



British Mycological
Society promoting fungal science

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio



Temperature and growth media affect conidia production, germination and virulence of *Metarhizium* spp. during simulated solid substrate fermentation conditions

Arnau Sala^{a,*}, Morgane Ourry^b, Nicolai V. Meyling^b

^a GICOM – Research Group on Bioconversion of Organic Residues and Advanced Materials, Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, Edifici Q, Campus de Bellaterra, Cerdanyola del Vallès, 08193, Spain

^b Department of Plant and Environmental Sciences, Section for Organismal Biology, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg, 1871, Denmark

ARTICLE INFO

Keywords:

Thermotolerant fungi
Conidia production
Conidia germination
Virulence
Tenebrio molitor
Principal component analysis

ABSTRACT

Solid State Fermentation (SSF) scale-up constraints (oxygen diffusion, heat accumulation, mass transfer and irreproducibility) pose significant obstacles to the production of biopesticides, such as entomopathogenic fungi (EPF). Understanding the responses of thermotolerant fungal strains is key to overcome these constraints. However, combination of relevant parameters for fungal growth and sporulation (temperature, growing media and time) may affect insecticidal activity (conidia production, germination and virulence), but this is not fully understood. Here, two thermotolerant strains of the EPF *Metarhizium* (*Metarhizium brunneum* EAMa 09/01-Su and *Metarhizium robertsii* EAMa 01/158-Su) were studied by combining the three parameters of relevance for SSF. Production conditions affected both quantity (conidia production) and quality (conidia germination and virulence) of the obtained bioproduct. Both strains were suitable for thermotolerant conidia production, with the *M. brunneum* strain being superior in *Tenebrio molitor* infection and conidial germination and the *M. robertsii* strain exhibiting faster germination and higher conidia production. These results provide valuable knowledge and serve as starting point for future works in production scale-up via SSF.

1. Introduction

Massive use of chemical pesticides in agriculture has led to several threats both to human health and to the environment due to their toxicity and mutagenic capabilities, demanding a shift to greener options (Rani et al., 2021). Biological control agents represent preferred alternatives to chemical pesticides within integrated pest management (Galli et al., 2024), among which entomopathogenic fungi can target a wide range of arthropod pests due to their ability to infect a range of species across insect orders while presenting no harm to humans or to the ecosystem (Mascarin and Jaronski, 2016). Fungal biopesticides can be produced by solid-state fermentation (SSF), being the only method able to obtain large quantities of aerial conidia, which are cheap and have higher resistance to temperature than propagules produced by submerged fermentation (De la Cruz Quiroz et al., 2015; Miranda-Hernández et al., 2017), thus requiring less formulation technology. However, SSF has drawbacks, mainly related to inherent constraints of oxygen diffusion, heat accumulation and mass transfer, which

are amplified during process scale-up. The main challenge lies in heat removal, being critical in every scale-up process regardless of the strain, reactor configuration or substrate used (Mitchell et al., 2023).

Use of thermotolerant fungal strains for aerial conidia production could be part of the solution to overcome challenges of heat generation. Thermotolerant fungi are those which can survive relatively well above temperatures close to or above 40 °C (Mouchacca, 2000). Thermotolerant fungal strains of different genera have been successfully cultivated, specially for enzymatic production (Arora et al., 2015; Casciadori et al., 2013; Hildén et al., 2009). Despite this, thermotolerant fungi have not been studied for conidia production by SSF. Effects of heat exposure in fungi have mostly been studied by means of heat shocks, involving exposure periods of elevated temperatures (around 37 °C) for 4–16 h (Keyser et al., 2014; Mattoon et al., 2021), conditions not reflecting the temperature rise that can be obtained when scaling up SSF (Mitchell et al., 2023).

Aside from high conidia production, ensuring viability of the conidia produced and maintaining virulence against arthropod pests also play

* Corresponding author.

E-mail address: arnau.sala@uab.cat (A. Sala).

<https://doi.org/10.1016/j.funbio.2026.101794>

Received 20 February 2026; Received in revised form 21 May 2026; Accepted 2 June 2026

Available online 3 June 2026

1878-6146/© 2026 The Authors. Published by Elsevier Ltd on behalf of British Mycological Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

crucial roles for successful strategies of fungal biopesticide fermentation. There is currently limited knowledge of comparative viability and virulence in SFF conidia production, as most studies have focused on single responses (Méndez-González et al., 2024; Sala et al., 2023; Yousef-Yousef et al., 2022). Therefore, existence of potential trade-offs in conidia produced remains unclear. Moreover, temperature effects should be studied in combination with growth substrate and production duration, which are all highly relevant during heat generation processes occurring during SSF (Mitchell et al., 2023). Furthermore, temperatures and exposure duration optimal for fungal hyphal growth may not correspond to optimal conditions for sporulation (Mattoon et al., 2021), indicating that different scenarios should be tested for growth and sporulation to sufficiently evaluate effects of SSF production conditions on conidia production, viability and virulence. Studies on these relationships and identification of trade-offs in thermotolerant fungal strains responses are mandatory to reproducibly produce effective biopesticides.

In this study, two selected strains of the entomopathogenic fungal genus *Metarhizium*, which were previously demonstrated to have thermotolerant traits (Lozano-Tovar et al., 2015; Yousef-Yousef et al., 2022), were used as model strains with an overall aim to clarify the ability of thermotolerant fungi strains to overcome heat generation constraints associated with SSF scale-up. To achieve this, the two *Metarhizium* strains were cultivated on a range of solid substrates at either constant or variable temperatures for quantification of resulting conidia production, viability (germination) and virulence against a model insect, *Tenebrio molitor*. We expected that the conidia traits of the two strains will show variability depending on growth substrate and temperature conditions. For substrates, different behaviour is expected depending on sugars present (higher growth and sporulation are expected in substrates with highest number of fermentable sugars). Higher temperature tolerance is expected from the strain of highest optimal growth temperature. However, their interaction cannot be predicted beforehand, highlighting the need of this study. This knowledge will be essential for identification of potential constraints during SFF production conditions.

2. Materials and methods

2.1. Fungal strains

The two entomopathogenic fungal strains were: *Metarhizium brunneum* EAMa 09/01-Su (also accessioned CECT20784) and *Metarhizium robertsii* EAMa 01/158-Su (also accessioned CECT20987). Both strains were obtained from the culture collection of the Agricultural Entomology Research Group AGR 163, at the Department of Agronomy, University of Cordoba, Spain. Both strains were isolated from soil in Sevilla, Spain, and have documented thermotolerant traits and pathogenicity towards several insect species (Garrido-Jurado et al., 2020; Resquín-Romero et al., 2016; Yousef-Yousef et al., 2022).

2.2. Media preparation

Six different solid media were used for cultivation of both strains (Table 1). Baseline agar media consisted of Sabouraud Dextrose Agar supplemented with Yeast (SDAY) represented a control substrate, while

same ingredients at quarter strength were tested as a depleted nutrient substrate (SDAY/4). Media representing sugar concentrations comparable to solid substrates relevant for SSF were prepared (Sala et al., 2023), including surrogate media for rice husk (RH) and beer draff (BDr). The agar media of RH contained relatively low concentrations of sugars, neopeptone and yeast extract, while media of BDr contained medium concentrations of the three components. As sugar for RH and BDr media, arabinogalactose was selected as it is more difficult to metabolise in comparison to glucose (Rangel et al., 2006). The media RH and BDr were always tested in comparison to SDAY. Media representing insect hosts (“trehalose” media) and plant rhizosphere (“raffinose” media) were also prepared to evaluate the responses of the two strains when cultivated on sugars predominantly represented in insect hemolymph (trehalose) and in root exudates of the rhizosphere of plants (raffinose) (Hu and St. Leger, 2002).

All media were autoclaved at 121 °C for 20 min and poured into 9 cm Petri dishes (approx. 25 mL per dish). The media were stored at room temperature for a maximum of 1 month before use.

2.3. Fungal growth and sporulation on solid substrates

Temperature, time and media were previously identified as key parameters affecting conidia production and viability during SSF (Sala et al., 2019). Four fungal growth and sporulation experiments were designed to represent either constant temperatures (CT experiments) or variable temperatures (VT experiments) during growth and sporulation phases; in VT experiments same total time but variable time periods for the two phases were investigated. Temperatures ranged between 15 and 35 °C for *M. brunneum* EAMa 09/01-Su, while temperatures between 15 and 40 °C were used for *M. robertsii* EAMa 01/158-Su. Incubation was performed in darkness inside climate cabinets. Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were used to obtain accurate temperature profiles during the experiments (maximum variation ±1 °C). Conditions and workflow of the four experiments are presented in Supplementary Materials (Figs. S1–S4). Due to the nature of the experiments, performed at the scale of petri dishes in large numbers, parameters that indicate fungal fermentative metabolism in SSF, such as oxygen consumption and CO₂ production, were not measured.

In all experiments, inoculation was achieved with pipetting 25 µL of conidia suspension at 10⁷ conidia/mL in 0.1% sterile Triton-X per plate and dispersing with a Drigalski spatula. All experiments were performed in triplicates with independent inoculation suspensions.

2.4. Conidia suspensions and conservation

Following each growth and sporulation experiment on the respective solid media (Table 1, Figs. S1–S4), conidia were harvested for evaluation of traits. From each Petri dish, conidia were suspended by adding 10 mL of sterile 0.1% Triton-X onto the sporulating culture and gently rubbing using a sterile Drigalski spatula to release conidia. The resulting suspension was transferred to a 50 mL Falcon tube using a 1 mL pipette with cut tip. The procedure was repeated until the Falcon tube contained 40 mL of suspension. The suspension was then poured into a new 50 mL Falcon tube through a glass funnel containing two layers of sterile gauze

Table 1
Detailed concentrations of all components in the six selected solid media.

Media/component	pH	Sugars (g/L)	Neopeptone (g/L)	Yeast extract (g/L)	Sugar concentration (%)
Control (SDAY)	6.5	40	10	10	100% glucose
SDAY/4	6.5	10	2.5	2.5	100% glucose
Rice husk (RH)	6.5	6	0.5	1.5	3% glucose, 97% arabinogalactose
Eq. beer draff (BDr)	6.5	20	3	5	50% maltose, 25% glucose, 25% arabinogalactose
Eq. insect (Trehalose)	6.5	34	8.50	8.5	100% trehalose
Eq. plant (Raffinose)	6.5	40	10.00	10	100% raffinose

to obtain a standardized conidia suspension without mycelia debris, representing a single Petri dish from the respective experiment. Each conidia suspension was aliquoted into 2 mL cryogenic tubes at 10% glycerol (0.4 mL 50% glycerol + 1.6 mL sample) and stored at -80°C for a maximum of 1 month before use.

2.5. Quantifying conidia production and germination using oCelloScope

Conidia counting and germination evaluation of conidia suspensions from both *Metarhizium* strains were always performed using an oCelloScope (model E5AA) and the Uni Explorer software v12.1.0.39 (Bio-Sense Solutions, Denmark).

