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Identification of native entomopathogenic fungi associated with *Mahanarva fimbriolata* Stahl in silvopastoral systems (*Urochloa brizantha* cv. MG-5 and *Eucalyptus* spp.)

H. Sarubbi[1*], G. Resquín-Romero[1] and I. Garrido-Jurado[2]

Abstract

Background Pastures are susceptible to attacks from various species of insects, with Cercopidae being one of the main responsible pests. The species *Mahanarva fimbriolata* has a wide distribution in the Eastern region of Paraguay, leading to pasture damage. The most promising alternatives to chemical use are bioinsecticides, which could offer effective control while minimizing negative environmental impacts. *Beauveria* and *Metarhizium* have been documented as biocontrol fungi for Cercopidae insects. Therefore, identifying and isolating virulent native strains presents potential alternatives for controlling the spittlebug *M. fimbriolata*.

Results Based on morphological and molecular characteristics, native strains of *Metarhizium anisopliae* and *Beauveria bassiana* were identified in the collected insects. Phylogenetic trees confirmed that the *Beauveria* sequence (*Beauveria* seq) obtained in this study aligned with the ARSEF 842 isolate of *B. bassiana*. The *Metarhizium* sequence (*Metarhizium* seq) was in the same clade as ARSEF 7450 and ARSEF 7487, which belong to the *Metarhizium anisopliae*. *Beauveria bassiana* displayed conidiophores that were broad at the basal part, forming synnemata or groups of conidiophores closely packed together, with a typical "zig-zag"-shaped rachis. The conidia were hyaline and smooth, ellipsoidal, and globose. On the other hand, *M. anisopliae* exhibited simple, straight conidiophores with bottle-shaped phialides. The conidia were elongated, ovoid to cylindrical, arranged in chains, and had an olive green color.

Conclusions This is the first report of *Metarhizium anisopliae* and *Beauveria bassiana* obtained from nymphs and adults of *Mahanarva fimbriolata* in Paraguay.

Keywords Biocontrol, Silvopastoral, *Mahanarva*, Entomopatogenic fungus, *Beauveria bassiana*, *Metarhizium anisopliae*

Background

Paraguay has an area of 15 million hectares dedicated to livestock production, with cultivated and natural pastures forming the primary basis for cattle feed. This is the most economical and practical method for meat production (Sarubbi and Ramírez 2020). Cercopidae, commonly known as pasture spittlebugs, are insects widely distributed in tropical regions and have the potential to cause severe damage on grasses (Valerio y Oliveira 2005). *Mahanarva fimbriolata* is one of the most significant species, with a broad distribution in the Eastern

*Correspondence:
H. Sarubbi
humberto.sarubbi@agr.una.py
[1] Faculty of Agricultural Sciences, National University of Asunción (FCA-UNA), San Lorenzo, Paraguay
[2] Agricultural Entomology, Department of Agronomy, Excellence Unit María de Maeztu (DAUCO), ETSIAM, University of Cordoba, Campus de Rabanales. C4 Building "Celestino Mutis", 14071 Córdoba, Spain

region of Paraguay, causing pasture damage (Sarubbi and Ramírez 2020). The nymphs feed on the roots by inserting their stylets into the xylem vessels, siphoning sap, and obstructing vascular vessels, impeding the transport of nutrients and water. This weakens the plant and induces physiological disorders (Grisoto et al. 2014). The foam produced by the nymphs hosts various bacteria, including Proteobacteria, Acidobacteria, and Actinobacteria (Tonelli et al. 2020), which shield the nymphs from entomopathogenic microorganisms due to the production of secondary metabolites with antibiotic properties (Zucchi et al. 2012). Controlling Cercopidae insects presents significant challenges. The use of chemical insecticides is restricted due to cost and environmental contamination concerns. One of the most promising alternatives is the use of bioinsecticides, which not only have a low environmental impact but can also offer more efficient control. Fungi from the genera *Beauveria* and *Metarhizium* have been reported as bio-controllers of cercopids. Therefore, identifying and isolating virulent native strains represent potential alternatives for controlling arthropods, such as the spittlebug *M. fimbriolata* (Cruz and Cajilema 2012).

Hence, the objective of this study was to identify and characterize potential native entomopathogens in silvopastoral systems (*Urochloa brizantha* MG-5 and Eucalyptus spp.).

