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Original Article

# Diversity of entomopathogenic fungi from soils of eucalyptus and soybean crops and natural forest areas

## Diversidade de fungos entomopatogênicos em solos de culturas de eucalipto e soja e área de mata nativa

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**Abstract**

Soils present high fungal diversity, including entomopathogenic species. These fungi are used in pest control, providing easy production, multiplication, application, and dispersion in the field. The objective of the present study was to evaluate entomopathogenic fungal diversity in soils from eucalyptus and soybean crops and natural forest areas. These fungi were isolated using the "Bait Method" with *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) larvae from 10 soil samples per area, collected at 10 cm deep in a zig-zag pattern. The isolated entomopathogenic fungi were cultivated in Petri dishes using PDA medium and their mycelia separated after seven days of incubation in a BOD-type chamber. Species of *Aspergillus, Beauveria, Cordyceps, Fusarium, Metarhizium, Penicillium* and *Purpureocillium* were identified. The "Bait Method" with *T. molitor* larvae is efficient to isolate entomopathogenic fungi with higher diversity from soils of the natural forest than the cultivated area.

**Keywords:** *Beauveria*, biological control, *Cordyceps*, *Fusarium*, *Metarhizium*.

**Resumo**

A diversidade de fungos, incluindo espécies entomopatogênicas, é alta nos solos. Esses fungos são utilizados no manejo de pragas com facilidade de produção, multiplicação, aplicação e dispersão no campo. O objetivo foi avaliar a diversidade de fungos entomopatogênicos em solos de culturas de eucalipto e soja e áreas de mata nativa. Fungos entomopatogênicos foram isolados pelo "Bait Method" com larvas de *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) de 10 amostras de solo por área, coletadas a 10 cm de profundidade em zig-zag. Os fungos isolados foram cultivados em três placas de Petri em meio BDA e seus micélios separados após sete dias de incubação em câmara tipo BOD. Fungos dos gêneros *Aspergillus*, *Beauveria*, *Cordyceps*, *Fusarium*, *Metarhizium, Penicillium* e *Purpureocillium* foram identificados. O "Bait Method" com larvas de *T. molitor* é eficiente para isolar fungos entomopatogênicos com maior diversidade em solos de área de mata nativa que naqueles com culturas de eucalipto e soja.

**Palavras-chave:** *Beauveria*, controle biológico, *Cordyceps*, *Fusarium*, *Metarhizium*.

## 1. Introduction

Soils present significant fungal diversity, including entomopathogenic species (Sharma et al., 2018). The soil protects these fungi from UV radiation and biotic and abiotic factors (Mascarin and Jaronski, 2016). Fungi of the genera *Aspergillus, Beauveria, Cordyceps, Fusarium, Metarhizium, Penicillium* and *Purpureocillium* are opportunistic, nematophagous, phytopathogenic, endophytic, and entomopathogenic with species reported for Brazil (Rocha and Luz, 2011; Corallo et al., 2019; Mussi-Dias et al., 2020). The fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps* spp. are used to manage insect pests in agricultural and forest crops (Soliman et al., 2019; Jordan et al., 2021; Khun et al., 2021). These microorganisms are easily produced, multiplied, applied, and dispersed in the field, and can be used, in combination or individually, with low impact on non-target organisms (Zimmermann, 2007a, b; Domingues et al., 2020).

The prospection and identification of fungi, especially those effective against agricultural and forest pests can complement integrated pest management methods with

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low environmental impact. Many studies of fungal diversity in soils have been realized, but few commercial products based on entomopathogenic fungi are available on the market due to the difficulty in finding virulent strains with insecticidal potential. Therefore, more prospective studies are needed. The objective was to evaluate the diversity of entomopathogenic fungi in soil samples from eucalyptus and soybean crops and natural forest areas using the "Bait Method" with *T. molitor* larvae.

## 2. Material and Methods

*Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) was fed on previously sterilized wheat bran, filling up to 2/3 of the tray, being changed when it reached powder form. Chayote, sugarcane or potato slices were used as a food supplement and a liquid source was provided for *T. molitor*. The rearing temperature was 28 ± 1 °C in a room with low light and relative humidity, favoring the insect's development (Ribeiro et al., 2018).

