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Isolation and Characterization of Native Isolates of *Metarhizium* sp. as a Biocontrol Agent of *Hypothenemus hampei* in Rodríguez de Mendoza Province—Peru

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Abstract: Entomopathogenic fungi represent the pinnacle of efficacy among biological control agents when combating insect pests within natural ecosystems, combating them without altering the environment. This study aimed to isolate and characterize the morphology, physiology, and pathogenicity of native isolates of *Metarhizium* sp. isolated from soil samples from the province of Rodriguez de Mendoza. Eighteen native isolates of *Metarhizium* sp. were isolated and characterized. Colony coloration varied between yellow-gray, white, brown, and olive, with feathery or wavy edges. As for radial growth, the highest averages were obtained by isolates LLM-M2 and TOR-M16, with 43.15 mm and 42.85 mm, respectively. Conidia production at 15 days was higher for isolate LLM-M2 with 9.8×10^7 conidia/mL; in the percentage of germination at 14 h, the treatment that reached 100% germination was TOR-M16. Isolate CMR-M7 reached 97.49% mycelial growth percentage at 288 h, being the best result; in the percentage of mortality in CBB adults, the treatments that reached 100% were TOR-M9, TOR-M16, and MNR-M1. In general, the results demonstrate the entomopathogenic potential of native isolates of *Metarhizium* sp. acting as biocontrol agents of *Hypothenemus hampei*, being a low-cost, easily accessible, and environmentally friendly alternative.

Keywords: biocontrol; bioassay; coffee rhizosphere; biological regulation; pathogenicity

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1. Introduction

Coffee (*Coffea arabica* L.) is among the most important and valued tropical crops in terms of exports worldwide, with an annual production of some 10.5 million tons [1]. In Peru, the coffee industry occupies a prominent position, representing the country's most important agricultural export product. It ranks third among the leading exporters in South America and twelfth worldwide [2]. However, production costs have increased due to phytosanitary problems, climate change, and other factors such as biotic and abiotic stress [3]. The greatest economic losses have been caused by *Hypothenemus hampei*, which affects the main coffee-producing areas of the globe [4].

The coffee berry borer [*Hypothenemus hampei* (Coleoptera: Curculionidae)] is a coleopteran deemed a devastating pest with great economic importance worldwide, often exceeding the threshold of economic damage and directly affecting the yield and quality of cherries [5].

This is because its progeny develops in galleries made by the female inside the coffee cherry, generating a decrease in bean weight; it also causes premature fruit drop due to early infestations and allows the opportunistic attack of other pests and diseases [6]. This pest can proliferate quickly, impacting as much as 95% of the cultivated zones worldwide [7]. In Peru, the spread of the coffee berry borer has increased in all coffee production regions due to a lack of technology implementation. In the province of Rodriguez de Mendoza, this pest affected around 35% of all farms [8].

Coffee berry borer control depends on chemical products, which impacts the organic production of Peruvian specialty coffees, representing a fairer price for farmers due to their high demand in the international market [9]. Biopesticides are presented as an option to develop sustainable agriculture. They will allow the substitution of chemical substances, be it an environmentally friendly product, and focus on integrating biological enemies of the pest in the same agroecosystem [10].

The fungus *Metarhizium* sp. is an option for the biocontrol of *H. hampei*, which is found as endophytes in woody plants such as coffee and in soils in agricultural fields [11]. The entomopathogenic potential of this genus lies in its preference for and effectiveness against coleoptera [12]. For this reason, strains of *Metarhizium* sp. represent 33.9% of the biopesticides used to control the coffee berry borer [13]. The mechanism it exerts lies in producing secondary metabolites that affect the pest, specifically destruxins, responsible for inducing paralysis [14].

In order to take advantage of the diversity of entomopathogenic fungi in different coffee-producing agroecosystems in Rodríguez de Mendoza province, the objective of this research was to isolate, identify, and evaluate the entomopathogenic capacity of different native isolates of *Metarhizium* sp. against *H. hampei* in the province of Rodríguez de Mendoza—Peru.

2. Materials and Methods

2.1. Collection of Isolates of Metarhizium sp.

Soil samples were collected from different organic coffee farms in Rodríguez de Mendoza province (Figure 1 and Table 1). Within each farm, five coffee trees were selected randomly; for each coffee tree, five soil subsamples (at a depth of 20 cm) were collected in previously coded polyethylene bags. All the subsamples collected from each farm were homogenized, and a final sample of 300 kg was obtained [15]. The samples were processed at the Laboratorio de Investigación en Sanidad Vegetal (LABISANV) of the Instituto de Investigación para el Desarrollo Sustentable de Ceja de Selva (INDES-CES) of the National University Toribio Rodríguez de Mendoza of Amazonas, Peru.

Table 1. Geographical location of sampling points of *Metarhizium* sp. isolates in Rodríguez de Mendoza province.

Collection Points	Code	District (Village)	UTM Coordinates		Altitude (Masl)
			East	North	
T1	MMR-M1	Mariscal Benavides (Michina)	221265	9292992	1565
T2	LLM-M2	Longar (Longar)	218471	9293560	1586
T3	CCR-M3	Cochamal (Cochama)	214885	9291139	1598
T4	MHR-M4	Huambo (Miraflores)	220603	9287599	1661
T5	PTR-M5	Totora (La Perla)	228487	9281802	1757
T6	MMR-M6	Milpuc (Milpuc)	230505	9280744	1662
T7	CMR-M7	Milpuc (Chontapampa)	233125	9281689	1689
T8	POR-M8	Omia (Pumamarca)	238132	9281904	1614
T9	TOR-M9	Omia (Tuemal)	232896	9286902	1431
T10	DHR-M10	Huambo (Dos Cruces)	218155	9289509	1645
T11	SRR-M11	Omia (Limón)	234775	9285337	1391
T12	PMR-M12	Mariscal Benavides (Pilancon)	222237	9293179	1585
T13	PHR-M13	Huambo (Puquio)	215684	9288975	1588

Table 1. *Cont.*

Collection Points	Code	District (Village)	UTM Coordinates		Altitude (Masl)
			East	North	
T14	TSR-M14	Santa Rosa (Trancaguaico)	224648	9287667	1729
T15	MMR-M15	Milpuc (Milpuc)	229996	9281264	1656
T16	TOR-M16	Omia (Gebil)	236088	9284363	1369
T17	MNR-M17	San Nicolás (Mito)	231472	9288826	1478
T18	GOR-M18	Omia (Gebil)	235997	9284919	1391

