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How temperature modulates the expression of pathogenesis-related molecules of the cross-kingdom pathogen *Lasiodiplodia hormozganensis*

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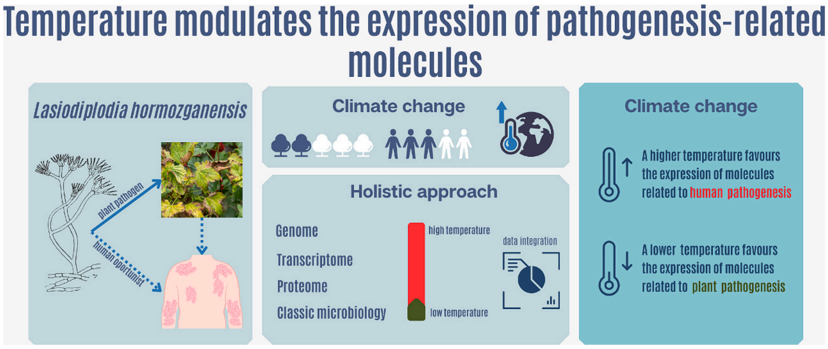
HIGHLIGHTS

- *Lasiodiplodia hormorzganensis* is able to infect both plants and humans.
- The cross-kingdom behavior is modulated by environmental temperature.
- Increased temperature shifts transcripts and expressed proteins towards human pathogenesis.
- Increasing environmental temperature may favor evolution towards human infection.

GRAPHICAL ABSTRACT



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ABSTRACT

Lasiodiplodia hormozganensis, initially recognized as a fungal plant pathogen, is recognized now acknowledged as a potential threat to humans. However, our understanding of the pathogenesis mechanisms of *Lasiodiplodia* species remains limited, and the impact of temperature on its pathogenicity is unclear. This study aims to elucidate the effects of temperature on the biology of *L. hormozganensis*, focusing on the expression of pathogenesis-related molecules and its ability to function as a cross-kingdom pathogen.

We conducted experiments at two different temperatures, 25 and 37 °C, analyzing the proteome and transcriptome of *L. hormozganensis*. Using strain CBS339.90, initially identified as *L. theobromae* but confirmed through ITS and *tef1-α* sequence analysis to be *L. hormozganensis*, we aimed to understand the fungus's protein expression under varying temperature conditions.

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Results from the functional analysis of the secretome at 25 °C showed a noteworthy presence of proteins related to carbohydrate metabolism, catabolism, plant cell wall degradation, and pathogenesis. However, when grown at 37 °C, the fungus exhibited an increased production of stress response and pathogenesis-related proteins. Our findings identified various pathways crucial for pathogenesis in both plants and humans, suggesting that *L. hormozganensis* possesses the genetic foundation to infect both hosts. Specific pathogenesis-related proteins, including the phytotoxin snodprot1, aspartic protease aspergillopepsin, and virulence protein SSD1, were also identified.

Concluding, we propose a possible mechanism of how *L. hormozganensis* adapts to different temperatures. The shift in temperature results in the expression of genes that favor human related pathogenesis molecules.

1. Introduction

Some microorganisms can infect members of different biological kingdoms (van Baarlen et al., 2007). The number of pathogens able to cross the barriers between kingdoms is limited and the rules of these mechanisms are still largely unknown (Sharma et al., 2014). The events that lead a pathogen to evolve and infect a species from a different kingdom are limited and include moving to a new habitat, (new) host stress, or exposure of the host by unusual vias. Some fungal plant pathogens have been reported as being able to infect humans (*e.g.*, *Alternaria alternata*, *Aspergillus flavus*, *Fusarium oxysporum*, *Microascus cinereus* and *Rhizopus arrhizus*) (Gauthier and Keller, 2013).

To cause an infection in the human body, a plant pathogenic fungus must overcome structural barriers (*e.g.*, skin) but also be able to grow under conditions different from their natural environment: elevated temperature, slightly alkaline pH of 7.4, and, of course, evade the human immune system (Gauthier and Keller, 2013). Human temperature is thought as being one of the main barriers for the germination of conidia and survival of fungal hyphae, but fungi like *A. flavus* are known for not only being able to germinate as to enhance germination rates at 37 °C (Araujo and Rodrigues, 2004). *Aspergillus fumigatus* alters its gene expression when grown at 37 °C of genes mainly involved in translation, amino acid, carbohydrate, lipid, and energy metabolism and heat shock proteins (Do et al., 2009). But further than adapting to elevated temperature, these cross-kingdom fungi need to overcome the 'new' host defenses. The velvet complex, MAPK, cAMP-PKA and G-protein signaling pathways are universally conserved signaling pathways important in the pathogenesis of both plants and humans (Kozubowski et al., 2009; Li et al., 2012; López-Berges et al., 2013; Montoya et al., 2008).. Besides the many enzymes that these pathogens can use to enter and feed themselves from human bodies, from proteases to lipases, the capacity to assimilate iron also contributes to pathogenesis.

Increased environmental temperature, due to climate changes are changing the plant-microbiome interactions (Trivedi et al., 2022) in their dynamics between pathogens and their hosts (leading to modifications in pathogen virulence) and will surely be a trigger for the infection of new hosts (Félix et al., 2016; Lindner et al., 2010).

The family *Botryosphaeriaceae* is widely distributed worldwide and comprises a wide range of endophytes and latent pathogens of mostly woody plants (Phillips et al., 2013). Species of the genus *Lasiodiplodia* are typically found in tropical and subtropical regions where they cause a vast number of diseases on a variety of plant hosts (Dou et al., 2017; Prasher, 2017; Rodríguez-Gálvez et al., 2015). As plant pathogens, *Lasiodiplodia* species cause branch dieback, stem canker, seed and fruit decay, gum exudation, neck rot, and foliage yellowing, leading to the plant death in several cases (Coutinho et al., 2017; Netto et al., 2014; Lima et al., 2013).

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., the type species of the genus, predominates in tropical regions (Phillips et al., 2013) and is a major pathogen of several plant species, especially crops (Rodríguez-Gálvez et al., 2015; Coutinho et al., 2017; Netto et al., 2014; Lima et al., 2013), counting with >500 hosts (Phillips et al., 2013). But, over the past decades, *L. theobromae* has been occasionally reported as a human opportunist pathogen causing rhino sinusitis, ocular and skin infections with different levels of severity (Kindo et al., 2010; Maurya et al., 2023; Saha et al., 2012a; Saha et al., 2012b; Summerbell et al., 2004) and one death was also reported (Woo et al., 2008).

Previous studies showed that *L. theobromae,* a closely related species of *L. hormozganensis*, has proteomic and metabolomic profiles that are temperature-dependent (Félix et al., 2016; Félix et al., 2019a; Félix et al., 2019b). Therefore, we aim to understand the effect of temperature at the cellular/molecular level. For that, we used a multi-omics approach combining genomics, transcriptomics, and proteomics.

2. Material and methods

2.1. Fungal strain and culture conditions

Strain CBS339.90 (Westerdijk Fungal Biodiversity Institute), isolated from a phaeohyphomycotic cyst of a patient from Jamaica and previously identified as *L. theobromae* (Alves et al., *2008)***,** was used in this study. The identification of the strain was confirmed through a phylogenetic analysis of concatenated sequences of the internal transcribed spacer region (ITS) of the rRNA gene cluster and translation elongation factor 1-alpha gene (*tef1-α*). Briefly, sequences from CBS339.90 were aligned with sequences from several *Lasiodiplodia* species retrieved from GenBank using ClustalX v. 1.83 (Thompson et al., 1997) and a Maximum Likelihood (ML) analysis was done using MEGA7 (Kumar et al., 2016). The best fitting DNA evolution model was determined with MEGA7 and selected based on the Akaike Information Criterium. ML analysis was performed on a Neighbor-Joining starting tree and the Nearest-Neighbor-Interchange was used as the heuristic method for tree inference. The robustness of the tree was assessed using 1000 bootstrap replicates.

