Universidade de Aveiro Departamento de Biologia
2019

Vanessa Agostinho Ferreira

Unravelling *Lasiodiplodia theobromae* and *Lasiodiplodia hormozganensis* virulence via functional genomics and metabolites isolation

Estudo da virulência de *Lasiodiplodia theobromae* e *Lasiodiplodia hormozganensis* por genómica funcional e isolamento de metabolitos

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
2019

Vanessa Agostinho Ferreira

Unravelling *Lasiodiplodia theobromae* and *Lasiodiplodia hormozganensis* virulence via functional genomics and metabolites isolation

Estudo da virulência de *Lasiodiplodia theobromae* e *Lasiodiplodia hormozganensis* por genómica funcional e isolamento de metabolitos

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e coorientação do Doutor Jochen Fischer, Investigador Associado do “Institut für Biotechnologie und Wirkstoff-Forschung”.

Apoio financeiro do FEDER através do programa COMPETE e do POR Lisboa e da FCT através de fundos nacionais no âmbito do projeto ALIEN.

PTDC/AGR-PRO/2183/2014
POCI-01-0145-FEDER-016788
LISBOA-01-0145-FEDER-016788

To love, hope and belief, which guide us every day.

To science, for making us jump, fall, and try harder.

‘For the impossible might be possible with a change of perspective.’

o júri

presidente	Prof. Doutora Sónia Alexandra Leite Velho Mendo Barroso professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro
arguente	Prof. Doutora Isabel Alexandra Marcos Miranda investigadora da Faculdade de Medicina da Universidade do Porto
	Prof. Doutor Artur Jorge da Costa Peixoto Alves professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Ao Dr. Jochen Fischer, por toda a orientação, a paciência extensa todos os dias, a disponibilidade a um click de distância, e tudo o que me ensinou. Foi de facto uma experiência única e enriquecedora!

Ao Prof. Dr. Eckhard Thines, por me ter aceite e me ter dado a oportunidade de aprender e trabalhar no seu grupo, e pela amabilidade que sempre demonstrou.

Ao Prof. Dr. Artur Alves e à Profª. Dr ª Ana Sofia Duarte, pela oportunidade de desenvolver este trabalho, pela confiança e apoio mesmo à distância, por toda a ajuda, e pelo otimismo que sobrepunha sempre o meu terrível pessimismo.

A toda a equipa do IBWF, por me terem acolhido de braços tão abertos, pela ajuda e as experiências trocadas mesmo em falta de idiomas compatíveis. Particularmente, um obrigada especial à Szabina e Azahara, pelas gargalhadas, os momentos inesquecíveis, os conhecimentos transmitidos, e por me ensinarem que a vida pode ser radiante em qualquer circunstância.

A toda a equipa do Microlab, pela união e ajuda a qualquer nível e momento, juntos chegamos longe! Um obrigada especial à Carina por tudo em que me ajudou, e também à Tânia, Fred e Rafael - Micro for the win!

À minha mãe, porque sem ela nada seria possível. Obrigada pelo orgulho que me faz querer atingir algo mais. Obrigada por me inspirares.

À minha família, porque me dão força todos os dias. Obrigada à minha tata Paula, à Janete e família, à Jéssica, ao meu pai, à Marisa e à Carina, por fazerem a experiência da “Alemanha” o melhor que eu podia pedir. Um obrigada também ao meu padrinho e aos meus manos por tudo, sempre.

Às minhas powerpuffs (Cat e Sara), à Márcia eterna, aos meus amigos e família de Aveiro (são muito e muitos!), por não me deixarem ficar em baixo e me puxarem para além do limite. Obrigada por tornarem a minha vida mais brilhante!

Ao David, por tudo o que é, todo o carinho, o apoio incansável, e a inspiração que me dá. Sem ti, não voava. Obrigada por acreditares mais em mim do que eu mesma.

Para finalizar, quero deixar novamente um grande ‘OBRIGADA’ a todas estas peças que me ajudaram a completar este “puzzle”.

palavras-chave

Transformação mediada por *Agrobacterium tumefaciens*, fermentação, fungos oportunistas, infeções humanas, metabolitos secundários.

resumo

As alterações climáticas globais podem levar a interações de fungos patogénicos com novos hospedeiros, incluindo de reinos diferentes. Para que isto ocorra, os fungos necessitam de desenvolver mecanismos específicos de virulência, por exemplo por produção de fatores de virulência. Alguns fungos fitopatogénicos da família Botryosphaeriaceae são patogénicos oportunistas humanos. Além disso, o género *Lasiodiplodia* tem sido relatado em várias infeções humanas. Contudo, os mecanismos de virulência por detrás desta capacidade ainda carecem de investigação. O objetivo desta tese consistiu no estudo da virulência em humanos por *L. theobromae* - estirpe LA-SOL3 - (isolado da videira) e *L. hormozganensis* - estirpe CBS339.90 - (isolado de paciente humano). No capítulo 1 realizou-se a substituição do gene *ssd1* nos fungos por transformação mediada por *Agrobacterium* tumefaciens. Consequentemente, a caracterização fenotípica dos fungos “selvagens” foi realizada após o seu crescimento em cinco meios diferentes. A citotoxicidade dos extratos brutos foi testada em células de mamíferos normais (Vero-B4) e cancerígenas (Hela S3), atividade antifúngica e antibacteriana. Os vetores de deleção (pSJ-SSD1-HPT) foram construídos com sucesso, porém não foram gerados mutantes. Os extratos brutos apresentaram atividade contra células normais e cancerígenas, bactérias, e fungos, embora os resultados variem de acordo com o meio de crescimento: em células Hela S3 e nos organismos teste, extratos de crescimento em meios ricos em nutrientes apresentaram maiores atividades. A análise dos extratos por HPLC revelou um número total de analitos maior no crescimento em meio BAF. No capítulo 2, este meio foi usado para fermentação em larga escala de *L. hormozganensis*, para isolar metabolitos secundários ativos. Após extração dos extratos brutos - micélio e meio extracelular - o último foi submetido a extração em fase sólida, HPLC preparativo e HPLC-MS. Compostos puros identificados como 4-etilanilina (27,3 mg) e ácido orselínico (1,5 mg) foram relatados pela primeira vez em *L. hormozganensis*. O composto 4-etilanilina não apresentou efeitos contra bactérias, fungos, plantas, células normais e cancerígenas, o que sugere não estar envolvido na virulência do fungo. O papel dos dois compostos ainda não está clarificado. O resultado da caracterização destes compostos revelou que são necessários mais estudos para compreender o seu envolvimento - assim como de outros metabolitos produzidos pelo fungo - em possíveis mecanismos de virulência para o hospedeiro humano.

keywords

Agrobacterium tumefaciens-mediated transformation, fermentation, opportunistic fungi, human infections, secondary metabolites.

abstract

Global climate change may lead to interactions of pathogenic fungi with new hosts, even from different kingdoms. In order for this to occur, fungi need to develop specific virulence mechanisms for the new host, for example through production of virulence factors. Some phytopathogenic fungi belonging to Botryosphaeriaceae are known human opportunistic pathogens. Moreover, the genus *Lasiodiplodia* has been reported in several human infections. Nonetheless, the virulence mechanisms behind this ability are still in need of research. The goal of this thesis consisted on the study of virulence towards human hosts by *L. theobromae* - strain LA-SOL3 - (isolate from grapevine) and *L. hormozganensis* - strain CBS339.90 - (isolated from human patient). In chapter 1, gene targeted replacement of *ssd1* gene via *Agrobacterium tumefaciens*-mediated transformation was conducted in both fungi. Consequently, phenotypic characterization of wild-type fungi was conducted after growth in five different media. Cytotoxicity of crude extracts was tested on both normal (Vero-B4) and cancer (Hela S3) mammalian cell lines, antifungal, and antibacterial activity. The deletion vectors (pSJ-SSD1-HPT) were successfully constructed, however, no mutants were generated. The crude extracts presented activity against normal and cancer cells, bacteria, and fungi, although these abilities varied according to growth media. In Hela S3 cells and test organisms, extracts from growth in nutrient-rich media presented higher activities. Also, HPLC analysis of the extracts revealed a total number of analytes higher when growth was on BAF medium. Thus, in chapter 2, this medium was used for large-scale fermentation of *L. hormozganensis*, to isolate active secondary metabolites. After extraction of crude extracts -mycelium and culture filtrate - the latter was submitted to solid phase extraction, preparative HPLC and HPLC-MS. Pure compounds identified as 4-ethylaniline (27.3 mg) and orsellinic acid (1.5 mg) were reported for the first time in *L. hormozganensis*. The compound 4-ethylaniline displayed no effects on bacteria, fungi, plants, mammalian and human cancer cells, suggesting that this compound is not involved in virulence by the fungus. The role of the two compounds remains unclear. The results on these compounds characterization reveal that further studies are required to understand their involvement - as well as other metabolites produced by this fungus - in potential virulence mechanisms towards the human host.

TABLE OF CONTENTS

List of abbreviations **IV**
List of figures **VII**
List of tables **IX**

General introduction **1**

1. Pathogen-host interactions: cross-kingdom host jumps 1

  1.1. From plant infections to human opportunistic diseases 1

2. Botryosphaeriaceae: opportunistic pathogens 2

  2.1. *Lasiodiplodia theobromae* 3

  2.2. *Lasiodiplodia hormozganensis* 4

3. Pathogenesis mechanisms: how do fungi do it? 4

4. Main goal 5

Chapter 1: ssd1 gene knockout in *Lasiodiplodia theobromae and Lasiodiplodia hormozganensis* **7**

1. Introduction 9

  1.1. Targeted gene replacement in filamentous fungi 9

    1.1.1. *Agrobacterium tumefaciens*-mediated transformation 9

  1.2. Virulence of *L. theobromae* and *L. hormozganensis*: the mystery remains 10

    1.2.1. *ssd1* – a target for gene knockout 11

  1.3. Goal 11

2. Material and methods 12

  2.1. Material 12

    2.1.1. Chemicals and media components 12

    2.1.2. Equipment 13

    2.1.3. Enzymes and PCR reagents 13

    2.1.4. Solutions, antibiotics and buffers 13

    2.1.5. Reaction kits 16

    2.1.6. Culture media 16

    2.1.7. Organisms 19

    2.1.8. Vectors 20

    2.1.9. Oligonucleotides 21

  2.2. Methods 22

    2.2.1. Measurements of DNA concentration and purity 22

        - 2.2.2. Isolation of genomic DNA from CBS339.90 and LA-SOL3 strains 22
        - 2.2.3. Production of electro-competent *Escherichia coli* cells 22
        - 2.2.4. Transformation of electro-competent *Escherichia coli* cells 23
        - 2.2.5. Phusion Polymerase Chain Reaction (PCR) 23
        - 2.2.6. Agarose gel electrophoresis 25
        - 2.2.7. Isolation of backbone vector via restriction enzyme digestion 25
        - 2.2.8. Vector construction for *ssd1* inactivation via Gibson Assembly 25
        - 2.2.9. Transformation of chemo-competent *Escherichia coli* cells 27
        - 2.2.10. Restriction enzyme digestion of pSJ-SSD1-HPT vector 28
        - 2.2.11. Transformation of *A. tumefaciens* cells with pSJ-SSD1-HPT vectors 28
        - 2.2.12. Obtaining fungal spores 29
        - 2.2.13. Transformation of *L. theobromae* and *L. hormozganensis* 29
        - 2.2.14. Characterization of wild-type fungi 30
            - 2.2.14.1. Small-scale fermentations 30
            - 2.2.14.2. Extraction of crude extracts 30
            - 2.2.14.3. Cytotoxicity assays 31
            - 2.2.14.4. Agar disk-diffusion assay 31
            - 2.2.14.5. Analytical HPLC of crude extracts 31
- 3. Results and discussion 32
    - 3.1. Generation of pSJ-SSD1-HPT deletion vectors 32
    - 3.2. Characterization of wild-type fungi 38
        - 3.2.1. Cytotoxicity of crude extracts 38
        - 3.2.2. Antimicrobial activity of crude extracts 41
        - 3.2.3. HPLC analysis of crude extracts 43
- 4. Conclusions 45

Chapter 2: Bioactivity-guided isolation of secondary metabolites of *Lasiodiplodia hormozganensis* **47**

- 1. Introduction 49
    - 1.1. Secondary metabolites in filamentous fungi: when and how? 49
    - 1.2. The role of secondary metabolites on human infections 50
        - 1.2.1. *Lasiodiplodia hormozganensis* and secondary metabolites 50
    - 1.3. Isolation of secondary metabolites 51
    - 1.4. Goal 52

2. Material and methods 52
  2.1. Material 52
    2.1.1. Chemicals and media components 52
    2.1.2. Equipment 53
    2.1.3. Solutions, antibiotics and buffers 53
    2.1.4. Reaction kits 54
    2.1.5. Culture media 55
    2.1.6. Organisms 57
  2.2. Methods 58
    2.2.1. Selection of growth medium 58
    2.2.2. Large-scale fermentation 58
    2.2.3. Extraction of crude extracts 59
    2.2.4. Characterization of crude extracts 59
      2.2.4.1. Agar disk-diffusion assay 59
      2.2.4.2. HPLC-based fractionation and cytotoxicity assays 60
      2.2.4.3. Cytotoxicity assay 60
    2.2.5. Solid phase extraction 61
    2.2.6. HPLC-based fractionation and cytotoxicity assay of fraction 3 61
    2.2.7. Preparative HPLC 62
    2.2.8. Semi-preparative HPLC 63
    2.2.9. Biological characterization of the compound 1 (4-ethylaniline) 63
      2.2.9.1. Antimicrobial activity 63
      2.2.9.2. Phytotoxicity assays 64
      2.2.9.3. Cytotoxicity assays 64
3. Results and discussion 65
  3.1. Fermentation parameters 65
  3.2. Preliminary screening of biological activity of crude extracts 66
  3.3. Isolation of metabolites 67
    3.3.1. Isolation of compounds from fraction 2 68
    3.3.2. Isolation of compounds from fraction 3 71
4. Conclusions 73

Future prospects 75
References 77
Appendix 88

LIST OF ABBREVIATIONS

°C	Celsium degrees
AIM	*Agrobacterium tumefaciens* inducing medium
Ala	Alanine
ANOVA	Analysis of Variance
APCI	Ionization at atmospheric pressure
ATMT	*Agrobacterium tumefaciens*-mediated transformation
bp	Base pair
CBS	Centraalbureau voor Schimmelcultures
CM	Complete medium
Da	Dalton
DAD	Diode Array Detector
DM	Double Malt
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dnTPs	Deoxynucleotides
$_{ds}$DNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal Bovine Serum
for	Forward
g	Gram(s)
h	Hour(s)
HiFi	High-Fidelity
HPLC	High Performance Liquid Chromatography
HPT	Hygromycin B phosphotransferase gene
IDZ	Inhibition diameter zones
kb	Kilobases
kV	Kilovolt
L	Litter(s)
LB	Luria Bertani
LLE	Liquid-Liquid Extraction

M	Molar
MAPKs	Mitogen-activated protein kinases
mAU	Milli-Absorbance Units
MeCN	Acetonitrile
MeOH	Methanol
mg	Milligrams
min	Minute(s)
mL	Milliliters
mm	Millimeter
mM	Millimolar
MM	Minimal Medium
MM+	Minimal Medium plus
mmol	Milimol
MS	Mass spectrometry
ng	Nanograms
nm	Nanometers
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PKS	Polyketide Synthases
rev	Reverse
RNase	Ribonuclease
rpm	Revolutions per minute
sec	Seconds
SPE	Solid Phase Extraction
T-DNA	Transfer DNA
T_A	Annealing temperature
TAE	Tris base, acetic acid and EDTA
T_E	Elongation time
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
UF	Ultra-filtrated ultrapure

UV	Ultraviolet radiation
V	Volts
v/v	Volume per volume
YMG	Yeast Malt Glucose
µF	Microfarad
µg	Micrograms
µL	Microliters
µm	Micrometer

LIST OF FIGURES

CHAPTER 1

Figure 1.1 | pSJ-basic plasmid map. 21

Figure 1.2 | Gibson assembly principle. 26

Figure 1.3 | pSJ-SSD1-HPT plasmid map for *Lasiodiplodia hormozganensis* strain CBS339.90 and *L. theobromae* strain LA-SOL3 27

Figure 1.4 | Scheme of pSJ-SSD1-HPT map of CBS339.90 and LA-SOL3, including restriction sites. 33

Figure 1.5 | Restriction analysis after digestion of pSJ-SSD1-HPT for CBS339.90 with *Nco*I. 33

Figure 1.6 | Restriction analysis after digestion of pSJ-SSD1-HPT for CBS339.90. The vector was digested with *Fsp*I, *Pvu*I, *Mlu*I, and a mixture of *Mlu*I with *Pvu*I or *Fsp*I. 34

Figure 1.7 | Restriction analysis after digestion of pSJ-SSD1-HPT for LA-SOL3. The vector was digested with *Bgl*II, *Nsb*I, *Nco*I, and a mixture of *Bgl*II and *Nco*I. 35

Figure 1.8 | Evaluation of cell viability (%) after 72 h exposure to crude extracts (500 µg/mL) from *Lasiodiplodia theobromae* and *L. hormozganensis* grown in 5 different media (BAF, DM, MM, MM+, YMG). 40

Figure 1.9 | Comparison of peak areas (mAU) of crude extracts from fungi grown in BAF, DM, YMG, MM+ and MM 45

CHAPTER 2

Figure 2.1 | Evaluation of pH, glucose (mmol/L) and mycelium dry weight (g/L) throughout large-scale fermentation of *L. hormozganensis* strain CBS339.90 in BAF medium. 66