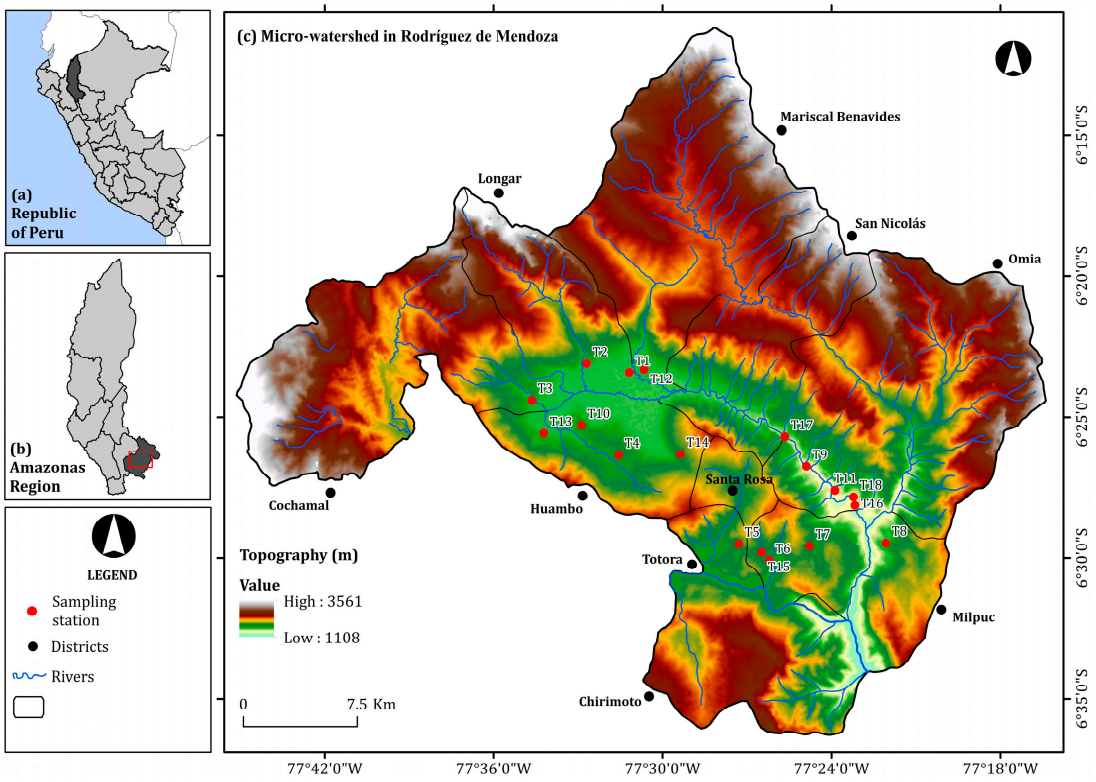


Figure 1. Collection sites of native isolates of *Metarhizium* sp. in Rodriguez de Mendoza province.

2.2. Isolation of Native Isolates of Metarhizium sp.

With some modifications, the "Galleria bait method" outlined by Zimmermann [16] was used for isolation. From each homogenized sample, 150 g of soil was taken and deposited in a sterile plastic container. Two adults of *Metamasius hemipterus* were immediately placed in the soil and incubated at 27 °C for seven days. Subsequently, dead larvae were removed and placed in humidity chambers to promote the growth of the entomopathogenic fungus. Adults with mycelial growth on their cuticle were used for the isolation and purification of the entomopathogenic fungus on potato dextrose agar (PDA) medium, which was incubated at 30 °C for seven days [17].

2.3. Morphological and Physiological Characterization of the Isolates

2.3.1. Macroscopic Morphology

Morphological characterization was carried out by observing macroscopic features such as color, texture, border, and type of spore production in the colonies. For this purpose, the purified isolates were grown on PDA medium and incubated at 30 °C for 30 days. The Munsell color chart [18] was used to describe color. Padilla et al.'s proposal [19] was used to describe color, texture, edge, and type of sporulation.

2.3.2. Microscopic Morphology

Microculture of Native Isolates of *Metarhizium* sp.

To carry out this procedure, 4 mm diameter discs were transferred from the edge of a PDA plate to a slide. The sporulated fungus was then inoculated onto the PDA discs using an inoculation rod. Subsequently, a paper towel was moistened with sterile distilled water, and the slides were incubated at 30 °C for five days [20].

After five days, semi-permanent slides were prepared using lactophenol blue. The coverslips with spores were carefully removed from the microcultures with forceps and transferred to previously prepared slides. This procedure was repeated for all slides of the microcultures. Subsequently, the mounts were dried with absorbent paper and sealed with clear nail polish for preservation. Microscopic structures were observed using a Leica® microscope (Leica Microsystems, Wetzlar, Germany), and images were captured to measure conidial length and width. Measurements of microscopic structures were performed using Image Tool 3.0 software.

2.3.3. Physiological Characterization of the Isolates

Radial Growth of Isolates of *Metarhizium* sp.

The procedure followed the methodology described by Torres et al. [21]. For physiological characterization, each growing colony's disk (4 mm in diameter) was transferred to a new Petri dish containing PDA medium and incubated at 30 °C for 20 days. According to the methodology of Padilla et al. [19], mycelial development was evaluated to determine radial growth; records were made millimetrically every two days until the twentieth day after sowing in the culture medium.

Measurement of radial growth is important, as it is considered a key indicator of the performance and growth capacity of *Metarhizium* sp. isolates. A faster radial growth rate may indicate a more robust and effective isolate in the field. This procedure maximizes conidial production and biological control efficacy under real-world conditions, ensuring that isolates are consistent and meet quality and efficacy standards.

Evaluation of Conidial Concentration in Isolates of *Metarhizium* sp.

The methodology proposed by Gómez et al. [22] was adapted to evaluate this variable, introducing slight modifications. The procedure began with preparing a stock solution, dissolving 1 g of *Metarhizium* sp. conidia in 100 mL of sterile water containing 0.01% Tween 80 in an Erlenmeyer flask. Using a magnetic stirrer, the conidia were homogeneously dispersed. Subsequently, 1 mL of this solution was extracted with a Pasteur pipette and transferred to a test tube for a 10^{-1} dilution. This procedure was repeated for the next dilution, taking 1 mL of the 10^{-1} dilution and adding it to 9 mL of sterile water, thus achieving the 10^{-2} dilution. To determine the concentration of conidia in the prepared dilutions, a 10 µL sample was extracted using a micropipette. This sample was carefully placed in a Neubauer chamber, allowing the conidia to settle for 2 min. The chamber was then examined under a Leica® microscope equipped with a 40X objective for conidial counting. The conidia in the Neubauer chamber were counted, and the concentration was calculated using Lipa and Slizynski's formula [23].

$$C = (Cc)(4 \times 10^6)(\frac{Fd}{80})$$

where

C = number of conidia. mL^{-1}.

Cc = average number of conidia counted in the Neubauer chamber.

Fd = dilution factor.

Evaluation of the Percentage Germination of *Metarhizium* sp. Spores

For the development of this activity, the methodology of Acuña et al. [24] was used, and serial dilutions of the spore suspension (10^{-1} to 10^{-2}) were prepared. To obtain

the 10^{-1} dilution, 1 mL of the spore suspension was taken, and 9 mL of sterile distilled water with a 0.01% solution of Tween 80 was added, vortexing the dilution for 1 min. This procedure was repeated until the necessary dilutions were obtained. From the spore concentrations, spores were taken for sowing in the laminar flow chamber, and 30 µL of the determined dilution of *Metarhizium* sp. were deposited with a micropipette in two spots of a Petri plate with PDA; the coverslip was placed on top and incubated at 30 °C. A total spore count, recording germinated and non-germinated spores, was performed 14 h after sowing.

