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First report on the natural occurrence of entomopathogenic fungi in populations of the leafhopper *Dalbulus maidis* (Hemiptera: Cicadellidae): Pathogen identifications and their incidence in maize crops

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ABSTRACT

The corn leafhopper *Dalbulus maidis* is one of the most important pests of maize in Latin America. Here we report, for the first time, the natural occurrence of two fungal species infecting the adult stage of this pest. In 2020, insects killed by a pale bluish green fungus in irrigated maize fields located in Northeast Brazil were found attached to the abaxial surface of leaves. Using morphological characters and multigenic phylogeny, it was identified as *Metarhizium brasiliense*. In the beginning of 2021, the same pathogen was seen on adults in a maize field in the Central-Western region, alongside an entomophthoralean fungus during an epizootic. The latter pathogen was molecularly identified as a species in the genus *Batkoa*. The number of *Batkoa*-infected leafhoppers, displaying the typical swollen abdomen and extended wings, reached an average of 1.88 per maize leaf (86.42% of the sampled adults). The incidence of *M. brasiliense* was higher in plots in the Northeastern region (0.22 and 0.53 adult per leaf) when compared to the Central-Western region (0.04 adult per leaf). The report of *D. maidis* adults infected by *M. brasiliense* in agricultural settings located in different geographic regions and over 550 km apart indicates probable widespread occurrence of this pathogen in Brazil. Moreover, this opens the possibility of more applied biological control studies and, perhaps, the development of new tools to manage *D. maidis* populations.

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1. Introduction

The corn leafhopper *Dalbulus maidis* (Hemiptera: Cicadellidae) is found in Latin America and some regions of the USA closely associated with maize (Santana Jr. et al., 2019; Summers et al., 2004); however, it may survive in other plants preferably in the same botanical family (Oliveira et al., 2020), and even reproduce in a few unrelated non-hosts infected with mollicute plant pathogens (Purcell, 1988; Sugio et al., 2011). *D. maidis* is capable of efficiently transmitting the causal agents of maize stunting diseases [*Spiroplasma kunkelli* (CSS) and the maize bushy stunt phytoplasma (MBSP)] and the Maize rayado fino virus (MRFV) (Nault, 1980; Oliveira et al., 2011). Maize stunting diseases are responsible for large economic losses, which may reduce 70% of grain production (Giménez-Pecci et al., 2002; Massola Jr. et al., 1999). Over the last five years, these diseases have been considered major phytosanitary issues in Brazil (Oliveira et al., 2020). The main control measure for *D. maidis* populations has been the routine use of systemic insecticides in the early stages of the crop. However, the environmental and safety hazards associated to this practice have increased the interest in the development of alternative control measures (Meneses et al., 2016, Santana Jr. et al., 2019).

A number of invertebrate-pathogenic fungi have been widely commercialised and used to control agricultural pests worldwide (Faria and Wraight, 2007; van Lenteren et al., 2018). Some fungal species within the taxa *Metarhizuim* and *Entomophthorales* have

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known associations with hemipterans in Auchenorrhyncha. For example, the generalist *Metarhizium anisopliae* has been annually sprayed on enormous acreages of sugarcane and pastures against spittlebugs (Cercopidae) (Toledo et al., 2008a; Li et al., 2010; Mascarin et al., 2019). Other *Metarhizium* species outside the *M. anisopliae* complex are adapted to specific hosts (Hu et al., 2014; Zhang et al., 2019), which include hemipteran-specific pathogens. For instance, *Metarhizium album*, first described by Petch (1931), was found closely associated with leafhoppers (Cicadellidae) in Asia, causing epizootics in field populations (Rombach et al., 1987). More recently, a phylogenetic study showed species of *Metarhizium* grouping in two distinct clades, one of them with typical *Metarhizium*-like morphology of the phialides and specific to small planthoppers, members of Delphacidae family (Mongkolsamrit et al., 2020). This clade contains mainly species originally isolated from leafhoppers, including *M. album* and *Metarhizium brasiliense*; the latter was originally isolated in Southeastern Brazil in the 1980s from an unidentified Cicadellidae (Kepler et al., 2014).

Entomophthoralean species have also been found naturally infecting and causing epizootics in different small hopper species. In South America, the genera *Pandora* and *Conidiobulus* were already reported occurring enzootically on adults of planthoppers associated with rice crops (Toledo et al., 2008b). Infections of spittlebugs by *Pandora* in Argentina (Foieri et al., 2018) and *Batkoa* and *Furia* in Brazil (Leite et al., 2002) were also reported in pastures. Similarly, cicadelids may also succumb to infections caused by this group of fungi. For instance, the occurrence of *Zoophthora* in populations of leafhopper has been described in several countries (Ben-Ze'ev and Kenneth, 1981; Galaini-Wraight et al., 1991; Mazzoglio et al., 2009).

Despite of the importance of *D. maidis* in maize production, there is a lack of studies on fungi associated to this pest. Species in the *M. anisopliae* complex can infect the corn leafhopper under laboratory conditions (Ibarra-Aparicio et al., 2005; Iwanicki et al., 2020), but naturally occurring infections in the field have not been reported. In surveys conducted by our team in 2020 and 2021, a number of dead *D. maidis* adults were found attached to maize leaves and colonised by two different fungi. Microscopic evaluations revealed that one of the pathogens belongs to the genus *Metarhizium*, with a candelabrum-like arrangement of phialides and conidia aggregated into prismatic columns. Cadavers colonized by the other fungus had swollen abdomen with fungal outgrowth from intersegmental membranes and extended wings, typical of an entomophthoralean infection. In the present study, we measured the incidence of both fungi in populations of *D. maidis* adults in maize fields located in two different regions in Brazil, and determined their identity through phylogenetic analyses.

2. Materials and methods

2.1. Incidence of entomopathogenic fungi in populations of D. maidis in maize fields

Dead *D. maidis* adults displaying evidence of fungal infection and attached to maize leaves were first collected in May 2020 from irrigated maize fields located in the municipality of Barreiras, Bahia, Northeastern Brazil (Field 1; S −12.1572 W −45.4538). The average temperature and relative humidity of the air were recorded daily by a weather station located 26.3 km from the field site. Mummified cadavers were also collected in a second location in January 2021 from a non-irrigated experimental area containing maize in Brasília, Federal District, Central-Western region (Field 2; S −15.5963; W −47.7178), approx. 550 km away from Field 1. The average temperature and relative humidity in Field 2 were also recorded by a weather station located 0.5 km from the site.