Figure 2.2 | Evaluation of cell viability (%) after 72 h exposure to crude extracts (500 µg/mL) of *L. hormozganensis* CBS339.90 grown in 20 L of BAF medium for 6 days, at 22 °C. 66

Figure 2.3 | Chromatogram (250 nm) and UV/vis and mass spectra of the compound 4-ethylaniline isolated from *L. hormozganensis* strain CBS339.90. 68

Figure 2.4 | Cytotoxicity of 4-ethylaniline in different concentrations (0.01, 0.025, 0.05, 0.1, 0.5 and 1 mg/mL) in Vero and A375 cells. 71

Figure 2.5 | Chromatogram (250 nm) and UV/vis and mass spectra of orsellinic acid isolated from *Lasiodiplodia hormozganensis* strain CBS339.90. .... **72**

APPENDIX

Figure S1 | Sensibility test of *Lasiodiplodia hormozganensis* strain CBS339.90 and *Lasiodiplodia theobromae* strain LA-SOL3 for hygromycin (0, 10, 20, 50, 75 and 100 mg/L). **88**

Figure S2 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate of *L. theobromae* LA-SOL3 grown in 500 mL of MM, DM medium, MM+, YMG medium, and BAF medium. **89**

Figure S3 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate of *L. hormozganensis* CBS339.90 grown in 500 mL of MM+, MM, DM medium, YMG medium, and BAF medium. **90**

Figure S4 | HPLC chromatograms (230 nm) of crude extracts from mycelia of *L. hormozganensis* CBS339.90 grown in 500 mL of YMG medium, MM, MM+, DM medium, and BAF medium. **92**

Figure S5 | HPLC chromatograms (230 nm) of crude extracts from mycelia of *L. theobromae* LA-SOL3 grown in 500 mL of YMG medium, DM medium, BAF medium, MM, and MM+. **94**

Figure S6 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate and mycelium of strain CBS339.90 obtained after large-scale fermentation. **95**

Figure S7 | HPLC chromatograms (230 nm) of fractions from culture filtrate of *L. hormozganensis* CBS339.90. .... **96**

Figure S8 | Scheme of isolation of metabolites from crude extracts of *Lasiodiplodia hormozganensis* CBS339.90. .... **97**

LIST OF TABLES

CHAPTER 1

Table 1.1 | Test organisms for antimicrobial activity characterization of *L. theobromae* and *L. hormozganensis*. **20**

Table 1.2 | Primers used for *ssd1* gene inactivation in *Lasiodiplodia hormozganensis* strain CBS339.90. .... **21**

Table 1.3 | Primers used for *ssd1* gene inactivation in *Lasiodiplodia theobromae* strain LA-SOL3. **22**

Table 1.4 | Phusion PCR master mix preparation. **24**

Table 1.5 | Phusion PCR program for the amplification of the flanking regions of LA-SOL3 and CBS339.90 strains, and HPT cassette amplification from pSJ-basic vector. **24**

Table 1.6 | Phusion PCR program for HPT cassette amplification from pCB1636 vector. **24**

Table 1.7 | Master mix used for Gibson Assembly of the vector pSJ-SSD1-HPT.......... **26**

Table 1.8 | Restriction reaction mix.......... **28**

Table 1.9 | Standard gradient for measurement of crude extracts on analytical HPLC. **32**

Table 1.10 | Antimicrobial activity assay of *L. hormozganensis* strain CBS339.90 and *L. theobromae* strain LA-SOL3 grown in different media (MM, MM+, YMG, BAF and DM media). **42**

CHAPTER 2

Table 2.1 | Test organisms for characterization of crude extracts and 4-ethylaniline. **58**

Table 2.2 | Standard gradient for measurement on analytical HPLC. **59**

Table 2.3 | Agar-disk diffusion assay of crude extracts of *Lasiodiplodia hormozganensis* strain CBS339.90 grown in 20 L of BAF medium for 6 days, at 22 °C. **67**

Table 2.4 | Summarized raw data of biological assays of 4-ethylaniline. **70**

GENERAL INTRODUCTION

1. Pathogen-host interactions: cross-kingdom host jumps

Pathogen-host interactions depend on mutual recognition, genetic compatibility, the host defenses and its resistance to the pathogen, fungal virulence, and the surrounding environment (Hube, 2009; van Baarlen *et al.*, 2007). Earth is facing climate change, which affects the environmental conditions, thus shifting microorganisms' biogeographic distribution (Bebber *et al.*, 2013), namely for Botryosphaeriaceae fungi (Slippers & Wingfield, 2007). Withal, pathogen-host interactions may also be altered during adaptation, possibly inducing new interactions with different hosts, even from different kingdoms (Félix *et al.*, 2016; van Baarlen *et al.*, 2007). Moreover, environmental adjustments may convert symbiotic or commensal relationships into pathogenic interactions (Tanaka *et al.*, 2006). Therefore, all the factors interrelated to microorganism-host interactions may promote new fungal diseases, resulting in cross-kingdom pathogenicity (Desprez-Loustau *et al.*, 2006). In cross-kingdom infections, the pathogenesis-related molecules produced by fungi target conserved regions of the host, thus, these pathogens tend to be generalists (Mahmood *et al.*, 2015). In addition, fungal survival on the new host depends on its adaptation to the host defenses, specifically the ability to target pathways that both hosts have in common (Mahmood *et al.*, 2015).

1.1. From plant infections to human opportunistic diseases

Regardless of the morphological and physiological differences between plants and humans, some phytopathogenic fungi are able to infect humans. This cross-kingdom jump may occur due to direct human contact with the infected plant, or indirect contact through the environment (van Baarlen *et al.*, 2007). In fact, human contact with new pathogens has been increasing due to changes in dietary habits, food production, handling and transport, travelling, and global international trades (van Baarlen *et al.*, 2007).

Most human pathogenic fungi are environmental and cause exogenous infections on the host, behaving as opportunists. Frequently, a temporary exposure of a human to an opportunistic fungus is unnoticed given its low virulence due to the host immune system (van Burik & Magee, 2001). For human infections to occur, pathogens need to recognize and ligate specific target receptors of the host to overcome the immune system (Mahmood *et al.*, 2015). In fact, fungi may become human pathogens when stimulated with resembling conditions to the human body, thus adjusting to adhere to surfaces, competing with other microorganisms and adapting to biotic and abiotic factors (Hube, 2009). Moreover, opportunistic fungi may become aggressive in injured or immunocompromised hosts (van Baarlen *et al*., 2007), increasing the susceptibility of these patients to fungal diseases (Kavanagh, 2011).

The human body displays several inherent barriers against exogenous fungi that may try to cause an invasive disease. Some of those barriers are the body temperature, which is higher than the common fungal optimum temperature, the immune system, and the inner neutral pH (van Burik & Magee, 2001). Although the human body has a neutral pH and fungi prefer more acidic environments, they are able to adapt by changing the pH in the environment (Hube, 2009). Herewith, fungi may overcome the structural barriers of the human body due to their virulence (van Burik & Magee, 2001).

The first stage for the establishment of fungal diseases is the attachment of the microorganism on the host, followed by tissue penetration, which depends on the specific pathogen (Kavanagh, 2011). Many pathogenic fungi are able to attach to the host through surface-associated adherence factors or adhesins, which sometimes can recognize different hosts from different kingdoms (Iyalla, 2017; van Baarlen *et al*., 2007). If the attachment is successful, the environmental fungus can cause an invasive infection. In fact, the infection may stay localized or even become systemic via dissemination on the bloodstream, requiring tissue damage by the fungus (van Burik & Magee, 2001). However, human pathogens mainly cause extracellular penetration. In the following step, the infection, the pathogen colonizes the host tissues to achieve nutrition and replication (Kavanagh, 2011). The progression of infection depends on virulence factors produced by the fungus and on the host defense system (van Burik & Magee, 2001). After this stage, the symptoms are developed. Finally, the last steps are the production of the inoculum by the filamentous fungus (which is usually a spore) and its dissemination (van Baarlen *et al*., 2007).

Opportunistic fungal diseases in humans and the number of individuals with acquired immune debilities have been increasing over the years (Lehrnbecher *et al*., 2010). Furthermore, current antifungal therapies are still scarce and the appearance of resistant fungal strains is becoming more urgent, affecting the success of the used approaches (Fisher *et al*., 2018). Consequently, studies regarding host-fungal interactions, fungal virulence and pathogenicity, and the discovery of antifungal targets are crucial (Templeton *et al*., 2018).

2. Botryosphaeriaceae: opportunistic pathogens

The family Botryosphaeriaceae (phylum Ascomycetes, class Dothideomycetes, order Botryosphaeriales) is composed of pathogenic, saprobic or endophytic fungi that infect mostly woody hosts (Dissanayake *et al*., 2016; Phillips *et al*., 2013). These fungi are spread around the world, with the exception of the polar regions (Phillips *et al*., 2013). Most of these fungi have a wide host range (Sakalidis *et al*., 2013; Slippers *et al*., 2013), cross-infecting native and introduced hosts (Parker & Gilbert, 2004).

Botryosphaeriaceae generally exist as endophytes in healthy plant tissues, which may lead to undetected dissemination, with potential infections of new hosts (Slippers & Wingfield, 2007). In stress conditions for the host, Botryosphaeriaceae latent stage can evolve to pathogenicity (Slippers & Wingfield, 2007). Therefore, some species can cause plant diseases or even death (Alves et al., 2013; Slippers & Wingfield, 2007; Úrbez-Torres, 2011), leading to economic losses in agriculture and forestry, which has brought an importance to Botryosphaeriaceae-related studies.

Based on phylogenetic studies, 24 genera are known to belong to Botryosphaeriaceae, including *Lasiodiplodia* (Burgess *et al*., 2018; Dissanayake *et al*., 2016; Phillips *et al*., 2013). *Lasiodiplodia* species form asexual reproductive spores, conidia, which are hyaline until pigmentation and septation occurs when maturity is reached, becoming dark-brown with longitudinal striations constituted by melanin deposits (Phillips *et al*., 2013). Moreover, research on human-Botryosphaeriaceae interactions, namely on the production of fungal pathogenesis-related molecules, is becoming more urgent since several of these fungi have been shown to cause human infections (Woo *et al*., 2008). For example, the genus *Lasiodiplodia* has been reported in several human infections (Carlet *et al*., 2019; Gu *et al*., 2016; Maslen *et al*., 1996; Mohan *et al*., 2016; Papacostas *et al*., 2015; Saha *et al*., 2012; Summerbell *et al*., 2004; Vanam *et al*., 2019; Woo *et al*., 2008).

2.1. *Lasiodiplodia theobromae*

Lasiodiplodia theobromae (Pat.) Griff. & Maubl. (sexual morph, *Botryosphaeria rhodian*) is the type species of the genus *Lasiodiplodia*. This phytopathogenic fungus has the ability to grow between 8 and 39 °C, with an optimum growth temperature ranging from 25-30 °C (Alam *et al*., 2001; Saha *et al*., 2008). Plant infections by this microorganism usually occur in tropical and subtropical areas in the world (Tsukada *et al*., 2010). Moreover, this species is known to display high virulence towards some hosts (Úrbez-Torres, 2011).

Lasiodiplodia theobromae is able to affect mammalian cells (Félix *et al*., 2016; 2018a; 2019a) and several reports regarding human infection have been made in immunocompetent and immunocompromised patients. Ergo, this fungus behaves as a human opportunistic pathogen. In fact, it has been reported to cause phaeohyphomycosis, pneumonia, keratitis, sinusitis, subcutaneous skin infections, ocular infections, and even human death (Gu *et al*., 2016; Maslen *et al*., 1996; Mohan *et al*., 2016; Papacostas *et al*., 2015; Saha *et al*., 2012; Woo *et al*., 2008).

2.2. *Lasiodiplodia hormozganensis*

Lasiodiplodia hormozganensis Abdollahz., Zare & A.J.L. Phillips is known to infect several plants, such as *Mangifera indica* (Al-sadi *et al*., 2013), *Olea* sp. (Abdollahzadeh *et al*., 2010), *Ricinus communis* (Custódio *et al*., 2018), *Bougainvillea spectabilis* (Li *et al*., 2015) and *Vitis vinifera* (Correia *et al*., 2016). This fungus has been reported in Asia, South America, South Africa and Europe (Abdollahzadeh *et al*., 2010; Al-sadi *et al*., 2013; Correia *et al*., 2016; Custódio *et al*., 2018; Li *et al*., 2015). Moreover, *Lasiodiplodia hormozganensis* strain CBS339.90, previously misidentified as *L. theobromae*, has been described as a human opportunistic pathogen (unpublished data). In fact, several studies have showed that CBS339.90 exhibits high cytotoxicity towards mammalian cells, at 37 °C (Félix *et al*., 2016; 2018a). Furthermore, strain CBS339.90 was reported to cause subcutaneous phaeohyphomycosis on a human patient (Summerbell *et al*., 2004).

3. Pathogenesis mechanisms: how do fungi do it?

The term “pathogenesis” refers to the ability of an organism to cause disease. Some pathogenic microorganisms can be more virulent than others (Cramer Jr & Perfect, 2009). A higher virulence is enhanced by the production of virulence factors. Indeed, production of different virulence factors aids the pathogenesis of fungal infection on immunocompromised humans (Kavanagh, 2011; van Baarlen *et al*., 2007; van Burik & Magee, 2001). Each virulence factor plays a specific role in pathogenesis (Cramer Jr & Perfect, 2009). In addition, secondary metabolites, which are active small molecular weight compounds produced by microorganisms during secondary metabolism, can aid microbial pathogenesis (Cramer Jr & Perfect, 2009). In some plant-fungal interactions, these compounds are considered the primary fungal virulence factors (reviewed by Cramer Jr & Perfect, 2009).

Various approaches have been used to uncover the pathogenicity mechanisms of fungi. Among these, different omics techniques are commonly used, providing relevant information regarding the pathogenesis-related molecules, including virulence factors (Félix *et* al., 2019a; van de Wouw & Howlett, 2011). Transcriptomics and proteomics can help uncovering differences on the pathogenicity mechanisms during fungal interaction with different hosts, e.g. plants and humans. Subsequently, the relevance of a candidate virulence factor on pathogenesis can be demonstrated through knockout of its coding gene, followed by a decrease in virulence, with a respective recover when the gene expression is reestablished (Kavanagh, 2011). However, if the opposite is observed, the involvement of the gene is inconclusive given that it belongs to a family, thus some of the belonging products may have a role on pathogenesis (van Burik & Magee, 2001).

4. Main goal

The pathogenesis mechanisms and virulence-related compounds produced by *L. theobromae* and *L. hormozganensis* are still in need of some clarification. Hence, the main goal of this thesis consisted on the study of *L. theobromae* (strain LA-SOL3) *and L. hormozganensis* (strain CBS339.90) pathogenicity, particularly related to human infection. For this purpose, the thesis was divided in two chapters. In chapter 1, transformation methods were conducted to generate fungal mutants with the deletion of *ssd1* gene, to understand its role in virulence of these fungi. In chapter 2, fermentation assays followed by bioactivity-guided isolation and characterization of secondary metabolites were performed in *L. hormozganensis*.

CHAPTER 1

ssd1 gene knockout in *Lasiodiplodia theobromae* and *Lasiodiplodia hormozganensis*

1.1. Targeted gene replacement in filamentous fungi

Microorganisms adapt to new environmental conditions (e.g. temperature) through adjustments on the genome, proteome or secondary metabolism (Bleuven & Landry, 2016; Braga *et al*., 2016), specifically metabolites directly involved in host-fungal interactions. Upon that, genomics, proteomics, metabolomics and transcriptomics have been used to discover fungal biological functions. Also, functional genomics, e.g. through the use of targeted gene replacement, can further help verifying if the sequence annotations are accurate (Utermark & Karlovsky, 2008). In this method, homologous DNA recombination allows the substitution of the target gene (the gene to be silenced) with an inserted gene. This gene needs to contain flanking regions, which are homologous to the surrounding regions of the target gene. Furthermore, gene knockout increases the knowledge of biological functions, through the comparison of phenotypes between mutant and wild-type fungi. A critical prerequisite for targeted gene replacement is a successful genetic fungal transformation (Frandsen *et al*., 2012). Several transformation methods are known to be used in filamentous fungi, such as biolistic protocols, electroporation, *Agrobacterium tumefaciens*-mediated transformation (ATMT), and treatment of protoplasts with polyethylene glycol (He *et al*., 2017; Li *et al*., 2017). It has been reported that some fungi from family Botryosphaeriaceae can be successfully transformed (Chen *et al*., 2016a; Muniz *et al*., 2014). To date, only two transformation protocols for *Lasiodiplodia* species have been described, specifically in *L. theobromae*, using Restriction Enzyme-Mediated Integration (Zhang *et al*., 2014) and ATMT (Muniz *et al*., 2014).