The percentage of germination was calculated using the following formula:

$$\% \text{ Germination} = \frac{\text{number of germinated spores}}{\text{Total number of spores observed}}(100)$$

This test is crucial because it allows us to determine if *Metarhizium* sp. spores are alive and can give rise to active fungal growth. A high germination rate suggests a higher probability of success in pest control. In addition, they help to identify optimal conditions of temperature, moisture, and nutrients for *Metarhizium* sp. growth and development, which can be crucial for monitoring the quality of the final product and ensuring its efficacy in the field.

2.4. Determination of Pathogenicity and Mycelial Growth of Native Isolates of Metarhizium sp.

To carry out the pathogenicity evaluation in the laboratory, 18 isolates of *Metarhizium* sp. were used as the different treatments and a control treatment, as detailed in Table 1. The experimental procedure was carried out following the immersion method [25], which consists of immersing the borers for 1 min inside a grid in a 0.5% commercial sodium hypochlorite solution for disinfection; subsequently, they were immersed in sterile distilled water for 1 min three times to eliminate chlorine residues. Once disinfected, the borers were immersed for 1 min in concentrations adjusted for each treatment of *Metarhizium* sp. at 1×10^7 CFU/mL of water (CFU = colony-forming unit).

After inoculation, new Petri dishes were prepared, and sterile, dampened filter paper was positioned at the bottom of each one. Then, using forceps, ten inoculated borers were deposited on each plate along with three coffee beans to avoid cannibalism and starvation. Each Petri dish was hermetically sealed with parafilm and carefully labeled with treatment and replicate codes to facilitate subsequent evaluations. The Petri dishes were then carefully placed in an incubator at 27 °C for 12 days, ensuring that the filter paper received a controlled amount of sterile distilled water to maintain humidity levels without reaching saturation.

The pathogenicity evaluations of the fungal isolates were recorded for 12 days [25], recording the time of pathogenic mortality and the fungal cycle. The berry borers, whether deceased or living, were maintained within the Petri dishes to preserve the natural dynamics of the insects and the progression of fungal development. However, the last mortality record of the burs was made on day 8, so the evaluation ended at that time.

Once the pathogenicity test was completed, daily evaluations of treatments inoculated with the fungus were conducted by observing the insects for symptoms of infestation. These symptoms included the presence of hyphae and an initial white coloration that evolved to olive with sporulation. Evaluations of fungal mycelial growth were made on the bodies of the insects in each treatment, determining the percentage of infected individuals showing evidence of mycosis. In the control treatment, where insects were treated with sterile distilled water, no mortality greater than 10% and no signs of mycosis were expected.

Experimental Design

To evaluate the pathogenicity of 18 isolates of *Metarhizium* sp. against *H. hampei*, a completely randomized experimental design with a factorial arrangement was implemented, where 18 isolates and three evaluation times (24, 48, and 72 h) were considered. Each isolate and time combination was replicated three times, totaling 162 observations. In addition, a

negative control was included at each evaluation time to establish a baseline for pathogenicity, making a total of 171 observations for 57 experimental units. Each experimental unit was represented by a 9 cm diameter Petri dish. The experiment was performed only once.

2.5. Data Analysis

We began by contrasting the assumptions of normal distribution of the data and homoscedasticity of variances; the contrasts were performed with the Shapiro–Wilk and Levenne tests, respectively. Data meeting these assumptions underwent analysis of variance (ANOVA) followed by the ScottKnott test to identify significant differences between treatments. Data that did not conform to the assumption of a normal distribution were transformed using the square root function. Mortality data were analyzed by factorial analysis of variance (ANOVA) to determine the effects of isolate, time, and isolate–time interactions on pathogenicity. Means were compared using Tukey's test to identify significant differences between isolates and time combinations. In addition, the Kaplan—Meier survival analysis with its subsequent log-rank test was performed to compare the curves generated in search of significant differences. All statistical tests were run in the statistical program InfoStat/Professional v. 2018p.

3. Results

3.1. Morphological and Physiological Characterization of the Isolates

3.1.1. Macroscopic Characteristics of *Metarhizium* sp.

Table 2 presents the morphological characteristics of the 18 isolates studied. Macroscopic features were characterized by slow growth on the PDA medium at 30 °C. Colony coloration ranged from dark olive gray to yellow, gray, white, light reddish-brown, and olive, with feathery or wavy edges.

Table 2. Macroscopic morphological characterization of *Metarhizium* sp. isolates.

Labeled Sample	Type of Sporulation	Colony Edge	Colony Texture	Colors	Pigmentation
MMR-M1	Rings in the whole colony	Wavy	Cottony	Dark olive gray	Reddish yellow
LLM-M2	Rings in the whole colony	Wavy	Powdery	White	White
CCR-M3	Rings in the whole colony	Wavy	Powdery	Dark olive gray	Red
MHR-M4	Rings in the whole colony	Feathery	Cottony	Dark olive gray	Pale yellow
PTR-M5	Rings in the whole colony	Wavy	Powdery	Reddish yellow	Reddish yellow
MMR-M6	Rings in the whole colony	Wavy	Cottony	Dark olive gray	Yellowish brown
CMR-M7	Uniform with central rings	Feathery	Powdery	Dark olive gray	Yellow
POR-M8	Uniform with terminal rings	Feathery	Cottony	Dark olive gray	White
TOR-M9	Rings in the whole colony	Wavy	Powdery	Dark olive gray	Red
DHR-M10	Rings in the whole colony	Wavy	Powdery	Pale yellow	Pale yellow
SRR-M11	Uniform with central rings	Feathery	Cottony	Dark olive gray	Pale yellow
PMR-M12	Rings in the whole colony	Feathery	Cottony	Dark olive gray	Pale yellow
PHR-M13	Uniform with central rings	Feathery	Powdery	Dark olive gray	Red
TSR-M14	Rings in the whole colony	Wavy	Powdery	Dark olive gray	Pale yellow
MMR-M15	Rings in the whole colony	Feathery	Powdery	Dark olive gray	Pale yellow
TOR-M16	Rings in the whole colony	Wavy	Powdery	Dark olive gray	Pale yellow
MNR-M17	Uniform with central rings	Wavy	Powdery	Pale yellow	Pale yellow
GOR-M18	Uniform with central rings	Wavy	Powdery	Dark olive gray	Yellow

The type of sporulation observed was uniform with terminal rings, rings throughout the colony, and uniform with central rings. The texture exhibited by the isolates was cottony or powdery, with pigmentation ranging from pale yellow to yellowish red, yellowish brown, white, and reddish yellow (Table 2, Figures 2–7), all typical characteristics of the genus *Metarhizium* sp.