A survey to evaluate the disease incidence in adult and nymph populations of *D. maidis* was conducted in both maize fields. In Field 1, two areas (115 ha each) with non-*Bt* maize hybrid IPV 2122 cultivated for seed production and distant from each other by at least 1.2 km were surveyed. Applications of chemical fungicides were conducted as needed. Field 2 consisted of a single area (1.25 ha) with Bt maize hybrid Syn488Vip3, and chemical pesticides were not sprayed. Between 120 and 200 leaves, one per plant, were randomly verified in each plot for the presence of fungus-killed *D. maidis* attached to the abaxial side of fully expanded leaves between 80 and 120 cm above the ground. Plants were in their reproductive stage, at around 90 days after seedling emergence. Fungal incidence in each field was calculated as the average number of cadavers per leaf showing signs of infection. In addition, the proportion of fungus-killed and living adults per leaf was estimated in Field 2 and individual maize plants (n = 18) was also verified from 20 cm above the ground level up to the top of the plant for the presence of fungus-killed adults.

2.2. Fungal morphology and growth characteristics

The morphology of fungal microstructures (rhizoids, conidiogenous cells, and spores) from infected insects were examined by brightfield or phase contrast microscopy (Nikon Eclipse Ci, Nikon Corporation, Tokyo, Japan). Spores (n = 15) were measured directly from microphotographs using the software NIS-Elements BR Analysis (Nikon Corporation, Tokyo, Japan). *Metarhizium*-like structures were isolated by transferring conidia from sporulated cadavers directly onto quarter-strength Sabouraud dextrose yeast agar medium (SDYA ¼) plus streptomycin (0.5 g L^{-1}). Plates were incubated for up to 15 days at 26 ± 0.5 °C and 12 h photophase. Morphological traits of purified colonies cultivated on potato dextrose agar (PDA) were also recorded. Two fungal strains, coded as CG1446 and CG1447, were isolated in Field 1, whereas a strain coded as CG1450 was collected in Field 2. All three *Metarhizium* isolates were preserved in liquid nitrogen in the Invertebrate-Associated Fungal Collection (CFI), at EMBRAPA Genetic Resources and Biotechnology (Brasilia, DF, Brazil). For the entomophthoralean fungus, coded as CG1454, the DNA was extracted and purified directly from fungal mass from infected cadavers, and then maintained in the culture collection at −80 °C.

Additionally, growth characteristics in liquid culture and on solid substrate were evaluated for isolate CG1447. In the first case, 250-mL Erlenmeyer flasks containing 150 mL of liquid SDY were inoculated with ca. 1 mg of conidia scraped from 15-day old colonies on PDA and placed in an orbital shaker at 26 ± 0.5 °C and 250 rpm for five days. Mycelium was harvested on filter paper by vacuum filtration and air-dried at room temperature (22–24 °C, ca. 30% RH) overnight. Dry mycelium samples (ca. 0.04 g) were then placed on glass coverslips in a moistened chamber at 26 ± 0.5 °C and complete darkness for four days to promote conidiation. In the second case, 150 g of autoclaved parboiled rice loaded into polypropylene bags (15 × 20 cm) was inoculated with 10 mL of a conidial suspension (1×10^7 conidia.mL^{-1}) and incubated in the dark for 12 days at 26 ± 0.5 °C. Fungus-colonized rice was then predried at room temperature for 48 h to reach ca. 14% moisture content, and after this period, samples (1.5 g) were harvested. Conidia were harvested from four independent samples of dry mycelium and colonized rice grains by vortexing the material in the surfactant Tween® 80 at 0.05%. The number of conidia per gram of substrate was determined with the aid of a Neubauer chamber. Additionally, the number of *Metarhizium* conidia produced on colonized *D. maidis* was estimated by vortexing four individual cadavers found in Field 2, and previously kept within a moisture

chamber for five days, in 1 mL of Tween 0.05%. The number of conidia per cadaver was also estimate using a Neubauer chamber.

2.3. Molecular identification

Molecular identification of fungal isolates was performed by direct comparison with key genomic sequences. For *Metarhizium*, strains were grown in 150 mL liquid SDY for five days in an orbital shaker at 125 rpm and 25 ± 1 °C. Mycelium was harvested and ground into a powder in liquid nitrogen using a mortar, and DNA extraction was performed following the method described by Raeder and Broda (1985). Partial sequences of the genes LSU, SSU, rpb1, rpb2, and 3′tef were amplified and sequenced (Mongkolsamrit et al., 2020). For the entomophthoralean species, which could not be cultured on artificial media, the fungal mass was previously separated from colonized cadavers using syringe needles under a stereoscope, and DNA was extracted using an extraction kit (PureLink Genomic DNA Mini Kit, Invitrogen™). In this case, identification was performed using only the partial sequence of the gene LSU (Gryganskyi et al., 2013).

Multiple sequence alignments were assembled and compared to reference sequences selected from GenBank (Table 1). Analysis of the alignment was carried out under the Maximum Likelihood criterion using the software W-IQTree (Trifinopoulos et al., 2016), and bootstrap support (ML) values were provided. Additionally, we used Bayesian phylogenetic inference by MrBayes v. 3.2.1 (Ronquist et al., 2012), and bootstrap support (BS) values were included in the ML tree.

3. Results

3.1. Incidence of entomopathogenic fungi in populations of D. maidis in maize crops

In Field 1 (Northeastern region), the average number of adults per leaf killed by *Metarhizium* reached averages of 0.22 ± 0.02 in the first area and 0.53 ± 0.15 in the second area. This was considerably higher than the average observed in Field 2 (Central-Western region), with 0.042 infected adults per leaf, which corresponded to only 1.87 ± 0.28% of the adult population. In both fields, feeding stages of *D. maidis* on leaves were represented mostly by nymphs, which were not found infected by fungi.

The entomophthoralean fungus was not detected in Field 1, but it was found causing epizootic in a *D. maidis* population in Field 2. Only adults were seen infected by this fungus and its incidence reached an average of 1.88 ± 0.26 cadavers per maize leaf, corresponding to 86.42 ± 3.04% of the adult population. Cadavers were found within plant canopy around 40 cm above the ground and near upper leaves, and only 2.71% of infected insects were found on leaves near the ground. The average number of *Entomophthorales*-infected adults per maize plant was 12.28 ± 1.90.

In Field 1, daily average temperatures in May 2020 showed little variation, between 19.2 and 24.9 °C, but temperatures usually varied 13.6 °C within the same day, from almost 30 °C in the afternoons to less than 17 °C at night. No rainfall was recorded during this same period, and the average relative humidity recorded was 75.1% (63.2–90.3%). Actual RH levels were probably higher since the field was irrigated twice a week during the experimental period, and presence of dew on plants was usually evident until 9 am. In Field 2, average temperatures in January 2021 were similar to Field 1, varying from 17.3 to 27.7 °C. Differently, rainfall was persistent in Field 2 (total of 339.64 mm), keeping the average relative humidity at 82.7% (67.2–94.7%).