Entomopathogenic fungi were isolated using the "Bait Method" with *T. molitor* larvae (Zimmermann, 1986) in soil samples collected from eucalyptus (three areas) and soybean (one area) crops and in a natural forest (two areas) in the states of Paraná and São Paulo, Brazil (Table 1) (Safitri et al., 2018).

Ten soil samples per area were collected at 10 cm deep in a zig-zag pattern and taken, separately in a thermal box under cold conditions, to the "Laboratório de Controle Biológico de Pragas Florestais" (LCBPF) of the "Universidade Estadual Paulista (UNESP)" in Botucatu, state of São Paulo, Brazil.

Soil samples were separated into 100 mL plastic pots with holes in the lid for aeration, and moistened when necessary, up to a field capacity (UCC) of 40%, calculated with the formula: UCC= [(Soil mass with 100% moisture - dry soil mass) *40/100], inside the laminar flow chamber with 10 replications (100 mL plastic pots) per sample (total of 100 replications per area).

Five *T. molitor* larvae were placed per pot (total of 500 insects per area), which were stored at 25 ± 1 °C, 70 ± 10% RH and 12 h photophase, inverted in the first five days, due to the positive phototropism of *T. molitor* to maintain direct contact with the soil.

The mortality of *T. molitor* larvae was evaluated for five days, observing those with signs or symptoms of fungal infection. The *T. molitor* larvae from the soil prospection were disinfected and kept in a humid chamber, until fungus sporulation, with mycelia being transferred to nutrient medium potato-dextrose-agar (PDA) for isolation.

The fungi were cultivated in Petri dishes with PDA medium autoclaved at 121 °C and 1 atm for 20 minutes. The mycelia of these fungi were separated seven days after incubation in a BOD-type chamber in three Petri dishes per dead *T. molitor* pupa. The fungi structures were transferred to microscopy slides and the characteristics of the mycelial colony and conidia observed under an optical microscope for morphological identification through keys (Domsch et al., 1980; Samson et al., 1988).

Fungus identification was performed with the molecular analysis of one species for each genus. The fungal colonies on the PDA medium in Petri dishes with 100 uL of 10% Chelex and 10 uL of proteinase K (20 mg/mL) was scraped, incubated in a thermal block at 100 °C for five minutes, and the genomic DNA of the fungi extracted. The PCR was amplified with a reaction in the ITS1-5.8S-ITS2 region of the rDNA using a total volume of 50 µL with 1X Taq DNA polymerase buffer, 1.5 mM MgCl2; 0.4 µM of each primer ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), 0.2 mM of dNTPs and 0.2 U of Taq DNA polymerase and 25ng of DNA. This amplification was carried out in a thermocycler, programmed for an initial denaturation of 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30s; annealing at 62 °C for 1min; extension at 72 °C for 2 min and final extension at 72 °C for 5 min (Domingues et al., 2022). The products from DNA extractions and PCR reactions (50 µL) were submitted to electrophoresis in 1% agarose gel and analyzed under UV light, with the amplified samples being purified using magnetic beads and sent to IBTEC at UNESP/Botucatu for Sanger sequencing.

## 3. Results

The fungi identified using morphological characteristics and confirmed by molecular analysis were species of the *Aspergillus*, *Beauveria*, *Cordyceps*, *Fusarium*, *Metarhizium*, *Penicillium*, and *Purpureocillium* genera (Figure 1, Table 2). *Aspergillus*, *Fusarium,* and *Metarhizium* were found at all collection points and were the only fungi in the soybean crop; *Cordyceps* and *Penicillium* were only found in the natural forest soil and *Purpureocillium* was only found in

**Table 1.** *Eucalyptus* (EU) and soybean (SO) crops and natural forest (NA) areas, municipalities (Mun.), latitude, longitude and altitude (meters) of soil sampling points in the states of São Paulo (SP) and Paraná (PR), Brazil in 2020 for the entomopathogenic fungi isolation.

| Crop | Mun., States | Latitude | Longitude | Altitude |
|---|---|---|---|---|
| EU | Agudos, SP | 22º 32' 1–2"S | 49º 2' 11–13" O | 580–620 |
| EU | Botucatu, SP | 22º 48' 11,6–13,59"S | 48º 25' 48,35–51,15" O | 740–750 |
| EU | Cruzeiro do Sul, PR | 23º 1–2'0,2–60" S | 52º 07'42,6–44,8" W | 450 |
| SO | Botucatu, SP | 22º 48-50' 31-51" S | 48º 25-26' 5–43" O | 740–804 |
| NA | Botucatu, SP | 22º 50' 15–31" S | 48º 25-26' 26–35" O | 721–808 |
| NA | Mandaguaçu, PR | 23º 23'5,8–8,5" S | 52º 08'43,4–44,7" W | 544 |