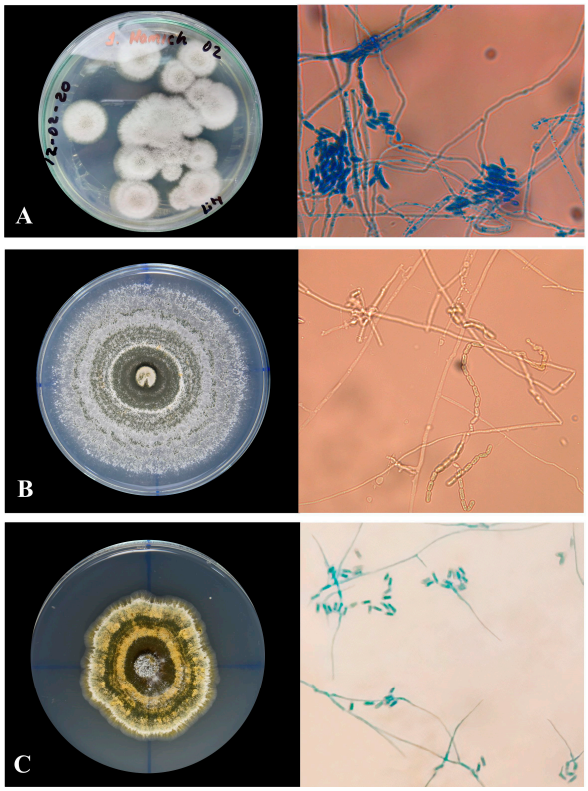


Figure 2. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of MMR-M1 (**A**), LLM-M2 (**B**), and CCR-M3 (**C**).

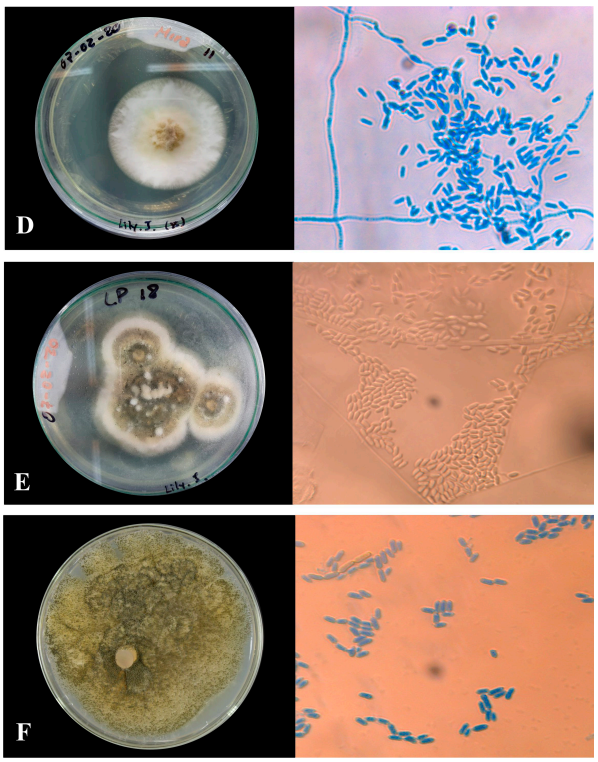


Figure 3. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of MHR-M4 (**D**), PTR-M5 (**E**), and MMR-M6 (**F**).

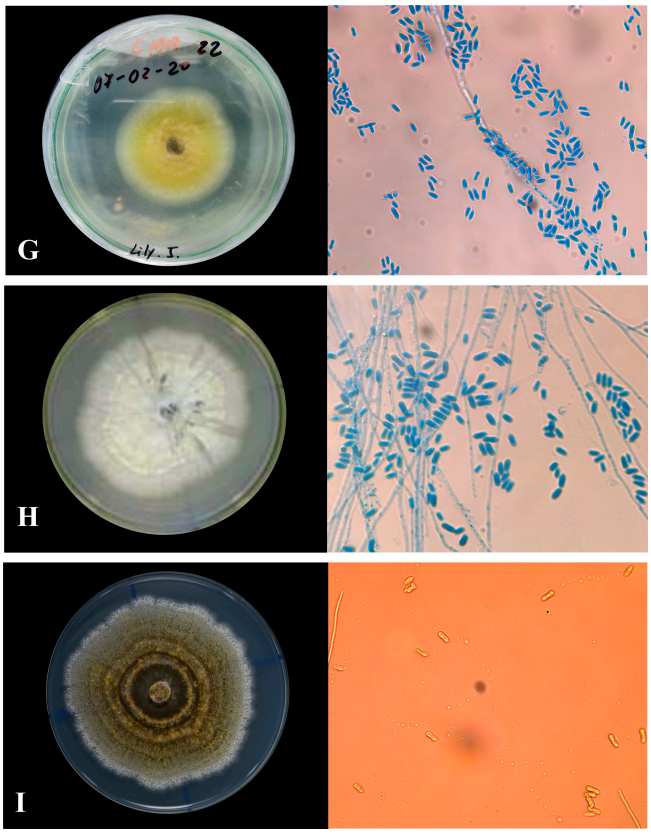


Figure 4. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of CMR-M7 (**G**), POR-M8 (**H**), and TOR-M9 (**I**).

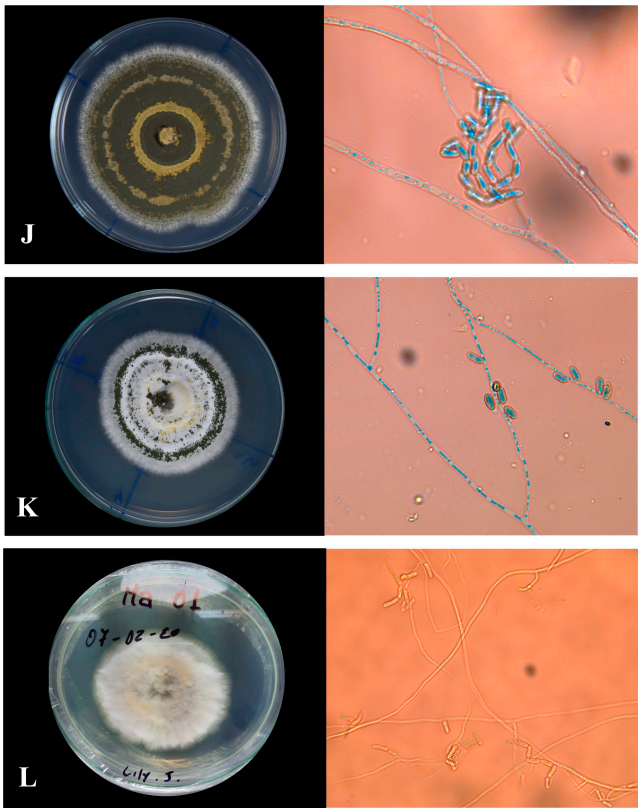


Figure 5. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of DHR-M10 (**J**), SRR-M11 (**K**), and PMR-M12 (**L**).