1.1.1. *Agrobacterium tumefaciens*-mediated transformation

Agrobacterium tumefaciens is a gram-negative soil-borne phytopathogenic bacterium able to induce random genetic alterations in the host genome, through the introduction of bacterial DNA on the plant genome. *A. tumefaciens* possesses a Ti plasmid, which contains T-DNA, the transferred fragment to the plant that contains genes involved in the infection. DNA transference occurs by means of a pilus that retracts and a DNA transfer channel that is formed until the host cytoplasm (Baron & Zambryski, 1996). This bacterium was found to be able to transform species from different kingdoms (reviewed by Idnurm *et al*., 2017). In fact, ATMT was discovered to be successful in filamentous fungi in 1998 by Groot and colleagues (de Groot *et al*., 1998). Currently, this is the most common applied method for genome manipulation of filamentous fungi, given that it presents several advantages. For example, it provides high efficiency and insertion of target genes, flexibility regarding the fungal sample (spores, hyphae or protoplasts), and T-DNA is inserted randomly in the host genome as a single copy (de Groot *et al*., 1998; reviewed by He *et al*., 2017). Hence, it is possible to produce genetic engineered fungi through the substitution of the T-DNA genes with a cassette that contains a selection marker and the target genes. ATMT is composed by three steps: i) transformation of the bacteria with the plasmid, ii) co-incubation of the bacterial culture with the fungal suspension on solid medium, and iii) selection of transformants in a selection medium (Utermark & Karlovsky, 2008). For this procedure, a binary vector system is mainly used (Frandsen *et al*., 2012), constituted by a small plasmid with the target gene and selection marker(s), and a large plasmid with the *vir* genes, which are involved in the mobilization of the plasmid into the fungus. The success of the transformation can be evaluated through Southern Blot analysis (Zhang *et al*., 2014). This technique reveals DNA identity, size and abundance after size-based separation of DNA fragments by electrophoresis, followed by DNA transference to a membrane, hybridization with a probe and labeled DNA detection. Thereupon, the accuracy of the insertion of the vector on the fungus can be achieved through the use of this approach.

1.2. Virulence of *L. theobromae* and *L. hormozganensis*: the mystery remains

Lasiodiplodia theobromae and *L. hormozganensis* are phytopathogenic fungi known as opportunistic pathogens to human hosts. Several pathogenesis-related compounds, including virulence factors, are produced by these organisms. However, there is still a lack of knowledge on the pathogenesis mechanisms and virulence of these pathogens, specifically during human-fungus interactions. Hence, research that allows the understanding of the virulence factors produced by these fungi and their role on human pathogenesis is crucial.

Lasiodiplodia theobromae LA-SOL3 (Félix *et al*., 2019a) and *L. hormozganensis* CBS339.90 (Félix *et al*., 2018b) secrete several enzymes, such as hydrolases, which participate as virulence factors for some organisms (Olivera *et al*., 2016; Schaller *et al*., 2005). Also, aspartic proteases, known to be involved in human pathogenesis (dos Santos, 2011), were found to be produced by both fungi (Félix *et al*., 2016; 2019a). Félix and colleagues (2019a) also demonstrated, through the use of multi-omics approaches, that *L. theobromae* strain LA-SOL3 produces a higher amount of pathogenesis-related proteins at 25 °C - environmental mean temperature -. In fact, proteins associated with siderophores production, toxins, allergens, mitogen-activated protein kinases (MAPKs), heat shock proteins, nudix effectors, and proteins belonging to the velvet complex were identified in the transcriptome and proteome of this fungus. MAPKs and the velvet complex proteins are important for full virulence of filamentous fungi (Zhao *et al*., 2007) and secondary metabolites’ production (Bayram & Braus, 2012), respectively. In addition, nudix effectors are relevant for shared mechanisms of virulence of pathogens in cross-kingdom infections (Dong & Wang, 2016). Unlike strain LA-SOL3, *L. hormozganensis* strain CBS339.90 produces a higher percentage of pathogenesis-related proteins at 37 °C - human body temperature -, such as snodprot1 protein, aspergillopepsin and the virulence protein SSD1 (unpublished data). Nonetheless, strain LA-SOL3 only expressed virulence factors at 37 °C, e.g. the virulence protein SSD1 (Félix *et al*., 2019a).

Several proteins with direct roles in pathogenesis are produced by both fungi, namely virulence protein SSD1, SnodProt1 protein (phytotoxin), and 4-aminobutyrate aminotransferase (Félix *et al*., 2019a; unpublished data). However, since virulence protein SSD1 was only produced when fungal growth occurred at 37 °C, the authors suggested that this protein might be specific for human pathogenesis. Indeed, SSD1 protein helps the colonization of human tissues by *Candida albicans*, contributing to the resistance of the human immune defense, enhancing fungal virulence (Gank *et al*., 2008).

1.2.1. *ssd1* - a target for gene knockout

As mentioned previously in this chapter, molecular techniques such as targeted gene replacement can enlighten the gene role on the infection mechanism of a microorganism. Hence, the knockout of *ssd1* gene, which encodes the virulence protein SSD1, could allow the understanding of its role in the virulence of *L. theobromae* and *L. hormozganensis* towards humans. SSD1 contributes to the resistance of *C. albicans* to human antimicrobial peptides (e.g. protamine, RP-1, human beta-defensin-2) and membrane permeability, and maintenance of mitochondrial membrane potential after host defense (Jung *et al*., 2013). In fact, *ssd1* gene knockout in *Candida albicans* led to a decrease of virulence by this pathogen (Gank *et al*.,2008).

1.3. Goal

In the present chapter, the goal was to transform *Lasiodiplodia theobromae* strain LA-SOL3 and *Lasiodiplodia hormozganensis* strain CBS339.90 with a constructed vector (psJ-SSD1-HPT) to achieve *ssd1* gene knockout. Consequently, the phenotype of mutant and wild-type fungi would be characterized to evaluate the effect of the knockout on virulence: antimicrobial activity, phytotoxicity and cytotoxicity of crude extracts against mammalian (Vero-B4 cells) and human cancer cells (Hela S3 cells), and comparison of compounds production via HPLC analysis.

2.1. Material

2.1.1. Chemicals and media components

a) Water

$H_2O_{deionized}$ ($_{di}H_2O$) [Seradest SD 2000, SERAL - Reinstwasser - System, GmbH, Ransbach-Baumbach] was used to prepare media, solutions and buffers. $H_2O_{Ultra\text{-}filtrated\ ultrapure}$ ($_{UF}H_2O$) [Milli-Q® Synthesis, Quantum® EX, Q-Gard® 2, Millipore GmbH, Schwalbach] was used for molecular experiments.

b) Solvents

Acetonitrile (Sigma-Aldrich Chemie GmbH), ethyl acetate (Fisher Chemical, UK), isopropanol (Sigma-Aldrich Chemie GmbH), methanol (Fisher Chemical, UK), DMSO (VWR).

c) Antibiotics

Ampicillin sodium salt (Sigma-Aldrich Chemie GmbH), cefotaxime sodium (Fresenius Kabi GmbH), cycloheximide (Sigma-Aldrich Chemie GmbH), hygromycin B Gold (InvivoGen), kanamycin sulfate (Carl Roth GmbH), penicillin G (Serva, Boehringer), rifampicin (Carl Roth GmbH), streptomycin sulfate (PanReac AppliChem ITW Reagents).

d) Media components and reagents

Acetosyringone (Sigma-Aldrich Chemie GmbH), Bacto™ Malt Extract (Becton Dickinson GmbH, Heidelberg), Bacto™ Nutrient Broth (Becton Dickinson GmbH, Heidelberg), Bacto™ Peptone (Becton Dickinson GmbH, Heidelberg), Bacto™ Tryptone (Becton Dickinson GmbH, Heidelberg), Bacto™ Yeast Extract (Becton Dickinson GmbH, Heidelberg), Biozym LE Agarose (Biozym Scientific, Oldendorf), Camptothecin (Alfa Aesar), d-Biotin (Sigma-Aldrich Chemie GmbH), Difco™ Agar Bacteriological (Becton Dickinson GmbH, Heidelberg), Difco™ Potato Dextrose Broth (Becton Dickinson GmbH), Dulbecco's Modified Eagle Medium (Gibco), Ethidium bromide (Carl Roth GmbH & Co. KG, Karlsruhe), Fetal Bovine Serum (Sigma-Aldrich), Glucose (SHS, Gesellschaft für klinische Ernährung, GmbH, Heilbronn), Glycerol (Sigma-Aldrich), Kartoffel Püree (Edeka, Germany), Maltose (Alfa Aesar, ThermoFisher GmbH, Erlenbachweg), MES – Hydrate (PanReac AppliChem ITW Reagents), Phosphate Buffered Saline (Gibco), RNase A solution (Thermo Scientific GmbH), SOC Outgrowth Medium (New England Biolabs), Thiamine chloride (Sigma-Aldrich Chemie GmbH), Tris

(Carl Roth GmbH), Trypsin EDTA (Corning), H_3BO_3 (Merck KGaA), $CaCl_2.2H_2O$ (Sigma-Aldrich Chemie GmbH), $CoCl_2.6H_2O$ (Riedel-de Haën AG), $CuSO_4.5H_2O$ (Fluka Chemie AG), $FeSO_4.7H_2O$ (Merck), KCl (Sigma-Aldrich Chemie GmbH), KH_2PO_4 (Bernd Kraft, Germany), K_2HPO_4 (Merck KGaA), $MgSO_4.7H_2O$ (Merck), $MnCl_2.4H_2O$ (Merck GmbH), NaCl (Carl Roth GmbH), $NaNO_3$ (VWR Chemicals), $Na_2EDTA.2H_2O$ (AppliChem GmbH), NH_4NO_3 (Riedel-de-Haën), $ZnSO_4.7H_2O$ (Merck).

2.1.2. Equipment

37 °C incubator (Memmert GmbH), Bath Sonicator (Elma), Analytical HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn), Centrifuge 5415 D (Eppendorf AG, Hamburg), Centrifuge Jouan GR4 22 (Axon), DNA-Concentrator 5301 (Eppendorf GmbH), Electroporation system Gene Pulser II (Bio-Rad, München), Forma Orbital Shaker (Thermo Electron Corporation), Freeze dryer (Christ® Gamma 1-20), GeneAmp PCR System 9700 (Applied Biosystems, USA), Mini centrifuge (Axon), NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA), PowerPac 300 (Bio-Rad), Qubit™ 4 Fluorometer (Invitrogen), Rotary Evaporator Laborota 4002-Digital (Heidolph), Speedvac Jouan RC10.22 (Thermo Fisher Scientific), Speedvac RVC 2-33 CD-plus (Christ GmbH), Thermal Cycler C1000 Touch (Bio-Rad GmbH, Medard), ThermoShaker (Biometra), TissueLyser II (Qiagen), UV reader (peqLAB GmbH, Erlangen), Vacuum Concentrator Jouan RC 10.22 (Labfish).

2.1.3. Enzymes and PCR reagents

10X Buffer O (Thermo Fisher Scientific), 10X Buffer Tango (Fermentas), 10X Fast Digest Green Buffer (Thermo Fisher Scientific, USA), 5X Buffer HF (New England Biolabs), 5X Phire Reaction Buffer (Thermo Fisher Scientific), 6X DNA Loading Dye (Thermo Fisher Scientific, USA), *Bgl*II 10 U/µL (Fermentas), dnTPs (MBI Fermentas), FastDigest *Hin*dIII (Thermo Fisher Scientific), GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific), GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA), NEBuilder HiFi DNA Assembly Master Mix - 2X Concentrated (New England Biolabs), *Nco*I 10 U/µL (Thermo Fisher Scientific), Phusion High-Fidelity DNA Polymerase (New England Biolabs), *Pst*I 1 U/µL (Fermentas).

2.1.4. Solutions, antibiotics and buffers

a) Solutions

Nitrate Salt Solution (20x)

$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4.7H_2O$	10.4 g
KH_2PO_4	30.4 g

Fill with 1 L of deionized water.

MES solution (pH 5.5)

MES-Hydrate	195 g

Deionized water is added after MES-Hydrate, filling the flask up to 1 L of total volume.

Trace elements solution (pH 6.5)

$CoCl_2.6H_2O$	1.7 g
$CuSO_4.5H_2O$	1.6 g
$FeSO_4.7H_2O$	5 g
H_3BO_3	11 g
$MnCl_2.4H_2O$	5 g
$Na_2EDTA.2H_2O$	50 g
$Na_2MoO_4.2H_2O$	1.5 g
$ZnSO_4.7H_2O$	22 g

Fill with 1 L of deionized water.

Acetosyringone solution (200 mM)

Acetosyringone	392 mg

Fill with DMSO up to 10 mL of total volume.

Camptothecin solution (1 mg/mL)

Camptothecin	10 mg

Fill with DMSO up to 10 mL of total volume.

b) Antibiotics

Antibiotic solutions were filtered with 0.2 µm pore size sterile filters (Whatman) and stored at -20 °C.

Ampicillin solution (100 mg/mL)

Ampicillin sodium salt	1 g

Fill with $_{UF}H_2O$ up to 10 mL of total volume.

Cefotaxime solution (100 mg/mL)

Cefotaxime sodium	1 g

Fill with $_{UF}H_2O$ up to 10 mL of total volume.

Cycloheximide solution (1 mg/mL)

Cycloheximide	10 mg

Fill with EtOH up to 10 mL of total volume.

Hygromycin solution (100 mg/mL)

Hygromycin B Gold	1 g

Fill with $_{UF}H_2O$ up to 10 mL of total volume.

Kanamycin solution (100 mg/mL)

Kanamycin sulfate	1 g

Fill with $_{UF}H_2O$ up to 10 mL of total volume.

Rifampicin solution (10 mg/mL)

Rifampicin	100 mg

Fill with MeOH up to 10 mL of total volume.

Streptomycin solution (100 mg/mL)

Streptomycin sulfate	1 g

Fill with $_{UF}H_2O$ up to 10 mL of total volume.

c) Buffers

K Buffer

K_2HPO_4	1.15 M
KH_2PO_4	1.15 M

Fill with 1 L of deionized water.

MN Buffer

$MgSO_4$	120 mM
NaCl	255 mM

Fill with 1 L of deionized water.

TAE buffer 50x (pH 8)

Tris	242 g
Na_2EDTA	64.3 g

Fill with 1 L of deionized water.

2.1.5. Reaction kits

NucleoSpin® Gel and PCR Clean-Up (Macherey-Nagel, Düren), Glucose Urine Test Strip, DUS G (DFI), GeneJET^{TM} Plasmid Miniprep Kit (Thermo Fisher Scientific), GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific), Gibson Assembly® HiFi DNA Cloning Kit (New England Biolabs), Qubit^{TM} dsDNA HS assay kit (Invitrogen, USA).

2.1.6. Culture media

The following media were used in this work, in which the amounts given for each composition are calculated for a total of 1 L of medium. For solid media, 18 g/L of agar were added before autoclaving. All media were autoclaved after preparation, at 121 °C for 20 minutes.

a) Media for *L. theobromae and L. hormozganensis* culture

BAF medium

Maltose	20 g
Glucose	10 g
Peptone	2 g
Yeast extract	0.2 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	1 g
$CaCl_2$ (0.1 M)	5 mL
$FeCl_3$ (10 mg/mL)	1 mL
$ZnSO_4.7H_2O$ (1 mg/mL)	1 mL

Double malt (DM) medium

Malt extract	40 g

Minimal medium (MM, pH 6.5)

Nitrate Salt Solution (20x)	50 mL
Trace elements	1 mL
Glucose	10 g
Thiamin (1 % v/v)	1 mL
Biotin (0.01 %)	250 µL

Minimal medium plus (MM+, pH 6.5)

Nitrate Salt Solution (20x)	50 mL
Trace elements	1 mL
Glucose	10 g
Thiamin (1 % v/v)	1 mL
Biotin (0.01 %)	250 µL
Starch	10 g

Potato Dextrose Agar (PDA) medium

Potato Dextrose Broth	24 g

Yeast malt glucose (YMG) medium (pH 5.5)

Yeast extract	4 g
Glucose	10 g
Malt extract	10 g

½ PDA medium

Potato Dextrose Broth	12 g

b) Media for *Escherichia coli* culture

Luria-Bertani (LB) medium (pH 7.4)

Tryptone	10 g

Yeast extract	5 g
NaCl	5 g

b) Media for *Agrobacterium tumefaciens* culture

***Agrobacterium tumefaciens* induction medium (AIM)**

$CaCl_2$ (1 %)	1 mL
$FeSO_4$ (0.01 %)	10 mL
Glucose (20 %)	5 mL
Glycerol (50 %)	5 mL
MES solution (1 M)	40 mL
NH_4NO_3 (20 %)	2.5 mL
Trace elements	5 mL
Thiamin (1 % v/v)	1 mL
K Buffer	0.8 mL
MN Buffer	20 mL

The components listed above were added to 100 mL of $_{di}H_2O$, followed by filter-sterilization (0.2 µm pore size; Whatman) and addition of 900 mL of previous autoclaved $_{di}H_2O$ cooled to 55 °C. For the production of AIM-solid medium, agar was added prior to autoclaving. For AIM medium with acetosyringone, when the medium was cooled at 55 °C, 1 mL of 200 mM acetosyringone solution was added.

b) Media for test organisms' culture

HA medium (pH 5.5)

Yeast extract	4 g
Glucose	4 g
Malt extract	10 g

NB medium (pH 6.5)

Bacto™ Nutrient Broth	8 g
NaCl	1 g

2.1.7. Organisms

2.1.7.1. *Agrobacterium tumefaciens*

Strain: *Agrobacterium tumefaciens* AGL1 (BAA-101) (ATCC, Manassas, USA)

Genotype: AGL0 recA::bla pTIBo542deltaT Mop+ CbR (Lazo *et al*., 1991).

Competence: chemo-competent.

The strain possesses resistance to rifampicin and carbenicillin.

2.1.7.2. *Escherichia coli*

Strain: *Escherichia coli* 5-alpha (High Efficiency) [New England Biolabs Inc., Beverly, USA]

Genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.*

Competence: chemo-competent.

The strain possesses resistance to streptomycin.

Strain: *Escherichia* coli XL1-Blue (Stratagene, La Jolla, USA)

Genotype: endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F' [::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+) (Bullock *et al*., 1987).

Competence: electro-competent.

The strain possesses resistance to tetracycline.

