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Endophytic Capacity of Entomopathogenic Fungi in a Pasture Grass and Their Potential to Control the Spittlebug *Mahanarva spectabilis* (Hemiptera: Cercopidae)

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Abstract: Pests in pastures have compromised the production of biomass for feeding livestock herds. Many strategies have been applied to sustainably solve this problem. One viable and innovative technique is the delivery of entomopathogenic fungi through endophytes. Therefore, this study aimed to (i) evaluate the endophytic capacity of two entomopathogenic fungi, *Fusarium multiceps* UFMGCB 11443 and *Metarhizium anisopliae* UFMGCB 11444, in *Urochloa brizantha* [(Hochst. ex A. Rich.) Stapf] (Poaceae) cultivar 'Marundu') via foliar inoculation or seed treatment, and (ii) measure their efficiency in controlling *Mahanarva spectabilis* Distant, 1909 (Hemiptera: Cercopidae) in *U. brizantha*. In the greenhouse, the fungi colonized the tissues of *U. brizantha* plants when inoculated via foliar spraying or seed treatment. The fungi *F. multiceps* and *M. anisopliae* caused 88% and 97.1% epizootic effects via seed inoculation, respectively, and 100% epizootic effects via foliar inoculation. In the field, the lowest fungal dose of 0.5 kg/ha had the same effect as a fourfold greater dose, with a >86% decrease in insect pest infestation observed. In summary, the fungi *F. multiceps* and *M. anisopliae* have endophytic effects and can effectively control *M. spectabilis* in *U. brizantha* pastures.

Keywords: biological control; endophytes; entomopathogenic fungi

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1. Introduction

In 2021, Brazil had the largest commercial cattle herd globally [1]. In export rankings, Brazil ranks third in bovine milk production [2] and first in beef production [3–6]. Most Brazilian cattle are raised in an extensive livestock system comprising native and cultivated pastures that represent approximately 45% of the agricultural area in the country [7], and these pastures must be of good quality to serve as animal feed [8–10]. Cattle ingest a large amount of pasture biomass supplemented with salt and minerals [11,12]. However, Brazil faces several problems due to these extensive systems. Extensive *Urochloa* spp. (Hochst. ex A. Rich.) R. D. Webster (synonymous with *Brachiaria* spp. [Hochst. ex A. Rich Stapf]) [13]

monocultivation and high temperatures in the country have led to outbreaks of spittlebugs (Hemiptera: Cercopidae), the main pests in pastures in tropical America [14–18]. Nymphs and adults of this insect pest damage host plants by sucking the sap and injecting toxins that induce phototaxis and reduce photosynthetic rates [19–23], leading to a loss of biomass availability for cattle and reducing the support capacity by 60% on average [15,16,19,24–26].

Outbreaks of spittlebugs are controlled by spraying agrotoxics. This approach is economically unfeasible in large areas [21,27] and carries the risks of residues accumulating in the final product, contamination of the production chain and environment, and harm to consumer health. These pesticides leave environmental residues, leading to contamination [28]. Therefore, other strategies to address spittlebug attacks on forages in a sustainable manner are being developed. These include the induction of plant resistance to spittlebug attacks [15,21,29], the use of plant compounds as biocontrol agents [30,31], pasture diversification [15], soil fertilization [21,32] and the use of entomopathogenic fungi [17,22,33].

However, all these sustainable strategies introduce challenges in production. With regard to the use of fungi, the challenge is the instability caused by abiotic factors. Fungi are most effective at mild temperatures [34]. Therefore, it is important to consider fungal species that live in endophytes, which can alleviate this instability. Endophytic entomopathogenic fungi are plant mutualists and insect pathogens [35,36] that live within the tissues of healthy plants without causing disease [37–40]. They can colonize plants, act as endophytes for part of their life cycle [41], and resist abiotic and biotic stresses [42–45] that reduce the viability of fungal conidia [46]. Colonization by endophytic fungi can be systemic, localized in specific parts [47–50] or subdivided among plants [51,52]. Entomopathogenic fungi can potentially regulate the populations of various pest insects directly [48]. Microorganisms can be introduced into plants via seed treatment [53,54] or foliar inoculation.

The entomopathogenic fungi UFMGCB 11443 and UFMGCB 11444 were previously isolated from the spittlebug *Mahanarva spectabilis* Distant (Hemiptera: Cercopidae) in a silvopastoral system in the State of Maranhão, Brazil. Both strains caused greater insect pest mortality than did the commercial strain *Metarhizium anisopliae* (Metschn.) Sorokin [55]. Therefore, it was hypothesized that these fungi, UFMGCB 11443 and UFMGCB 11444, colonize *Urochloa brizantha* plants, making the forage an efficient vector for the biological control of *M. spectabilis* via different inoculation methods. Therefore, this study aimed to (i) evaluate the endophytic capacity of the fungi UFMGCB 11443 and UFMGCB 11444 in *U. brizantha* via foliar or seed inoculation, (ii) measure their efficiency in the sustainable control of *M. spectabilis* on *U. brizantha* in a greenhouse via different inoculation methods, and (iii) verify their efficiency in controlling *M. spectabilis* outbreaks in the field by foliar spraying.

2. Materials and Methods

2.1. Fungal Origin

The endophytic experiments were conducted at the Federal University of São João del Rei (UFSJ), CTAN Campus, in São João del Rei (MG), Brazil (21°06′13.0″ S 44°14′52.5″ W, 908 m), in a greenhouse (25 m × 7 m) without climate control, covered with transparent waterproof plastic and closed with an anti-aphid net. The average temperature inside the greenhouse was 28 °C ± 4 °C, and the relative humidity was 65% ± 30%. *Urochloa brizantha* plants were grown in 1 L plastic pots filled with oxisol fertilized with NPK (formula 4-14-8 (Agroadubo, Brazil)). Six seeds were sown per pot, and the tests were conducted 90 days after seed planting. Each experimental unit (EU) comprised a pot with plants and nymphs. *Mahanarva spectabilis* nymphs of the second generation from the rearing maintained by the Laboratory of Entomology of Embrapa Gado de Leite, which had plants of *U. brizantha* as hosts, were used with or without fungal treatment according to the description below.

