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TITLE:

Comparison of Methods for Isolating Entomopathogenic Fungi from Soil Samples

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KEYWORDS:

Metarhizium, *Beauveria*, soil microbiota, insect bait, biological control, bioprospecting, *Tenebrio*, *Galleria*, selective medium.

SUMMARY:

Entomopathogenic fungal colonies are isolated from tropical soil samples using *Tenebrio* bait, *Galleria* bait, as well as selective artificial medium, i.e., potato dextrose agar enriched with yeast extract supplemented with chloramphenicol, thiabendazole, and cycloheximide (CTC medium).

ABSTRACT:

The goal of the present study is to compare the effectiveness of using insect baits versus artificial selective medium for isolating entomopathogenic fungi (EPF) from soil samples. The soil is a rich habitat for microorganisms, including EPF particularly belonging to the genera *Metarhizium* and *Beauveria*, which can regulate arthropod pests. Biological products based on fungi are available in the market mainly for agricultural arthropod pest control. Nevertheless, despite the high endemic biodiversity, only a few strains are used in commercial bioproducts worldwide. In the present study, 524 soil samples were cultured on potato dextrose agar enriched with yeast

extract supplemented with chloramphenicol, thiabendazole, and cycloheximide (CTC medium). The growth of fungal colonies was observed for 3 weeks. All *Metarhizium* and *Beauveria* EPF were morphologically identified at the genus level. Additionally, some isolates were molecularly identified at the species level. Twenty-four out of these 524 soil samples were also surveyed for EPF occurrence using the insect bait method (*Galleria mellonella* and *Tenebrio molitor*). A total of 51 EPF strains were isolated (41 *Metarhizium* spp. and 10 *Beauveria* spp.) from the 524 soil samples. All fungal strains were isolated either from croplands or grasslands. Of the 24 samples selected for comparison, 91.7% were positive for EPF using *Galleria* bait, 62.5% using *Tenebrio* bait, and 41.7% using CTC. Our results suggested that using insect baits to isolate the EPF from the soil is more efficient than using the CTC medium. The comparison of isolation methods in addition to the identification and conservation of EPF has a positive impact on the knowledge about biodiversity. The improvement of EPF collection supports scientific development and technological innovation.

INTRODUCTION:

Soil is the source of several microorganisms, including entomopathogenic fungi (EPF). This particular group of fungi is recognized by their ability to colonize and often kill arthropod hosts, especially insects[1]. After isolation, characterization, selection of virulent strains, and registration, EPF are mass-produced for arthropod-pest control, which supports their economical relevance[2]. Accordingly, the isolation of EPF is considered the first step to the development of a biopesticide. *Beauveria* spp. (Hypocreales: Cordycipitaceae) and *Metarhizium* spp. (Hypocreales: Clavicipitaceae) are the most common fungi employed for arthropod-pest control[3]. EPF have been successfully isolated from soil, arthropods with visible mycosis, colonized plants, and plant rhizosphere[4,5].

Isolation of EPF can also be useful to study the diversity, distribution, and ecology of this particular group. Recent literature reported that the use of EPF is underestimated, citing several unconventional applications of EPF such as their capacity to improve plant growth[4], to remove toxic contaminants from the soil, and to be used in medicine[6]. The present study aims to compare the efficiency of isolating EPF from soil using insect baits versus artificial culture medium[7–9]. The use of *Galleria mellonella* L. (Lepidoptera: Phyralidae) as an insect bait in the context of EPF isolation has been well accepted. These larvae are used worldwide by the scientific community as an experimental model to study host-pathogen interactions[10,11]. *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larva is considered another insect model for studies involving virulence and for isolation of EPF since this insect is easy to rare in the laboratory at a low cost[7,12].

Culture-independent methods such as using a variety of PCR techniques can be applied to detect and quantify EPF on their substrates, including soil[13,14]. Nevertheless, to properly isolate these fungal colonies, their substrate should be cultured onto a selective artificial medium[9], or the fungi present in the samples can be baited using sensitive insects[15]. On one hand, CTC is a dodine-free artificial medium that consists of potato dextrose agar enriched with yeast extract supplemented with chloramphenicol, thiabendazole, and cycloheximide. This medium was developed by Fernandes et al.[9] to maximize the recovery of naturally occurring *Beauveria* spp. and *Metarhizium* spp. from the soil. On the other hand, *G. mellonella* and *T. molitor* larvae can also

to be successfully used as baits to obtain EPF isolates from the soil. Nevertheless, according to Sharma et al.[15], fewer studies reported the concomitant use and comparison of these two bait insects. Portuguese vineyards soils exhibited significant recoveries of *Metarhizium robertsii* (Metscn.) Sorokin using *T.* molitor larvae in comparison to *G. mellonella* larvae; in contrast, *Beauveria bassiana* (Bals. -Criv.) Vuill isolation was linked to the use of *G. mellonella* baits[15]. Therefore, the decision on which EPF isolation method to use (i.e., *G. mellonella* bait, *T. molitor* bait, or CTC medium) should be considered according to the study's goal and the laboratory infrastructure. The goal of the present study is to compare the effectiveness of using insect baits versus artificial selective medium for isolating EPF from soil samples.

PROTOCOL:

As the present study accessed Brazilian genetic heritage, the research was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Sisgen) under the code AA47CB6.

1. Soil sampling

1.1. Collect 800 g of soil (with or without incident secondary plant roots) to a depth of 10 cm using a small shovel. Store them in polypropylene bags at room temperature until the start of the experiment.

NOTE: Small roots can also be collected as EPF are reported to have rhizosphere competence. The faster the processing of the samples, the better because the fungal spores may be less viable over time. In the present study, samples were analyzed no more than 7 days after the collection.

1.2. Use a GPS to identify the location of the collected samples in latitude and longitude and classify the collected area according to the type of soil (for example, grasslands, native rainforest, lakeshores, or croplands).

2. Isolation methods for entomopathogenic fungi

2.1. Isolation using CTC selective artificial medium.

2.1.1. To prepare the CTC medium (potato dextrose agar plus yeast extract (PDAY) supplemented with 0.5 g/L of chloramphenicol, 0.001 g/L of thiabendazole, and 0.25 g/L of cycloheximide[9]), weigh all the reagents individually, mix them in distilled water, and sterilize the medium in autoclave. In a biosafety cabinet, plate 23 mL of the medium into 60 mm x 15 mm Petri plates.

CAUTION: While weighing CTC reagents, use a lab coat, mask, gloves, and goggles because cycloheximide and chloramphenicol are toxic.