3.2. Fungal morphology and growth characteristics

Leafhopper adults killed by *Metarhizium* sp. on maize leaves were found covered with pale blue-green conidia in both fields (Fig. 1A). All three isolates had a typical *Metarhizium*-like morphology of the phialides and conidia with two different sizes (Fig. 1B). Short conidia varied from 5.6 to 8.9 μm length × 2.4–3.2 μm width and long conidia from 11.2 to 16.8 μm length × 3.2–4.0 μm width, in accordance with previously reported measurements by Kepler et al. (2014). Colonies of *Metarhizium* were white at initial growth on PDA, becoming dark blue-green after producing conidia (Fig. 1C). In addition, *Spodoptera frugiperda* larvae killed by *Metarhizium rileyi* were also recorded in Field 1.

In Field 2, *Entomophthorales*-infected insects showed the typical swollen abdomen and extended wings of infections caused by this fungus (Fig. 1D). Primary spores (Fig. 1E) discharged from cadavers (24.1–28.6 μm in diameter) were frequently seen on the insect extended wings and on the leaf surface around the body. Cadavers were attached to maize leaves by thick rhizoids with discoid terminal holdfasts (Fig. 1F). Morphological characteristics of this isolate matched those reported by Ben-Ze'ev and Kenneth (1982) and Humber (1989) for the genus *Batkoa*.

Isolate CG1447, assessed in the production assays, aggregated in hyphal pellets (3–4 mm) when cultivated in SDY, which after filtration and air-drying formed a multi-pellet crust with a gelatinous matrix (mycelium fragments). An average of $7.4 \pm 0.43 \times 10^9$ conidia was produced per gram of mycelium fragments after five days under high humidity conditions, and 14.8 ± 2.16% of the conidia were in the long-size category. On rice, conidiation started seven days after inoculation and reached an average of $1.5 \pm 0.07 \times 10^9$ conidia per gram of substrate after 15 days, with 30.0 ± 1.63% of them in the long-size category. The average number of conidia produced on *D. maidis* was $9.7 \pm 1.25 \times 10^6$ conidia per cadaver, all of them falling in the small-size category.

3.3. Molecular identification

For the *Metarhizium* strains, the alignment of the characters obtained from partial sequencing of five loci comprised 4072 base pairs. The phylogenetic analysis of the combined partial LSU, SSU, rpb1, rpb2, and 3′tef gene dataset clearly placed all three isolates within a group which comprises the *Metarhizium* species that has hemipterans (cicadas and small hoppers) as hosts (Mongkolsamrit et al., 2020). Isolates CG1446, CG1447, and CG1450 were identified as *M. brasiliense*, since bootstrap values of 100% (MP) and 1 (Bayesian posterior probability) were obtained for the branch in which the type species of *M. brasiliense* (ARSEF 2948) is placed (Fig. 2). The similarity between the *M. brasiliense* strains collected in Northeastern (Field 1) and Central-Western (Field 2) regions was very high (99.7–100%). The entomophthoralean isolate found causing epizootics in Field 2 was identified as *Batkoa* based on the analysis of the LSU gene (1145 base pairs). The isolate clearly clustered with other species in this genus (Fig. 3) and is closely related (97.8% of similarity) to isolate ARSEF 328 of *Batkoa* sp.

4. Discussion

Based on phylogenetic analyses, we identified *M. brasiliense* and *Batkoa* sp. naturally infecting the corn leafhopper *D. maidis* in maize fields, which is the first report of microorganisms causing diseases in this important pest. We provided here description of some morphological traits of both fungi and information on the incidence of *M. brasiliense* in two different regions of the country. We also added new ecological traits of *M. brasiliense*, which are missing in

Table 1
Strains of *Metarhizium* spp. and Entomophthoraceae used in the phylogenetic analysis.

Species	Strain code[a]	Host	Origin	Accession codes				
				SSU	LSU	3′TEF	rpb1	rpb2
Strains in *Metarhizium*								
M. brasiliense	ARSEF 2948^T	cicadellid	Brazil	–	–	KJ398809	KJ398020	KJ398718
	CG1446	**cicadellid**	**Brazil**	**MZ128140**	**MZ145380**	**MZ153209**	**MZ153203**	**MZ153206**
	CG1447	**cicadellid**	**Brazil**	**MZ128141**	**MZ145381**	**MZ153210**	**MZ153204**	**MZ153207**
	CG1450	**cicadellid**	**Brazil**	**MZ128139**	**MZ145382**	**MZ153208**		**MZ153205**
M. candelabrum	BCC29224^T	cicadellid	Thailand	MN781952	MN781853	MN781708	MN781755	MN781804
M. cercopidarum	BCC31660^T	cicadellid	Thailand	MN781953	MN781854	MN781709	MN781756	MN781805
M. chaiyaphumense	BCC78198	cicada	Thailand	KX369596	KX369593	KX369592	KX369594	KX369595
M. cicadae	BCC48881^T	cicada	Thailand	MN781949	MN781849	MN781704	MN781752	–
M. cylindrosporae	TNS-16371	cicada	Japan	JF415963	JF415986	JF416027	JN049902	–
M. ellipsoideum	BCC12847	cicadellid	Thailand	MN781959	MN781860	MN781715	MN781761	MN781810
	BCC49285^T	cicadellid	Thailand	MN781957	MN781858	MN781713	MN781759	MN781808
M. huainamdangense	BCC44270^T	cicadellid	Thailand	MN781956	MN781857	MN781712	–	MN781807
	BCC7672	cicadellid	Thailand	MN781955	MN781856	MN781711	MN781758	MN781806
M. megapomponiae	BCC25100^T	cicada	Thailand	MN781947	MN781847	MN781702	MN781751	MN781799
M. minus	ARSEF 2037	cicadellid	Philippines	AF339580	AF339531	DQ522353	DQ522400	DQ522454
M. niveum	BCC52400^T	cicada	Thailand	MN781933	MN781832	MN781685	–	MN781785
M. ovoidosporum	BCC7634	cicadellid	Thailand	MN781962	MN781863	MN781718	MN781764	MN781811
M. prachinense	BCC47950	lepidopteran	Thailand	KC011172	KC011180	KC011186	KC011184	–
M. samlanense	BCC17091	cicadellid	Thailand	HQ165665	HQ165727	HQ165686	–	HQ165646
M. takense	BCC30934	cicadellid	Thailand	HQ165658	HQ165720	HQ165679	HQ165740	HQ165639
M. viridulum	BCC36261	cicada	Thailand	MN781930	MN781827	MN781680	MN781737	MN781781
Strains in Entomophthoraceae								
Batkoa apiculata	ARSEF 3130	aphid	USA	–	EF392404	–	–	–
Batkoa gigantea	ARSEF 214	dipteran	Switzerland	–	JX242591	–	–	–
Batkoa major	ARSEF 3102	cicadellid	USA	–	EF392403	–	–	–
	ARSEF 2936	cicadellid	USA	–	EF392401	–	–	–
Batkoa obscurus^T	ARSEF 74	aphid	USA	–	NG058743	–	–	–
	CBS182.60	aphid	USA	–	JX242595	–	–	–
Batkoa pseudapiculata	ARSEF 1662	–	–	–	EF392398	–	–	–
	ARSEF 395	hymenopteran	Switzerland	–	EF392378	–	–	–
Batkoa sp	ARSEF 328	cicadellid	Australia	–	EF392375	–	–	–
	CG1454	**cicadellid**	**Brazil**	–	**MZ145379**	–	–	–
Conidiobolus brefeldianus^T	ARSEF 452	–	USA	–	EF392382	–	–	–
Entomophaga aulicae	ARSEF 172	lepidopteran	USA	–	EF392372	–	–	–
Entomophaga maimaiga	ARSEF 1400	lepidopteran	Japan	–	EF392395	–	–	–
Entomophthora muscae	ARSEF 3074	dipteran	USA	–	DQ273772	–	–	–
Entomophthora scatophaga	ARSEF 6704	dipteran	Denmark	–	DQ481226	–	–	–
Erynia ovispora	ARSEF 400	dipteran	Switzerland	–	JX242601	–	–	–
Erynia rhizospora	ARSEF 1441	trichoptera	England	–	EF392397	–	–	–
Furia americana	ARSEF 742	dipteran	Brazil	–	EF392389	–	–	–
Furia gastropachae	ARSEF 5541	lepidopteran	USA	–	EF392407	–	–	–
Pandora delphacis	ARSEF 459	cicadellid	Philippines	–	EF392384	–	–	–
Pandora dipterigena	ARSEF 397	dipteran	Switzerland	–	EF392380	–	–	–
Zoophthora lanceolata	ARSEF 469	dipteran	Switzerland	–	EF392385	–	–	–
Zoophthora radicans	ARSEF 388	dipteran	Switzerland	–	JX242605	–	–	–