2.1.7.3. Fungi

Lasiodiplodia theobromae strain LA-SOL3 was isolated from *Vitis vinifera* in Peru (Rodríguez-Gálvez *et al*., 2015). *Lasiodiplodia hormozganensis* strain CBS339.90 was isolated from a phaeohyphomycotic cyst of a human patient from Jamaica (Alves *et al*., 2008). The strain CBS339.90 was obtained from the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre. Both cultures were maintained on PDA medium at 25 °C.

2.1.7.4. Mammalian cell lines

Vero-B4 cells (ACC 33, Lot 11, DSMZ)

This cell line was established from the kidney of an adult African green monkey (*Cercopithecus aethiops*). The cells are adherent-elongated epithelial cells that grow in a monolayer.

Hela S3 cells (ACC 161, DSMZ)

This cell line is a human cancer cell line established from cervical human adenocarcinoma. The cells are adherent and epithelial, growing and forming a monolayer.

2.1.7.5. Test organisms

Standard organisms were tested to uncover the spectrum of antimicrobial activity of crude extracts from *L. hormozganensis* and *L. theobromae* (section 2.2.14.4). For this purpose, a broad spectrum of organisms was used (Table 1.1).

Table 1.1 | Test organisms for antimicrobial activity characterization of *L. theobromae* and *L. hormozganensis*. Information regarding brand, phylogenetic group, and growth medium and temperature is included.

Species	Brand	Taxonomy	Growth medium	Growth temperature
Bacillus brevis	ATCC 9999	Gram-positive bacteria	NB medium	37 °C
Enterobacter dissolvens	LMG 2683	Gram-negative bacteria	NB medium	27 °C
Micrococcus luteus	ATCC 381	Gram-positive bacteria	NB medium	37 °C
Mucor miehei	Tü 284	Fungi	HA medium	37 °C
Nematospora coryli	ATCC 10647	Fungi	HA medium	27 °C
Paecilomyces variotii	ETH 114646	Fungi	HA medium	37 °C

2.1.8. Vectors

pCB1636 (4417 bp)

pcB3616 is a high copy plasmid that possesses ampicillin and hygromycin resistance. The latter resistance is due to the Hygromycin B resistance cassette (Sweigard *et al*., 1997), controlled by the TrpC promoter.

pSJ-basic (5923 bp)

pSJ-basic was used as a backbone vector for the construction of the vector used in gene target replacement via *Agrobacterium tumefaciens*-mediated fungal transformation. This plasmid (Figure 1.1) can be replicated in both *E. coli* and in *A. tumefaciens*. pVS1 StaA increases stability during the segregation of the vector in *A. tumefaciens*. pVS1 RepA helps the replication of low-copy plasmids in *A. tumefaciens*. During ATMT, the DNA fragment (HPT cassette and flanking regions of *ssd1* gene), located between the left- and right-border repeats, is transferred into LA-SOL3 and CBS339.90 strains genome. The vector contains the kanamycin resistance gene (encoding aminoglycoside phosphotransferase that inactivates kanamycin), which is important for the selection of bacterial transformants. Moreover, pSJ-basic carries the Hygromycin B resistance cassette (*hph* gene*,* which encodes for hygromycin phosphotransferase, controlled by the TrpC promoter), essential for the selection of the fungal transformants given that wild-type fungi are susceptible to hygromycin.

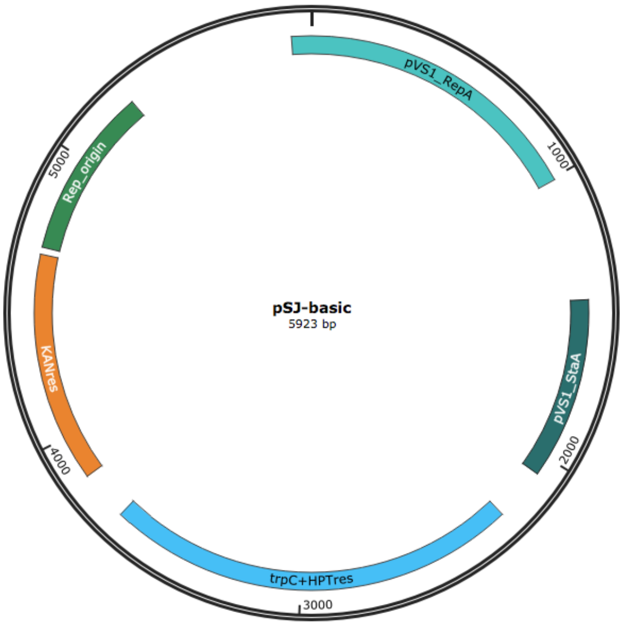


Figure 1.1 | pSJ-basic plasmid map.

2.1.9. Oligonucleotides

All Gibson primers used were created using the NEBuilder® HiFi DNA Assembly program (New England Biolabs). These primers were developed by the company Eurofins Genomics, Ebersberg, Germany (Tables 1.2 and 1.3).

Table 1.2 | Primers used for *ssd1* gene inactivation in *Lasiodiplodia hormozganensis* strain CBS339.90. Lowercase letters refer to overlapping oligonucleotides. Uppercase letters equal to gene-specific primer regions.

Oligoname	Sequence (5’-> 3’)	Annealing temperature (°C)
Flank1_SSD1_CBS_Fwd	gcaggatatattgtggtgtaaacaaCGGGGCCTTTTTGCTGAG	67.9
Flank1_SSD1_CBS_Rev	agttaacgtcgacCTCGCGCACCTACGAGGC	
HPT_pSJ_Fwd	taggtgcgcgagGTCGACGTTAACTGATATTGAAG	59.2
HPT_pSJ_Rev	tcgtcatgccgcGGTCGACGTTAACTGGTTC	
Flank2_SSD1_CBS_Fwd	gttaacgtcgaccGCGGCATGACGATCAGACG	70.5
Flank2_SSD1_CBS_Rev	aaacgctcttttctcttaggtttacctgcaTCTTCTGGCCGGAACGTGGGCGAGCCGACC	72

Table 1.3 | Primers used for *ssd1* gene inactivation in *Lasiodiplodia theobromae* strain LA-SOL3. Lowercase letters refer to overlapping oligonucleotides. Uppercase letters equal to gene-specific primer regions.

Oligoname	Sequence (5’-> 3’)	Annealing temperature (°C)
Flank1_SSD1_LA_Fwd	gcaggatatattgtggtgtaaacaaCGGGGCCTTTTTGCTGAG	67.9
Flank1_SSD1_LA_Rev	gtatcgataagcttgCTCGCGCACCTACGAGGCGTCGATGCACGC	72
HPT_cassette_Fwd	tcgtaggtgcgcgagCAAGCTTATCGATACCGTCGACGTTAACCT	71.7
HPT_cassette_Rev	tgatcgtcatgccgcCGTTAACGTCGAGAGAAGATGATATTGAAG	
Flank2_SSD1_LA_Fwd	ctctcgacgttaacgGCGGCATGACGATCAGACGTCTACATGGCCG	72
Flank2_SSD1_LA_Rev	aaacgctcttttctcttaggtttacctgcaTCTTCTGGCCGGAACGTGGGCGAGCCGACC	

2.2. Methods

2.2.1. Measurements of DNA concentration and purity

DNA quantification was evaluated with spectrophotometry (UV/vis) through the use of NanoDrop 1000 software V3.8.1. The absorbance ratio between 260 and 280 nm is useful to evaluate DNA purity, with values around 1.8 representing a higher level of purity. Qubit^{TM} 4 Fluorometer was also used for the measurement of DNA concentration in samples needed for the vector construction (sections 2.2.5, 2.2.7), i.e. the flanks, HPT cassette and backbone vector.

2.2.2. Isolation of genomic DNA from CBS339.90 and LA-SOL3 strains

Genomic DNA ($_g$DNA) of *Lasiodiplodia theobromae* strain LA-SOL3 and *L. hormozganensis* strain CBS339.90 was obtained. For this purpose, 5 plugs of each fungus were inoculated on 250 mL of YMG media and incubated at 22 °C, 220 rpm, for 3 days. Afterwards, both cultures were filtrated via vacuum filtration and mycelia were freeze-dried by lyophilizing. The material was then placed into safe-lock 2.0 mL tubes (Eppendorf) and turned into powder through the use of Tissuelyser II, according to the manufacturer’s user manual. The resulting material was used for $_g$DNA isolation with the GeneJET Plant Genomic DNA Purification Mini Kit, according to the manufacturer’s instructions. The amount of $_g$DNA was measured with NanoDrop 1000 software.

2.2.3. Production of electro-competent *Escherichia coli* cells

In order to generate electro-competent *Escherichia coli* XL1-Blue cells, the strain was inoculated on LB agar plates and incubated at 37 °C, 48 h. Afterwards, a colony was transferred to

5 mL of LB medium and incubated at 37 °C, 220 rpm, for 16-18 h. 500 mL of LB medium with glucose (20 mM) were inoculated with 2 mL of the culture and incubated at 37 °C, 220 rpm, until optical density at 600 nm reached 0.6-0.8. Thereupon, the culture was kept on ice, followed by centrifugation at 4000 rpm, for 15 min, at 4 °C. The supernatant was discarded and the pellet was resuspended in 200 mL of ice cold 10 % (v/v) glycerol. Centrifugation was repeated and the cell pellet resuspended in 50 mL of 10 % (v/v) glycerol, with a following centrifugation at 4000 rpm, for 10 min, at 4 °C. The cells were then resuspended in 2 mL of ice cold 10 % (v/v) glycerol and 80 µL were aliquoted to pre-chilled 1.5 mL tubes and stored at -80 °C.

2.2.4. Transformation of electro-competent *Escherichia coli* cells

Electro-competent *E. coli* XL1-Blue cells were transformed with the vectors pSJ-basic and pCB1636, according to several protocols (Fiedler & Wirth, 1998; Gonzales *et al.*, 2013), with some modifications. For this purpose, 1 µL of each vector was added to *E. coli* XL1-Blue electro-competent cells, and homogenized. The mixture was placed on a pre-chilled electroporation cuvette (1mm, Cell projects Ltd) and an electric chock (capacity 25 µF, resistance 200 Ω, voltage 1.3 kV) was applied with the Electroporation System Gene Pulser II. The time range was 4-5 ms. Then, 200 µL of SOC medium were added to each cuvette and mixed. The solution was placed in 10 mL tubes and incubated at 37 °C, for 1 h, in an orbital shaker at 220 rpm. Finally, the mixture was placed on LA medium plates (with 30 µg/mL kanamycin or 50 µg/mL of ampicillin for pSJ-basic and pCB1636 vectors, respectively) and incubated at 37 °C during 24 h. After bacterial growth, a colony, potentially transformed with pSJ-basic or pCB1636 vectors, was transferred to 5 mL of LB medium with 60 µg/mL kanamycin or 100 µg/mL of ampicillin, respectively. The solution was incubated at 37 °C, 220 rpm, overnight. In the end, each vector was isolated through the use of GeneJET™ Plasmid Miniprep kit and the concentration was measured using NanoDrop 1000 Spectrophotometer V3.8.1.

2.2.5. Phusion Polymerase Chain Reaction (PCR)

Phusion PCR was performed with Phusion High-Fidelity DNA Polymerase as a goal to amplify the Gibson assembly fragments. All amplifications occurred in GeneAmp PCR System 9700. The flanking regions were amplified from the genomic DNA of each fungus. For LA-SOL3 and CBS339.90 strains, HPT cassette was amplified from pCB1636 and pSJ-basic vectors, respectively. The PCR program and master mix were set up based on the general manufacturer's instructions (Tables 1.4, 1.5 and 1.6). For the amplification of HPT cassette and the flanking regions, elongation time was set at 45 and 15 seconds, respectively. Annealing temperature was set according to the primers' annealing temperature.

Table 1.4 | Phusion PCR master mix preparation.

PCR Reagent	Amount
Genomic DNA / plasmid DNA	100 ng / 10 ng
dnTPs	1 µL
Primer Forward	5 µL
Primer Reverse	5 µL
Buffer HF	10 µL
Phusion High-Fidelity DNA Polymerase	0.5 – 0.8 µL
$_{UF}H_2O$	Fill up to 50 µL

Table 1.5 | Phusion PCR program for the amplification of the flanking regions of LA-SOL3 and CBS339.90 strains, and HPT cassette amplification from pSJ-basic vector. T_A stands for annealing temperature. T_E stands for elongation time.

PCR step		Temperature (°C)	Time	Number of cycles
Step 1	Initial denaturation	98	30 seconds	1
Step 2	Denaturation	98	8 seconds	35
	Annealing	T_A	15 seconds	
	Extension	72	T_E	
Step 3	Final extension	72	10 minutes	1

Table 1.6 | Phusion PCR program for HPT cassette amplification from pCB1636 vector.

PCR step		Temperature (°C)	Time	Number of cycles
Step 1	Initial denaturation	98	30 seconds	1
Step 2	Denaturation	98	8 seconds	5
	Annealing	68	15 seconds	
	Extension	72	45 seconds	
Step 3	Denaturation	98	8 seconds	32
	Extension	72	30 seconds	
Step 4	Final extension	72	10 minutes	1

After PCR amplification, an agarose gel electrophoresis was carried out. The desired bands (1431 bp, 1473 bp, 788 bp, 750 bp, 752 bp, and 750 bp, for HPT from pSJ-basic vector, HPT from pCB1636 vector, flank 1 and 2 from CBS339.90, and both flanks from LA-SOL3, respectively) were excised from the gel with a clean scalpel and the DNA fragments were purified with NucleoSpin® Gel and PCR Clean-Up kit. A final concentration of 118 ng/µL, 16.9 ng/µL, 58.6 ng/µL, 85.6 ng/µL, 66.6 ng/µL, and 36.4 ng/µL were respectively obtained for HPT from pSJ-basic vector, HPT from pCB1636 vector, flank 1 and 2 from CBS339.90, and flank 1 and 2 from LA-SOL3.

2.2.6. Agarose gel electrophoresis

The separation of DNA fragments was performed in 1 % agarose gel at a continuous voltage of 90 V, during approximately 1 h. TAE buffer was used to dissolve the agarose. The assignment of each fragment size was possible due to the use of GeneRuler 1 kb DNA Ladder. All samples were dyed with 6X DNA Loading Dye before addition to the agarose gel. After each run, gels were stained with ethidium bromide solution (5 µg/mL), followed by washing with water for 15 min. Gel documentation and UV irradiation (365 nm) were evaluated using a UV Reader.

2.2.7. Isolation of backbone vector via restriction enzyme digestion

pSJ-basic was used as backbone vector for the construction of the binary vector. This vector was cut at 2242 and 3682 bp by *Bgl*II and *Pst*I restriction enzymes, respectively, generating two DNA fragments with 4483 and 1440 bp. The following reaction was prepared: 10 µL of pSJ-basic vector (313 ng/µL), 2.5 µL of *Bgl*II, 2.5 µL of *Pst*I, 5 µL of 10X Buffer O, and 30 µL of $_{UF}H_2O$. This reaction mixture was submitted to a water bath for 1 h at 37 °C, in agreement with the manufacturer's recommendations. Then, an electrophoresis gel (1 % agarose in TAE buffer) was performed, at 90 V, for 1 h. The desired band (4483 bp) was cut and the DNA fragment was extracted with NucleoSpin® Gel and PCR Clean-Up kit. A final concentration of 66.6 ng/µL was obtained.

2.2.8. Vector construction for *ssd1* inactivation via Gibson Assembly

The Gibson Assembly method was created in 2009 by Daniel Gibson (Gibson *et al*., 2009). This seamless technique allows the assembly of several DNA fragments by overlaps through the use of DNA polymerase, 5' exonuclease and DNA ligase, in an isothermal single-reaction (Figure 1.2) [Li & Elledge, 2007].

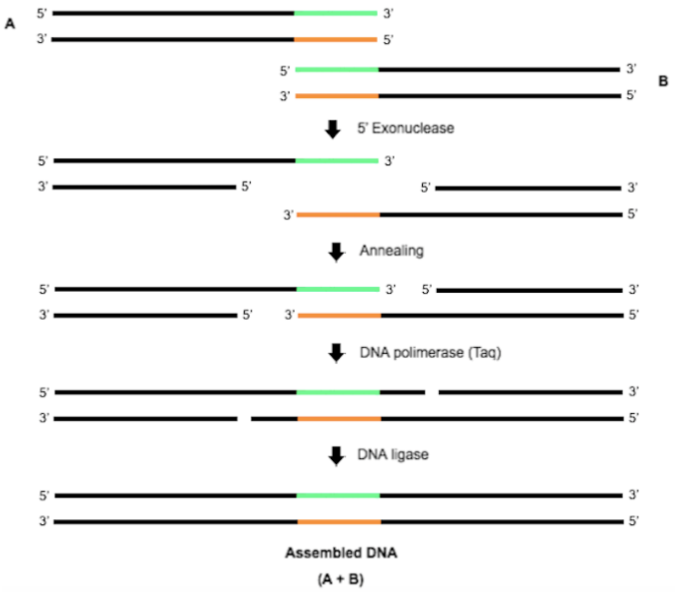


Figure 1.2 | Gibson assembly principle. The 5' ends of the $_{ds}$DNA fragments are degraded by 5' exonuclease, resulting in complementary overlaps between the fragments after annealing. DNA polymerase extends the 3' ends of the strands and DNA ligase seals the nicks. This results in a $_{ds}$DNA fragment that is constituted by the assembled fragments.