2.1.2. Weigh 0.35 ± 0.05 g of each soil sample (with or without roots) and place it in a 1.5 mL microtube.

2.1.3. In a biosafety cabinet, add 1 mL of sterile 0.01% (vol/vol) polyoxyethylene sorbitan monooleate aqueous suspension to the microtube containing soil and vortex for 30 s.

2.1.4. Remove 50 µL of the supernatant and pipette it onto the center of Petri plates with CTC medium. Homogenously disperse the suspensions onto the surface of the medium using a sterile Drigalski spatula (6 mm in diameter).

NOTE: At least three replicates for each soil sample should be prepared.

2.1.5. Incubate the plates in climate chambers (25 ± 1 °C, relative humidity ≥80%) in the dark and observe the growth of fungal colonies after 7, 14, and 21 days of incubation.

2.1.6. Observe the macromorphology and micromorphology of the fungal colonies seeking EPF. Transfer the EPF cultures to potato dextrose agar medium plus 0.05% chloramphenicol (PDAC) until pure cultures are obtained.

NOTE: Use the description keys presented below in step 3 for the identification of EPF colonies.

2.2. Isolation using insect baits

2.2.1. Use surface-disinfected *G. mellonella* and *T. molitor* late-stage larvae. Immerse the larvae into 0.5% sodium hypochlorite for 1 min for sterilization. Wash the larvae twice using sterile water.

NOTE: *G. mellonella* larvae from the fourth stage were used in the present study. *T. molitor* larval stages were not standardized.

2.2.2. Use plastic pots to assemble the baits. Add 250 g of collected soil to each plastic pot (98 mm width x 47 mm height x 142 mm length). Separate 15 larvae of each species (*T. molitor* and *G. mellonella*) and deposit five larvae per plastic pot. Store the pots at 25 ± 1 °C and relative humidity ≥ 80% in the dark.

NOTE: Drill 10 small holes (2 mm in diameter) in the pot lids to allow ventilation. A sharp heated iron device can be used to drill the holes.

2.2.3. Homogenize the soil every other day to allow maximum contact of larvae with soil.

NOTE: Moisture is important to support the fungal infection of larvae. To maintain moisture in the soil, spray sterile distilled water on the soil surface whenever necessary. Do not soak the soil sample in water.

2.2.4. Analyze the pots daily seeking dead insects.

NOTE: Observe the remaining larvae in the colony daily for invertebrate pathological signs to make sure the insects are not infected. As an alternative, control pots with sterile soil can be included in the study to check the health status of the insect larvae.

2.2.5. Remove dead insects and superficially sterilize them with 0.5% sodium hypochlorite for 1 min. Place the sterile insects in a humid chamber (relative humidity ≥ 80%) at 25 ± 1 °C for 7 days to favor the exteriorization of entomopathogenic fungi (mycosis).

2.2.6. Upon mycosis, harvest the conidia from the insect surface. Use a microbiological loop to place the conidia on PDAC medium under a stereoscopic microscope. As an alternative, place the whole infected larvae on the PDAC medium. Incubate the culture plates in a climate chamber at 25 ± 1 °C and relative humidity ≥ 80%.

2.2.7. Observe the macromorphology and micromorphology of the fungal colonies on the plates to confirm the identity of EPF. Repeat culturing on PDAC until pure fungal colonies are obtained.

NOTE: Use the description keys presented below in step 3 for the identification of EPF colonies.

3. Identification of EPF (*Metarhizium* spp. and *Beauveria* spp.)

3.1. Analyze the macromorphological characteristics of the fungal cultures on the plates (i.e., surface and reverse of colonies, their shape, edge, growth rate, color, texture, diffusible pigment, exudates, and aerial conidia) after 14 days at 25 ± 1 °C and relative humidity ≥ 80%.

3.2. Transfer the aerial conidia to slide cultures (microculture technique)[16] for 3 days at 25 ± 1 °C and relative humidity ≥ 80% and stain with lactophenol blue to observe the microscopic features (i.e., arrangement of conidia, conidiophores, shape, and size of conidia)[17–20].

3.3. Observe the microscopic fungal structures at 400x using an optical microscope to confirm the EPF identification.

NOTE: Morphological keys for EPF are described in the reports by Bischoff et al., Rehner et al., Seifert et al., and Humber[17–20]. The macro and micromorphology of fungal colonies are the most frequent criteria used to identify filamentous fungi at the genus level. Depending on the genus of the EPF, these morphological characteristics will change. Humber[20] presents an identification key to major genera of fungal entomopathogens. *Metarhizium* spp. colonies, for example, are usually circular, powdery, exhibiting varying shades of green, and can present exudate. Microscopically, these colonies have conidiogenous cells apical on broadly branched, densely intertwined conidiophores forming a compact hymenium, and cylindrical to ellipsoid conidia in parallel chains forming columns or plate-like masses. *Beauveria* spp. colonies are usually white, powdery, or cotton-like. They exhibit conidiogenous cells with a dilated basal portion extending apically in a zigzag direction. *Beauveria* conidiophores form dense clusters of globe-shaped conidia. Molecular analyses are needed for the identification of EPF at the species level.

3.4. Perform molecular analyses on the isolates for taxonomic identification at the species level. For the EPF strains isolated in this study, namely, *Metarhizium* spp. and *Beauveria* spp., perform molecular analyses based on the reports of Bischoff et al.[17] and Rehner et al.[18].

3.5. After confirming the isolates to be EPF, deposit the isolates in the collection of entomopathogenic fungal cultures from the Laboratory of Microbial Control (LCM) at the Federal Rural University of Rio de Janeiro.

REPRESENTATIVE RESULTS:

A total of 524 soil samples were collected from grassland: livestock pasture (165 samples), native tropical forest (90 samples), lakeside (42 samples), and cultivated/cropland (227 samples) between 2015 and 2018 in the Rio de Janeiro State, Brazil. Details of geographic coordinates of samples positive for EPF are given in **Supplementary Table 1**.

Of the 524 soil samples, 500 samples were analyzed only using CTC medium, and 24 samples were concomitantly analyzed using three forms of isolation (*Galleria*-bait, *Tenebrio*-bait, and the selective CTC culture medium), so the relative efficiency of these methods could be evaluated. A total of 51 EPF strains were isolated from 524 samples (41 *Metarhizium* spp. and 10 *Beauveria* spp.) (**Figure 1**). Micromorphological characteristics of some isolates are shown in **Figure 2**. All fungal strains were isolated from grassland or cropland (**Supplementary Table 1**). The results revealed that *Metarhizium* spp. is more prevalent than *Beauveria* spp. (**Supplementary Table 1**). Nine of the *Metarhizium* isolates (LCM S01 to LCM S09) were molecularly identified using the ef1-a (eukaryotic translation elongation factor 1-alpha) gene[21]. Of these, seven isolates (LCM S01–LCM S06 and LCM S08) were identified as *Metarhizium anisopliae* sensu stricto while two isolates (LCM S07 and LCM S09) were identified as *Metarhizium pingshaense*[21].

