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Microsclerotia from *Metarhizium robertsii*: Production, ultrastructural analysis, robustness, and insecticidal activity

Jennifer Lorena García Riaño [a,b,*], Gloria Patricia Barrera [a], Leonardo Castellanos Hernández [b], Laura Fernanda Villamizar [c]

[a] Corporación Colombiana de Investigación Agropecuaria – Agrosavia. Centro de Investigación Tibaitatá, Cundinamarca, Mosquera, 250047, Colombia
[b] Universidad Nacional de Colombia, Sede Bogotá, Facultad de Ciencias, Departamento de Química, Carrera 30 # 45, Bogotá, D.C., 111321, Colombia
[c] Microbial Solutions, AgResearch Ltd. Lincoln Research Centre, Christchurch, 8140, New Zealand

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ABSTRACT

Microsclerotia (MS) are considered one of the most promising propagules for use as active ingredients in biopesticides due to their tolerance to abiotic factors and ability to produce infective conidia for the control of pests. Therefore, the objective of this research was to establish the conditions required to induce the formation of microsclerotia in *Metarhizium robertsii* Mt004 and to study its development process, tolerance to abiotic factors and insecticidal activity of MS-derived conidia. *M. robertsii* started to form hyphal aggregates after 2 days and looked more compact after 8 days. MS were mature and pigmented after 20 days. The final yield was 2.0×10^3 MS/mL and MS size varied between 356.9 and 1348.4 μm. Ultrastructure analysis revealed that mature MS contained only a few live cells embedded in an extracellular matrix. Mature MS were more tolerance to UV-B radiation, heat and storage trials than conidia from Solid State Fermentation. MS-derived conidia were as virulent as conidia against *Diatraea saccharalis* larvae. These results showed that MS are promising propagules for the development of more persistent and efficient biopesticides for harsh environmental conditions. Our findings provide a baseline for production and a better understanding of microsclerotia development in *M. robertsii* strains.

1. Introduction

Metarhizium spp. (Ascomycota: Clavicipitaceae) are widely recognized as cosmopolitan fungi and biological control agents for soil-inhabiting insects (Mascarin et al., 2014; Patel, 2020). There are approximately 200 species of *Metarhizium* spp. with a wide host range, including arachnids and most other insect orders (Patel, 2020). *Metarhizium* is the second most common entomopathogenic fungus after *Beauveria bassiana* and is used in biopesticide development (Li et al., 2010; Patel, 2020).

Metarhizium robertsii J.F. Bisch., Rehner & Humber (Clavicipitaceae), formerly known as *Metarhizium anisopliae*, is a dimorphic entomopathogenic and endophytic fungus frequently reported to have high insecticidal activity against a broad range of agricultural pests and the ability to colonize roots, stimulating the plant defense system and the production of secondary plant metabolites (Mukherjee and Vilcinskas, 2018; Lira et al., 2020).

This fungus can produce different propagules, such as conidia, blastospores, chlamydospores, microsclerotia and mycelia pellets (Corval et al., 2020; Paixão et al., 2021). Conidia are asexual structures responsible for the primary infection of pest insects and the generation of epizootics (Beys-da-Silva et al., 2020; Patel, 2020).

Therefore, aerial conidia are the propagules most used as active ingredients in biopesticides due to their high infectivity to insect pests and ease of production by solid fermentation using low-cost substrates such as rice and wheat. However, it is known that conidia are susceptible to environmental conditions such as sunlight, heat, and desiccation, which can affect their insecticidal activity and persistence when applied in the field (Braga et al., 2001; Rangel et al., 2005; Fernandes et al., 2007; Jackson and Jaronski, 2009; Rangel et al., 2015; Song, 2018; Villamizar et al., 2018). Sunlight is one of the most detrimental factors affecting viability and can kill fungal conidia when they are directly exposed to solar radiation for a few hours due to the induction of dimerization between adjacent pyrimidine bases on DNA (Braga et al., 2015). Heat is

* Corresponding author. Corporación Colombiana de Investigación Agropecuaria – Agrosavia. Centro de Investigación Tibaitatá, Cundinamarca, Mosquera, 250047, Colombia.
E-mail address: jgarciar@agrosavia.co (J.L. García Riaño).

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another factor that affects conidial viability, causing DNA damage through base loss and leading to depurination (Li and Feng., 2009).

Conidia are generally produced by Solid State Fermentation (SSF), reaching high yields that depend on fermentation conditions, substrate, fungal isolate, etc. However, these systems require large volumes of substrate, complex downstream processes, high labor demand, and generate large volumes of solid residues, which increase the costs of production (Jackson and Jaronski, 2012; Villamizar et al., 2018).

To overcome these limitations, alternative strategies such as increasing endogenous reserves during fermentation (Mejía et al., 2020), developing complex formulations with the addition of adjuvants, genetic improvements, and the use of alternative and more robust fungal propagules (Mascarin et al., 2018), have been used to improve the persistence of fungal biopesticide in the field.

Microsclerotia (MS) in entomopathogenic fungi are defined as dark-pigmented and compact aggregates of hyphal threads with or without a distinct core (Paixão et al., 2021). This structure is considered the most interesting and promising fungal propagule for replacing conidia as the active ingredient in biopesticides due to its resistance and capacity to produce infective conidia (Song, 2018).

MS are produced naturally by phytopathogenic fungi, allowing them to survive for long periods (approximately 10 years). When soil conditions are suitable, MS can germinate and produce conidia that infect susceptible tissues (Griffin et al., 1978; Song, 2018). In entomopathogenic fungi, these structures must be artificially induced in liquid fermentation by using nutritional/oxidative stress (Jackson and Schisler, 1992; Jackson and Jaronski, 2009; Song, 2018). Numerous studies have reported MS formation with different fungal species, including *B. bassiana, M. robertsii, M. anisopliae, Metarhizium rileyi* (formerly known as *Nomuraea rileyi*) and *Purpureocillium lilacium*, among others (Jackson and Schisler, 1992; Jackson and Jaronski, 2009; You-Ping et al., 2012; Song et al., 2013; Song et al., 2014; Kobori et al., 2015; Fan et al., 2017; Song, 2018; Villamizar et al., 2018).

Structurally, MS comprise an outer layer composed of multiple layers of pigment parenchyma cells and an inner layer comprising loose tissue consisting of colorless cells interwoven with the medulla (Song, 2018). Pigment production (mainly melanin) has been associated with resistance characteristics and is considered “the fungal armor” (Pal et al., 2014). Fungal pigmentation is involved in tolerance against abiotic and biotic stress, and pigment formation depends on nutrition, culture age, and the presence of antioxidants (Song, 2018). Although the MS are not infectious propagules, they can germinate and produce thousands of infective conidia, which, in addition to their resistance characteristics, make these fungal propagules ideal for developing more stable biopesticides (Jackson and Jaronski, 2009; Jackson and Jaronski, 2012; Kobori et al., 2015; Villamizar et al., 2021).

In our previous work, *M. robertsii* Mt004 was selected for infection, causing more than 50% mortality in *Rhammatocerus schistocercoides, Ancognatha scarabaeoides* and *Diatraea saccharalis* (insects belonging to the orders Orthoptera, Coleoptera and Lepidoptera, respectively) (Correal et al., 2018a,b; Torres-Torres et al., 2020). Considering the high potential of this strain to be developed as a biopesticide with a broad spectrum of activity, the shelf-life and field persistence can be improved by using MS as a suitable propagule for delivery. The aim of this research was to establish the conditions required to induce the formation of microsclerotia in *M. robertsii* Mt004 and to study its development process, tolerance to heat, UV-B radiation and storage conditions as well as its insecticidal activity.

2. Materials and methods

2.1. Fungal strain

M. robertsii Mt004 was obtained from the Bank of Microorganisms for Biological Control of Agrosavia (Colombia). This strain was isolated from a larva of *A. scarabaeoides* Erichson, 1847 (Coleoptera: Melolonthidae) in Rionegro, Antioquia (Colombia). This work was carried out under the RGE0229-2 (No. 168) contract for access to genetic resources and their derived products in Colombia.

The strain was maintained at −80 °C and cryopreserved in 0.1% w/v peptone (Millipore®, Merck KGaA, Germany) and 10% w/v glycerol (Emsure®, Merck KGaA, Germany) solution. For reactivation, the fungal suspension was inoculated on Petri dishes with potato dextrose agar (PDA; Oxoid®, Thermo Fischer Scientific, U.S.A.) and incubated at 25 ± 0.5 °C for 10 days.

2.2. Screening of culture medium composition for the production of microsclerotia in M. robertsii Mt004

Four culture media (A, B, C, D) previously reported for MS production with other *Metarhizium* sp. strains were tested (Table 1).

Erlenmeyer flasks (500 mL) containing 100 mL of culture medium (3 flasks/medium) were inoculated with 1 mL of a 1.0×10^8 conidia/mL suspension to obtain a final concentration of 1.0×10^6 conidia/mL. Inoculated flasks were incubated in a rotary shaker (MaxQ™ 4000; Thermo Fisher Scientific) at 25 °C and 200 rpm for 8 days. To determine the MS concentration, 50 μL of broth were collected after 8 days for microscopic analysis. The samples were placed onto a glass slide and overlaid with a large 24 × 50 mm glass coverslip. The concentration of MS was determined by counting across the entire coverslip area under a microscope (ZEISS Axio), and images were acquired using a ZEISS AxioCam ERc 5s digital camera.