The culture was maintained on Potato Dextrose Agar (PDA) medium (Merck, Germany) at 25 °C. The mycelium of a culture grown on Potato Dextrose Broth (PDB) at 25 °C for 3 days was ground in liquid nitrogen and DNA was extracted according to Möller and co-workers (Moller et al., 1992). For protein and RNA extraction, two plugs (5 mm diameter each) from a culture with 4 days were inoculated into a 250 mL flask containing 50 mL of PDB and incubated at 25 °C and 37 °C for 4 days. All assays were performed in triplicate. Culture supernatants were collected by gravitational filtration through filter paper and stored at −80 °C for extracellular protein extraction. Mycelia were washed with distilled water and kept at −80 °C for cellular protein extraction. For RNA extraction, the supernatants were discarded, and the fresh mycelia were ground.

2.2. Genome sequencing and assembly

The genome sequence of *L. hormozganensis* CBS339.90 was obtained using Illumina paired-end sequencing technology by NXTGNT (Ghent University, Ghent, Belgium), as reported previously (Félix et al., 2019a). First, paired reads from sequencing were filtered by quality, then trimmed based on quality scores (modified Mott trimming algorithm, threshold = 0.05) and reads shorter than 100 nt were discarded. Detected adaptor sequences were also trimmed and reads mapping to the Illumina internal control phage phiX were discarded. Assembly and scaffolding were achieved with CLC Genomics Workbench 9.0.1, *de novo* assembly module with default settings, except for the minimum contig length (2000 nt) or word length (60 nt). The optimal word size was estimated using KmerGenie28 before the assembly. The primary scaffolds were further refined using the SSPACE2 scaffolder. The gapped regions in the re-scaffolded assembly were (partially) closed using GapFiller.

2.3. *Gene prediction and annotation*

Gene prediction and annotation were performed according to Félix et al (Félix et al., 2019a) using the softwares RepeatModeler v1.0.8, RepeatMasker v4.0.5, BRAKER1 v1 and HISAT2 v2.0.5.

Gene product names were assigned based on predicted protein sequences with BLASTP against the UniProt-KB/SwissProt database, with an *E*-value threshold of $1^{e\text{-}3}$. Other functional annotations were added with InterProScan v5.21–60 (Mulder and Apweiler, 2007), as well as GO terms, which were further annotated with Blast2GO (pipeline v2.5.0). Further functional analyses were carried out as described (Morales-Cruz et al., 2015).

2.4. *Genome functional analyses*

Different functional analyses were performed, specifically secreted proteins, enzymes, carbohydrate-degrading enzymes, fungal peroxidases, synthetic gene clusters, transporters, cytochrome P450 and pathogen-host interactions. All analyses were carried out as previously described by Félix et al. (Félix et al., 2019a). Effectors were predicted using the default algorithm of EffectorP 3.0 (Sperschneider and Dodds, 2022) (https://effectorp.csiro.au/).

2.5. *RNA extraction, library preparation, and sequencing*

The mycelium of each replicate (three replicates per condition) was ground in liquid nitrogen and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma), according to the manufacturer's instructions. Samples were incubated for 15 min with the DNase I digestion set (RNase-Free DNase Set, Qiagen). Integrity and quality analysis were carried on a 2100 Bioanalyzer RNA (Agilent Technologies). After RNA isolation and quality assessment, samples were stored at −80 °C until sequencing library preparation. Illumina mRNA sequencing libraries were made from 500 ng total RNA of each sample using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturer's protocol. Fourteen PCR cycles were used during the enrichment PCR. The size distribution, purity (absence of free adaptors) and quantity of the resulting libraries were measured using a High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, US). The libraries were equimolarly pooled and sequenced in an Illumina Nextseq 500 high throughput flow cell, generating single-end 75 bp reads. After sequencing, the data was demultiplexed using the sample specific nucleotide barcodes. Per sample, on average $38.3 \times 10^6 \pm 2.9 \times 10^6$ reads were generated. First, these reads were trimmed using Cutadapt v1.11 to remove the "QuantSEQ FWD" adaptor sequence. The trimmed reads were mapped to the genome of CBS339.90 using the STAR aligning software v2.5.3a (Dobin et al., 2013). The RSEM software, v1.2.31, was used to generate count tables. Only genes with a counts per million (cpm) above 1 in at least 3 samples were retained. EdgeR (Robinson et al., 2010) was used to normalize gene counts and identify the differentially expressed genes (DEGs) using the empirical Bayes quasi-likelihood F-test, adjusted using the Benjamin-Hochberg False Discovery Rate (FDR) correction to account for multiple comparisons.

2.6. *Extracellular and cellular protein extraction*

For the extraction of both cellular and extracellular proteins, the methods used were based on the work of Félix et al. (Félix et al., 2019a) Briefly, the extracellular protein extraction was based on the TCA/Acetone method using one volume of cold TCA/Acetone [20 %/80 % (*w/v*)] with 0.14 % (w/v) DTT to add to the supernatant. Proteins were precipitated by centrifugation (15,000 *g*, 20 min, 4 °C) and the pellet washed with cold acetone (twice) and with 10 mL of cold 80 % acetone (*v*/v). Protein pellet was resuspended in 200 μL of a Tris-based buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris-base) and stored at −80 °C. For the intracellular fraction of the proteome, mycelia were grinded to a fine powder in a mortar under liquid nitrogen. A 10 mM potassium-phosphate buffer (pH 7.4) containing 0.07 % DTT and cOmplete™ protease inhibitor cocktail (Roche) was added to the ground tissue and all the samples were sonicated [1 min sonication, 2 min pause (3 min of sonication in total)] (Branson, Sonifier 250). The supernatant was then submitted to the same procedure described above for extracellular protein extraction.

2.7. *Protein quantification and separation*

Protein concentration was determined with the 2-D Quant Kit (GE Healthcare, USA), according to the manufacturer's instructions. Each biological independent replicates was quantified in triplicate. Proteins were separated by electrophoresis as described (Félix et al., 2019a): proteins were electrophoresed and concentrated at the top of the separation gel (visualized as a unique band). This band was manually excised and destained in 200 mM ammonium bicarbonate (AB)/50 % acetonitrile for 15 min followed by 5 min in 100 % acetonitrile.

