following the descriptions proposed by [25–27]. For identification by molecular techniques, the surface of each sporocarp was cleaned with 75% ethanol and ultrapure water; then, with a sterile scalpel, the outer layer of both the pileus and the stipe was removed; moreover, sections of approximately 1 cm were taken, stored in 15 mL Falcon™ tubes, sealed with Parafilm® M, and lyophilized for three days in a Labconco 6L Freeze Dry brand lyophilizer; finally, they were stored at −80 °C until the DNA extraction. For this, the traditional CTAB (cetyl trimethyl ammonium bromide) protocol was used [28,29]. DNA was suspended in 50 µL of AE buffer (Qiagen commercial kit for plant tissue (Qiagen, Inc., Valencia, CA, USA)), checked for integrity on agarose gel, and quantified on a NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, US) They were identified by sequencing a region of the nuclear gene for the 28S large ribosomal region with the primers LSU200-F (5′-AACKGCGAGTGAAGMGGGA-3′) and LSU481-R (5′-TCTTTCCCTCACGGTACTTG-3′) [30]. PCR was performed in a volume of 50 µL; each reaction contained 5 µL of 10X HF taq buffer, 2 µL of $MgSO_4$ (50 mM), 1 µL of dNTP (10 mM), 1 µL of Primer F (10 µM), 1 µL of Primer R (10 µM), 0.2 µL of Phusion High-Fidelity Taq DNA Polymerase (5 U/µL) Thermo Fisher Scientific, 38.8 µL of MilliQ autoclaved water, and 1 µL of DNA (∼20 ηg). The amplification conditions were as follows: initial denaturation for 1 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 55 °C, 40 s at 68 °C, followed by 5 min at 68 °C for final extension. PCR products were purified with a SureClean Plus kit (Bioline) and sent to Macrogen Inc. (Seoul, Republic of Korea) for Sanger sequencing using the primers LSU200-F and LSU481-R.

2.3. Collection and Identification of Cysts

After collecting the sporocarps, each coffee plant was dug up to take the roots with cysts and the mealybugs inside them. All samples were cleaned with distilled water, wrapped in damp paper towels, and packed in labeled Ziploc bags and then transported in

Styrofoam coolers with cooling gels to the Cenicafé Entomology Department laboratory. The cyst-covered roots were initially washed with sufficient water, followed by rinsing with sterile water, ultrapure water, and, finally, with 75% ethanol. In addition, for each root, the morphological characteristics of the cysts were recorded using a Nikon SMZ1500 stereomicroscope with a SIGHD DS-5M digital camera and the NIS-Elements software. Sections were then taken from the interior of five cysts for each plant, stored separately in 15 mL Falcon™ tubes, sealed with Parafilm® M, and stored at −80 °C until DNA extraction. DNA extraction and identification by molecular techniques were carried out using the same protocol, primers, and procedures for sporocarps.

2.4. Collection and Identification of Mealybugs

To collect the samples of the mealybugs from the inside of each of the five cysts for each plant in the previous section, up to 10 adult insects were taken, and 2 were placed separately in 2 mL Eppendorf tubes; they were marked with the respective information and were stored at −80 °C until DNA extraction. The remainder were placed in 2 mL Eppendorf tubes with 96% alcohol and then mounted on slides using the methodology described by Sirisena et al. [31] and were identified following the taxonomic keys of [5,32–35].

For identification by molecular techniques, DNA was extracted from adult females (one for each sample) with a Qiagen commercial kit for animal tissue (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol. After extraction, the DNA was suspended in 50 µL of AE buffer, and the integrity was checked in agarose gel and quantified in a NanoDrop 2000 device. Identification was performed by sequencing the subunit I (COI) region of mitochondrial cytochrome c oxidase with the primers CIF (5′-CAACATTTATTTTGATTTTTTGG-3′) and CIR (5′-GCWACWACRTAATAKGTATCATG-3′) [36,37]. PCR was performed in a volume of 50 µL; each reaction contained 5 µL of 10X HF taq buffer, 2 µL of $MgSO_4$ (50 mM), 1 µL of dNTPs (20 mM), 1 µL of Primer F (10 µM), 1 µL of Primer R (10 µM), 0.2 µL of Taq DNA Polymerase (Phusion High-Fidelity), 36.8 µL of MilliQ autoclaved water, and 3 µL of DNA (∼20 ηg). The amplification conditions were as follows: initial denaturation for 2 min at 98 °C, followed by 34 cycles of 10 s at 98 °C, 30 s at 46 °C, 30 s at 72 °C, followed by 7 min at 72 °C as a final extension. All reactions were carried out in a thermocycler (TECHNE TC-512, Bibby Scientific, Burlington, NJ, USA). A Bioline SureClean Plus kit was used to purify the samples, and the manufacturer's instructions were followed.