Two strains of entomopathogenic fungi, UFMGCB 11443 and UFMGCB 11444, isolated in a silvopastoral system were used and were identified using molecular biological methods. Briefly, total DNA extraction was performed. The internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal DNA gene was amplified using the primers ITS1 and ITS4

along with RNA polymerase II using the primers RBP2 5F and RPB2 7R. The amplicons of these marker regions (ITS and Pol II) were evaluated using 1% agarose gel electrophoresis, purified, and sequenced by the Sanger method using an ABI automated system (Applied Biosystems Life Technologies, Austin, TX, USA). The generated nucleotide sequences were submitted for BLASTn (Basic Local Alignment Search Tool) analysis through alignment and comparison of their similarities with the sequences of fungal type species deposited in GenBank and are available on the NCBI portal (http://www.ncbi.nlm.nih.gov/blast/ accessed on 15 January 2024). These fungi were deposited in the Microorganisms Collection of the Federal University of Minas Gerais, Brazil (CM-UFMG; World Data Center for Microorganisms [WDCM] 1029) with the following accession numbers: *Fusarium multiceps* UFMGCB 11443 (GenBank accession number ON831395) and *Metarhizium anisopliae* UFMGCB 11444 (GenBank accession number ON831396).

The ability of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 to persist within the tissues of *U. brizantha* was tested. Two techniques were used to inoculate the fungi in the endophytic test: seed treatment and spraying on plants before pest infestation. The batches of conidia produced were tested for viability according to the methodology proposed by Lopes et al. [56] before starting the experiments.

2.2. Production of Fungi for Bioassays

To conduct the bioassays, the *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 isolates were reactivated and prepared to produce conidia for the experiments. They were subcultured in 9.0 cm × 1.5 cm Petri dishes containing potato dextrose agar (Kasvi®, Lehigh County, PA, USA) culture medium in a vertical laminar flow chamber previously sterilized with 70% alcohol. These dishes were incubated in germination chambers [to meet the biochemical oxygen demand (BOD) EletroLab®, Campinas, Brazil] at 25 ± 2 °C and 70 ± 10% RH with a 12 h photoperiod for vegetative growth and conidiogenesis. After incubation for 7–10 days, the conidia produced were removed from the surface of the culture medium with a sterile metal spatula and inoculated separately to prepare suspensions containing sterile water and an ionic surfactant (Tween 80 [0.001%], Sigma-Aldrich®, Steinheim, Germany) at a concentration of 1×10^8 conidia/mL.

2.3. Fungal Inoculation via Seed Treatment

Urochloa brizantha seeds were washed with 2.5% hypochlorite for 2 min and rinsed with distilled water for surface disinfestation, according to a methodology adapted from Carvalho et al. [57] and Ferreira et al. [58]. Then, they were inoculated in 100 mL of a suspension containing 1×10^8 conidia/mL and 0.05% Tween 80 as a surfactant for each fungus, plus the control suspension (sterile distilled water plus 0.05% Tween 80) without the presence of fungi, for a total of 3 treatments. The seeds were placed in contact with the suspension for 30 min as adapted from the methodology of Keyser et al. [59] and then sown. After seed germination, the plants were allowed to develop for approximately 45 days to evaluate the presence of fungi in the plant tissues.

2.4. Fungal Inoculation via Foliar Spraying

After germination and 30 days of growth from untreated seeds, plants were subjected to foliar spraying with either suspensions containing 1×10^8 conidia/mL of either fungus or a suspension with water and Tween without fungi using a manual sprayer with small nozzles and a calibrated spray volume of 50 L/ha. According to the methodology adapted from Ahmad et al. [60], the bases of the plants were protected to avoid contact with the spray and to prevent cross-contamination. After foliar inoculation with fungi, the plants were allowed to develop for approximately 45 days to evaluate the presence of the inoculated fungi in the plant tissues.

2.5. Analysis of Fungal Persistence in Urochloa brizantha Tissue

This bioassay used a randomized block design (DBC) with five treatments: plants from seeds treated with the fungus *F. multiceps* UFMGCB 11443; plants from seeds treated with the fungus *M. anisopliae* UFMGCB 11444; plants sprayed 30 days after planting with the fungus *F. multiceps* UFMGCB 11443; plants sprayed 30 days after planting with the fungus *M. anisopliae* UFMGCB 11444; and control plants without fungal inoculation.

Three leaves were taken randomly from each EU (one new, one young, and one senesced), totaling 150 leaf samples (3 leaves × 5 treatments × 10 replications). These leaves were placed in sterile plastic in a thermal box and taken to the DEPEB at UFSJ for testing. The surface of each leaf was disinfected by immersion in 70% ethanol (1 min) and 2% sodium hypochlorite (1 min), followed by washing with sterile distilled water (2 min) [57,58]. Disinfection was carried out to remove possible epiphyte microorganisms on the surfaces of leaves. After disinfection, a fragment of each leaf was placed in a Petri dish containing Sabouraud agar (Kasvi®) and incubated at 26 °C for approximately 15 days in a climate chamber (to meet the BOD). The macromorphologies and micromorphologies of the fungi present in these fragments were compared to those of inoculated *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 and with those of the fungi isolated from the control plants (<5%). The fungal species were grouped using identification keys [34], and 45 samples were selected for subsequent molecular analysis. The experimental design used was completely randomized. For each treatment, leaves were collected randomly from ten EUs to assess the presence of the applied fungi. The presence of endophytes was confirmed when the growth of the applied fungi from leaf samples of plants for each treatment was observed according to the methodology adapted from Ahmad et al. [60].