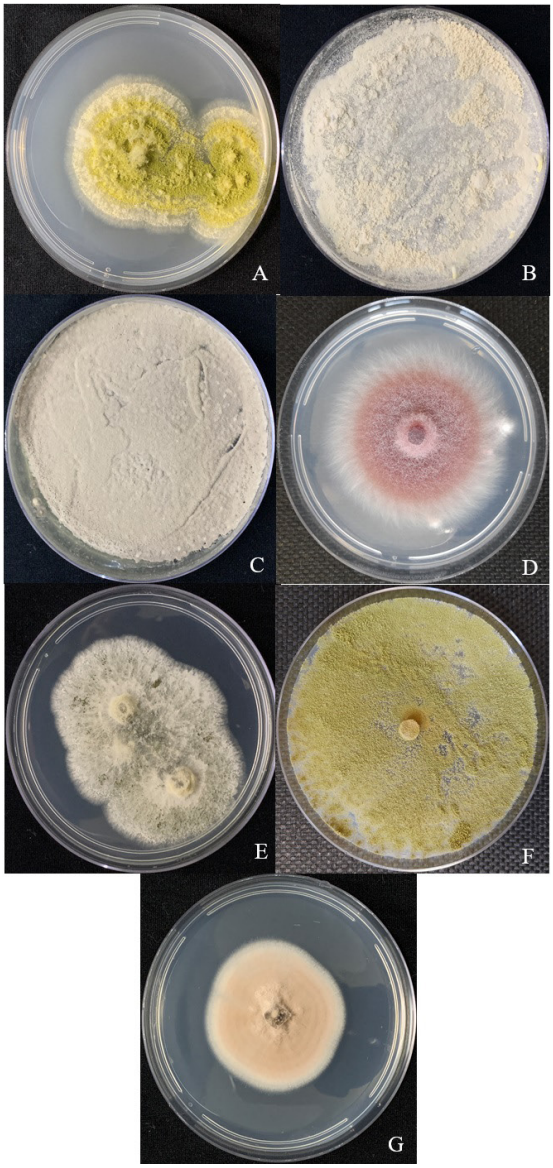


**Figure 1.** Entomopathogenic fungi of the genera *Aspergillus* (A), *Beauveria* (B), *Cordyceps* (C), *Fusarium* (D), *Metarhizium* (E), *Penicillium* (F) and *Purpureocillium* (G) cultivated in Petri dishes with PDA medium.

the eucalyptus soil. The diversity of entomopathogenic fungi was higher in the natural forest soil, with emphasis on the genus *Beauveria*, which was also found in the eucalyptus soils (Table 3).

## 4. Discussion

The identification of entomopathogenic fungi of the *Aspergillus, Beauveria, Cordyceps, Fusarium, Metarhizium, Penicillium*, and *Purpureocillium* genera corroborate reports of high diversity of these microorganisms in Brazil as observed for *Cordyceps cateniannulata*, *Cordyceps* sp., and *Metarhizium* isolates from soils collected in Brazil's central region (Rocha and Luz, 2011), *Aspergillus, Cordyceps, Fusarium,* and *Penicillium* in soil from citrus agroforestry systems (Prade et al., 2007), and *Beauveria*, *Cordyceps*, *Fusarium*, and *Purpureocillium* from individuals of *Thaumastocoris peregrinus* (Carpintero & Dellapé, 2006) (Heteroptera: Thaumastocoridae) collected in *Eucalyptus* plantations in Uruguay (Corallo et al., 2019).

The collection of *Aspergillus*, *Fusarium,* and *Metarhizium* from all environments confirms the wide distribution of these fungi in most regions of the world in different habitats, soil types, plants, and plant residues, in both air and water (Edel-Hermann et al., 2015; Heo et al., 2019; Glare et al., 2021). The presence of *Metarhizium* in all habitats, especially soybean, confirms reports of a strong association of species of this this fungus genus with soils in cultivated habitats, particularly field crops (Quesada-Moraga et al., 2007), and its relationship with different hosts, including forest and agricultural pests (Sullivan et al., 2022). The lower diversity of entomopathogenic fungi in soybean, with the presence only of this genus is, probably, due to fungicide use to manage diseases in this crop (Alves and Juliatti, 2018).