The purified PCR products obtained from the mealybugs were sent for Sanger sequencing to Macrogen Inc. (Seoul, Republic of Korea) using the CIF and CIR primers.

Additionally, in each of the plots where the samples of sporocarps and mealybugs were collected, a soil sample was taken for subsequent macronutrient analysis in the Multilab Agroanalítica laboratory, Chinchiná, Caldas, Colombia.

2.5. Description of the Damage Caused by the Association Between Fungi and Mealybugs in Coffee Plants

Twenty secondary roots were taken from three coffee plants with cysts and mealybugs. They were washed with enough water until the soil was removed. Subsequently, with a sterile scalpel, 1 mm thick cross-sections were made for each root, mounted on glass plates, and analyzed directly with a Nikon SMZ1500 stereomicroscope with a SIGHD DS-5M digital camera and the NIS-Elements software.

2.6. DNA Sequence Processing and Analysis

The chromatograms of the DNA sequences were visualized and processed using the Chromas v.2.6.6 software (http://technelysium.com.au/wp/chromas/, accessed on 19 December 2024) to discard low-quality sequences and remove sequences corresponding to

the primers for PCR. High-quality filtered chromatograms for each sample were assembled as consensus sequences using the GeneStudio V.2.2.0.0 software (http://genestudio.com/ accessed on 3 March 2023). The consensus sequences of each sample were used to perform a similarity search with sequences in the GenBank® sequence database.

3. Results

3.1. Identification, Collection, and Description of Sporocarps

A total of 71 sporocarps associated with 27 coffee plants were collected: 53 at La Esperanza farm, 11 at El Agrado farm, and 7 at El Panteón. The comparison of the sequences through GenBank analysis (Table 2) revealed that the sporocarps matched the species *Phlebopus beniensis*, *P. portentosus*, *Pseudolaccaria pachyphylla*, *Xerophorus olivascens*, and *Boletinellus rompelii*. This identification was corroborated by classical taxonomy. The recorded fungal species are described below.

Table 2. Identification of sporocarps through GenBank analysis.

Identified Species	DNA (pb)	GenBank Accessions	Similarity %	E-Value
Phlebopus beniensis	248	AY612822	100	7.8165×10^{-125}
Phlebopus portentosus	716	HQ687218	99.1	3.7127×10^{-128}
Pseudolaccaria pachyphylla	254	MN017488	97.2	6.2725×10^{-116}
Boletinellus rompelii	250	EU718159	99.6	8.7423×10^{-104}
Xerophorus olivascens	249	MN017498	98.4	1.0231×10^{-118}

Basidiomycota: Boletales: Boletinellaceae

Phlebopus beniensis (Singer & Digilio) Heinem. & Rammeloo, 1982.

Synonymy: *Phaeogyroporus beniensis* Singer & Digilio 1960 [25].

The material reviewed: thirty-seven sporocarps and mycelium from five coffee roots. **COLOMBIA**: Quindío, Quimbaya, El Laurel county, La Esperanza farm, 4°34′42″ N; 75°47′44″ O, 1251 m.a.s.l., manual collection, November 2021, coll. Carmona V., L. A, det. By sequencing and classical taxonomy. Eleven sporocarps and mycelium of five coffee roots. **COLOMBIA**: Quindío, Montenegro, Pueblo Tapao county, El Agrado farm, 4°31′4″ N; 75°48′30″ O, 1275 m.a.s.l., manual collection, September and November 2021, coll. Carmona V., L. A., det. By sequencing and classical taxonomy. One sporocarp and mycelium of a coffee root. **COLOMBIA**: Norte de Santander, Lourdes, Los Naranjos county, El Panteón farm, 7°56′54″ N; 72°49′51″ O, 1398 m.a.s.l., manual collection, May 2022, coll. Benavides M., P., det. By sequencing and classical taxonomy.