The occurrence of EPF (% of EPF positive samples spp) in the 24 soil samples studied using the three different methods of isolation is shown in **Table 1**. Recovery rates of EPF were analyzed by chi-square test. As shown in **Table 1**, *Galleria* bait proved to be more efficient in the isolation of EPF (91.7% (22/24) of positive samples) followed by *T. molitor* bait (62.5% (15/24) of EPF positive samples) and CTC medium (41.7% (14/24) of EPF positive samples). These 24 soil samples showed no recovery of *Beauveria* spp., but only *Metarhizium*.

FIGURE AND TABLE LEGENDS:

Figure 1: **Entomopathogenic fungal colonies of strains isolated from soil samples**. Colonies were cultivated on CTC artificial medium. (1) Petri plate exhibiting fungal colonies from soil samples 14 days after incubation on CTC selective medium before pure cultures are obtained; (2–42) Pure *Metarhizium* spp. colonies; (43–52) Pure *Beauveria* spp. colonies.

Figure 2: **Micromorphological characteristics of entomopathogenic fungi isolated from soil samples**. Colonies were incubated for 3 days on potato dextrose agar at 25 ± 1 °C and relative humidity ≥ 80%. The microscope slide was stained with lactophenol blue solution. Images show conidiophores and conidia of (**A**) *Metarhizium anisopliae* sensu stricto (s.s) isolate LCM S01; (**B**) *Metarhizium anisopliae* s.s. isolate LCM S03; (**C**) *Metarhizium* sp. isolate LCM S27; (**D–F**) *Beau-*

veria spp. isolates LCM S23, LCM S24, and LCM S20, respectively. All strains represented here were isolated using the CTC medium. LCM S27 was also recovered from soil using insect baits. * Conidiophores and conidia. ** Conidial chains show the characteristic side-by-side placement of *Metarhizium* spores in adjacent chains. Black arrows indicate *Metarhizium* cylindrical to ellipsoid conidia. Red arrows indicate *Beauveria* globe-shaped conidia.

Table 1: **Occurrence of entomopathogenic fungi (% of positive samples) in 24 soil samples using different isolation methods.**

Supplementary Table 1: **Geographical coordinates, isolation method, code, year of collection, and land-use types of samples positive for entomopathogenic fungi**.

DISCUSSION:

Natural and agricultural soil habitats are typical environments for EPF[22] and an excellent natural reservoir. In the present study, two methods of EPF isolation using insect baits versus selective medium were addressed. The first step for isolation is the collection of the soil samples. Proper storage and identification of soil samples are crucial. Information on the latitude, longitude, soil type, and biome is essential while depositing cultures and for studies involving epidemiological, modeling, and geospatial subjects[23,24]. After collection, it is recommended that the samples are processed as soon as possible (preferably within 7 days) because the viability of conidia in these soil samples can eventually decrease. Critical steps in the EPF isolation using CTC include: a) investigation of CTC plates 1 and 2 weeks after incubation (the first weeks are critical because, at later stages, other fungal colonies can narrow EPF development), and b) accurately identifying EPF colonies based on their macro- and micro-morphology. For isolation using insect baits, it is essential to keep the soil sample humid but not soak it in water.

The results reported by several studies have led to an interpretation that *M. anisopliae* is more common in cultivated soils than natural ecosystems[8,25,26]. Differences in the distribution and occurrence of these fungi can occur. In the present study, all strains were isolated either from cultivated soil (crops) or grasslands, and there was a predominance of *Metarhizium* spp. over *Beauveria* spp. It is suggested that cultivation practices and the high content of organic matter favor the presence of saprophytic fungi in the soil[27]. Accordingly, effective isolation techniques seeking EPF should consider reducing fungal contaminants.

Selective artificial media are commonly used for isolation because they are easy to use and have proven efficient in isolating entomopathogenic fungi, mainly *Metarhizium* spp. and *Beauveria* spp.[28]. These selective media use specific chemicals to reduce the growth of contaminants. In the 1980s and 1990s, the fungicide dodine became a widely used selective medium to isolate *Metarhizium* spp. and *Beauveria* spp.[29,30]. Although these artificial media are effective, some EPF species such as *Metarhizium acridum* can be susceptible to dodine[31]. That is why the dodine-free CTC medium was chosen in the present study. According to Fernandes et al.[9], CTC was developed to maximize the isolation of naturally occurring entomopathogenic fungi, including *M. acridum*. Using a selective medium rather than insect baits in the isolation of EPF is convenient because the former requires less space in the sample processing. The main disad-

vantage in CTC use relies on the fact that some of its components (i.e., cycloheximide and chloramphenicol) are toxic, so the use of personal protection equipment is mandatory.

As observed in the present study, a higher percentage of positive samples has been reported with insect baits as compared to artificial selective media for isolation of EPF[15,32–35]. The use of insect baits is considered a low-cost and high-efficiency alternative in the search for new EPF. Despite this, there are disadvantages associated with the use of insect baits over selective media. As the amount of soil to analyze using insects is higher, it is also necessary to have more physical space to store the samples and incubate the pots. The acquisition of insects can also be a limitation. In Brazil, for example, *G. mellonella* is not commercially available, so it is necessary to establish a colony in the lab to use this insect as bait. It is essential to keep the salubrity of the insects' colonies, avoiding natural infection by EPF. An EPF infection in the colony can make the isolation results unreliable. Therefore, one has to observe the remaining larvae in the colony seeking invertebrate pathological signs. As an alternative, control pots with sterile soil can be included in the study to check the health status of the insect larvae.

Seeking new fungal isolates with outstanding biocontrol traits is crucial to increase the effectiveness of fungi in arthropod-pest control. Fungi isolated from soil can be well adapted to growing in this environment[22], and they are likely to have high field persistence, which is an essential characteristic of successful EPF in pest control[21]. Accordingly, locally isolated EPF can improve the biological control of local pests because of their geographic and temporal congruence, increasing the chances of success and reducing the environmental impacts otherwise caused by the application of synthetic insecticides.

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DISCLOSURES:
The authors have no conflicts of interest.