All Gibson primers were created using the NEBuilder® HiFi DNA Assembly program (New England Biolabs) [section 2.1.9]. The first step was the extraction of the DNA fragments using Phusion PCR (section 2.2.5). The fragments were isolated with the NucleoSpin® Gel and PCR Clean-Up kit. Gibson Assembly was performed according to the instructions of the Gibson Assembly Cloning Kit Manual from New England Biolabs. Each mixture (Table 1.7) was placed at 50 °C for 1 h, in order to construct the binary vector for CBS339.90 and LA-SOL3 strains (Figure 1.3). The vectors were entitled pSJ-SSD1-HPT.

Table 1.7 | Master mix used for Gibson Assembly of the vector pSJ-SSD1-HPT.

Components	**CBS339.90 strain**		**LA-SOL3 strain**	
	Initial concentration (ng/µL)	Used volume (µL)	Initial concentration (ng/µL)	Used volume (µL)
Backbone vector	66.6	6.5	66.6	2.7
HPT cassette	118	1.63	16.9	4.5
Flank 1	58.6	0.78	66.6	0.66
Flank 2	85.6	0.91	36.4	1.95
NEBuilder HiFi DNA Assembly Master Mix	-	10	-	10

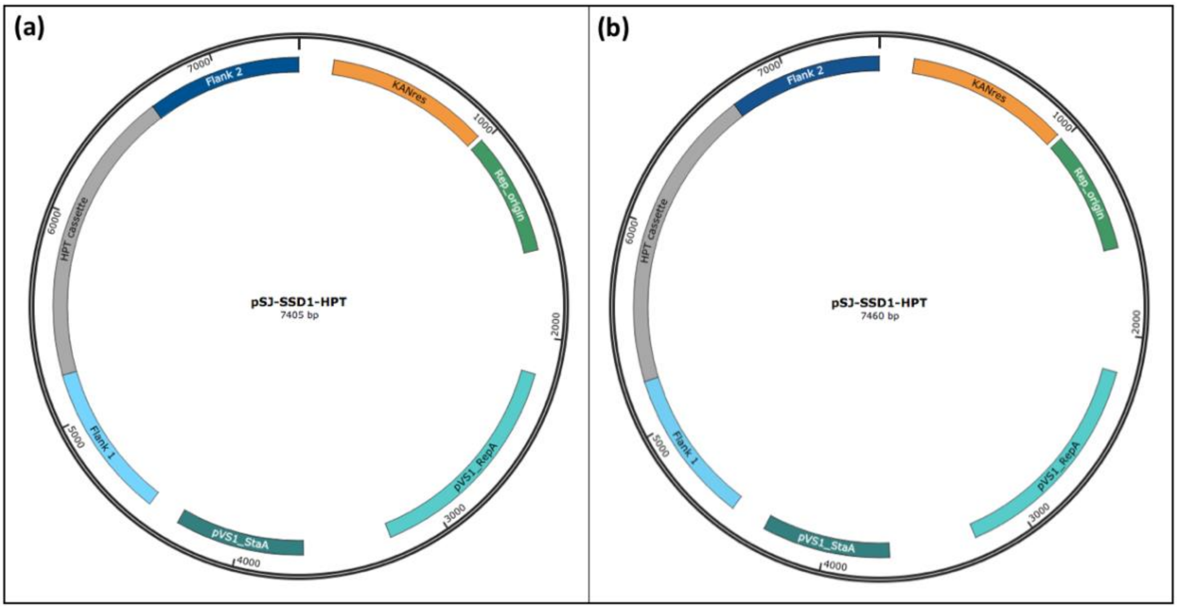

(a)
pSJ-SSD1-HPT
7405 bp
Flank 2
KANres
Rep_origin
pVS1_RepA
pVS1_StaA
Flank 1
HPT cassette
1000
2000
3000
4000
5000
6000
7000
(b)
pSJ-SSD1-HPT
7460 bp
Flank 2
KANres
Rep_origin
pVS1_RepA
pVS1_StaA
Flank 1
HPT cassette
1000
2000
3000
4000
5000
6000
7000

Figure 1.3 | pSJ-SSD1-HPT plasmid map for *Lasiodiplodia hormozganensis* strain CBS339.90 **(a)** and *L. theobromae* strain LA-SOL3 **(b)**.

2.2.9. Transformation of chemo-competent *Escherichia coli* cells

Chemo-competent *E. coli* 5-alpha (High Efficiency) cells were obtained from New England Biolabs. The cells were transformed with the vectors pSJ-SSD1-HPT prepared for the strains CBS339.90 and LA-SOL3, according to the manufacturer's instructions. First, 2 µL of each vector were added to previously thawed *E. coli* cells, and homogenized. Secondly, the mixtures were submitted to subsequent heat-shock reactions, through the respective placement on ice for 30 min, followed by incubation at 42 °C for 30 sec, and relocation to ice for 2 min. Afterwards, 950 µL of SOC medium were added and the homogenized mixtures were transferred to 10 mL tubes, and incubated at 37 °C, 220 rpm, for 1 h. The bacterial suspensions were applied on LA medium plates (with 30 µg/mL kanamycin) and incubated at 37 °C, for 24 h. After bacterial growth, a colony was transferred to 5 mL of LB medium with 30 µg/mL kanamycin and incubated at 37 °C, 220 rpm, overnight. A total of 10 colonies were used in the last step. The plasmids were isolated with GeneJET^{TM} Plasmid Miniprep kit and the concentration was measured using NanoDrop 1000 Spectrophotometer V3.8.1 and Qubit. The amounts considered to the following steps were the values collected with Qubit, given that in general values were lower compared to the ones obtained with NanoDrop 1000 Spectrophotometer.

2.2.10. Restriction enzyme digestion of pSJ-SSD1-HPT vector

The success of the assembly was evaluated through restriction of plasmid DNA by fast digest restriction enzymes. Incubation conditions were carried out according to the manufacturer's instructions, in specific proportions (Table 1.8). Hence, incubation was conducted at 37 °C for 20 min, followed by electrophoresis gel (1 % agarose in TAE buffer) to observe the amplified bands.

Table 1.8 | Restriction reaction mix.

Components	**Strain CBS339.90**						**Strain LA-SOL3**			
Restriction enzyme(s)	*Nco*I	*Fsp*I	*Pvu*I	*Mlu*I	*Mlu*I *Pvu*I	*Mlu*I *Fsp*I	*Bgl*II	*Fsp*I	*Nco*I	*Bgl*II *Nco*I
Amount of restriction enzyme	1 µL				1 µL each		1 µL			1 µL each
DNA (250 – 500 ng)	1.5 µL						2.5 µL			
10X Fast Digest Green Buffer	2 µL									
$_{UF}H_2O$	Fill up to 20 µL									

The pSJ-SSD1-HPT vector for CBS339.90 was digested with *Nco*I, *Pvu*I, *Mlu*I and *Fsp*I restriction enzymes. *Nco*I originates 2 DNA fragments with 6136 and 1269 bp, since it cuts in Flank 2 and HPT cassette. *Pvu*I cuts in Flank 1 and HPT cassette, originating 2 DNA fragments with 6012 and 1393 bp. *Mlu*I and *Fsp*I are able to cut in Flank 2 and in the backbone vector, respectively, originating one DNA fragment that corresponds to the total plasmid size, 7405 bp. A mixture of *Mlu*I with *Pvu*I was used, as a goal to obtain 3 DNA fragments of 4785, 1393 and 1227 bp. Multiple digestion with *Mlu*I and *Fsp*I, results in 2 fragments, 4881 and 2524 bp, if the DNA sequences are correct. On the other side, the constructed vector for LA-SOL3 was digested with *Bgl*II, *Fsp*I and *Nco*I. Digestion by *Nco*I on HPT cassette and Flank 2 originates DNA fragments of 6168 and 1292 bp. *Fsp*I and *Bgl*II are able to cut DNA in the backbone vector and Flank 2, respectively, originating a linear DNA fragment of 7460 bp. Multiple digestion with *Nco*I and *Bgl*II leads to the appearance of 3 DNA fragments with 5328, 1292 and 840 bp.

2.2.11. Transformation of *A. tumefaciens* cells with pSJ-SSD1-HPT vectors

An aliquot of chemo-competent *Agrobacterium tumefaciens* AGL1 cells (50 µL) was thawed on ice. Two µg of each pSJ-SSD1-HPT vector was added to the cells and gently mixed. The cells were submitted to heat-shock reactions, by incubation at 37 °C for 5 min followed by placement of the cells on ice for 2 min. Subsequently, the cells were transferred to 10 mL tubes with 300 µL of LB medium, and incubated at 28 °C, 220 rpm, for 3-4 h. Then, 100 µL of bacterial suspension were transferred to LA medium plates (with 60 µg/mL of rifampicin and kanamycin) and incubated at 28 °C. After bacterial growth, several colonies were transferred to 10 mL tubes with 5 mL of LB medium supplemented with 60 µg/mL of rifampicin and kanamycin. The bacterial suspension was incubated at 28 °C, 120 rpm, overnight.

2.2.12. Obtaining fungal spores

Spores (conidia) from *L. theobromae* strain LA-SOL3 and *L. hormozganensis* strain CBS339.90 were obtained according to Muniz *et al*. (2014), with slight modifications. Fungi were grown on Minimal medium plates with autoclaved pine needles, at 25 °C, under artificial light, for about 8 days, until transparent spores were observed on the macroscope. Afterwards, sterile $_{di}H_2O$ was added to 10 plates of each fungus and with the help of a L-shaped cell spreader, spores were gently released. The fungal suspension was filtered with a Myracloth filter (Calbiochem) and centrifuged at 4000 rpm for 10 min (Centrifuge Jouan GR4 22, Axon) to separate fungal spores from hyphae and media. The pellet was resuspended in sterile $_{di}H_2O$ and through the use of a Neubauer chamber, spores were counted and spore density was adjusted with $_{di}H_20$ to 10^7 spores/mL.

2.2.13. Transformation of *L. theobromae* and *L. hormozganensis*

Transformation of conidia of *L. theobromae* strain LA-SOL3 and *L. hormozganensis* strain CBS339.90 was performed via *Agrobacterium tumefaciens*-mediated transformation method (de Groot *et al*., 1998; Utermark & Karlovsky, 2008). The method was performed according to Muniz *et al*. (2014), with slight modifications. The bacterial cultures transformed with pSJ-SSD1-HPT vectors (section 2.2.11) were centrifuged at 6000 rpm, for 5 min (Centrifuge Jouan GR4 22, Axon). The supernatants were discarded, the pellets resuspended in 500 µL of AIM medium and subsequently transferred to 10 mL tubes with 4.5 mL of AIM medium, followed by incubation at 28 °C, 160 rpm, for 6-7 h. Thereafter, each fungal suspension (section 2.2.12) was mixed in a proportion of 1:1 with the bacterial suspension, and the mixtures were placed on the edges of cellulose nitrate filters (0.45 µm pore size, Sartorius Stedim Biotech GmbH, Goettingen) on top of agar plates of AIM medium with 200 mM acetosyringone. Each mixture was applied on 5 plates. After evaporation, the plates were incubated at 28 °C for 48 h in dark conditions. Then, the filters were transferred to plates with selection medium to inhibit bacterial growth – PDA or Minimal medium with 100 mg/L of hygromycin, 300 mg/L cefotaxime, 200 mg/L streptomycin - followed by incubation at 28 °C, for 15 days. Previously, a hygromycin susceptibility test was performed in both fungi, based on hyphae growth. The fungi were inoculated in 6-well plates on PDA, with different concentrations of hygromycin: 0, 10, 20, 50, 75 and 100 mg/L, and incubated at 25 °C for 15 days (Appendix). The concentration of hygromycin used on the selection media was defined according to these results. The assay was performed in triplicate.

2.2.14. Characterization of wild-type fungi

Cytotoxicity and antimicrobial activity assays were performed with the crude extracts from both fungi grown on different media (section 2.2.14.1). HPLC analysis of the crude extracts was also performed, in order to understand the metabolic profile of each fungus.

2.2.14.1. Small-scale fermentations

Small-scale fermentations of LA-SOL3 and CBS339.90 strains were performed in five different media, according to Schoettler *et al*. (2006). For this purpose, each fungus was grown on PDA plates until optimum growth. Then, 5 plugs of mycelium from the leading edge of the plate were inoculated in 500 mL of BAF, YMG and DM media, MM, and MM+ in 1 L baffled erlenmeyer flasks, and incubated at room temperature in agitation (220 rpm). The pH and glucose level of the cultures were checked each couple of days using a pH meter and Glucose Urine Test Strip, DUS G, respectively. When glucose was absent, the fungal culture was submitted to centrifugation at 4000 rpm for 10 min (Centrifuge Jouan GR4 22, Axon), leading to the separation of mycelia and culture filtrate.

2.2.14.2. Extraction of crude extracts

Liquid-liquid extraction of crude extracts (Schoettler *et al*., 2006) from the culture filtrate was performed with ethyl acetate (1:1), using a separation funnel. The organic phase was dried with Na_2SO_4 and the ethyl acetate fraction was collected and evaporated using a rotary evaporator *in vacuo* at 40 °C. Mycelia (section 2.2.14.1) was freeze-dry lyophilized on a freeze dryer (Christ® Gamma 1-20). Thereafter, solid-liquid extraction of the mycelium crude extracts was performed with methanol. For this purpose, first, mycelia covered on methanol in erlenmeyer flasks were incubated at room temperature, for 30 min, in agitation (220 rpm). Afterwards, the mycelia were discarded through filtration, and the methanol fractions were evaporated with a rotary evaporator.

Methanol was used to re-dissolve all dry crude extracts. The amount of each crude extract was measured after methanol evaporation in Speedvac Jouan RC10.22.

2.2.14.3. Cytotoxicity assays

Morphological observation-based cytotoxicity was evaluated in mammalian (Vero-B4) and human cancer (Hela S3) cell lines, according to Zapf *et al*. (1995), with slight modifications. First, 200 µL of cells (2×10^4 cells/mL of Vero-B4 cells; 1.5×10^4 cells/mL of Hela S3 cells) in supplemented Dulbecco's Modified Eagle's Medium (DMEM) [with 10 % Fetal Bovine Serum (FBS), 65 µg/mL penicillin G and 100 µg/mL streptomycin sulfate] were seeded in 96-well plates. The plates were incubated for 24 h in 5 % CO_2, 37 °C. 100 µg of each crude extract in methanol were applied on wells of 96-well plates. After solvent evaporation, 200 µL of supplemented DMEM were added to each well, and the plates were gently agitated for 5 min. Then, cells were exposed to the samples, in duplicate, through media replacement, followed by incubation for 72 h, at 37 °C, in 5 % CO_2. Cell viability was evaluated under microscopic observation of cell morphology, comparing with a positive (cells exposed with cycloheximide - 10 µg/mL – and camptothecin – 5 µg/mL) and negative (non-exposed cells) control.

2.2.14.4. Agar disk-diffusion assay

One common approach to screen *in vitro* antimicrobial activity is the agar disk-diffusion assay (Balouiri *et al*., 2016). This method was developed in 1940 (Heatley, 1944). For this protocol, agar plates were inoculated in optimum conditions, with test organisms (section 2.1.7.5): *Bacillus brevis*, *Micrococcus luteus*, *Enterobacter dissolvens*, *Nematospora coryli*, *Paecilomyces variotii* and *Mucor miehei*. Each crude extract was previously solved in 500 µL of methanol. Antibiotic assay disks (diam. 6 mm, Macherey-Nagel GmbH) containing 10 µL of the crude extracts were applied to the agar plates. The samples obtained when fungal growth was on Minimal medium were an exception given the low amount of crude extract, thus, 10 µL were continually applied 5 times to the disks. As a positive control, streptomycin was used for *Bacillus brevis*, *Micrococcus luteus* and *Enterobacter dissolvens*, and hygromycin was used for *Nematospora coryli*, *Paecilomyces variotii* and *Mucor miehei*. The agar plates were incubated for 24 h, at 37 °C for *Bacillus brevis*, *Micrococcus luteus*, *Paecilomyces variotii* and *Mucor miehei*, or 27 °C for *Enterobacter dissolvens* and *Nematospora coryli*. Inhibition growth zones were measured by diameter, given that antimicrobial agents diffuse into the agar and inhibit the microorganism growth.

2.2.14.5. Analytical HPLC of crude extracts

HPLC is a technique used to separate, identify and quantify components in a given mixture. A pump moves the solvent, which creates a mobile phase that allows sample transport in the HPLC system. On the column, the phase becomes stationary so that separation by chromatography is possible. Compounds identification is based on retention times on the column. A chromatogram constituted by several peaks, which may correspond to different sample components, is obtained. Furthermore, the components are eluted from the column by decreasing the polarity of the mobile phase. A UV spectra of the components can be obtained given that a source of UV light is applied during their elution, with a UV detector. Therefore, peak areas can be used to determine the nature of the components.

HPLC was used to analyze the crude extracts (section 2.2.14.2). The system was coupled with a diode array detector (DAD), which allowed retrieving a visual wavelength and UV-spectra. The separation was carried out in a RP18 separation column (LiChroCART® 125-4, LiChrospher® 100, 125 x 4 mm; 5µm, Merck, Darmstadt) at 40 °C. The gradient (Table 1.9) was performed at a flow rate of 1 mL/min using the eluents $_{UF}H_2O$ with 0.1 % TFA (eluent A) and MeCN (eluent B). The injection volume was 5 µL. The dry crude extracts were solved in 500 µL of methanol, followed by centrifugation (Centrifuge 5415 D, Eppendorf AG, Hamburg) at 13200 rpm, for 2 min. Then, 50 µL of each sample were transferred to 0.2 mL HPLC vials.

Table 1.9 | Standard gradient for measurement of crude extracts on analytical HPLC.