Morphological description: Veil: absent; **Pileus:** (Figure 2A) irregular, with convex-type elevation, entire margin and wavy edge, surface (cuticle) of velvety texture, dark brown in the center and light brown towards the margin, polar diameter 3.4–12.7 cm, equatorial diameter 3.7–11.5 cm; **Hymenophore:** (Figure 2B,C) tubulose, with obtuse margin, adnate, the context has a thickness of 0.36–1.05 cm, yellow in color, when cut, it oxidizes and changes to dark blue, bilateral tubes that form pores less than 1 mm in diameter, with a polygonal-to-irregular shape; **Annulus:** absent; **Stipe:** (Figure 2D) has a claviform shape, with a central or eccentric position with respect to the pileus and a confluent, radiating insertion, it is between 1.5–9.5 cm in length and between 0.8–3.5 cm in diameter, with a velvety brown surface. **Volva type**: absent; **Spores:** (Figure 2E) basidiospores ellipsoidal, smooth, dark olive-brown in 1% KOH, wall slightly thickened; **Mycelium:** (Figure 2F) measures up to 35 cm long.

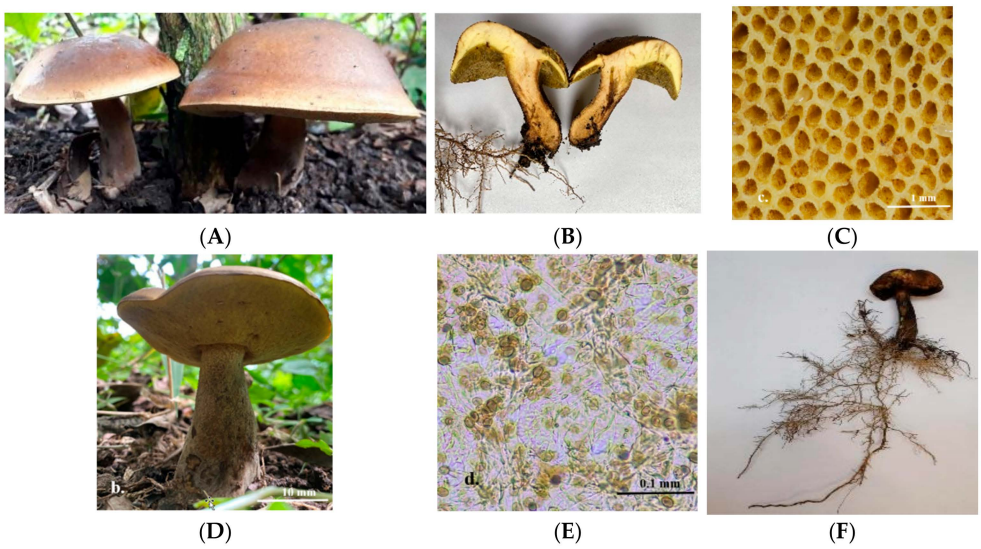

Figure 2. *Phlebopus beniensis*. (**A**) Pileus, (**B**,**C**) hymenophore, (**D**) stipe, (**E**) spores, (**F**) mycelium.

Comments: *Phlebopus beniensis* is mainly a Neotropical species, although it is also recorded in Liberia (Africa) [38]. In Colombia, it was reported by Franco–Molano et al. [22] in the department of Magdalena and by Palacio et al. [25] in the Besotes forest reserve in the municipality of Valledupar, department of Cesar. In the present study, the sporocarps were found in coffee crops under sun exposure; they grow around the base of the tree and in places with leaf litter. They are gregarious, and each coffee plant has up to ten sporocarps. The mycelium of the fungus wholly or partially covers the primary and secondary roots. They predominate during the rainy seasons.

Phlebopus portentosus (Berk. & Broome) Boedijn

Synonymy: *Boletus portentosus* Berk. & Broome, 1873¸ *Gyroporus portentosus* (Berk. & Broome) G. Stev., 1982, *Phaeogyroporus portentosus* (Berk. & Broome) McNabb, 1968, *Suillus portentosus* (Berk. & Broome) [39].

The material reviewed: one sporocarp and mycelium of three coffee roots. **COLOMBIA**: Quindío, Quimbaya, El Laurel county, La Esperanza farm, 4°34′42″ N; 75°47′44″ W, 1251 m.a.s.l., manual collection, September 2021, coll. Gil P., Z. N, det. By sequencing.

Morphological description: not carried out since the only sporocarp collected was incomplete.