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Figure 1

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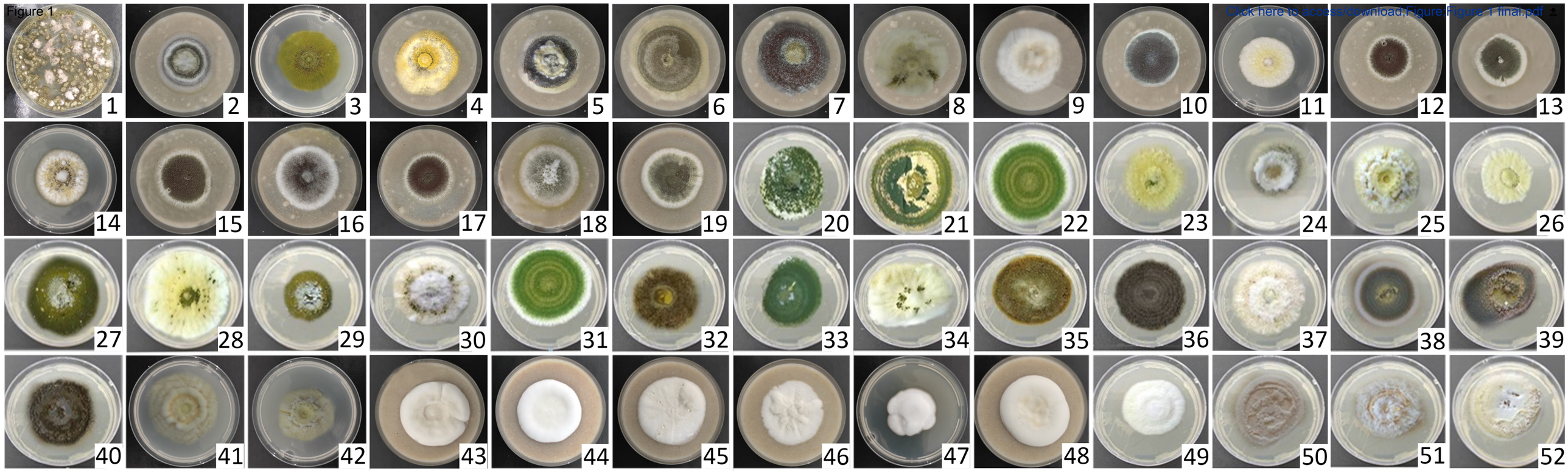

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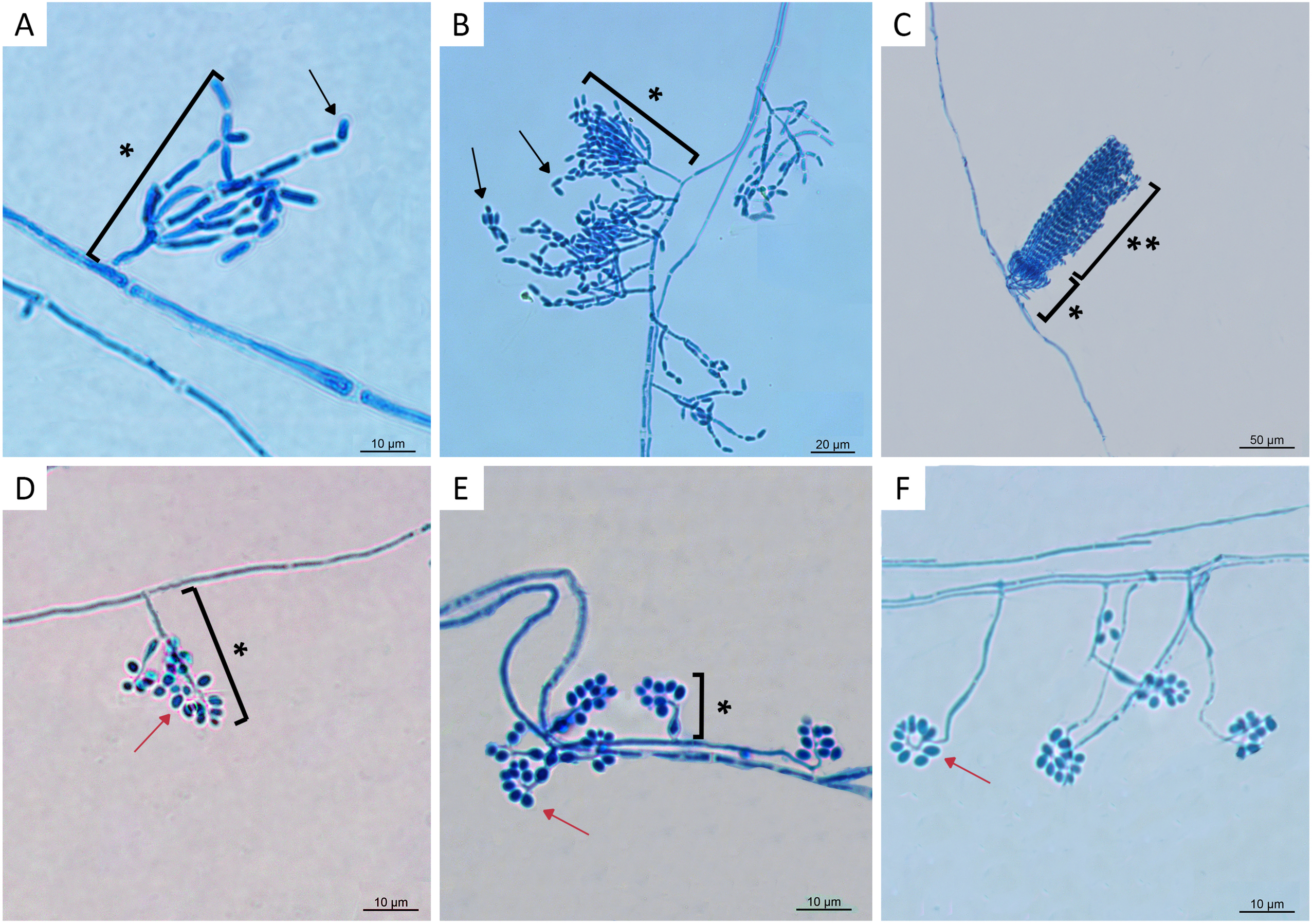

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Method of isolation	Entomopath
	Positive
Galleria -bait	91.7% (22/24)
Tenebrio -bait	62.5% (15/24)
CTC selective medium	41.7% (10/24)

* Only *Metarhizium* spp. were isolated

** Chi-square analysis, DF_2. $P = 0.0013$

ogenic fungi*	χ^2**
Negative	
8.3% (2/24)	
37.2% (9/24)	13.4
58.3% (14/24)	

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Table of Materials
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TITLE:

Comparison of Methods for Isolation of Entomopathogenic Fungi from Soil Samples

AUTHORS AND AFFILIATIONS:

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KEYWORDS:

Metarhizium, *Beauveria*, soil microbiota, insect bait, biological control, bioprospecting, *Tenebrio*, *Galleria*, selective medium.