2.6. Analysis of Fungal Persistence in the Sap of Urochloa brizantha

The EUs were taken to the DEPEB for plant sap collection. Ten repetitions of each treatment were performed (ten repetitions × five treatments = 50 samples total). Before cutting, the plants were disinfected with 2% hypochlorite and rinsed with distilled water [60]. Then, the plants were cut at the sheath, close to the first ligule and below the first node, with sterile scissors. The sap was collected with a sterile 1000 µL pipette 5 min after cutting. The collected sap was allocated and spread with Drigalski loops on a Petri dish containing Sabouraud dextrose agar. The plates were incubated at 26 °C for approximately seven days in a climate chamber (to meet the BOD), and the samples were purified by successive plating. The macromorphologies and micromorphologies of the fungi present in the fragments were compared with those of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 and of fungi isolated from control plants (<5%), and three samples from each treatment were randomly selected for subsequent molecular analysis. The experimental design used was completely randomized. Each treatment was represented by saps collected from ten random EUs to assess the presence of the applied fungi. The presence of endophytes was confirmed when we observed the growth of the applied fungi from leaf samples of plants for each treatment according to the methodology adapted from Ahmad et al. [60].

2.7. Greenhouse Bioassay with Mahanarva spectabilis

This bioassay used a randomized block design (DBC) with five treatments: plants from seeds treated with the fungus *F. multiceps* UFMGCB 11443; plants from seeds treated with the fungus *M. anisopliae* UFMGCB 11444; plants sprayed 30 days after planting with the fungus *F. multiceps* UFMGCB 11443; plants sprayed 30 days after planting with the fungus *M. anisopliae* UFMGCB 11444; and control plants without fungal inoculation. There were 20 plants or repetitions for each treatment, totaling 100 EUs (five treatments × 20 replicates). Each plant was grown in a 1 L pot previously fertilized with NPK. These plants received five third- or fourth-instar nymphs of *M. spectabilis* spittlebugs. The pots were individually covered in voile bags to avoid cross-contamination between the treatments and insect escape. Starting 24 h after release, insect mortality was evaluated daily for 15 days. The

dead nymphs were removed daily, placed in 1.5 mL Eppendorf tubes, and frozen in a −20 °C freezer to determine their cause of death [61].

2.8. Field Bioassay with Mahanarva spectabilis

The field trial was conducted in cultivated pastures with a predominance of *U. brizantha* grass (Hochst. ex A. Rich.) R.D. Webster (synonymous with *Brachiaria brizantha* [Hochst. ex A. Rich.] Stapf) in the municipality of Açailândia in the southwest of the State of Maranhão (Brazil), with the map coordinates 04°57′48.2″ S and 047°08′58.0″ W Gr and an altitude of 274 m.

This region is an ecotone between the Cerrado and the Amazon Forest. At the start of the experiment, the pasture had an average infestation density of 42.5 nymphs/m^2, which is above the minimum level of 20–25 nymphs/m^2 recommended for controlling spittlebugs [62,63]. The temperature was approximately 34.9 ± 3 °C, and the relative humidity was 89%, with winds of up to 6 km/h and solar radiation of 4 MJ/m^2. The average rainfall in February and March 2019 was 175 mm [64]. *Brachiaria* pastures were used under a rotation system for producing Nelore beef cattle, with a history of intense infestation by pasture spittlebugs.

After the start of the experiment, the height of the plants was maintained between 25 and 35 cm through continuous grazing. The experiment used a DBC with eight treatments (Table 1) and six replications each. The EUs of each trial treatment covered an area of 80 m^2 (8 × 10 m), separated from each other by 50 m wide corridors. The methodology used to count insects and foam masses [65] determined the infestation and fluctuation levels of adults and nymphs in each EU before and after fungal application. The population survey for nymphs was conducted by counting the number of nymphs per foam at the base of the plant (ground level) using a square measuring 0.25 × 0.25 m (0.0625 m^2). This square was randomly dropped within each area to define a sampling point [66], with 10 drops (sampling points) per area. The fluctuation in adults was assessed by sampling the number of adult spittlebugs using a scanning net comprising five transects in the central portion of each EU to minimize edge effects. The spittlebugs captured by the net were transferred into 2 mL Eppendorf tubes for subsequent species identification.

Table 1. *Fusarium multiceps* UFMGCB 11443 and *Metarhizium anisopliae* UFMGCB 11444 doses in kilograms per hectare in the field trials.

Treatments	Dose (kg/ha)
Fusarium multiceps	0.5
Metarhizium anisopliae	0.5
Fusarium multiceps	1.0
Metarhizium anisopliae	1.0
Fusarium multiceps	2.0
Metarhizium anisopliae	2.0
Fusarium multiceps + *Metarhizium anisopliae*	1.0 + 1.0
Control	0.0

The conidium batches were produced in parboiled rice with 1 × 10^{12} conidia/g, and suspensions were prepared at different concentrations for the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 belonging to CM-UFMG (WDCM 1029). In all suspensions, 0.05% refined soybean oil and 0.01% neutral detergent were added as adhesives, and the fungi were added according to the treatment (Table 1).

The control area was sprayed with a pure suspension without fungi. For application, the sporulated fungi in rice were washed in water and strained through rice sieves. Then, the solution was poured into a coastal sprayer tank with low-flow fan nozzles and a volume of 50 L/ha for spraying. The application was conducted at a height of approximately 50 cm from the ground after counting the insects to allow contact between the biological pesticides with the spittlebug nymphs, the stage most susceptible to the entomopathogenic agent [67].

Zimmermann [68] emphasized that the contact-based action of the microbial insecticide is more effective at lower temperatures. Following these guidelines, the application time was between 7:00 am and 5:00 pm. Nymphs and adults were counted before and after 6, 15, 20, and 30 days of application.