Comments: In South America, *P. portentosus* is present in Brazil [14], Costa Rica [40], and Mexico in shaded coffee plantations [15]. In this study, a sporocarp associated with *Coffea arabica* was collected in crops under sun exposure, becoming the first record for Colombia. The mycelium of the fungus wholly or partially covers the primary and secondary roots. At the site where the samples were collected, the most rainy months of 2021 were between March and May and September and November [41], which coincides with the appearance of the sporocarps in the base of the trees. The native vegetation of the area is a transition between premontane humid forest and tropical dry forest, with some elements of premontane very humid forest. The average temperature is 21 °C [42].

Boletinellus rompelii (Pat. & Rick) Watling, 1997

Sinonimia: *Gyrodon rompelii* (Pat. & Rick) Singer, 1938, *Phylloporus rompelii* Pat. & Rick, 1907

The material reviewed: one sporocarp and mycelium of a coffee root. **COLOMBIA**: Norte de Santander, Lourdes, Los Naranjos county, El Panteón farm, 7°56′54″ N; 72°49′51″ W, 1398 m.a.s.l., manual collection, May 2022, coll. Benavides M., P., det. By sequencing.

Morphological description: not carried out because the only sporocarp collected was in an advanced state of maturation (Figure 3).

Figure 3. Sporocarp of *Boletinellus rompelii*, associated with Pseudoccoccidae species in coffee roots.

Comments: *Boletinellus rompelii* is recorded in Argentina, Brazil, Mexico, and the United States [43]. In this study, a sporocarp associated with Pseudococcidae species present in roots of *Coffea arabica* was collected in the coffee crop with a shade of *Albizia carbonaria* Britton (Fabaceae), becoming the first record for Colombia. The mycelium of the fungus wholly or partially covers the primary and secondary roots. The area has tropical dry forest, premontane humid forest, and montane humid forest vegetation [42].

Basidiomycota: Agaricales: Catathelasmataceae

Pseudolaccaria pachyphylla (Fr.) Vizzini & Contu, 2015

Sinonimias: *Agaricus pachyphyllus* Fr. 1815, *Clitocybe pachyphylla* (Fr.) Gillet, (1878), *Camarophyllus pachyphyllus* (Fr.) P. Karst, (1879), *Omphalia pachyphylla* (Fr.) Quél (1886), *Pseudoomphalina pachyphylla* (Fr.) Knudsen, (1992) [27].

The material reviewed: fifteen sporocarps and mycelium from five coffee roots. COLOMBIA: Quindío, Quimbaya, El Laurel county, La Esperanza farm, 4°34′42″ N; 75°47′44″ W, 1251 m.a.s.l., manual collection, November 2021 and April 2022, coll. Carmona V., L. A., det. By sequencing and classical taxonomy. Three sporocarps and mycelium from three coffee roots. COLOMBIA: Norte de Santander, Lourdes, Los Naranjos county, El Panteón farm, 7°56′54″ N; 72°49′51″ W, 1398 m.a.s.l., manual collection, May 2022, coll. Benavides M., P., det. By sequencing and classical taxonomy.

Morphological description: Veil: absent; **Pileus:** (Figure 4A) irregular, with elevation from convex to plane convex, a polar diameter of 1.5–5.7 cm, equatorial diameter of 1.3–5.9 cm, curved margin, entire, dry and fibrillose cuticle, covered with small reddish scales, flattened on the surface, which are grouped towards the center; homogeneous context, pinkish; **Hymenophore:** (Figure 4B,C) with lamellae attached to the stipe, slightly decurrent with a serrated edge, pale pink in color and presence of lamelules, 0.15–1.08 cm; **Annulus:** absent; **Stipe:** (Figure 4A) cylindrical, 1.4–5.4 cm in length and 0.4–1.5 cm in diameter, central to the crown, solid context, fibrillose texture, purplish red in color; **Volva type**: absent; **Spores**: (Figure 4D) basidiospores ovoid to ellipsoidal, smooth, some with a slightly thickened edge, darken in contact with 1% KOH; Mycelium (Figure 4E) measures up to 23 cm.

Comments: In the Americas, *Pseudolaccaria pachyphylla* is registered in the United States. In the present study, sporocarps associated with *Coffea arabica* were collected in crops under sun exposure and semi-shade of *Albizia carbonaria*, becoming the first record for South America and Colombia. The sporocarps grow around the base of the tree, and in sites with leaf litter, and they are gregarious; up to seven were found per plant, and they predominate during the rainy seasons. Sometimes, *P. pachyphylla* is associated with *P. beniensis*; the sporocarps of both species were observed in the same coffee root (Figure 5). The mycelium of both fungi wholly or partially covers the primary and secondary roots.