2.5.1. Preparation and software

Each conidia suspension was prepared from a cryogenic tube kept at -80°C using 0.1% Triton-X as diluent and adjusting concentrations to 10^3 – 10^5 conidia mL^{-1} (dilutions of 1:10, 1:100 or 1:1000 depending on initial sample concentration), as recommended by the oCelloScope provider. Using a sterile 96 well-plate (Nunc Delta Surface, Thermo Fisher Scientific, Denmark), 100 μL of each suspension were transferred per well in laminar air flow chamber. Three biological replicates were prepared in all experiments, each representing a separate conidia suspension obtained from independent *Metarhizium* cultures. For each run, control treatments (100 μL of 0.1% Triton-X) were prepared in triplicate. After loading the wells, the 96-well plate was placed in the oCelloScope and secured laterally with side screws to avoid movement of samples. To ensure homogeneous temperature during incubation, the oCelloScope instrument was placed inside an incubator (TS 1008-i, Xylem, Denmark) with the temperature set according to each *Metarhizium* strain, 25°C for *M. brunneum* EAMa 09/01-Su and 28°C for *M. robertsii* EAMa 01/158-Su (Yousef et al., 2013; Yousef-Yousef et al., 2022). Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were placed inside the incubator during the experiments to obtain accurate temperature profiles (maximum variation $\pm 0.5^{\circ}\text{C}$). When temperatures stabilized at $\pm 0.5^{\circ}\text{C}$ of the intended experimental temperature, the 96-well plates were left for 1h before recording started to allow for settlement of conidia in the wells. Uni Explorer 12.1 software was used in all oCelloScope experiments.

2.5.2. Conidia counting

For conidia counting, image acquisition was performed from biological triplicates. The following parameters were used: image distance of 4.08 μm , scan area of 337.5 μm , illumination of 167 and 2 ms, with 10 images per well. The detection limit of the oCelloScope for conidia was determined as ca. 1000 conidia mL^{-1} (or 1 conidium μL^{-1}). The analysis of the images was done in the Uni Explorer software v12.1.0.249 using the 'Fungal Spore Count' plugin v4. The count plugin has different models to detect and count conidia, the 'Metharizium' model was used at a threshold between 0.5 and 0.8 depending on how many 'objects' were present on the image and by ensuring that only few non-conidia objects were counted. A mean count value of conidia per well was then obtained from the triplicates of each sample.

For data recording, the 'acquire' and 'counting' functions were used sequentially. A total of five repetitions were selected for a total time of 1h 20min, corresponding to acquiring images every 20 min including time 0. Conidia counts and calculation of concentrations (i.e., conidia production) were obtained using the software. These values were used to determine the necessary dilution (1:10, 1:100 or 1:1000) to achieve the correct concentration (10^3 – 10^5 conidia mL^{-1}) to evaluate conidia germination.

2.5.3. Conidia germination

The same set-up as in conidia counting experiments was used, although longer duration and data acquisition intervals were required for obtaining germination rates. Samples were diluted to a concentration between 10^3 – 10^4 conidia mL^{-1} (corresponding to approximately 20–200

conidia per well) using 0.1% Triton-X, according to conidia counts previously performed. Total time was set to 40 h and data was acquired every 1.5 h. Conidial viability was expressed as germination %. Objects counted as conidia by the oCelloScope diminish over time as the conidia germinate, allowing an estimation of the germination % based on initial counts. Germination was calculated for each time point based on the number of objects counted at that time point in comparison to the highest number initially counted, producing a germination % for each sample for each time point. Germination-based metrics are based on conidia counting, with the number of conidia identified by the oCelloScope software's algorithm decreasing as the conidia germinate. As such, 100% germination occurs at the time point where the conidia count is zero. Time to 50% germination, however, is calculated as the time it takes for 50% of the conidia to germinate. The minimum number of conidia counted is subtracted from the maximum number of conidia counted and divided by two to calculate 50% germination. Time to 50% germination is thus the time at which this germination rate occurs. This calculation thus considers that 100% germination may not occur under all circumstances.

2.6. Infection bioassays against insects

Larvae and/or adults of the yellow mealworm *T. molitor* (Coleoptera: Tenebrionidae) were exposed to conidia obtained from the different growth and sporulation conditions to evaluate the relative effect on virulence of the conditions on each of the two *Metarhizium* strains. Conditions and workflow of the bioassays are presented in Supplementary Materials (Figs. S5 and S6).

2.6.1. Insect culture

Insect culture maintenance, size, conditions and production of adult beetles were performed as in previous works (Sala et al., 2023).

2.6.2. Insect bioassays set-up

Different set-ups were used depending on *T. molitor* developmental stage. For larvae, the setup was adapted from (Herren et al., 2023). Larvae were introduced in plastic cups (5.2 cm diameter, 155 mL) in groups of 10 per experimental treatment, with each experiment repeated three times, resulting in total of 30 larvae per treatment. Each cup was closed with a perforated lid and contained a filter paper at the bottom (3×3 cm) moistened with 0.15 mL sterile water and a soaked filter paper (2×5 cm) on the inner side of the cup to maintain humidity. Larvae were exposed individually to conidia suspensions by pipetting 2 μL of the suspension (see 2.6.3 and 2.6.4) onto the anterior part of the body. Larvae were then incubated in the cups at 25°C (*M. brunneum* EAMa 09/01-Su) or 28°C (*M. robertsii* EAMa 01/158-Su) in darkness for 24h. Then, each group of 10 larvae were moved to a new plastic cup without lid containing 6 g diet and 1 g bacteriological water agar (1% w/v), which was maintained at same conditions as during the first 24 h. Cups were checked daily for shed exuviae during first two days. Twice a week, 1 g bacteriological water agar (1% w/v) was added to each cup. Mortality was monitored daily for 14 days.

In the infection experiments with adults of *T. molitor*, medicine cups of 30 mL were used. Beetles were individually exposed by pipetting 5 μL of the conidia suspension (see 2.6.3 and 2.6.4) onto the intersegmental membrane between the head and the pronotum. Each beetle was then incubated individually (1 beetle per cup) at 25°C in darkness in the lidded medicine cup with a potato slice for 24h to maintain high moisture for conidia germination. Potato slices were then removed and replaced with a piece of moist filter paper and each beetle was provided with 1.5–2 g organic oatmeal (Coop Denmark A/S, Denmark) and left at 25°C in the sealed medicine cup for up to 14 days. Mortality was monitored daily. Moist filter papers were replaced if dry.

In all experiments, dead individuals were collected daily and individually surface sterilized by immersion in 5% sodium hypochlorite for 30 s, followed by two rinses of 30 s in deionized water. The rinsed

cadavers were then incubated inside parafilm-closed Petri dishes with moist filter paper at 25 °C for 2–7 days to stimulate fungal emergence as sign of mycosis.

2.6.3. Dose-response of two *Metarhizium* strains

Dose-response tests were conducted on *T. molitor* larvae for each of the two *Metarhizium* strains. The experiments were performed with conidia suspensions harvested from standard SDAY plates grown at the following conditions: 25 °C, 21 d for *M. brunneum* EAMa 09/01-Su; 30 °C, 21 d for *M. robertsii* EAMa 01/158-Su. Four concentrations of *M. brunneum* EAMa 09/01-Su (10^5 , 10^6 , 10^7 , 10^8 conidia mL⁻¹) and five concentrations of *M. robertsii* EAMa 01/158-Su (10^5 , 10^6 , 5×10^6 , 10^7 and 5×10^7 conidia mL⁻¹) were prepared using Triton-X 0.1% as diluent and control. In addition, dose-response bioassays against adults were also performed based on previous experiments (Sala et al., 2023). Procedures for preparation of conidia suspensions were done as in 2.6.1, with inoculation and incubation of insects as described in 2.6.2. The dose-response tests were repeated three times for each condition with conidia harvested from independent cultures (10 individuals per run).

2.6.4. Comparative virulence of conidia produced at different media and temperature conditions

Comparative virulence tests of conidia obtained from eight (*M. brunneum*) or six (*M. robertsii*) selected production conditions were performed against *T. molitor* (Figs. S5 and S6), using setup as previously described (Keyser et al., 2016; Seid et al., 2019). The virulence bioassays were performed using the estimated LC₅₀ (based on dose-response bioassays, section 2.6.3), using 10 individuals of larvae or adults of *T. molitor*. Conidia suspensions used were the same as prepared for the oCelloScope analyses, adjusted to LC₅₀ (i.e., 6.6×10^6 conidia mL⁻¹ for larvae and 1×10^7 conidia mL⁻¹ for adults with *M. robertsii* EAMa 01/158-Su and 5.0×10^7 conidia mL⁻¹ with *M. robertsii* EAMa 01/158-Su). The virulence tests were repeated three times with independent conidia samples.

2.7. Statistical analysis

The data obtained from the oCelloScope and insect infection bioassays were analysed using the R software version 4.5.1 (R Core Team, 2025), a threshold of 0.05 for the significance and the “tidyverse” package (Wickham et al., 2019) while plots were generated with the “ggplot2” package (Wickham, 2016).

2.7.1. OCellScope assays

Four variables from the oCelloScope assays were analysed: conidia production, maximum germination %, maximum germination time and 50% germination time. All variables were tested against the media, the temperature and their interaction in CT experiments and against the media, the temperature, the time and the bi- and tri-way interactions between these factors in VT experiments, using generalized linear models (GLM) with the Poisson family, GLM with the Binomial family or linear models (LM), depending on data distribution. VT experiments contained both constant and variable temperature conditions, which were analysed separately to assess the effects due to media and temperature in the former and due to media, temperature and time in the latter, where bi- and tri-interactions were removed from the final model whenever not significant. Models were validated using the “plotresid” function of the “RVAide Memoire” package (Herve, 2025) for independency of the residues for GLM and for independency, normality and homoscedasticity of the residues for LM. Some models required transformation on the response variable to be validated (log or fourth root). The significance of each term of each model was tested using a Type II analysis of variance (“Anova” function, “car” package (Fox and Weisberg, 2019)) with different tests such as likelihood-ratio chi-square test for GLM or F-test for LM. Whenever a term was significant, pairwise comparisons were performed using the estimated marginal

means and p-values were corrected using the false discovery rate (“emmeans” function and package (Lenth, 2025), “cld” function of the “multcomp” package (Hothorn et al., 2008)).

2.7.2. Insect survival assays

Survival curves and mycosis rate (proportion of dead individuals showing fungal emergence) were tested against the concentration for the dose-response experiments, against the combination of media and temperature when using conidia produced in CT experiments and against the combination of media, the temperature and the time in VT experiments, using survival regressions and Cox model (“survreg” and “coxph” function from the “survival” package (Therneau, 2024)) for survival curves and LM (with the square root transformation when necessary) for the mycosis rate. The significance of each term of each model was tested using a Type II analysis of variance with different tests such as likelihood-ratio chi-square test for survival regressions, Wald test for Cox models and F-test for LM. The pairwise comparisons and p-value correction were performed as described above. Survival curves were plotted using the “survminer” package (Kassambara et al., 2024). Dose-response models were performed using the “drm” function from the “drc” package (Ritz et al., 2015) with the three-parameter Weibull function for *M. brunneum* EAMa 09/01-Su and the four-parameter log-logistic function for *M. robertsii* EAMa 01/158-Su.

2.7.3. Multivariate analysis

Principal component analysis (PCA) was performed for both strains using data collected in *M. brunneum* EAMa 09/01-Su VT experiment (25 in total) and *M. robertsii* EAMa 01/158-Su CT experiment (12 in total) with the following parameters divided in two groups: experimental parameters (growth temperature, sporulation temperature, growth time, sporulation time, total time and media used) and response parameters (conidia production, 50% germination time, maximum germination rate, time to reach maximum germination and mortality against larvae of *T. molitor*). Selected experiments correspond to the ones where mortality against *T. molitor* larvae were tested. Minitab 17 software was used for PCA.

