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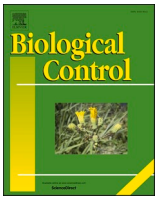

The Eucalyptus snout beetle in Colombia: Selection and evaluation of entomopathogenic fungi as bioinsecticides against *Gonipterus platensis*

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HIGHLIGHTS

- We confirmed the presence of *G. platensis* in Colombia, using molecular identification.
- From *G. platensis* adults, we isolated native fungi from *Beauveria* spp. and *Metarhizium* spp.
- The *B. pseudobassiana* isolate showed to be the best isolate according to the evaluations.

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ABSTRACT

The Eucalyptus snout beetles, which belong to the *Gonipterus scutellatus* species complex, cause heavy damage to *Eucalyptus* spp. worldwide. The species was reported in Antioquia, Colombia for the first time in 2016, threatening more than 115,000 ha of planted *Eucalyptus* spp. Elsewhere, its damage has been controlled using *Anaphes* spp. as classical biological control, but *Anaphes* spp. are not available in Colombia, where there are no documented control methods for this species to date. We identified the *Gonipterus* species using molecular methods and isolated entomopathogenic fungi from naturally infected beetles collected from the current distribution area in Colombia. We characterised the fungal isolates, selecting the best to develop a biological control agent. We considered insecticidal activity, UV-B radiation tolerance, yield, germination, and enzyme activity of conidia in making our selection. We confirmed the presence of *G. platensis* in Colombia, with adults naturally infected by *Beauveria* spp. and *Metarhizium* spp. From the isolated native fungi, *M. brunneum* (CA-3), *M. robertsii* (RI-1), *B. bassiana* (CA-1) and (CA-2), *B. pseudobassiana* (SP-1) were identified. We found remarkable differences in response to different variables between the selected isolates and SP-1 and CA-3 were the most virulent. The SP-1 isolate stood out from the rest for being the most tolerant to UV-B radiation, alkaline media, and low temperatures, germinating faster and producing elevated levels of lipases, chitinases, and proteases. The identified promising native fungus isolate, naturally occurring on field individuals, could be used to develop a biopesticide for controlling *G. platensis* in *Eucalyptus* spp. plantations in Colombia.

1. Introduction

The Eucalyptus snout beetles, referred to as *Gonipterus scutellatus* Gyllenhal, 1833 (Coleoptera: Curculionidae: Gonipterini), is a *Eucalyptus* spp. (Myrtaceae) pest that has spread to America, Africa and Europe (Mapondera et al., 2012; Hurley et al., 2016; Schröder et al., 2020) and has a high economic impact on *Eucalyptus* spp. production worldwide (Mapondera et al., 2012). Taxonomic studies have shown that *G. scutellatus* is a complex of species. The *G. scutellatus* species are native to Australia and include the species *G. balteatus*, *G. notographus*, *G. platensis*, *G. pulverulentus*, *G. scutellatus*, *Gonipterus* sp. 1, *Gonipterus* sp. 2, *Gonipterus* sp. 3, *Gonipterus* sp. 4, and *Gonipterus* sp. 5 (Mapondera

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et al., 2012).

Gonipterus spp. feed on developing eucalypt leaves, buds, and shoots of the upper crown and cause defoliation and stunted growth, which results in significant losses in forest growth and wood production (Loch and Matsuki, 2010; Schröder et al., 2020). Reis et al. (2012) predicted that the defoliation caused by *Gonipterus* could reach 50 %, resulting in up to 21 % wood production loss.

The Eucalyptus snout beetle's devastating impact is related to its capacity for dispersal, facilitated both by its great flight capability and the transport of forest products (Hanks et al., 2000). Within a year of the identification of *G. scutellatus* in Chile in 1998, its distribution reached 1,156 km^2 (Estay et al., 2002). The pattern of dispersal was similar in South Africa and the United States of America during the first five years since the detection in these countries, with the snout beetle's range growing at a rate of 160 km/year (Hanks et al., 2000). The rapid dispersal and high devastation potential suggest that the *G. scutellatus* species complex should be considered a global threat to reforestation (FAO, 2009).

The recent occurrence of the *G. scutellatus* species complex in *Eucalyptus* spp. plantations in Colombia—which has approximately 115,570 ha of planted *Eucalyptus* spp. forest (MADR, 2022)—and the subsequent declaration of a phytosanitary alert (ICA, 2016), demonstrate the urgency of developing strategies for its control and to avoid financial losses. There is no evaluation of economic losses available in Colombia, so far. The most common *Eucalyptus* spp. species planted in Colombia is *Eucalyptus grandis* (42,385 ha; MADR, 2022), mostly used of paper pulp.

Classical biological control of *Gonipterus* spp. using *Anaphes nitens* (Hymenoptera: Mymaridae) has been one of the most impressive examples of biological control in Europe (Reis et al., 2012; Valente et al., 2018), Africa (Tribe, 2005) and the Americas (Hanks et al., 2000; Medeiros de Souza et al., 2016). This could be the end of the story, but parasitism rates do not frequently reach financially sustainable levels (Pereira et al., 2023); and are affected by climate and population dynamics fluctuations of the host and the parasitoid (Reis et al., 2012; Valente et al., 2018). Furthermore, it has been suggested that the *G. scutellatus* species complex could differentially respond to parasitism (Mapondera et al., 2012). Efforts are currently being made to reduce the damage caused by *Gonipterus*, such as the evaluation of *Eucalyptus* spp. with low susceptibility to the pest (Gonçalves et al., 2019).

The use of entomopathogenic fungi (EF) is a promising method for insect pest control and several biopesticides have been developed worldwide (Jiang and Wang, 2023). The mycoinsecticides belonging to the fungal genera *Beauveria* and *Metarhizium* are the most commonly produced and commercialised for these purposes (Jiang and Wang, 2023; Sullivan et al., 2022), however limited research using EF to control *Gonipterus* populations is available. Echeverri-Molina and Santolamazza-Carbone (2010) evaluated five biological insecticides with active ingredients of *Beauveria bassiana*, *Metarhizium anisopliae*, and a mixture of *Brevibacillus laterosporus*, *Bacillus licheniformis,* and *Bacillus chitinosporus.* The findings of the study showed that *B. bassiana* was highly effective both by contact and ingestion with a mortality of 100 %, while *M. anisopliae* and the *Bacillus* mixture caused low mortalities, ranging from 2.5 to 5 %. In Brazil, Berti Filho et al. (1992) reported the occurrence of *B. bassiana* in *G. scutellatus* adults in a commercial *Eucalyptus* spp. plantation, and recent research described the virulence of previously isolated indigenous *Beauveria* spp. and *Metarhizium* spp. strains against *G. platensis* finding that a *B. bassiana* isolate had a lower median lethal concentration LC_{50} (2.3×10^7 con/mL) than *M. anisopliae* isolate (6.6×10^7 con/mL) (Jordan et al., 2021). Although *B. bassiana* and *Bacillus thuringiensis* are recommended for the control of the Eucalyptus snout beetle in Colombia (MADR, 2019), there are no registered biopesticides (active ingredient: microorganisms) to control *Gonipterus* in the country (ICA, 2023).

Despite the benefits of using mycoinsecticides to control pests in agroecosystems, there are still limitations and a slow adoption of this bioproducts. These microorganisms are exposed to abiotic stress in the form of UV radiation, pH, and temperature fluctuations that may alter their persistence and performance in the field (Fernandes et al., 2015; Jackson et al., 2010). As a result, the growth ability under variations in these conditions is a relevant characteristic when selecting for massive production and tolerance to environmental stress. Microorganisms that are adapted to agroclimatic conditions where *Gonipterus* are established have the potential to be an friendly pest control solution. In this context, we used a molecular approach to identify the *Gonipterus* species occurring in Colombia and isolated EF from naturally infected insects, characterised the fungi, and selected those with high insecticidal activity to be used as an active ingredient in biopesticides for the control of the Eucalyptus snout beetle.

2. Materials and methods

2.1. Eucalyptus snout beetle collection

The Eucalyptus snout beetle were collected from six separate locations in the Department of Antioquia: Santa Elena (SE) (6°12′50,7″N; 75°30′28,7″W), San Pedro de los Milagros (SP) (6°24′4,7'N; 75°35′52,1″W), Santa Rosa de Osos (SR) (6°44′45″N; 75°23′44″W), Don Matías (DM) (6°29′34″N; 75°24′33″W), Caldas (CA) (6°05′40,4″N; 75°39′13,1″W), and Rionegro (RI) (6°05′51,4″N; 75°26′18,1″W). Healthy-appearing adults were placed in plastic cages (20 × 20 × 8 cm) with eucalypt leaves as feeding substrate and transported to the laboratory. Collected adults were fed *Eucalyptus* spp. leaves and kept in controlled conditions (20 °C, 60 % RH). All individuals were collected according to the guidelines in the collection framework permit number 1466 of 2014, granted by the National Authority of Environmental Licenses (ANLA) to the Colombian Corporation for Agricultural Research (AGROSAVIA).