[a] Abbreviations for collections: *ARSEF* - USDA-ARS Collection of Entomopathogenic Fungal Cultures, USA; *BCC* - BIOTEC Culture Collection, BIOTEC, National Science and Technology Development Agency, Thailand; *CBS* - CBS-KNAW Collections, Fungal Biodiversity Centre, Netherlands; *CG* - Invertebrate-Associated Fungal Collection, Embrapa Genetic Resources and Biotechnology, Brazil; *TNS* - NBRC Culture Collection, National Institute of Technology and Evaluation, Japan.
Accession codes in bold were obtained and sequenced by our team; $^{(T)}$ indicates ex-type culture.

the original descriptions of the ex-type material collected in Brazil from leafhoppers in 1989 (Driver et al., 2000; Kepler et al., 2014).

Increasing the amount of molecular data for *Metarhizium* species allows a better understanding of their diversity and genetic relationship with close taxa (Bischoff et al., 2009; Kepler et al., 2012; Kepler et al., 2014; Kortsinoglou et al., 2020; Luangsa-ard et al., 2017). The host range for *Metarhizium* species contains insects found in different orders (Kepler et al., 2014; Lopes et al., 2014). Most species are usually found in soils, as endophytes or infecting insects with at least part of their lifecycle in this habitat (Bamisile et al., 2018; Stone and Bidochka, 2020; van Lenteren et al., 2018). For instance, the generalist species *M. anisopliae* sensu stricto has been successfully isolated and used to control feeding-root cercopids in sugarcane and pastures (Iwanicki et al., 2019; Mascarin et al., 2019; Rezende et al., 2015). A recent comprehensive study revealed that *Metarhizium* species can be grouped into well genetically supported clades, which in some cases may have a narrower host range (Mongkolsamrit et al., 2020). The host-specific *Metarhizium acridum* and *Metarhizium cylindrosporum* are found infecting mostly orthopterans (Driver et al., 2000) and cicadas (Tzean et al., 1993), respectively, and both groups of insects have developmental stages in the soil. Much less common are *Metarhizium* species naturally associated with insects living aboveground, such as the caterpillar killer *M. rileyi* (Fronza et al., 2017) and the leafhopper pathogens *Metarhizium minus* and *M. album* (Rombach et al., 1986, 1987). *M. brasiliense* is adapted to infect a specific group of aboveground insects in the order Hemiptera, all belonging to a genetic group of small planthopper-infecting fungal pathogens, as the recently described *Metarhizium cercopidarum*, *Metarhizium candelabrum*, *Metarhizium huainamdangense,* and *Metarhizium ellipsoideum* (Mongkolsamrit et al., 2020).