3. Results

3.1. Effect of temperature, media and time conditions on conidia production and germination of thermotolerant *M.* strains

Model results varied depending on the analysed response and strain. Complete results of statistical outputs are presented in Supplementary Material (Table S1). Overall, conidia production was the most significantly influenced response by experimental conditions in all models, whereas 50% germination time and max germination rate were less influenced. In contrast, the time to reach maximal germination time was only affected to a limited extend by the experimental conditions. For conidia production, max germination rate and 50% germination time, significant effects of both media and temperature were seen in all experiments, with time representing most significant influence for *M. robertsii* (VT experiment). Below, only results associated to the most significant factors (i.e., media, temperature and time) and to the responses most affected are presented.

3.1.1. Constant (CT) and variable (VT) temperature experiments with *M. brunneum* EAMa 09/01-Su

Previous experiments (data not shown) demonstrated that *M. brunneum* EAMa 09/01-Su produced hyphae and conidia up to 33 °C, while at 34 °C or above no growth was observed, independent of media. Thus, 33 °C was used as maximum temperature for this strain.

Conidia production and germination were achieved in all tested conditions of the CT experiment, including 33 °C. The production of conidia ranged between 6.6×10^6 and 3.68×10^8 conidia mL⁻¹ (Fig. 1a), which was significantly influenced by the interaction between

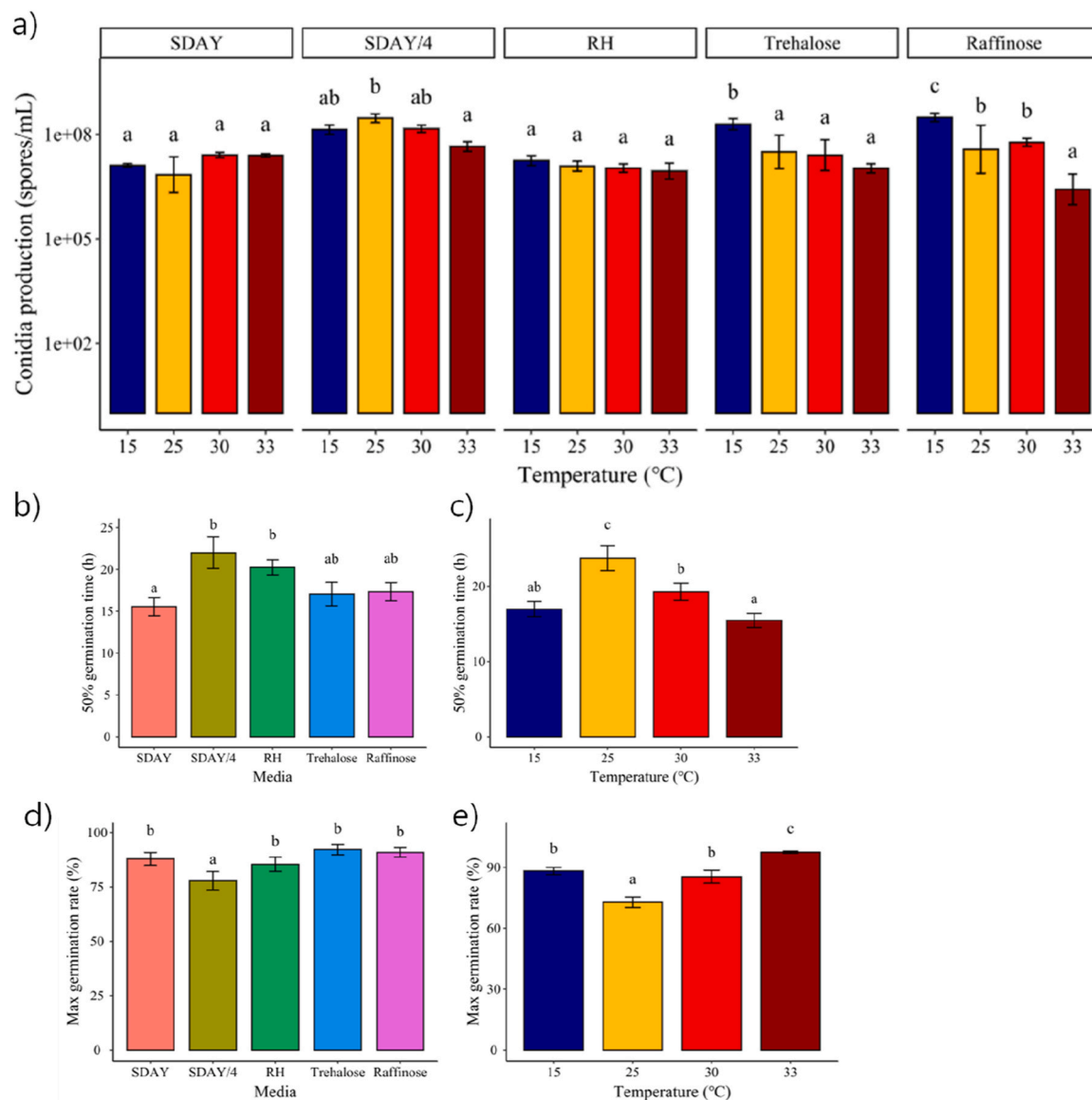


Fig. 1. Conidia production and viability of *M. brunneum* EAMa 09/01-Su obtained in CT (constant temperature) experiment. a) Conidia production by temperature for each medium; b) and c) 50% germination time by media and by temperature, respectively; d) and e) maximum germination rate by media and by temperature, respectively. Bars correspond to means $\pm$ standard errors. Different lowercase letters indicate significant differences between experimental conditions within each facet. The experimental design associated to these results is presented in Fig. S1.

media and temperature (Table S1). Highest production was observed at 15 °C on Raffinose media, followed by 15 °C on Trehalose media, and 25 °C on SDAY/4 media, while other temperature and media combinations produced comparable amounts of conidia (Fig. 1a). Conidial germination traits were significantly affected by media and temperature individually (Table S1). Shortest time to reach 50% germination was seen for conidia grown at SDAY although not significantly faster than on Trehalose or Raffinose (Fig. 1b), while longer time was necessary for SDAY/4 and RH. Although conidia produced at 33 °C were relatively few, they germinated as fast as those produced at 15 °C (Fig. 1c) and reached highest germination rate across media (Fig. 1e). The slow germinating conidia of SDAY/4 also reached lowest maximum germination (Fig. 1d).

In VT experiments with *M. brunneum* EAMa 09/01-Su, conidia production and germination were achieved at all tested conditions, both at constant and variable temperatures. All conidial traits were affected by complex two- or three-way interactions (Table S1). Amounts of conidia produced were reduced by high constant temperature at 33 °C for SDAY

and RH media, while it was lowest at 25 °C for BDr (Fig. 2a). At variable temperatures, BDr in general produced relatively high amounts of conidia (Fig. 2b), and for all three media most conidia were produced after 7 d at 25 °C for growth and 14 d at 33 °C for sporulation (Fig. 2b). At constant temperature, 50% germination was reached rapidly on SDAY irrespective of incubation temperature, while time to achieve 50% of conidia germination was prolonged on RH at 25 °C and on BDr at both 15 and 25 °C (Fig. 2c). The time to reach 50% germination at variable temperatures was not affected by temperatures, but individually by the media and by the period for growth vs. sporulation (Table S1). SDAY produced conidia of which half had germinated in the shortest time, while only 7 days at high temperature for sporulation yielded longer time to achieve 50% germination (Fig. 2d). Comparably, maximum germination rates were high and unaffected by temperatures on SDAY, while the germination rates were lowest at 25 °C on RH and BDr (Fig. 2e). At variable temperatures, SDAY similarly gave highest max germination rates, and 10.5–14 days at 33 °C gave relatively high germination rates (Fig. 2f). Heatmap trends are shown in Fig. S7.

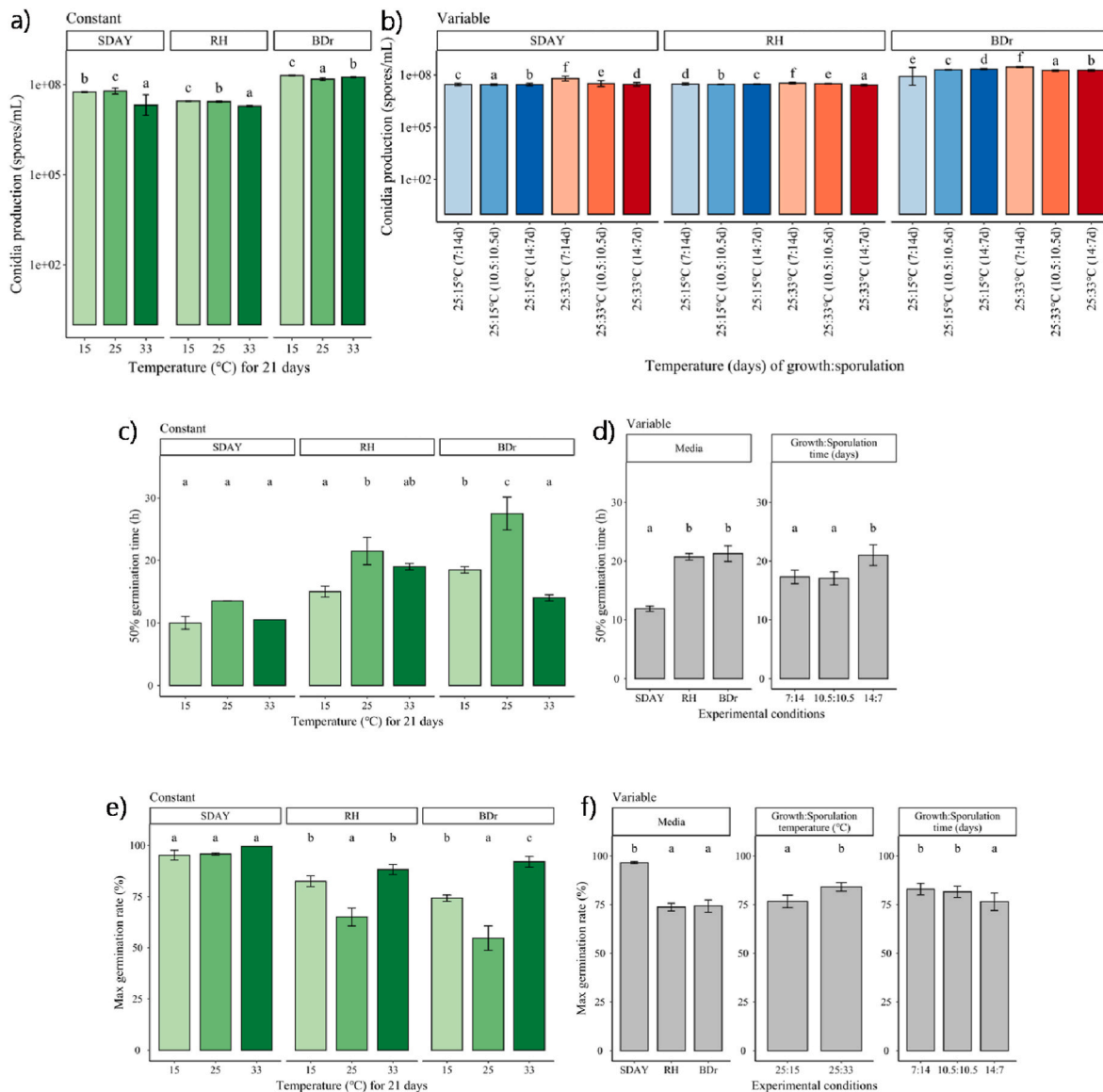


Fig. 2. Conidia production and viability of *M. brunneum* EAMa 09/01-Su obtained in VT (variable temperature) experiment. Constant temperature values are shown in a), c) and e) by media × temperature interactions, with the media as facets. Variable values are shown in b) by media × temperature × time interactions, with the media as facets; in d) by media and by time and in f) by media, by temperature and by time. Panels a) and b) display conidia production; c) and d) 50% germination time; e) and f) maximum germination time. Bars correspond to means ± standard errors. Different lowercase letters indicate significant differences between experimental conditions within each facet. The experimental design associated to these results is presented in Fig. S2.