SUMMARY:

Entomopathogenic fungal colonies are isolated from tropical soil samples using *Tenebrio* bait, *Galleria* bait, as well as selective artificial medium, i.e., potato dextrose agar enriched with yeast extract supplemented with chloramphenicol, thiabendazole, and cycloheximide (CTC medium).

ABSTRACT:

The goal of the present study is to compare the effectiveness of using insect baits versus artificial selective medium for isolating entomopathogenic fungi (EPF) from soil samples. The soil is a rich habitat for microorganisms, including EPF particularly belonging to the genera *Metarhizium* and *Beauveria*, which can regulate arthropod pests. Biological products based on fungi are available in the market mainly for agricultural arthropod pest control. Nevertheless, despite the high endemic biodiversity, only a few strains are used in commercial bioproducts worldwide. In the present study, 524 soil samples were cultured on potato dextrose agar enriched with yeast extract supplemented with chloramphenicol, thiabendazole, and cycloheximide (CTC medium).

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The growth of fungal colonies was observed for three weeks. All *Metarhizium* and *Beauveria* EPF were morphologically identified at the genus level. Additionally, some isolates were molecularly identified at the species level. 24 out of these 524 soil samples were also surveyed for EPF occurrence using the insect bait method (*Galleria mellonella* and *Tenebrio molitor*). A total of 51 EPF strains were isolated (41 *Metarhizium* spp. and 10 *Beauveria* spp.) from the 524 soil samples. All fungal strains were isolated either from croplands or grasslands. Of the 24 samples selected for comparison, 91.7% were positive for EPF using *Galleria* bait, 62.5% using *Tenebrio* bait, and 41.7% using CTC. Our results suggested that using insect baits to isolate the EPF from the soil is more efficient than using the CTC medium. The comparison of isolation methods in addition to the identification and conservation of EPF has a positive impact on the knowledge about biodiversity. The improvement of EPF collection supports scientific development and technological innovation.

INTRODUCTION:

Soil is the source of several microorganisms, including entomopathogenic fungi (EPF). This particular group of fungi is recognized by their ability to colonize and often kill arthropod hosts, especially insects[1]. After isolation, characterization, selection of virulent strains, and registration, EPF are mass-produced for arthropod-pest control, which supports their economical relevance[2]. Accordingly, the isolation of EPF is considered the first step to the development of a biopesticide. *Beauveria* spp. (Hypocreales: Cordycipitaceae) and *Metarhizium* spp. (Hypocreales: Clavicipitaceae) are the most common fungi employed for arthropod-pest control[3]. EPF have been successfully isolated from soil, arthropods with visible mycosis, colonized plants, and plant rhizosphere[4,5].

Isolation of EPF can also be useful to study the diversity, distribution, and ecology of this particular group. Recent literature reported that the use of EPF is underestimated, citing several unconventional applications such as their capacity to improve plant growth[4], to remove toxic contaminants from the soil, and to be used in medicine[6]. The present study aims to compare the efficiency of isolating EPF from soil using insect baits versus artificial culture medium[7-9]. The use of *Galleria mellonella* L. (Lepidoptera: Phyralidae) as an insect bait in the context of EPF isolation has been well accepted. These larvae are used worldwide by the scientific community as an experimental model to study host-pathogen interactions[10,11]. *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larva is considered another insect model for studies involving virulence and for isolation of EPF since this insect is easy to rare in the laboratory at a low cost[7,12].

Culture-independent methods such as using a variety of PCR techniques can be applied to detect and quantify EPF on their substrates, including soil[13,14]. Nevertheless, to properly isolate these fungal colonies, their substrate should be cultured onto a selective artificial medium[9], or the fungi present in the samples can be baited using sensitive insects[15]. On one hand, CTC is a dodine-free artificial medium that consists of potato dextrose agar enriched with yeast extract supplemented with chloramphenicol, thiabendazole, and cycloheximide. This medium was developed by Fernandes et al.[9] to maximize the recovery of naturally occurring *Beauveria* spp. and *Metarhizium* spp. from the soil. On the other hand, *G. mellonella* and *T. molitor* larvae can also be successfully used as baits to obtain EPF isolates from the soil. Nevertheless, according to

Sharma et al.[15], fewer studies reported the concomitant use and comparison of these two bait insects. Portuguese vineyards soils exhibited significant recoveries of *Metarhizium robertsii* (Metscn.) Sorokin using *T.* molitor larvae in comparison to *G. mellonella* larvae; in contrast, *Beauveria bassiana* (Bals. -Criv.) Vuill isolation was linked to the use of *G. mellonella* baits[15]. Therefore, the decision on which EPF isolation method to use (i.e., *G. mellonella*-bait, *T. molitor*-bait, or CTC medium) should be considered according to the study's goal and the laboratory infrastructure. The goal of the present study is to compare the effectiveness of using insect baits versus artificial selective medium for isolating EPF from soil samples.

PROTOCOL:

As the present study accessed Brazilian genetic heritage, the research was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Sisgen) under the code AA47CB6.

1. Soil sampling

1.1. Collect 800 g of soil (with or without incident secondary plant roots) to a depth of 10 cm using a small shovel. Store them in polypropylene bags at room temperature until the start of the experiment.

NOTE: Small roots can also be collected as EPF are reported to have rhizosphere competence. The faster the processing of the samples, the better because the fungal spores may be less viable over time. In the present study, samples were analyzed no more than seven days after the collection.

1.2. Use a GPS to identify the location of the collected samples in latitude and longitude and classify the collected area according to the type of soil (for example, grasslands, native rainforest, lakeshores, or croplands).

2. Isolation methods for entomopathogenic fungi

2.1. Isolation using CTC selective artificial medium.

2.1.1 To prepare the CTC medium [~~(~~potato dextrose agar plus yeast extract (PDAY) supplemented with 0.5 g/L chloramphenicol, 0.001 g/L thiabendazole, and 0.25 g/L cycloheximide[9]~~)~~], weigh all the reagents individually, mix them in distilled water, and sterilize the medium in an autoclave. In a biosafety cabinet, plate 23 mL of the medium into 60 mm × 15 mm Petri plates.

CAUTION: While weighing CTC reagents, use a lab coat, mask, gloves, and goggles because cycloheximide and chloramphenicol are toxic.

2.1.2 Weigh 0.35 ± 0.05 g of each soil sample (with or without roots) and place it in a 1.5 mL microtube.

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Commented [A6]: What is the volume taken?