MS viability was evaluated by determining myceliogenic and conidiogenic germination. Fifty (50) MS were recovered from the broth, rinsed with distilled water, and individually placed on the surface of water agar plates. The plates were incubated at 25 °C, and after 48 h, MS were examined under a Carl Zeiss™ Stemi 508 stereomicroscope to determine the occurrence of hyphal growth (myceliogenic germination) (Villamizar et al., 2018). Conidium production (conidiogenic germination) was determined after 8 days of incubation by adding 5 mL of Tween® 80 solution (0.1% w/v) to each Petri dish. Conidia were dislodged by scraping the agar surface with a sterile inoculation loop. The liquid (conidial suspension) was recovered, and the concentration was determined using a hemocytometer (Neubauer Chamber) (Rivas- Franco et al., 2020).

2.3. Microsclerotia differentiation process

MS were produced using medium D described by Mascarin et al. (2014) with some modifications. The production was carried out as described in section 2.2 for 20 days. The MS concentration was determined by collecting 50 μL samples at days 2, 4, 6, 8, 12, 16 and 20 of fermentation. The samples were subjected to glucose consumption

Table 1
Medium culture composition selected for production of MS from *M. robertsii* Mt004.

Ingredient	Medium A (Song et al., 2013)	Medium B (Jackson and Jaronski, 2009)	Medium C (Song et al., 2014)	Medium D (Mascarin et al., 2014)
Glucose (g/L)	40	75	32	36
Peptone (g/L)	2.5			
Yeast Extract (g/L)	5			3.6
Acid-hydrolyzed casein (g/L)		15		
Ammonia citrate (g/L)			2	

All media were supplemented with salts solution with the follow composition per liter: KH_2PO_4: 4 g, $CaCl_2 \cdot 2H_2O$: 0.8 g, $Fe_2(SO_4)_3 \cdot 7H_2O$: 0.2 g*, $MgSO_4 \cdot 7H_2O$: 0.6 g $CoCl_2 \cdot 6H_2O$: 37 mg, $MnSO_4 \cdot H_2O$: 16 mg, $ZnSO_4 \cdot 7H_2O$: 14 mg *El $Fe_2(SO_4)_3 \cdot 7H_2O$ in medium B: 0.1 g; medium C: 0,15 g; and medium D: 0.1 g.

determination following the DNS technique (Miller, 1959) and microscopic analysis. The MS concentration and viability were determined according to the methodology described above (Section 2.2).

Electron microscopy: MS from samples collected after 4, 8, 16 and 20 days of fermentation were processed and observed by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). For TEM, MS were directly fixed with glutaraldehyde 2.5% (pH 7.4) and osmium tetraoxide (1%) and dehydrated with ethanol in ascendant concentrations. Then, the samples were embedded in acrylic resin LR White (London Resin Company, Reading, U.K.), cut into ultrathin sections (40 nm), contrasted with uranyl acetate and lead citrate (Villamizar et al., 2021) and observed under a JEOL JEM-1400 plus microscope.

For SEM, MS were directly fixed with 2.5% glutaraldehyde (pH 7.4), dehydrated with ethanol in ascendant concentrations, sputtered with colloidal gold (Villamizar et al., 2021) and observed under a JEOL JSM 6490-LV microscope.

2.4. Evaluation of microsclerotia and aerial conidia tolerance to abiotic factors

MS were produced in medium D and harvested after 20 days of fermentation by adding 5 g of diatomaceous earth to each 100 mL of culture broth. The suspension was vacuum filtered in a Buchner funnel through Whatman No. 1 filter paper to remove the spent medium (Villamizar et al., 2018). The resulting filter cake was manually granulated using a 1 mm mesh, layered in Petri dish plates, and air-dried overnight under laminar flow at 22 °C. Dry granules (<10% moisture) containing MS were used to determine tolerance to abiotic factors. Samples were prepared by dispersing 0.25 g of dry granules in 1 mL of Tween® 80 solution (0.1% w/v). The mixture was allowed to settle for 30 s and then, 500 μL of the supernatant was removed and replaced with 500 μL of Tween® 80 solution (0.1% w/v). This process was repeated three times to remove as much diatomaceous earth as possible and finally, 100 μL samples were inoculated and spread on water agar plates. Using fine-tipped forceps, clean MS were removed from the surface of the agar and transferred to another plate with water agar (rinsed MS) for evaluation.

Aerial conidia were produced by solid fermentation using three aluminum trays (10.5 × 17.5 cm) containing 100 g of white rice and 80 mL of water. The trays were covered with aluminum foil and autoclaved at 121 °C and 15 psi for 30 min. Then, the trays were inoculated with 10 mL of conidial suspension adjusted to a concentration of 1.0×10^6 conidia/mL and covered with a film of plastic food wrap. The trays were incubated at 25 ± 2 °C and a relative humidity of 40 ± 10% for 10 days. After the incubation, conidia were recovered by successively washing the sporulated rice grains with a Tween® 80 solution (0.1% w/v). The conidial suspension was used for the evaluation.

2.4.1. Tolerance to UV-B radiation

The tolerance of MS and aerial conidia to UV-B radiation was tested according to the technique described by Song et al. (2016) with modifications. An 8-W monochromatic lamp UVP® 95-0343-01 3UV™ model 3UV-38 Handheld UV Lamp fitted 20 cm above the base in an irradiation chamber was used to provide UV radiation (UV–B output at 302 nm). Fifty rinsed MS were placed on the surface of water agar plates that were then irradiated for 0, 1, 2, 3 and 4 h (total irradiation doses of 0, 12.61 kJ/m^2, 25.55 kJ/m^2, 37.83 kJ/m^2 and 50.44 kJ/m^2, respectively). Three replicate plates were used per irradiation time, which were incubated at 25 °C in the dark after irradiation. MS viability was measured by determining the occurrence of myceliogenic and conidiogenic germination following the previously described methodology.

For aerial conidia, the suspension obtained after washing the sporulated rice was adjusted to 1×10^6 conidia/mL and inoculated on plates containing malt extract agar (0.1% w/v) supplemented with benomyl at 0.00015% w/v. The plates were irradiated following the same conditions used for the MS samples, and three replicate plates were used for each irradiation time (0, 1, 2, 3 and 4 h). The irradiated plates were incubated for 24 h at 25 °C, after which lactophenol blue solution (Sigma–Aldrich®, Merck®, Germany) was added directly to the agar surface to stop germination. Germinated and ungerminated conidia were counted until a minimum quantity of 100 conidia per plate was reached. Conidia were considered germinated when the germ tubes were at least as long as the diameter of the conidia (Braga et al., 2001).

2.4.2. Tolerance to heat

Groups of 50 rinsed MS were placed on the surface of water agar plates (30 plates). The three plates were incubated at 25 °C for 48 h. MS viability was determined by assessing the myceliogenic germination and conidium production following the previously described methodology. The other 27 plates were divided into three groups of 9 plates that were exposed to heat shocks at 30, 40, or 50 °C in an incubator/oven for 1, 2, and 3 h. After each exposure time, the three plates were removed from each temperature treatment and transferred to an incubator at 25 °C. Viability was evaluated after 48 h of incubation.

The conidial suspension obtained after washing the sporulated rice grains was adjusted to 1×10^6 conidia/mL and inoculated on 30 plates containing malt extract (0.1% w/v) supplemented with benomyl at 0.00015% w/v. The three plates were incubated at 25 °C for 24 h, after which the conidial germination percentages were determined following previously described methods. The other 27 plates were exposed to the same heat shocks used for the MS samples. After each exposure time, three plates were removed from each temperature and transferred to an incubator at 25 °C for 24 h for further germination assessment.

2.4.3. Storage stability studies

Samples (250 mg) of dry granules containing MS and aerial conidia were packaged in 1.5 mL Eppendorf tubes and stored at 8 °C and 18 °C. The viability of the stored MS and conidial formulations was determined at time zero and after 2 and 4 months of storage. Three samples of MS granules were assessed per temperature/storage time. Granules were dispersed by adding 1 mL of Tween® 80 solution (0.1% w/v) to Eppendorf tubes and mixing with a micropipette. The samples were allowed to settle for 30 s, after which 500 μL of the supernatant was removed and replaced with 500 μL of Tween® 80 solution (0.1% w/v). This process was repeated three times to remove as much diatomaceous earth as possible. Then, 100 μL samples were inoculated and spread on water agar plates. Using fine-tipped forceps, 50 rinsed MS were removed from the surface of the agar and transferred to another plate with water agar (50 MS/plate and 3 plates/temperature/storage time). The plates were incubated at 25 °C ± 2 °C, and MS viability was determined by assessing myceliogenic germination and conidium production following previously described methods.

For aerial conidia, 1 mL of Tween® 80 solution (0.1% w/v) was added to Eppendorf tubes containing the dry samples (3 tubes/temperature/storage time). The granules were dispersed by mixing with a micropipette, and 100 μL were inoculated on Petri dishes containing water agar supplemented with 0.00015% w/v benomyl. The inoculated plates were incubated at 25 ± 2 °C for 24 h.

Conidial germination percentages were then determined by following the previously described methodology.

2.5. Insecticidal activity of MS-derived conidia and aerial conidia against *D. saccharalis* larvae

***D. saccharalis* rearing:** The experiments were carried out using second-instar larvae of *D. saccharalis* provided by the Agrosavia rearing facility (Entomology Laboratory Tibaitatá Research Center), where larvae are maintained on a semisynthetic diet (Lastra and Gómez, 2006).

Lethal concentrations: The bioassay was carried out following the methodology described by García et al. (2018). Conidial suspensions were prepared with conidia from SSF and conidia derived from

germinated MS.

MS-derived conidia were obtained from rinsed MS inoculated on water agar plates that were incubated at 25 ± 2 °C for 8 days to allow them to sporulate. Conidia were subsequently recovered following the methods described in section 2.2.