2.8. *Tryptic digestion, mass spectrometry analysis, and protein identification*

Proteins were reduced by 20 mM dithiothreitol in 25 mM AB and incubated for 20 min at 55 °C. The mixture was cooled to room temperature, followed by alkylation of free thiols by of 40 mM iodoacetamide in 25 mM AB in the dark, for 20 min. After that, protein bands were washed twice in 25 mM AB. Proteolytic digestion was performed adding trypsin (Promega, Madison, WI), 12.5 $\text{ng.}\mu\text{L}^{-1}$ of enzyme in 25 mM AB, and incubated at 37 °C overnight. Protein digestion was stopped by the addition of trifluoroacetic acid (TFA, 1 % final concentration). Digested samples were dried in a speedvac. A nano LC analysis was performed in a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) with a C18 75 μm × 50 Acclaim Pepmam column (Thermo Scientific). Previously, peptide mixture was loaded in a 300 μm × 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in 2 % acetonitrile/0.05 % TFA for 5 min at 5 $\mu\text{L.min}^{-1}$. Peptide separation was performed at 40 °C for all runs. Mobile phase A was composed of water acidified with 0.1 % (*v/v*) formic acid. Mobile phase B was composed of 20 % (v/v) acetonitrile acidified with 0.1 % formic acid. Samples were separated at 300 nL.min^{1}. Mobile phase B increased to 4–45 % B for 60 min; 45–90 % B for 1 min, followed by a 5 min wash at 90 % B and a 15 min re-equilibration at 4 % B. The total time of chromatography was 85 min.

Eluting peptide cations were converted to gas-phase ions by nano electrospray ionization and analyzed in a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific). Mass spectrometer was operated in positive mode. Survey scans of peptide precursors from 400 to 1500 *m/z* were performed at 120 K resolution (at 200 m/z) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 1 Th with the quadrupole, CID fragmentation with normalized collision energy of 35, and rapid scan MS analysis in the ion trap. The AGC ion count target was set to 102 and the max injection time was 75 ms. Only those precursors with charge state 2–6 were sampled for MS2. The dynamic exclusion duration was set to 15 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles, meaning the instrument would continuously perform MS2 events until the list of non-excluded precursors diminishes to zero or 3 s, whichever is shorter.

The raw data was processed using Proteome Discoverer v 2.1.0.81 (Thermo Scientific). MS2 spectra were searched with SEQUEST engine against an in-house database of proteins deduced from the genomic sequence. Peptides were generated from tryptic digestion with up to one missed cleavage, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines as variable modifications. Precursor mass tolerance was 10 ppm and product ions were searched at 0 Da tolerance. Peptide spectral matches (PSM) were validated using percolator based on q-values at a 1 % false discovery rate (FDR). With Proteome Discoverer software v2.1 (Thermo Scientific), peptide identifications were affiliated into proteins according to the law of parsimony and filtered to 1 % FDR. The identified proteins were also filtered and considered for analysis only if present in 3 replicates and using at least 3 peptides for identification (Tables S1, S2, S3 and S4). The expression level was obtained considering the temperature of 25 °C as control ($0.5 \geq FC \geq 2$).

2.9. Siderophores' production

Siderophores' production was tested according to Pérez-Miranda et al. (Pérez-Miranda et al., 2007) with slight modifications. Strain CBS339.90 was grown in PDA medium for 24 h at 25 °C and 37 °C. After this period the plates were overlaid with CAS medium (Chrome azurol S) and incubated at 25 °C and 37 °C, respectively, for 4 h. Production of siderophores was detected as a change of color of the overlay from blue to orange, purple, or light yellow, allowing differentiating the type of siderophore production, hydroxamate, catechol or carboxylate, respectively.

2.10. Data availability

This whole-genome shotgun project has been deposited in the Genome (NCBI) database under accession number ASM3051232v1 (PRJNA470903 for BioProject and SAMN09112320 for BioSample).

3. Results

3.1. Fungal strain identity

Phylogenetic analysis of concatenated ITS and *tef1-α* nucleotide sequences showed that strain CBS339.90, originally identified as *L. theobromae* (Alves et al., *2008*)**,** does belong to the closely related but distinct species *L. hormozganensis*. As depicted in Fig. 1, strain CBS339.90 groups in the same clade as strain CBS124709 the ex-type strain of *L. hormozganensis*.

3.2. Genome sequencing and assembly

The genome of *L. hormozganensis* CBS339.90 was sequenced into 36.7 million matched paired-end reads by Illumina sequencing. Assembly led to a genome size of 43.2 Mb with approximately 222× coverage (Table 1). The genome sequence was assembled into 356 scaffolds with a minimum size of approximately 2.2 Mb, a total of 86 gaps and 6015 N's (undetermined nucleotides), as well as a G + C content of 54.58 % (Table 1). The N50 value was approximately 291 kb. RepeatMasker analysis, coupled with RepeatModeler and RepeatProteinMask, discovered an overall repeat content of 2.26 % of the genome. Gene prediction with BRAKER identified 12,652 genes, with an average length of 1606 bp, thus accounting for a gene density of 293 genes per Mb and a total of 47.0 % of the genome covered by protein-coding genes. There was a mean relation of 1 mRNA per gene, with an average of 2.9 exons and 1.9 introns per mRNA, and thus per gene, similar to what was found by Félix et al. (Félix et al., 2019a) and Yan et al. (Yan et al., 2018) for *L. theobromae*.

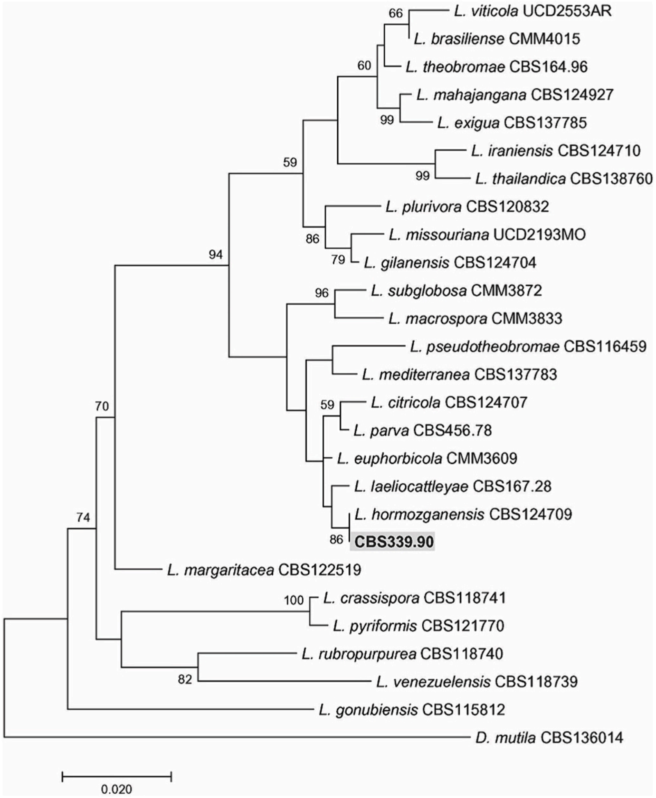

Fig. 1. Phylogenetic relationship of strain CBS339.90 and ex-type strains of several *Lasiodiplodia* species inferred using the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap values (> 50 %) are shown at the nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Diplodia mutila* was used as outgroup to root the tree.

Table 1
General features of the *Lasiodiplodia hormozganensis* CBS339.90 genome assembly and gene prediction.

L. hormozganensis CBS339.90	
Size (Mb)	43.2
Coverage	222×
% G + C content	54.58
% Repeats	2.26
Number of genes	12652
Average gene length (bp)	1606
% Genes	47.0
Gene density (genes/Mb)	293
mean exons per mRNA	2.9
Average exon length (bp)	507
mean introns per mRNA	1.9
Average intron length (bp)	86
mean mRNAs per gene	1

3.3. Genome functional analysis

The analysis of *L. hormozganensis* genome was focused mainly on those genes/functions related to virulence and pathogenesis in plants and animals. We investigated genes coding for CAZymes, transporters, heat shock proteins, secondary gene clusters for effectors, and other pathogenicity-related proteins.