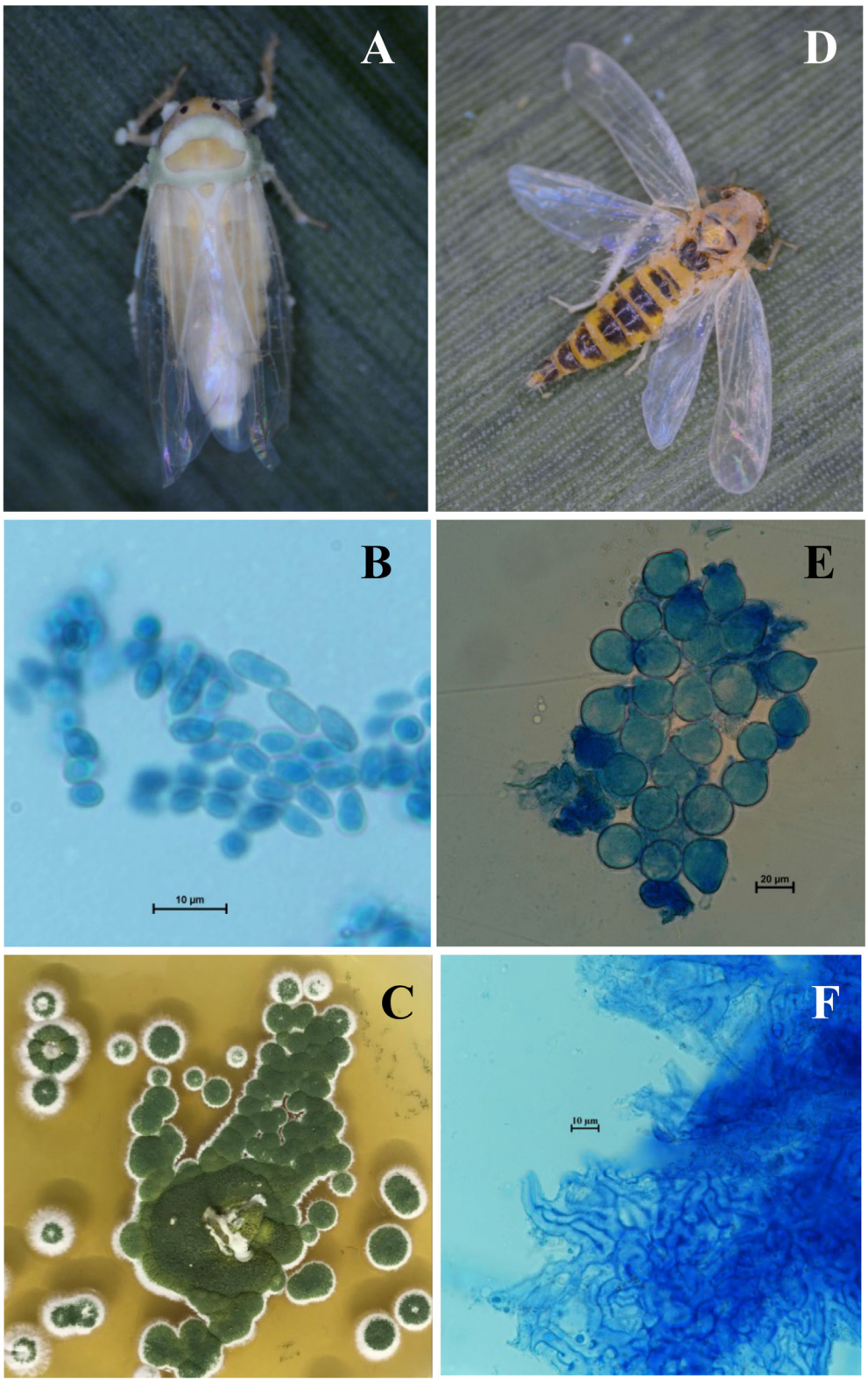

Fig. 1. *Metarhizium brasiliense* – Fungus-killed adults of *Dalbulus maidis* attached to leaves covered with pale blue-green conidia (A), two different size categories for conidia (x1000) (B) and 10-day old colony on potato dextrose agar medium showing dark blue-green conidia (C). *Batkoa* sp. – Dead adults of *Dalbulus maidis* with swollen abdomen and extended wings attached to leaves (D), primary spores, (E) and rhizoids (F). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

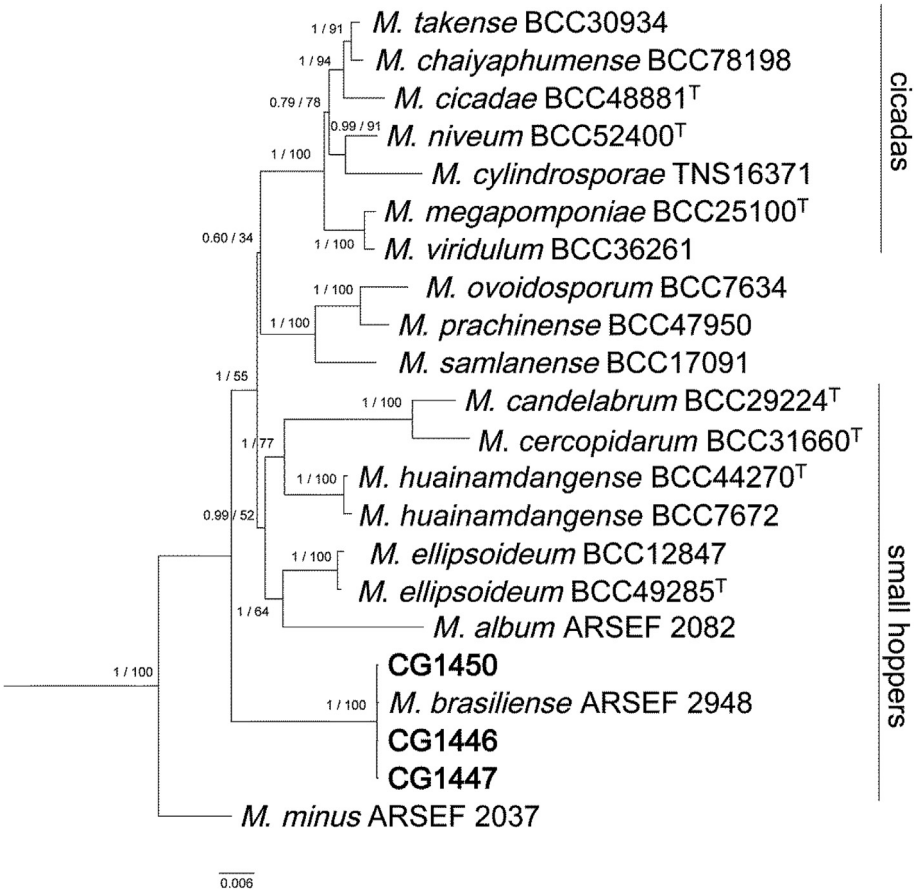

Fig. 2. Maximum likelihood phylogenetic tree generated from analyses of a combined dataset of LSU, SSU, rpb1, rpb2, and 3′tef sequences for *Metarhizium* strains. *Metarhizium minus* is the outgroup taxon. Support values were given as the Bayesian prior probability (first number) and percentage of bootstrap support derived from a ML analysis (second number). Ex-type strains are indicated with superscript T.

The placement of isolates CG1446, CG1447, and CG1450 with the ex-type culture ARSEF 2948 in the concatenated phylogenetic tree is strongly supported by the bootstrap values and in agreement with the morphological characteristics of this species. The blue-dark green color of the colonies on culture medium after conidia formation was also described by Kepler et al. (2014) for the ex-type culture. Interestingly, dead leafhopper adults found in maize fields were initially covered with pale blue-green conidia, resembling the color of *M. rileyi* conidia. However, aged conidia and those produced on PDA or rice grains displayed darker green color. This morphological variation was not reported by Kepler et al. (2014), since their description was solely based on a strain recovered from a culture collection. Indeed, as informed by L.G. Leite (personal communication), who collected the ex-type material from an unidentified cicadellid, at the time the cadavers were also covered by pale blue-green conidia. According to his report, a significant number of infected adults were found attached to leaves of non-grass weeds growing between rows of banana trees, and it was described as an epizootic event. Curiously, Driver et al. (2000) reported an Australian isolate from coleopteran larva as being *M. brasiliense* (formerly treated as *Metarhizium flavoviride* type E), but this material was not available for our study, and therefore this information requires further investigation. Noteworthy is the relatively high yield of this fungus on cooked rice (1.5×10^9 conidia per gram), which is exactly the same yield reported by Jenkins et al. (1998) for the commercially available species *M. acridum* (formerly known as *M. flavoviride*).