2.9. Analysis of the Cause of Mahanarva spectabilis Death

From each EU, in the greenhouse, five nymph or adult spittlebug (*M. spectabilis*) samples placed in sterile Eppendorf tubes were randomly chosen (500 samples total) for the cause-of-death analysis and taken to the Microbiology Laboratory of the Department of Bioengineering (DEPEB) at UFSJ. After 24 h, these samples were incubated in culture medium to assess the presence or absence of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444, which were inoculated in *U. brizantha* plants, to confirm the cause of mortality. The insects were superficially disinfected by immersion in 70% ethanol (1 min) and 2% sodium hypochlorite (1 min) and then washed with sterile distilled water (2 min) according to an adapted methodology [57,58,69] to remove external impurities. The samples were placed individually in Petri dishes containing Sabouraud agar and kept for seven days in a BOD chamber at 25 °C to allow the emergence of the fungi inside the dead insects [70]. After macroculture, microculture was conducted on a fraction of the macroculture, followed by microscopy according to a methodology adapted from Kuzhuppillymyal-Prabhakarankutty et al. [71].

The colonies of all the fungal isolates were photographed (front and back) and grouped according to their macromorphological characteristics, such as colony color (front and back), surface texture (front and back), edge appearance, and growth time. These isolates were compared with the macromorphology of the applied fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 [69,72]. Micromorphology was assessed by comparing the fungal reproductive structures after the samples were microcultured under an optical microscope. The fungal species were identified with the help of identification keys [34]. Afterward, molecular analysis was carried out to confirm the results, as described in the next section. The experimental design used was completely randomized. Each treatment consisted of spittlebugs randomly collected from each of the 20 EUs to assess the presence of the applied fungi.

2.10. Molecular Analysis of Fungal Samples Isolated from Mahanarva spectabilis Corpses

After macro- and micromorphological analyses, the fungal isolates from all treatments (including the control) were grouped, and from each group, a random sample was selected with characteristics similar to those of the fungi applied for molecular analysis, totaling 18 samples for molecular analysis. Filamentous fungi were inoculated on Sabouraud dextrose agar for seven days. The DNA of the isolates was extracted according to the method described by Doyle and Doyle [73] from microorganisms grown in culture medium. The extracted genomic DNA sample was subjected to polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) region of the rDNA using the SR6R (5′-AAGWAAAAGTCGTAACAAGG-3′) and LR1 (5′-GGTTGGTTTCTTTTCCT-3′) primer oligonucleotides [74]. The PCR mixture consisted of 1 µL of DNA, 1 µL of each primer at 10 µM, 10 µL of 5X PCR buffer, 1 µL of dNTPs at 10 mM, 0.2 µL of GoTaq DNA polymerase (5 U/µL; Promega, Madison, WI, USA), and 35.8 µL of autoclaved Milli-Q H2O, for a final volume of 50 µL. The amplification program was as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s and a final extension at 72 °C for 4 min. The amplified products were verified by electrophoresis on a 0.8% agarose gel stained with ethidium bromide. The amplified products were purified by precipitation with polyethylene glycol [75], subjected to sequencing by the chain termination method using Big Dye 3.1 reagent (Applied Biosystems, Waltham, MA, USA), and analyzed in a 3500 xL automatic capillary sequencer (Applied Biosystems).

2.11. Statistical Analysis

In the greenhouse bioassay evaluating the efficiency of different methods in controlling *M. spectabilis*, the treatment mortality data were corrected for natural control mortality, where there was no presence of pathogens other than those applied, using Abbott's formula [76] with 95% confidence intervals based on the average of 20 repetitions per treatment: $Ma = (Mt - Mc)/(100 - Mc) \times 100$, where Ma is the mortality corrected for the control treatment, Mt is the mortality observed in the treatment with the insecticide, and Mc is the mortality observed in the control treatment [77]. Before the statistical analyses, all the data were tested for normality and homogeneity of variance. The data were compared among treatments using analysis of variance (ANOVA), and the Scott–Knott test was used to compare the means at a 5% ($p < 0.05$) error probability.

Data on the cause of death and the presence of fungi applied to the plant tissue and sap of *U. brizantha* plants were compared among treatments using Pearson's chi-square test. Their associations were assessed using Cramer's V.

The normality of the data was verified using the Kolmogorov–Smirnov test, and the homogeneity of variance was assessed by Bartlett's test. Data obtained in the field were extrapolated to the number of spittlebugs per m^2, tabulated, and compared between treatments using ANOVA and a nonparametric Kruskal–Wallis analysis followed by Dunn's test (5% significance level). All the statistical analyses were performed with SPSS Statistics (version 22).

3. Results

3.1. Fungal Strain Identification

BLASTn analysis revealed that the fungal strains UFMGCB 11443 and UFMGCB 11444 were *F. multiceps* and *M. anisopliae*, respectively. The fungi displayed ITS and Pol II sequences with high query cover and identified ≥99% of the respective species.

3.2. Analysis of the Persistence of Fungi in the Tissue and Sap of Urochloa brizantha

Analysis of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in *U. brizantha* revealed no significant differences ($p = 0.880$) among cultures of fungi isolated from the leaves or sap, regardless of whether they were inoculated via the leaves or seeds. Fungi with characteristics similar to those of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 were absent in non-inoculated plants (controls) (Table 2). The degree of association among fungi present in the leaves or sap was 23.4% according to Cramer's V test, but this difference was not significant according to Pearson's chi-square test ($\chi^2 = 6.601$, df = 7, $p = 0.880$).

Table 2. The percentage of fungi present in the sap or leaves of *U. brizantha* plants treated with *Fusarium multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 via foliar or seed treatment, and percentages of fungi present in *U. brizantha* leaves at different ages.