3.1.2. Constant (CT) and variable (VT) temperature experiments with *M. robertsii* EAMa 01/158-Su

As inherent thermotolerance of strain *M. robertsii* EAMa 01/158-Su were expected to be higher than of *M. brunneum* EAMa 09/01-Su, temperatures up to 40 °C were tested to assess effect on growth and sporulation (Yousef-Yousef et al., 2022). At constant temperatures, conidia production was observed up to 35 °C on SDAY only, while no sporulation was seen at 40 °C (Fig. 3a). At lower temperatures, BDr, Trehalose and Raffinose media generally produced significantly more conidia than RH media (Fig. 3a). Conidia produced at 15 °C yielded equally high values for max germination rates across all media, while conidia from RH media generally showed high germination rates at 20–30 °C (Fig. 3b). Time for 50% germination and to reach maximum germination were only affected by temperature (Table S1).

Cultivation of *M. robertsii* EAMa 01/158-Su on three media SDAY, RH and BDr for 14 days at constant temperatures showed increased conidia production at 28 °C compared to 15 °C on all media, but no conidia were produced at 35 °C on SDAY in this experiment (Fig. 4a). When the fungal

strain was cultured for 14 days at variable temperatures, results varied depending on conditions. For conidia production, a significant three-way interaction was found (Table S1), leading to complex results. When growth was allowed at 28 °C for 4–7 days, successful sporulation was achieved even at 35 °C and at 40 °C on the three media (Fig. 4b and c). At these conditions of high sporulation temperatures, RH and BDr produced more conidia than the standard SDAY with periods of 7 days for growth and sporulation yielding high conidia production. However, the conidia produced at 40 °C took longer to reach 50% germination when cultivated on RH and BDr compared to SDAY (Fig. 5a). Aligned with this observation, significantly fewer conidia germinated when cultivated on RH and BDr at sporulation temperatures of 40 °C, while conidia cultivated on SDAY germinated almost completely when produced at all temperature and time combinations (Fig. 5b). Heatmap trends are shown in Fig. S8.

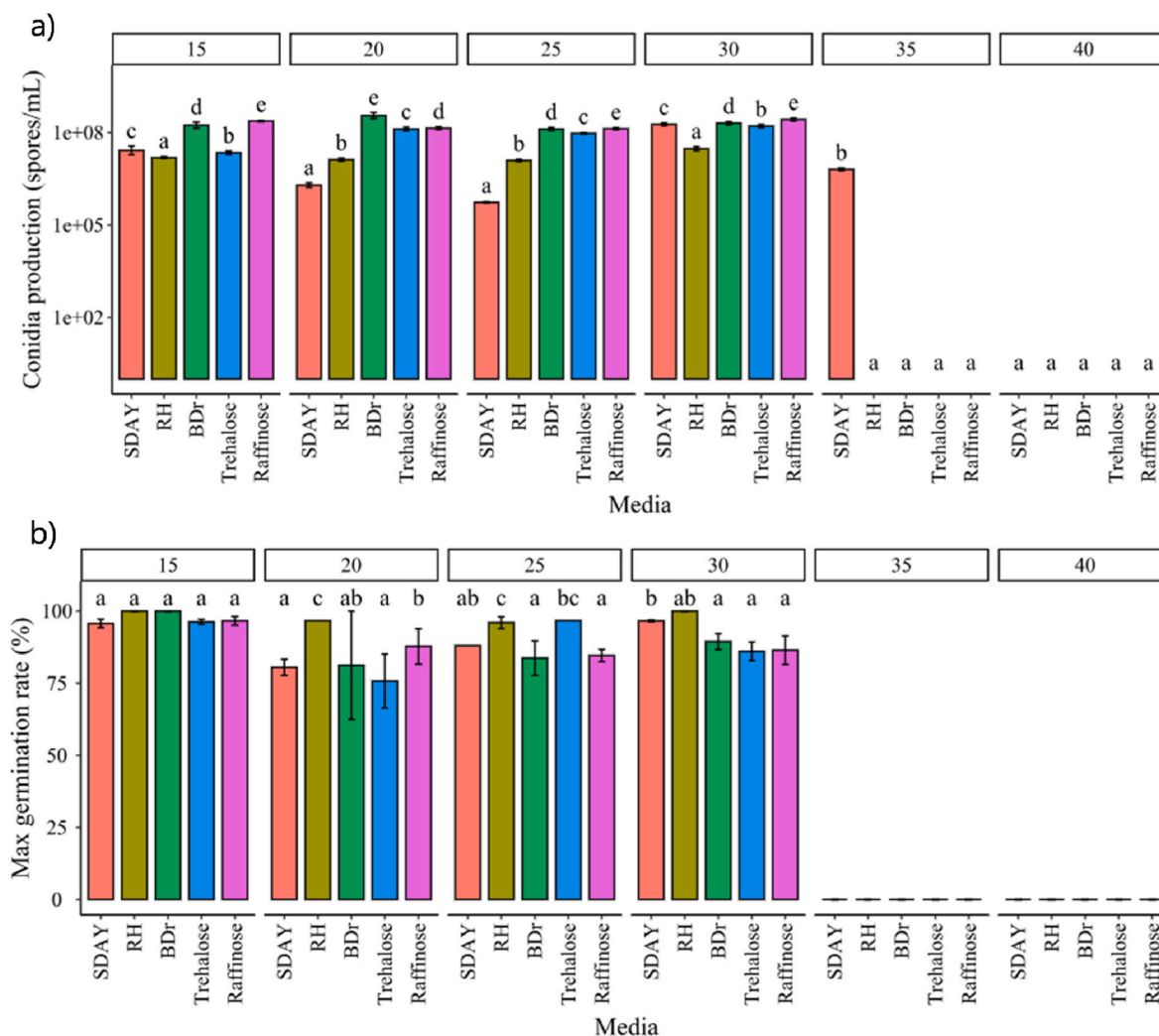


Fig. 3. Conidia production and viability of *M. robertsii* EAMa 01/158-Su obtained in CT experiment. Panel a) show conidia production and b) maximum germination rate by temperature $\times$ media interactions, with the temperature as facets. Bars correspond to means $\pm$ standard errors. Different lowercase letters indicate significant differences between experimental conditions within each temperature facet. The experimental design associated to these results is presented in Fig. S3.

3.2. Effect of production conditions on virulence against *T. molitor*

Complete results of all virulence experiments (statistical outputs) are presented in Supplementary data (Table S2).

3.2.1. Dose-response experiments on *T. molitor* larvae

Clear dose-dependent responses after exposure of *T. molitor* larvae to the two *Metarhizium* strains were observed, presenting different intensity depending on the strain used (Fig. S9). The LC50 values were clearly separated for each strain, being 3.1×10^5 conidia mL⁻¹ for *M. brunneum* EAMa 09/01-Su and 1.4×10^7 conidia mL⁻¹ for *M. robertsii* EAMa 01/158-Su. The *M. brunneum* strain was thus most virulent, achieving 90% mycosis (100% mortality) at 10^7 mL⁻¹. In contrast, *M. robertsii* achieved lower mycosis rate (53.3%) even at the highest concentration of 5×10^7 conidia mL⁻¹. In both strains, exposure to the two highest concentrations did not increase mortality or mycosis rates significantly. Mortality was also achieved most rapidly by *M. brunneum*, reaching LT₅₀ by 5 or 4.5 days at 10^7 or 10^8 conidia mL⁻¹ respectively, contrasting with *M. robertsii* where LT₅₀ was not achieved until 8 days for the highest concentration of 5×10^7 conidia mL⁻¹. As *M. brunneum* showed most potential against *T. molitor*, this strain was used in infection experiments against both stages (larvae and adults) to compare effects of all growth and sporulation parameters on conidia virulence, while *M. robertsii* was only tested against larvae stage.

3.2.2. Virulence of *M. brunneum* EAMa 09/01-Su against *T. molitor* larvae and adults

Concentration used against larvae was of 6.6×10^6 conidia mL⁻¹ and of 1×10^7 conidia mL⁻¹ against adults. When exposed to conidia produced at constant temperatures of 15, 25 or 33 °C on either of the three media SDAY, RH or BDr, larval mortality was only significantly increased when conidia were produced on SDAY and RH at 15 °C, compared to the control treatment (Fig. 6a). For these two treatments, LT₅₀ values were 8 and 10 days, respectively. Mycosis rates varied, but patterns generally followed the levels of mortality (Fig. S10). In contrast, conidia produced at variable temperatures caused significantly increased mortality in larvae except for conidia produced during 14 days at 33 °C for sporulation. Shorter incubation periods of 7 or 10.5 days for sporulation at 33 °C maintained virulence equal to sporulation temperatures of 15 °C (Fig. 6b). Here, LT₅₀ values ranged from 7 to 10 days after exposure. Mycosis rates were more clearly separating the sporulation treatments at 33 °C, with lower mycosis development in larvae exposed to conidia incubated for 14 days during sporulation than those incubate for 7 or 10.5 days (Fig. S10).

Exposure of adult *T. molitor* to conidia produced at a sub-set of constant temperatures on the three media separated mortality patterns more evidently than for larvae (Fig. 6c). Highest mortalities were obtained for conidia produced at 15 °C on RH and SDAY, leading to LT₅₀ values of 9 and 11, respectively. In contrast, no significant mortality was