Methods

Collection and maintenance of samples

Sampling was conducted in a silvopastoral field featuring *Urochloa brizantha* cv. MG-5, situated in Rancho 68, Department of San Pedro, within the Santa Rosa del Aguaray district (S23° 43′ 39′′; W56° 27′ 17′′). Thirty sampling points were established within a one-hectare silvopastoral cultivation area. Specimens of *Mahanarva fimbriolata* (Hemiptera: Cercopidae), including both nymphs and adults, were manually collected during the months of February and March 2019 (Fig. 1).

The insects were photographed, placed in plastic containers, and transported in coolers to the Entomology Laboratory for the identification and morphological characterization of the entomopathogenic fungi (EF) present. EF found in the infected insects were isolated using standard isolation procedures. Pure isolates were obtained in Petri dishes with nutrient medium (Sabouraud Dextrose Agar European Pharmacopoeia) with the addition of streptomycin sulfate (Sigma-Aldrich, China). They were then incubated at 28 °C in dark conditions for eight days. To preserve the fungi, routine transfers were made to new plates with PDA + antibiotic nutrient medium. Paraffin oil was used for preserving sterile cultures, covering 1 cm above the PDA medium in test tubes and sealing them hermetically. These tubes were stored at − 4 °C. The fungi were examined with the assistance of Zeiss stereomicroscope and Zeiss ICC50 optical microscope, and digital images were obtained. An average of 30 conidia and conidiophores of each species were analyzed, and with the help of the LAS EZ image processing software (Zeiss), measurements of length and width were taken.

Molecular identification was carried out in the Applied Molecular Biology laboratory of the Biological Institute of São Paulo, SP, CEP 04014-002. DNA extraction of the isolates followed the method using the CTAB reagent (hexadecyltrimethylammonium bromide) as described by Rawat et al. (2016). Mycelium from the fungus, grown in potato-dextrose medium, was ground in microtubes. Genomic DNA underwent a polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) region located between the genes encoding 18S and 28S ribosomal RNAs, as well as the segment of the gene encoding translation elongation factor 1α (EF1α) and the beta-tubulin gene. The oligonucleotide primers for the ITS region were ITS1 (5′-TCCGTAGGTGAACCTGCG G-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Chanu et al. 2022). PCR was conducted using primers at a final concentration of 0.2 μM, dNTPS at 0.2 mM, 1U

Fig. 1 Foam produced by nymphs and macromorphology of *Mahanarva fimbriolata* (Hemiptera: Cercopidae) nymphal and adult stages under field conditions

of GoTaq Green enzyme (Promega), in a final volume of 50 µl. The program used for ITS and EF was as follows: initial denaturation at 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 4 min. The verification of the amplified products was performed through electrophoresis in a 0.8% agarose gel stained with ethidium bromide. The amplified products were purified by precipitation with polyethylene glycol (Arbeli and Fuentes 2007), subjected to the sequencing reaction using the chain termination method with the Bigdye 3.1 reagent (Applied Biosystems), and analyzed on an automatic capillary sequencer 3500 XL (Applied Biosystems). The sequences obtained were compared to sequences of specimens identified by specialists deposited at the NCBI (National Center for Biotechnology Information—www.ncbi.nlm.nih.gov) using the Blast tool. For the phylogenetic analysis, the sequence was compared to sequences of 11 *Beauveria* spp., 8 *Metarhizium* spp., and one *Cordyceps militaris* (L.) Fr. strain used as an outgroup. The sequence data from all 11 *Beauveria* and 8 *Metarhizium* strains, along with the *Beauveria* and *Metarhizium* sequences obtained in this study, were aligned using the MegAlign program (DNASTAR package, London, UK). The phylogenetic analysis was conducted using the MEGA 11 program (Tamura et al. 2021). Phylogenetic trees were constructed using the neighbor-joining method, and the bootstrap consensus tree was generated from 1000 replicates. Evolutionary distances were calculated using the Poisson correction method, and positions with gaps and missing data were removed from the dataset.

Results

Based on the morphological and molecular characteristics, the native EF *Metarhizium anisopliae* and *Beauveria bassiana* were identified in the collected insects (Figs. 2 and 3).