Applied Fungi	Analyzed Site	Inoculation Site	% Presence	Chi-Square Test	Ages	% Presence	Chi-Square Test
Fusarium multiceps UFMGCB 11443	Sap	Seeds	80	$X^2 = 6.601$ $p = 0.880$ V = 0.234 GL = 7	New	80	$X^2 = 1.867$ $p = 0.867$ V = 0.176 GL = 5
		Leaves	80		Young	80	
	Leaves	Seeds	80		Senescent	60	
		Leaves	60				
Metarhizium anisopliae UFMGCB 11444	Sap	Seeds	80		New	80	
		Leaves	70		Young	80	
	Leaves	Seeds	70		Senescent	70	
		Leaves	70				
Control	Sap	Seeds	0		New	0	
		Leaves	0		Young	0	
	Leaves	Seeds	0		Senescent	0	
		Leaves	0				

Similarly, no significant differences were found in the presence of *F. multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 at different leaf ages (p = 0.867). The degree of association among leaf ages in the treatments was 17.6% according to Cramer's V test, and this was not significant according to Pearson's chi-square test (χ^2 = 1.867; df = 5, p = 0.867). Notably, ≥60% of the analyzed plants contained the inoculated fungi (Table 2), showing the endophytic capacity and persistence of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 fungi in the plants. The presence of the inoculated fungi was confirmed by molecular analysis after morphological grouping and microculture slide analysis of the *U. brizantha* plant isolates used in this study and analysis of the sequences deposited in GenBank (Supplementary Table S1).

3.3. Greenhouse Bioassay with Mahanarva spectabilis and Cause of Death

All *M. spectabilis* samples collected from plants inoculated with *F. multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 showed conidia of the inoculated fungi on their corpses. The isolates from the control plants were not used in this analysis because they did not contain any isolates from dead insects with macro- or micromorphology similar to that of the inoculated fungi, representing <5% of the samples. The degree of association between the tested fungi was 100% according to Cramer's V test and significant according to Pearson's chi-square test (χ^2 = 100.0, df = 4, $p \leq 0.001$). After grouping 205 fungal isolates extracted from spittlebug nymphs based on the macromorphological and microscopic characteristics of their microcultures, the morphotypes of the applied fungi predominated, indicating that the insect deaths were caused by the applied fungi, which was confirmed by molecular analysis and the sequence deposited in GenBank (Supplementary Table S1).

In *U. brizantha* plants inoculated via foliar spraying, *M. spectabilis* nymph mortality from the sixth day after the start of the experiment was 83% for *F. multiceps* UFMGCB 11443 and 73.6% for *M. anisopliae* UFMGCB 11444. After 10 days, both fungi showed 100% efficiency in controlling *M. spectabilis* via foliar inoculation. In *U. brizantha* plants inoculated via seed treatment, *M. anisopliae* UFMGCB 11444 and *F. multiceps* UFMGCB 11443 caused 58.2% and 65.6% epizootic effects in the spittlebugs exposed to the plants for six days, respectively, with the values increasing to 97.9% and 91.9%, respectively, by the tenth day (Table 3). These results highlight the efficiency and ability of these fungi to control spittlebug populations through the endophytic route (Figure 1).

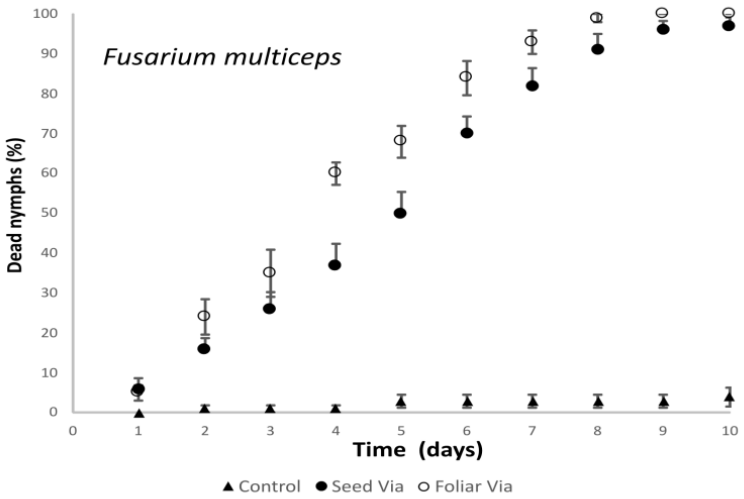

Figure 1. *Cont.*

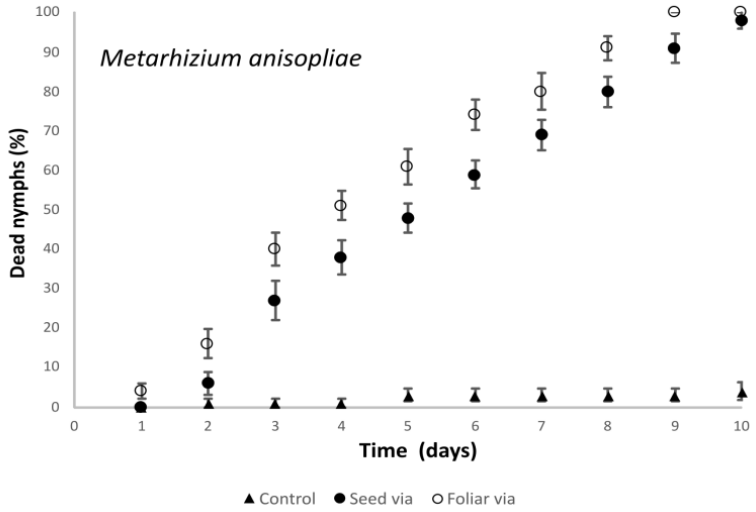

Figure 1. The cumulative percentage mortality of *Mahanarva spectabilis* nymphs fed on *Urochloa brizantha* plants inoculated via seeds or foliar spraying with the entomopathogenic fungus *Fusarium multiceps* UFMGCB 11443 or *Metarhizium anisopliae* UFMGCB 11444 or on non-inoculated control plants. The dots and bars indicate the means ± standard errors of 20 pots/fungi, with five nymphs per plant.