Commented [A7]: The volume of distilled water will depend on the final volume of the artificial medium that has been prepared. Please observe that the text gives the reagents' weights and their respective volume (in liters). For example, for each liter of artificial medium, the researcher should weigh 0.5 of chloramphenicol, 0.001g of thiabendazole, and 0.25g of cycloheximide. If, for example, the researcher intends to prepare 100 plates, he/she will need 2.3 liters of final medium. That is why the volume of water was not added, only the proportion of gram of reagents per liter.

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2.1.3 In a biosafety cabinet, add 1 mL of sterile 0.01% (vol/vol) polyoxyethylene sorbitan monooleate aqueous suspension to the microtube containing soil and vortex for 30 s.

2.1.4 Remove 50 µL of the supernatant and pipette it onto the center of Petri plates with CTC medium. Homogenously disperse the suspensions onto the surface of the medium using a sterile Drigalski spatula (6 mm in diameter).

NOTE: At least three replicates for each soil sample should be prepared.

2.1.5 Incubate the plates in climate chambers (25 ± 1 °C, relative humidity ≥ 80%) in the dark and observe the growth of fungal colonies after 7, 14, and 21 days of incubation.

2.1.6. Observe the macro- and micromorphology of the fungal colonies seeking EFP. Transfer the EPF cultures to potato dextrose agar medium plus 0.05% chloramphenicol (PDAC) until pure cultures are obtained.

NOTE: Use the description keys presented in item 3 for identification of EPF colonies.

2.2 Isolation using insect baits

2.2.1 Use surface-disinfected *G. mellonella* and *T. molitor* late-stage larvae. Immerse the larvae into 0.5% sodium hypochlorite for 1 min for sterilization. Wash the larvae twice using sterile water.

NOTE: *G. mellonella* larvae from the fourth stage were used in the present study. *T. molitor* larval stages were not standardized.

2.2.2 Use plastic pots to assemble the baits. Add 250 g of collected soil to each plastic pot (98 mm width x 47 mm height x 142 mm length). Separate 15 larvae of each species (*T. molitor* and *G. mellonella*) and deposit five larvae per plastic pot. Store the pots at 25 ± 1 °C and relative humidity ≥ 80% in the dark.

NOTE: Drill ten small holes (2 mm in diameter) in the pot lids to allow ventilation. A sharp heated iron device can be used to drill the holes.

2.2.3 Homogenize the soil every other day to allow maximum contact of larvae with soil.

NOTE: Moisture is important to support fungal infection of larvae. To maintain moisture in the soil, spray sterile distilled water on the soil surface whenever necessary. Do not soak the soil sample in water.

2.2.4 Analyze the pots daily seeking dead insects.

Commented [A12]: Are these EPF cultures already? How do you exclude other fungal contamiants at this stage?

Commented [A13]: Yes. Selection of EPF colonies should follow the keys included in the lines 207 and 208.

Commented [A14]: Do you repeatedly streak in fresh medium until pure cultures are obtained? How do you identify EPF fungi from other fungal strains? Please clearly discuss this in a NOTE.

Commented [A15]: The sentence "Observe the macro- and micromorphology of the fungal colonies seeking EFP" and the note "Use the description keys presented in item 3 for identification of EPF colonies" were added. Identification of EFP should follow the keys included in the lines 207 and 208.

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Commented [A19]: Instructions are in the following sentence "Immerse the larvae into 0.5% sodium hypochlorite for 1 min for sterilization."

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NOTE: Observe the remaining larvae in the colony daily for invertebrate pathological signs to make sure ~~EPF did not previously infect~~ the insects are not infected. As an alternative, control pots with sterile soil can be included in the study to check the health status of the insect larvae.

2.2.5 Remove dead insects and superficially sterilize them with 0.5% sodium hypochlorite for 1 min. Place the sterile insects in a humid chamber at 25 ± 1 °C and relative humidity ≥ 80% for 7 days to favor the exteriorization of entomopathogenic fungi (mycosis).

2.2.6 Upon mycosis, harvest the conidia from the insect surface. Use a microbiological loop to place the conidia on PDAC medium under a stereoscopic microscope. As an alternative, place the infected larvae on PDAC medium. Incubate the culture plates in a climate chamber at 25 ± 1 °C and relative humidity ≥ 80%.

2.2.7. Observe the macro- and micromorphology of the fungal colonies on the plates to confirm the identity of EPF. Repeat culturing on PDAC until pure fungal colonies are obtained.

NOTE: Use the description keys presented in item 3 for identification of EPF colonies.

3. Identification of ~~isolated fungi~~EPF (*Metarhizium* spp. and *Beauveria* spp.)

3.1. Analyze the macromorphological characteristics of the fungal cultures on the plates (i.e., surface and reverse of colonies, their shape, edge, growth rate, color, texture, diffusible pigment, exudates, and aerial conidia) after 14 days at 25 ± 1 °C and relative humidity ≥ 80%.

3.2. Transfer the aerial conidia to slide cultures (microculture technique)[16] for 3 days at 25 ± 1 °C and relative humidity ≥ 80% and stain with lactophenol blue to observe the microscopic features (i.e., arrangement of conidia, conidiophores, shape, and size of conidia)[17-20].

3.3. Observe the microscopic fungal structures at 400 x using an optical microscope to confirm the EPF identification.

NOTE: Morphological keys for EPF are described in the reports by Bischoff et al., Rehner et al., Seifert et al., and Humber[17-20]. The macro and micromorphology of fungal colonies are the most frequent criteria used to identify filamentous fungi at the genus level. Depending on the genus of the EPF, these morphological characteristics will change. Humber[20] presents an identification key to major genera of fungal entomopathogens. *Metarhizium* spp. colonies, for example, are usually circular, powdery, exhibiting varying shades of green, and can present exudate. Microscopically, these colonies have conidiogenous cells apical on broadly branched, densely intertwined conidiophores forming a compact hymenium; cylindrical to ellipsoid conidia in parallel chains forming columns or plate-like masses. While *Beauveria* spp. colonies are usually white, powdery, or cotton-like. They exhibit conidiogenous cells with a dilated basal portion extending apically in a zigzag direction. *Beauveria* conidiophores form dense clusters of globe-shaped conidia. Molecular analyses are needed for the identification of EPF at the species level.

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Commented [A32]: Do you repeatedly streak in fresh medium until pure cultures are obtained? How do you identify EPF fungi from other fungal strains? Please clearly discuss this in a NOTE.

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The identification of the colonies should follow the keys included in item 3.