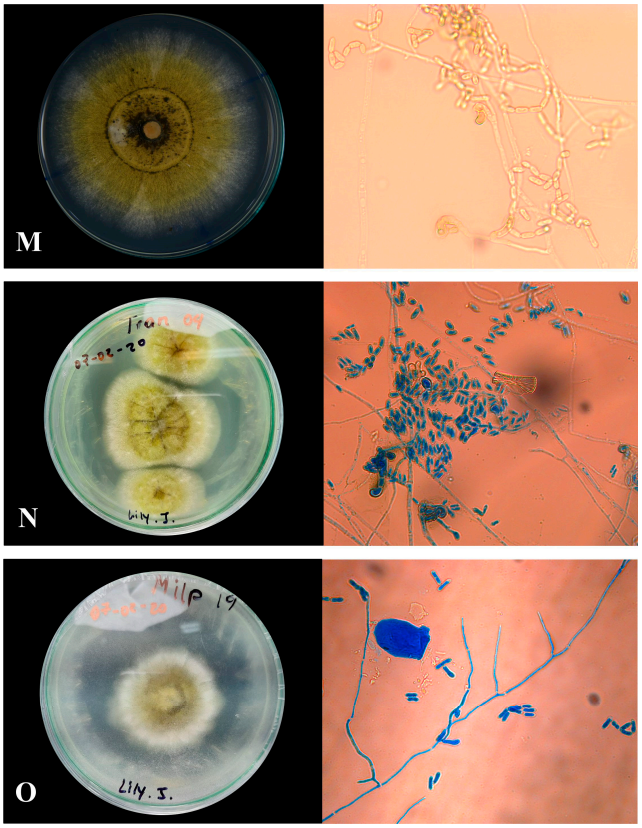


Figure 6. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of PHR-M13 (**M**), TSR-M14 (**N**), and MMR-M15 (**O**).

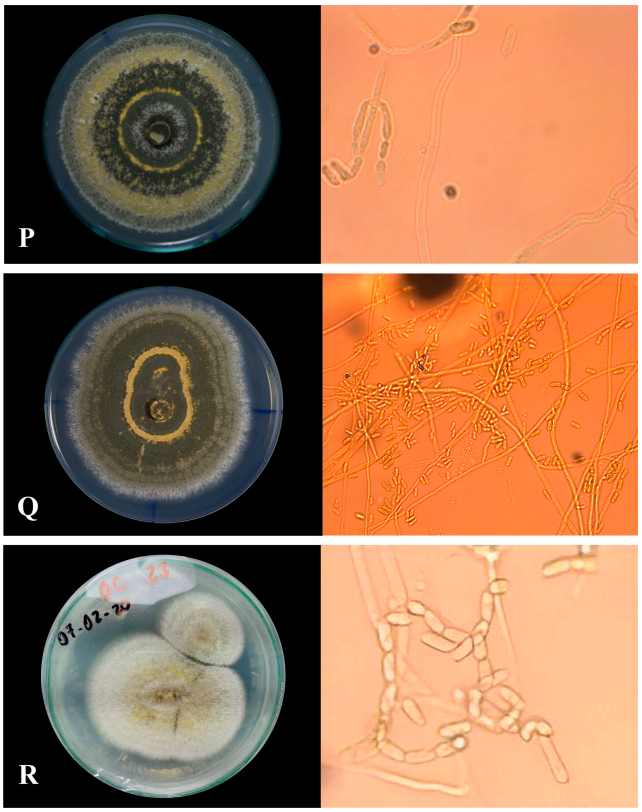


Figure 7. Colonies of 20-day-old *Metarhizium* sp. on potato dextrose agar (PDA) medium, showing the macroscopic and microscopic characteristics of TOR-M16 (**P**), MNR-M17 (**Q**), and GOR-M18 (**R**).

3.1.2. Microscopic Characterization

(A) Conidial length

For this variable, three isolates of *Metarhizium* sp. reached the highest averages and were significantly different from the others: MHR-M4, POR-M8, and PHR-M13, with values of 7.2, 7.6, and 7.8 µm, respectively (Figure 8).

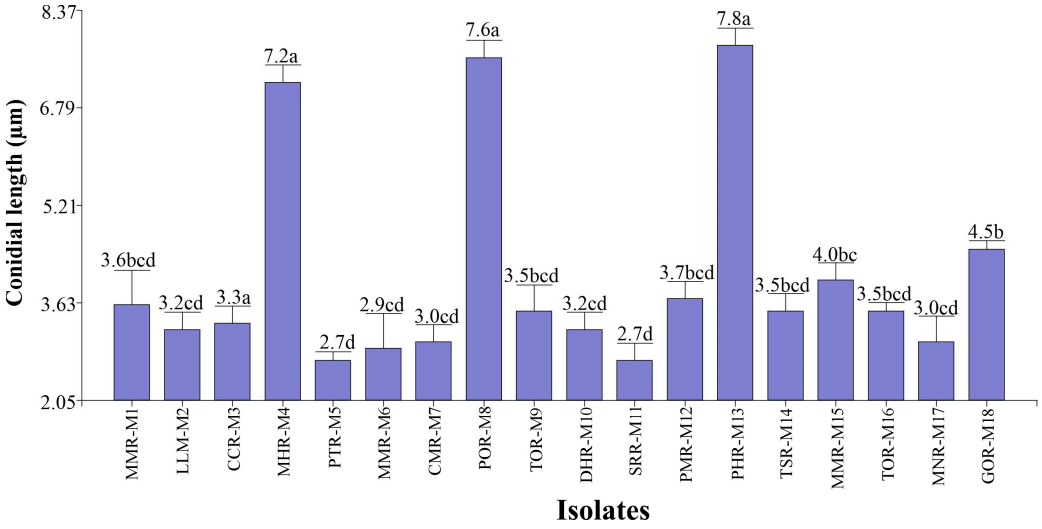


Figure 8. Conidial length of 18 isolates of *Metarhizium* sp. Significant differences between groups were denoted by distinct letters, as determined by the Skott—Knott test ($p \leq 0.05$).

(B) Conidial width

Figure 9 shows the width of the isolates of *Metarhizium* sp. Isolates MHR-4, POR-M8, and PHR-M13 stood out with the highest averages, recording 2.8, 2.1, and 2.3 µm, respectively.

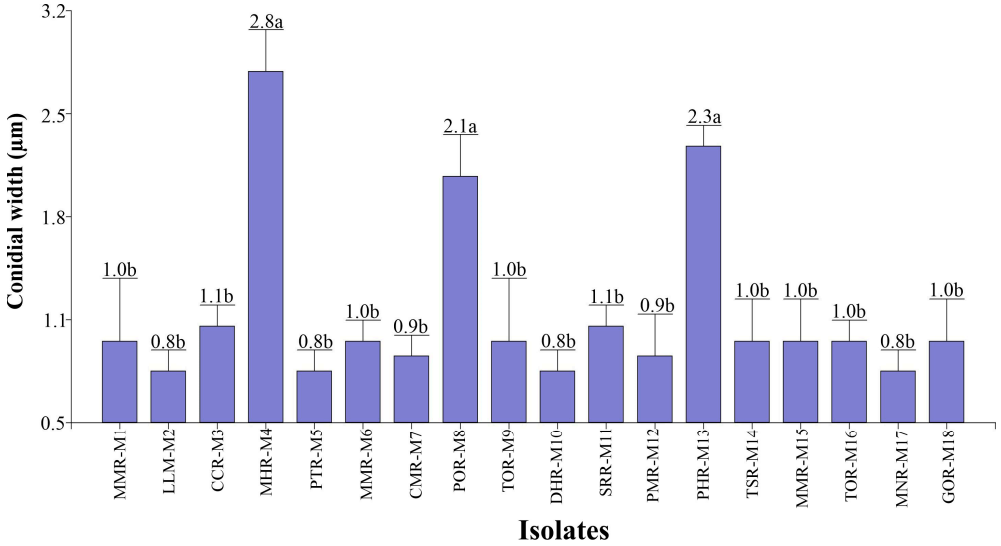


Figure 9. Conidial width of 18 isolates of *Metarhizium* sp. Significant differences between groups were denoted by distinct letters, as determined by the Skott—Knott test ($p \leq 0.05$).