2.2. Molecular identification of the Eucalyptus snout beetle

Insect DNA extraction was performed following a methodology based on the use of cetyltrimethylammonium bromide (CTAB-PVP) (Calderón-Cortés et al., 2010). The *in vitro* amplifications of the partial cytochrome *c* oxidase subunit I (COI) gene were carried out via the polymerase chain reaction (PCR) method in standard conditions (final volume 25 μL) using Taq polymerase (PROMEGA), 50 ng of a template and the primers previously used in *Gonipterus*: GON-F 5′-GGAGTACTCGGGATAATTTACG-3′ (Mapondera et al., 2012) and TL2-N-3014 (PAT) (reverse) 5′-TCCAATGCACTAATCTGCCATATT A-3′ (Simon et al., 1994). The PCR thermal cycling consisted of one cycle at 94 °C for 5 min, followed by 35 cycles of 92 °C for 10 sec, 55 °C for 15 sec, 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. The PCR-amplified products of 47 collected insects were visualised in 1 % agarose gel and sequenced by the Sanger method.

The sequences of the partial COI mitochondrial gene were inspected manually and used to infer phylogeny by the neighbour-joining method (Saitou and Nei, 1987) using MEGA 11 software (Tamura et al., 2021) with a bootstrap consensus tree inferred from 1000 replicates. The evolutionary distances were computed using the Kimura two-parameter model (Kimura, 1980). The rate variation among sites was modelled with a Gamma distribution (shape parameter = 1). This analysis involved 95 nucleotide sequences, with a total of 417 positions in the final dataset. Estimates of the average evolutionary divergence over sequence pairs within species were then calculated and compared to the representative sequences of different *Gonipterus* species from the National Center for Biotechnology Information (NCBI) database.

2.3. Isolation of entomopathogenic fungi

We isolated EF from naturally infected adults with signs of mycosis using the methodology described by Mar et al. (2012) with modifications. The mummified insects were collected in the field and transported

to the laboratory in individual 2-mL tubes. Fungal sporulation was then promoted by placing the insects in humidity chambers (60 % RH). The fungus was scraped from the insect cadaver, cultured on potato dextrose agar (Sigma®) supplemented with 0.05 mg/l of chloramphenicol, and incubated at 25 ± 2 °C for 7 days. After incubation, the fungus from a single colony was placed on a new PDA plate to obtain a pure culture and incubated for an additional 96 h. Preliminary morphological identification of fungal isolates was done using a slide culture (Harris, 1986). All isolates were identified using a Zeiss Axio Lab. A1 microscope following the methodology of Humber (1997).

2.4. Molecular identification of entomopathogenic fungi

Genomic DNAs were extracted from conidia which were ground in liquid N_2. DNA extraction was conducted using the *Quick*-DNA Fungal/Bacterial MiniPrep system (Zymo Research) according to the manufacturer's recommendations. Identification was based on the partial sequencing of two regions: the internal transcribed spacer region of nuclear ribosomal DNA (ITS) and elongation factor 1-alpha (EF-1α). ITS was amplified using the primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). EF-1α was amplified using the primers EF1T (5′-ATGGGTAAGGARGACAAGAC) and EF2T (5′-GGAAGTACCAGTGATCATGTT-3′) (Rehner and Buckley, 2005). PCR reactions were carried out in 25 μL volumes using 5 μL reaction buffer, 6.25 mM dNTPs, 2.5 mM MgCl2, 1 μL of each primer (10 μM), 0.2 μL DNA taq polymerase (Promega), and 10 μL of genomic DNA. The thermal conditions were: 94 °C for 4 min, followed by 35 cycles of 10 s at 92 °C, 20 s at 58.5 °C for ITS primers and 56 °C for EF1T primers, 60 s at 72 °C, and a final extension of 5 min at 72 °C. The products were cleaned with Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced by Sanger method at Agrosavia's laboratory.

The phylogenetic trees were inferred by using the neighbour-joining method (Saitou and Nei, 1987) with a bootstrap consensus tree inferred from 1000 replicates using MEGA 11 software (Tamura et al., 2021). The evolutionary distances were computed using the Kimura two-parameter model (Kimura, 1980). There was a total of 1033 positions in the final dataset for concatenated sequences of ITS, β-tubulin and EF-1α. The sequences were then compared to the representative sequences of different *Metarhizium* and *Beauveria* species from the NCBI database (Table 1).

2.5. Insecticidal activity of the entomopathogenic fungi

To evaluate the insecticidal activity of the five EFs, healthy-appearing adult Eucalyptus snout beetles were kept in plastic cages (20x20x8 cm) with *Eucalyptus* spp. leaves as feeding substrate under controlled conditions (20 °C, 60 % RH); only those individuals that remained healthy after one week were used for the experiment. The *Gonipterus* spp. adults were prepared by superficial disinfection with a 0.05 % NaClO solution for 30 sec followed by three rinses with distilled water and were allowed to dry at room temperature. After drying, the adult beetles were immersed in a conidial suspension (1×10^7 con/mL) in 0.1 % Tween 80® for 30 s. The control group was immersed in 0.1 % Tween 80®. The insects were once again placed in plastic cages (20 × 20 × 8 cm) with *Eucalyptus* spp. leaves in humidity chambers and incubated at 25 °C, with 60 % relative humidity (RH) and a light/darkness photoperiod of 12 h. New fresh *Eucalyptus* spp. leaves were added every two days. The experimental unit consisted of 10 adults per cage and three repetitions. The mortality of the treatment was corrected with the mortality of the control according to the Schneider-Orelli formula (Zar, 1999). The isolates of each genus that presented the greatest efficacies were selected and evaluated in a new trial. In the new trial, the comparison of the two selected isolates was made by evaluating lower conidia concentrations of 1×10^5, 1×10^6 and 1×10^7 con/mL. The experimental design and conditions were the same as those previously described.

Table 1
Sequences used in phylogenetic analysis of the fungal isolates.

Species and strain code	GenBank accession number
Metarhizium acridum CQMa 102	PRJNA245139
Metarhizium album ARSEF1941	PRJNA72731
Metarhizium anisopliae JEF-290	PRJNA530366
Metarhizium brunneum ARSEF 3297	PRJNA608152
Metarhizium guizhouense ARSEF 977	PRJNA184755
Metarhizium majus ARSEF297	PRJNA302308
Metarhizium robertsii ARSEF23	PRJNA245140
Beauveria bassiana ARSEF 2860	PRJNA225503
Beauveria brongniartii RCEF 3172	PRJNA72729
Beauveria pseudobassiana RGM2184	PRJNA799395

2.6. Ecophysiological characterisation of the entomopathogenic fungi

2.6.1. UV-B tolerance

UV-B tolerance was tested according to the methodology described by Fernandes et al. (2007) with modifications. The fungal isolates were grown on PDA in Petri dishes for 7 days at 25 °C. Conidia were harvested with a spatula and suspended in distilled water containing 0.1 % Tween 80® (Sigma®), vigorously agitated, and filtered through a fine-mesh sieve to remove the hyphal aggregates. The conidial suspension (1×10^7 conidia/mL) was inoculated onto water agar (WA) supplemented with 0.00005 % (w/w) benomyl and potato dextrose agar (PDA) to assess relative germination and culturability, respectively. Relative germination and culturability were calculated according to Braga et al. (2001). To estimate relative germination, the WA plates were exposed to 191.8 kJ/m^2 weighted UV-B (UVP 3UV-38 Lamp, 302 nm wavelength) at 20 cm of distance for 2.4, 4.8, 7.2, 9.6, 14.4, and 19.2 min resulting to final doses of 7.7, 15.4, 23.1, 30.8, 46.2 and 61.6 kJ/m^2. Control plates were not exposed. The plates were then incubated in darkness at 25 °C for 24 h and germination was determined at 400× magnification. Conidia with germ tubes longer than their width were considered germinated (Braga et al., 2001). To estimate relative culturability, the PDA plates were exposed under the conditions mentioned above and were incubated at 25 °C for seven days, after which colony-forming units (UFC/mL) were counted. The experiment consisted of three repetitions.

2.6.2. Effect of temperature on growth

The effect of temperature was evaluated on germination and radial extension. Fungal isolates were grown on potato dextrose agar and incubated at 25 °C for 7 days. A 5-mm disc of fungal culture was placed on PDA in Petri dishes and was incubated at a temperature of 15, 25, 30, and 35 °C (memmert IF55 incubator- temperature sensor is 1 Pt100 sensor DIN class A in 4-wire-circuit). Perpendicular measures of the radial extension were made every 72 h for 15 days. The slope of the linear regression curves of the radial extension data against time was used to estimate the growth rate (Hallsworth and Magan, 1999). Also, 100 μL of a conidial suspension was cultured on malt extract agar (0.1 %) supplemented with 0.0005 % benomyl and incubated at the temperatures mentioned above for 24 h to determine percentage of germination. Conidia with germ tubes longer than their width were considered germinated (Villamizar et al., 2018). The experiment consisted of three repetitions.