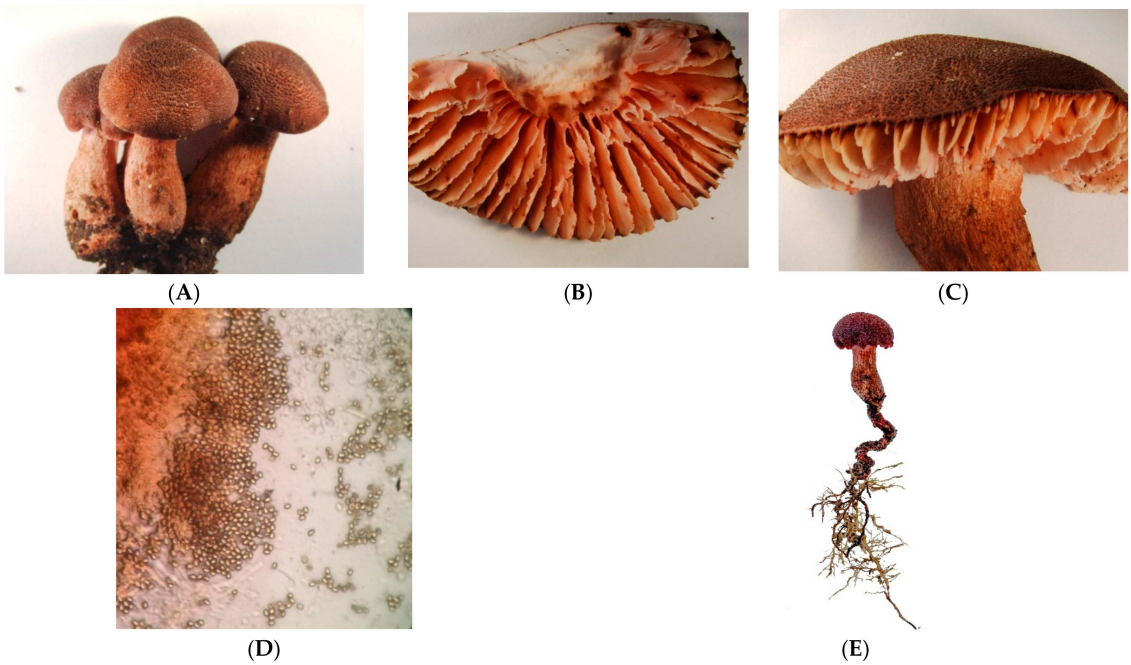

Figure 4. *Pseudolaccaria pachyphylla.* (**A**) Sporocarps, (**B**) lamellae, (**C**) pileus and lamellae, (**D**) spores, (**E**) mycelium.

Figure 5. Sporocarps of *Phlebopus beniensis* and *Pseudolaccaria pachyphylla* in the same root of Coffea arabica.

Basidiomycota: Agaricales: Callistosporiaceae

Xerophorus olivascens (Boud.) Vizzini, Consiglio & M. Marchetti, 2020

Synonymy: *Callistosporium olivascens* (Boud.) Bon, 1976, *Clitocybe aerina* (Quél.) Bigeard & Guillemin, *Collybia aerina* Quél., 1884, *Tricholoma olivascens* Boud., 1917 [44].

The material reviewed: Two sporocarps and mycelium in a coffee root. COLOMBIA: Norte de Santander, Lourdes, Los Naranjos county, El Panteón farm, 7°56′54″ N; 72°49′51″ W, 1398 m.a.s.l., manual collection, May 2022, coll. Benavides M., P., det. By sequencing.

Morphological description: Veil: absent; **Pileus:** (Figure 6A) convex to widely convex, fissured edges, without umbo, glabrous surface, white, polar diameter 0.7–1.05 cm, equatorial diameter 0.85–1.0 cm; **Hymenophore:** (Figure 6B) with thick and spaced lamellae, attached to the stipe, slightly decurrent with the serrulate edge, with partial lamellae, between 1.1–1.2 cm; **Annulus:** absent; **Stipe:** (Figure 6A) cylindrical, with tapering at the

base, sometimes curved, 0.85 to 1.3 cm in length and 0.3 cm in diameter, central to the crown, solid context, with a fibrillose texture, creamy white coloring; **Volva type**: absent; **Spores:** no data; **Mycelium:** no data.