Dry aerial conidia produced on rice and MS-derived conidia were used to prepare suspensions adjusted to five concentrations of 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 conidia/mL. *D. saccharalis* larvae (L2) were inoculated by pipetting 2 μL of suspension on the dorsal thoracic region of each larva. Each inoculated larva was placed individually into a plastic cup containing one fresh grain of corn as a feeding substrate. Ten cups were placed into a 473 mL plastic container and incubated at 26 ± 2 °C and 70% RH, after which mortality and sporulation were evaluated every two days. The control treatment consisted of no inoculated larvae. The experimental unit consisted of 10 larvae, and three replicates (30 larvae) were used per treatment. The experiment was repeated twice at different times. The mortality data were subjected to a Probit analysis with the software POLO-PLUS 2.0 (LeOra Software) to determine the lethal concentrations.

2.6. Statistical analysis

The homogeneity of variances and the normality of the results were determined through the Bartlett and Shapiro–Wilk tests (95% confidence), respectively. After demonstrating normality, differences between treatments were determined by variance analysis (ANOVA) and Tukey's test (95% confidence). For nonparametric analysis, we used the Kruskal–Wallis's test (95% confidence) with the statistical program Statistix (8.0). Data without variance were not included in the statistical analysis and were considered significantly different.

3. Results

3.1. Screening of culture medium composition for the production of microsclerotia from M. robertsii Mt004

After 8 days of fermentation, *M. robertsii* Mt004 formed MS-like structures (MS-ls) in all the culture media; these structures were compact, variable in size and slightly pigmented (Fig. 1). The maximum yield of MS-ls was obtained with media A and D (Table 2), with values significantly higher than those obtained with media B and C ($F_{3,58} = 108$, df = 61, p = 0.0000). Glucose consumption varied among the culture media, reaching more than 72.7% of the total available glucose consumed in media A, B and D and only 39.4% consumed in medium C after 8 days of fermentation ($F_{3,11} = 64.4$, df = 14, p = 0.0000) (Table 2). With all media, a high viscosity was observed.

MS-ls recovered from the four evaluated culture media presented 100% myceliogenic germination after 48 h of incubation on water agar plates (Fig. 1). MS-ls produced in media A, C and D produced approximately 10^5 conidia/structure (Table 2), values significantly higher than the yield obtained with structures produced in medium B ($F_{3,8} = 20.9$, df = 11, p = 0.0004).

Due to the poor pigmentation observed in MS-ls harvested after 8 days, a second fermentation was carried out, and the culture time was extended until dark pigmented structures were observed (characteristic associated with microsclerotia maturation). Pigmentation increased with all media by extending the fermentation time, and structures were harvested after 20 days. The structures in medium A were compact and heterogeneous in size. Some aggregates were completely pigmented, while others only showed a dark pigmented zone in the center (Fig. 2a); the yield in medium A was 1.7×10^3 MS-ls/mL. MS-ls obtained from media B and C were spherical with mycelia around the structure and sizes >1000 μm. The yields for media B and C were 2.1×10^2 and 2.2×10^2 MS-ls/mL, respectively (Fig. 2b and c). The structures from medium D were pigmented and compact, with sizes ranging from 352.6 μm to 1187.8 μm (Fig. 2d), and the yield reached 1.9×10^3 MS-ls/mL. It is important to note that the viscosity of media A and D decreased during fermentation.

The culture medium D (Mascarin et al., 2014) was selected to continue this work and produce MS from *M. robertsii* Mt004 based on three factors: i) high MS yield, ii) homogeneous dark pigmentation, and iii) a greater benefit/cost ratio in comparison with the other culture media due to the use of a single component as a nitrogen source (Table 1).

3.2. Microsclerotium differentiation process

After 2 days of fermentation, a high density of loose mycelia and few loose hyphal aggregates were observed (Fig. 3a), which increased in size and density on day 4 postinoculation (Fig. 3b).

The ultrastructural analysis showed an intertwined hyphal structure after 4 days of fermentation (Fig. 4a and b). These aggregates showed brownish pigmentation, and the presence of granular particles adhered to the hyphae, which possibly corresponded to residues from the culture medium (Fig. 4c). The internal analysis showed differentiation between two zones (Fig. 4d): the external zone was composed of loose hyphae, and the internal zone contained different cells, such as submerged conidia, spores in gemmation and some segmented hyphae (Fig. 4e–h). The micrographs showed the beginning of the formation of an extracellular matrix in the internal zone (Fig. 4e and g).

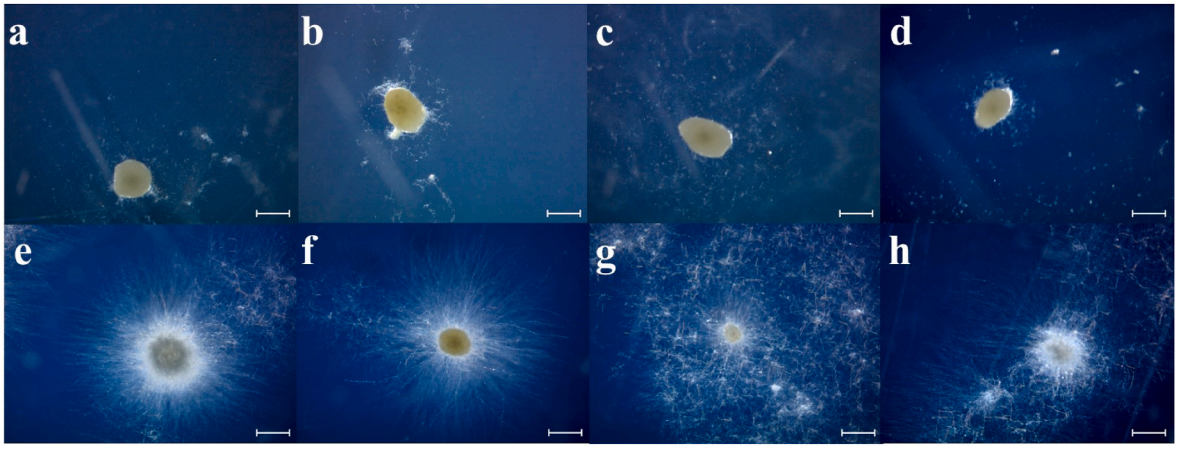

Fig. 1. MS-like structures (MS-ls) harvested after 8 days of fermentation. (a, b, c, d) MS-ls immediately after harvesting from each culture medium, (e, f, g, h) MS-ls after 48 h of incubation on water agar (myceliogenic germination). Medium A: a, e, Medium B: b, f, Medium C: c, g, Medium D: d, h. Photographs taken under a stereoscope with objective 2.5×. Scale bar: 500 μm.

Table 2
Yield, glucose consumption and conidiogenic germination of MS-ls of *M. robertsii* Mt004 produced in different culture media after 8 days of submerged fermentation (MS-ls) MS-like structure. Different letters indicate significant differences according to Tukey mean comparison test (95%) for each parameter evaluated.

Medium	Carbon (g L^{-1})	Nitrogen (g L^{-1})	C:N	Yield (MS-ls/mL)	Conidium production (Conidia/MS-ls)	Glucose consumption (%)
A	18.0	0.6	28:1	1.6×10^{3a}	1.0×10^{5b}	80.1^{a}
B	37.9	1.2	32:1	1.7×10^{2b}	3.7×10^{4c}	78.1^{a}
C	13.1	0.2	54:1	1.2×10^{2b}	1.2×10^{5ab}	39.4^{b}
D	16.0	0.3	55:1	1.4×10^{3a}	1.6×10^{5a}	83.0^{a}

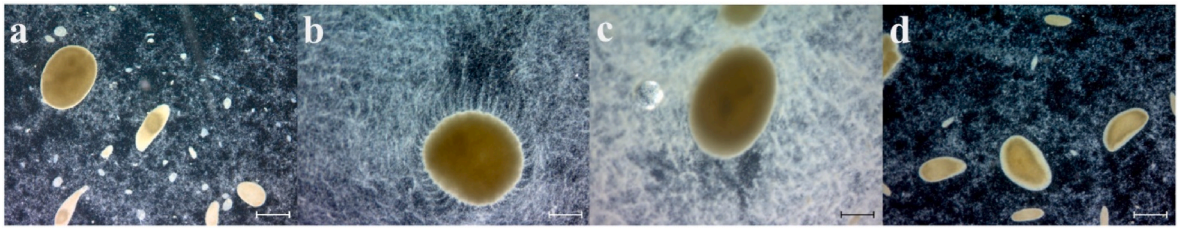

Fig. 2. MS-ls after 20 days of fermentation. (a) Medium A, (b) Medium B, (c) Medium C, (d) Medium D. Photographs were taken under a stereoscope with objective 2.5×. Scale bar: 500 μm.

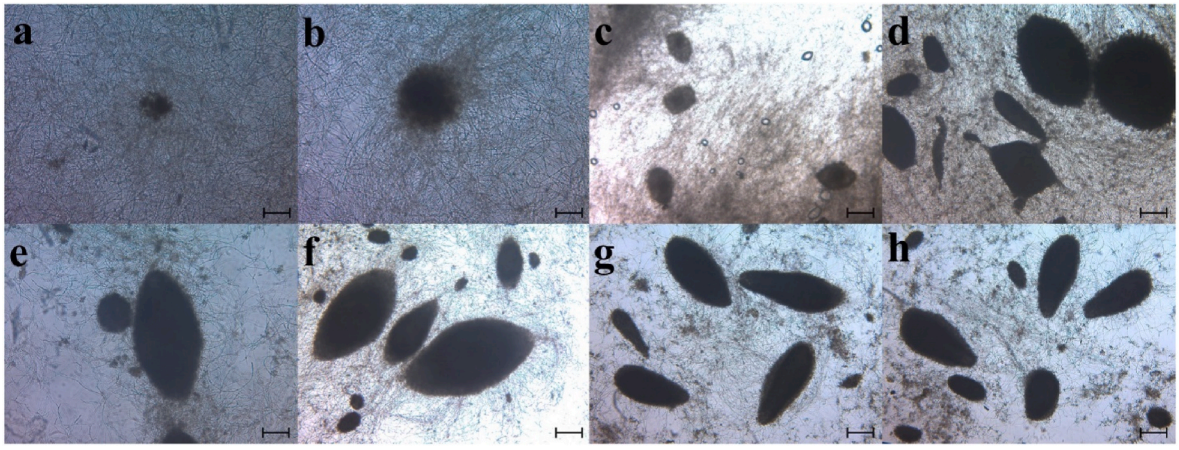

Fig. 3. Morphogenesis of MS formed by *M. robertsii* Mt004 during 20 days of fermentation in culture medium D. Beginning of mycelial aggregation observed at day 2 (a) and 4 (b). Aggregates increasing size, pigmentation, and density between at days 6 (c) 8 (d) and 12 (e). Mature MS observed at days 16 (f) and 20 (h). Photographs were taken under an optical microscope with the objective 5×. Scale bar: 200 μm.