2.6.3. Effect of pH on growth

The effect of pH on morphology, germination, and radial extension was also evaluated. Fungal isolates were grown on potato dextrose agar and incubated at 25 °C for 7 days. The pH of the PDA and Sabouraud dextrose agar (SDA) media was adjusted to 3, 5, 7, and 9 with either 0.1 N HCl or 0.1 N NaOH before autoclaving. Perpendicular measures of the radial extension were made every 72 h for 15 days. The slope of the linear regression curves of the radial extension data against time was used to estimate the growth rate (mm/day) (Hallsworth and Magan,

1999). To determine percentage of germination, 100 μL of a conidial suspension was cultured on extract malt agar (0.1 %) supplemented with 0.0005 % benomyl and adjusted to a pH of 3, 5, 7, and 9 with 0.1 N HCl or 0.1 N NaOH and incubated at 25 °C for 24 h. Conidia with germ tubes longer than their width or diameter were considered germinated (Villamizar et al., 2018). The experiment consisted of three repetitions.

2.7. Growth parameters of entomopathogenic fungi

2.7.1. Semisolid-state fermentation of B. bassiana SP-1

The conidial production of *B. bassiana* was performed in semisolid-state fermentation (Mejía et al., 2020) using a rice powder substrate supplemented with oat (3:1), potato (3:1), wheat bran (19:1), and soy protein (19:1). Trays (180x115x40 cm) were filled with 120 mL of culture media (10 % solid substrate) and sterilised. The trays were then inoculated with 5 mL of a 1×10^6 con/mL conidial suspension, covered with plastic film, and incubated at 25 °C for 7 days. After the incubation time, the plastic film was later changed to a paper towel to induce drying at same temperature of incubation for 7 days. Dry conidia were harvested through a sieve of 150 μm and stored at 4 °C. The fermentation consisted of three repetitions.

2.7.2. Solid-state fermentation of M. anisopliae CA-3

Conidial production of *M. anisopliae* was performed in solid-state fermentation using rice as the substrate supplemented with oat (3:1), potato (3:1), wheat bran (19:1), and soy protein (19:1). Plastic bags were filled with 100 g of the substrate and 70 mL of water and inoculated with 10 mL of a 1×10^6 con/mL conidial suspension. Plastic bags were incubated at 25 °C for 7 days. The colonised substrate was placed in trays ($180 \times 115 \times 40$ cm) and covered with a paper towel to induce drying at the same temperature as during incubation for 7 days. Dry conidia were harvested through a sieve of 150 μm and stored at 4 °C. The fermentation consisted of three repetitions.

2.7.3. Germination

10 mg of dry conidia were suspended in 990 μL of 0.1 % Tween 80®, serial dilutions were made to a final concentration of 1×10^6 con/mL, and 100 μL of the suspension was poured on water agar supplemented with 0.1 % malt extract and 0.00005 % benomyl. The Petri dishes were incubated at 25 °C and germination was measured at 16, 24, and 48 h. Conidia with germ tubes longer than their width were considered germinated (Villamizar et al., 2018). The experiment consisted of three repetitions.

2.7.4. Enzymatic activity

To obtain the crude extract used to measure enzymatic activity, 50 mg of dry conidia were suspended in 1 mL 1.0 % Tween 80®. The suspension was stirred at 3000 rpm for 1 h and centrifuged at 8000 rpm for 10 min. The supernatant was recovered and stored at −20 °C.

Lipase activity was determined according to Santos Díaz et al. (2017). The crude extract (20 μL) was mixed with 100 μL of p-nitrophenyl-palmitate (Sigma®) (1 mg/mL in dimethyl sulfoxide). The reaction was incubated at 35 °C for 30 min. 150 μL of NaOH-glycine (pH 10.4) was then added and the absorbance at 400 nm was determined. One unit was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per minute. The experiment consisted of three repetitions.

Chitinase activity was measured following the methodology described by Santos Díaz et al. (2017). 20 μL of crude extract was mixed with 100 μL of p-nitrophenyl-N-acetylglucosamine (Sigma®) (1 mg/mL in citrate buffer, 0.1 M, pH 5) and incubated at 35 °C for 30 min. 150 μL of NaOH-glycine (pH 10.4) was then added and the absorbance at 400 nm was determined. One unit was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per minute. The experiment consisted of three repetitions.

Protease activity was determined according to Cupp-Enyard (2008). 130 μL of 0.65 % casein were mixed with 25 μL of crude extract. The reaction was incubated at 37 °C for 10 min, after which 130 μL of trichloroacetic acid (110 mM) was added, the solution was mixed, and incubated at 37 °C for 20 min. The mixture was then centrifuged at 10,000 rpm for 5 min. 250 μL of supernatant was placed in a new microtube to which 625 μL of sodium carbonate (500 mM) and 125 μL of Folin-Ciocalteu reagent (0.5 M) were added. The mix was incubated at 37 °C for 30 min. The absorbance at 660 nm was measured to determine μmoles of tyrosine. One unit was defined as the amount of enzyme that released 1 μmol tyrosine per minute. The experiment consisted of three repetitions.

2.8. Data analysis

The experimental design was completely randomised with three repetitions per treatment and measures across time. All data were checked for normality (Shapiro–Wilk test) and variance homogeneity (Bartlett test). Data were analysed by one-way ANOVA and significant differences were determined with the Tukey test (95 % confidence) using Statistix software (Version 7.0 Analytical Software, Florida, U.S. A.).

3. Results

3.1. Molecular identification of the Eucalyptus snout beetle

Our phylogenetic analysis of the *Gonipterus* COI gene fragment sequences from the 47 insects confirmed the presence of *Gonipterus platensis* in Antioquia, Colombia. In the elaboration of the phylogenetic tree, a representative insect from each geographical site sampled was used for simplicity, since the found diversity was very low. We achieved a high accuracy of identification (98 %), as demonstrated by the high bootstrap values of each clade. The analysis included other related species belonging to the *G. scutellatus* species complex that were clearly separated into different clades (Fig. 1). We found very low diversity among *G. platensis* individuals when considering the analysis of genetic distance in both our samples and those reported from Spain, Australia, and Portugal. This implies an average evolutionary divergence value of 0.003 (please see Online Resource 1 for more information) within the group, compared to much higher values for the populations of *G. pulverulentus* (0.023), *G. notographus* (0.038) and *Gonipterus* sp. 2 (0.030). The divergence was even smaller (0.0006) when it was estimated using only our insects collected in Colombia.

3.2. Selection and identification of entomopathogenic fungi isolates

We recovered five isolates from the collected cadavers of *Gonipterus* individuals. The selected EF isolates were termed according to the locality and the preliminary genus identification. They were SP-1 (*Beauveria* sp.), CA-1 (*Beauveria* sp.), CA-2 (*Beauveria* sp.), CA-3 (*Metarhizium* sp.), and RI-1 (*Metarhizium* sp.).

3.3. Molecular identification of entomopathogenic fungi

Phylogenetic analysis based on concatenated ITS sequences and elongation factor showed that isolates CA-1, CA-2, and SP-1 clustered in the *Beauveria* clade (bootstrap value = 100), while CA-3 and RI-1 clustered in the *Metarhizium* clade (bootstrap value = 97). The isolate CA-3 clustered with *M. brunneum* and RI-1 with *M. robertsii* (Fig. 2). The genetic distances among species of the genus *Metarhizium* showed a very close relationship between *M. anisopliae, M. brunneum,* and *M. robertsii* with values between 0.007 and 0.008 (please see Online Resource 2 for more information). This was reflected in bootstrap values of <90 % for the classification of Colombian isolates. In contrast, the isolates CA-1 and CA-2 clustered with *B. bassiana* and SP-1 with *B. pseudobassiana*, also with high bootstrap values. The estimated genetic distances within the *Beauveria* genus ranged from 0.01 to 0.03, showing greater