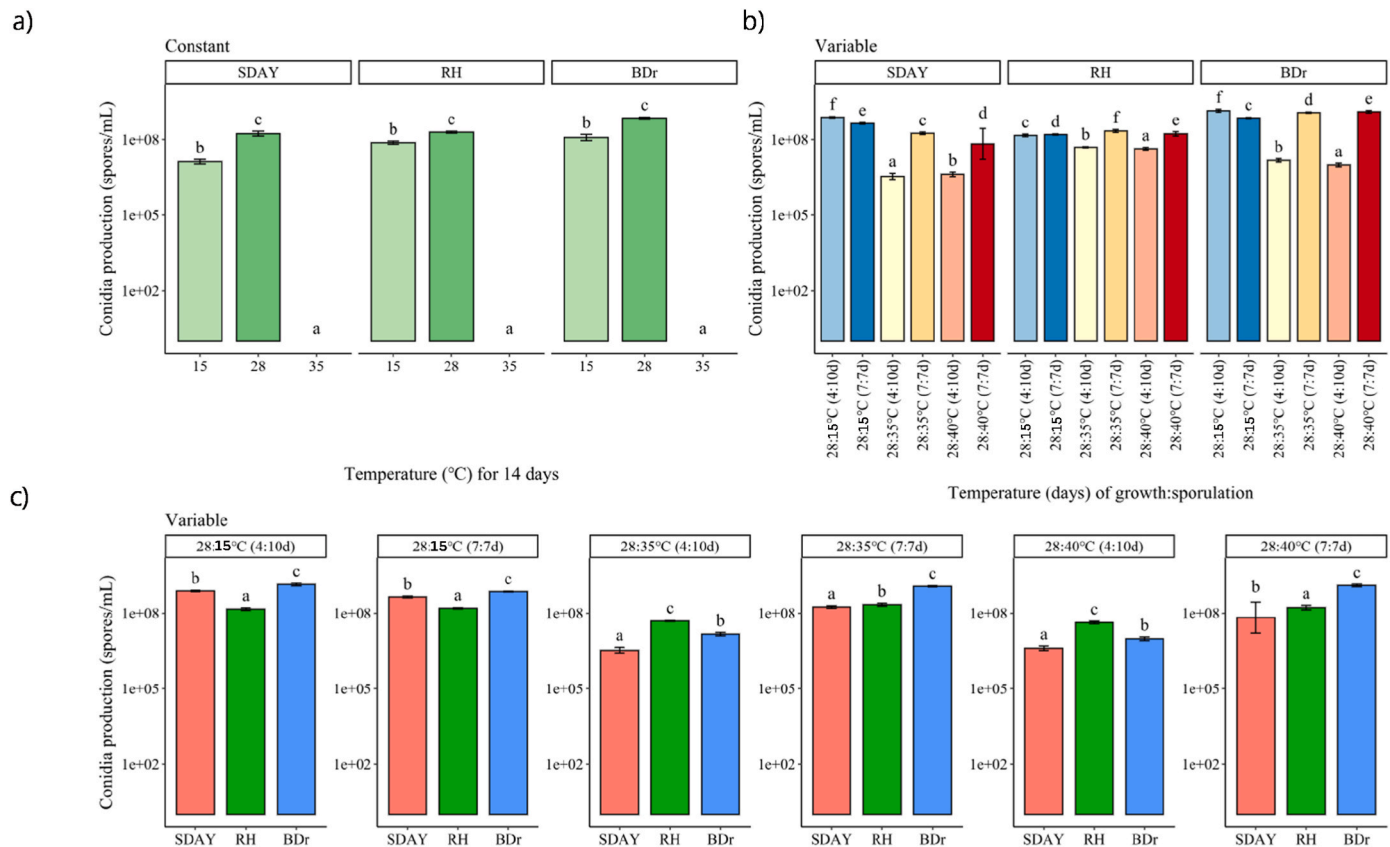


Fig. 4. Conidia production of *M. robertsii* (EAMa 01/158-Su) obtained in VT experiment. Constant temperature values are shown in a) by media $\times$ temperature interactions, with the media as facets. Variable experiment values are shown media $\times$ temperature $\times$ time interactions in b) with the media as facets and in c) with temperature $\times$ time as facets. Bars correspond to means $\pm$ standard errors. Different lowercase letters indicate significant differences between experimental conditions within each facet. The experimental design associated to these results is presented in Fig. S4.

observed for conidia produced on RH at 25 and 33 °C. Conidia from BDr at 15 °C caused intermediate level of mortality with LT₅₀ of 15 days, which was the only temperature for BDr tested against adults. Adults exposed to conidia incubated at 15 °C resulted in the highest rates of mycosis after incubation of cadavers (Fig. S10). Conidia produced at a sub-set of the variable temperature conditions all caused comparable levels of significant mortality in adults, irrespective of the incubation period for sporulation at 15 or 33 °C (Fig. 6d). The corresponding LT₅₀ values ranged between 9 and 11 days. However, mycosis rates of dead beetles were similar for the four groups of conidia (Fig. S10).

3.2.3. Virulence of *M. robertsii* EAMa 01/158-Su against *T. molitor* larvae

In comparison to conidia production of *M. brunneum* EAMa 09/01-Su, the total period for growth and sporulation for strain *M. robertsii* EAMa 01/158-Su was reduced to 14 days, with temperatures at 15, 28 or 35 °C, at a concentration of 5.0×10^7 conidia mL⁻¹. Exposure of *T. molitor* larvae to conidia of *M. robertsii* EAMa 01/158-Su produced at constant temperatures of 15 or 28 °C on either RH or BDr for 14 days only led to significant mortality compared to the control treatment for RH incubated at both temperatures and for BDr at 15 °C (Fig. 7a) with LT₅₀ values of 5-6 days. Conidia from either temperature produced on SDAY media did not cause significant mortality in the larvae. However, only larval cadavers exposed to SDAY at 28 °C failed to develop mycosis (Fig. S11). When conidia were produced at variable temperatures, all four conditions for sporulation caused significant mortality towards *T. molitor* larvae (Fig. 7b). Exposure of larvae to conidia produced at sporulation temperature of 35 °C for 4 days showed lower mortality (LT₅₀ = 12 days) than 10 days incubation at 35 °C and both sporulation periods at 15 °C (LT₅₀ = 5 days). Similarly, the 4 days of sporulation at 35 °C also produced lower mycosis rate than the other treatments

(Fig. S11).

3.3. Thermotolerant strains relation with germination and conidia production

To assess the relationship of the analysed parameters on various responses, PCA analysis was performed using the following six parameters: growth temperature, sporulation temperature, growth time, sporulation time, total time and media; coupled with five responses: conidia production, 50% germination time, max. germination rate, time to reach max. germination and mortality against larvae of *T. molitor*.

More than 81% of the total variance was explained by four components, with the majority (57.7%) being explained by the first and second components. Thus, PC1 and PC2 were plotted against each other in biplot form (Fig. 8), with data distributed for the two *Metarhizium* strains tested.

Concerning the response parameters, dependence between conidia production and time for germination can be observed, mainly along PC1. In contrast, mortality against *T. molitor* and max germination rate of conidia are related, but in opposite direction of PC1.

The observed patterns of experimental variables differed between the two strains (colour codes in Fig. 8), implying strain-specific effects of the analysed parameters. For strain *M. brunneum* EAMa 09/01-Su, the most important parameter affecting the responses was temperature, indicating same direction along PC2 for effects of growth and sporulation temperatures. Incubation periods (total, growth and sporulation time) were in opposite direction mainly along PC1 for *M. brunneum* EAMa 09/01-Su, while media represented limited importance on both strains. Concerning strain *M. robertsii* EAMa 01/158-Su, mainly incubation periods showed relationships along same trajectory, with limited

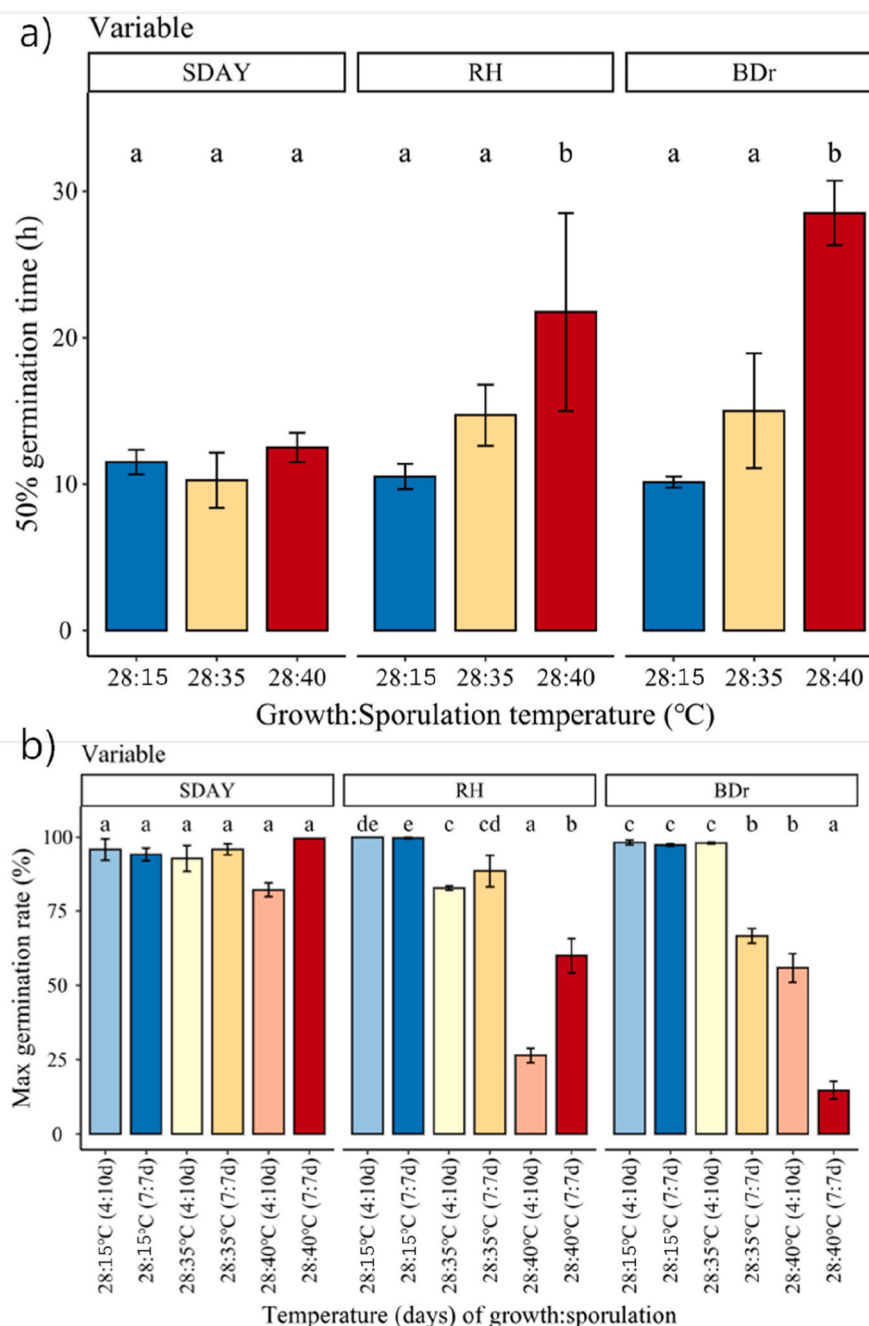


Fig. 5. Conidia viability of *M. robertsii* (EAMa 01/158-Su) obtained in VT experiment, corresponding to variable experimental values. a) 50% germination time and b) max germination rate are shown by media $\times$ temperature and media $\times$ time interactions respectively, with the media as facets. Bars correspond to means $\pm$ standard errors. Different lowercase letters indicate significant differences between experimental conditions within each facet. The experimental design associated to these results is presented in Fig. S4.

differences among total, growth and sporulation times. Temperature trajectories were opposite, indicating no relevant effect on responses of the strain, implying that the selected range was suitable.

4. Discussion

In the present study, we evaluated the effect of several production parameters (temperature, growing media and time) on different response parameters (conidia production, germination and virulence against *T. molitor*) of two thermotolerant *Metarhizium* strains. Our findings demonstrate that differences in conidia traits, mainly in germination and virulence, are observed depending on the simulated

SSF conditions during conidia production. Moreover, the feasibility of using the thermotolerant strains in fungal SSF scale-up is demonstrated, while showing evidence that responses of the strains were different. Our results confirm that *M. robertsii* EAMa 01/158-Su showed superior thermotolerant potential over *M. brunneum* EAMa 09/01-Su, and that the tested strategies at variable temperatures simulating SSF conditions obtained relatively high conidia concentrations coupled with high germination values and resulting virulence against *T. molitor*.