The hyphal aggregates became larger, with an average size of 422.7 μm, and more compact and pigmented from day 6–8 of fermentation (Fig. 3c and d). When the concentration reached 1.2×10^3 aggregates/mL on day 8, the viscosity of the culture medium started to increase.

After 8 days of fermentation, hyphal aggregates had formed (Fig. 5a), and an increased amount of extracellular matrix surrounding the cells (blastospores, submerged conidia, etc.) was observed (Fig. 5b and c). The two zones mentioned above became more differentiated (Fig. 5d). Internally, there was an increase in the number of cells undergoing autolysis (Fig. 5i), and electrodense deposits were detected in the internal zone (Fig. 5j).

The free mycelium in the culture media and medium viscosity decreased after 12 days of fermentation, while the mycelial aggregates increased in pigmentation and size (428.8–1182.4 μm), exhibiting characteristics more consistent with the MS description (Fig. 3e). At this fermentation time, the concentration reached 1.7×10^3 MS/mL.

Between 8 and 16 days of fermentation, the morphological changes in the hyphal aggregates were more evident (Fig. 5). SEM micrographs showed hyphae and some cells on the aggregate surface and an increase in extracellular matrix formation (Fig. 5b), and the internal structure showed some spaces in the internal zone (Fig. 5e). After 16 days of fermentation, the MS reached sizes between 352.6 and 1068.0 μm and had a defined edge, compact appearance, and darker pigmentation (Fig. 3f) than the 8-day-old structures (Fig. 3d). Ultrastructural analysis revealed that the extracellular matrix covered almost the entire structure (Fig. 5e), but some hyphae were still observed on the surface (Fig. 5f). The internal analysis showed no differences between zones with a drastic decrease in cellular density and lysed cells. Electrodense deposits inside the cells were also observed (Fig. 5g and h).

After 20 days of fermentation, the MS were more compact and darker than they were after 8 days of fermentation (Fig. 3d and g). These structures reached sizes between 356.9 and 1348.4 μm (Fig. 3g and h), and the surface was completely covered with the extracellular matrix (Fig. 6a–c). Few live cells with debris produced during cellular lysis were observed inside the MS. The TEM micrographs showed abundant electrodense deposits suggesting the presence of pigments in the intercellular space, which reached a maximum size of 3 μm (Fig. 6f and g). In the MS inner zone, a network of "*threads*" was observed that could help to support the structure (Fig. 6h).

The final MS concentration was 2.0×10^3 MS/mL, and the glucose consumption was 98.8%. The mature MS presented myceliogenic germination of 100% after 48 h of incubation and conidiogenic germination of 6.0×10^5 conidia/MS, a value that was significantly higher than that obtained from MS harvested after 8 days of fermentation ($F_{1,4}$

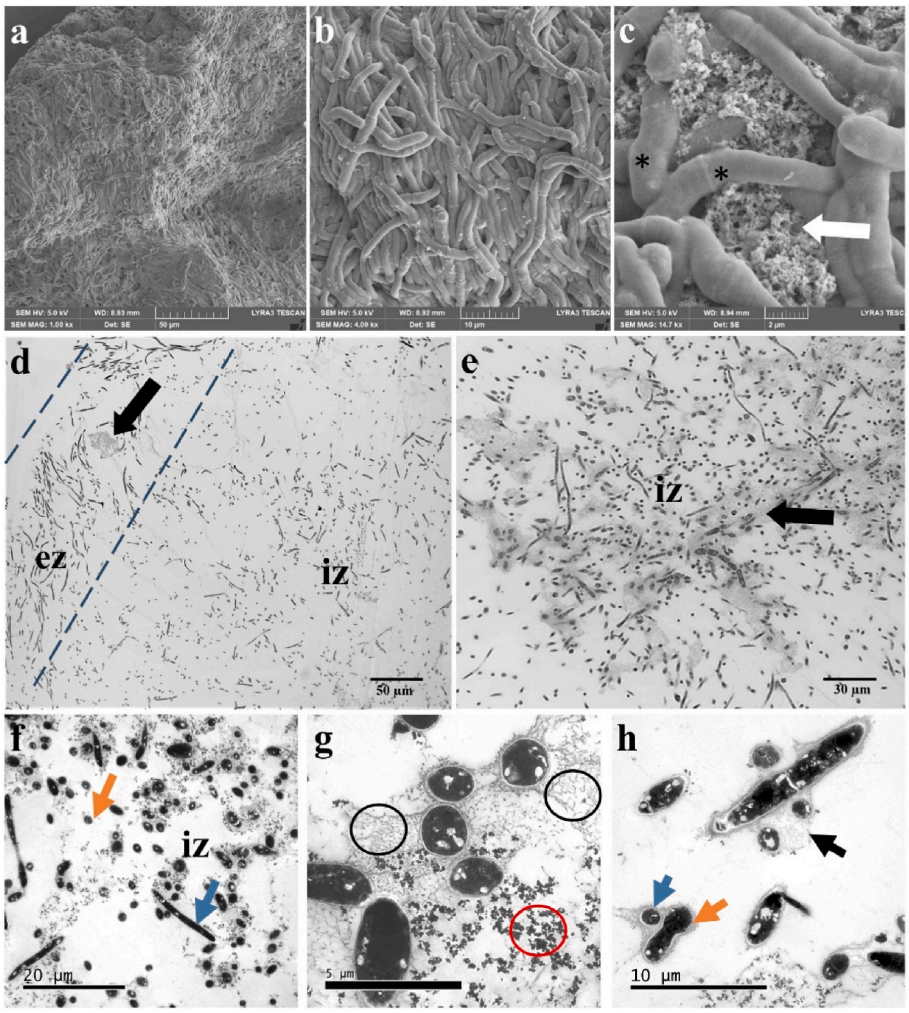

Fig. 4. Surface (SEM micrographs a, b, c) and internal structure of hyphal aggregates (Light microscopy d, e) after 4 days of fermentation. (a, b) SEM micrograph from hyphal aggregate surface (c) SEM micrograph shows granular particles (white arrow) and hyphae (black asterisk), (d, e) Internal structure showing extracellular matrix (black arrows) formation (f–h) TEM micrographs of internal structure of hyphal aggregates (f) Loose cells and hyphae in the internal zone (iz) Hyphae (blue arrow), Loose cells (orange arrow), (g) Extracellular matrix (black circles), granular material (red circles) (h) extracellular matrix (black arrow), germinating spore (orange arrow), conidia (blue arrow), ez: external zone, iz: internal zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

= 195, df = 5, p = 0.0002).

3.2.1. *Evaluation of microsclerotia tolerance against abiotic factors*

Microsclerotia tolerance to UV: Aerial conidia not exposed to UV-B radiation presented 100% germination, while conidia irradiated for one hour were not able to germinate (0% germination). In contrast, MS presented 100% myceliogenic germination before irradiation and maintained survival, with 84.6% germination after 4 h of irradiation with UV-B light (Fig. 7).

After the first hour of irradiation, MS presented 99.6% myceliogenic germination and produced 2.2 × 10^5 conidia/MS, presenting no significant differences in comparison with nonirradiated MS (2.9 × 10^5 conidia/MS). After 2 h, both the myceliogenic and conidiogenic germination rates significantly decreased to 90.3% ($F_{4,25}$ = 24.9, df = 29, p = 0.0001) and 1.1 × 10^5 conidia/MS ($F_{4,25}$ = 24.9, df = 29, p = 0.0000), respectively, which continued to decrease during the following 2 h, reaching a final value of 84.6% myceliogenic germination and conidium production of up to 6.8 × 10^4 conidia/MS. It is important to mention that even though MS were able to germinate after 4 h of irradiation, the mycelial density/vigor decreased (Fig. 8).

Microsclerotia tolerance to heat: The germination of MS remained stable after 3 h of incubation at 30 °C, with 100% myceliogenic germination and conidial production higher than 10^5 conidia/MS (Table 3). In contrast, the percentage of germinated aerial conidia exposed for one hour to 30 °C significantly decreased from 100% to 90.0%, a value that remained stable after two and three hours of exposure to this temperature (Fig. 9A).

MS exposed to 40 °C reduced germination up to 97.0% and 91.6% after one and three hours of exposure respectively, values significatively lower (p = 0.0172) than those obtained with MS not exposed to heat (Fig. 9B). Conidiogenic germination remained stable for MS exposed for up to 3 h to 40 °C (Table 3). The germination of aerial conidia exposed at 40 °C decreased to 87.6% after one hour and to 84.3% after 3 h, values that were significantly lower than those of conidia not exposed to heat ($F_{8,45}$ = 12.7; df = 53; p = 0.0000). After 3 h of exposure to 40 °C, no significant differences were detected between the germination of aerial conidia and MS ($F_{1,10}$ = 1.75; df = 11; p = 0.2150) (Fig. 9A and B).