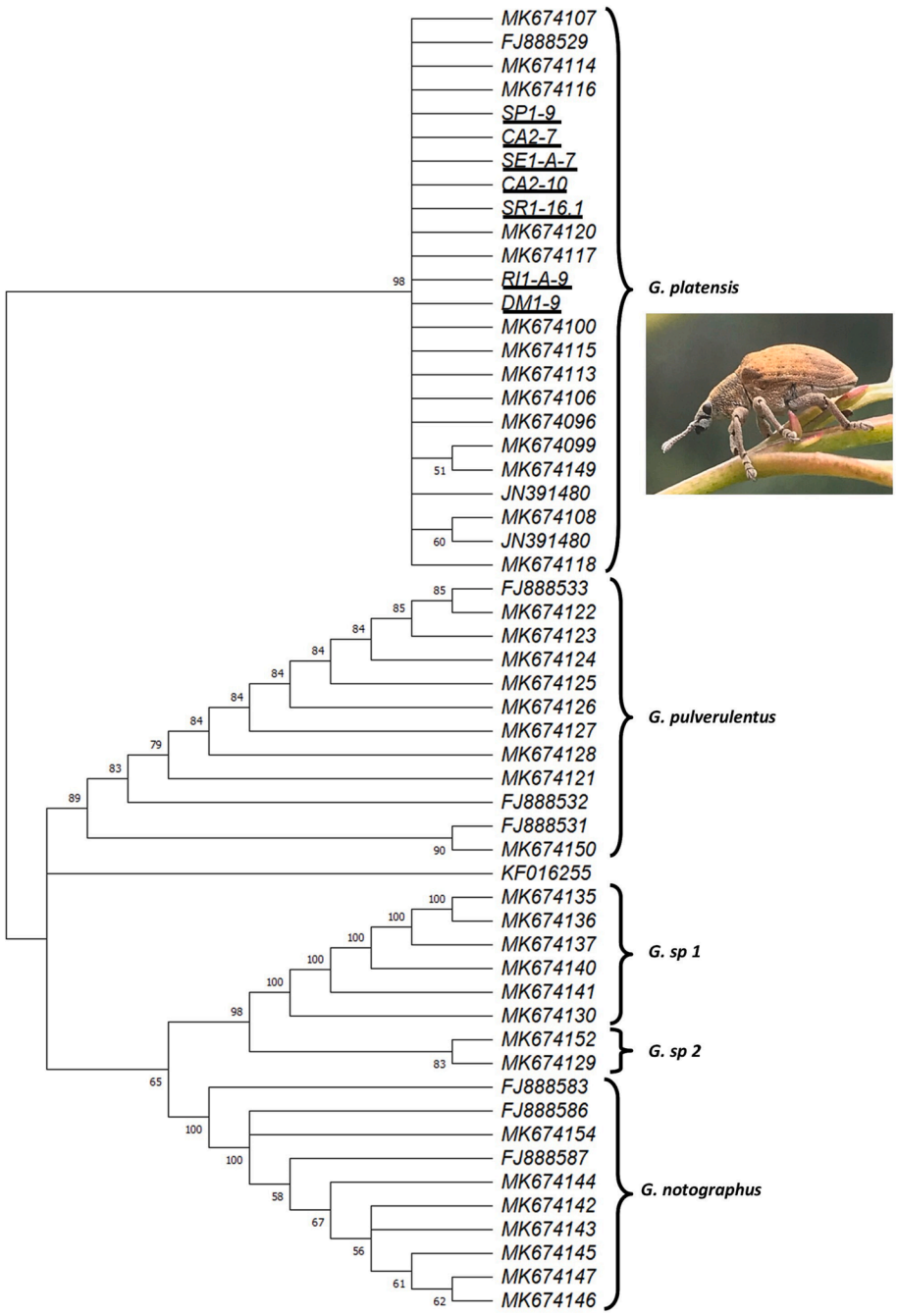

Fig. 1. Phylogenetic tree constructed with Kimura two-parameter distance and the neighbour-joining method showing the *Gonipterus* species belonging to the *G. scutellatus* species complex. Bootstrap values higher than 50 are shown for each branch. Accession numbers for insects from GenBank are included in the tree. Colombian insects are underlined.

divergence among species than within the *Metarhizium* genus.

3.4. Insecticidal activity of the entomopathogenic fungi

The mortality of *Gonipterus* adults varied across the isolates. At 7 days post-inoculation, the highest efficacies were achieved with isolates *B. pseudobassiana* SP-1 (92.5 %), *B. bassiana* CA-1 (90.0 %), and *M. brunneum* CA-3 (85.0 %). The *M. robertsii* RI-1 and *B. bassiana* CA-2 isolates achieved a much lower efficacy of 74.2 % and 43.3 %, respectively ($F = 7.09$, $df = 14$, $p < 0.05$) (Fig. 3). On Day 10, the efficacy for all isolates reached values of 96.7–100 %, except for the isolate CA-2 which again exhibited the lowest mortality (73.6 %) ($F = 8.10$, $df = 14$, $p < 0.05$). We selected the isolates SP-1 and CA-3 for further analysis as they belong to different genus and resulted in the highest efficacy.

Regarding the assessment of different conidial concentrations of *B. bassiana* against *G. platensis* adults, an efficacy of 100 % was achieved with a conidial concentration of 1 × 107 con/mL and 43.3 % with a conidial concentration of 1 x 106 con/mL on day 9. ($F = 45.7$, $df = 8$, $p < 0.05$). However, after 15 days of inoculation, the efficacy reached the 92.6 % compared to 37.0 % using the conidial concentration of 1×10^5 con/mL. When a conidial suspension of 1×10^7 con/mL of *M. brunneum* CA-3 was used, an efficacy of 83.3 % was determined on day 9, whereas efficacies of 23.3 and 3.3 % were obtained using conidial suspensions of 1×10^6 and 1×10^5 con/mL, respectively ($F = 156$, $df = 8$, $p < 0.05$). In contrast, on day 15 after inoculation, the efficacies were 60.0 and 20.0 %, respectively.

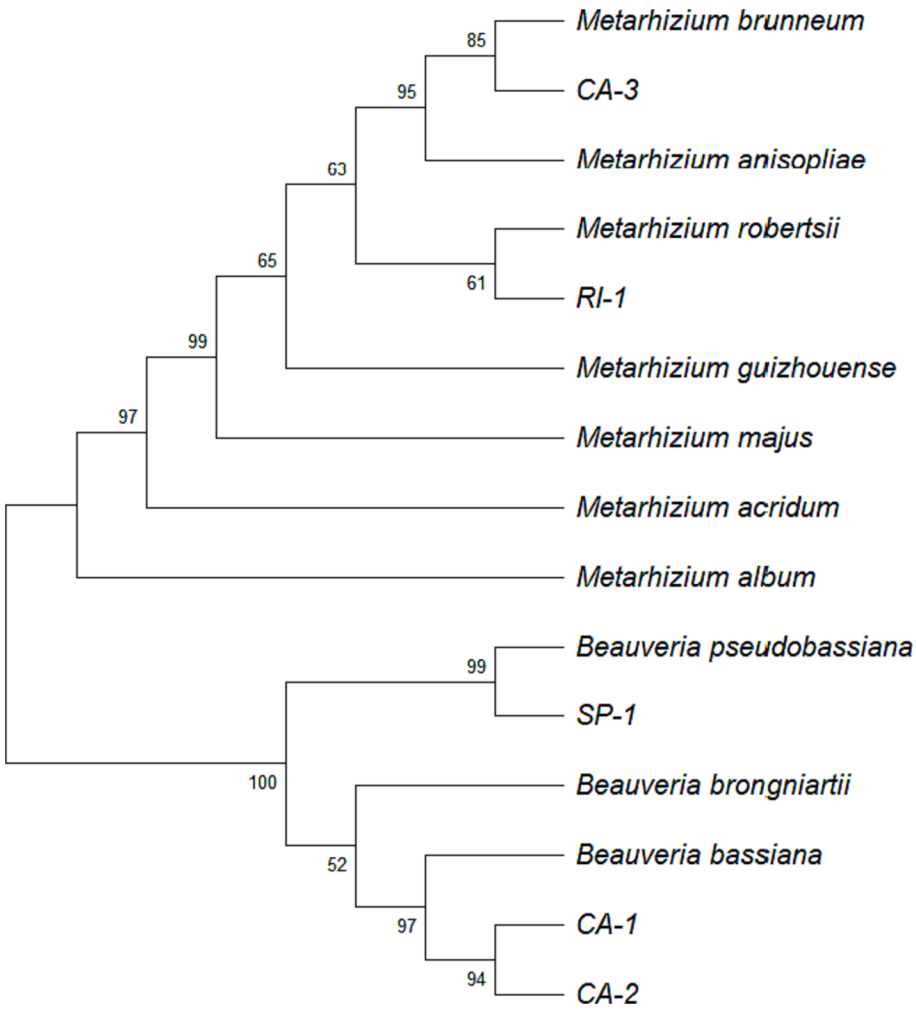

Fig. 2. Phylogenetic tree constructed with Kimura two-parameter distance and the neighbour-joining method, showing the isolated entomopathogenic fungi. It was built using concatenated sequences of ITS and EF-1α. Bootstrap values are shown above/below each branch.

