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Comparative transcriptomics of growth metabolism and virulence reveal distinct morphogenic profiles of yeast-like cells and hyphae of the fungus *Metarhizium rileyi*

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ABSTRACT

Metarhizium rileyi is an entomopathogenic fungus with a narrow host range which distinguishes it from other *Metarhizium* species with broad host ranges. This species is also unique because the initial yeast-like growth on solid media is only observed in liquid culture in other *Metharizium* species. A lack of knowledge about the metabolism and genetic signatures of *M. rileyi* during this yeast-like phase on solid and in liquid media is a bottleneck for its large-scale production as a commercial biocontrol agent. In this study we found that *M. rileyi* yeast-like cells produced on solid medium infected and killed the important insect pest *Spodoptera frugiperda* with comparable efficiency as yeast-like cells grown in liquid medium. Secondly, we used comparative transcriptomic analysis to investigate the active genes and genomic signatures of the *M. rileyi* yeast-like morphotypes produced on solid and in liquid media. Yeast-like cells grown in liquid medium had upregulated genes relating specifically to signal transduction and particular membrane transporters. Thirdly, we compared the transcriptomic profiles of yeast-like phases of *M. rileyi* with those of *M. anisopliae*. The yeast-like phase of *M. rileyi* grown on solid medium upregulated unique genes not found in other *Metarhizium* species including specific membrane proteins and several virulence factors. Orthologous genes associated with heat shock protein, iron permease, membrane proteins and key virulence traits (e.g. collagen-like protein Mcl1) were upregulated in both species. Comparative transcriptome analyses of gene expression showed more differences than similarities between *M. anisopliae* and *M. rileyi* yeast-like cells.

1. Introduction

Entomopathogenic fungi in the genus *Metarhizium* (Ascomycota: Hypocreales: Clavicipitaceae) have been used worldwide for biological control of pests. In Brazil, aerial conidia of *Metarhizium anisopliae* have been successfully applied for many years to more than 2 million hectares of sugarcane annually (Parra, 2014), while in Europe, the species *M. brunneum* is used more commonly (Fischhoff et al., 2017). Fungi in the genus *Metarhizium* are dimorphic and, depending on conditions, grow either in a filamentous or in a single-celled yeast-like form (usually referred to as 'blastospores' when grown artificially in liquid culture). Cell dimorphism represents a strategy developed by many fungi to produce infective or dispersal structures and maximize nutrient uptake in diverse environments (Gauthier, 2015). In the genus *Metarhizium,* yeast-like cells are an essential adaptation to enhance dispersion and colonization of the insect body (Lu and St. Leger, 2016). Under natural conditions, fungi in the genus *Metarhizium* grow as yeast-like cells during proliferation in the insect haemolymph (Butt et al., 2016; Pedrini, 2017). The morphogenic switch from filamentous to yeast-like growth is a response to environmental changes between the external host tissue and the internal liquid haemolymph which has a high osmolarity (Lovett and St. Leger, 2014; Mascarin et al., 2021). Unique features of yeast-like cells, such as specific cell wall surface epitopes, thinner cell walls, and the activation of genes responsible for expression of a protective collagenous coat (*Mcl1*), enable them to remain undetected by the host immune system (Wang and St Leger, 2006).

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For industrial applications, *Metarhizium* species are mainly produced in biphasic systems. Fungal biomass (mycelia, blastospores and/or submerged conidia) is cultivated initially in liquid culture media and then inoculated on to solid substrates (grains such as rice or wheat) with a large surface area to shorten the production time of conidia (Jaronski and Jackson, 2012). However, blastospores grown in liquid culture are infective and can be used as the active ingredient of biological control products (Jackson et al., 1997; Alkhaibari et al., 2016, 2017; Mascarin and Jaronski, 2016; Wassermann et al., 2016; Iwanicki et al., 2021). For the biological control industry, it would thus be desirable to identify an inexpensive liquid medium that supports high *Metarhizium* blastospore yield that can be used either for biphasic production of conidia or commercialized directly as a blastospore-based biological control agent.

Biphasic production of the generalist pathogens *M. anisopliae* and *M. brunneum* is well established, and conidia-based biological control agents of these species are commercialized and used worldwide (de Faria and Wraight, 2007; Behle et al., 2013; Brunner-Mendoza et al., 2019; Iwanicki et al., 2019; Rauch et al., 2017, Zottele et al., 2021). In comparison, the specialist *M. rileyi,* with a narrow host range primarily infecting insects in the order Lepidoptera, is more fastidious and, currently, not sufficiently stable for continuous production by solid substrate fermentation. Although *M. rileyi* belongs to the same genus as *M. anisopliae* and *M. brunneum*, it has different characteristics, including being more nutritionally demanding and needing a longer cultivation time (Goettel and Roberts 1991). Aerial conidia of *M. anisopliae* and *M. brunneum* germinate on solid media to form hyphae and mycelium. In contrast, *M. rileyi* is the only *Metarhizium* species that produces yeast-like cells directly from aerial conidia on solid agar medium before switching to hyphae (Pendland and Boucias, 1997). The underlying genetic mechanism governing formation of this unique solid-state yeast-like morphotype in *M. rileyi* is unknown. Furthermore, the virulence of blastospores produced in liquid medium by *M anisopliae, M. robertsii, M. brunneum* and *M. rileyi* is well documented (Riba and Glandard, 1980, Ignoffo et al., 1982; Alkhaibari et al., 2016, 2017; Wassermann et al., 2016, Iwanicki et al., 2018, 2019,2020; Li et al., 2021), whereas the virulence of *M. rileyi* yeast-like cells produced on solid media is unknown.

Cellular dimorphism in *M. rileyi* ultimately depends on expression of specific genes and metabolic processes. These processes are usually studied using genome-wide transcriptomic and protein analyses (Song et al., 2013, 2016; Boucias et al., 2016). One of the first comparative transcriptome studies involving *M. rileyi* aimed at understanding microsclerotia formation and how to enhance their formation during fermentation (Song et al., 2013). Microsclerotia are resistant structures formed by compact and melanized threads of hyphae and are produced in specific liquid media (Jackson and Jaronski, 2009; Paixão et al., 2021); comparative transcriptomics have demonstrated that many genes involved in oxidative stress responses are active during microsclerotia formation (Song et al., 2013). Genetic transformation techniques, later showed that two mitogen-activated protein kinases, Hog1, and Slt2-type, are involved in microsclerotia formation; mutants without these genes had delayed germination and vegetative growth and were more sensitive to various stresses (Song et al., 2016). Recently, Li et al., (2021) showed that in *M. rileyi*, the pacC/Pal signal transduction pathway was involved in the transition to a yeast-like stage under alkaline conditions and associated with virulence of the yeast-like phase produced in liquid culture. To induce production of specific fungal structures, such as these yeast-like cells, and to overcome bottlenecks in the industrial production of fungi, it is essential to understand the underlying metabolic mechanisms involved in induction and maintenance of desired fungal structures in the culture medium. This knowledge can then be used to improve production systems by optimizing the conditions necessary to stimulate expression of metabolic pathways and genes related to fermentation. Furthermore, candidate genes related to yeast-like cell production may serve as molecular markers for selection of highly productive strains in the future.

The fungus *M. rileyi* has many desirable characteristics for biological control purposes, being a specialist pathogen of Lepidoptera and producing yeast-like cells in solid media that have the potential to speed up industrial fermentation pipelines. In order to fully take advantage of these characteristics and provide a basis for optimizing the production of *M. rileyi* yeast-like cells, more knowledge of the underlying metabolism and phenotypic characteristics of *M. rileyi* yeast-like cells grown on solid media is needed. In the present study, we sought to first resolve key phenotypic questions about the functional significance of cell-wall thickness and virulence of yeast-like cells of *M. rileyi* grown on solid media before embarking on a molecular analysis of the genes regulating such fungal cells during their in vitro formation and growth. Specifically, we asked whether: (I) yeast-like cells from solid and liquid media have similar cell-wall thickness and are equally virulent, (II) genome-wide gene expression profiles of yeast-like cells from solid and liquid media are more similar to each other than either of them are to hyphal cells of *M. rileyi,* (III) genes specifically expressed in *M. rileyi* yeast-like cells on solid media are unique or orthologous to genes in other *Metarhizium* species, and (IV) genes that are differentially-expressed in yeast-like cells and hyphae of the generalist species, *M. anisopliae*, are comparable to those in the Lepidoptera-specific pathogen, *M. rileyi*.

To answer these questions, we first performed phenotypic analyses of *M. rileyi* yeast-like cells produced in solid and liquid medium. After establishing that yeast-like cells from solid media were virulent to the *Spodoptera frugiperda* larvae, we then proceeded with RNA-sequencing to obtain genome-wide transcriptomes during in vitro growth. Using comparative transcriptomics, we compared changes in gene expression between the three *M. rileyi* morphotypes: yeast-like cells produced in liquid media (YL treatment), yeast-like cells produced in solid media (YS treatment), and hyphae (H treatment), to determine the extent to which YS differed from YL. Thirdly, we used orthologous analysis of common genes in *M. rileyi* and related species to determine the novelty of *M. rileyi* genes involved in production of yeast-like cells on solid media. Finally, we used comparative transcriptomics to compare gene expression patterns of yeast-like and hyphal morphotypes across the broad host range species *M. anisopliae* and the host specialist, *M. rileyi,* to identify differences and commonalities between these two related dimorphic fungi. Our analyses showed extensive regulation and activity in genes that were only present in *M. rileyi,* which was consistent with the hypothesis that yeast-like cells produced on solid media are a unique evolutionary innovation in *M. rileyi* compared with other species in the genus *Metarhizium.*

2. Material and methods

2.1. Isolate selection and cultivation

The present study used *M. rileyi* isolate ESALQ4948 which originated from an infected *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larva collected in Itaara, Rio Grande do Sul State, Brazil. This isolate is held in the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) under accession number ESALQ4948. A conidial monospore culture was obtained by growing the fungus in agar (15 g/L), maltose (40 g/L), Bacto® neopeptone (10 g/L) yeast extract (20 g/L)(SMAY) for two weeks at 28 °C in a 12:12 h light:dark regime. A fungal stock culture was established by preserving sporulating agar chunks in a sterile 10 % glycerol solution at −80 °C.