The entomophthoralean genus *Batkoa* has been reported naturally infecting cicadellid and fulgorid species in the Philippines (Villacarlos and Keller, 1997) and India (Baiswar and Firake, 2021; Keller and Yubak Dhoi, 2007). In Brazil, this fungus was described infecting cercopids in sugarcane and pastures (Batista Filho et al., 1997; Leite et al., 2002). However, molecular data for phylogenetic studies in *Entomophthorales* are less abundant in open access databases, and morphological similarity among some genera in *Entomophthoraceae* has led to misidentifications, suggesting the need for a robust molecular-based revision (Gryganskyi et al., 2013; Nie et al., 2020). The strain CG1454 was clearly placed within the genus *Batkoa* and is closely related to the strain ARSEF 328 collected in Australia also from Cicadellidae, according to the USDA-ARSEF Collection Catalog (Castrillo and Wheeler, 2017). These two strains, which represent an unidentified species of *Batkoa*, clustered with the species *Batkoa major*, recently found causing epizootics on the invasive planthopper *Lycorma delicatula* in North America (Clifton et al., 2019). Similar to our study, a coepizootic by two unrelated entomopathogenic fungi in a population of *L. delicatula* adults was reported in the USA, with most dead planthoppers being killed by *B. major* (73%) and only 27% by the hypocrealean *Beauveria bassiana*. Interestingly, a greater percentage of *D. maidis* adults killed by *Batkoa* sp. in relation to *M. brasiliense* in the coepizootic was also seen in Field 2, confirming

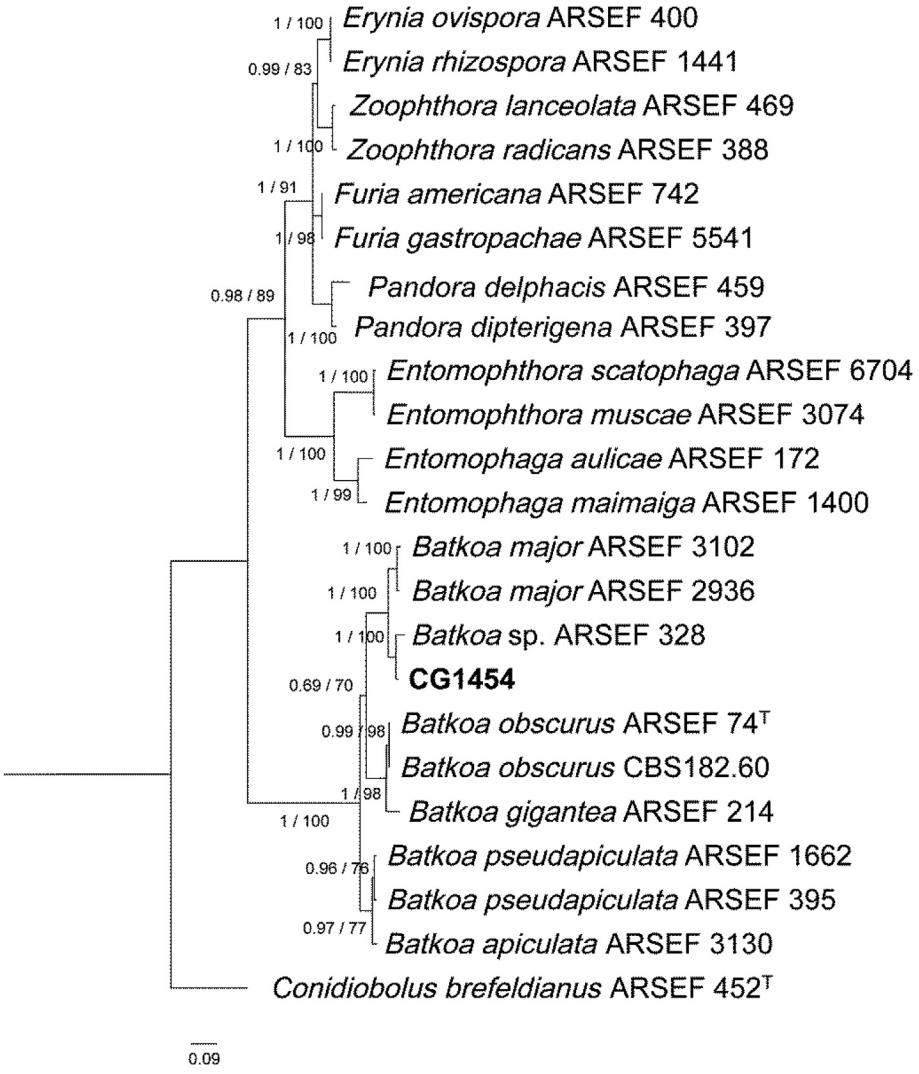

Fig. 3. Maximum likelihood phylogenetic tree generated from analysis of LSU sequence dataset for strains within the family Entomophthoraceae. *Conidiobolus brefeldianus* is the outgroup taxon. Support values were given as the Bayesian prior probability (first number) and percentage of bootstrap support derived from a ML analysis (second number). Ex-type strains are indicated with superscript T.

the great epizootic potential of the former genus. Our surveys targeted maize plants in the reproductive stage when the microenvironmental conditions and insect density were favorable for fungal establishment in the host population. At this stage, damage caused by pant pathogens is minimal and control strategies targeting the vector *D. maidis* are usually no longer applied, allowing high incidence levels of both diseases reported in this study.

We did not find infected *D. maidis* nymphs in both fields. Infections by *Metarhizium* spp. and entomophthoralean fungi in cercopids and cicadellids has been usually observed in adults (Gryganskyi et al., 2013; Mongkolsamrit et al., 2020). Another interesting behavioral characteristic of the corn leafhopper is its ability to migrate over long distances. Studies in Brazil have suggested that at the end of the maize cycle, part of the *D. maidis* population leaves the senescent plants and migrates to new maize fields at distances greater than 20 km (Oliveira et al., 2013). This behavioral characteristic may contribute to the dissemination of fungal entomopathogens potentially allowing them to infect *D. maidis* populations in distant maize fields.

The use of these biological control agents could be an important tool to manage these diseases by reducing the migrating population of the vector able to colonize new maize crops. Both pathogens probably play a role in regulating the density of migrant adults to other areas, which, as reported here, is particularly significant in epizootics caused by the fastidious *Batkoa*. The fungus *M. brasiliense*, a species apparently restricted to South America, might be present in most of the maize producing regions in Brazil that share similar environmental conditions with the areas evaluated in our study. Based on the ability of *M. brasiliense* to disseminate infective propagules through continuous maize fields and to produce significant amounts of conidia under laboratory conditions (a prerequisite for applied biological control), we foresee the potential of this fungus as a new tool against *D. maidis* populations. Further investigations on the biocontrol potential of this species,

including its impact on migrant populations of *D. maidis*, are needed.