Table 3. The Abbott-corrected mean efficiency for the mortality of infected *M. spectabilis* nymphs in *U. brizantha* plants treated with *Fusarium multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 six and 10 days after insect contact.

Application via	*Fusariummulticeps*		*Metarhizium anisopliae*	
	6 days	10 days	6 days	10 days
Control	2.7 ^{a} ± 1.5	3.8 ^{a} ± 2.2	2.7 ^{a} ± 1.5	3.8 ^{a} ± 2.2
Seeds	65.6 ^{b} ± 5.5	91.9 ^{b} ± 5.3	58.2 ^{b} ± 3.4	97.9 ^{b,c} ± 2.1
Foliar	83.8 ^{c} ± 4.3	100.0 ^{c} ± 0.0	73.6 ^{c} ± 3.9	100.0 ^{c} ± 0.0
ANOVA	$F_{(119,5)} = 136.48$; $p \leq 0.05$		$F_{(119,5)} = 292.81$; $p \leq 0.05$	

Means with distinct letters in the columns indicate significant differences according to Tukey's test ($p < 0.05$).

3.4. Field Bioassay of Mahanarva spectabilis and Cause of Death

In the field, the applied fungi could maintain the nymphal and adult *M. spectabilis* populations at values lower than those in the control treatment group with only one spraying. A significant reduction in the insect pest population was observed after 15 days at all doses of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 used (Figure 2A–G). This period reflected the highest number of live nymphs/m^2 in the control treatment (Figure 2H). Therefore, using the lowest dose of either fungus is recommended because it is more economical, facilitates application, and is sufficient to contain the spittlebug infestation cycle in pastures.

The lowest fungal dose of 0.5 kg/ha had the same effect as the fourfold higher dose, with an appreciable decrease in insect pest infestation observed (Table 4). The effect of mixed suspension of *F. multiceps* and *M. anisopliae* at 1.0 kg/ha did not significantly differ from that of the same concentration of each fungus alone after six (p = 0.686) and 15 (p = 0.520) days of application (Table 4). Isolates extracted from dead spittlebug nymphs from the field were grouped based on the macromorphological and microscopic characteristics of their microcultures. The morphotypes of the applied fungi predominated, indicating that the insect deaths were due to the applied fungi, which was confirmed by molecular analysis and the sequence deposited in GenBank (Supplementary Table S1).

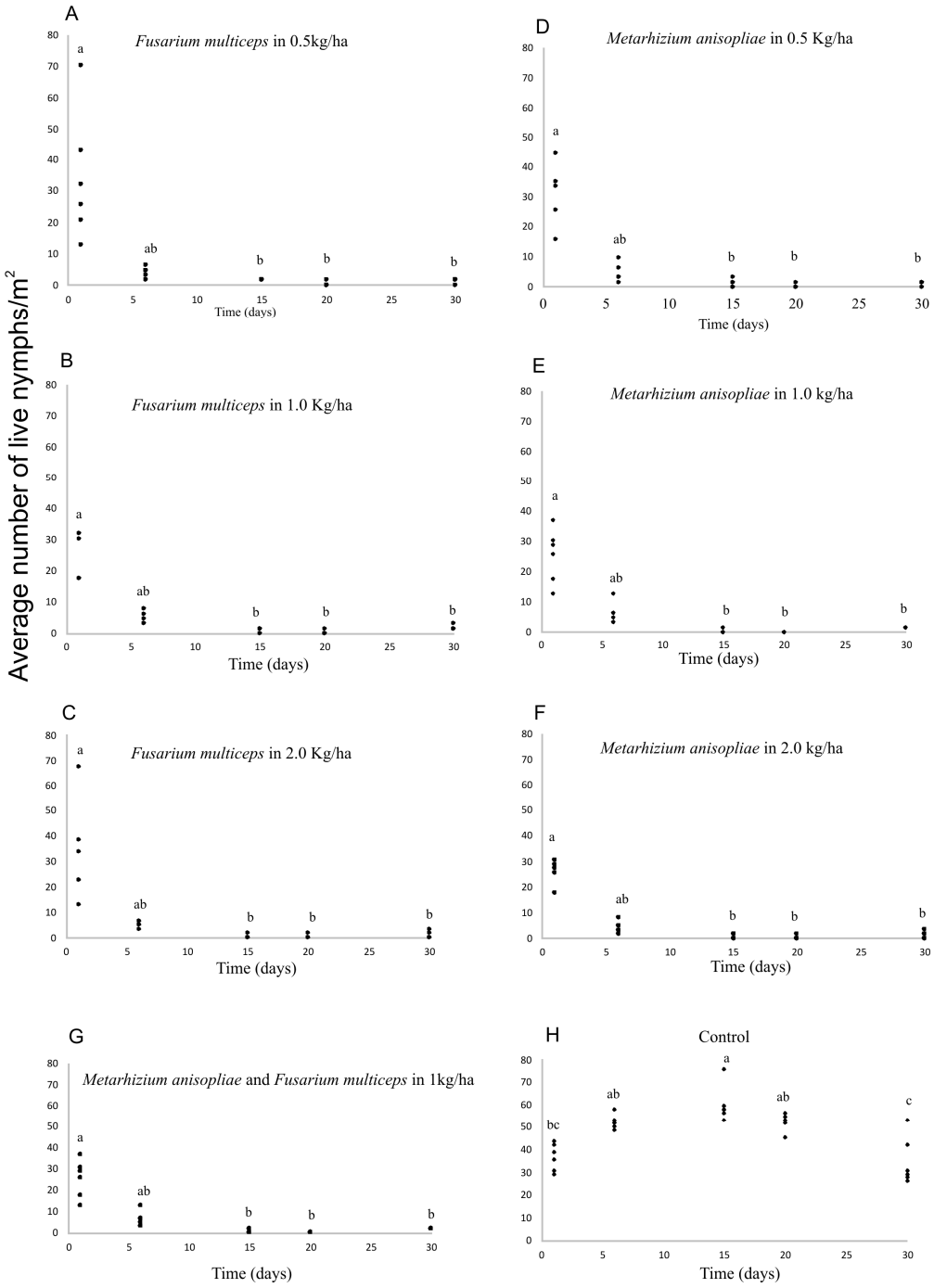

Figure 2. Average number of *Mahanarva spectabilis* nymphs/m^2 in *Urochloa brizantha* pastures sprayed with the entomopathogenic fungus *Fusarium multiceps* UFMGCB 11443 (**A**–**C**) or *Metarhizium anisopliae* UFMGCB 11444 (**D**–**F**) at different concentrations (0.5, 1.0 and 2.0 kg/ha), with both fungi combined in the same solution (**G**), or with the control solution (**H**). The numbers of nymphs/m^2 at different times with different letters were significantly different according to Dunn's test (5% significance level) following the Kruskal–Wallis test, with six replicates per time point.