2.2. Induction of filamentous and yeast-like growth

Isolate *M. rileyi* ESALQ4948 was grown on modified Jackson's medium (Jaronski and Jackson, 2012) with the following nutritional composition per liter: 45 g yeast extract, minerals, trace metals, and vitamins at the following concentrations per litre: KH_2PO_4, 2.5 g; $CaCl_2{\cdot}2H_2O$, 1.0 g; $MgSO_4{\cdot}7H_2O$, 0.83 g; $FeSO_4{\cdot}7H_2O$, 0.3 g; $CoCl_2{\cdot}6H_2O$, 29.6 mg; $MnSO_4{\cdot}H_2O$, 12.8 mg; $ZnSO_4{\cdot}7H_2O$, 11.2 mg; 0.2

mg each of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid; and 0.02 mg each of folic acid, biotin and vitamin B12. The medium was amended with 45 g/L of glucose solution that had been autoclaved separately. Sterile solutions of vitamins and metals were added to the autoclaved medium before the pH was adjusted to 5.5. Growth and formation of hyphae during the filamentous phase of the fungus and yeast-like states were induced by growing *M. rileyi* ESALQ4948 on solid and in liquid Jackson's medium (Jaronski and Jackson, 2012), respectively. Thus, identical nutritional compositions were used to grow mycelial hyphae and yeast-like states in a liquid medium. The only difference was that 15 g/L of agar was added to solidify the medium used for inducing hyphal growth. We used a SMAY medium to induce growth of the yeast-like state in a solid medium (see 2.1) as this medium provides excellent conditions for development of this morphotype.

Conidia of *M. rileyi* ESALQ4948 were obtained by growing agar chunks from the stock culture in Petri dishes containing SMAY for fourteen days at 28 °C in a 12:12 h light:dark regime. Conidia were harvested by washing Petri dishes containing actively sporulating fungal cultures with 10 mL of a sterile aqueous solution of 0.02 % polyoxyethylene sorbitan monooleate (Tween® 80, Sigma). The conidial concentration was determined using haemocytometer adjusted to 5×10^6 conidia mL^{-1} and used to inoculate 50 mL liquid Jackson's medium in 250-mL baffled Erlenmeyer flasks to achieve a final concentration in the culture broth of 5×10^5 conidia mL^{-1} (i.e., 10 % v/v inoculum). Liquid cultures were incubated at 28 °C in a rotatory incubator shaker at 350 rpm for 72 h. Four Erlenmeyer flasks were inoculated; each flask was considered as a biological replicate.

A volume of 120 μL of the 5×10^6 conidia mL^{-1} suspension was spread using sterile Drigalski handles in Petri dishes (9 cm diameter) containing solid Jackson's medium (to induce hyphal growth) or SMAY medium (to generate yeast-like growth). There were four Petri dishes for each solid media and they were incubated at 28 °C for six days to obtain mycelial hyphae or three days to obtain the yeast-like state, in a 12:12 h light:dark regime. Each Petri dish was considered as one biological replicate. This yielded three different growth treatments with four replicates each: hyphae from solid Jackson's media (H treatment), yeast-like cells from liquid Jackson's media (YL treatment), and yeast-like cells from solid SMAY media (YS treatment).

2.3. Phenotypic analyses of yeast-like cells

Yeast-like cells produced on solid medium, were washed in 1 mL potassium buffer saline (PBS) at pH 6,0 and transferred to a 1.5 mL sterile Eppendorf® tube, while 1 mL of YL were transferred directly to 1.5 mL tubes. Cell suspensions were centrifuged at 8000 rpm for 10 min and the supernatant discarded. The cell pellet was washed thrice in 1.0 mL of PBS at pH 6.0 before being suspended in distilled water for staining. Chitin staining was done by adding 1.5 μL of calcofluor white stock solution (10 mg mL^{-1}, Sigma, 18909) to 40 μL of the yeast-like phases that had been placed on glass slides and incubated for one minute at room temperature in darkness, after which cells were immediately observed using a filter (excitation 405, emission 450) in a Nikon *C2* + confocal laser scanning microscope at 600 × magnification. The intensity of the laser applied was the same for all samples.

2.4. Virulence bioassay

The virulence of *M. rileyi* ESALQ4948 yeast-like cells produced in liquid and solid media was assessed against second instar larvae of the fall armyworm *S. frugiperda*. Yeast-like cells were cultivated for three days in liquid and solid media (see 2.2). Yeast-like cells produced on solid media were washed in 100 μL of 0.05 % Tween® 80 solution and transferred to a 1.5 mL sterile Eppendorf® tube, while cells produced in liquid media were transferred directly to 1.5 mL tubes. Next, cell suspensions were centrifuged at 8000 rpm for 10 min and the supernatant discarded. Cell pellets, formed after centrifugation, were washed thrice in 1.0 mL PBS at pH 6.0 and centrifuged. Washed cells were resuspended in 1 mL of 0.05 % Tween® 80 solution, transferred to a 15 mL tube for homogenization and counted using a haemocytometer and phase-contrast microscope. We then adjusted to achieve four concentrations of each yeast-like cell morphotype: 5×10^6, 1×10^7, 5×10^7, 1×10^8 cells/mL. Tween® 80 0.05 % solution was used as a control treatment. For each treatment, we established four Petri dishes plates (12 cm diameter) containing moistened filter paper and twelve second-instar larvae previously fed on maize leaves. A volume of 2 mL of the yeast-like cell suspensions corresponding to the different treatments was applied per plate using a Potter Tower. After spraying, larvae were fed with fresh maize leaves and Petri dishes were incubated for 24 h at 26–28 °C in a 12:12 h light:dark regime; relative humidity was not controlled. Larvae were then transferred individually to plastic trays with filter paper. Larvae were observed daily for mortality and fresh maize leaves provided; the filter paper was replaced as necessary. Trays were incubated at 26–28 °C, in a 12:12 h light:dark regime for ten days. Dead larvae were transferred to a humid chamber at 26 °C until mycosis (if present) could be observed as an indication of infection. The entire experiment was done on three occasions using new batches of fungi and *S. frugiperda* cohorts.

2.5. Statistical analysis of virulence bioassay

Larval mortality data were fitted to a generalized linear model (GLM) with quasi-binomial distribution for errors, and treatment means were separated by Tukey's HSD test at $P < 0.05$. Survival analysis was done with censored data for dead larvae until day 11 and fitted to a parametric Weibull survival model using the package 'flexsurv' (Jackson, 2016) in the statistical software R (R Core Development, 2015). In addition, Weibull survival curves were compared based on the log-likelihood ratio test at $P < 0.05$. Median lethal concentrations of YS and YL (LC_{50} with 95 % confidence limits) were calculated by fitting data to GLMs with binomial distribution and using the function 'dose.p' in the MASS package in R. Plots were built on SigmaPlot® 14.0 (Systat Software Inc., San Jose, CA, USA) or in R.

2.6. RNA extraction and sequencing

Yeast-like cells from liquid culture (YL) were harvested by filtering three-day-old fungal cultures grown in liquid Jackson's medium. The total culture broth of each replicate was filtered in a vacuum pump coupled to a Buchner funnel lined with a 7 cm diameter filter paper disk (Whatman®, n°1; 11 μm pore size) to remove hyphae. Each replicate was examined on microscope slides at 400x magnification using optical light microscopy to verify that the filtrate contained only yeast-like cells. To separate yeast-like cells from the culture medium, 30 mL of the filtrate was placed in 50 mL Falcon® tubes and centrifuged at 2500 rpm, 4 °C for 5 min. The supernatant was discarded, and the pellet of yeast-like cells quickly transferred to a pre-cooled porcelain mortar using a pre-cooled scoop. Liquid nitrogen was added immediately, and the pellet macerated with a pre-cooled pestle. The resulting powder was not allowed to thaw and was transferred to an Eppendorf tube containing 1 mL TRIzol® reagent (Invitrogen, USA) and kept on ice. Mycelia and yeast-like cells grown on Jackson's agar and SMAY medium, were harvested from six and three days old fungal cultures, respectively. Hyphae and yeast-like cells were removed from the medium using a sterilized and pre-cooled spatula and placed immediately in an Eppendorf tube containing 1 mL of TRIzol® and kept on ice. Care was taken to avoid collecting the solid media when scraping off the hyphae and yeast-like cells.

Total RNA was extracted from fungal samples immersed in TRIzol® following the manufacturer's instructions. Eppendorf tubes containing 1 mL of TRIzol® with either yeast-like cells or hyphae were incubated for 5 min at room temperature before homogenizing the samples by

pipetting up and down. This was followed by centrifugation for 5 min at 12000x g and 4 °C. The supernatant was transferred to a new clean Eppendorf tube, and the samples homogenized for 5 min in a tissue homogenizer to disrupt the cell walls. Chloroform (200 μL) was added to each sample following agitation for 15 s and incubated at room temperature for 5 min. After another centrifugation (12,000 × g, 15 min at 4 °C) to separate the mixture, total RNA was precipitated from the upper aqueous phase with half a volume of isopropanol (0.5 mL isopropanol per 1 mL of TRIzol®) and centrifugation. The pellet was washed in 1 mL of 75 % ethanol and dried for 30 min at room temperature, followed by resuspension of total RNA in 20 μL of pre-cooled DEPC-treated water. Total RNA was quantified fluorometrically using a Qubit® (Invitrogen), and the purity and quality evaluated in a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA). RNA integrity was estimated by 1 % agarose-formaldehyde gel capillary electrophoresis using a Bioanalyzer (Agilent); only samples with a RNA integrity measure (RIN) higher than eight were used.

Messenger RNA libraries were prepared using an Illumina TruSeq Stranded mRNA Library Prep kit (Illumina Inc., San Diego, CA) and quantified by qPCR using the Illumina KAPA Library Quantification kit. Samples were sequenced using Illumina HiSeq 2500 technology, which yielded at least 20 million 100-bp paired-end reads per library. Library preparation and sequencing were done by the 'Laboratório Multiusuários Centralizado de Genômica Funcional Aplicada à Agropecuária e Agroenergia', Piracicaba-SP, Brazil.