Fig. 2 Macromorphology of the fungal colonization of *Metarhizium anisopliae* on *Mahanarva fimbriolata* (Hemiptera: Cercopidae) nymphal and adult stages under field conditions in situ

Fig. 3 Macromorphology of the fungal colonization of *Beauveria bassiana* on *Mahanarva fimbriolata* (Hemiptera: Cercopidae) nymphs, under field conditions in situ

Generic description

Metarhizium anisopliae (Metsch.) Sorok

The fungus grew on insects, both nymphs and adults, exhibiting a dark green mycelium. It developed simple conidiophores that were straight and featured bottle-shaped phialides. The conidia were elongated, ovoid to cylindrical, arranged in parallel compact chains, and had an olive green color. They measured approximately 5–6 µ in length and 2 µ in width. Initially, the fungus had white mycelium on the Sabouraud medium, but it later transitioned to a dark green color, forming conidia after 15 days at 25 °C and under a 12-h photoperiod (Fig. 4).

Beauveria bassiana (Bals.-Criv.)Vuill.

The fungus infected both nymphs and adults, resulting in a white mycelial growth on the insects. Microscopic observation revealed that the conidiophores were densely clustered, forming synnemas or groups of conidiophores very close together. They were broad at the basal part and featured a "zig-zag"-shaped rachis from which the spores developed. The conidia were hyaline, smooth, and ellipsoidal-globose, measuring approximately 2.5–2.6 µ in length and 2.2–2.1 µ in width. Initially, the fungus exhibited white mycelium in the Sabouraud medium, later forming conidia after 20 days at 25 °C under a 12-h photoperiod (Fig. 4).

Molecular identification of *Beauveria* and *Metarhizium* strains

Two *Beauveria* and one *Metarhizium* isolates were molecularly identified. The two *Beauveria* isolates showed the same sequence when were identified with both ITS and EF primers. The phylogenetic trees confirmed that the *Beauveria* sequence (*Beauveria* seq) obtained in this work was aligned with the ARSEF 842 isolate of *B. bassiana* and in the same clade of the rest strains from this species (Fig. 5). On the other hand, the *Metarhizium* sequence (*Metarhizium* seq) obtained in the present work was in the same clade that the ARSEF 7450 and ARSEF 7487 of *Metarhizium anisopliae* species (Fig. 6).

Discussion

There are several worldwide reports on the use of entomopathogenic fungi (EF), particularly of the genus *Metarhizium*, for the control of *M. fimbriolata*, such as *M. anisopliae* and *M. anisopliae* var. *anisopliae*. However, these have shown limited control (Thiago et al. 2011). In