3.5. Ecophysiological characterisation of the entomopathogenic fungi

3.5.1. UV-B tolerance

The germination of entomopathogenic fungal isolates decreased with increasing UV-B exposure time (Fig. 4a). The isolate of *B. bassiana* SP-1 was the most tolerant fungus to UV-B radiation. At the exposure time of 2.4 min, no significant change on relative germination of *B. pseudobassiana* SP-1 was found with a value of 99.3 % whereas the relative germination of *M. brunneum* CA-3 decreased to 82.9 % ($F = 110$, $df = 11$, $p < 0.05$). At an exposure time of 4.8 min, the relative germination of *B. pseudobassiana* SP-1 was 97.7 %, whereas for *M. brunneum* CA-3 germination was 60.8 % ($F = 238$, $df = 11$, $p < 0.05$). When exposure time was doubled to 9.6 min, the relative germination was 74.1 % for SP-1 and 8.4 % for CA-3 ($F = 2244$, $df = 11$, $p < 0.05$). At the final exposure time (19.2 min), the germination for *B. pseudobassiana* SP-1 was 28.3 % and 1.2 % for *M. brunneum* CA-3 ($F = 513$, $df = 11$, $p < 0.05$). Non-irradiated isolates had germination above 90 %. The relative culturability also diminished as exposure time increased (Fig. 4b). The highest relative culturability was reached by isolate SP-1 exhibited the highest relative culturability of 70 % at an exposure time of 4.8 min versus 56 % for CA-3 at the same exposure time ($F = 79.5$, $df = 11$, $p < 0.05$). The relative culturability fell drastically to below 22 % for both isolates at exposure times greater than 9.6 min ($F = 165$, $df = 11$, $p < 0.05$).

3.5.2. Effect of pH and temperature on growth

Differential growth of *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 was evidenced when fungi were exposed to different pH values in PDA and SDA (Fig. 5). Typical greenish sporulation of *M. brunneum* was only observed when culturing in PDA. *B. pseudobassiana* growth was reduced in SDA and the appearance of aerial mycelium changed according to the pH. The germination of *B. pseudobassiana* was reduced at pH 3, but differences between pH 5, 7, and 9 were not statistically significant ($F = 5581$, $fd = 47$, $p < 0.05$) (Fig. 6a). Isolate *M. brunneum* differed in that it presented the maximum germination at pH 7 and the lowest at pH 3 and 9 (Fig. 6a). Despite *M. brunneum* germination being inhibited at pH of 3 and 9, the isolate was still able to grow under these conditions (Fig. 6c). *B. pseudobassiana* did not present differences in growth rate at pH 5, 7, and 9, but a lower growth rate was observed at pH 3 ($F = 44{,}3$ $fd = 23$, $p < 0.05$) (Fig. 6b). Interestingly, *M. brunneum*, which had germinated poorly at pH 9, had the highest growth rate (6.2–6.4 mm/day) under the same condition ($F = 38{,}2$, $fd = 23$, $p < 0.05$) (Fig. 6c). Regardless of the effect of pH in germination, the five isolates were all able to grow in the culture media.

We did not find statistically significant differences in the germination of isolates at 25 °C and 35 °C for either *B. pseudobassiana* SP-1 or *M. brunneum* CA-3 (Fig. 6d). *B. bassiana* SP-1 was more tolerant to 15 °C than *M. anisopliae* CA-3 with germinations of 65 and 15 %, respectively ($F = 1045$, $fd = 47$, $p < 0.05$). Neither of the isolates germinated at 35 °C. The radial growth rate varied among isolates and across the evaluated temperatures. At the lowest temperature (15 °C),

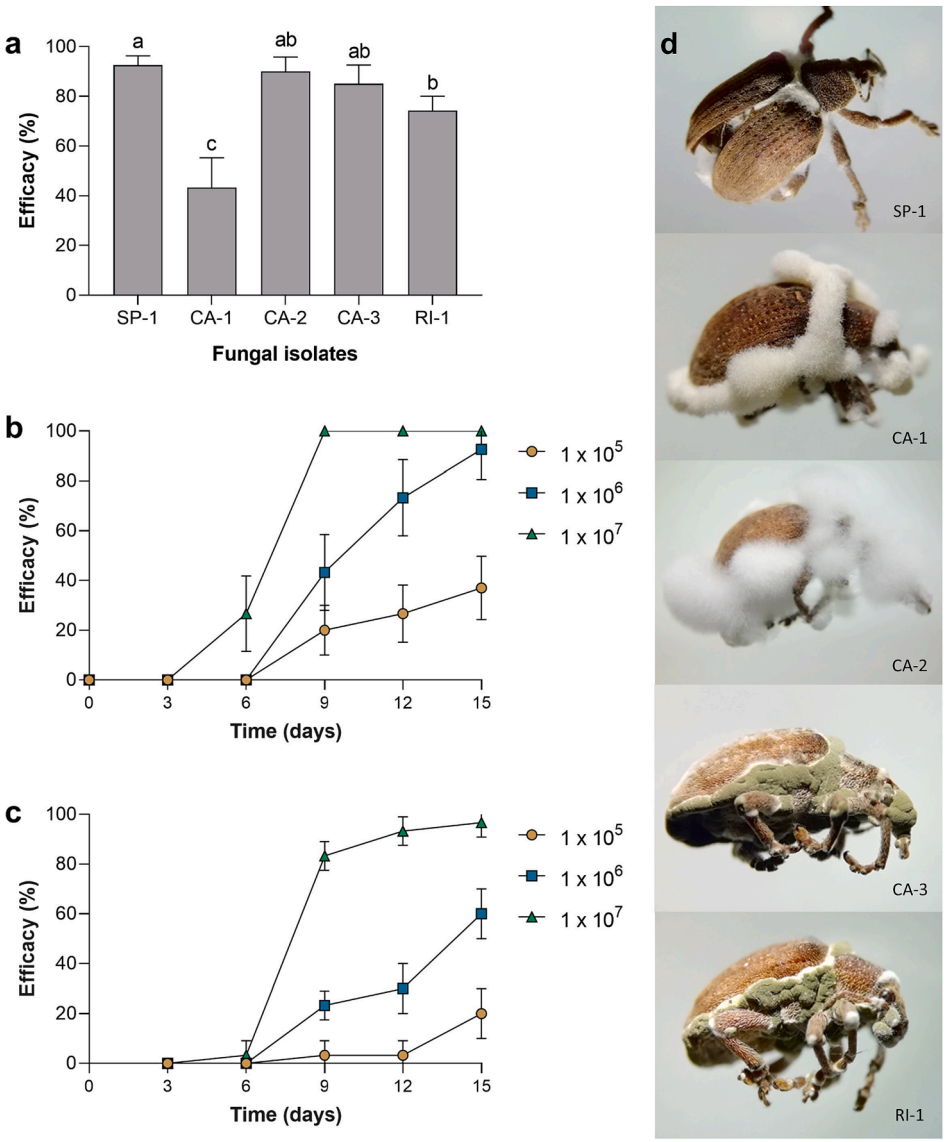

Fig. 3. Assessment of entomopathogenic fungi (EF) against *G. platensis* adults. **a)** Efficacy of EF (*B. psudobassiana* SP-1, *B. bassiana* CA-1, *B. bassiana* CA-2, *M. brunneum* CA-3 and *M. robertsii* RI-1) using a conidial suspension of 1×10^7 con/mL 7 days post-inoculation. Mean values (±SD) followed by different letters are significantly different according to the Tukey test (95 %). **b)** Efficacy of *B. pseudobassiana* SP-1 and **c)** efficacy of *M. brunneum* CA-3 against *G. platensis* using three conidial suspensions of 1×10^5, 1×10^6, and 1×10^7 con/mL under laboratory conditions. **d)** *G. platensis* adults colonised by entomopathogenic fungi.

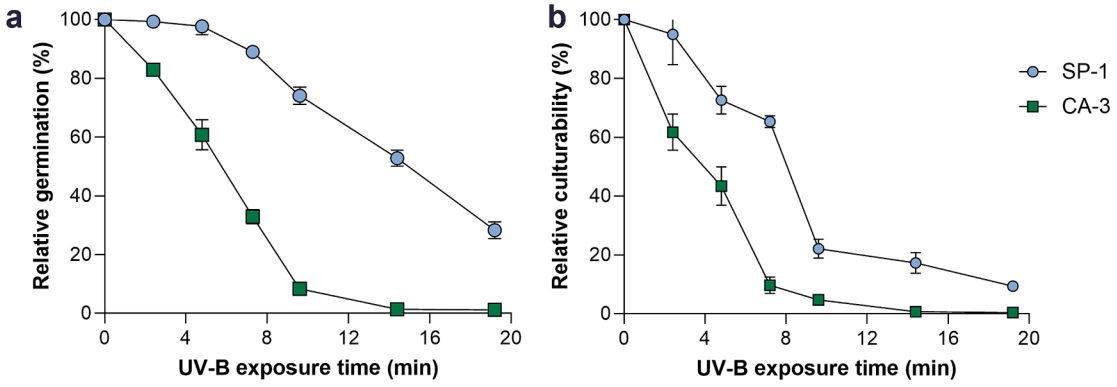

Fig. 4. **a)** Relative germination of the fungal isolates *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 and **b)** relative culturability of fungal isolates after exposure to 978 mW/m^2 weighted UV-B for 2.4, 4.8, 7.2. 9.6, 14.4, and 19.2-min. Mean values (±SD) of relative germination and culturability were calculated in relation to non-irradiated controls.