2.7. Mapping RNA-Seq reads and quantitative differential expression analysis

Trimming and removal of low-quality sequences were done using Trimmomatic V0.32 (Bolger et al., 2014) with the following options: HEADCROP:7 TRAILING:20 MINLEN:36. The quality of raw reads before and after quality control was assessed using fastQC (Babraham Bioinformatics). Quality trimmed reads were aligned to the reference genome (*M. rileyi* RCEF4871 from NCBI) using HISAT2 (Pertea et al., 2016). First, we used python scripts included in the HISAT2 package: extract_splice.py and extract_exons.py, to extract the splice-site and exon information from the annotation file, respectively. Then, we built the indexes for the reference genome with the program hisat2-build and the options: –ss and –exon, to provide outputs from splice sites and exons, respectively. Finally, we aligned RNA-seq reads to the reference genome with the program hisat2 and the options: –dta and –p 8. Gene quantification was done with StringTie v1.3.3 (Pertea et al., 2016) using gene annotations from the *M. rileyi* RCEF4871 reference genome. The StringTie program was used with the following options: -b, -B and –G. The gene count matrix was obtained with python script: prepDE.py, provided by John Hopkins University, center for computational biology, CCB (https://ccb.jhu.edu/software/stringtie/index.shtml?t=manual#deseq). Differential expression analysis was done using the DESeq2 library (Love et al., 2014) for the statistical software R (R Core Team, 2017). Only genes with a false discovery rate (FDR) adjusted p-values < 0.001, and a $\log_2$ fold change (FC) > 2 were considered as differentially expressed. Individual gene expression was not re-validated by qPCR because: previous studies have shown a close correlation between qPCR and RNAseq data (Asmann et al., 2009; Griffith et al., 2010; Wu et al., 2014); our biological samples were robustly replicated, highly similar within treatments, and distinctly different between treatments (Fig. 3A); and there is little evidence that qPCR analysis of a few specific genes from the same samples adds any new utility to the data or changes the major conclusions drawn from the much larger groups of genes analyzed in the RNAseq dataset. Heatmaps of differentially-expressed genes were made using the web application 'shinyheatmap' (Khomtchouk et al., 2017) with the following parameters: apply clustering: column, Distance metric: Euclidian; Linkage algorithm: complete.

2.8. Gene-set enrichment analysis

Gene set enrichment analysis (GSEA) is software that determines whether an *a priori*-defined set of genes is statistically significantly different between two biological states (Subramanian et al., 2005). GSEA ranks gene sets by enrichment magnitude and indicates classes of genes that are over-represented in a gene set. As recommended for RNA-seq datasets, GSEA was used in the GSEAPreranked mode with a list of all genes pre-ranked according to expression. Then, GSEAPreranked calculates an enrichment score by matching genes from gene sets to those in the user ranked list. Next, the gene set's enrichment score indicates how often members of that gene set occur at the top or bottom of the ranked data set. This study used GSEAPreranked mode with gene sets categorized by gene ontology (GO) annotation. The metric used in the GSEA input file was the sign (+or -) of the fold change multiplied by its inverse *p-value*. We used the *p-value* provided as an output of DESeq2. When the *p-value* output from DESeq2 was '0′, the '0′ value was replaced by artificially high or low values '+1E + 308′ or '-1E + 308′ for up and down-regulated genes, respectively, according to the sign of fold change. The parameter adopted for running the GSEAPreranked for GO terms was: minlengh ten and maxlengh 500, enrichment statistic: 'classic', and FDR-correction for multiple testing <0.25 for enriched gene sets. The unusually high FDR threshold of <0.25 is recommended by GSEA because it indicates that the result is likely to be valid three out of four times (Subramanian et al., 2005). The web server REVIGO (Supek et al., 2011) was used to analyze GO terms in the categories: biological process, cellular component and molecular function.

2.9. Orthologous analysis of shared genes in M. rileyi and related species

To elucidate the novelty of *M. rileyi* genes involved in yeast-like cell formation on solid agar medium (YS) we estimated orthologous gene groups from 13 fungal species that also exhibit dimorphic development, including eight *Metarhizium* species (*Metarhizium robertsii* ARSEF 23 and ARSEF 2575, *Metarhizium anisopliae* strain E6 and ARSEF 549, *Metarhizium album* strain ARSEF 1941, *Metarhizium guizhouense* ARSEF 977, *Metarhizium brunneum* ARSEF 3297, *Metarhizium rileyi* strain Cep018-CH2, *Metarhizium acridum* strain CQMa 102 and *Metarhizium majus* strain ARSEF 297), a closely related species *Pochonia chlamydosporia* (Hypocreales: Clavicipitaceae) (strain 170), the entomopathogenic fungus *B. bassiana* (Hypocreales: Cordycipitaceae) (strain D1-5 and JEF-007), the human pathogen *Candida albicans* (Saccharomycetales: Saccharomycetaceae) (strain SC5314), and plant pathogens *Ustilago maydis* (Ustilaginales: Ustilaginaceae) (strain 521) and *Zymoseptoria tritici* (Capnodiales: Mycosphaerellaceae) (strain IPO323). The strains are deposited at the USDA-ARS Collection of Entomopathogenic Fungal Cultures, United States of America (ARSEF 23, ARSEF 2575, ARSEF 549, ARSEF 1941, ARSEF 977, ARSEF 3297, ARSEF 297), The Jeonbuk (Chonbuk) National University Collection of Entomopathogenic Fungi, Korea (JEF-007), the Centro de Estudios Parasitológicos y de Vectores, Argentina (Cep018-CH2), the China General Microbiological Culture Collection Center (CQMa 102 (CGMCC, number 0877), (*Pochonia chlamydosporia* 170 (CGMCC, number 8860)), American Type Culture Collection (ATCC) (SC5314), the German Collection of Microorganisms, (*Ustilago maydis* 521 (DSMZ number 14603)), CBS-KNAW Fungal Biodiversity Centre of the Royal Netherlands (IPO323 (accession numbers CBS 115943)).Orthologous gene groups were identified using OrthoFinder (Emms and Kelly, 2019) with default parameters. Protein sequences of these genomes were obtained in the NCBI database and used as input in Orthofinder. To get the evolutionary relationships between orthogroups, we analyzed a phylogenetic tree of orthologous genes inferred by the Maximum Likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001) (Ma et al., 2010), and the bootstrap percentage was inferred from 1000 replicates. The alignment and the phylogenetic tree were conducted in MEGA6 (Tamura et al., 2013).

2.10. Comparative transcriptome analysis of yeast-like phases of M. rileyi and M. anisopliae

To identify similarities and differences between gene expression patterns of yeast-like cells in *M. anisopliae* ESALQ4676 and *M. rileyi* ESALQ4948, we compared the yeast-like transcriptomes of the two species. We used *M. anisopliae* data from (Iwanicki et al., 2020a), which is available in the European Nucleotide Archive repository [https://https://www.ebi.ac.uk/ena, under accession number: PRJEB30948]). Growth and formation of *M. anisopliae* hyphae (filamentous phase) and yeast-like cells had been induced by growing on solid and in liquid modified Adamek medium (Iwanicki et al., 2018), respectively and, as in our current study, the nutritional compositions used to grow hyphae and yeast-like cells were identical; the only difference was that 15 g/L of agar had been added to solidify the medium used for inducing hyphal growth.

We first compared enriched biological processes, based on GSEA results, across yeast-like phases for the two *Metarhizium* species and highlighted the similarities and differences between them. The parameter adopted for running the GSEA (minlength ten and maxlength 500, enrichment statistic: 'classic' and FDR- correction for multiple testing, 0.25 for enriched gene sets) were the same for both *M. anisopliae* and *M. rileyi* transcriptomes. Next, we determined the orthologous genes upregulated (FDR adjusted $p < 0.001$, Log2FC > 2) in yeast-like phases in *M. anisopliae* and *M. rileyi*.

3. Results

3.1. Phenotypic analysis and insect virulence of yeast-like cells

Phenotypic analysis of yeast-like cells produced in solid and liquid medium was achieved by microscopic observation of calcofluor white stained cells. We observed that yeast-like cells were pleomorphic and have different sized spots of chitin accumulation (Fig. 1). Most significant deposits of chitin were observed in the cell walls, in the growth regions of cells, and in septa, which appeared more common in YS than in YL (Fig. 1). We also found a greater accumulation of chitin in the central part of the cell walls in YL than in YS where the deposits of chitin were more homogenous throughout the cell wall or accumulated in one of cell extremities along the longitudinal axis.

We assessed the virulence of *M. rileyi* ESALQ4948 YL and YS against second instar of *S. frugiperda* larvae. Our data showed a significant difference in *S. frugiperda* mortality between control and fungus treatments ($F_{8,110} = 16.31$, $P < 0.0001$) and a longer survival time of untreated *S. frugiperda* than those sprayed with YS or YL, regardless of the concentration tested ($\chi^2 = 204.56$, df = 8, $P < 0.0001$) (Fig. 2). At a high concentration of 1×10^8 YL or YS/mL, yeast-like cells killed 62 % and 59 % of the larvae, respectively, compared with 42 % and 36 % at a low concentration of 5×10^6 of YL or YS/mL (Fig. 2B). Although we found no clear correlation between dosage and mortality, the estimated median concentration (LC_{50}) suggested that YL had higher virulence than YS, with an LC_{50} of $8.12 \pm 1.58 \times 10^6$ cells/mL compared with $6.08 \pm 1.55 \times 10^7$ cells/mL for YS. Only larvae sprayed with YS exhibited fungal outgrowth after host death (mycosis) (Fig. 2C) in 1.5–12 % of exposed insects and depending on YS concentration (Fig. 2B).

3.2. Transcriptomic analysis

To determine the main genes related to *M. rileyi* yeast-like cell formation, we compared the genome-wide expression profile of yeast-like cells grown in liquid and solid media to hyphae. A total of 165.6 million paired-end 100-bp clean reads were obtained from yeast-like cells from liquid medium (YL), solid medium (YS), and hyphae (H) (Table S1). The mean alignment rate of YL, YS and H reads to the reference genome were 94 %, 90 % and 94 %, respectively. Clustering analysis of YL, YS, and H showed clear differences in gene expression between biological treatments (Fig. 3A). The first principal component differentiated the three treatments and explained 64 % of variation. Pairwise analysis showed that out of 8,764 genes identified in the *M. rileyi* genome (RCEF 4871), 1,024 (11.68 %), 986 (11.26 %) and 893 (10.18 %) of genes were differentially expressed between YS and H, YS and YL and YL and H, respectively (FDR adjusted $p < 0.001$, $Log_2FC > 2$ or <-2) (Table S2). Of these, 417 genes (4.75 % of the genome) were upregulated in YS compared with H and 145 genes (1.65 % of the genome) compared with YL. Additionally, 186 genes were upregulated in YL compared with H (Fig. 3). We found 276 genes (3.14 % of the genome) that shared up-regulation in YS compared with H and YL (Fig. 3B). The fungus thus exhibited comprehensive differences in genome-wide gene expression patterns during filamentous growth compared with the yeast phases in either liquid medium (YL) or on solid medium (YS). This is further emphasized since we found 647 genes (7.38 %) upregulated in H compared with YS and 340 genes (3.87 %) upregulated in H compared with YL (Fig. 3B).

3.3. Functional gene set enrichment analysis of yeast-like cells

To investigate overall patterns of gene function amongst differentially-expressed genes, we performed gene set enrichment analysis of the main biological processes, cellular components and molecular function using gene ontology terms (GO). It was possible to assign GO terms to 5,818 (66.3 %) out of 8,763 genes in the *M. rileyi* RCEF4871 genome. First, yeast-like cells grown in solid (YS) and liquid media (YL) were compared with hyphae (H) as a control before YS and YL were compared with each other.