Table 4. Mortality of *Mahanarva spectabilis* nymphs and adults/m^2 at six and 15 days after applying the fungus *Fusarium multiceps* UFMGCB 11443 or *Metarhizium anisopliae* UFMGCB 11444 at three different concentrations or mixed application of both fungi at 1.0 kg/ha each in *Urochloa brizantha* pastures. Kruskal–Wallis nonparametric analysis was performed.

Fungus	Dose	Median	Kruskal—Wallis
Fusarium multiceps 6 days	0.5 kg/ha 1.0 kg/ha 2.0 kg/ha	85.94 77.17 85.37	H = 4.58 *df* = 2 *p* = 0.101
Fusarium multiceps 15 days	0.5 kg/ha 1.0 kg/ha 2.0 kg/ha	95.31 93.48 99.80	H = 5.58 *df* = 2 *p* = 0.062
Metarhizium anisopliae 6 days	0.5 kg/ha 1.0 kg/ha 2.0 kg/ha	89.92 77.89 85.00	H = 3.43 *df* = 2 *p* = 0.180
Metarhizium anisopliae 15 days	0.5 kg/ha 1.0 kg/ha 2.0 kg/ha	97.48 99.00 97.00	H = 0.05 *df* = 2 *p* = 0.977
Fusarium multiceps 6 days	1.0 kg/ha	**77.17**	**H= 0.753** ***df* = 2** ***p* = 0.686**
Metarhizium anisopliae 6 days	1.0 kg/ha	**77.89**	
Fusarium multiceps + *Metarhizium anisopliae* 6 days	1.0 kg/ha/each fungus	81.63	
Fusarium multiceps 15 days	1.0 kg/ha	93.48	H= 2.81 *df* = 2 *p* = 0.52
M. anisopliae 15 days	1.0 kg/ha	99.00	
Fusarium multiceps + *Metarhizium anisopliae* 15 days	1.0 kg/ha/each fungus	96.23	

4. Discussion

Endophytic entomopathogens can influence herbivore population dynamics by reducing the populations of pests [78]. This observation corroborates other studies on the endophytic establishment of entomopathogenic fungi in a variety of cultivated plants [22,79–81]. Some of these fungi are vertically transmitted via seeds from one generation to another. Some endophyte genera form a distinct group of fungi related to the ecological requirements and discrete adaptations of grasses [82]. Various *Metarhizium* spp. naturally associate with the roots of grasses, shrubs, herbs, and trees in the field [79,83] and can control various insect pests.

This study revealed that the use of entomopathogenic fungi as endophytes in plants provides diverse opportunities that can benefit the production chain of these plants in agroecosystems at likely more affordable costs. Therefore, the inoculation of forages with the entomopathogenic fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 via the endophytic route has promise as a defensive strategy. Thus, these fungi could be candidates for new bioproducts. Unlike synthetic insecticide products, according to Zimmermann, the fungi likely do not affect the production chain, the environment, or human health [68]; generate less chemical waste; and add value to the final product. Reducing agrochemical use is a trend in developed countries that are increasingly using sustainable healthy agriculture. These organizations aim to participate in the competitive market for organic products that are increasingly appreciated by society.

Seed treatment with entomopathogenic fungi is a promising technology for the protection and sustainable production of crops without adverse effects on plant performance or

final products [45,79,84–86]. However, the main advantage of using biocontrol agents is their ability to regulate insect pests in a more stable manner and direct or indirect biocontrol activity. Therefore, different methods of inoculating plants with fungi that could act as endophytes can effectively reduce the spittlebug population in pastures.

This study confirmed the presence of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 as endophytes. The plants were inoculated via foliar spraying or seed treatment, and the fungi were then reisolated from the leaves or sap since they were possibly present systemically. Some fungi, such as *Metarhizium robertsii* Bischoff, Rehner & Humber and *Beauveria bassiana* (Bals.) Vuill., penetrate and colonize their hosts systemically. These proteins penetrate through the stomata and persist in the epidermal cells of *Zea mays* L., growing through the air spaces between the plant parenchymal cells [87] or roots and leaves [79].

There were no significant differences between inoculation by foliar spraying and seed treatment, indicating that both techniques are efficient for plant colonization. Fungal propagation by passive transmission leads to random diffusion into plant tissue [80]. This might explain the action of the fungi used in this study when applied via seeds. The potential of seed treatment with entomopathogenic fungi is a promising technology for the protection and sustainable production of crops without adverse effects on plant performance or final products [45,79,86]. In addition, the greatest barriers to the use of products based on entomopathogenic fungi, e.g., abiotic effects, should be mitigated. Endophytes protect fungi from these effects, as they reside within plant tissues. Therefore, different methods of inoculating plants with fungi that act as endophytes can effectively reduce the spittlebug population in pastures.