(**A**) (**B**) (**C**)

Figure 6. *Xerophorus olivascens.* (**A**) Pileus and stipe, (**B**) lamellae, (**C**) sporocarps on the coffee tree base.

Comments: *Xerophorus olivascens* is a recently described species [44]; therefore, there are only European records. In the present study, it was recorded for the first time for the Neotropics and Colombia. According to Vizzini et al. [44], this species grows under cedar trees in parks and gardens; we report it growing in the base of *Coffea arabica* trees (Figure 6C) in crops with the shade of *Albizia carbonaria*. The mycelium of the fungus wholly or partially covers the primary and secondary roots. The vegetation of tropical dry forests, premontane humid forests, and montane humid forests predominate in the region [42].

3.2. Collection, Description, and Identification of Cysts

The results of the sequences (Table 2) indicated that the mycelium and cysts present in the roots of the 27 coffee plants corresponded to the species of sporocarps recorded in the base of the trees, except for the species *Pseudolaccaria pachyphylla*, where the cyst corresponded to *Phlebopus beniensis*

Regarding the morphology of the cysts, all fungal species exposed similar characteristics. In general, the cysts are globose or semi-globose, 1–3.5 mm thick, brown–yellow in color (Figure 7A) (color 15-0960 TCX according to Pantone®), and become darker as they progress through the stage of maturation, at which time they lose humidity. Internally, they are light yellow to cream in color and velvety in appearance (Figure 7A). At first, they develop individually (Figure 7B), and over time, they form clusters that surround the coffee plants' main root and secondary roots. Once the groups of nodules are formed, small holes, like chimneys, are observed that can serve as ventilation ducts and entrance cavities for insects (Figure 7C). Inside each cyst, between 1 and 16 mealybugs were found in all stages of development, predominantly females (Figure 7D).

(**A**) (**B**) (**C**) (**D**)

Figure 7. Cysts on the roots of *Coffea arabica*. (**A**) Cross-section of the group of cysts, (**B**) cysts in their initial stages of formation, (**C**) ventilation ducts and insect entry and exit, (**D**) individuals of *Dysmicoccus* inside the cysts on coffee roots.

3.3. Identification of Mealybugs

By comparing the DNA sequences obtained with those from GenBank (Table 3), the mealybug species *Dysmicoccus brevipes* (Cockerell, 1893), *D. neobrevipes* Beardsley, 1959, *Pseudococcus elisae*, and *P. nr. sociabilis* Hambleton, 1935, and two species of Pseudococcidae (Hemiptera: Pseudococcidae) were associated with the cysts that covered the roots of the coffee trees and the sporocarps in the base of the trees (Table 4). The identification of the mealybug species was corroborated by classical taxonomy.

Table 3. Sequences used in the analysis for mealybugs insect species.

Identified Species	DNA (pb)	GenBank Accessions	Similarity %
Dysmicoccus brevipes	385	LC121504	98.2
Dysmicoccus neobrevipes	385	MF966992	98.0
Pseudococcus nr. sociabilis	356	KJ530627	97.2
Pseudococcus elisae	385	KX639740	100

Table 4. Pseudococcidae mealybug species are associated with the cysts that cover the coffee roots and sporocarps in the tree base.

Pseudococcidae Species	Species of Fungi (Sporocarp)	Species of Fungi (Cysts)	Farm
Pseudococcus elisae	*Phlebopus beniensis*	*Phlebopus beniensis*	El Agrado
Dysmicoccus neobrevipes	*Phlebopus beniensis*	*Phlebopus beniensis*	El Agrado, La Esperanza
Pseudococcus nr. sociabilis	*Phlebopus beniensis*	*Phlebopus beniensis*	El Panteón
Dysmicoccus neobrevipes	*Phlebopus portentosus*	*Phlebopus portentosus*	La Esperanza
Dysmicoccus neobrevipes	*Pseudolaccaria pachyphylla*	*Phlebopus beniensis*	El Agrado, La Esperanza
Dysmicoccus brevipes	*Pseudolaccaria pachyphylla*	*Phlebopus beniensis*	El Panteón
Pseudococcidae	*Xerophorus olivascens*	*Xerophorus olivascens*	El Panteón
Pseudococcidae	*Boletinellus rompelii*	*Boletinellus rompelii*	El Panteón

Phlebopus beniensis was the most commonly found species associated with the four mealybug species, while *P. pachyphylla* was not found to be associated with any species of mealybug, since only the sporocarps were identified, and the cysts corresponded to *P. beniensis*. *Dysmicoccus neobrevipes* was associated with three species of fungi, while *P. elisae* was only found in association with *P. beniensis* both in the analyses of the sporocarps and the cysts. Finally, mealybugs associated with the fungi *X. olivascens* and *B. rompelii* were not identified as a species, since the stages collected were first-instar nymphs, and the PCR products did not amplify. They belonged to insects of the family Pseudococcidae.