We found 15 and 25 GO terms in the biological process category that were significantly enriched in yeast-like cells grown in solid medium (YS) and liquid medium (YL), respectively, compared with hyphae (H) (Fig. 4; Table S3). The most enriched GO terms in YS were for: metabolic processes (*GO:0008152*) such as chemical reactions and pathways (e.g. anabolism, catabolism) related to cell growth, the transformation of small molecules, macromolecular processes (e.g. DNA repair and replication), and protein synthesis and degradation; intracellular protein transport (*GO:0006886*) and vesicle-mediated transport (*GO:0016192*) both involved in the transport of substances within the cell or enclosed in membrane-bounded vesicles; and ATP hydrolysis (*GO:0015991*) (Fig. 4A). In contrast, the most enriched GO terms in YL were related to DNA (*GO:0006281*, *GO:0006260*) and RNA (*GO:0006364*, *GO:0006396*) activities, and intracellular protein transport (*GO: 0006886*) (Fig. 4B). Furthermore, we found eight GO terms in the biological process category that were significantly enriched in yeast-like cells grown in solid medium (YS) compared with yeast-like cells grown in liquid medium (YL); and 21 GO terms in the biological process category in YL compared with YS (Fig. 5A and 5B). In YS more upregulated genes were associated with cell redox homeostasis (*GO:0045454*), carbohydrate transport (*GO:0008643*) and lipid metabolic processes (*GO:0006629*). In comparison, in YL more upregulated genes were associated with intracellular signal transduction (*GO:0035556*) and intracellular protein transport (*GO:0006886*).

3.4. Different nutrient acquisition, transport, and membrane-associated genes active in yeast-like cells from liquid and solid culture medium

To investigate the active genes associated with induction or maintenance of yeast-like cell growth in liquid culture media, we focused on the 186 genes that were upregulated in YL compared with H and the 340 genes that were upregulated compared with YS. Based on domain sequences, homology protein families (PFAM) could be assigned to 6185 (70.5 %) out of 8763 genes in the *M. rileyi* genome, which revealed that several active genes were attributed to nutrient transport, absorption, and membrane and cell wall components (Table S2). Most nutrient transporters are expressed at the plasmatic membrane, and their expression levels were strongly regulated by substrate availability

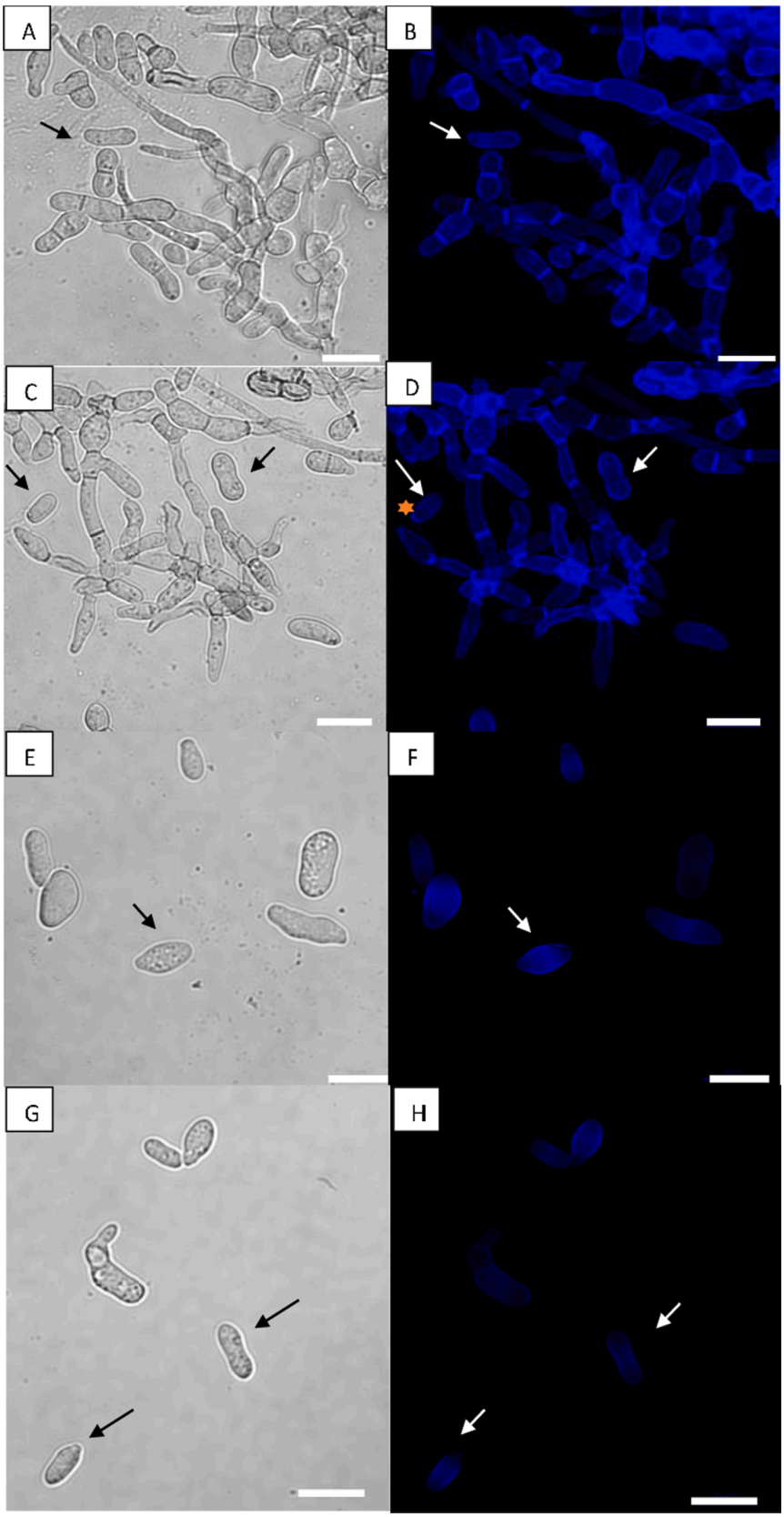

Fig. 1. Calcofluor white fluorescence staining of chitin from *Metarhizium rileyi* (ESALQ4948) yeast-like cells produced on solid medium (B and D) and liquid medium (F and H); and bright-field images of the same (A, C, and E, G). Scale white bar = 10 μm. Black and white arrows indicate yeast-like cells. The orange star indicates a spot of chitin accumulation.

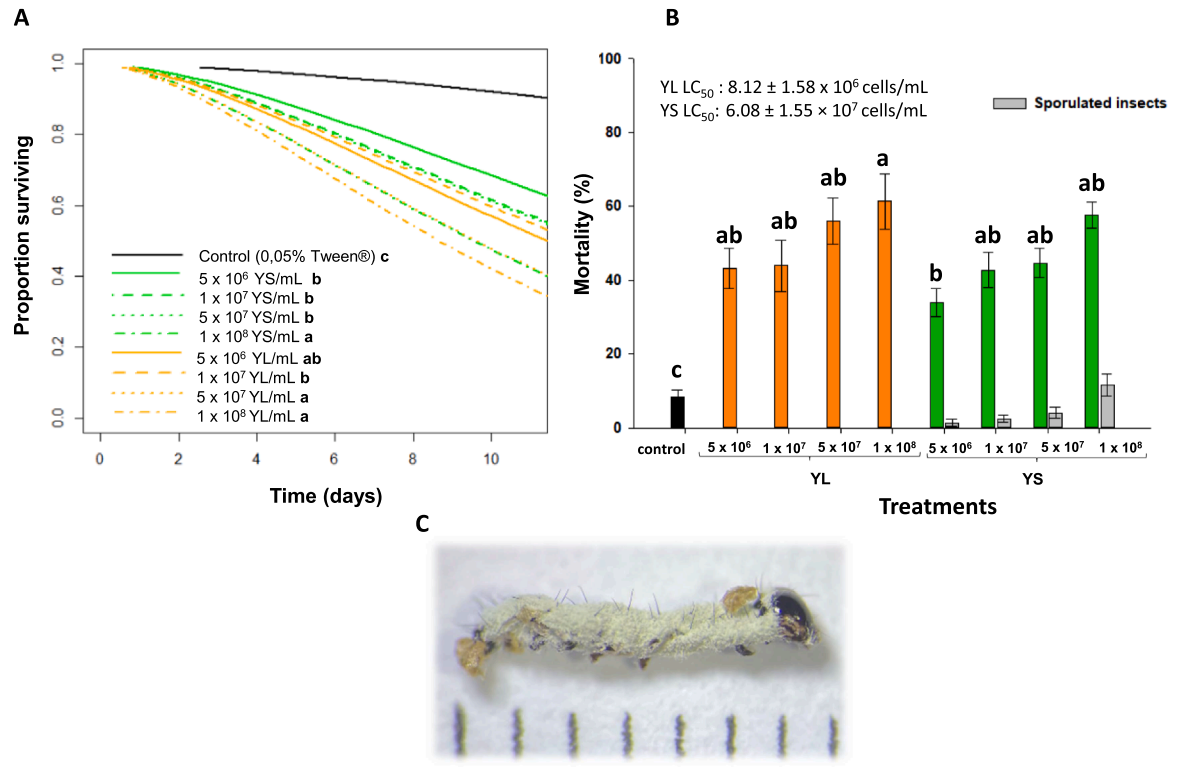

Fig. 2. A) Proportion of *Spodoptera frugiperda* larvae surviving after exposure to different concentrations of *Metarhizium rileyi* (ESALQ4948) yeast-like cells produced on solid (YS) and liquid (YL) medium. Survival curves with different lower case letters are significantly different to each other ($P < 0.05$). B) Percent mortality and sporulating larvae of *S. frugiperda.* Bars (means ± standard error [SE]) with different lower case letters are significantly different to each other, according to Tukey's HSD test ($P < 0.05$). Estimated median concentration (LC_{50}) of YL and YS ± standard error. C) *S. frugiperda* larvae sporulating with *M. rileyi* conidia after being sprayed with YS.