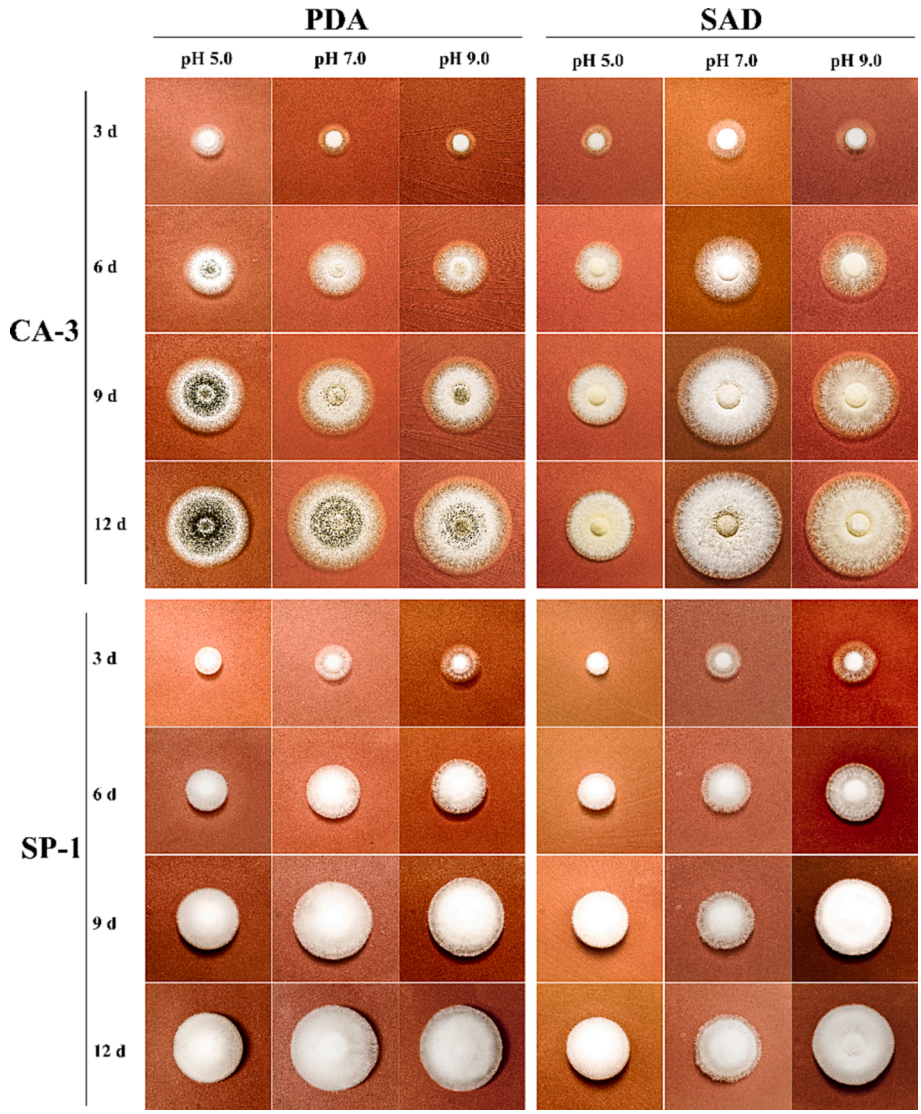

Fig. 5. Fungal growth pattern of isolates *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 on PDA and SDA culture media with different pH values after 3, 6, 9, and 12 days of incubation at 25 °C.

B. pseudobassiana SP-1 had a higher radial growth rate (5.4 mm/day) than *M. brunneum* CA-3 (2.4 mm/day) (Fig. 6e, 6f). The *B. pseudobassiana* SP-1 isolate had its highest radial growth rate (5.8 mm/day) at 25 °C; in contrast, the isolate of *M. brunneum* CA-3 had its highest growth rate (9.0 mm/day) at 30 °C. Fungal growth was inhibited at 35 °C.

3.5.3. Conidial production and enzymatic activity

The conidial production of the EF presented statistically significant differences according to the isolate and substrate evaluated ($F = 105$, $fd = 29$, $p < 0.05$) (Fig. 7a). The highest conidia yield of 4.3×10^9 con/mL and 6.4×10^9 con/mL for isolates *B. pseudobassiana* SP-1 and *M. brunneum* CA-3, respectively, were achieved using rice powder supplemented with wheat bran. Statistically significant differences were also found in *Beauveria* and *Metarhizium* germination. Notably, *B. pseudobassiana* germination was above 90 % after only 16 h, while *M. brunneum* germination was below 10 % at 16 h and did not germinate above 70 % until 48 h of incubation (Table 2).

The *B. pseudobassiana* SP-1 isolate produced higher levels of lipases, chitinases, and proteases than *M. brunneum* CA-3 isolate. The highest enzymatic activity was frequently achieved with supplemented rice (Fig. 7). The lipase activity was statistically significantly higher in conidia of *B. bassiana* SP-1 harvested from rice supplemented with either oat or potato with values of 0.25 and 0.27 U/mL, respectively ($F = 731$, $fd = 29$, $p < 0.05$) (Fig. 7b). In contrast, the isolate CA-3 produced values of lipases near 0.04 U/mL in all evaluated substrates.

Overall, higher enzymatic chitinase activity was reached in rice supplemented with oat, potato, or soy protein for both fungal isolates. The highest overall chitinase activity of 1.6 U/mL was found in *B. pseudobassiana* SP-1 isolate harvested from the substrate of rice-potato, followed by rice-oat and rice-soy protein, both with values of 1.4 U/mL ($F = 952$, $df = 29$, $p < 0.05$) (Fig. 7c). In contrast, the *M. brunneum* CA-3 isolate produced lower chitinase activity ranging between 0.10 and 0.35 U/mL, though activity was still the highest in the rice-potato substrate.

Similarly, the protease activity increased when fungi were produced on rice with oat, potato, or soy protein. *B. pseudobassiana* SP-1 produced high levels of proteases across all treatments, but the highest activity (4.5 dU/mL) was achieved on rice-soy protein followed by rice-oat (4.3 U/mL) (Fig. 7e). Protease activity in conidia recovered from the non-supplemented rice substrate was 2.2 U/mL for *B. pseudobassiana* SP-1. *M. brunneum* CA-3 produced lower chitinase activity with values of 1.3–2.0 U/mL; the highest protease activity of 2.0 U/mL was obtained by using rice-soy protein as a substrate.