3.4. Description of the Damage Caused to Coffee Plants by the Association Between Fungi and Mealybug Insects

The presence of cysts and mealybugs in the coffee roots affects the epidermis, part of the bark, and sometimes the vascular cylinder (Figure 8A). Symptoms appear in the aerial part, such as chlorosis, wilting, and sometimes death (Figure 8B).

(**A**) (**B**)

Figure 8. Cross-section of a secondary root of *Coffea arabica.* (**A**) Root with affected epidermis, cortex, and vascular cylinder. (**B**) Coffee plant with chlorosis.

4. Discussion

The tripartite association between *Coffea arabica*, basidiomycetes, and Pseudococcidae was reported by Fang et al. and Zhang et al. [7,8], who found the fungus *P. portentosus* and the three mealybugs *Dysmicoccus neobrevipes*, *Formicococcus polysperes* Williams, 2004, and *Planococcus minor* (Maskell, 1897) (Pseudococcidae) on the roots of coffee plants in China. In our study, four species of fungi were identified in a tripartite relationship with four species of Pseudococcidae; additionally, one fungus, *P. pachyphylla*, present in the sporocarps in the base of the coffee trees, was not in cysts containing insects; therefore, its role remains unknown.

This work reports, for the first time, *P. portentosus* in Colombia after this species has been documented in a tripartite relationship with 37 species of plants, involving 13 species of mealybugs in several countries on four continents [7,8,12–18]. Regarding the ecology of *P. portentosus*, Zhang et al. [12] reported that the greatest fruiting occurs during the rainy seasons and adapts to soils with a pH between 4.5 and 6.5; however, it prefers soils with a high nitrogen content. In the present study, the greatest fruiting also occurred in the rainy seasons, and the soils contained a pH of 5.4; however, the nitrogen (0.21%) and organic matter (4.7%) contents were low in the site where the samples were collected.

The *P. beniensis* species was the most abundant in this study with 49 sporocarps collected, representing 69% of the total sample, and it was present in the three sampled sites. It was also associated with the most significant number of mealybugs species: *P. elisae*, *P. nr. sociabilis*, *D. neobrevipes*, and *D. brevipes*; in addition, this is the first record of this fungus in a tripartite association with insects and the first in *Coffea arabica*. Palacio et al. [25] recorded it in a forest reserve in Colombia; the sporocarps were close to plant species of the genera *Hymanaea*, *Andira*, and *Bauhinia* (Fabaceae), and Sulzbacher et al. [38] recorded its presence in Brazil near *Laguncularia racemosa* (Combretacae) and *Hibiscus tiliaceus* (Malvaceae), two cultivated botanical species.

In this study, *Boletinellus rompelii* was another fungal species recorded in a tripartite association with Pseudococcidae insects. Although only one sporocarp was collected in the coffee tree base, the molecular analysis of the cysts that covered the roots of the plant allowed us to complement the identification and determine the association with the mealybugs inside the cysts. This finding becomes the first record of *B. rompelii* for Colombia and the first record of the association with Pseudococcidae species, which agrees with what was described by Zhang et al. [8]. These authors indicate that part of the fungus that is associated with soil insects, mainly Pseudococcidae, belongs to the Boletinellaceae family of the order Boletales, which includes two genera, *Phlebopus* (R. Heim) Singer (1936) and *Boletinellus* Murrill (1909). According to Watling and Meijert [45], *B. rompelli* is represented by solitary basidiomes, which explains why only one sporocarp was found in the coffee plant. In Brazil, these same authors reported the presence of sporocarps near *Inga* sp. (Fabaceae) and *Allophylus edulis* (Sapindaceae) but did not present evidence of any association with insects.