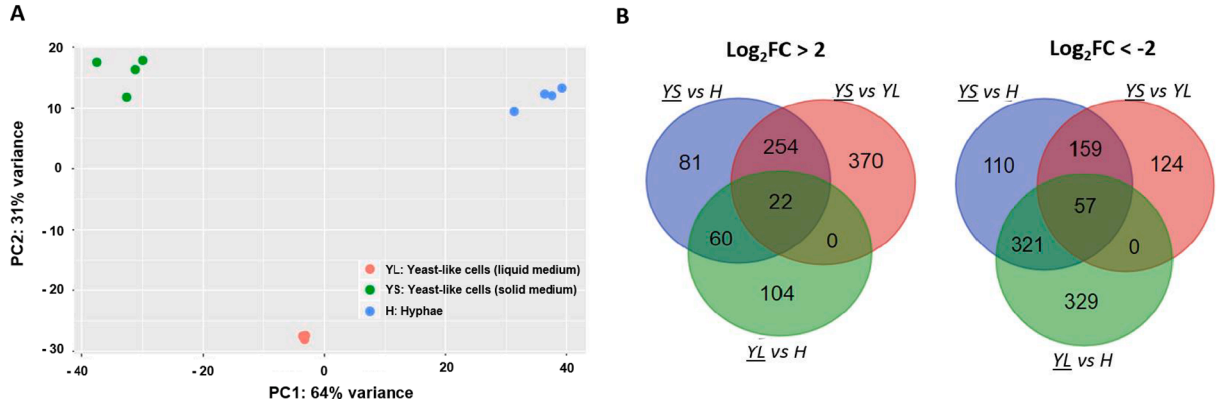

Fig. 3. A) Principal component analysis of regularized-logarithmic (rlog) transformed gene counts of four biological samples of yeast-like cells grown in liquid medium (YL), yeast-like cells grown in solid medium (YS), and hyphal samples (H) of *Metarhizium rileyi* ESALQ4948. YL, YS, and H samples are represented by pink, green and blue dots, respectively (n = 4 per treatment). B) Venn diagrams showing differentially-expressed genes of *Metarhizium rileyi* ESALQ4948, up (left) and down (right) regulated in yeast-like cells produced in liquid (YL), on solid (YS) medium, and hyphae (H). Log2FC > 2 or $Log_2FC < -2$. FDR-*p-value* < 0.001. Subscribed letters indicate phenotypes in which genes were up (left) or down (right) regulated.

(Busto and Wedlich-Söldner, 2019). Genes associated with transporters were differently upregulated in YL with the substrate transporter and the major facilitator superfamily domain (MFS) (pfam: *PF07690*), being the largest group of genes upregulated. Compared with hyphae, we found more genes containing domains related to the major facilitator superfamily domain (MFS) (n = 5) and amino acid/polyamine transporter (pfam: *PF01490*) (n = 2) were upregulated compared with YS (n = 3) and (n = 1), respectively. Additionally, we found one gene related to iron permease (pfam: *PF03239*) that was upregulated in YL compared with H. In contrast, compared with YS, we found two upregulated genes associated with ABC transport and one compared with H.

We found integral membrane proteins and CFEM domain genes were upregulated in YL compared with YS and H. The CFEM domain represents proteins involved in different functional categories such as cell wall biogenesis and integrity and fungal pathogenesis (Zhang et al., 2015). Two genes related to hydrophobins and one to mannosidase (an enzyme that hydrolyses mannose), and one to a fungal cell wall polymer, were upregulated compared with YS. In comparison, two genes related to cell wall glucan synthesis and one to endoglucanase were upregulated in YL compared with H. However, of the differentially-expressed genes

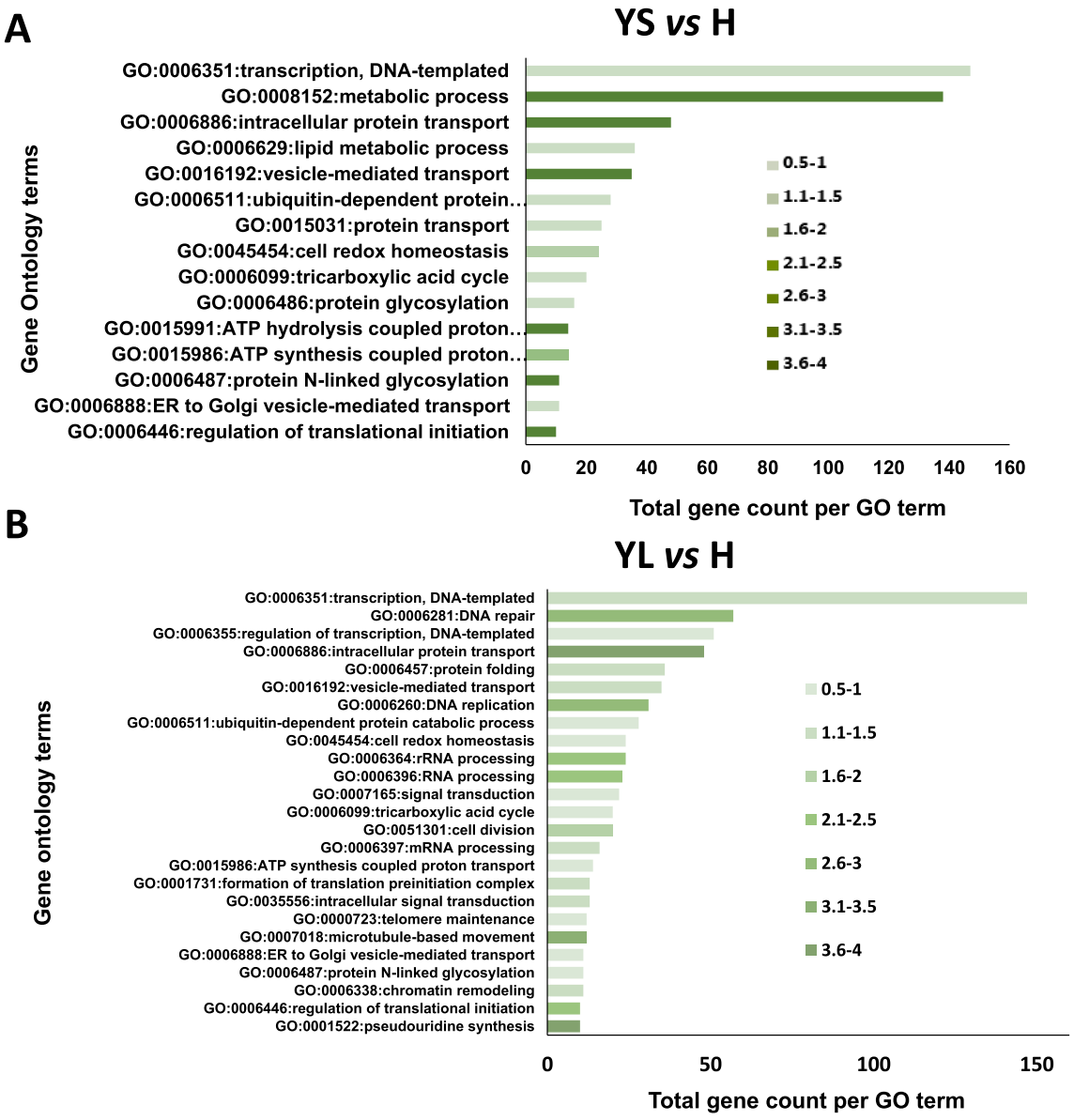

Fig. 4. (A) Gene ontology (GO) terms related to biological processes enriched in yeast-like cells grown on solid medium (YS) and (B) yeast-like cells grown in liquid medium, considering hyphae as a control baseline. Horizontal bars show the total gene counts in the genome per GO term. The bar colour denotes $-\log_{10}$(FDR-*p-value* from enrichment score), with darker green colours showing significantly more enrichment.

in YL compared with YS and H, 77 and 131 were attributed to 'uncharacterized protein' (Table S2) and were not considered further.

To elucidate the prominent signature of yeast-like cells grown in a solid medium, we first analyzed the most highly upregulated genes ($Log_2FC > 4$, FDR-*p-value* < 0.001) and their respective protein families (pfam) in YS compared with H and YL (Fig. 6, Table S4). In total, we found 66 highly upregulated genes in YS, of which a significant proportion (42.64 %) were classified as 'uncharacterized protein' with no available annotation related to protein family (pfam) and gene ontology (GO terms). The remaining 57.64 % of genes were related to enzymes that mediate nutrient acquisition such as trehalase (gene id: OAA35535), taurine catabolism dioxygenase (gene id: OAA51918), nitroreductase (gene id: OAA51918), amino acid permeases and lipase. Additionally, various genes involved in nutrient and substance transport, such as the major facilitator superfamily domain, carboxylic acid protein transporter, and oligopeptide transporter; and metalloproteases such as peptidase M43 (gene id: OAA35764) and deuterolysin (gene id: OAA34772) were highly expressed in YS (Fig. 6, Table S4). Our results show that YL have a distinct repertoire of active genes compared with YS and H, primarily related to nutrient transport and membrane/cell wall modification. The unique genes active in YS compared with YL and H are also associated with nutrient acquisition and transport but different compared with YL.

3.5. Orthogroup comparison across fungi with yeast-like growth forms

To determine whether genes commonly upregulated in YS compared with YL and H were *M. rileyi*-specific and to what extent they were exclusively involved in *M. rileyi* YS morphogenesis, we conducted a protein orthology analysis of those genes across other dimorphic fungi, including all *Metarhizium* species with available genomes. Our rationale being that the orthogroups (genes derived from a common ancestral gene) conserved in *M. rileyi* and in species that grow with a yeast phase on solid surfaces such as *Z. tritici*, *B. bassiana,* and *C. albicans* might provide some insights about conserved molecular mechanisms that trigger the formation of YS.