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Authors' contributions

RBL, CMO and MAT designed the study; CMO, MAT, RBL and DAS performed the research; RBL and MF analyzed the data and wrote the paper.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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References

Baiswar, P., Firake, D.M., 2021. First record of *Pandora formicae* on ant, *Camponotus angusticollis* and *Batkoa amrascae* on white leaf hopper, *Cofana spectra* in rice agroecosystem of India. J. Eco-Friendly Agricult. 16, 27–31.

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Wang, L., 2018. Fungal endophytes: beyond herbivore management. Front. Microbiol. 9, 544.

Batista Filho, A., Leite, L.G., Takada, H.M., Lamas, C., Ramiro, Z.A., 1997. Incidência do fungo entomopatogênico *Batkoa apiculata* (Entomophthorales) sobre cigarrinhas das pastagens em Pindamonhangaba, SP, 64. Arquivos do Instituto Biológico, p. 82.

Ben-Ze'ev, I., Kenneth, R.G., 1981. *Zoophthora radicans* and *Zoophthora petchi* sp. nov. (Zygomycetes: Entomophthorales), two species of the "sphaerosperma group" attacking leaf-hoppers and frog-hoppers (Horn.). Entomophaga 26, 131–142.

Ben-Ze'ev, I., Kenneth, R.G., 1982. Features-criteria of taxonomic value in the Entomophthorales: I. A revision of the Batkoan classification. Mycotaxon 14, 393–455.

Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101, 512–530.

Castrillo, L.A., Wheeler, M.M., 2017. ARSEF Database. http://ARSEF.fpsnl. cornell.edu/4DACTION/W_List_Tables/param. (Accessed 10 February 2020).

Clifton, E.H., Castrillo, L.A., Gryganskyi, A., Hajek, A.E., 2019. A pair of native fungal pathogens drives decline of anew invasive herbivore. Proc. Natl. Acad. Sci. Unit. States Am. 116, 9178–9180.

Driver, F., Milner, R.J., Trueman, J.W.H., 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol. Res. 104, 134–150.

Faria, M., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Contr. 43, 237–256.

Fronza, E., Specht, A., Heinzen, H., Barros, N.M., 2017. *Metarhizium* (*Nomuraea*) *rileyi* as biological control agent. Biocontrol Sci. Technol. 27, 1243–1264.

Foieri, A., Pedrini, N., Toledo, A., 2018. Natural occurrence of the entomopathogenic genus *Pandora* on spittlebug pests of crops and pastures in Argentina. J. Appl. Entomol. 142, 363–370.

Galaini-Wraight, S., Wraight, S.P., Carruters, R.I., Magalhães, B.P., Roberts, D.W., 1991. Description of a *Zoophthora radicans* (zygomicetes: Entomophthoraceae) epizootic in a population of *Empoasca kraemeri* (Homoptera: Cicadellidae) on beans in Central Brazil. J. Invertebr. Pathol. 58, 311–328.

Giménez-Pecci, M.P., Laguna, I.G., Avila, A.O., Remes-Lenicov, A.M.M., Virla, E., Borgogno, C., Nome, C.F., Paradel, S., 2002. Difusión del Corn Stunt Spiroplasma del maíz (*Spiroplasma kunkelii*) y del vector (*Dalbulus maidis*) en la República Argentina. Rev. Fac. Agron. 105, 1–8.

Gryganskyi, A.P., Humber, R.A., Smith, M.E., Hodge, K., Huang, B., Voigt, K., Vilgalys, R., 2013. Phylogenetic lineages in entomophthoromycota. Persoonia 30, 94–105.

Humber, R.A., 1989. Synopsis of a revised classification for the Entomophthorales (zygomycotina). Mycotaxon 34, 441–460.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., Leger Rj, St, Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl. Acad. Sci. Unit. States Am. 111, 16796–16801.

Ibarra-Aparicio, G., Moya-Raygoza, G., Berlanga-Padilla, A., 2005. Efecto de *Beauveria bassiana* y *Metarhizium anisopliae* sobre la chicharrita del maíz (*Dalbulus maidis*) (Delong y Wolcott, 1923) (Hemiptera: Cicadellidae). Folia Entomol. Mexic. 44, 1–6.

Iwanicki, N.S.A., Pereira, A.A., Botelho, A.B.R.Z., Rezende, J.M., Moral, R.A., Zucchi, M.I., Delalibera Jr., I., 2019. Monitoring of the field application of *Metarhizium anisopliae* in Brazil revealed high molecular diversity of *Metarhizium* spp. in insects, soil and sugarcane roots. Sci. Rep. 9, 4443. https://doi.org/10.1038/s41598-019-38594-8.

Iwanicki, N.S.A., Mascarin, G.M., Moreno, S.G., Eilenberg, J., Delalibera Jr., I., 2020. Growth kinetic and nitrogen source optimization for liquid culture fermentation of *Metarhizium robertsii* blastospores and bioefficacy against the corn leafhopper *Dalbulus maidis*. World J. Microbiol. Biotechnol. 36, 71. https://doi.org/10.1007/s11274-020-02844-z.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J., Lomer, C.J., 1998. Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News Inf. 19, 21N–31N.

Keller, S., Yubak Dhoi, G.C., 2007. Insect pathogenic Entomophthorales from Nepal and India. J. Swiss Entomol. Soc. 80, 211–215.

Kepler, R.M., Sung, G.H., Ban, S., Nakagiri, A., Chen, M.-J., Huang, B., Li, Z., Spatafora, J.W., 2012. New teleomorph combinations in the entomopathogenic genus *Metacordyceps*. Mycologia 104, 182–197.

Kepler, R.M., Humber, R.A., Bischoff, J.F., Rehner, S.A., 2014. Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. Mycologia 106, 811–829.

Kortsinoglou, A.M., Saud, Z., Eastwood, D.C., Butt, T.M., Kouvelis, V.N., 2020. The mitochondrial genome contribution to the phylogeny and identification of *Metarhizium* species and strains. Fungal Biol. 24, 845–853.

Leite, L.G., Alves, S.B., Takada, H.M., Batista Filho, A., Roberts, D.W., 2002. Occurrence of Entomophthorales on spittlebugs pests of pasture in Eastern São Paulo state, Brazil, 69. Arquivos do Instituto Biológico, pp. 63–68.

Li, Z., Alves, S.B., Roberts, D.W., Fan, M., Delalibera Jr., I., Tang, J., Lopes, R.B., Faria, M., Rangel, D.E.N., 2010. Biological control of insects in Brazil and China: history, current programs and reasons for their successes using entomopathogenic fungi. Biocontrol Sci. Technol. 20, 117–136.

Lopes, R.G., Faria, M., Souza, D.A., Bloch, C., Silva, L.P., Humber, R.A., 2014. MALDI-TOF mass spectrometry applied to identifying species of insect-pathogenic fungi from the *Metarhizium anisopliae* complex. Mycologia 106, 865–878.