For the species *Pseudolaccaria pachyphylla*, no previous studies report interactions with insects, and this study failed to present evidence of any direct association with insects. Even though the fungus was present in coffee plants containing the mealybugs *Dysmicoccus brevipes* and *Dysmicoccus neobrevipes*, no cysts containing this insect species were inside. On the contrary, every time there was a sporocarp of this fungus, the cysts of the roots of the same coffee tree belonged to *P. benieneses*. However, *P. pachyphylla* was the second most abundant species, with 18 sporocarps collected, representing 25.3% of the total sample, besides being near coffee roots infested by Pseudococcidae. These findings illustrate the first record for this fungi species in Colombia and the Neotropics and the first record for *C. arabica* as a host. Existing studies also report *P. pachyphylla* in Europe

being associated with perennial species such as *Pinus pinea*, *Cistus salvifolium*, *Quercus suber*, *Quercus ilex*, *Cedrus atlantica* and *Pinus pinaster*, and *Cistus ladanifer* [46].

We found *P. pachyphylla* and *P. beniensis* in the same coffee roots in eight of the evaluated coffee trees. When performing the molecular analyses of both the cysts and the sporocarps, we found that the cysts always corresponded to *P. beniensis*; also, two species of Pseudococcidae were found in the same roots: *P. elisae* and *Dysmicoccus neobrevipes*. This association remains unsolved, and no reports in the literature support these findings.

The information available for the species *Xerophorus olivascens* is scarce; a study by Vizzini et al. [44] indicated that specimens of this species were found growing under cedar trees, but their trophic state and relationships remained unknown. In this study, we did find mealybugs inside cysts formed by this fungus; however, this species of Pseudococcidae needs to be identified in the adult stages.

We found that the mycelium that covered the roots of the coffee plants and the mealybug insects protected inside the cysts caused damage to the epidermis, part of the bark, and the vascular cylinder. These findings confirm those recorded by Zhang et al. [8], who performed an anatomical analysis of the roots of *Coffea arabica* and found that *P. portentosus* hyphae invaded the epidermis and even the cortical cells. The same authors indicate that these findings suggest a parasitic nature of *P. portentosus*, which could damage the host plant's roots. In the present study, it was also found that the tripartite association generated chlorosis, defoliation, and even death in coffee plants, contrary to what was reported by Zhang et al. [8], who did not detect apparent symptoms in the aerial part of the plants evaluated. Meanwhile, Gonçalves et al. [47] reported that *Phlebopus tropicus* formed cysts with *Pseudococcus comstocki* (Kuwana, 1902) on the roots of citrus plants in Brazil, and this association caused the extinction of citrus cultivation due to the massive reproduction of *P. comstocki*. The extent of the damage caused in coffee plants in Colombia requires attention to avoid the consequences reported in Brazil.

On the other hand, it could be thought that the occurrence of fungi, in relation to mealybugs and the plant, was of an ectomycorrhizal type; however, in the case of *P. portentosus*, the study [48] carried out field and laboratory tests and concluded that this fungus does not form mycorrhizae. Meanwhile, Zhang et al. [8] indicated that *P. portentosus* has a biotrophic relationship with mealybugs through the formation of cysts and a parasitic relationship with plant roots. In this biotrophic relationship, mealybugs produce sugary substances that attract and stimulate mycelial growth and cyst formation, while cysts provide mealybugs with favorable habitats, including protection [8]. For *Phlebopus beniensis*, Palacio et al. [25], in a study of macrofungi from Colombia, indicated that they did not find a Hartig network associated with this species, corroborating what was stated by Tedersoo et al. [49], where they suggest that species of the genus *Phlebopus* are not ectomycorrhizal but rather facultative or obligate biotrophic. In the case of *Boletinellus rompelli*, Singer et al. [50] reported the plant species *Allophylus edulis* as an ectomycorrhizal partner of this fungus, although it is the only record and requires experimental support.

5. Conclusions

The results of this study allowed us to determine a tripartite association involving four species of fungi (sporocarps-cysts), four species of mealybugs, and the roots of coffee plants.

The tripartite association between the fungi, insects, and coffee roots causes damage at the cellular level of the plants and, as a consequence, plant death.

This work shows basidiomycete fungal species in a tripartite association with Pseudococcidae species that had not been reported in previous studies; it is also the first record of coffee plants as a host of these fungi.

Mealybug insects are a pest in coffee cultivation, as they affect both plant development and production. Identifying the basidiomycete fungi associated with these insects and their influence on their populations is essential for their control and management.

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