3.4. Enzymes, P450 and CAZymes

The genome of *L. hormozganensis* contains 4548 genes annotated as coding for enzymes with EC numbers by the Ensemble Enzyme Prediction Pipeline (E2P2 v3.0, Table 2). The most represented enzymes were

Table 2
Genes predicted to encode enzymes, by E2P2 v3.0 tool, in the genome of *L. hormozganensis* CBS339.90.

Enzyme type	Total (n)	Secreted (n)
Hydrolases	1515	247
Oxidoreductases	1282	44
Transferases	1002	7
Lyases	282	30
Ligases	194	0
Isomerases	120	8
Total genes	4548	335

oxidoreductases, transferases, and hydrolases. It should be noticed that some genes were annotated with more than one function, while others were annotated as enzymes by E2P2 but with no EC numbers assigned. From these, 335 genes are predicted to code for secreted enzymes (approximately 50 % of the predicted secreted proteins). The majority of these were described as hydrolases.

Lasiodiplodia hormozganensis genome encodes for 788 CAZymes and carbohydrate-binding modules (CBM, Table 3). The largest number were annotated as glycoside hydrolases, accounting for 316 genes. From these, 271 genes that code for secreted proteins were annotated as extracellular CAZymes. Most of these were glycoside hydrolases (128), while no glycoside transferases were identified.

One of the main functions of peroxidases in plant pathogenic fungi is related to the degradation of the plant cell wall lignin, contributing to the penetration of the pathogen into the plantaury (Kellner et al., 2014). The genome of strain CBS339.90 includes 41 genes annotated as coding for fungal peroxidases, with a total of 69 functions (Table S5). Several of these genes (13) were annotated with two or more peroxidase functions.

The function of cytochrome P450 enzymes in pathogenic fungi is usually related to fungal defense against toxic substances produced by the hosts, synthesizing metabolites such as mycotoxins, as aflatoxins, and gibberellins (Cresnar and Petric, 1814). A total of 283 genes were annotated as coding for fungal cytochrome P450, within 92 different P450 families (Table S6).

3.5. *Transporters and heat shock proteins*

Several studies suggest that transporters (membrane transport proteins) play a crucial role in the pathogenicity of fungi, contributing to different functions, such as the export of drugs from the cell (Yan et al., 2018) or the transport of molecules involved in appressoria formation (Hamel et al., 2012). A total of 1944 genes were annotated in the genome of strain CBS339.90 as coding for transporters (Table 4).

Different families of Heat Shock Proteins (HSP) known to be involved in responses to heat stress were identified in the genome of *L. hormozganensis* (Table 5).

3.6. *Secondary gene clusters*

A total of 52 secondary metabolite gene clusters were identified: 9 terpene, 16 t1pks (type 1 polyketide synthases), 11 nrps (nonribosomal peptide-synthetase), 5 t1pks-nrps and 11 “other”. From these clusters, 7 were found to have known homology to other described gene clusters (from the internal antiSMASH database, Table 6).

Table 3
Genes predicted to code for CAZymes in the genome of *L. hormozganensis* CBS339.90. CAZymes were predicted with the web-based application dbCAN (HMMs 5.0).

CAZyme type	Total	Secreted enzymes
Glycoside Hydrolases (GH)	316	128
Carbohydrate Esterases (CE)	150	38
Auxiliary Activities (AA)	135	52
Glycoside Transferases (GT)	96	0
Carbohydrate-Binding Modules (CBM)	66	33
Polysaccharide Lyases (PL)	25	20
Total	788	271

Table 4
Genes predicted to code for transporters in the genome of *L. hormozganensis* CBS339.90, using the Transporter Classification Database.

Type	Number
Electrochemical potential-driven transporters	766
Primary active transporters	340
Incompletely characterized transport systems	323
Channels and pores	312
Accessory factors involved in transport	128
Group translocators	56
Transport electron carriers	19
Total	1944

Table 5
Predicted heat shock proteins involved in responses to heat stress in the genome of *L. hormozganensis* CBS339.90.

Accession Code	HSP name	HSP Family
P40920	30 kDa heat shock protein	HSP 30
P31540	Heat shock protein hsp98	HSP clpA/clpB
P22943	12 kDa heat shock protein	HSP 20
Q96UX5	Heat shock protein 78, mitochondrial	HSP clpA/clpB
O14368	Heat shock protein 16	HSP 20

3.7. *Effectors and other pathogenicity-related proteins*

Apoplastic and cytoplasmic effectors were predicted using EffectorP v3.0 (Sperschneider and Dodds, 2022), developed for fungi and oomycete effectors prediction. We identified 157 extracellular apoplastic and 29 extracellular cytoplasmic potential effector proteins (Supplementary Table S7). These potential effectors include proteins from the Necrosis Inducing Protein family ($n = 13$), Hydrophobin Protein Family ($n = 5$) - related to plant-pathogenic fungi pathogenicity but with roles still unknown (Bayry et al., 2012), proteins from the Cerato-Ulmin Hydrophobin Family (n = 1), proteins from the Nis1-like Family (n = 1), Egh16-like Virulence Factor Family (n = 1), proteins from the Copper Acquisition Factor BIM1-like family ($n = 11$), known from facilitating copper acquisition in mammalian hosts (Garcia-Santamarina et al., 2020), proteins from the Cerato-Platanin Family ($n = 10$) and numerous carbohydrate degrading enzymes. We also predicted the existence of 6 proteins from the Diedel Protein Family, typical of insect immune response, and whose function in fungi is not known.

Additionally, 4021 genes were annotated as proteins described in PHI-base (the Pathogen Host Interactions Database), of which 1848 are known to be involved in pathogenicity processes (Table 7).

Table 6
Metabolic gene clusters identified in the genome of *L. hormozganensis* CBS339.90. NRPS - nonribosomal peptide-synthetase; T1PKS - type 1 polyketide synthases. Percentage corresponds to the percentage of genes that present similarity to known gene clusters.

Type	Most similar known cluster
Terpene	PR toxin (50 %)
NRPS	Aflatoxin (8 %)
NRPS	Hexadehydro-astechrome (HAS) (25 %)
T1PKS	Brefeldin (20 %)
T1PKS	Emericellin (50 %)
T1PKS	Lasiodiplodion (71 %)
T1PKS-NRPS	Fusaridone A (12 %)

Table 7
Genes predicted to have a role in the interaction pathogen-host in the genome of *L. hormozganensis* CBS339.90 and the respective method used for identification.

	Number (n)	Method/Software/Tool
Total proteins	12,658	BRAKER1
Secreted proteins	698	FunSec
Enzymes	4548	E2P2 v3.0
Secreted Enzymes	335	FunSec + E2P2
CAZymes	788	dbCAN
Secreted CAZymes	271	FunSec + dbCAN
Secondary metabolism gene clusters	52	fungiSMASH
Peroxidases	41	fPoxDB
Transporters	1944	Transporter Classification Database
Pathogen-Host Interactions	4021 (1848)[1]	PHI-base

[1] 1,848 genes were annotated with evidence of being involved in pathogenicity mechanisms.

3.8. *Transcriptome and proteome of Lasiodiplodia hormozganensis CBS339.90 at two temperatures*

Temperature modulated both the transcriptome (Fig. 2 and Fig. S1) and the proteome (Fig. 2 and Fig. S2) of *L. hormozganensis* CBS339.90. 1056 differential transcripts (Differentially Expressed Genes - DEGs) were identified: 602 up-regulated and 454 down-regulated at 37 °C (Fig. S1). From those, only the annotated DEGs (806 in total) were used in the functional analysis (Fig. 2).