MS and conidia exposed to 50 °C progressively and significantly reduced the germination capacity after the first hour of exposure, with values of 90.6% and 748%, respectively (MS: $F_{5,30}$ = 6.71; df = 35; p = 0.0003) (Conidia: $F_{8,45}$ = 12.7; df = 53; p = 0.0000). After 3 h of exposure to this temperature, the germination of MS decreased by 17.0%, while conidial germination decreased by 42.0% (Fig. 9B). The conidiogenic germination of MS was significantly ($F_{9,50}$ = 12.7, df = 59;

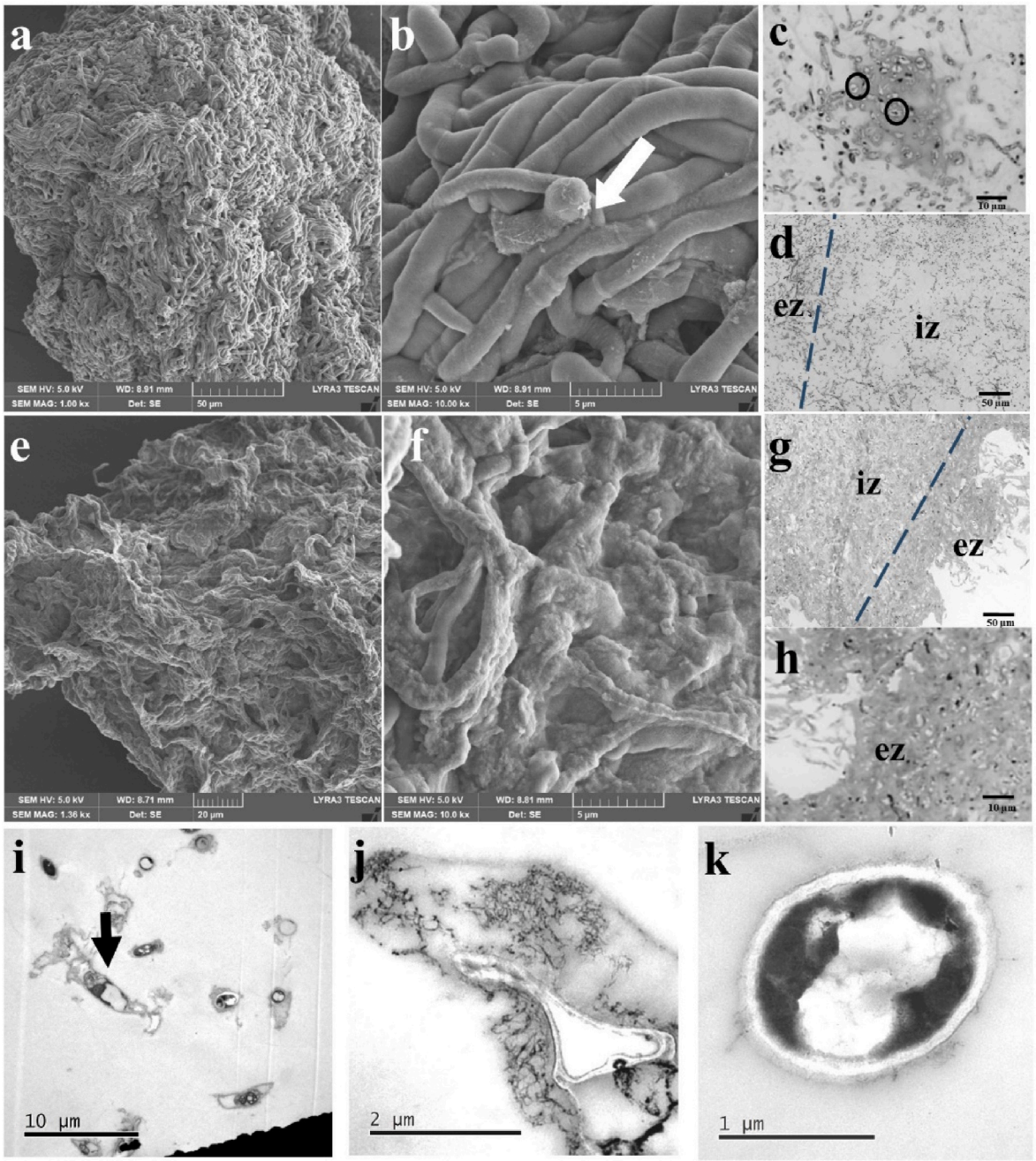

Fig. 5. Surface (SEM micrographs a, b, e, f) and internal structure (Light microscopy c, d, h, g) of hyphal aggregates after 8 and 16 days of fermentation. (a) SEM micrograph shows hyphal aggregate surface at 8 days of fermentation (b) SEM micrograph shows loose cell (white arrow) with extracellular matrix on the surface at 8 days (c) Swollen cell (black circle) embedded in matrix extracellular zone (d) Differentiation between external zone (ez) and internal zone (iz) of hyphal aggregates at 8 days (e, f) SEM micrograph shows MS surface at 16 days of fermentation (g,h) Internal structure of MS at 16 days of fermentation (i–k) TEM micrographs of internal structure of MS formed after 8–16 days of fermentation. (i) Hyphae in lysis (black arrow), (j) Extracellular matrix formation (k) Conidia.

$p = 0.0000$) affected by this temperature with MS exposed for 3 h reaching 8.5×10^4 conidia/MS, which was significantly lower than the initial yield of 2.0×10^5 conidia/MS (Table 3).

Storage stability of MS and conidia from SSF: Before storage, conidia and MS presented 100% myceliogenic germination, and MS reached conidiogenic germination with a yield of 2.2×10^5 conidia/MS. Conidia from SSF were not able to germinate after 2 months of storage at either of the evaluated temperatures. The stored MS remained alive with more than 90.0% myceliogenic germination (Fig. 10) and conidial production of 2.8×10^5 conidia/MS after 4 months of storage at 8 °C. However, myceliogenic germination significantly decreased ($F_{3,8} = 18.5$; $df = 11$; $p = 0.0006$) to 70.6% after 4 months at 18 °C without reducing conidium production (1.5×10^5 conidia/MS), with yields similar to the initial yields before storage ($F_{1,5} = 0,91$, $df = 6$; $p = 0.3948$) (Fig. 10).

3.3. Insecticidal activity of MS-derived conidia and aerial conidia against *D. saccharalis* larvae

A bioassay was carried out to compare the insecticidal activity of conidia from SSF- and MS-derived conidia by determining their lethal concentrations. In general, mortality was directly proportional to conidial concentration. The infected larvae presented characteristic signs of infection by entomopathogenic fungi, exhibiting purple to reddish coloration and a hard body after death. Then, white mycelia

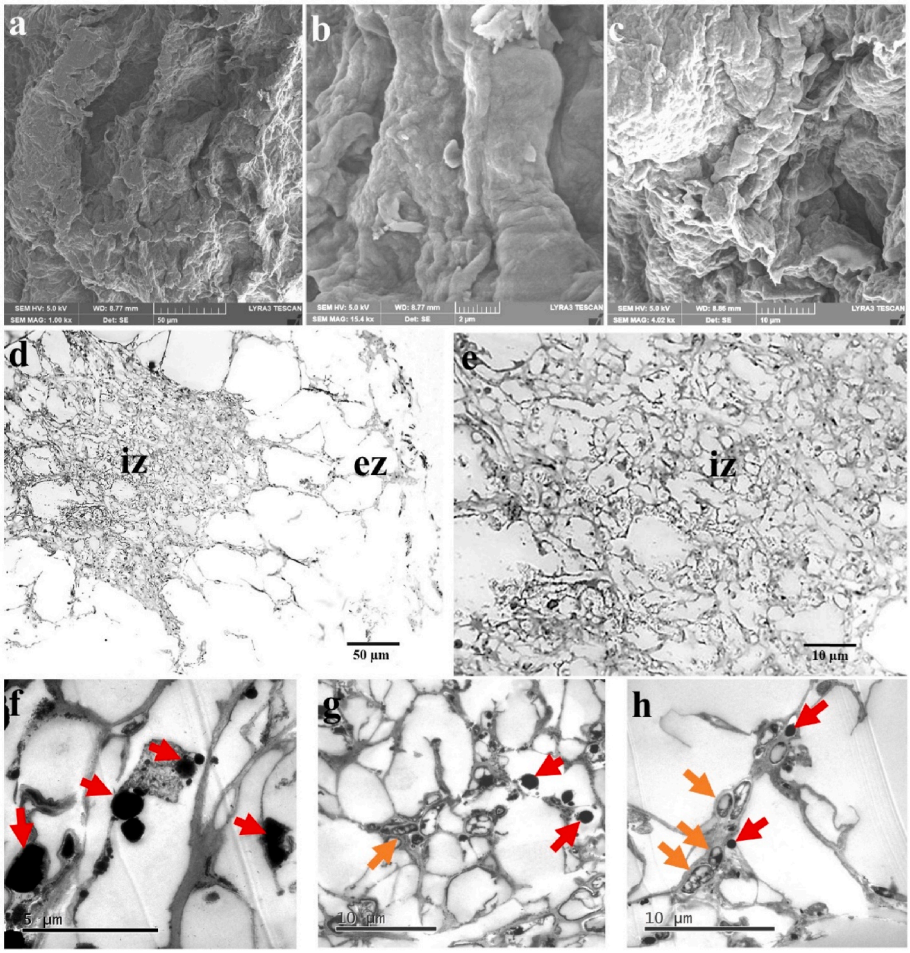

Fig. 6. Surface (SEM micrographs a, b, c) and internal structure (Light microscopy d, e) of mature MS formed after 20 days of fermentation. (a–c) MS surface. (d, e) Internal structure of MS. ez: external zone, iz: internal zone. (f–h) TEM micrographs of mature MS formed at 20 days of fermentation showing pigment deposits (red arrow) and few live cells (orange arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