Time (min)	Eluent A (%)	Eluent B (%)
0	99	1
20	0	100
24	0	100
25	99	1

3. RESULTS AND DISCUSSION

3.1. Generation of pSJ-SSD1-HT deletion vectors

The pSJ-SSD1-HPT vectors were designed for *ssd1* gene inactivation in strains CBS339.90 and LA-SOL3, by replacing this gene with Hygromycin B resistance cassette. The homologous recombination would be mediated by DNA sequences homologous to the surrounding regions of SSD1 gene (Flank 1 and 2), which were bordering the HPT cassette in the designed vectors.

The vectors were generated and the success was evaluated through restriction analysis of the plasmid DNA (Figure 1.4). With regards to the deletion vector for CBS339.90, 9 samples (of a total of 10) obtained after cloning in *E. coli* cells and subsequent plasmid isolation were used, given that sample 2 had a null amount of plasmid DNA. The vectors were first digested with *Nco*I (Figure

1.5). The sizes of the restriction fragments were correct for all samples except the tenth. Hence, the correct samples (1; 3-9) were further digested with *Pvu*I, *MIu*I and *Fsp*I restriction enzymes (Figure 1.6). All restriction fragments obtained corresponded to the expected fragment sizes. The 10 vector samples for LA-SOL3 were digested with *Bgl*II, *Fsp*I and *Nco*I restriction enzymes (Figure 1.7). All the restriction fragments corresponded to the expected sizes, except sample 4 and 5. Sample 9 -well B- was not correctly applied on the gel, therefore, no bands appeared. However, all remaining restriction fragments' size of this sample were correct.

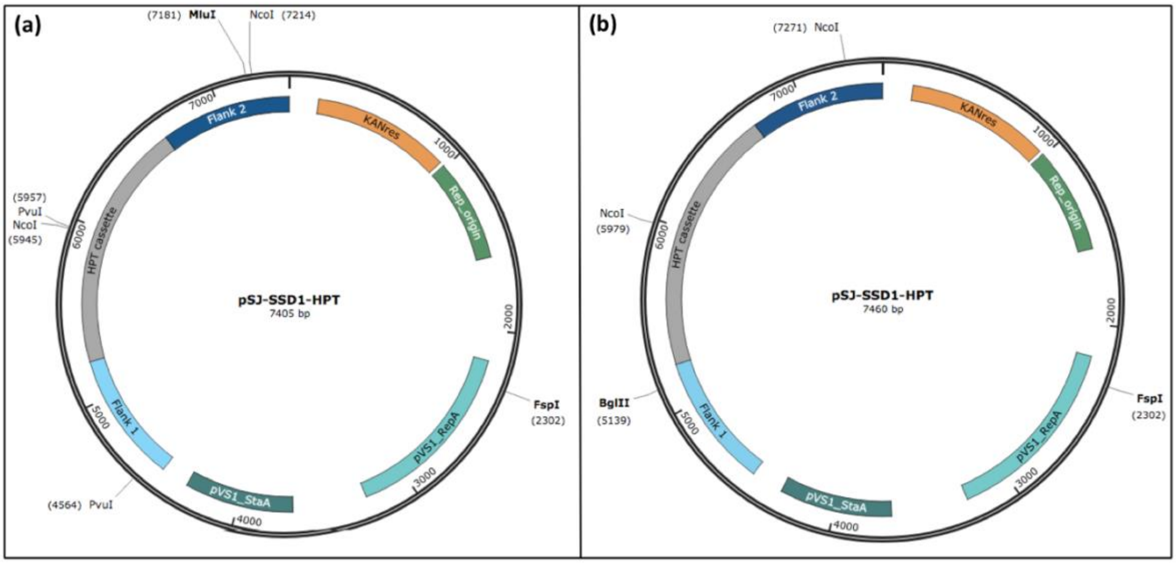

(a)
(7181) MluI
NcoI (7214)
(5957) PvuI
NcoI (5945)
Flank 2
KANres
Rep_origin
HPT cassette
pSJ-SSD1-HPT
7405 bp
FspI (2302)
Flank 1
pVS1_RepA
pVS1_StaA
(4564) PvuI
7000
1000
2000
3000
4000
5000
6000
(b)
(7271) NcoI
NcoI (5979)
Flank 2
KANres
Rep_origin
HPT cassette
pSJ-SSD1-HPT
7460 bp
FspI (2302)
BglII (5139)
Flank 1
pVS1_RepA
pVS1_StaA
7000
1000
2000
3000
4000
5000
6000

Figure 1.4 | Scheme of pSJ-SSD1-HPT map, of CBS339.90 **(a)** and LA-SOL3 **(b)**, including restriction sites.

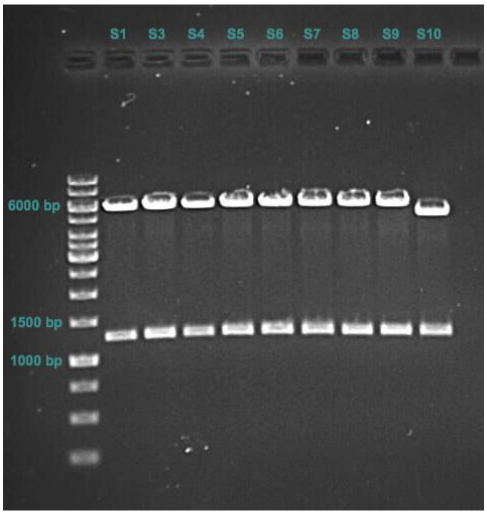

S1
S3
S4
S5
S6
S7
S8
S9
S10
6000 bp
1500 bp
1000 bp

Figure 1.5 | Restriction analysis after digestion of pSJ-SSD1-HPT for CBS339.90 with *Nco*I. The first well corresponds to 1 Kb ladder. S1-S10 correspond to the 9 samples containing the vector.

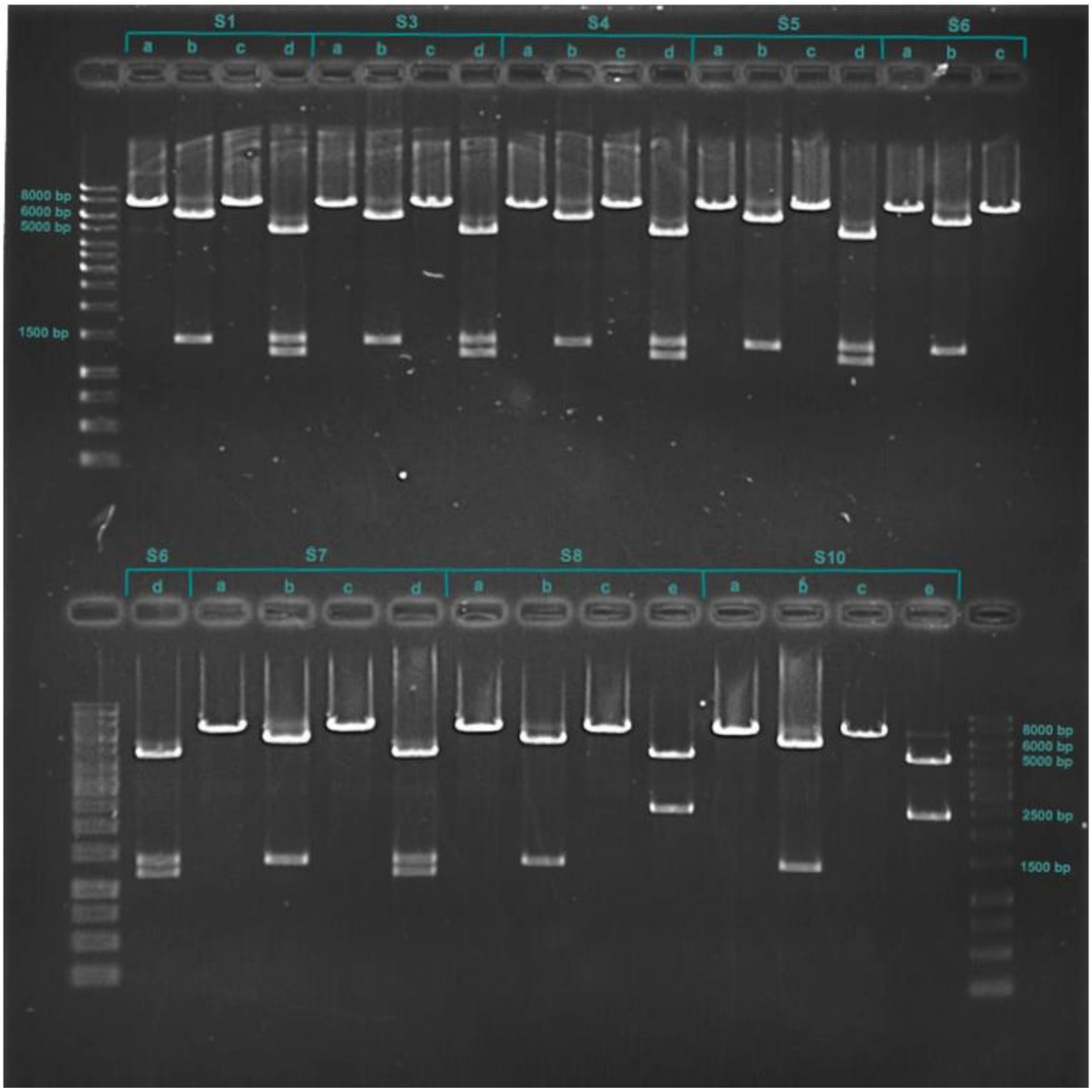

S1
a
b
c
d
S3
a
b
c
d
S4
a
b
c
d
S5
a
b
c
d
S6
a
b
c
8000 bp
6000 bp
5000 bp
1500 bp
S6
d
S7
a
b
c
d
S8
a
b
c
e
S10
a
b
c
e
8000 bp
6000 bp
5000 bp
2500 bp
1500 bp

Figure 1.6 | Restriction analysis after digestion of pSJ-SSD1-HPT for CBS339.90. The vector was digested with *Fsp*I (letter a), *Pvu*I (letter b), *Mlu*I (letter c), and a mixture of *Mlu*I with *Pvu*I (letter d) or *Fsp*I (letter e). The top left and bottom left and right wells correspond to 1 kb ladder. S1-S10 refer to all 9 vector samples.

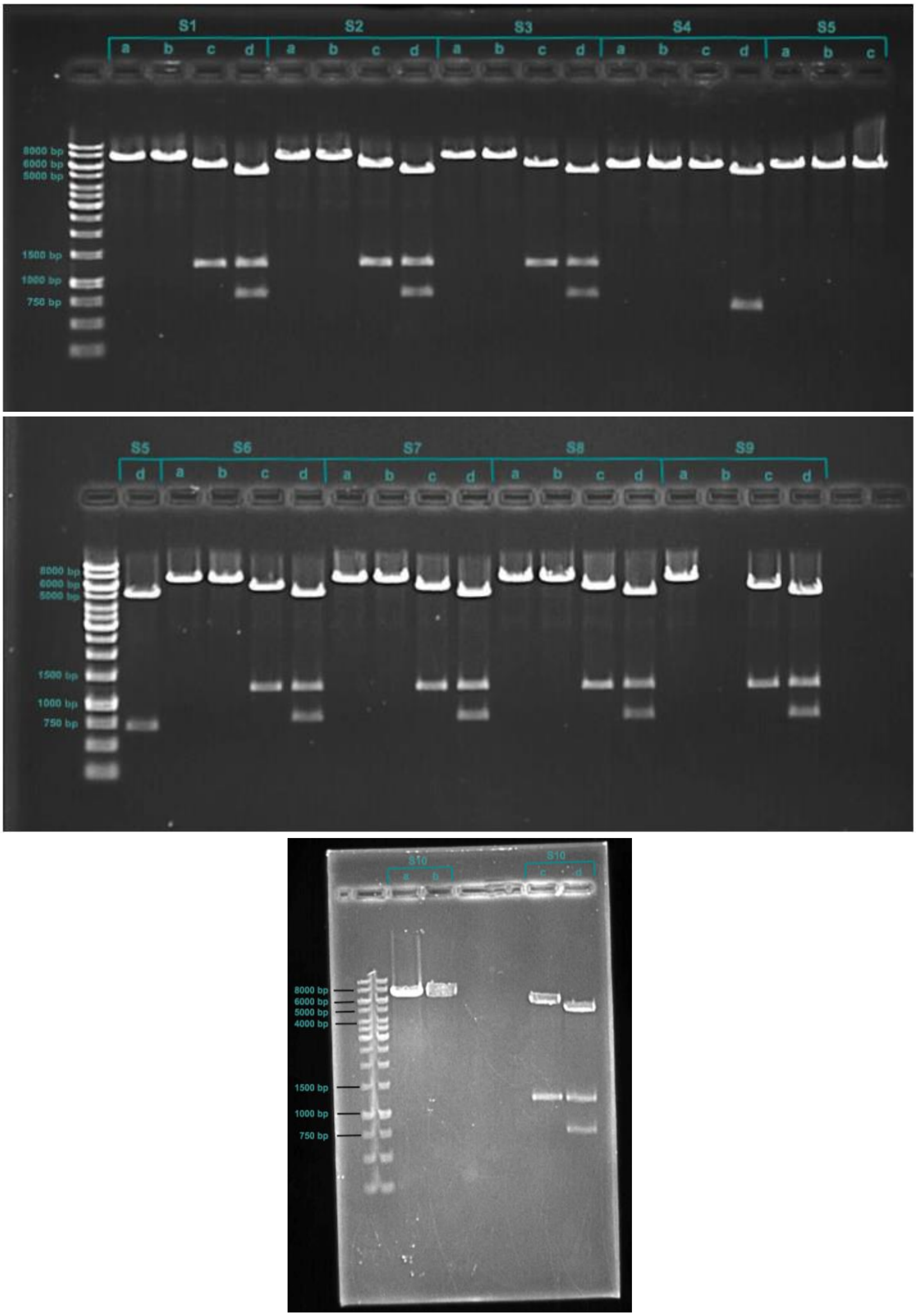

S1
a
b
c
d
S2
a
b
c
d
S3
a
b
c
d
S4
a
b
c
d
S5
a
b
c
8000 bp
6000 bp
5000 bp
1500 bp
1000 bp
750 bp
S5
d
S6
a
b
c
d
S7
a
b
c
d
S8
a
b
c
d
S9
a
b
c
d
8000 bp
6000 bp
5000 bp
1500 bp
1000 bp
750 bp
S10
a
b
S10
c
d
8000 bp
6000 bp
5000 bp
4000 bp
1500 bp
1000 bp
750 bp

Figure 1.7 | Restriction analysis after digestion of pSJ-SSD1-HPT for LA-SOL3. The vector was digested with *Bgl*II (letter a), *Nsb*I (letter b), *Nco*I (letter c), and a mixture of *Bgl*II and *Nco*I (letter d). The left wells correspond to 1 Kb ladder while the remaining correspond to the 10 vector samples (S1-S10).

As mentioned in the last paragraph, the constructed vectors were successfully cloned and their sequences were confirmed by restriction analysis. The correct vectors were further used for *Agrobacterium tumefaciens*-mediated transformation of *L. theobromae* strain LA-SOL3 and *L. hormozganensis* strain CBS339.90. However, so far, no transformants were isolated. In this section, several hypotheses that could explain this fact, will be discussed.

The pitfalls of ATMT in fungi are the optimization of the protocol and the fact that it is time-consuming, since the optimal conditions vary among different species (Li *et al*., 2017; Michielse *et al*., 2005; Tzima *et al*., 2014). Several parameters are important for protocol optimization, e.g. the concentration of acetosyringone, the pH of the co-cultivation medium, the fungal inoculum, the ratio of bacterial and fungal cells, and the duration, temperature and type of membranes used during co-cultivation (Li *et al*., 2017; Michielse *et al*., 2005; Sørensen *et al*., 2014; Tzima *et al*., 2014). In this workflow, acetosyringone (0.2 mM) was added to the co-culture medium, in order to fully induce virulence gene expression by *Agrobacterium* and consequently lead to the transfer of T-DNA into each fungus. Acetosyringone could be one of the factors that lead to the null transformation rate in this work. In fact, several authors have suggested that the concentration of acetosyringone influences ATMT efficiency in fungi: e.g., *Fusarium avenaceum*, *Lentinula edodes* and *Thielaviopsis basicola* (Sørensen *et al*., 2014; Tzima *et al*., 2014; Yan *et al*., 2019). Moreover, pre-induction (addition of acetosyringone before co-culture) has been correlated to transformation efficiency (Mullins *et al*., 2001; Rho *et al*., 2001; reviewed by Tzima *et al*., 2014).

Muniz and coworkers (2014) showed that *L. theobromae* was successfully transformed via ATMT, with 48 h of co-culture at 22 °C. Temperature and period of co-culture have been suggested to affect the efficiency of ATMT protocol (Li *et al*., 2017; Michielse *et al*., 2005; Sørensen *et al*., 2014). For example, Sørensen and colleagues (2014) observed that higher co-culturing times increase the transformation frequency in *F. avenaceum*. In the present work, co-culturing was performed for 48 h at 28 °C. Hence, possible alterations for protocol optimization could rely on assays with co-culturing for 48, 72 and 96 h. The temperature of co-incubation is known to be generally optimal at 20-25 °C (reviewed by Hooykaas *et al*., 2018), which is in accordance with the results from Muniz *et al*. (2014) for *L. theobromae*. Hence, co-culture of strains LA-SOL3 and CBS339.90 could be attempted at this range of temperatures.