Differentially expressed genes at 37 °C were characterized concerning their GO-Biological Process category (Table S8). Heat stress induced an increase of transcripts related to primary metabolism (PM) and pathogenesis (P). Heat stress also induced a decrease of transcripts (Fig. 2) related to amino acid metabolism and catabolism (AMC), stress response (SR), carbohydrate metabolism and catabolism (CMC), transport (T), and cell wall organization (CWO).

We identified 95 proteins in the secretome of *L. hormozganensis* grown at 25 °C and 59 in the secretome of *L. hormozganensis* grown at 37 °C (Fig. 3A, Fig. S2, and Table S9). From these, a total of 18 proteins were less abundant and 2 proteins more abundant (Fig. 3B, 0.5 ≥ FC ≥ 2), compared to 25 °C. Regarding the cellular proteome, 636 proteins were identified at 25 °C and 482 at 37 °C (Fig. 2 and Table S10). A total of 23 proteins were more abundant and 47 less abundant at 37 °C than at 25 °C (0.5 ≥ FC ≥ 2, Fig. 3B).

As expected, a higher number of proteins was identified on the cellular proteome when compared with the secretome, especially at 25 °C (Fig. 3).

Functional analysis of extracellular proteins (GO-Biological Process, Fig. 2B), show that at 25 °C, *L. hormozganensis* mainly expresses proteins related to CMC, possibly related both to nutrition and pathogenesis, pathogenesis and, plant cell wall degradation (PCWD). At 37 °C, there is

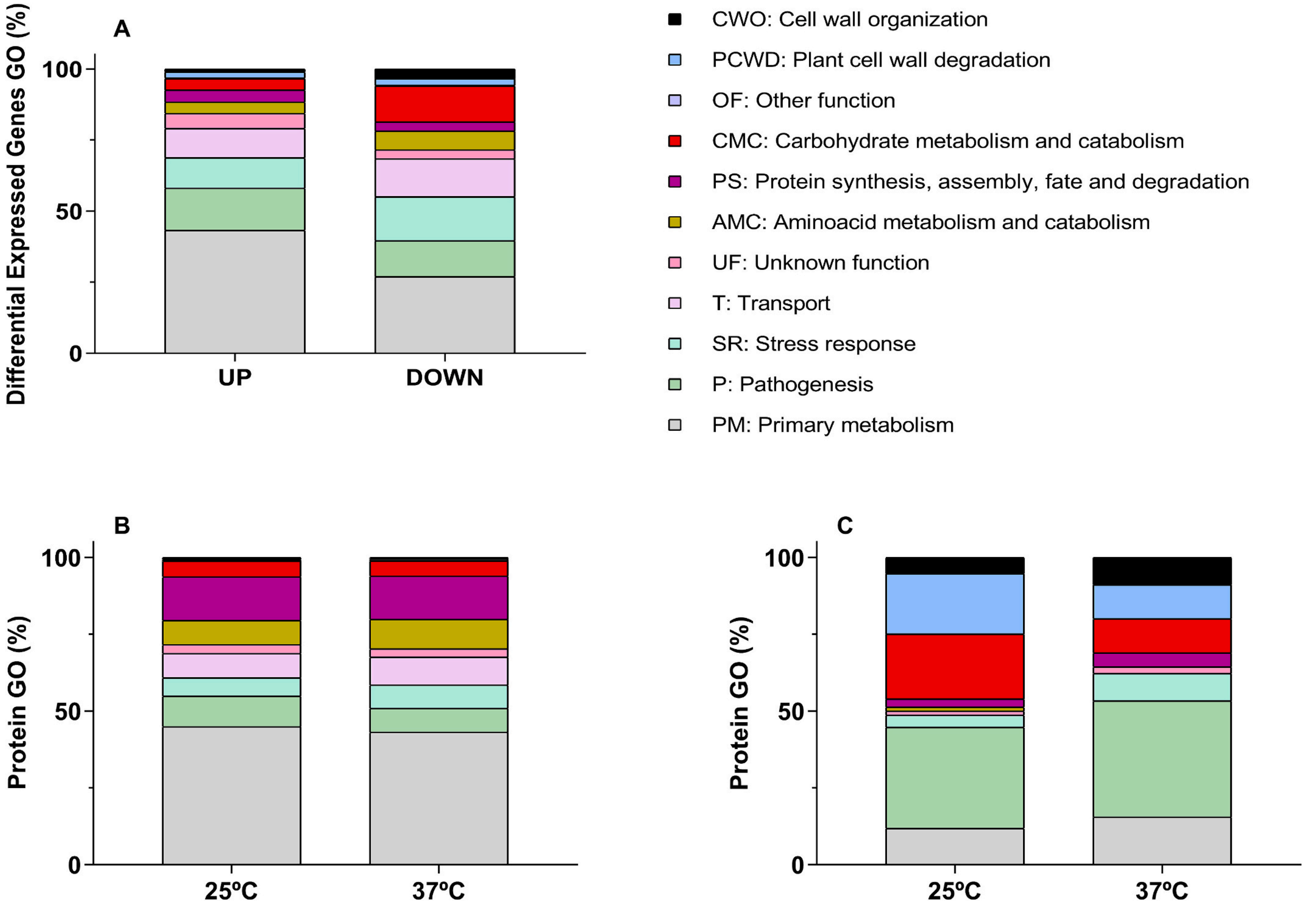

Fig. 2. Gene Ontology classification of the transcripts identified in strain CBS339.90. A-Percentage of distinct species present in up and down-regulated transcripts in comparison with the fungus grown at 25 °C. B- Gene Ontology (Biological Processes) classification of the extracellular proteins identified in *L. hormozganensis* CBS339.90. Percentage of distinct species present in extracellular proteins at 25 °C and 37 °C. C- Gene Ontology classification of the cellular proteins identified in CBS339.90. Percentage of distinct species present in extracellular proteins at 25 °C and 37 °C. GO classifications were obtained from biological process of each gene product according to the UniProt database (http://www.uniprot.org/).

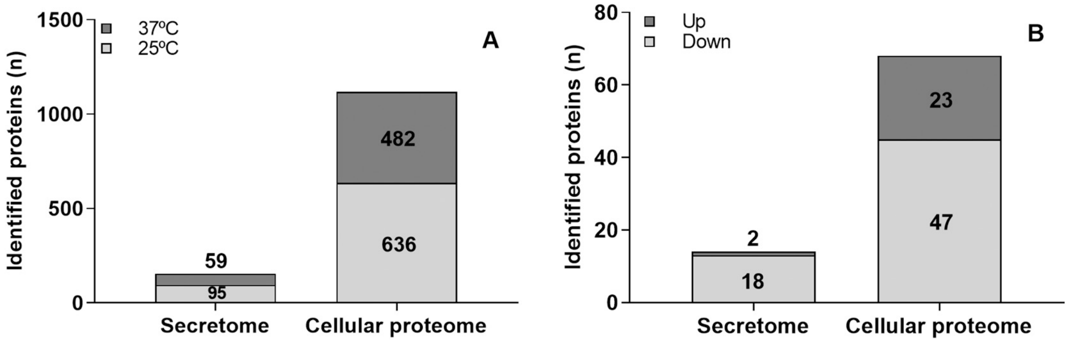

Fig. 3. Proteins identified in the secretome and in cellular proteome of L. *hormozganensis* CBS339.90 (Tables S7 and S8). A- Number of identified proteins at 25 and 37 °C; B- Difference in the number of proteins at 37 °C, in comparison to 25 °C.

a decrease of proteins related with CMC and PCWD, as well as an increase in the stress response (SR) and the pathogenesis-related proteins.