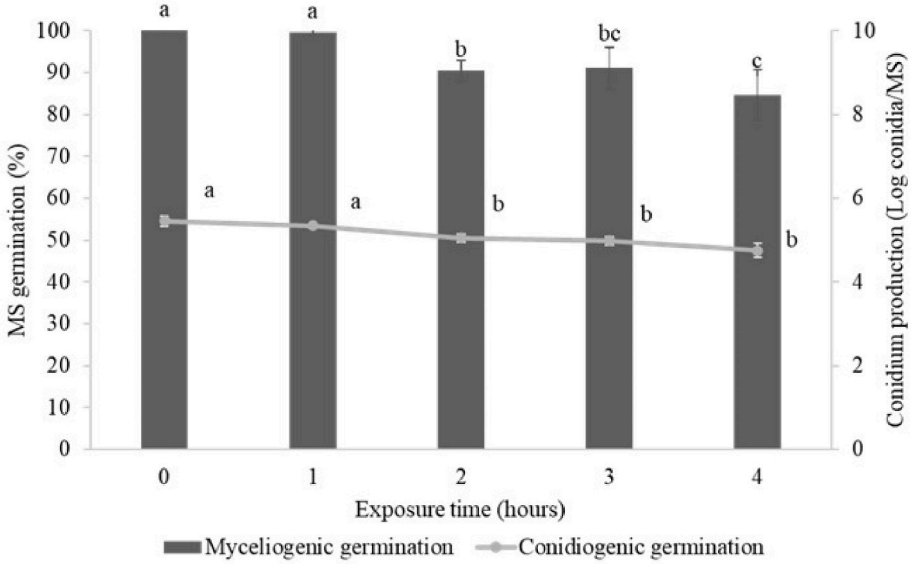

Fig. 7. Myceliogenic (bars) and conidiogenic (line) germinations of MS exposed to UV-B radiation. Different letters indicate significant differences according to Tukey mean comparison test (95%) for conidiogenic germination and according to Kruskal–Wallis test (95%) for myceliogenic germination. The error bars represent the standard deviation of the mean from six replicates.

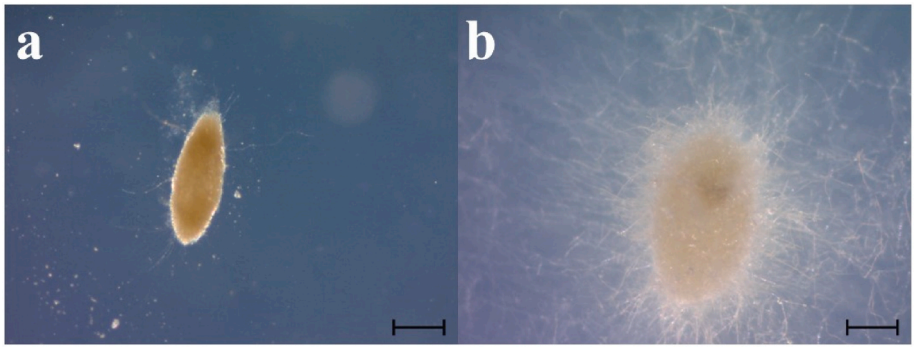

Fig. 8. Myceliogenic germination of MS exposed to UV-B radiation. (a) MS non-radiation. (b) MS after 4 h irradiation. Photographs were taken under a stereoscope with objective 2.5×. Scale bar: 500 μm.

Table 3
Conidiogenic germination (conidia/MS) from MS incubated at 30, 40, and 50 °C. Different letters indicate significant differences according to Tukey mean comparison test (95%).

Temperature (°C)	Incubation time (hours)			
	0	1	2	3
30	2.0×10^{5bc}	1.7×10^{5bc}	2.8×10^{5a}	1.7×10^{5bc}
40	2.0×10^{5bc}	2.1×10^{5ab}	1.7×10^{5bc}	2.0×10^{5bc}
50	2.0×10^{5bc}	1.9×10^{5bc}	1.3×10^{5cd}	0.8×10^{5d}

covered the cadaver, and after a couple of days, green sporulation occurred, confirming that *M. robertsii* was the cause of death.

The *Probit* analysis presented a P value > 0.05, indicating a linear correlation between fungus dose and larval mortality. The mean lethal concentrations were 9.8×10^4 conidia/mL and 5.6×10^4 conidia/mL for the aerial conidia (from SSF) and MS-derived conidia, respectively (Table 4).

Although the LC_{50} determined for conidia produced by MS was 1.7 times higher than the LC_{50} obtained for aerial conidia produced by SSF, these values did not significantly differ according to the comparison of their confidence limits (95%). This result was confirmed by the POLO-PLUS program, which determined a value of $P > 0.05$ for the hypotheses of equality and parallelism, suggesting that MS-derived conidia were as virulent as conidia produced from SSF.

4. Discussion

We have shown that the Colombian strain of *M. robertsii* Mt004 can produce MS in submerged fermentation and that the C/N ratio and type of nitrogen source in the culture medium play a determining role in microsclerotia yield and morphogenesis.

The maximum yields were obtained with media A and D, which contained yeast extract. Yeast extract is prepared mainly from waste brewer's yeast, which is rich in nucleotides, proteins, amino acids (glutamic acid, aspartic acid, glycine, and alanine), sugars and a variety of trace elements and vitamins (Tao et al., 2023) and has been demonstrated to stimulate the growth and sporulation of *Metarhizium* spp. strains (Li and Holdom, 1995). Culture medium A also contained peptone, a rich source of amino acids such as glutamic acid, proline, leucine, aspartic acid, and lysin, which has also been frequently used as a source of nutrients for *Metarhizium* spp. production (Kleespies and Zimmermann, 1992; Jaronski, 2023). Although media A and D had very different C/N ratios (28:1 and 55:1, respectively), both reached the maximum MS yields after 8 days of fermentation. This finding suggested that the source of nitrogen, common for A and D, was more relevant for MS production than was the C/N ratio (Jackson and Jaronski, 2009; Kobori et al., 2015).

The nitrogen source in Medium B was acid-hydrolyzed casein (CAS). CAS has been demonstrated to promote rapid growth in microorganisms, including *Metarhizium* spp., by providing essential amino acids such as glutamic acid, proline, leucine, lysine, serine, valine, and aspartic acid (Kerr et al., 1983; Li and Holdom, 1995). This nitrogen source has been commonly used for MS production by different authors (Jackson and Jaronski, 2009; Kobori et al., 2015; Rivas-Franco et al., 2020), who obtained yields of approximately 1×10^5 MS/mL, which is greater than our yield (1.7×10^3 MS/mL). These differences could be related to the specific nutritional requirements of each fungal species or strain (You-Ping et al., 2012).

Culture medium C was the only medium containing an inorganic nitrogen source (ammonia citrate) and was used in previous work to optimize MS production with an *M. rileyi* strain (Song et al., 2014). However, ammonia citrate was not as effective as organic nitrogen sources for the induction of MS formation in *M. robertsii* Mt004, suggesting that this strain needs an organic nitrogen source for carrying out protein and nucleic acid synthesis and stimulating proper cellular functioning and fasting growth (Griffin, 1981). Generally, culture media with inorganic nitrogen sources must be supplemented with amino acids such as glutamic acid and aspartic acid that promote transamination processes (Griffin, 1981). In this sense, the poor yields obtained with medium C suggested that ammonia citrate did not provide the nitrogen required to induce MS formation in Mt004.

The critical effect of nitrogen sources on fungal aggregate formation was also demonstrated by Shearer and Jackson (2006) when MS were produced from *Mycoleptodiscus terrestris*. The highest yield was obtained with cottonseed meal (1.5×10^3 MS/mL), followed by corn steep liquor powder, yeast extract and casamino acids that reached concentrations of 7.2×10^2, 4.5×10^2 and 1.5×10^2 MS/mL, respectively. These authors reported that nitrogen sources affected not only yields but also the pigmentation, conidial production, germination, and morphology of MS.

The highest MS yields obtained in the present work were approximately 10^3 MS/mL at 8 days of fermentation, a value like that reported by other authors, e.g., Lira et al. (2020), who used the strain ESALQ2459 of *M. robertsii* and a culture medium with a C:N ratio of 10:1 and obtained a yield of 1.8×10^3 MS/mL after 4 days of fermentation. Mascarin et al. (2014) obtained slightly higher yields after 5 days of fermentation with the strain CG632 of *M. robertsii* and the same culture medium used in the present work (referred to here as medium D), reaching 7.0×10^3 MS/mL.