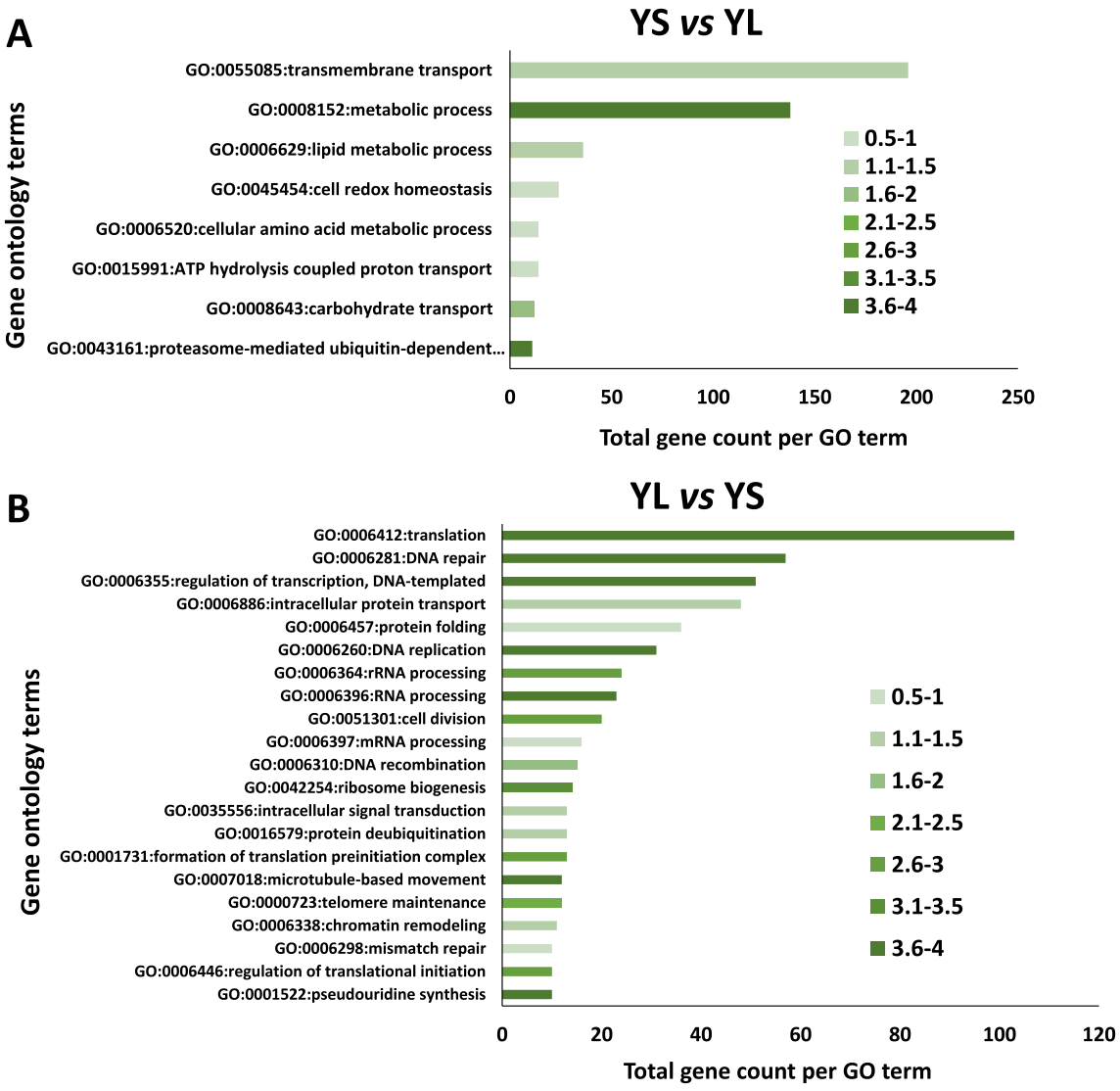

Fig. 5. (A) Gene ontology (GO) terms related to biological processes enriched in yeast-like cells grown on solid medium (YS) and (B) yeast-like cells grown in liquid medium. Horizontal bars show the total gene counts in the genome per GO term. The bar colour denotes $-\log_{10}$(FDR-*p-value* from enrichment score), with darker green colours showing significantly more enrichment.

A total of 264 orthogroups were assigned to the 276 genes commonly upregulated in YS ($Log_2FC > 2$, FDR-*p-value* < 0.001). Orthogroups were shared with *C. albicans* (n = 46), *U. maydis* (n = 79), *Z. tritici* (n = 126), *B. bassiana* (n = 142), *P. chlamydosporia* (n = 167), and *Metarhizium* species (n = 222). Furthermore, 29 out of 264 orthogroups were shared only with at least one *Metarhizium* species, and three (OG0000924, OG0008162, OG0008163) orthogroups had at least one gene representative in all *Metarhizium* species (Table S5). One orthogroup was shared only with *B. bassiana* (OG0010358), two were shared exclusively with *Z. tritici* (OG0009257, OG0011214), and one shared with *B. bassiana* and *Z. tritici* (OG0008298) exclusively (Table S5).

The orthogroup OG0008298, shared only with *B. bassiana* and *Z. tritici,* was composed of ten orthologous genes related to amino acid permeases in which two genes were found for each *B. bassiana* isolate, four genes were from Z. *tritici* and one gene found for each *M. rileyi* isolate (gene id of studied isolate: OAA36856, protein family PF13520: amino acid permeases). Nitroreductases and permeases are enzymes that play a role in reducing nitrogen-containing compounds and nutrient acquisition, respectively. We found that *M. rileyi* isolates had in their genomes two orthologous genes related to nitroreductase (gene ids: OAA39368, OAA44961) grouped in orthogroup OG0004805. In contrast, all *Metarhizium* species, both isolates of *P. chlamydosporia*, Z. *tritici* and *U. mayidis,* had only one gene and none were found in *B. bassiana* isolates. The ML phylogenetic analysis showed that the sequence of the nitroreductase found highly upregulated in YS (gene id: OAA39368) compared with H and YL were grouped with that found in *Z. tritici,* while the other nitroreductase gene, not differentially expressed in this study, grouped with all *Metarhizium* species genes (Fig. S1).

The enzyme peptidase M43 pregnancy-associated plasma-A, was shown to be involved in sporulation, cell wall integrity and virulence factors in *M. robertsii* (Zhou et al., 2018). We found that *M. rileyi* had three orthologous genes related to peptidase M43 in the orthogroup OG0007192 while *M. album, M. brunneum,* and *M. robertsii* had only one gene, and other *Metarhizium* species had none. One of the *B. bassiana* isolates had two genes in this orthogroup, while the other isolate has four genes. Additionally, we found a protein, Pal1 cell morphology (gene is: OAA40599 and orthogroup: OG0005147) was upregulated in YS. This membrane-associated protein is involved in maintaining cylindrical cellular morphology (Ge et al., 2005). All species had one gene in the OG0005147 orthogroup except *C. albicans* and *U. maydis,* which had none (Table S5).

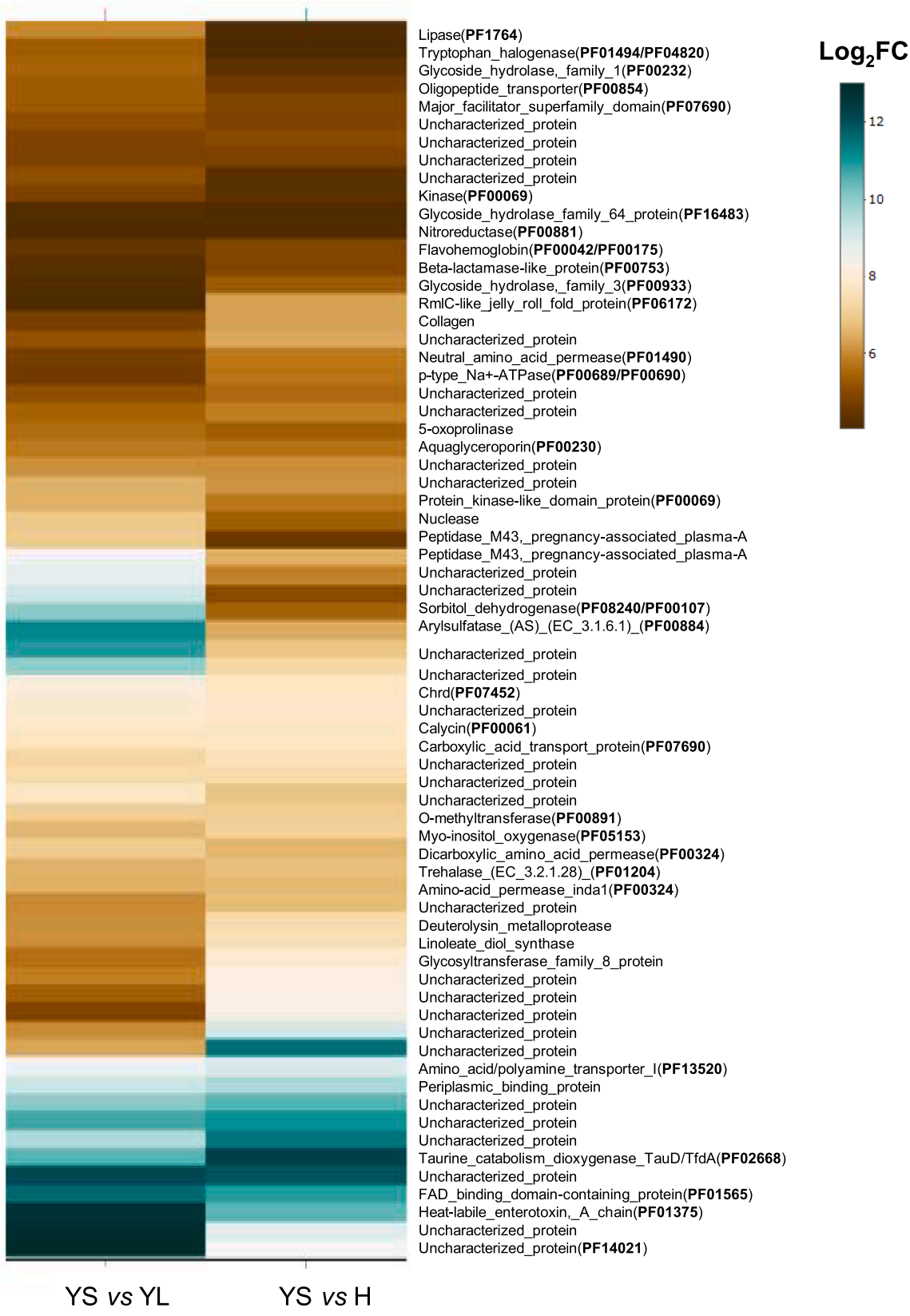

Fig. 6. Heatmap most highly upregulated genes in yeast-like cells of *Metarhizium rileyi* ESALQ4948 grown on solid medium (YS) compared to yeast-like cells (YL) grown in liquid medium and hyphae (H). Log2FC > 4 and FDR-*p-value* < 0.001).

Additionally, YS had two upregulated genes for heat-labile enterotoxins, one from the orthogroup OG0004298 (gene id: OAA43809) and the other representing one of the genes most up-regulated in YS compared with YL (Log_2FC 12.7, FDR-*pvalue* < 0.001) from the orthogroup OG0000918 (gene id: OAA35032); both are enzymes related to virulence. It is noteworthy that *M. rileyi* had three genes in orthogroup OG0000918 while other *Metarhizium* species had only one and species from different genera had none. Nonetheless, a total of 38 orthogroups were found to be *M. rileyi*-specific and were not shared with any fungi analyzed in this study, and included an unknown cell wall protein (orthogroup: OG0012636, gene id: OAA43728) and a highly upregulated collagen protein (orthogroup: OAA42706, gene id: OG0012597). We found that 2/3 of the collagen protein gene sequence was made up of multiple repeats of a sequence with different GC content. Notably, the coding part of this gene was unique to *M. rileyi* and had no hits with any other species. All these findings suggest that induction of yeast-like cells in a solid agar medium requires expression of genes found exclusively in *M. rileyi*.