Treating seeds in conidial suspensions is an alternative artificial colonization method to induce successful endophyte colonization in many cultivated plants [88]. *Metarhizium* spp. have been studied as insect pathogens and are increasingly being investigated for their beneficial effects on host plants [79,89–93]. The seeds of corn plants were treated with this fungus, indicating that *M. robertsii* was systemically established in the maize plants [79]. Kabaluk and Ericsson [91] obtained promising results with maize seeds treated with *M. anisopliae*, including purportedly minimized damage caused by maize wireworms (*Agriotes obscurus* L.) (Coleoptera: Elateridae). Members of this genus also have effects when applied to the seeds of *U. brizantha* to control *M. spectabilis*.

Santos et al. (2019) [94] identified 29 isolates of entomopathogenic fungi of the genus *Fusarium* associated with Hemiptera. The species *F. multiceps* belongs to the *Fusarium incarnatum-equiseti* species complex (FIESC) [95]. This group is also associated with insects [96]. Several of these strains have the ability to control agricultural pests with high mortality rates of insect pests, exhibiting rapid action and abundant sporulation, making them ideal for use in microbial control [97]. Several species of the genus *Fusarium* are endophytes associated with *Rosa* (Rosaceae), many of which have not yet been identified at the species level [98]. An endophytic strain of *F. multiceps* was isolated from *Acanthus ilicifolius* L. (Acanthaceae), which produces secondary metabolites with biological characteristics suitable for use in agriculture and medicine [99]. This work is the first report of *F. multiceps* inoculated into *U. brizantha* as a vector for controlling *M. spectabilis*.

Among other genera, fungi such as *Beauveria bassiana* and *Isaria fumosorosea* (Wize) Brown & Smith Mantzoukas and Lagogiannis [85] have been reported to have high pathogenicity in insects when inoculated into plants [86]. Fungi grow systemically on all aboveground plant organs, resulting in the vertical transmission of endophytes via seeds [79,100]. Other studies have reported findings similar to those of the current study on the ability of fungi to adapt to plants and promote mutualistic interactions, inducing defense mechanisms against herbivores in plants and thereby decreasing their insect population [76,101].

For entomopathogenic fungi to successfully colonize both plants and insects, they must be able to invade and establish themselves in both organisms [35]. Our findings revealed that the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 cause

epizootic effects in greenhouses and in the field. The results of this work are supported by studies demonstrating the systemic colonization of several plant species by *Metarhizium* spp. [79,102].

The mortality of spittlebugs in a greenhouse exposed to plants inoculated with *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 indicates that the induction of various defense responses, which act directly as insect pest mortality factors, provides protection to plants and reduces herbivory. This fungus—plant—insect interaction was also observed by Branine et al. [36], who used *M. robertsii* as an endophyte to control cotton leafworm (*Spodoptera littoralis* Boisduval) (Lepidoptera: Noctuidae). However, few studies have examined treating other seed species with entomopathogenic fungi as endophytes [103,104]. This is the first study in which *U. brizantha* seeds were treated with entomopathogenic fungi.

This study showed that the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in the field attacked nymphs at the base of the plants when inoculated through foliar spraying. Based on these considerations, *Metarhizium brunneum* (Metchnikoff) and *B. bassiana* are widely used as bioprotectors and infest soil-dwelling or larval-dwelling insects [105,106]. Therefore, these means of infestation are ideal for reaching spittlebug nymphs living in foams at the base of *U. brizantha* plants. The population of *M. spectabilis* spittlebugs in the field was controlled by only one application of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444, which, in addition to the lower dose, was more economical for the producer. Consistent with this study, a product based on *M. anisopliae* had the necessary efficiency to maintain the size of the spittlebug population below its actionable limit after *M. anisopliae*-based biopesticide application in the field [22].

The fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 were reisolated from the corpses of nymphs or adult spittlebugs of *M. spectabilis* from both field and greenhouse samples. Klieber and Reineke [69] also reisolated *B. bassiana* from corpses of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) after the fungus was originally inoculated into *Solanum lycopersicum* L. cv. Harzfeuer (Solanaecae) via foliar spraying.

Bioinputs, including those based on fungi, are a trend in developed countries where integrated and ecologically friendly agriculture has already been implemented [106]. In this context, Brazil is starting to use sustainable products, such as entomopathogenic fungi, for pest control. In 2023 alone, 90 products were registered for biological control in Brazil [101]. Using grasses as hosts for entomopathogenic fungi is a low-cost innovation for producers, in addition to its benefits in promoting sustainable meat and milk production without harming the environment and generating products without toxic residues [68]. The demonstration of the efficiency of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in the endophytic pathway of *U. brizantha* provides biological control alternatives that producers might be more willing to use, given their properties. Given the efficiencies obtained in greenhouses and in the field, and the possibility of using *U. brizantha* as a host for entomopathogenic fungi, these results provide insights for mitigating some challenges in pastures.

5. Conclusions

The entomopathogenic fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 are endophytic and colonize the tissues of *U. brizantha*, which makes them a viable and effective alternative for reducing the population of *M. spectabilis* spittlebugs, whether inoculated via seeds or foliar spraying. Therefore, these fungi are promising candidate bioproducts for controlling this pest.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050943/s1, Table S1: Confirmation of fungi applied via foliar or seed of Urochloa ruziziensis isolated from nymphs feeding on these plants in the field or greenhouse, and from plant tissue after infestation. GenBank accessions of the sequences.

Author Contributions: M.O.C. conceived and designed the study; A.M.A. identified the spittlebugs, designed the study, and reviewed and edited drafts; R.M.M. collaborated on the study and revised the manuscript; A.P.M. analyzed and interpreted the data; M.A.C. funded and provided the site for the field experiments; L.H.R. identified the fungi; M.F.A.P. collaborated on the methodology; M.M., S.R.O.S., N.B.M.S. and A.C.R.S. collaborated on the experiments; W.G.C. designed and supervised the study and analyzed and interpreted the data. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The author Michelle Oliveira Campagnani is a CNPq fellow. The other authors declare that the research was carried out in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. We confirm that all collection of plant material was conducted in accordance with relevant institutional, national, and international guidelines and legislation.

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