This study reports new insights on the effect of prolonged heat stress on conidia production and germination, prolonging these effects for several days (4 to 14 depending on the experiment). Temperature has been described as the most critical environmental factor for reducing

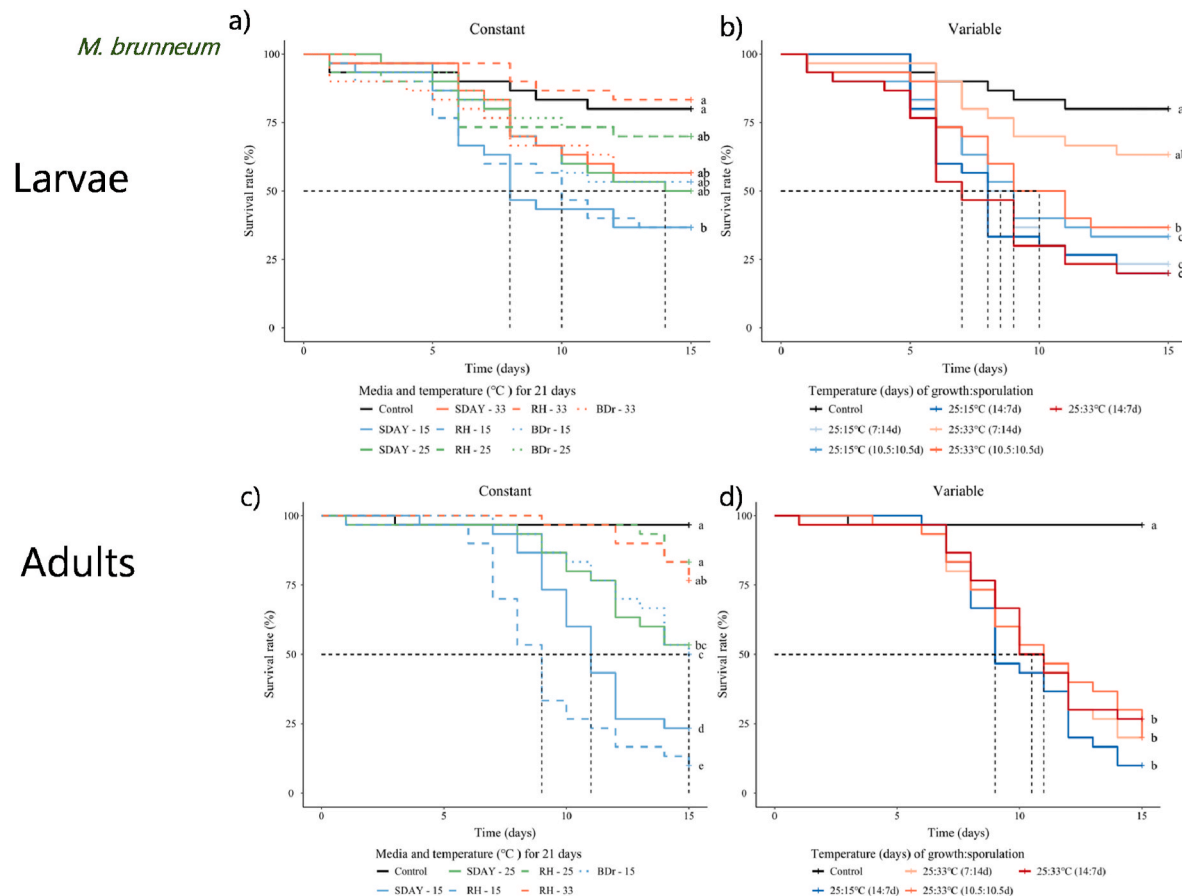


Fig. 6. Comparative virulence of *M. brunneum* EAMa 09/01-Su from CT and VT temperature experiments on *T. molitor* larvae (a-b) and adults (c-d). Results of CT experiment are displayed in panels a and c, while those of VT experiment on BDr media are displayed in panels b and d. The dashed black lines correspond to the median lethal time. Different lowercase letters indicate significant differences between experimental conditions within each plot. The experimental design associated to these results is presented in Fig. S5.

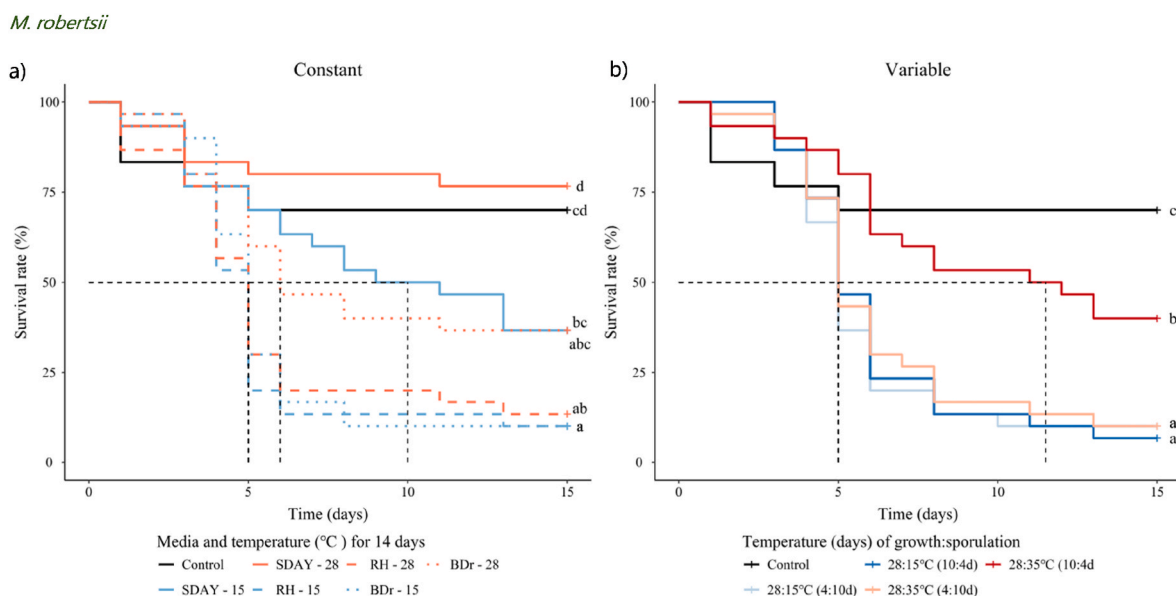


Fig. 7. Comparative virulence of *M. robertsii* EAMa 01/158-Su from CT and VT temperature experiments on *T. molitor* larvae. Results of CT experiment are displayed in panel a, while those of VT experiment on BDr media are displayed in panel b. The dashed black lines correspond to the median lethal time. Different lowercase letters indicate significant differences between experimental conditions. The experimental design associated to these results is presented in Fig. S6.

virulence in EPF (Quesada-Moraga et al., 2024). Temperatures above optimal have produced highly germinative conidia, albeit at lower

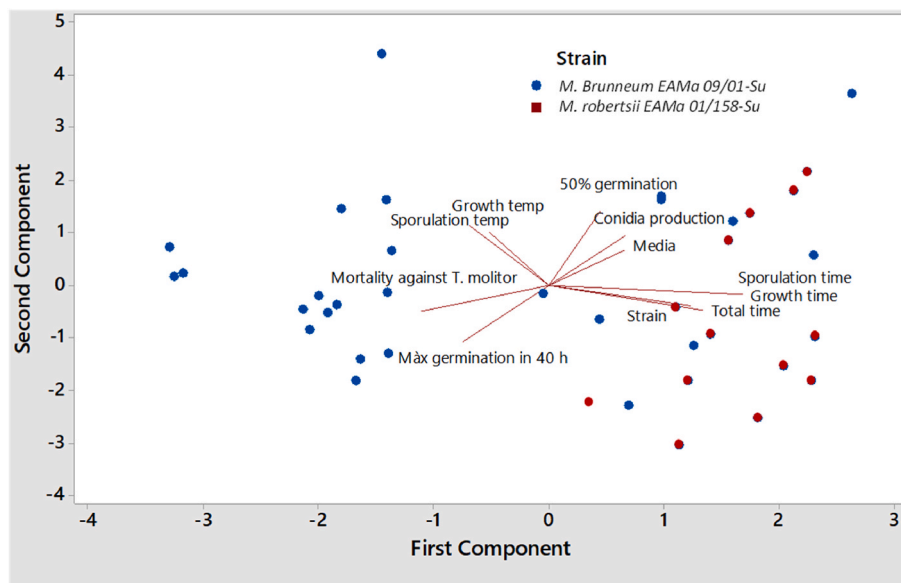


Fig. 8. PCA plot (component 1 vs component 2) obtained in the PCA analysis of experiments from VT experiment (25 conditions using *M. brunneum* EAMa 09/01-Su) and CT experiment (12 conditions using *M. robertsii* EAMa 01/158-Su). The following parameters were analysed: growth temperature, sporulation temperature, growth time, sporulation time, total time, media, strain, conidia production, 50% germination, max germination rate, max germination time and mortality against *T. molitor* larvae. Blue dots correspond to strain *M. brunneum* and red dots to strain *M. robertsii*.

absolute productions than optimal values. Most studies report heat stress effect on entomopathogenic fungi at shorter periods than those tested here. For example, Keyser et al. (2014) reported periods of heat stress of 4–20 h in contrast to several days in the present study, which represent an approach indicative of scaling conidia production during SSF, where heat stress can last for days (Perez et al., 2019).

In terms of germination, although best results were obtained overall when working at or close to optimal temperatures (25 °C for *M. brunneum* EAMa 09/01-Su and 28 °C for *M. robertsii* EAMa 01/158-Su), relevant decrease was only found when working at 4 days of growth temperature in VT strategies, meaning more time was required for proper fungal growth before changing to sporulation temperature. Comparable findings were reported when working with *Trichoderma atroviride*, although not being defined as thermotolerant (Daryaei et al., 2016). Temperature is a key parameter in SSF conidia production, thus has been extensively studied, either by testing different temperatures for the same process or via its radial variation through packed beds (Sala et al., 2019; da Cunha et al., 2020). However, influence of production temperature on conidia traits such as germination and virulence have not been equally researched. Most studies performed with agar media involve effects of changes in ambient conditions on fungal growth and sporulation, mostly by means of heat stress (Keyser et al., 2014; Rangel et al., 2015; Seid et al., 2019), surpassing temperatures considered “optimal” for each strain. Exposure to extreme temperatures for longer times always reduces production and germination, highlighting the relevance of controlling this parameter. Detrimental effect of high temperatures (temperatures of 35 °C or higher) on germination has been previously reported, with slightly less values required to reduce germination in comparison to conidia production (Omuse et al., 2022).

We observed interesting variation between effects of growth media. Media representing intermediate sugar availability (SDAY/4, BDr, trehalose and raffinose) tended to yield higher conidia production but reduced germination traits, whereas SDAY and RH (high and low sugar availability, respectively) tended to have high germination rates but relatively low conidia production. Particularly BDr maintained conidia production at high temperatures. This observation could indicate carbon source starvation, which was previously reported as one main factor influencing heat-tolerant conidia formation in EPF (Maheshwari et al., 2000; Montesinos-Matías et al., 2021). Other studies have

demonstrated that nutrient stress on minimal media is a factor inducing formation of heat-tolerant conidia (Medina et al., 2020). Although our findings are in accordance with this, sugar starvation did not cause differences in germination traits. We hypothesize that media with low or medium sugar concentration are beneficial to the fungal strain at extreme temperatures (temperatures of 35 °C or higher), favouring fast growth and sporulation. During SSF, the specific substrate will highly influence maximum temperature generation and the period during which this temperature is achieved (Sala et al., 2021). Balancing sugar consumption and fermentation time must be achieved for optimized SSF processing.