*Cordyceps* and *Penicillium*, found only in the natural forest area, highlights the presence of these fungi in less impacted areas, as observed for *Cordyceps* in *Citrus sinensis* orchards with organic management (Prade et al., 2007), *Cordyceps* in Cerrado soils (Rocha and Luz, 2011) and *Penicillium* sp. in Caatinga and Atlantic Forest soils (Barbosa et al., 2016). This may be related to texture, acidity, and organic matter content, which facilitate the presence of these entomopathogenic fungi (Quesada-Moraga et al., 2007).

**Table 2.** Coverage, identity, and closest GenBank access of entomopathogenic fungi of the genera *Aspergillus*, *Beauveria*, *Cordyceps*, *Fusarium*, *Metarhizium*, *Penicillium*, and *Purpureocillium* to confirm morphological identification.

| Genera | Coverage | Identity | Closest GenBank access |
|---|---|---|---|
| *Aspergillus* sp. | 100% | 99% | MT538324.1 |
| *Beauveria* sp. | 100% | 100% | MK918495.1 |
| *Cordyceps* sp. | 100% | 100% | KX034378.1 |
| *Fusarium sp.* | 97% | 99% | MT 114705.1 |
| *Metarhizium* sp. | 100% | 99% | MH859067.1 |
| *Penicillium* sp. | 95% | 95% | MH048884.1 |
| *Purpureocillium* sp. | 100% | 98% | MK290901.1 |

**Table 3.** Frequency of occurrence of entomopathogenic fungi of the genera *Aspergillus*, *Beauveria*, *Cordyceps*, *Fusarium*, *Metarhizium, Penicillium,* and *Purpureocillium* isolated from 10 soils sample collected per eucalyptus and soybean crops and in natural forest areas sites using larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae).

| Genera | Eucalyptus | Soybean | Natural Forest |
|---|---|---|---|
| *Aspergillus* | 2 | 2 | 3 |
| *Beauveria* | 3 | - | 8 |
| *Cordyceps* | - | - | 5 |
| *Fusarium* | 3 | 2 | 2 |
| *Metarhizium* | 2 | 5 | 4 |
| *Penicillium* | - | - | 2 |
| *Purpureocillium* | 2 | - | - |

The presence of *Purpureocillium* in soil from eucalyptus crops is due to its relationship with some insect species, including forest pests, as reported for *T. peregrinus* (Corallo et al., 2019).

The *Beauveria*, *Cordyceps,* and *Metarhizium* genera, with registered commercial products (Brasil, 2022), are the most suitable to formulate and produce mycoinsecticides, due to their greater safety for human health and entomopathogenic potential (Zimmermann, 2007a, b; Chen et al., 2020). The use of these entomopathogenic agents should be evaluated because the genera *Aspergillus*, *Fusarium, Penicillium,* and *Purpureocillium* include entomopathogenic species (Kataria et al., 2018; Ahmad et al., 2019; Sun et al., 2021), but also some with mycotoxins that can be pathogenic to humans (Antas et al., 2012; Mendonça et al., 2009; Li et al., 2020).

The diversity of fungal genera identified in soils of eucalypts and soybean crops and natural forest is similar to that reported in soil samples from agroforestry citrus orchards, with 28 genera identified, Brazilian Caatinga, with 42 species, including those of the genus *Aspergillus* and *Penicillium*, and Restinga, with 14 genera of fungi (Prade et al., 2007; Barbosa et al., 2020; Mussi-Dias et al., 2020). The higher diversity of fungal isolates collected from natural forest soil reflects an absence of agricultural practices such as chemical use, including fungicides, and soil manipulation (Quesada-Moraga et al., 2007). The predominance of *B. bassiana* in agricultural and natural habitats, with greater diversity in the latter, may be related to soils with higher pH and clay content (Quesada-Moraga et al., 2007) and diversity of host insect species (Amobonye et al., 2020).

The diversity of entomopathogenic fungi was higher in soils from the natural forest area and indicated that they should be prospected in such areas to use in pest management programs.

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