3.6. Comparative transcriptomics of M. Rileyi and M. Anisopliae yeast-like cells

Comparative transcriptome analyses of gene expression showed more differences than similarities between *M. anisopliae* and *M. rileyi* yeast-like cells from liquid media. We identified seven and 11 unique enriched GO terms in *M. anisopliae* and *M. rileyi,* respectively (Fig. 7). The enriched biological process associated with *M. anisopliae* YL involved glycolytic processes, carboxylic acid metabolic processes, response to oxidative stress and cellular amino acid metabolic processes. At the same time, those in *M. rileyi* YL were more associated with primary metabolisms such as DNA activities, cell division, intracellular signal transduction and mRNA processing (Fig. 7). We found seven enriched GO terms in the biological processes category that were common in both *M. anisopliae* and *M. rileyi* YL, and were associated with DNA and RNA activities, tricarboxylic acid cycle and protein folding (Fig. 7). Considering YS, this morphotype the genes that were highly expressed were related to protein transport, lipid metabolic process and protein glycosylation. Our data also indicated that the primary biological processes in *M. rileyi* YS and YL were associated with cell redox homeostasis, vesicle-mediated transport and intracellular transport; these were not active in *M. anisopliae* YL (Fig. 7).

We found 27 orthologous genes that were upregulated in yeast-like phases of both *M. anisopliae* and *M. rileyi* (Table 1). Amongst these, we highlight the collagen-like protein *Mcl1,* known to form a collagenous protective coat around fungal cells to evade insect immune responses and membrane-related proteins. It is noteworthy that, in general, fold changes in gene expression were more remarkable in *M. anisopliae* than in *M. rileyi*.

4. Discussion

In this study, we showed that *M. rileyi* yeast-like cells produced on solid media were just as virulent against an insect species as yeast-like cells produced in liquid media. Specifically, YS and YL were equally good at killing *S. frugiperda* larvae regardless of concentration, indicating that yeast-like cells from solid media have the potential to be used in biological control of lepidopteran pests. To further investigate the genetic control of the YS cells uniquely produced by *M. rileyi,* we identified the main biological processes that were active based on upregulated genes involved in maintaining yeast-like cells. We found a unique set of genes associated with enzymes such as amino acid permease that were active in yeast-like cells on solid media and not found in other *Metarhizium* species. YS also had upregulated genes for a unique nitroreductase, specific membrane proteins and several virulence factors, whereas YL had specific genes upregulated that were related to signal transduction, and may be associated with cell growth and activation of particular membrane transporters related to iron acquisition. Comparative transcriptomic analyses revealed that genes associated with heat shock protein, iron permease, membrane proteins and key virulence traits such as collagen-like protein Mcl1 were upregulated in both yeast-like phases in *M. rileyi* and *M. anisopliae*. Transcriptomic profiles of yeast-like cells from solid media thus exhibited a unique gene expression pattern involving membrane proteins, nutrient acquisition and virulence factors, where several of the key genes were unique to *M. rileyi* with no homologues in other *Metarhizium* species that did not form yeast-like cells on solid media.

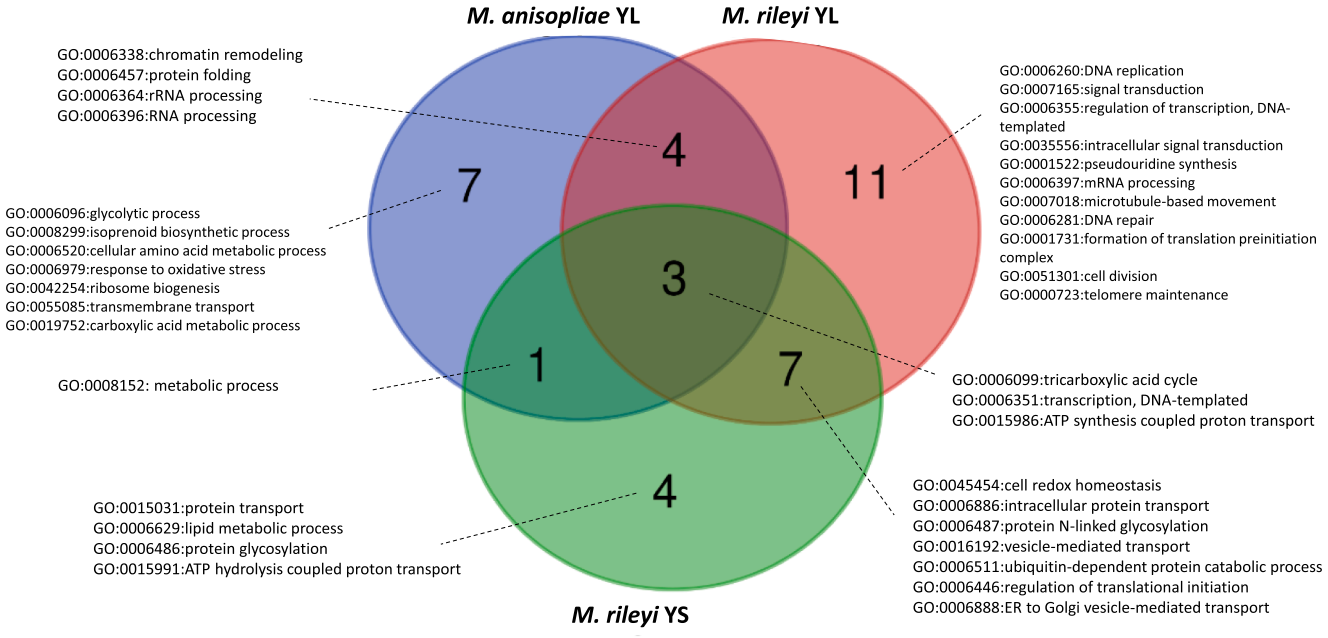

Fig. 7. Venn diagrams showing enriched biological processes in *Metarhizium anisopliae* ESALQ4676 yeast-like cells produced in liquid medium (YL) and *Metarhizium rileyi* ESALQ4948 yeast-like cells produced in liquid (YL) and solid (YS) medium considering hyphae as the baseline.

Table 1
Orthologous genes that were upregulated (FDR adjusted $p < 0.001$, Log2FC > 2) in yeast-like cells produced in liquid medium by *Metarhizium rileyi* ESALQ4948 (NOR) and *Metarhizium anisopliae* ESALQ4676 (MAN) (Iwanicki et al., 2020a).

Gene ID NOR	Log_2FC	Gene ID MAN	Log_2FC	Protein name
NOR_07034	8,01	MAN_05613	10,12	Glycosyl transferase, group 2 family protein
NOR_02753	5,90	MAN_06393	10,88	WSC domain containing protein
NOR_07033	5,11	MAN_05612	11,22	Nucleotide sugar dehydrogenase
NOR_00787	4,56	MAN_02994	3,30	Heat shock protein
NOR_01055	4,21	MAN_04442	3,19	FAD binding domain-containing protein
NOR_00099	4,19	MAN_04020	3,70	Amidase
NOR_08581	4,11	MAN_04550	2,09	Extracellular membrane protein, CFEM domain protein
NOR_07861	3,84	MAN_07474	2,10	Iron permease FTR1
NOR_08084	3,76	MAN_01699	3,00	Multidrug resistance protein 1
NOR_00174	3,58	MAN_04103	4,13	Uncharacterized protein
NOR_03903	3,37	MAN_07818	5,69	BZIP transcription factor
NOR_07384	3,22	MAN_01644	9,56	NAD(P)-binding domain protein
NOR_07171	3,11	MAN_05845	2,65	Gti1/Pac2 family protein
NOR_06346	3,03	MAN_07950	11,45	Uncharacterized protein
NOR_04913	2,95	MAN_01210	3,57	Trypsin
NOR_00173	2,92	MAN_04102	3,40	Amino acid/polyamine transporter I
NOR_02837	2,86	MAN_01263	5,04	Beta-1,3-endoglucanase
NOR_04343	2,77	MAN_07719	8,28	Collagen-like protein Mcl1
NOR_05674	2,48	MAN_00237	2,59	Xylitol dehydrogenase
NOR_02092	2,41	MAN_00671	7,14	Concanavalin A-like lectin/ glucanase
NOR_05520	2,39	MAN_08210	4,20	ATPase, P-type, transmembrane domain protein
NOR_00100	2,32	MAN_04019	2,66	Protein related to integral membrane protein pth11
NOR_08369	2,18	MAN_09205	4,35	Cerato-platanin
NOR_08152	2,14	MAN_08832	3,55	Short-chain dehydrogenase
NOR_07982	2,12	MAN_01755	2,81	Permease, cytosine/purine, uracil, thiamine, allantoin
NOR_07346	2,07	MAN_07121	4,15	Amine oxidase (EC 1.4.3.-)
NOR_03300	2,06	MAN_06917	3,34	Peroxidase (EC 1.11.1.-)

4.1. Yeast-like cells from liquid and solid media both express genes associated with virulence

We expected to find more genes related to virulence factors in YL than in YS. This was because, to some extent, liquid medium mimics the insect haemolymph, which, as far as we know, is the only environment where the yeast-like cells of *Metarhizium* species are naturally produced. However, we found evidence that YS resembles the yeast-like cells naturally produced within the insect more closely than YL. For example, genes for a trehalase enzyme were much more upregulated in YS than in YL or H. This enzyme degrades trehalose, the primary sugar component of insect haemolymph (Wyatt 1961) and is not present in SMAY culture medium. Another example of a potential virulence factor is collagen-like protein (gene id: OAA42706) which had no orthologous genes in other species in the *Metarhizium* genus, or other fungi. However, it's tempting to suggest that the collagen-like protein function is analogous to the collagen-Like protein (gene: Mcl) expressed in *M. robertsii* yeast-like cells (Wang and St Leger, 2006) and the orthologous gene expressed in YL of *M. anisopliae* (Iwanicki et al., 2020a) and *M. rileyi*. Mcl protein masks antigenic components of the cell wall of yeast-like cells, allowing them to evade the insect's immune response (Wang and St Leger, 2006). Pendland and Boucias (1998) speculated that a particular epitope found in *M. rileyi* cell wall hyphal bodies may have the unique 'ability' to 'mimic' host cell molecules and thus allow the fungus not to be recognized by haemocytes in the host haemolymph. The collagen-like protein found exclusively in *M. rileyi* and up-regulated in YS may be one of such epitopes previously mentioned. Additionally, the higher similarity of the collagen-like protein gene sequence to that of lepidopteran species may be related to the specificity of this species to lepidopteran hosts. Nonetheless, the proximity of the coding region to a repeat sequence could indicate that the gene can be, or has been, fast evolving.