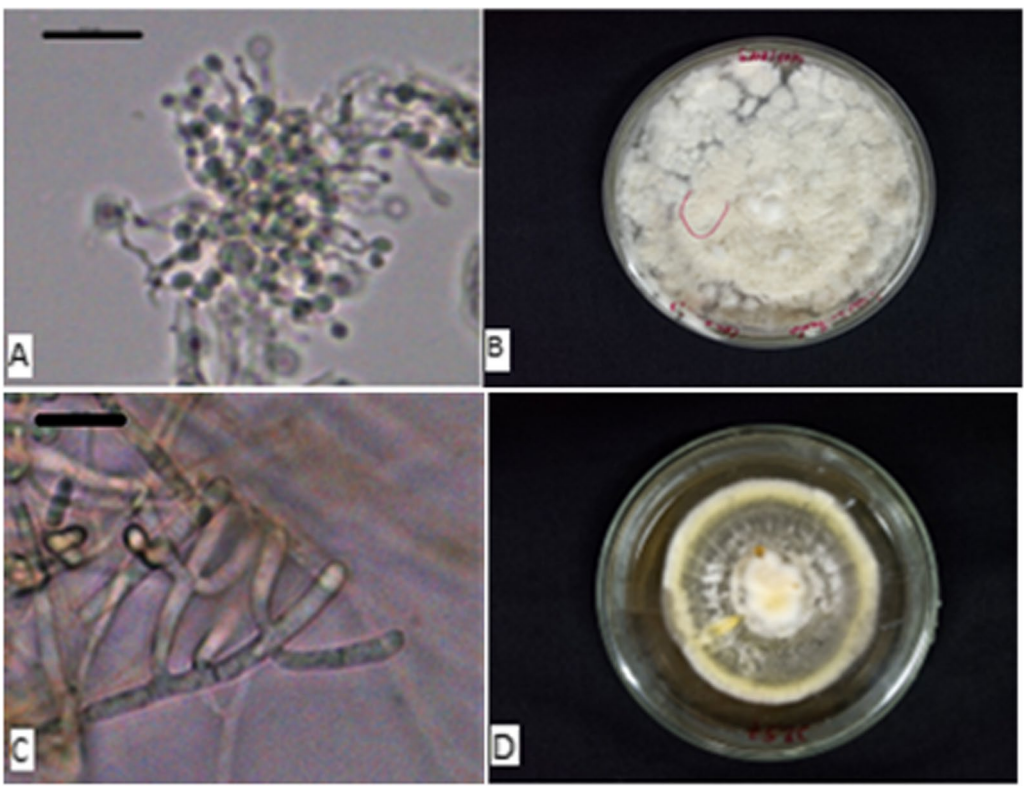

Fig. 4 **A**. *Beauveria bassiana,* conidiophores and conidia **B**. *B. bassiana* in Sabouraud dextrose agar medium **C** *Metarhizium anisopliae*, conidiophores **D** *M. anisopliae* in Sabouraud dextrose agar medium. Bar: A = 20 µ, C = 10 µ

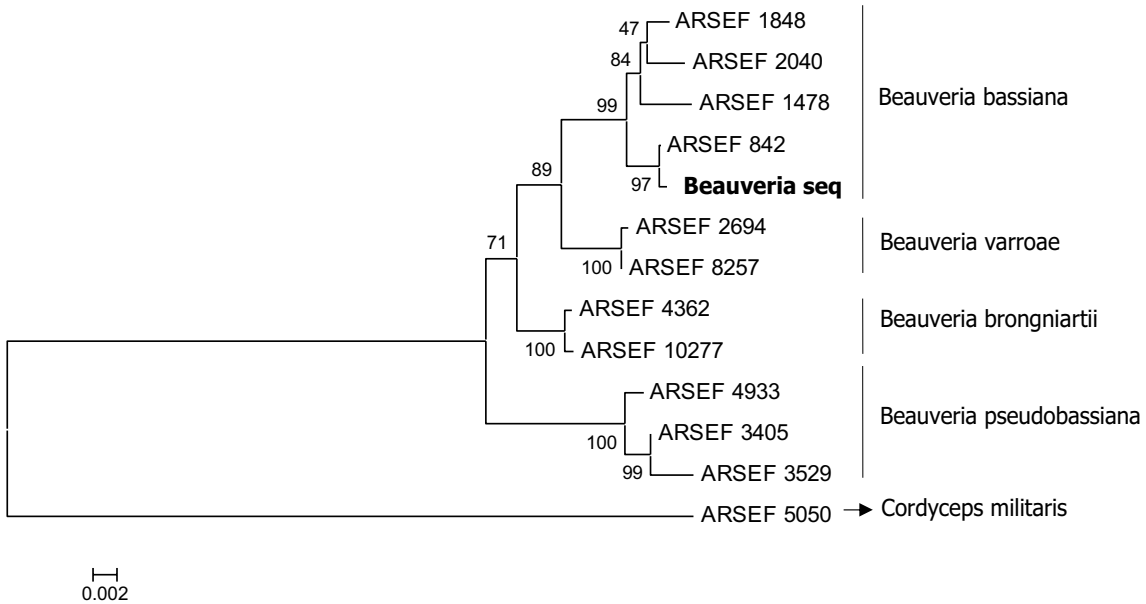

Fig. 5 Phylogenetic tree of the ITS and EF1α sequences from the *Beauveria bassiana* isolated and 11 reference strains retrieved from NCBI database based on the neighbor-joining method. The numbers at nodes show the level of bootstrap support based on data for 1000 replications