3.1.3. Physiological Characterization of Isolates of *Metarhizium* sp.

(a) Radial growth of isolates of *Metarhizium* sp.

The treatments that achieved the highest radial growth were LLM-M2 and TOR-M16, with 43.15 mm and 42.85 mm, respectively, with significant differences concerning the other isolates (Supplementary Table S1).

(b) Conidial concentration in isolates of *Metarhizium* sp.

After 15 days, the LLM-M2 treatment had the highest concentration of conidia, with 9.8 $\times 10^7$ spores/mL, significantly different from the other treatments evaluated (Supplementary Table S1).

(c) Germination percentage of isolates of *Metarhizium* sp.

The treatments that exceeded 95% germination were DHR-M10 with 95.5%, LLM-M2 with 98%, and TOR-M16 with 100% at 14 h; however, they did not show significant differences (Supplementary Table S1).

3.2. Pathogenicity and Mycelial Growth of Native Isolates of Metarhizium sp.

The factorial analysis of variance performed to evaluate the pathogenicity of different isolates of *Metarhizium* sp. at different times yielded statistically significant results (Table 3). The overall model was highly significant (F = 32.54, $p < 0.0001$), indicating that both main and interaction effects contribute significantly to the variability in observed pathogenicity.

Table 3. Factorial analysis of variance.

F.V.	SC	gl	CM	F	*p*-Value
Model	186,499.42	56	3330.35	*32.54*	*<0.0001*
Isolate	29,210.53	18	1622.81	*15.86*	*<0.0001*
Time	139,945.03	2	69,972.51	*683.73*	*<0.0001*
Isolate * time	17,343.86	36	481.77	*4.71*	*<0.0001*
Error	11,666.67	114	102.34		
Total	198,166.08	170			

F.V.: source of variation, SC: sum of squares, gl: degrees of freedom, CM: mean squares, and F: F-statistic. * indicates the interaction of both factors.

The main effect of the isolate was significant (F = 15.86, $p < 0.0001$), suggesting notable differences in pathogenicity among the isolates studied. Similarly, the main effect of time was also highly significant (F = 683.73, $p < 0.0001$), showing that time is a critical factor in pathogenicity variability.

Furthermore, the interaction between isolate and time was significant (F = 4.71, $p < 0.0001$), indicating that the effect of time on pathogenicity varies depending on the isolate considered. This suggests a complex interaction between isolates and time, where certain isolates may show a different pattern of pathogenicity over time compared to other isolates.

Evaluation of the pathogenicity of 18 isolates of *Metarhizium* sp. over three days showed significant variations in the effectiveness of the isolates over time, as detailed in Table 4.

Table 4. Pathogenicity of 18 isolates of *Metarhizium* sp. up to three days of evaluation.

Isolates	Time	Mortality Percentage (Media ± SD)	Isolates	Time	Mortality Percentage (Media ± SD)
MMR-M1	24 h	20.00 ± 10.00 g–j	SRR-M11	24 h	3.33 ± 5.77 ij
MMR-M1	48 h	36.67 ± 15.28 e–i	SRR-M11	48 h	10.00 ± 0.00 h–j
MMR-M1	72 h	100.00 ± 0 a	SRR-M11	72 h	36.67 ± 15.28 e–i
LLM-M2	24 h	13.33 ± 5.77 h–j	PMR-M12	24 h	6.67 ± 5.77 h–j
LLM-M2	48 h	33.33 ± 5.77 f–j	PMR-M12	48 h	40.00 ± 0.00 d–h
LLM-M2	72 h	70.00 ± 10.00 a–e	PMR-M12	72 h	83.33 ± 11.55 a–c
CCR-M3	24 h	6.67 ± 11.55 h–j	PHR-M13	24 h	0.00 j
CCR-M3	48 h	30.00 ± 10.00 f–j	PHR-M13	48 h	36.67 ± 5.77 e–i
CCR-M3	72 h	80.00 ± 10.00 a–c	PHR-M13	72 h	86.67 ± 5.77 a–c
MHR-M4	24 h	13.33 ± 5.77 h–j	TSR-M14	24 h	6.67 ± 5.77 h–j
MHR-M4	48 h	40.00 ± 0 d–h	TSR-M14	48 h	16.67 ± 20.82 h–j
MHR-M4	72 h	90.00 ± 0 ab	TSR-M14	72 h	83.33 ± 11.55 a–c

Table 4. *Cont.*

Isolates	Time	Mortality Percentage (Media ± SD)	Isolates	Time	Mortality Percentage (Media ± SD)
PTR-M5	24 h	16.67 ± 5.77 h–j	MMR-M15	24 h	16.67 ± 5.77 h–j
PTR-M5	48 h	70.00 ± 10.00 a–e	MMR-M15	48 h	40.00 ± 0.00 d–h
PTR-M5	72 h	100.00 ± 0.00 a	MMR-M15	72 h	96.67 ± 5.77 a
MMR-M6	24 h	3.33 ± 5.77 ij	TOR-M16	24 h	6.67 ± 5.77 h–j
MMR-M6	48 h	23.33 ± 20.82 g–j	TOR-M16	48 h	13.33 ± 5.77 h–j
MMR-M6	72 h	80.00 ± 26.46 a–c	TOR-M16	72 h	100.00 ± 0.00 a
CMR-M7	24 h	10.00 ± 10.00 h–j	MNR-M17	24 h	10.00 ± 0.00 h–j
CMR-M7	48 h	33.33 ± 5.77 f–j	MNR-M17	48 h	33.33 ± 11.55 f–j
CMR-M7	72 h	96.67 ± 5.77 a	MNR-M17	72 h	73.33 ± 23.09 a–d
POR-M8	24 h	10.00 ± 10.00 h–j	GOR-M18	24 h	20.00 ± 10.00 g–j
POR-M8	48 h	20.00 ± 10.00 g–j	GOR-M18	48 h	23.33 ± 5.77 g–j
POR-M8	72 h	53.33 ± 20.82 c–g	GOR-M18	72 h	76.67 ± 5.77 a–c
TOR-M9	24 h	10.00 ± 0.00 h–j	Control	24 h	0.00 j
TOR-M9	48 h	40.00 ± 0.00 d–h	Control	48 h	0.00 j
TOR-M9	72 h	100.00 ± 0.00 a	Control	72 h	6.67 ± 5.77 h–j
DHR-M10	24 h	3.33 ± 5.77 ij			
DHR-M10	48 h	23.33 ± 5.77 g–j			
DHR-M10	72 h	60.00 ± 26.46 b–f			

SD: standard deviation. Significant differences between groups are indicated by different letters (Tukey's test, $p \leq 0.05$).

The results showed that isolate MMR-M1 presented significant pathogenicity, reaching 100% at 72 h. Other isolates, such as PTR-M5, TOR-M9, and TOR-M16, also showed high pathogenicity simultaneously. In contrast, SRR-M11, DHR-M10, and the control isolate showed significantly lower pathogenicity across the evaluated times.