Mature MS produced by entomopathogenic fungi were recently defined as condensed survival structures (Liu et al., 2022) composed of hyphal aggregates that are completely compact, darkly pigmented and with or without a distinct core (Paixão et al., 2021). In this sense, Rivas-Franco et al. (2020) proposed that longer fermentation times result in higher melanization of MS, which could be associated with MS maturation. Based on these findings, the fermentation time for the production of MS from *M. robertsii* Mt004 was extended to 20 days, when dark pigmentation of the structures was observed. At this point, the

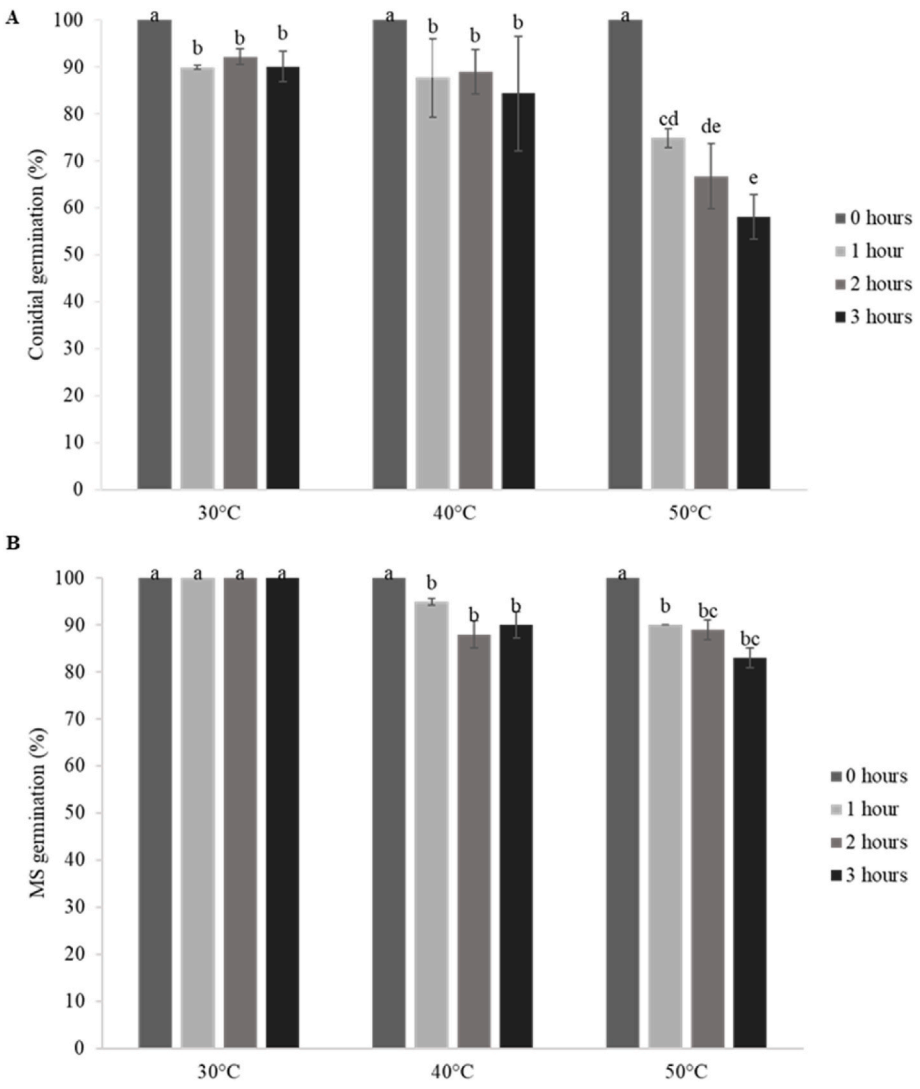

Fig. 9. Germination of conidia and MS (48 h of incubation) exposed to heat (30, 40 and 50 °C). Different letters indicate significant differences between treatments according to Tukey mean comparison test (95%). (A) Conidia from SSF, (B) MS. Error bars represent the standard deviation of the mean across six replicates.

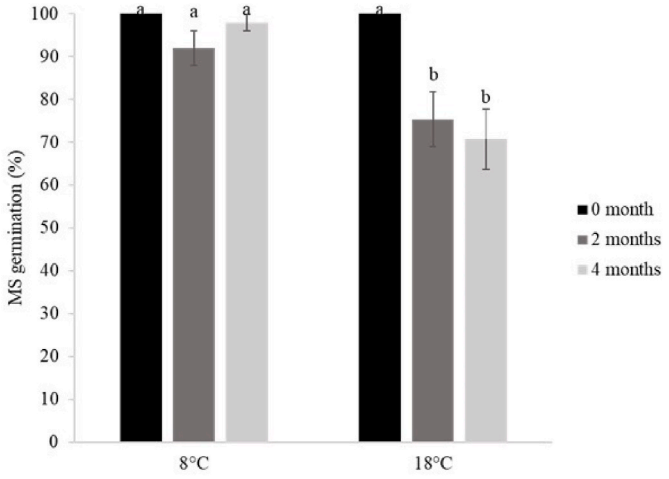

Fig. 10. Myceliogenic germination of MS granules stored during 4 months at 8° and 18 °C. Different letters indicate significant differences between treatments according to the Tukey mean comparison test (95%). Error bars represent the standard deviation of the mean across three replicates.

Table 4
Probit analysis of conidia from SSF and MS-derived conidia against *D. saccharalis* larvae.

Treatments	LC_{50} (conidia/mL)	Limits of confidence (95%)		χ^2	df	P
		Lower	Upper			
MS-derived conidia	9.8×10^4	7.9×10^3	1.2×10^6	1.399	3	0.466
Conidia from SSF	5.6×10^4	6.8×10^3	3.3×10^5	1.307	3	0.436

structures were considered mature, which could be related to better tolerance to abiotic factors and longer survival under stress conditions (Rivas-Franco et al., 2020).

After 20 days of fermentation, we obtained a final concentration of 2.4×10^3 MS/mL, which was lower than that reported by other authors who have reached yields $>10^4$ MS/mL after shorter fermentation times of approximately 4–8 days (Jackson and Jaronski 2009, 2012; Kobori et al., 2015; Rivas-Franco et al., 2020). However, this research demonstrated the feasibility of inducing MS formation with the *M. robertsii* Mt004 strain and allowed the establishment of preliminary growth conditions that will constitute the basis for further optimization and

scale-up of this process.

Fungi that normally form sclerotia during their life cycle produce consistent structures of closely intertwined hyphae that often form parenchyma-like tissue, where it is possible to distinguish three concentric zones identified as the rind, core and medulla (Willetts and Bullock, 1992). Entomopathogenic fungi need to be artificially induced to form MS-ls, which present a different structure that has been described as an outer layer of pigmented parenchymal cells and an inner layer of poorly pigmented or colorless medullary cells (Song, 2018). Commonly, MS formation is divided into three stages: initiation, development, and maturation (Georgiou et al., 2006; Song et al., 2013), and the initiation has been associated with low availability of nitrogen sources during the initial days of fermentation (Jackson and Schisler, 1992).

In the present study, hyphal aggregates and the extracellular matrix began to form during the initial stages of MS formation, similar to what has been reported for other sclerotia-forming fungi, such as *Grifola umbellata* (Choi et al., 2002) and *Ophiocordyceps sinensis* (Xing and Guo., 2008). The presence of blastospores and submerged conidia inside the aggregates suggested that these free cells were also produced during fermentation; these cells were then trapped by hyphae and became entangled with each other to form aggregates.

Cell lysis within the structures possibly contributes to the production of material for extracellular matrix formation. This matrix forms the majority of MS and potentially plays an important role as an energy and nutrient source for MS germination (Bullock et al., 1980; Willetts and Bullock, 1992; Choi et al., 2002; Xing and Guo, 2008; Villamizar et al., 2021). Although the composition of the extracellular matrix was not determined in this study, other authors have reported that fungal sclerotia produce and contain high amounts of low-molecular-weight compounds such as carbohydrates, monosaccharides, disaccharides, polysaccharides, and polyols. These authors have also attributed different functions to this matrix, including morphogenesis, cell adhesion and water storage to prevent dehydration, as well as work as energy reserves and help in structure compaction (Willetts and Bullock, 1992; Choi et al., 2002; Villamizar et al., 2021).

The granular material observed inside the structures suggested that some solid components of the culture medium were also trapped during MS formation; this was also described by Villamizar et al. (2021), who observed crystalline structures during the formation of cellular aggregates of *Beauveria pseudobassiana*. These authors concluded that the encapsulation of medium components inside the aggregates could constitute another fungal strategy for storing nutrients and energy reserves that can be used to survive under stress and revive when conditions are suitable for fungal development.

Some structural changes occurred between days 8 and 16 of fermentation, when a more compact structure with some hyphae exposed on the surface and others embedded in the extracellular matrix was observed. Cell death also increased, which could be related to oxidative stress caused by fermentation conditions or due to nutrient depletion in the broth, which was also correlated with an increase in extracellular matrix density (Bullock et al., 1980; Willetts and Bullock, 1992; Choi et al., 2002; Xing and Guo, 2008).

During the maturation process (16–20 days of fermentation), MS reached high compaction and the surface was completely covered with an extracellular matrix, which could act as a protective barrier encapsulating the cells in the interior and shielding them from damaging abiotic and biotic conditions (Villamizar et al., 2021). Internally, we differentiated two zones, a central/inner zone with a few live cells embedded in an extracellular matrix that could correspond to the medulla zone previously described by other authors (Song et al., 2013; Song, 2018; Liu et al., 2022) and an outer zone composed of pigmented parenchyma cells (Song, 2018).

Sunlight, temperature, desiccation, and precipitation are factors that affect the field persistence and survival of fungal propagules produced by entomopathogenic fungi. Therefore, these conditions should be considered when developing stable biopesticides to guarantee their efficacy under field conditions (Corval et al., 2021). In this context, we assessed the robustness and biological activity of MS in comparison with aerial conidia produced by solid fermentation, and in general terms, MS were more tolerant to heat and UV radiation than conidia, confirming the findings of other authors (Song et al., 2014, 2016; Corval et al., 2021).

The tolerance of MS to UV-B radiation could be related to the increase in dark pigmentation that occurred when the fermentation time was extended. Some pigments, such as melanin, can absorb light energy via the conversion of photon energy (Gessler et al., 2014) through a process called ultrafast internal conversion (Tseng et al., 2014). In accordance with Song (2018), during MS formation, a gelatinous pigmented matrix is deposited in outer layer cells and is influenced by nutritional status, age of culture and presence of antioxidants. Fungal pigmentation confers protection against UV radiation and heat and enhances cell rigidity (Song, 2018). Recently, Espín-Sánchez et al. (2023) studied the dark pigments produced by *M. robertsii* ARSEF 2575 during microsclerotia formation and suggested that DHN-melanin could play a role in microsclerotia differentiation and environmental stress tolerance. However, further research is needed to understand the role of pigments in microsclerotia from entomopathogenic fungi.