The fungal inoculum is also a major factor for the success of ATMT in fungi (Li *et al*., 2017; Sørensen *et al*., 2014). The number of spores used in the experiment can alter the transformation efficiency. According to literature, high transformation rates are usually obtained with 10^5-10^7 spores/mL (reviewed by Li *et al*., 2017). Moreover, Muniz *et al.* (2014) used 10^7 spores/mL of *L.*

theobromae. Upon this, in this thesis, the final amount of conidia of *L. theobromae* LA-SOL3 and *L. hormozganensis* CBS339.90 used was 10^7 spores/mL, with 10^5 spores/mL as a first experiment for transformation (with no success). In another perspective, the stage of maturity of the spores could have been one of the causes for the lack of efficiency of the protocols. Throughout this work, firstly, dark-walled conidia obtained after 15-18 days of culture were used for transformation. However, the protocol had no success. Since mature conidia of *Lasiodiplodia* have melanin deposits on the inner surface of the walls (Phillips *et al*., 2013), this was admitted as a likely cause for the lack of success of transformation. Therefore, the protocol was optimized and immature hyaline conidia were obtained after 8 days of culture. However, when the spores were acquired, several dark brown conidia (mature) were seen in suspension through the microscope. This leads to the suggestion that the final suspension corresponded to a mixture of immature and mature conidia. Consequently, this could also affect the ratio of bacterial and target fungal cells. Hence, this could be one of the causes for the null transformation rate. Furthermore, the protocol to obtain spores should be further optimized and several experiments should be used within the range of 10^5-10^7 spores/mL.

In addition, the co-cultivation medium could also need some modifications. Some authors have indicated that the pH of the medium can alter the transformation rate (Li *et al*., 2017; Michielse *et al*., 2005), given that the activation of the *vir* genes of *A. tumefaciens* depends on pH. The optimal pH is usually 5-5.3, depending on *Agrobacterium* strain (reviewed by Hooykaas *et al*., 2018). With regards to the induction medium, in Muniz *et al*. (2014) work, the induction medium contained the selective antibiotics used for the replication of the vector in *A. tumefaciens*. However, in the present work, the induction medium (AIM) had no selective antibiotics, which could have led to the lack of success of transformation. Indeed, the absence of the antibiotics may have resulted in stress conditions for the bacterium and consequently interfering on the transfer of the T-DNA to the fungal genomes.

As another hypothesis, even though restriction digestion confirmed that all fragments were correct in the constructed vectors, the corresponding nucleotide sequence could have errors. In fact, replication errors could have occurred during cloning in *E. coli* or transformation in *A. tumefaciens*, resulting in disruption of homologous recombination in fungi. Therefore, sequencing of the vectors could be conducted to validate the nucleotide sequences, as well as restriction digestion of the vectors after replication in *A. tumefaciens*, to reassure the bacterial colonies incorporated the correct vectors.

3.2. Characterization of wild-type fungi

3.2.1. Cytotoxicity of crude extracts

A total of 10 crude extracts were obtained from *L. theobromae* LA-SOL3 and *L. hormozganensis* CBS339.90 after growth in five different media: BAF medium, minimal medium (MM), minimal medium plus (MM+), double malt (DM) medium and yeast malt glucose (YMG) medium. In order to characterize the phenotype of these fungi, cytotoxicity assays with crude extracts from both isolates were conducted in mammalian (Vero-B4) and human cancer (Hela S3) cell lines (Figure 1.8). Vero cells are non-tumorigenic and therefore, have been successfully used as substrates for human vaccines and cytotoxicity assays with microorganisms with cytotoxic potential (Duarte *et al*., 2015; Félix *et al.,* 2016; 2018a; 2019b; Steil *et al*., 2015). Vero-B4 is a sub-clone cell line that mimics the morphological and growth parameters of the parent cell line (Vero), with a different karyotype (Yasumura *et al*., 1988). Hela S3 cells are human cervical cancer cells frequently used in cytotoxicity assays to study potential anticancer properties of samples, e.g. for anticancer therapeutics (Handayani *et al*., 2018; Schüffler *et al*., 2009).

In search for potential anticancer compounds, several crude extracts from the fungus LA-SOL3 exhibited high activity in Hela S3 cell line cytotoxicity screen (Figure 1.8). Samples from fungal growth on BAF and YMG media showed the highest activities, with crude extracts from the culture filtrate (CE_{CF}) and mycelium (CE_{MY}) leading to decreases of 100 and 75 % on viability, respectively. However, the crude extracts of MM+ and CE_{MY} of MM and DM medium showed no activity. The results suggest that *L. theobromae* LA-SOL3 might produce compounds with toxic properties towards cancer cells, particularly in nutrient-rich environments (BAF and YMG media). Our findings are in agreement with previous publications that showed *L. theobromae* possesses *in vitro* anticancer activity (Deshmukh *et al*., 2018; Jin *et al*., 2011). Indeed, this species has been shown to produce cholestenol glycosides (Valayil *et al*., 2015), taxol (Purwandari, 2018), lasiodiplodin (Félix *et al*., 2019a), preussomerins and analogues (Chen *et al*., 2016b), which present anticancer activity towards different cancer cell lines (Hahismoto *et al*., 2008; Pandi *et al*., 2010).

Crude extracts of strain CBS339.90 grown in YMG medium, MM+ and MM led to a total loss of Hela S3 cells' viability (Figure 1.8), which indicates this fungus might also produce substances with toxicity towards cancer cells. All samples from the culture filtrate showed higher activity than mycelium samples, with the exception of BAF medium, where CE_{CF} had no effect on cells' viability. This is the first report of *L. hormozganensis* demonstrating a potential anticancer activity. However, it is known that several compounds produced by strain CBS339.90, namely jasmonic acid, furanones and 4-hydroxymelleins (Félix *et al.*, 2018a), are able to affect human cancer cells (Cimmino *et al.*, 2016; Elkhayat & Goda, 2017; Fingrut & Flescher, 2002; Rotem *et al.*, 2005; Tawfike *et al.*, 2019). Further studies are required to evaluate the potential anticancer activity of *L. hormozganensis*, namely through bioactive guided-assays to search for bioactive secondary metabolites produced by this fungus.

Regardless of the growth media, all CE_{CF} of strain LA-SOL3 were shown to induce 100 % of Vero-B4 cells' mortality (Figure 1.8). On the other side, CE_{MY} of this fungus induced lower levels of damage on cellular viability. Among these, the CE_{MY} of strain LA-SOL3 from growth in MM+ and YMG medium caused higher levels of mortality (75 %), followed by CE_{MY} of BAF medium and MM (25 %), with no effect from DM medium sample. These results suggest that *L. theobromae* is able to produce cytotoxic substances in the extracellular media, which could interact with the host and aid in fungal infection. This is consistent with previous published findings that showed the culture filtrate of this species, as well as several produced metabolites, present cytotoxic activity towards mammalian cells (Vero and 3T3 cells) [Félix *et al.*, 2016; 2018a; 2019b]. Moreover, through the use of omics tools, several compounds with human pathogenesis-related roles were identified in the transcriptome and proteome of *L. theobromae* (Félix *et al.*, 2019a). In fact, *L. theobromae* is a known human opportunistic pathogen that has been previously reported in several human infections (Gu *et al.*, 2016; Maslen *et al.*, 1996; Mohan *et al.*, 2016; Papacostas *et al.*, 2015; Saha *et al.*, 2012; Woo *et al.*, 2008). Furthermore, since cytotoxic activity has been reported in different strains (LA-SOL3, LA-SV1, CAA019), one may speculate that the ability to affect mammalian cells might be a species characteristic, although the quantitative effect of cytotoxicity appears to be strain-dependent (consistent with results from: Félix *et al.*, 2018a; 2019b).

Regarding *L. hormozganensis* strain CBS339.90, all crude extracts led to at least 50 % mortality of Vero-B4 cells (Figure 1.8). Overall, the CE_{CF} generated a higher percentage of non-viable cells than CE_{MY}. The crude extracts of CBS339.90 grown in DM and YMG media were the samples with less effect on cell viability. These findings are in line with those of Félix and colleagues (2016), which showed that culture filtrates from this strain exhibit cytotoxicity to Vero cells. This was also demonstrated by Félix *et al.* (2018a) when fungal growth was conducted at 37 °C, in Vero and 3T3 cells. Additionally, these authors showed cytotoxicity of compounds produced by the microorganism. Moreover, Félix *et al.* (2016) suggested that cytotoxic activity of *L. hormozganensis* CBS339.90 could be a result of its production of aspartic proteases, which are involved in human pathogenicity (Monod *et al.*, 2002).

Upon this, the results obtained in the current work support the hypothesis that *L. hormozganensis* and *L. theobromae* are able to produce substances that aid these fungi on the induction of toxic effects to mammalian cells. Also, it has been suggested that the production of pathogenesis/virulence-related compounds for mammals and humans, as well as the fungal cytotoxic activity towards mammalian cells, might be related to adaptation to temperature increase (Félix *et al*., 2016; 2018a; 2018b; 2019b; Yan *et al*., 2018). Ergo, further research is required to study the biological activity of these microorganisms, namely through the pursuit of pathogenesis- or virulence-related compounds important for human infections.

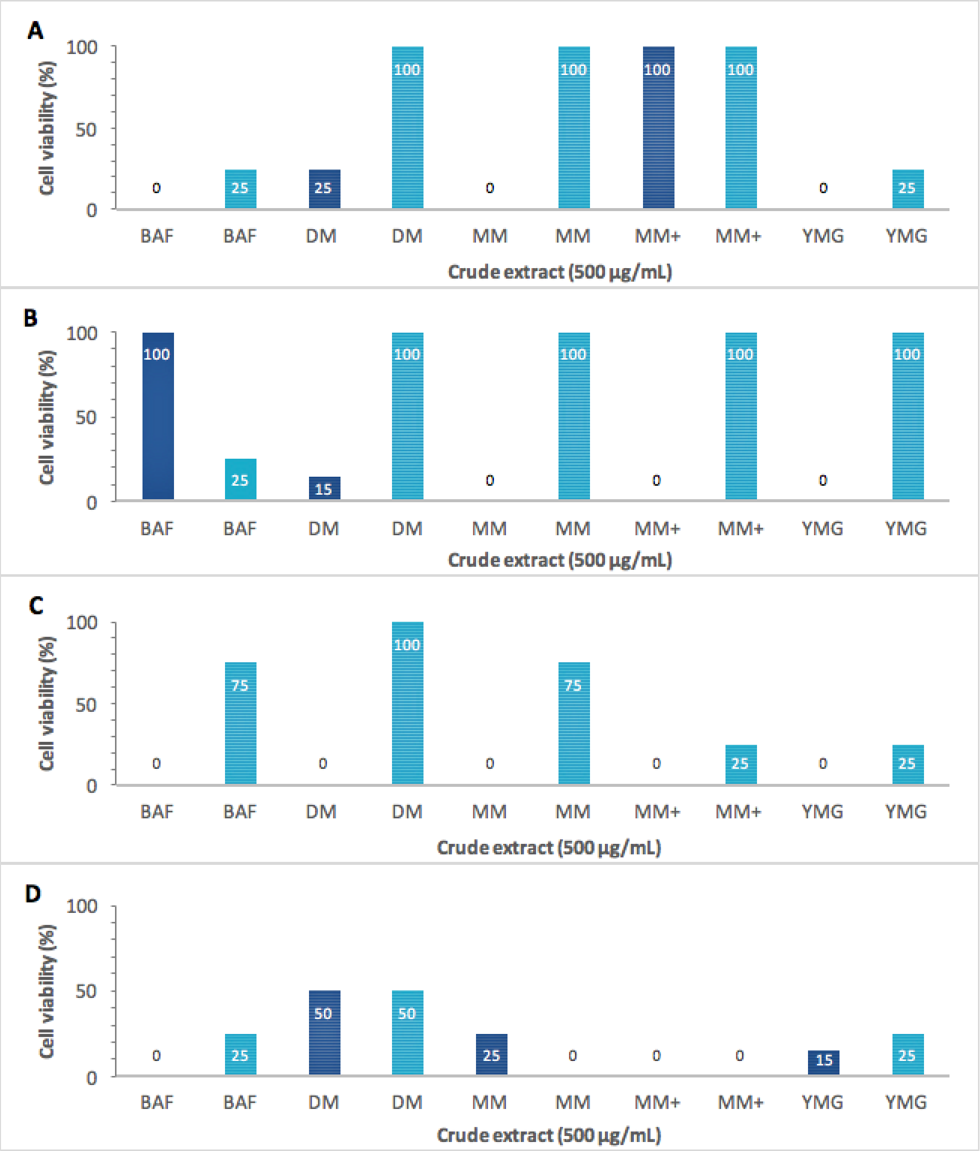


Figure 1.8 | Evaluation of cell viability (%) after 72 h exposure to crude extracts (500 µg/mL) from *Lasiodiplodia theobromae* and *L. hormozganensis* grown in 5 different media (BAF, DM, MM, MM+, YMG). Hela S3 cells were exposed to *L. theobromae* strain LA-SOL3 (**A**) and *L. hormozganensis* strain CBS339.90 (**B**). Vero-B4 cells were exposed to *L. theobromae* LA-SOL3 (**C**) and *L. hormozganensis* CBS339.90 (**D**). Dark and light blue bars correspond to crude extracts from culture filtrate and mycelium, respectively.

3.2.2. Antimicrobial activity of crude extracts

Agar disk-diffusion assays were performed with the crude extracts from both fungi to investigate their biological activity towards several microorganisms (Table 1.10). These organisms were used to provide a broad antimicrobial profile, including gram-positive bacteria, gram-negative bacteria and fungi. The inhibition diameter zones (IDZ) were separated in three categories according to Chagas *et al*. (2017): IDZ < 10 mm – low active; 10 ≤ IDZ < 20 mm – moderately active; IDZ ≥ 20 mm – very active.

Crude extracts from strain LA-SOL3 had no effect on the growth of the fungi *Paecilomyces variotti* and *Mucor miehei*. However, antifungal activity towards the fungus *Nematospora coryli* was observed, with a higher inhibition from samples of BAF medium (IDZ CE_{CF} = 28 ± 0.5 mm; IDZ CE_{MY} = 36 ± 0.5 mm). In fact, the majority of active samples were shown to be highly active towards *N. coryli*, presenting higher activity than positive control (IDZ = 20 ± 0.5 mm). Exceptions were CE_{CF} of MM and CE_{MY} of YMG medium, which showed moderate (IDZ = 18 ± 0.5 mm) and low (IDZ = 8 ± 0.5 mm) activity, respectively. In literature, extracts from *L. theobromae* have been demonstrated to be active towards yeasts and dimorphic fungi, namely *Candida glabrata*, *Saccharomyces cerevisiae,* and *Malassezia furfur* (Chagas *et al*., 2017; de Souza *et al*., 2013; Moron *et al*., 2018). Ergo, *L. theobromae* appears to have antifungal properties towards specific organisms, which may depend on growth conditions. In fact, in this thesis, higher activity was observed towards fungi, which could be related to fungal competition by the fungus. In addition, crude extracts from growth in DM and BAF media were moderately active towards all tested bacteria, with the exception of CE_{MY} from BAF medium. Overall, a higher antimicrobial activity was derived from the fungus LA-SOL3 grown in BAF and DM media, followed by YMG medium, MM+, and MM. The current results are in agreement with previous reports that display that *L. theobromae* is able to affect gram-negative and -positive bacteria growth: *Bacillus* sp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Citrobacter freundi*, *Escherichia coli*, *Salmonella* sp., *Micrococcus luteus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* (Chagas *et al*., 2017; Chen *et al*., 2016b; de Souza *et al*., 2013, Orlandelli *et al*., 2012). Although growth conditions were different (e.g. media, temperature, fermentation period, among others), Orlandelli and colleagues (2012) demonstrated that *L. theobromae* exhibited moderate activity (IDZ = 14.08 ± 0.5 mm) against *M. luteus*. This is in concordance with the current findings that show the majority of the extracts from nutrient-rich media (YMG, BAF and DM media) are moderately active towards *M. luteus*.

Regarding strain CBS339.90, only the CE_{CF} of growth in BAF medium showed inhibition of *P. variotii* and *M. miehei* growth. The inhibition halo obtained for *M. miehei* was equal to the positive control (IDZ = 15 ± 0.5 mm), thus the extract was considered moderately active. This extract was also moderately active (IDZ = 11 ± 0.5 mm) towards *P. variotii*. In addition, the crude extracts from this fungus were more active when fermentation was conducted in BAF medium, suggesting that the isolate is able to produce substances with antimicrobial activity. To date, no evidence of *L. hormozganensis* possessing antimicrobial activity has been established, thus this is the first report enlightening the potential antimicrobial properties of this species. However, further research is necessary to fully comprehend these characteristics.

In general, both species showed higher inhibition towards *Nematospora coryli*, with the majority of inhibition halos higher than positive control. Of all samples, the highest effect was seen towards *N. coryli* with an inhibition diameter of 52 ± 0.5 mm (CE_{CF} in BAF medium). Therefore, it is suggested that both isolates are able to produce one or more compounds to which *Nematospora coryli* is sensitive to. Furthermore, the results suggest that growth of both fungi on nutrient-poor media (MM and MM+) leads to the production of smaller quantities of active compounds or compounds with lower antimicrobial activity, than nutrient-rich media.

Table 1.10 | Antimicrobial activity assay of *L. hormozganensis* strain CBS339.90 and *L. theobromae* strain LA-SOL3 grown in different media (MM, MM+, YMG, BAF and DM media). Biological activity is represented via diameter (mm) of inhibition halo for each microorganism. 'CF' and 'MY' stand for crude extracts from the culture filtrate and mycelium, respectively. 'C+' corresponds to positive control.