Effects of media were observed in all experiments of the present study, implying that media composition is important for thermotolerant *Metarhizium* strains. However, additional studies with different genera should be performed to assess if such results are reproducible for other thermotolerant fungi.

Recent works have also demonstrated the effect of culture conditions on conidia traits (size, production, germination, thermotolerance and virulence) in EPF. Tamayo-Sánchez et al. (2026) observed that subculturing different EPF led to differences in conidia production, observing reduction in successive subculturing. Contrary to what we observed, germination and virulence (against larvae of the wax moth *Galleria mellonella*) were stable, with also no significant differences for thermotolerance (Tamayo-Sánchez et al., 2026). These results complement our findings on temperature and media effect on conidia traits.

This study presented clear strain specific differences in virulence against *T. molitor* larvae. Both *Metarhizium* species are considered generalists, capable of infecting a wide range of insect hosts (Barelli et al., 2022; Couceiro et al., 2022). Particularly, several *M. brunneum* strains (including EAMa 09/01-Su) have been shown to produce high amounts of destruxin, a prominent insecticidal compound affecting fungal virulence (Lozano-Tovar et al., 2015). Previous studies have shown effects of *M. robertsii* EAMa 01/158-Su against soil dwelling insect pests with promising results (Garrido-Jurado et al., 2011; Yousef-Yousef et al., 2022), but the strain was not tested against *T. molitor*. Other *M. brunneum* strains have been demonstrated to infect *T. molitor* larvae and adults at various degrees (Dahal et al., 2022; Herren et al., 2023). Aside from *Metarhizium* virulence analyses, *T. molitor* larvae is widely considered as one of the most useful *in vivo* models to test the

effectiveness and safety of biological drugs, specially for pathogenic fungi and fungal virulence, with more than 5700 reports in the literature to the present day (de Souza et al., 2018; Santos et al., 2025).

Our results suggest an effect of SSF production conditions on the quality of the obtained conidial-based product, as clear differences in mortality and virulence were obtained depending on the sample used. Similar behaviour between larvae and adult *T. molitor* virulence assays suggests that virulence is not only dependent on insect stage (being superior in adult stage, as shown in Fig. 6) and concentration used but also on conidia production conditions. This trait had not been previously observed, as most studies focus on a single stage (mainly larvae) or comparison of different insect species (Praprotnik et al., 2021; Swathy et al., 2024). Differences in physiological responses between *T. molitor* stages have been extensively studied in recent years (Pedersen et al., 2020), and stage specific variation in gene expression patterns showed higher immune activation during the larval stage (Petronio et al., 2022). This is in accordance with the present results, where the larval stage exhibited higher proportional survival than adults when exposed to conidia from same growth and sporulation conditions. Moreover, the effect of sporulation temperature on conidia virulence seemed irrelevant, as high virulence was achieved with all temperatures tested during sporulation period (15, 25 and 33 °C). Consequently, to obtain an effective bioproduct, production condition effects must be considered alongside others that affect conidia virulence, such as several biological factors, e.g., the type of conidia used, the application method and the entry pathway into the insect hemolymph (Graf et al., 2025).

In both strains tested, infective conidia were obtained also at highest tested temperature conditions (33 °C for *M. brunneum* and 35 °C for *M. robertsii*). High stress conditions have been reported as inducers of higher virulence in conidia of different EPF genera (Rangel et al., 2015). The capabilities of *M. brunneum* EAMa 09/01-Su and *M. robertsii* EAMa 01/158-Su for producing infective conidia when sporulating at 33 °C for 7 to 14 days after growth (*M. brunneum*) or 35 °C for 4 to 10 days after growth (*M. robertsii*) were demonstrated. This response is highly relevant for conidia mass production in SSF packed-bed reactors, as temperatures surpassing 30 °C are reached when scaling fungal biopesticide production up to 22 L (Sala et al., 2021). Further, higher temperatures are reached when scaling up other SSF production processes (Cerdeja et al., 2017; Mitchell et al., 2023), demonstrating that *M. brunneum* EAMa 09/01-Su has potential for SSF fungal biopesticide scaling.

The PCA analysis demonstrates strain specific relationships with response parameters, mainly conidia production and germination. Specifically, conidia of *M. brunneum* EAMa 09/01-Su showed higher affinity for *T. molitor* infection and germination rate, while *M. robertsii* EAMa 01/158-Su had more relation with rapid germination and high conidia production. For both strains, conidia production was related to germination time, but highest production was obtained for *M. robertsii* EAMa 01/158-Su, indicating conidia production is mainly dependent on fast growth and germination rather than on maximum germination rates. Overall, *M. robertsii* EAMa 01/158-Su exhibited overall faster germination rate and conidia production in comparison to *M. brunneum* EAMa 09/01-Su, while also obtaining much lower mortality against *T. molitor* larvae, results validated by PCA.

Relationship between germination and successful infection was also demonstrated in the PCA, showing that conidia germination rate was more important for *T. molitor* infection than conidia quantity. This suggests that different production parameters should be optimized during SSF depending on the desirable response, implying that conidia quantity may not represent the best indicative parameter for biopesticide quality, whilst germination rate is more suitable proxy for efficacy. As presented by Seid et al. (2019), conidial germination is essential for entomopathogenicity, but germination parameters do not always reflect conidia infectivity against an insect. Thus, an approach with combined *in vitro* and *in vivo* assessment of the produced conidia is necessary for complete evaluation of the potential quality of fungal infective units produced by SSF.

5. Conclusions

Using two thermotolerant *Metarhizium* strains that have demonstrated biocontrol potential, effect of conidia production conditions on relevant traits regarding quality and quantity of conidia have been demonstrated, with emphasis on germination and virulence. This knowledge will be valuable for future works regarding production and scalability of the analysed strains by means of SSF.

CRedit authorship contribution statement

Arnau Sala: Conceptualization, Data curation, Funding acquisition, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Morgane Ourry:** Data curation, Writing – review & editing. **Nicolai V. Meyling:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Prof. Dr. Enrique Quesada-Moraga (Agronomy Department, Agricultural Entomology, ETSIAM, University of Córdoba) for providing the strains used in this work. Arnau Sala is grateful to Universitat Autònoma de Barcelona, the Spanish Ministry of Universities and the European Union (European Union-NextGenerationEU) for a Margarita Salas postdoctoral grant (2022/894792) and to University of Copenhagen for accommodating a one-year exchange stay. The authors also thank Henrik De Fine Licht for providing access to the oCelloScope platform, and Laura V. Florez for the valuable discussions on germination-metric analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funbio.2026.101794>.

References

- Arora, R., Behera, S., Kumar, S., 2015. Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: a future perspective. *Renew. Sustain. Energy Rev.* 51, 699–717. <https://doi.org/10.1016/j.rser.2015.06.050>.
- Barelli, L., Behie, S.W., Hu, S., Bidochka, M.J., 2022. Profiling destruxin synthesis by specialist and generalist metarhizium insect pathogens during coculture with plants. *Appl. Environ. Microbiol.* 88. <https://doi.org/10.1128/aem.02474-21>.
- Casciadori, Fernanda Perpétua, Aparecida, P., Frassatto, C., Thomeo, J., Casciadori, F.P., Casciadori, P.A., Thoméo, J.C., 2013. Cellulase production in packed-bed bioreactor by solid-state fermentation. <https://doi.org/10.5071/21stEUBCE2013-3DV.1.13>.
- Cerdeja, A., Mejías, L., Gea, T., Sánchez, A., 2017. Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: the consistency of the process and the microbial communities involved. *Bioresour. Technol.* 243, 1059–1068. <https://doi.org/10.1016/j.biortech.2017.07.076>.
- Couceiro, J. da C., De Fine Licht, H.H., Delalibera, I., Meyling, N.V., 2022. Comparative gene expression and genomics reflect geographical divergence in the plant symbiotic and entomopathogenic fungal genus *Metarhizium*. *Fungal Ecol.* 60. <https://doi.org/10.1016/j.funeco.2022.101190>.
- da Cunha, L.P., Casciadori, F.P., Vicente, I.V., Garcia, R.L., Thoméo, J.C., 2020. *Metarhizium anisopliae* conidia production in packed-bed bioreactor using rice as substrate in successive cultivations. *Process Biochem.* 97, 104–111. <https://doi.org/10.1016/j.procbio.2020.07.002>.
- Dahal, S., Jensen, A.B., Lecocq, A., 2022. Effect of probiotics on *Tenebrio molitor* larval development and resistance against the fungal pathogen *Metarhizium brunneum*. *Insects* 13. <https://doi.org/10.3390/insects13121114>.
- Daryaei, A., Jones, E.E., Ghazalibiglar, H., Glare, T.R., Falloon, R.E., 2016. Effects of temperature, light and incubation period on production, germination and bioactivity of *Trichoderma atroviride*. *J. Appl. Microbiol.* 120, 999–1009. <https://doi.org/10.1111/jam.13076>.
- De la Cruz Quiroz, R., Roussos, S., Hernández, D., Rodríguez, R., Castillo, F., Aguilar, C. N., 2015. Challenges and opportunities of the bio-pesticides production by solid-