In addition, variations in pathogenicity effectiveness were observed among the different isolates at different times, with some isolates showing a gradual increase in pathogenicity while others rapidly reached high levels at early times.

Mycelial growth at 288 h was higher for isolates MMR-M1, MHR-M4, CMR-M7, PHR-M13, and TSR-M14, exceeding 90% (Figure 10).

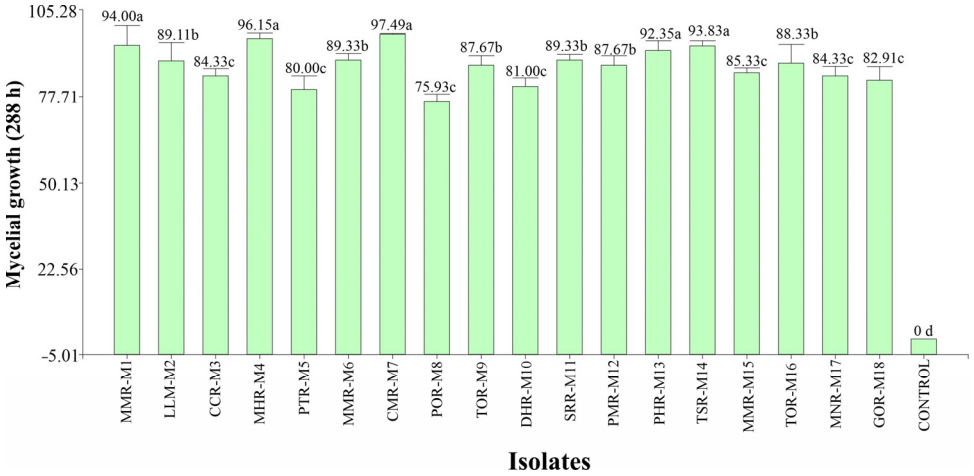


Figure 10. Mycelial growth of different isolates at 288 h, expressed as the mean ± standard deviation. Bars with different letters indicate significant differences between groups, determined by the Scott-Knott test ($p \leq 0.05$). Comparison of growth between the different treatments evaluated is shown.

The CRM-M7 treatment significantly differed from the other treatments; the inoculum on *H. hampei* covered 97.49% of the individual's body in the final phase (Figure 11B), having

greater dissemination over 288 h. This revealed the presence of hyphae and an initial white coloration.

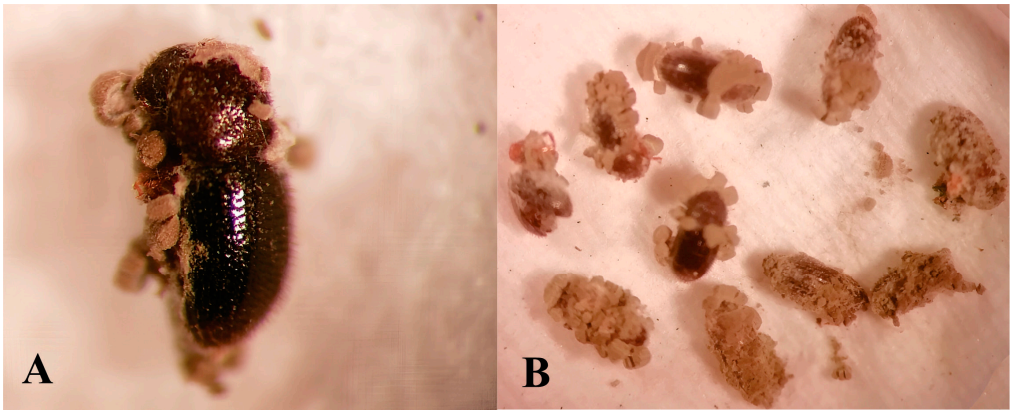


Figure 11. Faster mycelial growth of CRM-M7 treatment on *H. hampei* at 12 days (**A**), and infection with 97.49% (**B**).

In addition, the Kaplan—Meier survival curve (Figure 12) was estimated, and the mortality data were evaluated at up to 192 h. Except for isolate PHR-M13 and the control treatment, a reduction in the survival rate of *H. hampei* was observed from 24 h onwards. At 72 h, three isolates (MMR-M1, TOR-M9, and TOR-M16) managed to reduce the survival rate to 0%. The isolates that took the longest time to reduce the percentage survival of *H. hampei* to 0 were POR-M8 (192 h), DHR-M10 (144 h), CCR-M3 (120 h), SRR-M11 (120 h), and GOR-M18 (120 h). According to the log-rank statistic, there were highly significant differences between the isolates studied for the survival rate of *H. hampei* (Test = 83.954, $p = 0.000$).

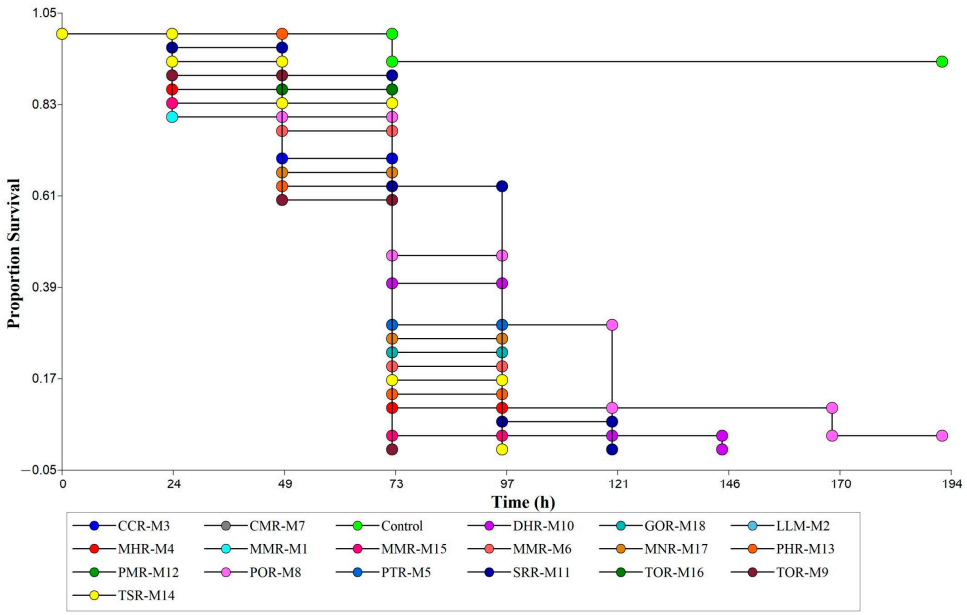


Figure 12. Kaplan—Meier curve showing the survival of subjects infected by different isolates of *Metarhizium* sp. Each curve represents a different isolate, with the vertical axis indicating the probability of survival and the horizontal axis showing the time elapsed in hours. Differences between the curves may indicate variations in the virulence of the isolates.