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Commented [A37]: The macro and micromorphology of fungal colonies are the most frequent criteria used to identify filamentous fungi at the genus level. Depending on the genus of the EPF, these morphological characteristics will change.

The purpose of analyzing the fungal morphological characteristics was added in the next note.

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Commented [A40]: For the benefit of new researchers, please discuss what microscopic features typically correspond to EPF. This is especially important to discuss in view of 3rd reviewer's concern of possible fungal contaminants.

Commented [A41]: The features that typically correspond to *Metarhizium* and *Beauveria* were added in the next note.

Commented [A42]: Please mention the specific strains of EPF analyzed in the cited reports. Also, briefly mention what the specific molecular key(s) are for the reported strains.

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3.4. Perform molecular analyses on the isolates for taxonomic identification at the species level. For the EPF strains isolated in this study, namely, *Metarhizium* spp. and *Beauveria* spp., perform molecular analyses based on the reports of Bischoff et al.[17] and Rehner et al.[18].

3.5. After confirming the isolates to be EPF, deposit the isolates in the collection of entomopathogenic fungal cultures from the Laboratory of Microbial Control (LCM) at the Federal Rural University of Rio de Janeiro.

REPRESENTATIVE RESULTS:

A total of 524 soil samples were collected from grassland: livestock pasture (165 samples), native tropical forest (90 samples), lakeside (42 samples), and cultivated/cropland (227 samples) between 2015 and 2018 in the Rio de Janeiro State, Brazil. Details of geographic coordinates of positive samples for EPF are given in **Supplementary Table 1**.

Of the 524 soil samples, 500 samples were analyzed only using CTC medium, and 24 samples were concomitantly analyzed using three forms of isolation (*Galleria*-bait, *Tenebrio*-bait, and the selective CTC culture medium), so the relative efficiency of these methods could be evaluated. A total of 51 EPF strains were isolated from 524 samples (41 *Metarhizium* spp. and 10 *Beauveria* spp.) (**Figure 1**). Micromorphological characteristics of some isolates are shown in **Figure 2**. All fungal strains were isolated from grassland or cropland (**Supplementary Table 1**). The results revealed that *Metarhizium* spp. is more prevalent than *Beauveria* spp. (**Supplementary Table 1**). Nine of the *Metarhizium* isolates (LCM S01 to LCM S09) were molecularly identified using the ef1-a (eukaryotic translation elongation factor 1-alpha) gene[21]. Seven isolates, LCM S01-LCM S06 and LCM S08, were identified as *Metarhizium anisopliae* sensu stricto while the two isolates, LCM S07 and LCM S09, were identified as *Metarhizium pingshaense*[21].

The occurrence of EPF (% of positive EPF samples) in the 24 soil samples studied using the three different methods of isolation is shown in **Table 1**. Recovery rates of EPF were analyzed by chi-square test. As shown in **Table 1**, *Galleria* bait proved to be more efficient in the isolation of EPF (91.7% (22/24) of positive samples) followed by *T. molitor* bait (62.5% (15/24) of EPF positive samples) and CTC medium (41.7% (14/24) of EPF positive samples). These 24 soil samples showed no recovery of *Beauveria* spp., only *Metarhizium* spp.

FIGURE AND TABLE LEGENDS

Figure 1: **Entomopathogenic fungal colonies of strains isolated from soil samples**. Colonies were cultivated on CTC artificial medium. (1) Petri plate exhibiting fungal colonies from soil samples 14 days after incubation on CTC selective medium before pure cultures are obtained; (2-42) Pure *Metarhizium* spp. colonies; (43-52) Pure *Beauveria* spp. colonies.

Figure 2: **Micromorphological characteristics of entomopathogenic fungi isolated from soil samples**. Colonies were incubated for 3 days on potato dextrose agar at 25 ± 1 °C and relative humidity ≥ 80%. The microscope slide was stained with lactophenol blue solution. Images show conidiophores and conidia of **(A)** *Metarhizium anisopliae* sensu stricto (s.s) isolate LCM S01; **(B)** *Metarhizium anisopliae* s.s. isolate LCM S03; **(C)** *Metarhizium* sp. isolate LCM S27; (**D-F)** *Beau-*

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Commented [A51]: No. For these pictures, EPF colonies were incubated in potato dextrose agar (PDA) without chloramphenicol because these colonies had already been isolated, so there is no need for an antibiotic.

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veria spp. isolates LCM S23, LCM S24, and LCM S20, respectively. All strains represented here were isolated using CTC medium. LCM S27 was also recovered from soil using insect baits. * Conidiophores and conidia. ** Conidial chains show the characteristic side-by-side placement of *Metarhizium* spores in adjacent chains. Black arrows indicate *Metarhizium* cylindrical to ellipsoid conidia. Red arrows indicate *Beauveria* globe-shaped conidia.

Table 1: **Occurrence (% of positive samples) of entomopathogenic fungi in 24 soil samples using different isolation methods.**

Supplementary Table 1: **Geographical coordinates, isolation method, code, year of collection, and land-use types of samples positive for entomopathogenic fungi**.

DISCUSSION:

Natural and agricultural soil habitats are typical environments for EPF[22] and an excellent natural reservoir. In the present study, two methods of EPF isolation using insect baits versus selective medium were addressed. The first step for isolation is the collection of the soil samples. A proper storage and identification of soil samples are crucial. Information on the latitude, longitude, soil type, and biome are essential while depositing cultures and for studies involving epidemiological, modeling, and geospatial subjects[23,24]. After collection, it is recommended that the samples are processed as soon as possible (preferably within seven days) because the viability of conidia in these soil samples can eventually decrease. Critical steps in the EPF isolation using CTC include a) investigation of CTC plates one and two weeks after incubation; the first weeks are critical because, at later stages, other fungal colonies can narrow EPF development; b) accurately identifying EPF colonies based on their macro- and micro-morphology. For isolation using insect baits, it is essential to keep the soil sample humid but not soak it in water.

The results reported by several studies have led to an interpretation that *M. anisopliae* is more common in cultivated soils than natural ecosystems[8,25,26]. Differences in the distribution and occurrence of these fungi can occur. In the present study, all strains were isolated either from cultivated soil (crops) or grasslands, and there was a predominance of *Metarhizium* spp. over *Beauveria* spp. It is suggested that cultivation practices and the high content of organic matter favor the presence of saprophytic fungi in the soil[27]. Accordingly, effective isolation techniques seeking EPF should consider reducing fungal contaminants.