Most of the genes upregulated in YL were related to cell wall synthesis and degradation which usually occurs during cell growth. The only exceptions were the two genes for hydrophobins that were upregulated in YL but not in YS. Hydrophobins are components of the rodlet layer of conidia cell surfaces, and play a role in virulence and resistance to environmental stress; they are not commonly found in yeast cells (Bayry et al., 2012). However, our findings showed that two hydrophobins were upregulated in YL but not in YS which indicates that YL has a more hydrophobic cell surface and is more resistant to environmental stress than YS (Bayry et al., 2012). In agreement with this result, Mascarin et al., (2021) and Iwanicki et al. (2020a) observed high expression of hydrophobins in YL of *B. bassiana* and *M. anisopliae,* respectively, indicating that specific hydrophobins are key proteins in yeast-like cells of entomopathogenic fungi produced in liquid medium.

The fact that yeast-like cells express many genes related to virulence factors without the presence of haemolymph for induction strongly indicates that this underexplored propagule has potential for pest control. For the first time we demonstrated that the virulence of YS against *S. frugiperda* was comparable to the virulence of YL; YS and YL kill similar numbers of *S. frugiperda* larvae regardless of concentration. The highest mortalities of 60 % and 58 % were achieved at a concentration of $1x10^8$ yeast-like cells/mL for YL and YS, respectively. *Metarhizium rileyi* ESALQ4948 was isolated from *A. gemmatalis* larvae, and one could hypothesize that higher mortality would be achieved if *A. gemmatalis* were used instead of *S. frugiperda*. Laboratory assays conducted by our team showed 85 and 90 % mortality of *S. frugiperda* larvae sprayed with aerial conidia of *M. rileyi* ESALQ4948 at a concentration of $5x10^7$ and $1x10^8$ conidia/mL, respectively; this indicates that yeast-like cells are less virulent than aerial conidia. In contrast to our findings, *B. bassiana* yeast-like cells induced in solid medium had higher virulence (lower medium lethal concentration and lethal time) than conidia against *Diatraea saccharalis* larvae, an economically important sugar cane pest (Alves et al., 2002).

4.2. M. Rileyi yeast-like cells from solid media have a unique transcriptomic profile compared with other single-celled yeast-like fungi

Orthology analysis showed evidence that induction of yeast-like cells in solid agar medium requires: 1) the expression of genes found exclusively in *M. rileyi*; 2) genes that are not orthologous with other *Metarhizium* species, or when they are, they are similar in all *Metarhizium* species; 3) activation of genes from orthogroups in which *M. rileyi* has several genes while other *Metarhizium* species have only one; 4) the expression of several genes assigned as 'unknown protein'.

We showed that *M. rileyi* yeast-like cells grown on solid media had highly upregulated genes for a specific amino acid, permease; these were orthologous genes found only in entomopathogenic *B. bassiana* and phytopathogenic *Z. tritici* fungi. While the ecological function of yeast-like cells in *Z. tritici* has been proposed as a strategy to increase initial inoculum on the leaf surface (Francisco et al., 2019), our findings indicate that both *M. rileyi* and *B. bassiana* yeast-like cells might be formed not only in the haemolymph but also on non-liquid parts of the insect. Furthermore, these yeast-like cells might be associated with other ecological niches in nature. As a membrane protein, the amino acid, permease, may have two functions: for transportation of amino acids into the cell or as an assessor that triggers signals in response to external amino acids (Boles and André, 2004). In the case of *B. bassiana* and *M. rileyi*, activation of permease in yeast-like cells may be related to the

detection of amino acids in insect haemolymph and, therefore, be interpreted as a constitutive response of this cell type. Nonetheless, in *M. rileyi,* permease might play a role in cell-to-cell signaling in a quorum-sensing system involved in switching from yeast-like cells to mycelial growth *in vivo* at a late stage of infection (Boucias et al., 2016). Through this membrane protein, yeast-like cells could detect an amino acid shortage in the haemolymph and signal induction of hypha formation. Conversely, in *Z. tritici,* this amino acid may be involved in the ability of yeast-like cells (blastospores) to use amino acids extracted from the surface of wheat leaves (Francisco et al., 2019).

Besides permease, we found that *M. rileyi* had genes for two nitroreductases in its genome while all *Metarhizum* species had only one. One of these nitroreductases was not differentially expressed and had a similar sequence to those from other *Metarhizum* species; the other one which was upregulated in YS was more similar to a nitroreductase found in *Z. tritici*. This may be related to the unique ability of *Z. tritici* and *M. rileyi* to degrade specific nitric compounds on the surface of wheat leaves and solid culture medium, respectively. Degraded compounds may be required to induce and maintain the yeast-like cell phase in those species. Nonetheless, the specific amino acid permease might be involved in transporting those degraded compounds by nitroreductase or signaling the presence of particular compounds. The complementary action of these genes in the process of induction of yeast-like cells in a solid medium requires further study.

We found that growth of *M. rileyi* yeast-like cells might be mediated by other membrane proteins in addition to permeases, such as Pa11 or the metalloproteases peptidase M43. Pa11 is associated with maintaining cylindrical cellular morphology and is known to be involved in cellular morphogenesis and cell wall integrity in fission yeast (Ge et al., 2005). Additionally, a recent study showed that one peptidase (M43) in *M. robertsii* is involved in sporulation, cell wall integrity and virulence (Zhou et al., 2018). In our study, the higher number of orthologous genes related to peptidase M43 in *M. rileyi* (n = 3) compared with only one gene found in three *Metarhizium* species, and the fact that we found that genes for two of these enzymes were highly upregulated in YS, increasing our knowledge about the specific role of these genes in yeast-like cell morphogenesis and virulence.

Another fact we wish to emphasize is the very high number of commonly up-regulated genes (42.64 %) in YS compared with YL and H that were associated with 'uncharacterized proteins'. Therefore, annotation of these genes should be addressed in future research to understand the formation of this fungal structure in a solid medium.

4.3. Signal transduction, heat shock proteins, and nutrient transporters genes associated with yeast-like cells

Using gene set enrichment analysis of GO terms we showed that in YL a set of genes related to cell growth, replication and signal transduction were upregulated. This indicates that an active metabolism is required by yeast-like cells during their growth phase. One aspect of such activated metabolism is the recognition of an external stimulus through a signal transduction process and translation into an internal trigger for a specific cellular response (Lengeler et al., 2000). Here, the up-regulated genes associated with signal transduction might be involved in transmitting signals to the cell, indicating that external conditions favour maintenance of basal metabolism and induction of cell replication. These candidate genes were grouped in enriched GO terms such as signal transduction (*GO:000716*) and internal signal transduction (*GO:0035556*). The involvement of signal transduction in cell development of *M. anisopliae* was shown by Fang et al., (2007). For *M. anisopliae,* gene disruption of a protein involved in signal transduction pathways (G protein, cag8) resulted in the formation of irregularly shaped yeast-like cells and, in some cases, lysis during growth in a liquid medium. Additionally, signal transduction processes regulate dimorphic growth in several fungi such as *Saccharomyces cerevisae*, *Aspergillus nidulans* and *Ustilago maydis* (Lengeler et al., 2000).

To support metabolism during fungal cell growth, we previously showed that liquid-cultivated yeast-like cells of *M. anisopliae* need to import nutrients from the outside environment (Iwanicki et al., 2020b), a process mediated by various types of membrane transporters (Busto and Wedlich-Söldner, 2019). Our results showed that YL yeast-like cells activated three types of nutrient transporters compared with H and YS. In YL the gene for an iron permease membrane protein that plays a role in the uptake of extracellular ferrous iron (Fe^{2+}) was much more highly expressed than in H. Notably, the protein family PF03239 is associated exclusively with the upregulated gene for iron permease (gene id: OAA35670), making it unique amongst all upregulated genes in YL. Cross-species comparisons confirmed that the orthologous gene for iron permease upregulation in *M. rileyi* YL was also upregulated more in *M. anisopliae* YL than in H (Table 1). Together these findings indicate that *M. rileyi* YL and H acquire iron by different mechanisms or that yeast-like cells have a greater need for this metal than hyphae, which correlate with the extensive growth of YL. Besides iron permease, other orthologous genes upregulated in *M. rileyi* and *M. anisopliae* YL is the collagen-like protein (gene: Mcl1), known to form a collagenous protective coat enables the fungus to evade insect immune responses and is associated with virulence (Wang and St Leger, 2006), and a heat-shock protein.

Heat shock proteins (Hsp) include a diverse range of proteins that have roles in various cellular responses to e.g. temperature increases, stress conditions, growth, and cell differentiation (Tiwari et al., 2015). We also found a higher response in YL than in H related to activation of heat shock proteins. This finding is supported by upregulation of three genes for heat shock protein 20 (pfam: *PF00011*) found in YL. In fungi, Hsp60 and small Hsp (e.g. Hsp20) control osmotic stress (Tiwari et al., 2015). The fact that we found more Hsp60 and Hsp20 up-regulated in YL than in YS and any up-regulated Hsp in YS compared with H may indicate that osmotic stress is involved in YL formation and maintenance but not in YS. It has been suggested that osmotic stress prompts blastospore production in *B. bassiana* (Mascarin et al., 2015; Mascarin et al., 2021) and *M. robertsii* (Iwanicki et al., 2018). Also, *B. bassiana* produced significantly more blastospores when cultivated in hyperosmotic conditions than in normal conditions. However, further studies are needed to identify genes related to major metabolic pathways involved in the osmotic stress response, such as the well-known high-osmolarity glycerol (HOG) pathway (Hohmann, 2002). The plasma membrane first recognizes stressful conditions, such as osmotic and oxidative stress, as it is the first barrier between a cell and its environment. Therefore, we expected to find different gene expression profiles related to membrane proteins in YL, H and YS. However, in the present study, we found few differences in the number of upregulated genes related to the cell wall and membrane components between YL and YS and between YL and H.

Our results showed that yeast-like cells produced on solid (YS) and in liquid (YL) medium are distinct in *M. rileyi* and activate different genes. We showed that in YS almost twice (n = 417) as many genes were upregulated as in YL (n = 186) compared with hyphae. Morphologically, yeast-like cells formed in a liquid medium were similar in size and shape to those formed on solid medium. Although we found more septate cells produced on solid medium, these characteristics may be a result of the physical condition of the culture, i.e. in the liquid medium cells separate quickly following binary fission but remain attached or nearby on the static solid medium.

In conclusion, the results presented here provide insights that contribute toward a comprehensive understanding of gene expression patterns and virulence in *M. rileyi* yeast-like cells. Additionally, our study lays the foundation for further exploration of the metabolism necessary for yeast-like cell growth in liquid media and identified candidate genes that will serve as a basis for future research on optimizing *M. rileyi* yeast-like cell production.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2022.103766.

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