On the other hand, the cellular proteomes of CBS339.90 grown at 25 °C and at 37 °C are very similar with the most represented cellular proteins being related to primary metabolism (PM) and protein synthesis, assembly, fate, and degradation (PS, Fig. 2).

3.9. Siderophores production

Lasiodiplodia hormozganensis produces siderophores at 25 °C and 37 °C (Fig. 4): at 25 °C siderophores of the type hydroxamate are produced and, at 37 °C both of the hydroxamate and the carboxylate types (Pérez-Miranda et al., 2007) were detected (Fig. 5).

The genome of *L. hormozganensis* CBS330.90 encodes siderophore iron transporters: as *MirA* (KAK0621063.1), *MirB* (KAK0609267.1) and *MirC* (KAK0654078.1).

4. Discussion

Lasiodiplodia hormozganensis was identified for the first time in 2010 in Iran (Abdollahzadeh et al., 2010) and later in Australia (Sakalidis et al., 2011), Brazil (Marques et al., 2013) and Oman (UAE) (Al-Sadi et al., 2014; Al-Sadi et al., 2013) as a plant pathogen. CBS339.90 was isolated from a phaeohyphomycotic cyst of a patient from Jamaica (Summerbell et al., 2004) but was incorrectly assigned to the close related species *L. theobromae* (Alves et al., *2008)*. Therefore, the assignment of CBS339.90 to *L. hormozganensis*, makes this the first time that *L. hormozganensis* is reported as a cross-kingdom pathogen (able to infect both plants and humans).

We identified several groups of genes relevant to the pathogenesis to plants and animals in the genome of *L. hormozganensis*. Gene clusters involved in toxin synthesis, and genes encoding hydrolases (1515 genes), glycoside-hydrolases (316 genes), peroxidases (41 genes), transporters (1944 genes), peroxidases (69), and P450 enzymes (283) were identified.

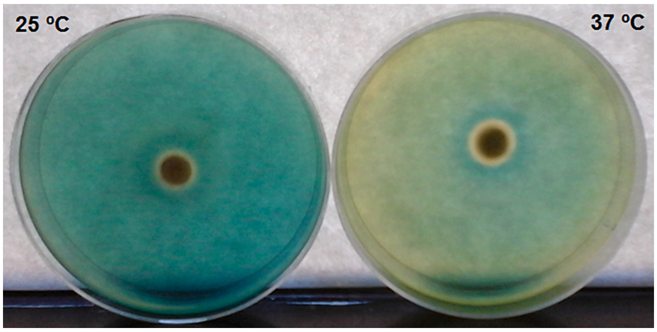

Fig. 4. Siderophores' production by strain CBS339.90 grown at 25 °C and 37 °C using O-CAS assay.

P450 enzymes are known for being involved in numerous processes in fungi from primary and secondary metabolism to toxins synthesis, and detoxification. *Lasiodiplodia hormozganensis* genome encodes enzymes from 92 families. The family with the higher number of genes is family CYP504 (44 genes). The enzymes from this family are known to be involved in the degradation of aromatic exogenous compounds (Cresnar and Petric, 1814). Interestingly, the second most populated P450 family, with 20 genes is family CYP65 whose enzymes are involved in the synthesis of the mycotoxins trichothecenes. *Lasiodiplodia hormozganensis* also encodes P450 enzymes involved in the synthesis of the mycotoxin fumonisins (CYP505, 10 genes) and enzymes involved in detoxification (CYP53) and denitrification processes (CYP55).

In plants, carbohydrate-active enzymes are known to participate in the breakdown, biosynthesis, and modification of glycoconjugates and oligo- and polysaccharides (Lyu et al., 2015). These enzymes are responsible for fungal nutrition, host cell wall degradation, and act as virulence factors in pathogen-host interactions (Chandrasekaran et al., 2016; Pour et al., 2022).

Comparing the genome of *L. hormozganensis* CBS339.90 with the most closely related available genomes of *L. theobromae (*Félix et al., *2019a;* Yan et al., *2018)***,** the main difference is related with the transporters identified. Although the total number of genes encoding for transporters in the genome of CBS339.90 is similar to the number found in the other genomes, their functions are different. *Lasiodiplodia hormozganensis* genome has more genes coding for channels and pores, translocators, and accessory factors, than what is described for *L. theobromae* genome. This suggests different requirements of the two species concerning the transport of molecules, which might be related to the specific isolation of CBS339.90 from a human.

The GO analysis of transcripts (Fig. 2A) and of cellular proteins (Fig. 2B) shows that most proteins are involved in primary metabolism (Ene et al., 2014). On the contrary, the secretome (Fig. 2C) of *L. hormozganensis* exhibits a high percentage of proteins involved in pathogenesis processes, which increases when the fungus grows at 37 °C. Interestingly, opposite results were found by Félix et al. (Félix et al., 2019a) for a strain of *L. theobromae* isolated from grapevine that did not express proteins directly related to pathogenesis at 37 °C.

Previously, we showed that the ability to cause toxicity to mammalian cells of *L. theobromae* (CAA019, isolated from a coconut tree) (Félix et al., 2016; Gonçalves et al., 2019) was restricted to the growth temperatures of 25 °C and 30 °C and limited in time (only for 96 h). On the contrary, *L. hormozganensis* CBS339.90 induced 90 % of cell mortality throughout the growth time investigated (Gonçalves et al., 2019). This agrees with transcriptomics and proteomics data showing a higher expression of proteins related to plant cell wall degradation at 25 °C and a higher expression of proteins related to pathogenesis at 37 °C. The optimal growth temperature of *L. hormozganensis (*Félix et al., *2016)* is 25 °C, so the stress induced by growing at 37 °C induced an up-