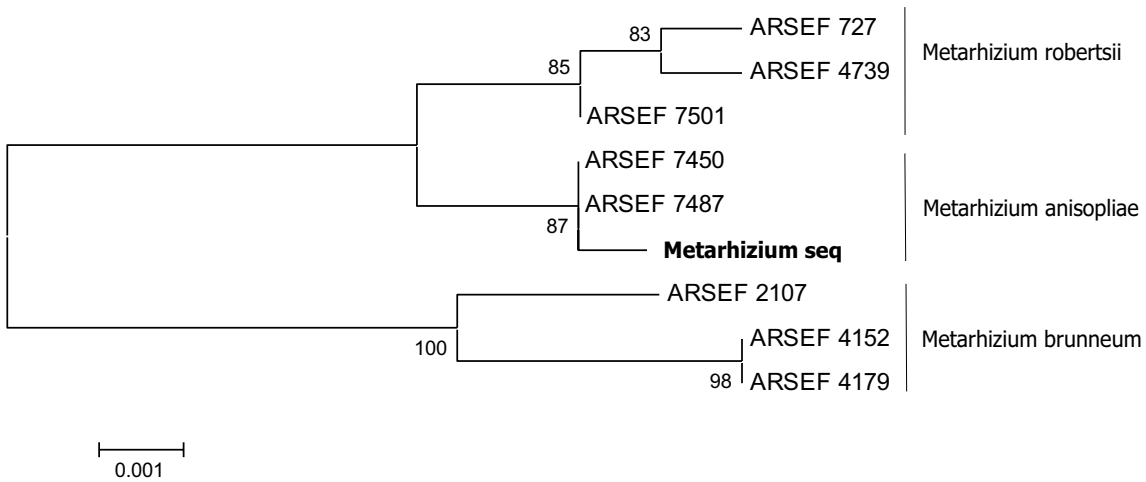

Fig. 6 Phylogenetic tree of the EF1α sequences from the *Metarhizium anisopliae* isolated and 8 reference strains retrieved from NCBI database based on the neighbor-joining method. The numbers at nodes show the level of bootstrap support based on data for 1000 replications

contrast, this study observed a substantial number of cercopidae insects with EF infections among both adults and nymphs in their natural habitat. Additionally, the effects of EF infection were noted on other insects, including cutter ants of the *Atta sexdens rubropilosa* and *Atta laevigata* species.

Throughout the research period, frequent rains were observed, and it was noted that the shading provided by the trees, along with the microenvironment created by the foliage of the pastures, resulted in a microclimate with a cooler temperature (approximately 27 °C) and high humidity (over 70%). This microclimate differed from pastures exposed to direct sunlight. This environment was highly conducive to insect reproduction, resulting in many nymphs with their characteristic foam at the base of the plant stem, as well as numerous adult insects. Another favorable factor for the insects was the excess of stubble at ground level within the pasture due to the lack of grazing by animals. Koller

and Valerio (1988) have indicated that this can lead to a significant increase in spittlebug nymphs.

The presence of grass stubble, combined with the shade of the trees, allowed the nymphs to avoid the dehydrating effects of direct sunlight, which can be lethal to them. Regarding the pasture (*U. brizantha* cv. MG-5), it was previously classified as resistant to common spittlebug species such as *Notozulia entreriana*, but recent research has shown it to be a susceptible host to *M. fimbriolata* attacks (Grisoto et al. 2014). With the highest incidence of these cercopids, an extensive epizootic event was observed in these field conditions, a situation similar to what Leger (2008) reported, stating that *M. anisopliae* can remain in pasture soil for years with infective capacity. It is evident that the favorable temperatures and high relative humidity favored fungal growth over the insects.

In studies on the development of *B. bassiana* and *M. anisopliae*, it has been found that temperatures ranging from 25 to 32 °C and humidity exceeding 75% are optimal conditions for their growth (Lanza et al. 2009). Conidia need high humidity (around 70% for 14 h) to germinate, hydrate, and form germ tubes, which then grow on the surface of the insect to locate receptor sites and initiate penetration of the cuticle (Dillon and Charnley 1990).

The climatic conditions recorded during this investigation align with the ideal temperature and humidity levels for the development of EF, underscoring that a silvopastoral field provides a unique environment for effective biocontrol of spittlebugs. From a biological control perspective, the native EF strains identified in this study are highly relevant for the development of a biological insecticide, as their control effectiveness is directly linked to the local temperature and relative humidity (Quedraogo et al. 1997). Gebremariam et al. (2021) argue that selecting isolates adapted to the local climate presents the best opportunities for control. However, some studies suggest that combining commercial products with native strains may still yield superior control of cercopidae insects in the field (Iwanicki et al. 2019).

B. bassiana and *M. anisopliae* isolates could be developed as a potential biocontrol agent against spittlebugs in integrated pest management (IPM) programs within silvopastoral systems.

Conclusion

The identification of *Metarhizium anisopliae* and *Beauveria bassiana*, obtained from nymphs and adults of *Mahanarva fimbriolata*, is reported for the first time in Paraguay.

Abbreviations

ITS Internal transcribed spacer
EF Entomopathogenic fungi

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Author contributions

HS made contributions to the conception, design of the work; the acquisition, analysis, interpretation of data and have drafted the work. GR-R made contributions to the conception and design of the work; the acquisition, analysis, interpretation of data and substantively revised it. IG-J performed the interpretation of data and substantively revised it.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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