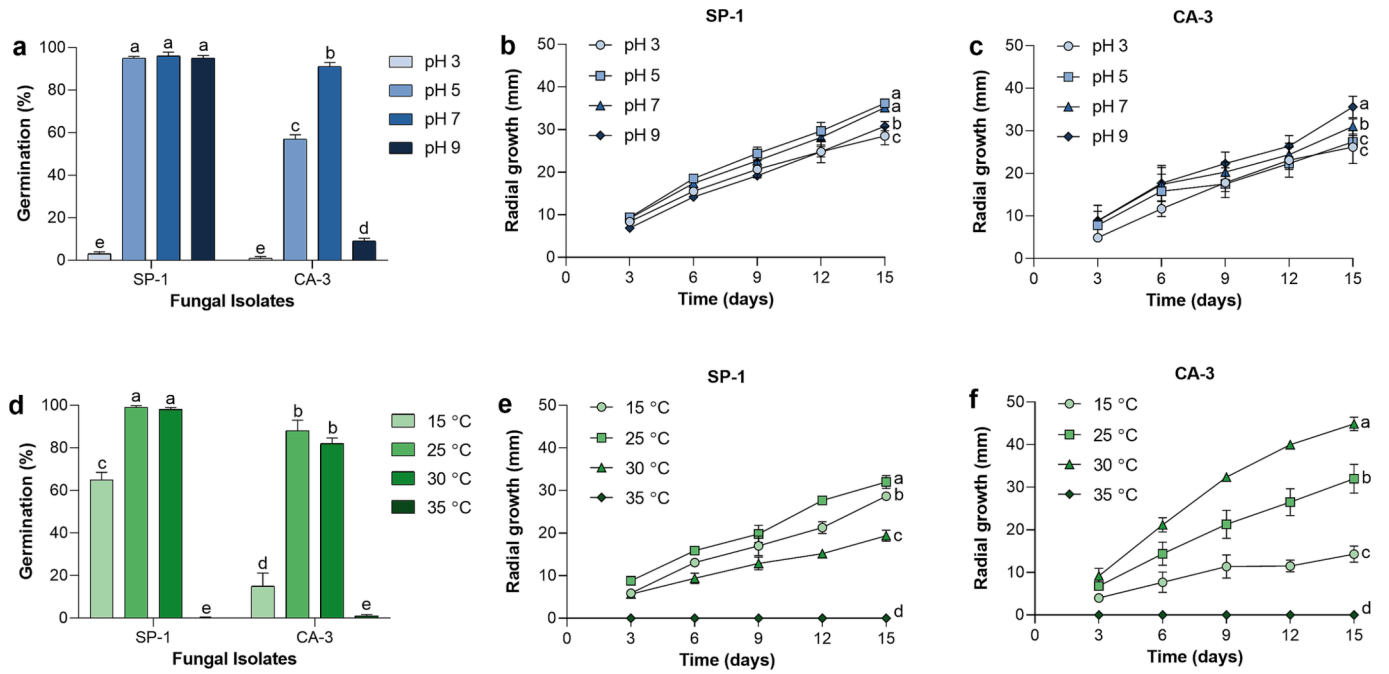

Fig. 6. Effect of pH and temperature on germination and radial growth of *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 on PDA. **a)** Germination (%) of *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 evaluated at 24 h of incubation; **b)** radial growth of *B. pseudobassiana*; and **c)** radial growth of *M. brunneum* on PDA with adjusted pH values and incubated at 25 °C. **d)** Germination (%) of *B. pseudobassiana* and *M. brunneum* after 24 h of incubation; **e)** radial growth of *B. pseudobassiana*; and **e)** radial growth of *M. brunneum* on PDA at different incubation temperatures. Mean values (±SD) followed by different letters are significantly different according to the Tukey test (95 %). The linear portion of the regression curves slope, drawn from the generated radial extension data, was used to estimate the growth rate.

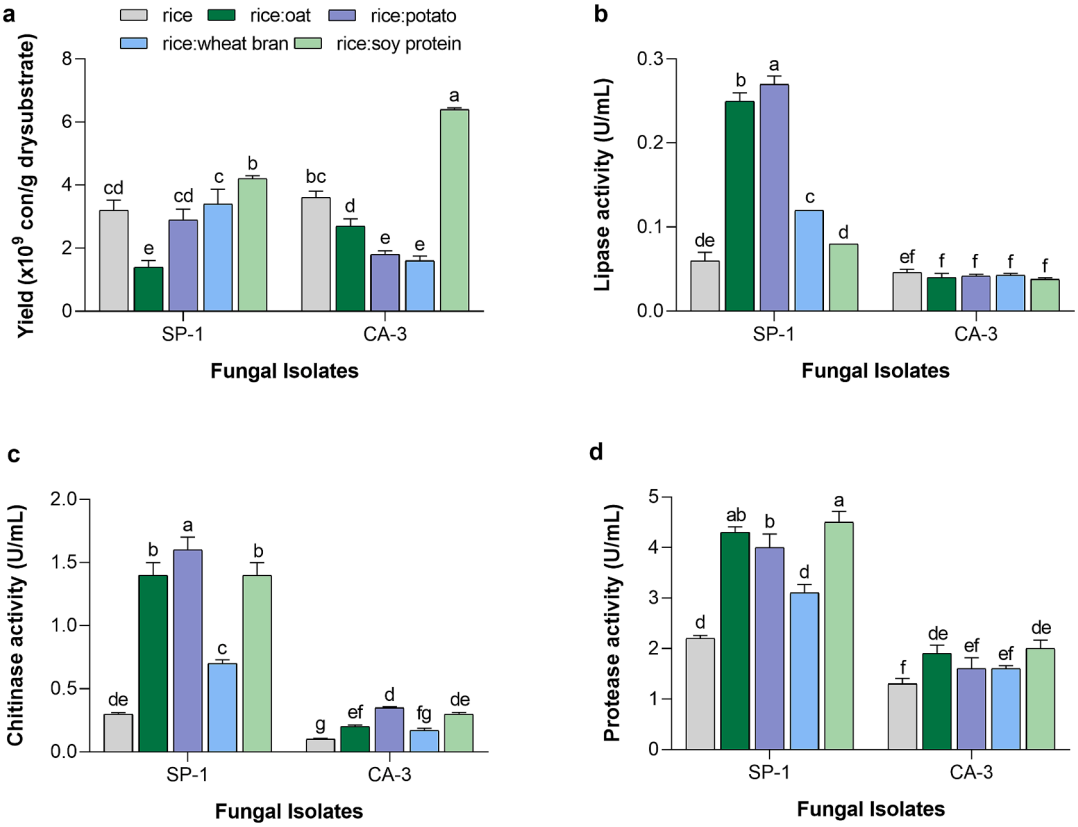

Fig. 7. Production of *B. pseudobassiana* SP-1 in semisolid-state fermentation and *M. brunneum* CA-3 solid-state fermentation. **a)** conidial yield, **b)** germination, **c)** lipase activity, **d)** chitinase activity, **e)** protease activity. Mean values (±SD) followed by different letters are significantly different according to the Tukey test (95 %).

Table 2
Germination of *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 conidia produced in semisolid-state and solid-state fermentation, respectively. Mean values (±SD) followed by different letters are significantly different according to the Tukey test (95 %).

Fungal isolates	Incubation time (h)	Germination (%)				
		Rice	Rice: Oat	Rice: Potato	Rice: Wheat bran	Rice:Soy protein
SP-1	16	94.6 ± 1.3	95.6 ± 1.4	96.1 ± 1.0	96.3 ± 1.6	97.4 ± 0.4
	24	94.8 ± 2.0	96.1 ± 1.0	95.6 ± 0.8	96.1 ± 0.6	95.7 ± 0.8
CA-3	16	4.3 ± 1.1	2.1 ± 1.4	3.5 ± 0.7	2.5 ± 1.1	6.6 ± 2.9
	24	20.1 ± 1.7	20.5 ± 3.8	21.8 ± 2.4	20.8 ± 0.9	24.3 ± 3.2
	48	73.4 ± 1.9	73.4 ± 2.8	75.4 ± 0.9	75.1 ± 4.5	74.9 ± 1.8

4. Discussion

The first report of *Gonipterus* in Latin America was recorded in 1925 (Marelli, 1926) and it was later reported in Europe in 1975 and the United States of America in 1994 (Jeger et al., 2018). The initial classification was *G. scutellatus* Gyllenhal, but molecular and morphological analysis carried out by Mapondera et al. (2012) showed that the described insect populations corresponded to a *G. scutellatus* species complex of cryptic species which were identified by genital-morphological analysis and molecular data. Among *G. scutellatus,* there is a high predominance of three species: *G. platensis* (Marelli, 1926) in America, Spain, and Portugal; *G. pulverulentus* (Lea, 1897) in Argentina, Brazil, and Uruguay (Nanini et al., 2022); and undescribed *Gonipterus* sp.2 in France and Italy. *G. platensis* is the member of the *G. scutellatus* complex with both the greatest presence in South America—first reported in Argentina (1925), Uruguay (1943), Brazil (1979), and Chile (1998)—and the largest distribution worldwide (Jeger et al., 2018). In Colombia, *Gonipterus* spp. were detected in 2016 in several localities within the Department of Antioquia and reported as *G. scutellatus* complex. Through this research, we have confirmed the presence of *G. platensis* in the studied areas by molecular methods, based on partial sequences of the cytochrome *c* oxidase subunit I gene. The *G. platensis* collections in this work showed sequences with low divergence, corroborating similar studies of *G. scutellatus* complex populations in Brazil, which have suggested low insect dispersal capacity and haplotype fixation (Nanini et al., 2022).

The EF we isolated represent a promising friendly strategy for controlling *G. platensis*. We found strains of *B. bassiana*, *B. pseudobassiana*, *M. brunneum* and *M. robertsii*. Fungal isolates from the genera *Beauveria*, *Metarhizium* and *Hirsutella* have previously been isolated from *Gonipterus* insects (Dara et al., 2019). Although they have the potential for use as entomopathogens, their virulence is strongly influenced by stimuli and stress conditions during fermentation, storage, and the infection process. In nature, variations of pH in the cuticle and hemolymph of the target insect and soil, temperature fluctuations, and UV radiation are the most detrimental factors (Abdul Qayyum et al., 2021; Quesada-Moraga et al., 2023). Given this context, the ecophysiological characterisation of isolates is a mandatory step to select EF and enhance growth, virulence, and adaptation to stress factors.