- state fermentation: filamentous fungi as a model. *Crit. Rev. Biotechnol.* 35, 326–333. <https://doi.org/10.3109/07388551.2013.857292>.
- de Souza, P.C., Caloni, C.C., Wilson, D., Almeida, R.S., 2018. An invertebrate host to study fungal infections, mycotoxins and antifungal drugs: *tenebrio molitor*. *J. Fungi*. <https://doi.org/10.3390/jof4040125>.
- Fox, J., Weisberg, S., 2019. *An R Companion to Applied Regression*, third edition. Sage, Thousand Oaks CA. z01 978-1-5443-3647-3 01.pdf.
- Galli, M., Feldmann, F., Vogler, U.K., Kogel, K.H., 2024. Can biocontrol be the game-changer in integrated pest management? A review of definitions, methods and strategies. *J. Plant Dis. Prot.* <https://doi.org/10.1007/s41348-024-00878-1>.
- Garrido-Jurado, I., Resquín-Romero, G., Yousef-Naef, M., Riós-Moreno, A., Quesada-Moraga, E., 2020. Soil drenching with entomopathogenic fungi for control of the soil-dwelling life stages and adults of the same generation of *Spodoptera littoralis* (Boisd.) (Lepidoptera: noctuidae). *Bull. Entomol. Res.* 110, 242–248. <https://doi.org/10.1017/S000748531900052X>.
- Garrido-Jurado, I., Valverde-García, P., Quesada-Moraga, E., 2011. Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*. *Biol. Control* 59, 366–372. <https://doi.org/10.1016/j.biocontrol.2011.09.011>.
- Graf, T., Moser, F., Embleton, H., Niklaus, P.A., Grabenweger, G., 2025. Which factors influence the virulence of entomopathogenic fungi? Effect of spore type, oosporein, application method, and pathway of entry on the infectiveness of *Beauveria brongniartii* against *Melolontha melolontha*. *J. Appl. Microbiol.* 136. <https://doi.org/10.1093/jambio/xfaf259>.
- Herren, P., Hesketh, H., Dunn, A.M., Meyling, N.V., 2023. Heat stress has immediate and persistent effects on immunity and development of *Tenebrio molitor*. *J. Insects Food Feed* 10, 835–853. <https://doi.org/10.1163/23524588-20230095>.
- Herve, M., 2025. RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9-83-12. <https://CRAN.R-project.org/package=RVAideMemoire>.
- Hildén, K., Hakala, T.K., Lundell, T., 2009. Thermotolerant and thermostable laccases. *Biotechnol. Lett.* <https://doi.org/10.1007/s10529-009-9998-0>.
- Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. *Biom. J.* <https://doi.org/10.1002/bimj.200810425>.
- Hu, G., St Leger, R.J., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol.* 68, 6383–6387. <https://doi.org/10.1128/AEM.68.12.6383-6387.2002>.
- Kassambara, A., Kosinski, M., Biecek, P., 2024. *survminer: Drawing Survival Curves using 'ggplot2'*. R package version 0.5.0. <https://CRAN.R-project.org/package=survminer>.
- Keyser, C.A., Fernandes, É.K.K., Rangel, D.E.N., Roberts, D.W., 2014. Heat-induced post-stress growth delay: a biological trait of many *Metarhizium* isolates reducing biocontrol efficacy? *J. Invertebr. Pathol.* 120, 67–73. <https://doi.org/10.1016/j.jip.2014.05.008>.
- Keyser, C.A., Jensen, B., Meyling, N.V., 2016. Dual effects of *Metarhizium* spp. and *Clonostachys rosea* against an insect and a seed-borne pathogen in wheat. *Pest Manag. Sci.* 72, 517–526. <https://doi.org/10.1002/ps.4015>.
- Lenth, R., 2025. emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.11.2-8. <https://CRAN.R-project.org/package=emmeans>.
- Lozano-Tovar, M.D., Garrido-Jurado, I., Lafont, F., Quesada-Moraga, E., 2015. Insecticidal activity of a destruxin-containing extract of *metarhizium brunneum* against *Ceratitis capitata* (Diptera: tephritidae). *J. Econ. Entomol.* 108, 462–472. <https://doi.org/10.1093/jeet/tov041>.
- Maheshwari, R., Bharadwaj, G., Bhat, M.K., 2000. *Thermophilic Fungi: Their Physiology and Enzymes*. Microbiology and Molecular Biology Reviews.
- Mascarin, G.M., Jaronski, S.T., 2016. The production and uses of *Beauveria bassiana* as a microbial insecticide. *World J. Microbiol. Biotechnol.* <https://doi.org/10.1007/s11274-016-2131-3>.
- Mattoon, E.R., Casadevall, A., Cordero, R.J., 2021. Beat the heat: correlates, compounds, and mechanisms involved in fungal thermotolerance. *Fungal Biol. Rev.* <https://doi.org/10.1016/j.fbr.2021.03.002>.
- Medina, E.Q.A., Oliveira, A.S., Medina, H.R., Rangel, D.E.N., 2020. Serendipity in the wrestle between *Trichoderma* and *Metarhizium*. *Fungal Biol.* 124, 418–426. <https://doi.org/10.1016/j.funbio.2020.01.002>.
- Miranda-Hernández, F., Angel-Cuapio, A., Loera-Corral, O., 2017. Production of fungal spores for biological control. In: *Current Developments in Biotechnology and Bioengineering: Production, Isolation and Purification of Industrial Products*. Elsevier Inc., pp. 757–779. <https://doi.org/10.1016/B978-0-444-63662-1.00033-6>.
- Mitchell, D.A., Ruiz, H.A., Krieger, N., 2023. A critical evaluation of recent studies on packed-bed bioreactors for solid-state fermentation. *Processes*. <https://doi.org/10.3390/pr11030872>.
- Méndez-González, F., Loera, O., Saucedo-Castañeda, G., Buenrostro-Figueroa, J.J., Favela-Torres, E., 2024. Improved packed bed column bioreactor to produce fungal conidia for biological control. *Syst. Microbiol. Biomanuf.* <https://doi.org/10.1007/s43393-024-00308-1>.
- Montesinos-Matías, R., Ordaz-Hernández, A., Angel-Cuapio, A., Colin-Bonifacio, Y., García-García, R.E., Ángel-Sahagún, C.A., Arredondo-Bernal, H.C., 2021. Principal component analysis of the biological characteristics of entomopathogenic fungi in nutrient-limited and cuticle-based media. *J. Basic Microbiol.* 61, 147–156. <https://doi.org/10.1002/jobm.202000627>.
- Mouchacca, J., 2000. Thermotolerant fungi erroneously reported in applied research work as possessing thermophilic attributes. *World J. Microbiol. Biotechnol.* 16, 869–880. <https://doi.org/10.1023/A:1008979123304>.
- Omuse, E.R., Niassy, S., Wagacha, J.M., Ong'amo, G.O., Azrag, A.G.A., Dubois, T., 2022. Suitable models to describe the effect of temperature on conidial germination and mycelial growth of *Metarhizium anisopliae* and *Beauveria bassiana*. *Biocontrol Sci. Technol.* 32, 281–298. <https://doi.org/10.1080/09583157.2021.1993133>.
- Pedersen, K.E., Pedersen, N.N., Meyling, N.V., Fredensborg, B.L., Cedergreen, N., 2020. Differences in life stage sensitivity of the beetle *Tenebrio molitor* towards a pyrethroid insecticide explained by stage-specific variations in uptake, elimination and activity of detoxifying enzymes. *Pestic. Biochem. Physiol.* 162, 113–121. <https://doi.org/10.1016/j.pestbp.2019.09.009>.
- Perez, C.L., Casciari, F.P., Thoméo, J.C., 2019. Strategies for scaling-up packed-bed bioreactors for solid-state fermentation: the case of cellulolytic enzymes production by a thermophilic fungus. *Chem. Eng. J.* 361, 1142–1151. <https://doi.org/10.1016/j.cej.2018.12.169>.
- Petronio, G., Pietrangeli, L., Cutuli, M.A., Magnifico, I., Venditti, N., Guarnieri, A., Abate, G.A., Yewhalaw, D., Davinelli, S., Di Marco, R., 2022. Emerging evidence on *Tenebrio molitor* immunity: a focus on gene expression involved in microbial infection for host-pathogen interaction studies. *Microorganisms*. <https://doi.org/10.3390/microorganisms10101983>.
- Praprotnik, E., Lončar, J., Razingar, J., 2021. Testing virulence of different species of insect associated fungi against yellow mealworm (Coleoptera: tenebrionidae) and their potential growth stimulation to maize. *Plants* 10. <https://doi.org/10.3390/plants10112498>.
- Quesada-Moraga, E., González-Mas, N., Yousef-Yousef, M., Garrido-Jurado, I., Fernández-Bravo, M., 2024. Key role of environmental competence in successful use of entomopathogenic fungi in microbial pest control. *J. Pest. Sci.* <https://doi.org/10.1007/s10340-023-01622-8> (2004).
- Rangel, D.E.N., Anderson, A.J., Roberts, D.W., 2006. Growth of *Metarhizium anisopliae* on non-preferred carbon sources yields conidia with increased UV-B tolerance. *J. Invertebr. Pathol.* 93, 127–134. <https://doi.org/10.1016/j.jip.2006.05.011>.
- Rangel, D.E.N., Braga, G.U.L., Fernandes, É.K.K., Keyser, C.A., Hallsworth, J.E., Roberts, D.W., 2015. Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. *Curr. Genet.* 61, 383–404. <https://doi.org/10.1007/s00294-015-0477-y>.
- Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A.S., Srivastav, A.L., Kaushal, J., 2021. An extensive review on the consequences of chemical pesticides on human health and environment. *J. Clean. Prod.* <https://doi.org/10.1016/j.jclepro.2020.124657>.
- Resquín-Romero, G., Garrido-Jurado, I., Quesada-Moraga, E., 2016. Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisd.) (Lepidoptera: noctuidae). *Biol. Control* 92, 101–110. <https://doi.org/10.1016/j.biocontrol.2015.10.007>.
- Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. *PLOS ONE* 10 (12), e0146021. <https://doi.org/10.1371/journal.pone.0146021>.
- Sala, A., Barrera, R., Artola, A., Sánchez, A., 2019. Current developments in the production of fungal biological control agents by solid-state fermentation using organic solid waste. *Crit. Rev. Environ. Sci. Technol.* 49, 655–694. <https://doi.org/10.1080/10643389.2018.1557497>.
- Sala, A., Barrera, R., Meyling, N.V., Artola, A., 2023. Conidia production of the entomopathogenic fungus *Beauveria bassiana* using packed-bed bioreactor: effect of substrate biodegradability on conidia virulence. *J. Environ. Manag.* 341. <https://doi.org/10.1016/j.jenvman.2023.118059>.
- Sala, A., Barrera, R., Sánchez, A., Artola, A., 2021. Fungal biopesticide production: process scale-up and sequential batch mode operation with *Trichoderma harzianum* using agro-industrial solid wastes of different biodegradability. *Chem. Eng. J.* 425. <https://doi.org/10.1016/j.cej.2021.131620>.
- Santos, A.M., Santana Júnior, C.C., Nascimento Júnior, J.A.C., Oliveira, A.M.S., Rajkumar, G., Alves, I.A., Serafini, M.R., 2025. Beyond the standard: a comprehensive review of innovative experimental models using *Tenebrio molitor* larvae. *Biol. Futur.* <https://doi.org/10.1007/s42977-025-00284-0>.
- Seid, A.M., Fredensborg, B.L., Steinwender, B.M., Meyling, N.V., 2019. Temperature-dependent germination, growth and co-infection of *Beauveria* spp. isolates from different climatic regions. *Biocontrol Sci. Technol.* 29, 411–426. <https://doi.org/10.1080/09583157.2018.1564812>.
- Swathy, K., Parmar, M.K., Vivekanandhan, P., 2024. Biocontrol efficacy of entomopathogenic fungi *Beauveria bassiana* conidia against agricultural insect pests. *Environ. Qual. Manag.* 34. <https://doi.org/10.1002/tqem.22174>.
- Tamayo-Sánchez, F., Guzmán-Franco, A.W., Santillán-Galicia, M.T., Santillán-Ortega, C., 2026. Phenotypic and pathogenicity changes in *Beauveria* and *Metarhizium* isolates induced by serial subculturing and passage through different host species. *J. Invertebr. Pathol.* 215. <https://doi.org/10.1016/j.jip.2025.108520>.
- Therneau, T., 2024. A Package for Survival Analysis in R. R package version 3.8-3. <https://CRAN.R-project.org/package=survival>.
- Wickham, H., 2016. *Data Analysis. ggplot2: Elegant Graphics for Data Analysis*. Springer International Publishing, Cham, pp. 189–201. https://doi.org/10.1007/978-3-319-24277-4_9.
- Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemond, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H., 2019. Welcome to the tidyverse. *J. Open Source Softw.* 4, 1686. <https://doi.org/10.21105/joss.01686>.
- Yousef, M., Lozano-Tovar, M.D., Garrido-Jurado, I., Quesada-Moraga, E., 2013. Biocontrol of *Bactrocera oleae* (Diptera: tephritidae) with *Metarhizium brunneum* and its extracts. *J. Econ. Entomol.* 106, 1118–1125. <https://doi.org/10.1603/EC12489>.
- Yousef-Yousef, M., Romero-Conde, A., Quesada-Moraga, E., Garrido-Jurado, I., 2022. Production of microclerotia by *Metarhizium* sp., and factors affecting their survival, germination, and conidial yield. *J. Fungi* 8. <https://doi.org/10.3390/jof8040402>.