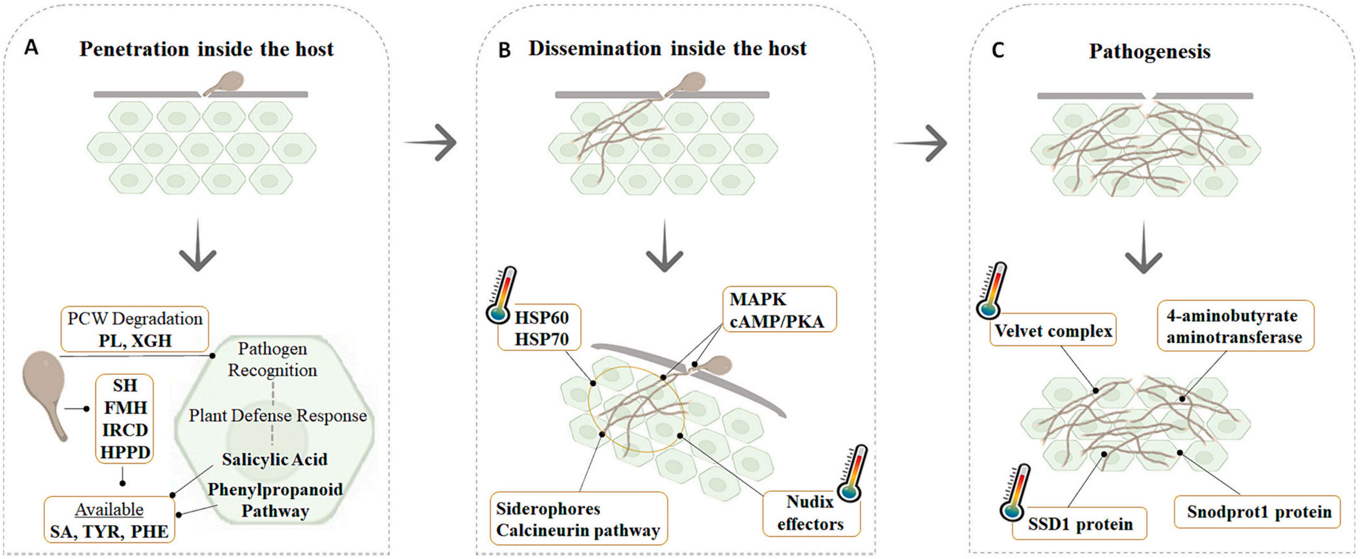

Fig. 5. Schematic representation of a process of infection of a filamentous fungus and its host and the respective molecules/pathways identified in this work related with each step of the process, showing the identified molecules that may be related not only with plant and human pathogenesis (highlighted with the thermometer symbol). A - Pathway of salicylic acid degradation by the fungus proposed by Paolinelli et al. (Félix et al., 2018) for *L. theobromae* and identified in *L. hormozganensis* CBS339.90. PCW: plant cell wall; PL: pectate lyase, XGH: xylosidase glycoside hydrolase; SH: salicylate hydroxylase; FMH: fumarylacetoacetate hydrolase; IRCD: intradiol-ring cleavage dioxygenase; HPPD: 4-hydroxyphenylpyruvate dioxygenase; SA: salicylic acid; Tyr: tyrosine; Phe: phenylalanine. B – Pathways and isolated molecules known to contribute to the dissemination of the fungal pathogen inside the host. HSP: Heat shock proteins; MAPK: mitogen activated protein kinases; cAMP/PKA: cyclic adenosine-$3',5'$-monophosphate/protein kinase A. C – Specific pathways/proteins directly involved in pathogenesis caused by fungi not only in plants but also in humans.

regulation of proteins related to the stress response. Data from *L. theobromae*, already suggested that increasing temperature (to 35 °C) led to larger lesions in grapevines infected with *L. theobromae* when compared with a lower temperature (Gonçalves et al., 2019; Urbez-Torres, 2011) (30 °C). Similarly, Yan et al. (Yan et al., 2018) tested the interaction between *L. theobromae* and grapevines, concluding that the larger lesions were also found for the highest temperature tested (35 °C) and not for the lower (25 °C) (Yan et al., 2018). The data obtained from both studies, which also included mammalian cells, suggest a molecular adaptation of *Lasiodiplodia* to higher temperatures and the trigger of specific molecules at each temperature. However, it should be highlighted that the *in vitro* testing (Yan et al., 2018; Urbez-Torres, 2011) excluded the mechanical barriers imposed by host penetration (since a mechanical opening was made to inoculate the fungus), which may be related to the higher percentage of proteins related to PCWD at 25 °C.

Nutrition/Dissemination inside the host.

A model based on transcriptomic data of *L. theobromae* suggests that, in the presence of host (grapevine), the fungus uses salicylic acid and phenylpropanoid pathway precursors as carbon sources (Paolinelli-Alfonso et al., 2016). In fact, a pathogen hitting the plant cell wall is the first impetus that triggers the phenylpropanoid pathway for plant defense (Yadav et al., 2020). We identified enzymes in the cellular proteome at 25 °C involved in the degradation of 4-hydroxylphenylpyruvate dioxygenase and fumarylacetoacetate hydrolase (degradation of tyrosine/phenylalanine) and salicylate hydroxylase (degradation of salicylic acid) (Fig. 7). Since our investigation did not involve the presence of plant material, data suggest that this mechanism is constitutively active even in the absence of the host.

For fungal pathogens, a rapid and fitting response of the fungus upon host tissue recognition is crucial (Choi et al., 2015). The first steps for a successful infection are the perception of the host conditions and the ability to adapt to the differences in nutrient availability, physical conditions (*e.g.* pH, oxygen, and temperature) and overcome the host defense responses (He et al., 2017) (Fig. 7). For this, several pathways involved in regulation processes are activated, particularly the mitogen-activated protein kinase (MAPK) pathways and the cyclic adenosine-$3',5'$-monophosphate (cAMP)/protein kinase A (PKA) pathway that play a key role in penetration and dissemination inside the host (Hamel et al., 2012; Choi et al., 2015; He et al., 2017). One of the responses caused by mitogen-activated protein kinase cascades is related to the penetration of the fungus inside the host, contributing to the formation of the appressorium (Hamel et al., 2012; He et al., 2017). Several proteins that belong to these pathways were identified in the transcriptome and proteome of *L. hormozganensis* at growth temperatures, as previously described for *L. theobromae (*Félix et al., *2019a)*.

After the successful penetration of the pathogen, it needs to overcome the stress originated by the new environment and by the defenses of the host. *Lasiodiplodia hormozganensis* expresses several transcripts and proteins related with Nudix effectors, specifically the *YSA1* gene, both at 25 °C and 37 °C. Another gene encoding for a Nudix hydrolase (*NUDT1*) was upregulated in the transcriptome at 37 °C and only detected as a protein at 37 °C in the cellular proteome. The relevance of the Nudix effectors being more abundant at 37 °C comes from recent data showing their contribution to the infection process across kingdoms (Dong and Wang, 2016). Nudix proteins and effectors have been reported in different plant pathogenic organisms, suggesting that these proteins might be relevant for a wide range of plant pathogens (Dong and Wang, 2016). Although the biological role of these effectors is poorly understood, they seem to manipulate the host defense systems. Nudix proteins are also relevant for animal and human pathogens. In *Cryptococcus neoformans*, a Nudix hydrolase gene, *YSA1*, contributes to the virulence of the fungus, modulating the oxidative stress response and susceptibility to drugs (Dong and Wang, 2016).

HSP60 and HSP70 were more abundant at 37 °C (present in the transcriptome, upregulated at the proteome level, Tables S8 and S11). While HSP60 up-regulation was expected, due to its role in the control of damages in proteins affected by the high temperature, HSP70 up-regulation comes with some surprise. HSP70 seems to facilitate human infection by yeasts (Brown et al., 2012; Tiwari et al., 2015**)**, and it has been described to be upregulated upon heat stress – due to its function in unfolding of denatured proteins, mediating thermotolerance in fungi. The information available relative to filamentous fungi is somewhat contradictory: in *A. fumigatus*, *A. terreus*, *C. cladosporioides* and *T. mentagrophytes* HSP70 facilitates human infection, but not in *P. chrysogenum* and *S. apiospermum (*Tiwari and Shankar, 2018*)*.

The nutrition of a pathogen inside a host is also of extreme importance. Without a proper up-take of nutrients, the development of the fungus is not possible. *Lasiodiplodia hormozganensis* expresses different transporters of iron (transcripts and proteins were identified by us) as well as different types of siderophores (Fig. 4). Iron is an essential nutrient required for almost microorganisms (Renshaw et al., 2002) and to up-take this element, microorganisms need to solubilize Fe (III), which is usually achieved by the production of siderophores. These iron-chelating ligands are an advantage to the producer, promoting the enhancement of its growth and limiting the available iron for other microorganisms (Renshaw et al., 2002).