4. Discussion

Agroecological insecticides or biopesticides are in proportion to the genetics and physiology of the insect pest, including its behavior, which allows determining its sensitivity

and efficiency as a biological control [26]. Entomopathogenic fungi fulfill the natural function of biopesticides or biological controllers in different ecosystems and, in some cases, are considered cosmopolitan, as occurs in coffee-growing areas with the presence of species of the genus *Metarhizium* sp., which naturally infect coleoptera harmful to coffee fruits, such as *H. hampei* [27]. The genus *Metarhizium* sp. species have been reported to inhabit altitudes of 1600 m a.s.l. [28], an altitude similar to the range of the sampling zones of the coffee farms in the province of Rodríguez de Mendoza, where the isolation and identification of 18 isolates scattered throughout the province was achieved.

The morphological and physiological characteristics evaluated by Padilla et al. [19] and Valle-Ramírez et al. [29] were considered for the identification of the isolate genus; these characteristics included colony edge and texture, color and pigmentation, spore length and width, radial growth, conidial concentration, and germination percentage. However, these characteristics may differ between isolates due to the genetic diversity observed when collecting and isolating in different geographical locations [30]. Macroscopic morphological characterization showed moderately rapid growth in most isolates incubated at 30 °C, results that differ from those of Dimbi et al. [31], who recommend that the optimum temperature is 25 °C for the growth of *Metarhizium anisopliae*. Villamizar et al. [32] also found that *M. novozealandicum* grows best at 25 °C. Temperature is an important factor because it determines the mycelial growth rate, but it is a highly variable condition among isolates, as demonstrated by Ghayedi and Abdollahi [33], who determined that the ideal temperature for the development of *Metarhizium anisopliae* isolates is in the range of 25 to 30 °C, specifying that at 30 °C they were able to obtain the maximum growth and the highest growth rate.

According to other characteristics, the colonies are known to have a cottony appearance and are rounded in shape. Their colors vary between green, dark green, and yellowish white on the obverse, and the reverse varies between orange, brown, and yellowish white [34]. It should be noted that the names of the colors exposed in this research are according to Munsell's table [20], but the shades correspond to each other; another aspect of the colony that we observed in some isolates was the olive green powdery type due to abundant sporulation [31]. Pigmentation was observed in different shades of white, yellow, brown, and red, the first three matching those described by Rachappa et al. [35]. In addition, the type of sporulation with rings in the PDA medium is observed in the work of Sepúlveda et al. [36], adding that seeding in this medium can cause problems in the virulence of the isolates and their development.

Regarding morphological characteristics, it was observed that the size of *Metarhizium* sp. spore conidia ranged from 2.7 to 7.8 µm in length and from 0.8 to 2.8 µm in width. However, Valle-Ramírez et al. [29] reported higher values ranging from 5.08 to 7.19 µm in length and between 2.02 and 3.24 µm in width for *Metarhizium* conidia isolated from sugarcane crops in Ecuador. These differences highlight the need for molecular analysis in future studies since, although the conidia belong to the same genus, they could be of different species. This identification is crucial to achieving accurate identification, evaluating efficacy in biological control, determining product quality, and optimizing commercial formulations. However, it is also necessary to thoroughly characterize the spores in color and shape to differentiate between isolates of the genus *Metarhizium*, as recommended by Aynalem et al. [37] and Fernandes et al. [38]. Despite this, they emphasize that temperature-based evaluations make it possible to identify tentatively and quickly.

On the other hand, in the search for entomopathogenic fungi with optimal qualities for biocontrol, germination tests, growth rate, and sporulation levels must be prioritized; these factors correlate with the potential for pathogenicity [34].

The highest spore concentration obtained was 9.8×10^7 spores/mL for isolate LLM-M2 after 15 days, coinciding with the work of Padilla et al. [19], where they obtained 8.6×10^7, also indicating that high spore production would not necessarily be associated with the virulence of the fungus. However, it is considered a good quality for mass production. While the determination of spore viability through their germination percentage shows that

germination speed is an important character for insect infection, this premise is confirmed by the result of the most virulent isolate, which obtained 100% germination 14 h after sowing by TOR-M16 treatment.

The results of the present investigation demonstrated the efficiency of native isolates of *Metarhizium* sp. as biopesticides for *H. hampei* isolated from different farms in the Rodriguez de Mendoza province. Our results indicated that the percentage of mortality of coffee berry borer adults reached 100% 72 h after inoculation by immersion with isolates TOR-M16, TOR-M9, and MMR-M1. This response is considered the one with the highest potential. These isolates should be selected for future studies since they work efficiently at low concentrations and differ from other isolates that need to increase their concentration to obtain the same potential [39]. The response varies due to the genetic diversity that exists within the genus, some of them with high pathogenicity and others not, as described by Mesquita et al. [40], since among their isolated isolates, precisely the LCM S04 isolate stands out as the most efficient with a concentration of 10^7, reaching values higher than 90% against *R. microplus*. On the other hand, Apriyanto and Nadrawati [41] reached lower results for isolated *Metarhizium anisopliae*, reaching a maximum efficacy of 80% with a concentration of 10^9, which could be due to the method of exposure of the entomopathogenic fungus since immersion increases contact and spraying mimics the dispersion of conidia in the field.

The Kaplan—Meier curve indicated which isolates have the highest entomopathogenic potential according to exposure times, highlighting the isolates MMR-M1, TOR-M9, and TOR-M16 with the shortest exposure time (72 h) and survival rates of 0%. This finding highlights the individual characteristics of the isolates in reducing *H. hampei* survival. Knowing which isolates reduce insect survival in the shortest possible time will have important implications for the integrated management of this pest in coffee cultivation.

Their origin must also express the difference in virulence of the isolates since the isolate of *M. anisopliae* reported by Samuels et al. [42] isolated in Brazil is considered to have the lowest potential due to its mortality rate of 4.3% after the third day; however, our results show that the isolates with the lowest potential obtained 36.67% and 53.33% with isolates SRR-M11 and POR-M8, respectively, under the same conditions of time and concentration. These findings not only extend what we already know but also give us a clearer vision of how native isolates of *Metarhizium* sp. can be an effective tool in the biocontrol of the pest *H. hampei* in our region and coffee-growing regions of Peru with similar edaphoclimatic conditions. Aware of the limitations of this research, future investigations should also focus on the molecular evaluation of the isolates that registered the highest percentages of pathogenicity to achieve species-level identification of the native isolates of the genus *Metarhizium* sp. isolated from the rhizosphere of coffee plantations in the Rodríguez de Mendoza province.

5. Conclusions

This study demonstrates the efficacy of native isolates of *Metarhizium* sp. as biopesticides against *H. hampei* in the province of Rodriguez de Mendoza—Peru. The morphological and physiological characteristics of the isolates showed considerable variability, underlining the importance of genetic diversity within the genus *Metarhizium*. In particular, isolates MMR-M1, TOR-M9, and TOR-M16 exhibited high entomopathogenic potential, reaching 100% mortality of CBB in 72 h, indicating their effectiveness at low concentrations and short exposure times.

The high pathogenicity of specific isolates suggests that they could be implemented as efficient biopesticides, decreasing dependence on chemical products and promoting the sustainability of coffee production. In addition, the Kaplan—Meier survival curve highlights the variability in virulence among isolates, highlighting the importance of carefully selecting the most promising ones for practical application.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071341/s1, Table S1. Variables of radial growth, conidial concentration and germination percentage of the different treatments with *Metarhizium* sp. isolates (mean ± standard deviation).

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