The susceptibility/tolerance of conidia to UV radiation has also been associated with cell color/pigmentation (Fernandes et al., 2015). This was demonstrated by Braga et al. (2006), who showed that conidia from *M. anisopliae* mutants without color were more susceptible to UV radiation than wild conidia with natural green coloration. Ignoffo and Garcia (1992) also reported that dark conidia (almost black) produced by *Aspergillus niger* survived after 16 h of exposure to simulated sunlight, while conidia produced by *M. anisopliae, M. rileyi, B. bassiana* and *A. niger subsp. cinnamomeus* (conidia colorless) survived for only a maximum of 2 h of irradiation.

The MS produced by *M. robertsii* Mt004 showed high photostability, with >80% germination remaining after 4 h of irradiation with a UB-V lamp (total energy of 50.4 kJ/m^2). In a similar work, MS produced by *Purpureocillium lilacinum* germination was reduced to 25.0% when exposed to a lower irradiation energy (19.4 kJ/m^2) (Song et al., 2016). These differences could be related to the inherent characteristics of each fungal species or strain but also to the different contents and compositions of pigments in the MS, as well as differences in culture history (nutrients and light quality) and water imbibition (dry or wet) (Corval et al., 2021). For example, Corval et al. (2021) demonstrated that MS produced by different species of *Metarhizium* sp. presented different germination rates (between 9.2 and 100%) after 1 h of exposure to UV-B radiation (4.0 kJ/m^2).

Temperature is a limiting factor during morphogenesis, germination, and fungal metabolic processes (Abrashev et al., 2008; Zhang and Zhang, 2016; Paixão et al., 2021), but the germination of both conidia and MS from *M. robertsii* Mt004 was not affected by heat shocks at 30 °C or 40 °C in the present study. However, MS performed better than conidia when the heat shock was at 50 °C and maintained >80.0% germination after three hours, while conidia decreased to 58.0% germination. Tolerance to heat stress in sclerotia/microsclerotia has been related to the type of carbohydrates accumulated during structural development and the formation of the extracellular matrix that embeds hyphae and provides protection against physicochemical and biological degradation (Willetts, 1997; Ellil, 1999; Fan et al., 2017). The thermotolerance observed in *M. robertsii* Mt004 could also be related to specific adaptation processes to the natural habit where this strain was isolated (Rionegro, Antioquia, Colombia) but could also be the result of repeated exposure to high temperatures in the field because infected hosts frequently attempt to control the disease by raising their body temperature by exposing it directly to the sun (Corval et al., 2021).

In the present work, the effect of temperature on the germination of MS and conidia was studied by exposing inoculated agar plates to dry heat (Fargues et al., 1997; Rangel et al., 2010; Jackson et al., 2011),

which could simulate what happens in the environment when fungal propagules are on the surface, on the leaf or soil or when the host insects are in environments with low relative humidity. However, several authors have reported that temperature can affect cells in different ways, and dry heat is usually less detrimental than wet heat (Rangel et al., 2005). For example, Song et al. (2014) reported that the germination of *M. rileyi* MS suspended in Tween 80 solution (0.1%) decreased from 93.5% to 34.5% when the plants were exposed to 50 °C for 45 min in a water bath. Later, Song et al. (2016) reported that MS produced by *P. lilacium* reduced its germination rate from 92.4% to 23.5% after 30 min of exposure to wet heat at 50 °C.

However, MS progressively produced fewer conidia (less sporulation) with increasing exposure to heat and UV-B radiation. A loss of sporulation capacity was also observed by Paixão et al. (2021), who studied the susceptibility of MS and mycelium pellets produced by *M. robertsii* ARSEF 2575 to UV radiation and 45 °C. MS and mycelium pellets had 83.0% and 89.0% reductions in conidium production after 4 h of UV irradiation and 36.0% and 52.0% reductions after 4 h of exposure to heat (45 °C). Based on these results, the authors concluded that MS possess the ability to withstand both stressful conditions better than mycelia, as shown by their greater conidium production than mycelium pellets.

The mechanism for the reduction in conidium production in MS is unknown. In this context, we propose two hypotheses. First, it is possible that some internal cells suffer the lethal effects of UV radiation and heat, leaving very few surviving cells that can germinate and produce mycelia and conidia (reduction in MS vigor). However, further studies are needed to determine the viability of MS internal cells after exposure to these stress conditions. Our second hypothesis is that a latency state is induced by exposure to radiation/heat. This behavior has been observed in conidia exposed to stressful conditions such as UV radiation, heat, or storage and is thought to be due to cells prioritizing repair systems and consequently delaying germination and sporulation processes (Fernandes et al., 2007). It is possible that the seven days of incubation needed to assess sporulation were not enough for MS to recover from abiotic stress, resulting in low conidia counts. This phenomenon was also observed by Paixão et al. (2021), who evaluated the production of conidia after 10 days of incubation and showed that this period was necessary to promote complete sporulation in MS from *M. robertsii* ARSEF 2575.

Compared with conidia, MS from *M. robertsii* Mt004 were more robust and more tolerant to desiccation and storage conditions. This tolerance is generally influenced by humidity and drying rate (Hong et al., 2000); as well as by nutritional and fermentation conditions, which determine the production and accumulation of endogenous reserves and/or polyols in cells (Hallsworth and Magan, 1996; Jackson et al., 2010; Kobori et al., 2015).

The decrease in MS germination after storage at 18 °C could have been the result of the negative effects of moisture content and atmospheric oxygen, which have been described as critical factors affecting the shelf life of fungal propagules (Jackson et al., 2010; Mascarin et al., 2018). These conditions must be kept as low as possible to avoid the growth of contaminants, activation of basal metabolism and the use of energy reserves as well as the production of toxic metabolites (Moore et al., 1996; Jackson and Jaronski, 2012). The significant decrease in MS viability during storage in the present work may also be related to the long fermentation time. During the differentiation process of MS formed by Mt004, we observed an increase in cell autolysis and extracellular matrix production along with a reduction in the live cell concentration in the internal structure by extending the fermentation time. Coley--Smith and Cooke (1971) and Liu et al. (2022) proposed that inner hyaline cells are autolyzed during differentiation and that these cells cannot be viable for germination. The low concentration of live cells in the mature MS may be responsible for the low storage stability, indicated by low germination and conidium production. Future studies are necessary to evaluate the performance of MS harvested at shorter fermentation times to test this hypothesis and establish the optimal duration at which MS need to be harvested to maximize their ecological fitness.

These results are similar to those reported by Villamizar et al. (2018) for MS produced by *B. pseudobassiana* AgR-F704, which retained a germination rate of 68.0% after six months of storage at 20 °C, in contrast to the germination rate of conidia, which decreased from 40.0% to 0%. Song et al. (2014, 2016) evaluated the stability of MS from *P. lilacinum* and *M. rileyi*, finding that both microorganisms maintained 85% germination after 12 months of storage at 25 °C. These authors considered that MS granules presented an "acceptable" shelf-life and highlighted the impact of the germination protocols applied to assess this parameter in filamentous fungi.

MS-derived conidia and aerial conidia caused larval mortalities of up to 77.9% and 84.2%, respectively, when *D. saccharalis* larvae were inoculated with a final concentration of 1.0×10^7 conidia/mL. These results were similar to the 82.6% reported by Torres-Torres et al. (2020) for conidia grown on potato dextrose agar (PDA) from the same strain of *M. robertsii* Mt004 against the same host (*D. saccharalis* larvae).

Conidia produced by MS were able to cause infection and mortality in *D. saccharalis* larvae, with the same level of virulence as conidia produced by solid fermentation. This ability of MS to produce infective conidia is an important and remarkable characteristic that can be used as a promising alternative in biopesticide development (Jaronski and Jackson, 2008; Jackson and Jaronski 2009; Rivas-Franco et al., 2020; Villamizar et al., 2021). Several authors have shown that MS applied to fields under suitable conditions can remain viable and infective while producing and releasing large amounts of conidia into the environment (Jackson and Jaronski, 2012; Correal et al., 2018a,b). Goble et al. (2016) estimated that each MS produced by *Metarhizium brunneum* F52 was able to produce approximately 100,000 conidia in the field, increasing over time, suggesting that reapplication of a product in the field might only need to occur after 6 weeks.

In this work, each MS was able to produce approximately 10^5 conidia, which would require a field application rate of approximately 2.0×10^7 MS per hectare, based on the application doses for *Metarhizium* spp. conidia reported in the literature that suggests approximately 10^{12} to 10^{13} conidia per hectare (Lomer et al., 1993; Peng et al., 2008; Pilz et al., 2011; Ansari and Butt, 2013). The smaller amount of propagules required for field application combined with the lower cost of production and formulation suggest greater technological and economic feasibility of using MS produced by liquid fermentation as the active ingredient for biopesticides in comparison with using aerial conidia produced by SSF.

In conclusion, *M. robertsii* Mt004 can produce MS in liquid culture using a medium containing yeast extract as the nitrogen source. Mature structures formed after 20 days of fermentation were more tolerant to heat and UV radiation than conidia from SSF. MS-derived conidia showed high virulence against *D. saccharalis,* reaching similar insecticidal activity as aerial conidia. These results suggest that MS from *M. robertsii* Mt004 are promising propagules that could replace conidia as the active ingredient in the development of novel biopesticides with high technical and economic feasibility.

Disclosure of statement

The authors report no conflicts of interest.

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Declaration of competing interest

This paper has not been published previously and is not under

consideration elsewhere. The authors are responsible for the reported research, and all of them have participated in the concept and design, analysis and interpretation of data, drafting or revising of the manuscript, and have approved the manuscript as submitted. The data, models, and methodology used in the research are the property of AGROSAVIA/AgResearch/Universidad Nacional de Colombia. There is no conflict of interest on the part of any of the authors with other authors or reviewers.

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