Selective artificial media are commonly used for isolation because they are easy to use and proven efficient in isolating entomopathogenic fungi, mainly *Metarhizium* spp. and *Beauveria* spp.[28]. These selective media use specific chemicals to reduce the growth of contaminants. In the 1980s and 1990s, the fungicide dodine became a widely used selective medium to isolate *Metarhizium* spp. and *Beauveria* spp.[29,30]. Although these artificial media are effective, some EPF species such as *Metarhizium acridum* can be susceptible to dodine[31]. That is why the dodine-free CTC medium was chosen in the present study. According to Fernandes et al.[9], CTC was developed to maximize the isolation of naturally occurring entomopathogenic fungi, including *M. acridum*. Using a selective medium rather than insect baits in the isolation of EPF is convenient because the former requires less space in the sample processing. The main disad-

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vantage in CTC use relies on the fact that some of its components (i.e., cycloheximide and chloramphenicol) are toxic, so the use of personal protection equipment is mandatory.

As observed in the present study, a higher percentage of positive samples has been reported with insect baits as compared to artificial selective media for isolation of EPF[15,32-35]. The use of insect baits is considered a low-cost and high-efficiency alternative in the search for new EPF. Despite this, there are disadvantages associated with the use of insect baits over selective media. As the amount of soil to analyze using insects is higher, it is also necessary to have more physical space to store the samples and incubate the pots. The acquisition of insects can also be a limitation. In Brazil, for example, *G. mellonella* is not commercially available, so it is necessary to establish a colony in the lab to use this insect as a bait. It is essential to keep the ~~celebrity~~ salubrity of the insects' colonies, avoiding natural infection by EPF. An EPF infection in the colony can make the isolation results unreliable. Therefore, observe the remaining larvae in the colony seeking invertebrate pathological signs. As an alternative, control pots with sterile soil can be included in the study to check the health status of the insect larvae.

Seeking new fungal isolates with outstanding biocontrol traits is crucial to increase the effectiveness of fungi in arthropod-pest control. Fungi isolated from soil can be well adapted to growing in this environment[22], and they are likely to have high field persistence, which is an essential characteristic of successful EPF in pest control[2,10]. Accordingly, locally isolated EPF can improve the biological control of local pests because of their geographic and temporal congruence, increasing the chances of success and reducing the environmental impacts otherwise caused by the application of synthetic insecticides.

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ACKNOWLEDGMENTS:

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The authors have nothing to disclose

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Soil sample*	Geographical coordinates		Method of isolation positive for EPF**	*Beauveria* spp. code
3	22° 46' 05" S	43° 40' 39" W	A	
5	22° 46' 04" S	43° 40' 41" W	A	
13	22° 45' 58" S	43° 40' 39" W	A	
14	22° 46' 04" S	43° 40' 48" W	A	
15	22° 45' 58" S	43° 40' 49" W	A	
16	22° 45' 58" S	43° 40' 49" W	A	
17	22° 45' 58" S	43° 40' 48" W	A	
18	22° 45' 58" S	43° 40' 49" W	A	
19	22° 49' 07" S	43° 40' 49" W	A	
234	22°49' 07.2" S	43°12' 09.3" W	A	
370	22°31' 25.4" S	43°00' 00.8" W	A	LCM S19
387	22°32' 28.5" S	42°35' 25.9" W	A	LCM S20
414	22°32'28.9" S	42°34'31.2" W	A	LCM S21

415	22°32'28.0" S	42°34'30.7" W	A	LCM S22
417	22°32'28.6" S	42°34'30.0" W	A	LCM S23
421	22°32'24.9" S	42°34'17.9" W	A	LCM S24
501	22°45'56.7" S	43°40'52.1" W	A,B,C	LCM S48
503	22°45'56.7" S	43°40'52.1" W	A,B,C	
504	22°45'59.5" S	43°40'47.1" W	A,B,C	LCM S49
505	22°45'59.5" S	43°40'47.1" W	B,C	LCM S50
506	22°46'01.1" S	43°40'42.7" W	B,C	
507	22°45'59.5" S	43°40'47.1" W	B,C	
508	22°46'01.1" S	43°40'42.7" W	B,C	
509	22°46'01.1" S	43°40'42.7" W	A,B,C	
510	22°46'01.1" S	43°40'42.7" W	C	
511	22°45'39.9" S	43°41'58.6" W	A,B,C	
512	22°45'38.1" S	43°41'57.9" W	B	
513	22°45'39.1" S	43°41'57.9" W	A,B,C	
514	22°45'40.0" S	43°41'58.1" W	A,B,C	

515	22°45'37.7" S	43°41'58.0" W	A,B,C	
516	22°45'40.0" S	43°41'58.1" W	A,B,C	
517	22°45'39.1" S	43°41'59.1" W	A,B,C	
518	22°45'40.0" S	43°41'58.1" W	B	
519	22°45'39.4" S	43°41'59.2" W	B	
520	22°45'39.7" S	43°41'58.7" W	B	LCM S51
522	22°57'22.6" S	42°49'44.6" W	B	
523	22°57'17.3" S	42°49'44.3" W	B	
524	22°57'13.7" S	42°49'40.7" W	B	

*Samples 1 to 500 were analyzed using only (A) CTC. Samples 501 to 524 were anal
(C) *Tenebrio* -bait.

** (A) CTC artificial medium; (B) *Galleria*- bait; (C)*Tenebrio*- bait

Metarhizium spp. code	Year of colletion	Land-use types
LCM S02	2015	Grassland
LCM S01	2015	Grassland
LCM S03	2015	Crop
LCM S04	2015	Crop
LCM S05	2015	Crop
LCM S06	2015	Crop
LCM S07	2015	Crop
LCM S08	2015	Crop
LCM S09	2015	Crop
LCM S10	2015	Crop
	2017	Crop
LCM S11 / LCM S12	2015	Crop
	2017	Crop

LCM S13 / LCM S14 / LCM S15 / LCM S16	2017	Crop
LCM S17	2017	Crop
LCM S18	2017	Crop
LCM S25	2018	Crop
LCM S26	2018	Crop
LCM S27	2018	Crop
LCM S28	2018	Crop
LCM S29	2018	Crop
LCM S30	2018	Crop
LCM S31	2018	Crop
LCM S32	2018	Crop
LCM S33	2018	Crop
LCM S34	2018	Crop
LCM S35	2018	Crop
LCM S36	2018	Crop
LCM S37	2018	Crop

LCM S38	2018	Crop
LCM S39	2018	Crop
LCM S40	2018	Crop
LCM S41	2018	Crop
LCM S42	2018	Crop
LCM S46	2018	Grassland
LCM S43/LCM S47	2018	Grassland
LCM S44	2018	Grassland
LCM S45	2018	Crop

yzed using (A) CTC, (B) *Galleria* -bait, and