Also, the entire pathway of calcineurin was identified in the transcriptome of *L. hormozganensis* at both temperatures. The calcineurin pathway consists of several transporters, channels, pumps, and other proteins/enzymes involved in the uptake of calcium. In fungal pathogens, the calcineurin pathway is used to survive and to effectively propagate within the host (either plants or humans), contributing to cell wall integrity, growth at elevated temperatures, alkaline pH, cation homeostasis, mating, azole resistance and morphogenesis (Chen et al., 2010; Liu et al., 2015).

The genome of *L. hormozganensis* CBS330.90 encodes the siderophore iron transporters: MirA, MirB and MirC. In *Aspergillus fumigatus*, *MirC* seems to be related with ferricrocin biosynthesis contributing to the maintenance of iron homeostasis (Mulvihill et al., 2017) rather than being involved in siderophore transport. In *L. hormozganensis*, this duality remains to be solved. The genome of *L. hormozganensis* also encodes L-Ornithine N(5)-Monooxygenase (*sidA*, KAK0662164). This enzyme is directly involved in the synthetic pathways of siderophores (Hissen et al., 2005)), whose synthesis depends on the hydroxylation of ornithine. CBS339.90 genome encodes several other proteins of the biosynthesis of extra- and intracellular siderophore pathways: *SidD* (the non-ribossomal peptide synthetase 4, gene *NRPS4_3*, KAK0615529), *SidC* (the non-ribossomal peptide synthetase 2, KAK0642682 and KAK0664701). *SidF* and *SidL* were not identified in the genome of *L. hormozganensis*, but the Putative Lysine N-Acyltransferase (KAK0642680) and N(6)-Hydroxylysine O-Acetyltransferase (KAK0645146) share, respectively, 63 and 57.5 % identity with *SidF* and *SidL* of *A. fumigatus*. In *A. fumigatus*, the biosynthesis of intracellular siderophores (ferricrocin and hydroferricrocin), involves the Transacylase sidF transfers anhydromevalonyl to N5-hydroxyornithine, which is acetylated by SidL to form ferrirocin. Assembly of the extracellular fusarinine C and of ferrirocin is catalyzed by two nonribosomal peptide synthetases, SidD and SidC, respectively (Schrettl et al., 2007).

MirC (Siderophore Iron Transporter mirC) was downregulated at 37 °C (Table S11), while *SidA* (L-ornithine N(5)-monooxygenase), *SidC* (Nonribosomal Peptide Synthetase 2), Lysine N-acyltransferase seem to be constitutively expressed regardless the temperature (Table S11). None of these proteins were identified by proteomics (Tables S2 and S4).

4.1. Pathogenesis

The Velvet complex, composed of several proteins, modulates chromatin accessibility and gene expression thus being involved in pathogenesis processes (López-Berges et al., 2013). *Lasiodiplodia hormozganensis* transcripts of three components of the Velvet complex at both temperatures tested were identified: *velB*, *velC* and *LaeA*. Although the component VeA was not identified by any of the approaches used in this study, we identified the homologous gene, *vel1*, in the genome of *L. hormozganensis*. This gene, *vel1*, is associated to the growth of the human opportunist *Aspergillus fumigatus* at 37 °C (Lind et al., 2016).

In *Fusarium oxysporum*, a plant pathogenic fungus with the ability to infect humans, the components VeA and LaeA are crucial for full virulence on a mammalian host. VeA and VelB components are in turn essential for early infection on tomato plants. Alone, LaeA acts during late infection stages, working in the colonization of the xylem vessels and consequently on vascular wilt symptoms (López-Berges et al., 2013).

Mycotoxins induce toxicity in the host, promoting damage in host tissues [8,71,72]. *Lasiodiplodia hormozganensis* expresses proteins related to toxin biosynthesis, namely aflatoxins. Although the similarity of the cluster gene associated with aflatoxins is not high, several up-regulated proteins participating in the synthesis of these toxins were identified in the transcriptome and proteome of *L. hormozganensis*.

Additional proteins implicated in pathogenesis, are the proteins involved in γ-aminobutyric acid degradation. In this work, a 4-aminobutyrate aminotransferase (P14010) was identified at 25 °C and 37 °C in the cellular proteome of the fungus. It has been suggested that the metabolization of γ-aminobutyric acid contributes to fulfilling pathogen nitrogen requirements during infection and manipulates the plant metabolism to maintain the necessary concentration of nitrogen for the survival of the fungus (Fernandes et al., 2014). In fact, GABA is one of the signal molecules inducing defenses and it is used in plants in priming (Guo et al., 2023). The expression of GABA degradation proteins is certainly an advantage for the pathogen.

SnodProt1 (O74238) is a phytotoxin, from the family of cerato-platanins, identified in the secretome of this CBS339.90. SnodProt1, from *Ceratocystis fimbriata*, can manipulate the immune response of plants inducing necrosis. A SnodProt1 homologue is required for full virulence in *Magnaporthe oryzae (*Brown et al., *2010)*.

The virulence protein SSD1 (Q5AK62) was identified in the cellular proteome of CBS339.90, but only at 37 °C. This protein is known to be crucial for the pathogenesis of *C. albicans* by promoting resistance to the host immune defense, facilitating the colonization of human tissue (Gank et al., 2008) (Fig. 7). In *L. theobromae*, these proteins were already identified in the same conditions (Félix et al., 2019a**)**, suggesting that *L. hormozganensis* has several mechanisms similar to other pathogens able to infect simultaneously plants and humans.

5. Conclusion

The main conclusion of this study is that not only *L. hormozganensis* CBS339.90 is rich in molecular mechanisms that allow it to infect both plants and humans, explaining its “cross-kingdom jump” behavior but that increasing culture temperature leads to higher expression of genes related to pathogenesis mechanisms of non-plant hosts.

The combination of different “omics” showed that environmental temperature modulates *L. hormozganensis* CBS339.90 molecular pathways, guaranteeing its survival and growth in stringent environments. At 25 °C, primary metabolism, metabolism of carbohydrates and plant cell wall degradation are favored. At 37 °C, there is an increase of proteins related with pathogenicity and stress response. Several transcripts and proteins related to pathogenesis in plants and in humans were identified: cerato-platanin SnodProt1, at 25 °C and 37 °C, known to contribute to the plant infection; and virulence proteins as SSD1 (expressed at 37 °C) known to participate in human infections. Also, several pathways known to contribute to pathogenesis were identified, as MAPKs, cAMP/PKA, calcium/calcineurin, or degradation of salicylic acid.

The increasing environmental temperature due to climate change is likely to induce an alteration on fungal behavior. In the case of *L. hormozganensis* CBC 339.90, heat stress induces molecular alterations (in the transcriptome and the proteome) that might favor human pathogenesis, which should be explored with close attention.

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CRediT authorship contribution statement

Carina Félix: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Rodrigo Meneses:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Micael F.M. Gonçalves:** Writing – review & editing, Investigation, Formal analysis. **Ana S. Duarte:** Writing – review & editing, Investigation, Formal analysis. **Jesus V. Jorrín-Novo:** Writing – review & editing, Supervision, Resources. **Yves van de Peer:** Writing – review & editing, Resources, Methodology. **Dieter Deforce:** Writing – review & editing, Resources. **Filip Van Nieuwerburgh:** Writing – review & editing, Resources. **Artur Alves:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Ana C. Esteves:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

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