Luangsa-ard, J.J., Mongkolsamrit, S., Thanakitpipattana, D., Khonsanit, A., Tasanathai, K., Noisripoom, W., Humber, R.A., 2017. Clavicipitaceous entomopathogens: new species in *Metarhizium* and a new genus *Nigelia*. Mycol. Prog. 16, 369–391.

Mascarin, G.M., Lopes, R.B., Delalibera Jr., I., Fernandes, E.K.K., Luz, C., Faria, M., 2019. Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. J. Invertebr. Pathol. 165, 46–53.

Mazzoglio, P.J., Dolci, P., Lessio, F., Alma, A., 2009. *Zoophthora radicans* affecting *Zyginidia pullula*. Bull. Insectol. 62, 143–148.

Massola Jr., N.S., Bedendo, I.P., Amorim, L., Lopes, J.R.S., 1999. Quantificação de danos causados pelo enfezamento vermelho e enfezamento pálido do milho em condições de campo. Fitopatol. Bras. 24, 136–142.

Meneses, A.R., Querino, R.B., Oliveira, C.M., Maia, A.H.N., Silva, P.R.R., 2016. Seasonal and vertical distribution of *Dalbulus maidis* (Hemiptera: Cicadellidae) in Brazilian corn fields. Fla. Entomol. 99, 750–754.

Mongkolsamrit, S., Khonsanit, A., Thanakitpipattana, D., Tasanathai, K., Noisripoom, W., Lamlertthon, S., Himaman, W., Houbraken, J., Samson, R.A., Luangsa-ard, J., 2020. Revisiting *Metarhizium* and the description of new species from Thailand. Stud. Mycol. 95, 171–251.

Nault, L.R., 1980. Maize bushy stunt and corn stunt: a comparison of disease symptoms, pathogens host ranges, and vectors. Phytopathology 70, 659–662.

Nie, Y., Yu, D.S., Wang, C.F., Liu, X.Y., Huang, B., 2020. A taxonomic revision of the genus *Conidiobolus* (Ancylistaceae, Entomophthorales): four clades including three new genera. MycoKeys 66, 55–81.

Oliveira, E., Sousa, S.M., Landau, E.C., 2011. Trasmission of maize bushy stunt phytoplasma by *Dalbulus maidis* leafhoopers. Bull. Insectol. 64, S153–S154.

Oliveira, C.M., Frizzas, M.R., Oliveira, E., 2020. Overwintering plants for *Dalbulus maidis* (DeLong and wolcott) (Hemiptera: Cicadellidae) adults during the maize off-season in central Brazil. Int. J. Trop. Insect Sci. 40, 1105–1111.

Oliveira, C.M., Lopes, J.R.S., Nault, L.R., 2013. Survival strategies of *Dalbulus maidis* during maize off-season in Brazil. Entomol. Exp. Appl. 147, 141–153.

Petch, T., 1931. Notes on entomogenous fungi. Trans. Br. Mycol. Soc. 16, 55–75.

Purcell, A.H., 1988. Increased survival of *Dalbulus maidis*, a specialist on maize, on non-host plants infected with mollicute plant pathogens. Entomol. Exp. Appl. 46, 187–196.

Raeder, J., Broda, P., 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1, 17–20.

Rezende, J.M., Zanardo, A.B.R., Lopes, M.S., Delalibera, I., Rehner, S.A., 2015. Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. BioControl 60, 495–505.

Rombach, M.C., Humber, R.A., Roberts, D.W., 1986. *Metarhizium flavoviride* var. *minus* var. nov., a pathogen of plant- and leafhoppers on rice in Philippines and Solomon Islands. Mycotaxon 27, 87–92.

Rombach, M.C., Humber, R.A., Evans, H.C., 1987. *Metarhizium album*, a fungal pathogen of leaf- and planthoppers of rice. Trans. Br. Mycol. Soc. 88, 451–459.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D., Darling, A., Höhna, S., Larget, B., Liu, L., Huelsenbeck, J.P., 2012. MrBayes v.3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Santana Jr, P.A., Kumar, L., Silva, R.S., Pereira, J.L., Picanço, M.C., 2019. Assessing the impact of climate change on the worldwide distribution of *Dalbulus maidis* (DeLong) using MaxEnt. Pest Manag. Sci. 75, 2706–2715.

Stone, L.B.L., Bidochka, M.J., 2020. The multifunctional lifestyles of *Metarhizium*: evolution and applications. Appl. Microbiol. Biotechnol. 104, 9935–9945.

Sugio, A., MacLean, A.M., Kingdom, H.N., Grieve, V.M., Manimekalai, R., Hogenhout, S.A., 2011. Diverse targets of phytoplasma effectors: from plant development to defense against insects. Annu. Rev. Phytopathol. 49, 175–195.

Summers, C.G., Newton, A.S., Opgenorth, D.C., 2004. Overwintering of corn leafhopper, *Dalbulus maidis* (Homoptera: Cicadellidae), and *Spiroplasma kunkelii* (mycoplasmatales: spiroplasmataceae) in California's san joaquin valley. Environ. Entomol. 33, 1644–1651.

Toledo, A.V., Remes Lenicov, A.M.M., López Lastra, C.C., 2008a. Host range findings on *Beauveria bassiana* and *Metarhizium anisopliae* (ascomycota: hypocreales) in Argentina. Bol. Soc. Argent. Bot. 43, 211–220.

Toledo, A.V., Giambelluca, L., Remes Lenicov, A.M.M., López Lastra, C.C., 2008b. Pathogenic fungi of planthoppers associated with rice crops in Argentina. Int. J. Pest Manag. 54, 363–368.

Trifinopoulos, J., Nguyen, L.T., von Haeseler, A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 44, W232–W235.

Tzean, S.S., Hsieh, L.S., Chen, J.L., Wu, W.J., 1993. *Nomuraea cylindrospora* comb. nov. Mycologia 85, 514–519.

van Lenteren, J.C., Bolckmans, K., Köhl, J., Ravensberg, W.J., Urbaneja, A., 2018. Biological control using invertebrates and microorganisms: plenty of new opportunities. BioControl 63, 39–59.

Villacarlos, L.T., Keller, S., 1997. *Batkoa amrascae* keller & villacarlos, a new species of Entomophthorales (zygomycetes) infecting the cotton leafhopper, *Amrasca biguttula* (ishida) (Homoptera: Cicadellidae) in the Philippines. Philipp. Entomol. 11, 81–86.

Zhang, Q., Chen, X., Xu, C., Zhao, H., Zhang, X., Zeng, G., Qian, Y., Liu, R., Guo, N., Mi, W., Meng, Y., St, Leger, R.J., Fang, W., 2019. Horizontal gene transfer allowed the emergence of broad host range entomopathogens. Proc. Natl. Acad. Sci. Unit. States Am. 116, 7982–7989.