In this research, the *Beauveria* and *Metarhizium* isolates identified as highly lethal to *Gonipterus* adults were collected in localities where *Eucalyptus* spp. is planted, so the adaptation of these EF to this ecosystem is expected. These two fungal genera have been reported as successfully controlling *G. platensis* (Jordan et al., 2021). In this research, we obtained efficacies above 90 % using a conidial concentration of 1×10^7 con/mL on day 10, in contrast, Jordan et al. (2021) found *Beauveria* and *Metarhizium* isolates with mortalities of 5–95 % after 20 days post-spraying using a higher conidial suspension of 1×10^8 con/mL.

The selected isolates showed differences in UV-B tolerance over time and growth rates under pH and temperature ranges evaluated. Interestingly, the isolate SP-1 that strongly adapted to these stressors was recovered from the location with the highest altitude (2,582 m.a.s.l). Other isolates were recovered in localities ranging from 1,974 to 2,217 m.a.s.l. This suggests that the fungus may be adapted to the environment, representing a possible advantage when used in field conditions. Rather than providing new information, these results confirm previous research indicating that *B. bassiana* strains were more tolerant to UV-B radiation than *M. anisopliae* strains, confirming a variation in UV-B tolerance according to the species (Mustafa and Kaur, 2012; Rodrigues et al., 2016). UV-B tolerance could be influenced by habitat and latitude, as well as by age of inoculum, pH and nutrients of the culture media, and temperature. Different culture media have been reported to induce tolerance of *M. anisopliae* to this stressor and pH and temperature have also been reported to influence UV-B radiation tolerance (Fernandes et al., 2007; Rangel et al., 2015).

Overall, the optimal germination and growth temperature for the isolates *B. pseudobassiana* SP-1 and *M. brunneum* CA-3 was between 25 and 35 °C. *B. pseudobassiana* SP-1 presented higher germination than *M. brunneum* CA-3 at the lowest tested temperature of 15 °C, indicating differential responses of conidia germination and mycelium growth rate for each fungal genus. This is supported by previous characterisation showing that these structures greatly differ in internal nutrient reserves that could influence the environmental adaptation of EF as described for *B. bassiana* (Liu et al., 2015). Tefera and Pringle (2003) found a decrease in growth for *B. bassiana* and *M. anisopliae* at 15 °C and 35 °C, though germination was more temperature-tolerant than radial growth. In other research, an isolate of *M. acridum* presented the highest germination and growth at 32 °C in comparison to *B. bassiana* at 26 °C (Berlanga-Padilla and Hernández-Velázquez, 2002). These results could be evaluated to optimise the fermentation process to increase propagule production and improve environmental tolerance and adaptation (Barra-Bucarei,et al., 2016). Temperature has been shown to not only affect growth but also virulence, for example, Mishra et al. (2015) established that temperature affected *B. bassiana* virulence against *Musca domestica* with the highest mortality at 30 °C. Similarly, a temperature of 20–30 °C was shown to increase mortality of *Chilo partellus* to 100 % when studying *B. bassiana* and *M. anisopliae* (Tefera and Pringle, 2003). *B. bassiana* has also been shown to cause a higher mortality of *Schistocerca piceifrons piceifrons* at 26 °C, while *M. anisopliae* causes higher mortality at 32 °C (Berlanga-Padilla and Hernández-Velázquez, 2002). An isolate of *B. bassiana* was also found to be more virulent against *D. saccharalis* at a temperature of 26 °C (Svedese,et al., 2013a).

In this research, *B. pseudobassiana* SP-1 was capable of growth at pH 5–10 with germinations above 90 % which differed to that of *M. brunneum* CA-3 with germination greater than 90 % at pH 7. Similar results from previous studies show that *B. bassiana* isolates were able to tolerate pH values from 5 to 13 (Padmavathi et al., 2003). Other *B. bassiana* isolates grew at a pH ranging from 3 to 10 with germination above 90 % (Perfetti et al., 2007). Despite *M. brunneum* CA-3 germinating poorly at pH 9, it was still able to colonise the culture media under these conditions in our research. This behaviour was previously reported for *M. anisopliae* that produce oxalic acid to counteract alkaline pH and enable themselves to grow (St Leger et al., 1999). pH is also a determining factor in enzyme expression involved in the virulence of EF. For example, chitinases that cleave the chitin present in the cuticle of insects are produced in higher proportion at an acidic pH, while proteases and haemolymph are mostly expressed at alkaline pHs (St. Leger et al., 1998; St Leger et al., 1999).

The characterisation of the massive production of EF is pertinent to producing high-quality propagules with high virulence. Virulence is associated with the speed of germination, level of enzymatic activity, and tolerance to stress conditions (Butt et al., 2016; Wang et al., 2016)). In this context, the composition of the culture media can influence these

variables to improve the ecophysiological response of fungi and enhance virulence. In the present work, the use of different substrates in semi-solid and solid-state fermentation had a significant effect on the growth and enzymatic activity of fungi. A rice substrate supplemented with wheat bran and soy protein in both fermentation systems increased the growth of *M. brunneum* and *B. pseudobassiana.* These results suggest that nitrogen content is crucial to enhancing the fungi sporulation, as described previously (Safavi et al., 2007). Mejía et al. (2020) reported that *B. bassiana* presented higher sporulation of 2.4×10^9 con/g dry substrate based on an oat substrate with a carbon:nitrogen (C:N) ratio of 30:1, compared to sporulation of 5.5×10^8 con/g dry substrate based on a rice substrate with a C:N ratio of 43:1. This implies that an augmentation of nitrogen content could increase the sporulation of fungi. The germination of the two fungi evaluated in this research did not change significantly in all the substrates evaluated. Furthermore, the *M. brunneum* fungi presented low germination compared to *B. pseudobassiana.* This could indicate that the isolate *M. brunneum* could be affected during the drying process.

The induction of enzymes is crucial to enhancing the virulence of EF (Mondal et al., 2016) (Svedese et al., 2013b). In this research, the highest enzymatic activity was achieved with rice supplemented with substrates such as oat, potato, wheat bran, and soy protein, which may be related to the high levels of carbon and nitrogen (Dhar and Kaur, 2009). Although the isolate *B. pseudobassiana* had the lowest conidial production in semisolid-state fermentation, it produced high levels of enzymes compared to the other *Beauveria* isolates. These results demonstrate the importance of selecting appropriate substrates to obtain not only high levels of conidia, but also high enzymatic activity related to virulence.

Among the selected isolates, *B. pseudobassiana* was the most virulent, had the highest insecticidal efficacy over time, was the most UV-B tolerant, and had the highest enzymatic activity in a majority of the evaluated substrates. This screening and characterisation allow us to identify a highly virulent EF that could be used to control the *Gonipterus* insect pest.

At the moment, developing the identified fungi as control agents for the Eucalyptus snout beetle is the best available choice for controlling the pest in Colombia. Another option is the introduction and liberation of the insect's main natural enemy (*Anaphes* spp.), but the approach has serious legal restrictions and requires long-term research efforts. Additionally, *Anaphes* spp. do not always achieve the ideal control levels (Reis et al., 2012; Valente et al., 2018). Plant resistance and chemical control are other choices that could be used.

The use of the identified fungi may contribute to sustainable forestry when used in integrated pest management. These two genera of entomopathogenic fungi are the most widely used worldwide and have been evaluated on a large number of pest insects (Sullivan et al., 2022). In general, there are no representative side effects on non-target species, but these may occur in a variable manner, depending on the type of application, the concentration applied and the stage of development of the non-target insect (Dias et al., 2020; Portilla et al., 2017; Sayed et al., 2021). Once the parasitoids are introduced, the compatibility of both methods should be tested, with the advantage that *B. bassiana* is reported as compatible with *A. nitens* (Pérez et al., 2003).

We confirmed the presence of *G. platensis* in Colombia by molecular methods. Using this method will help to find new introductions of tine insect in the future. The characterised EF represent a promising strategy to control *G. platensis*. These results highlight the predominance of *Beauveria* and *Metarhizium* genera as natural biological control agents. From the found strains—*B. bassiana, B. pseudobassiana, M. brunneum* and *M. robertsii*—we selected *B. pseudobassiana* for posterior studies controlling *G. platensis*, such as the development and evaluation of formulations in field tests. *B. pseudobassiana* isolate SP-1 stood out for its UV-B tolerance, produced the highest enzymatic activities, and had the highest insecticidal efficacy over time. Future research should focus on the development and evaluation of the isolated fungi formulations for field tests.

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CRediT authorship contribution statement

Cindy Mejía: Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Gloria Barrera:** Formal analysis, Writing – review & editing. **John Alexander Pulgarín Díaz:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition, Supervision. **Carlos Espinel:** Formal analysis, Funding acquisition, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105407.

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