Sample			**Inhibition diameter zone (± 0.5 mm)**					
			Bacillus brevis	*Micrococcus luteus*	*Enterobacter dissolvens*	*Nematospora coryli*	*Paecilomyces variotii*	*Mucor miehei*
LA-SOL3	**MM**	**CF**	-	-	-	18	-	-
		MY	-	-	-	-	-	-
	MM+	**CF**	8	-	-	21	-	-
		MY	-	-	-	-	-	-
	YMG	**CF**	15	-	-	30	-	-
		MY	-	13	-	8	-	-
	BAF	**CF**	13	11	13	28	-	-
		MY	17	10	-	36	-	-
	DM	**CF**	16	15	13	25	-	-
		MY	16	11	11	35	-	-
CBS339.90	**MM**	**CF**	-	-	-	10	-	-
		MY	-	-	-	-	-	-
	MM+	**CF**	-	-	-	7	-	-
		MY	-	-	-	-	-	-
	YMG	**CF**	-	-	12	20	-	-
		MY	-	-	-	11	-	-
	BAF	**CF**	20	10	18	52	11	15
		MY	13	27	12	23	-	-
	DM	**CF**	18	11	20	44	-	-
		MY	-	-	10	15	-	-
C+	Streptomycin		26	33	35			
	Hygromycin					20	24	15

3.2.3. HPLC analysis of crude extracts

Characterization of *L. theobromae* LA-SOL3 and *L. hormozganensis* CBS339.90 was also achieved through HPLC analysis. HPLC chromatograms were obtained for each crude extract (Appendix) and a comparison was made to discover which fermentation media leads to the most active extract. This was further relevant for large-scale fermentation in Chapter 2. The area of each peak is related to the concentration of the chemical, therefore, areas of matching peaks, i.e. with similar retention times, between the samples of each extract were compared (Figure 1.9). In general, for both fungi, the extracts corresponding to BAF medium appeared to be more active than the others, exhibiting a higher total number of analytes. The retention time of each peak seen in BAF medium samples was considered as the control for the comparison of areas throughout the analysis.

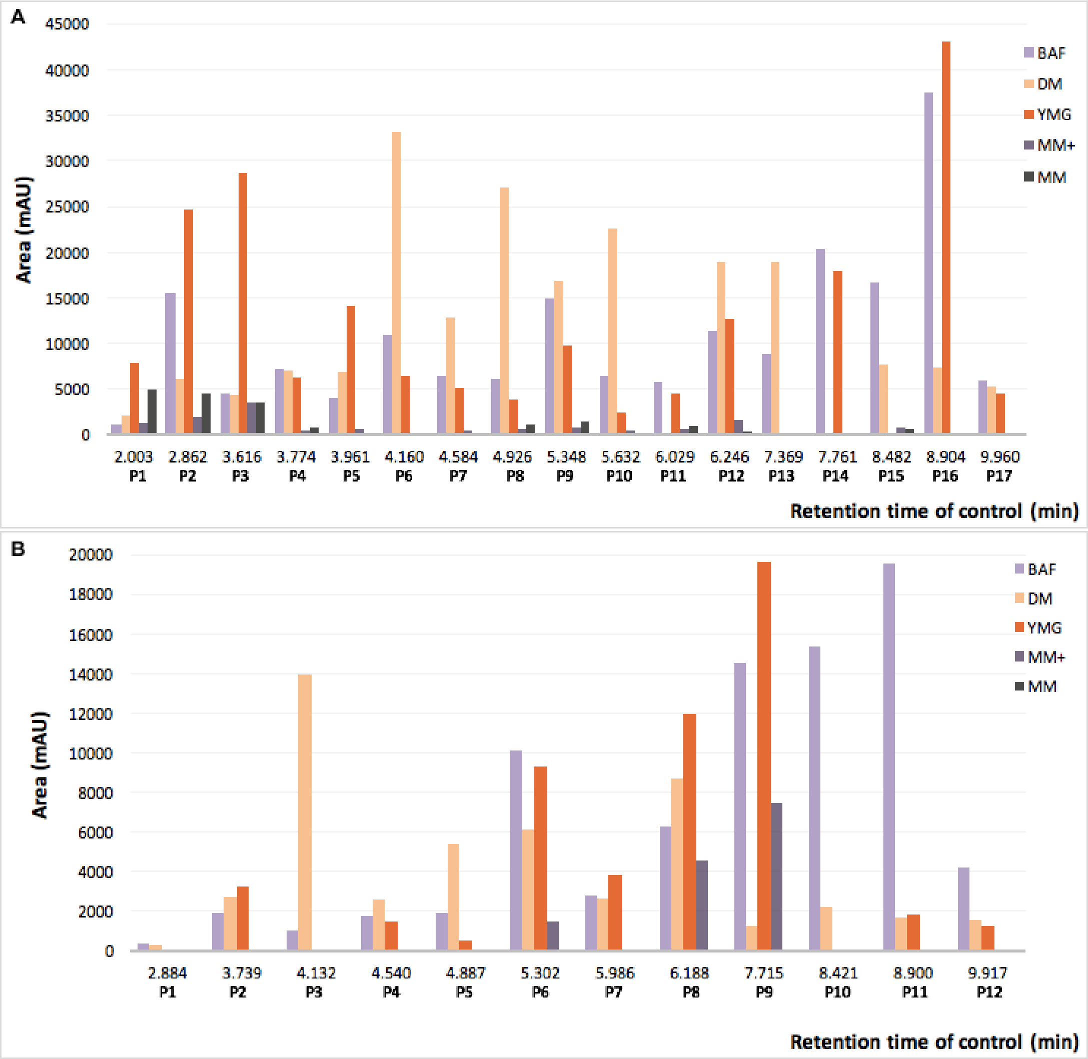
Seventeen peaks of CE_{MY} of LA-SOL3 were compared with the following retention times (on the control): 2.003 (P1), 2.862 (P2), 3.616 (P3), 3.774 (P4), 3.961 (P5), 4.160 (P6), 4.584 (P7), 4.926 (P8), 5.348 (P9), 5.632 (P10), 6.029 (P11), 6.246 min (P12), 7.369 (P13), 7.761 (P14), 8.482 (P15), 8.904 (P16), and 9.960 min (P17) [Figure 1.9A]. Unlike the culture filtrate, mycelia samples showed higher amounts of chemicals when growth was in DM medium (P6-10, P12-13). Nevertheless, peaks 4, 11, 14, 15 and 17 were present in higher amounts in $CE_{MY\ BAF}$. $CE_{MY\ YMG}$ exhibited higher quantities of analytes 1-3, 5 and 16. In general, data shows that growth of *L. theobromae* LA-SOL3 in MM and MM+ results in lower amounts of analytes (of CE_{MY} and CE_{CF}).

The CE_{CF} of strain LA-SOL3 showed low activity on MM up to retention time inferior to 5 min. Twelve peaks were compared among the CE_{CF} of LA-SOL3 with the following retention times (on the control): 2.884 (P1), 3.739 (P2), 4.132 (P3), 4.540 (P4), 4.887 (P5), 5.302 (P6), 5.986 (P7), 6.188 (P8), 7.715 (P9), 8.421 (P10), 8.900 (P11), and 9.917 min (P12) [Figure 1.9B]. In $CE_{CF\ BAF}$, analytes 1, 6, 10, 11 and 12 showed higher areas than all other samples containing the corresponding peaks. On the other side, analytes 2 and 3 were present in higher amounts in $CE_{CF\ DM}$ (1.45 and 1.34 times higher than control, for peak 2 and 3, respectively) and $CE_{CF\ YMG}$ (1.73 times higher for peak 2) than $CE_{CF\ BAF}$. Regarding peaks 4 and 5, higher amounts were observed in $CE_{CF\ DM}$, followed by $CE_{CF\ BAF}$ and $CE_{CF\ YMG}$. Also, higher amounts for peaks 7, 8 and 9 were seen in $CE_{CF\ YMG}$. Therefore, the majority of the chemicals were produced in higher amounts in $CE_{CF\ BAF}$, when comparing areas of all samples.

Thirteen peaks of CE_{CF} of CBS339.90 were compared with the following retention times (on the control): 3.747 (P1), 3.931 (P2), 4.130 (P3), 5.308 (P4), 6.157 (P5), 7.190 (P6), 7.402 (P7), 8.261 (P8), 8.818 (P9), 9.392 (P10), 9.930 (P11), 11.121 (P12), and 12.627 min (P13) [Figure 1.9C]. The growth of *L. hormozganensis* CBS339.90 in BAF medium contributed to the larger amount of the analytes in more than half of the compared peaks (P5, 6, 8-10, 12, 13). On the remaining peaks, bigger areas were obtained with $CE_{CF\ DM}$ (P1-3) or $CE_{CF\ YMG}$ (P4, 7, 11).

Twenty-five peaks of CE_{MY} of CBS339.90 were compared with the following retention times (on the control): 2.005 (P1), 2.898 (P2), 3.152 (P3), 3,484 (P4), 3,782 (P5), 4,170 (P6), 4,955 (P7), 5.354 (P8), 5.767 (P9), 6.237 (P10), 7.338 (P11), 8.060 (P12), 8.287 (P13), 8.857 (P14), 9.439 (P15), 9.958 (P16), 10.666 (P17), 11.279 (P18), 12.657 (P19), 13.186 (P20), 13.760 (P21), 14.266 (P22), 14.745 (P23), 15.571 (P24) and 16.444 min (P25) [Figure 1.9D]. *Lasiodiplodia hormozganensis* expressed higher quantities of 22 of the analytes when fungal growth was in BAF medium. Regarding peaks 1, 6 and 12, this was seen by CE_{MY} of YMG medium.

In summary, the total production of compounds was lower when LA-SOL3 and CBS339.90 were grown in MM and MM+. On the contrary, growth in BAF medium increased production, as well as the concentrations of the analytes.



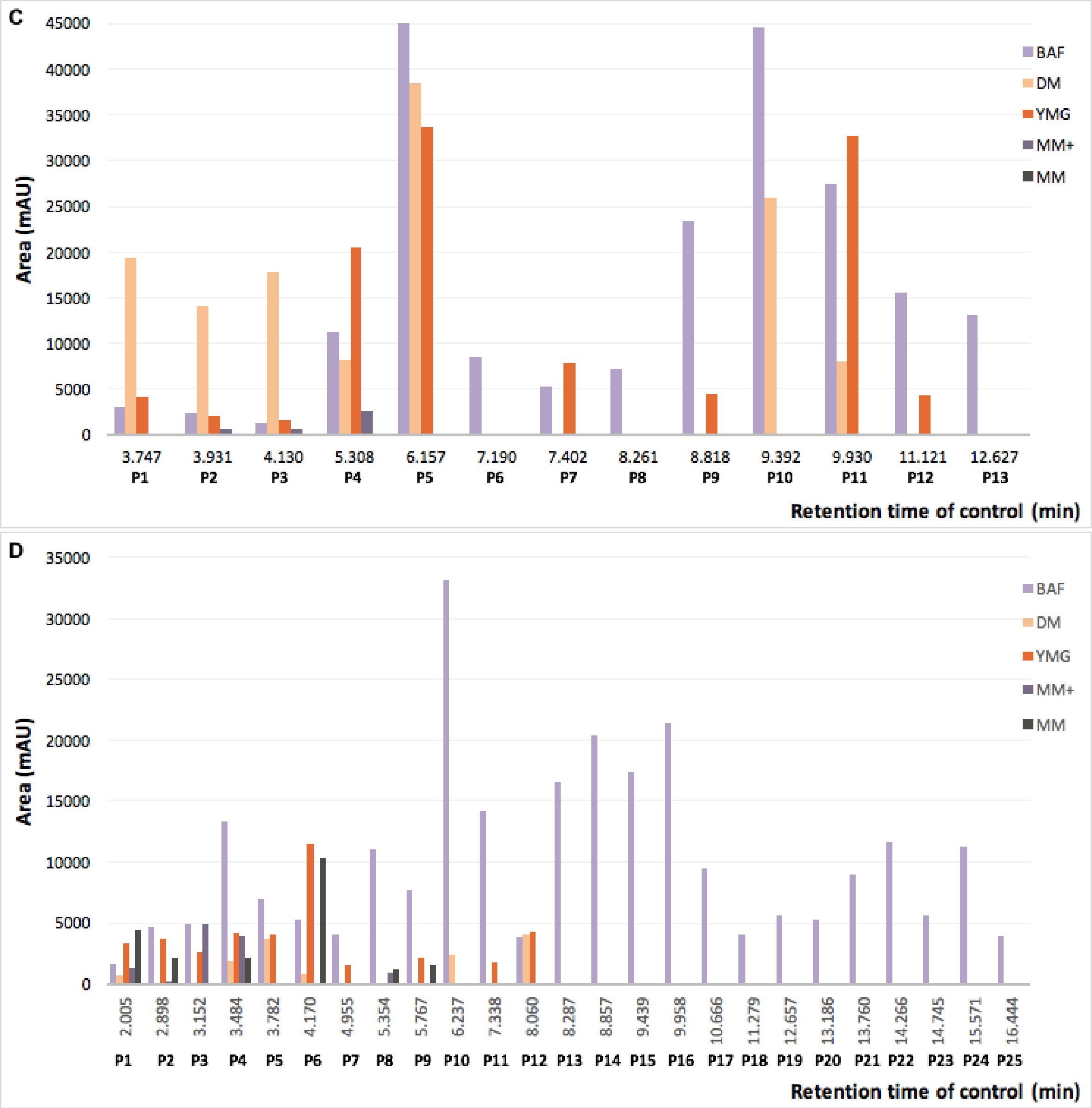


Figure 1.9 | Comparison of peak areas (mAU) of crude extracts from fungi grown in BAF, DM, YMG, MM+ and MM. Crude extracts from LA-SOL3 of mycelia (**A**) and culture filtrates (**B**), and extracts from CBS339.90 of culture filtrates (**C**) and mycelia (**D**) were analyzed via HPLC, and peak areas are presented according to retention time of peaks from fungal growth in BAF medium. P1-P25 correspond to the peaks.

4. CONCLUSIONS

To sum up, deletion vectors (pSJ-SSD1-HPT) were successfully constructed to achieve *ssd1* gene targeted replacement in *L. hormozganensis* CBS339.90 and *L. theobromae* LA-SOL3. Nonetheless, the integration of the vector into the genomes of both fungi was unsuccessful. Ergo, it is suggested that ATMT protocols for these fungi need further optimization. The crude extracts from wild-type fungi presented activity towards human cancer and mammalian cells, fungi, gram-positive and -negative bacteria. Furthermore, this was the first report of *L. hormozganensis* displaying potential anticancer and antimicrobial properties. Moreover, the attributes for biological activity of *L. hormozganensis* and *L. theobromae* appear to depend on growth media. In this line, further research is needed to study the biological activity of these microorganisms. As *L. theobromae* is a known human opportunistic pathogen and *L. hormozganensis* affects viability of mammalian cells, there is an increasing interest on research of their abilities to potentially infect humans, for example through characterization of pathogenesis- or virulence-related compounds.

CHAPTER 2

Bioactivity-guided isolation of secondary metabolites of *Lasiodiplodia hormozganensis*

---

1. INTRODUCTION

1.1. Secondary metabolites in filamentous fungi: when and how?

In optimal chemical and physical growth conditions, filamentous fungi grow according to a batch growth curve, which consists with a succession of lag – exponential – stationary phases. In the stationary phase, fungal cells are in a survivor state without added nutrients, thus fungi may experience secondary metabolism, which is involved in their survival through the production of secondary metabolites. Thereupon, industrial fermentations with the purpose of extracting secondary fungal metabolites require the sustenance of fungi at stationary growth phases. In batch fermentation systems, the medium is inoculated with a low cell density, and fungi grow according to the batch growth curve (Kavanagh, 2011). For this purpose, when fungi are actively growing, a transfer to a large fermenter is made, with control of medium composition, pH, temperature, agitation and glucose level (Thirumurugan *et al*., 2018).

Nutrient uptake by filamentous fungi occurs mainly through the plasma membrane, which functions as a permeable barrier, allowing nutrients to entry and metabolites to exit the cell. The absorption occurs at the tip of hyphae, nutrients enter the cell and a proton motive force is generated via the H^+-ATPase pump, leading protons to be exported to the extracellular media (Kavanagh, 2011). This process is often used in fungal fermentation to identify fungi on stationary phase for further metabolite isolation, with the control of pH and glucose level as mentioned in the last paragraph.

Identification of secondary metabolites relies on structure, function and biosynthesis (Thirumurugan *et al*., 2018). Currently, these compounds are divided in five classes: terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, enzyme cofactors and nonribosomal polypeptides. In fungi, secondary metabolites are encoded by biosynthetic gene clusters (Brakhage, 2013). Furthermore, each filamentous fungus can encode up to 70 secondary metabolism gene clusters and some of them act in combination (Scharf *et al*., 2014). The production of secondary metabolites depends on the environmental growth conditions of the microorganism and its genotype (Lind *et al*., 2016). The regulation of these compounds depends on global regulators, which function according to environmental signals, and pathway-specific transcription factors, specific to the respective biosynthetic gene cluster (Macheleidt *et al*., 2016). The signaling pathways in fungi are highly conserved, and several of them have been associated with the production of secondary metabolites, e.g. polyketide synthase (PKS) pathway (Macheleidt *et al*., 2016).

1.2. The role of secondary metabolites on human infections

Secondary metabolites have several subsets of functions, such as antimicrobial activity, anticancer activity, metal acquisition, UV protection, oxidative stress protection, competition with other microorganisms, among others. Moreover, some secondary metabolites can increase the virulence attributes of the pathogen on the host (Bignell *et al*., 2016; Scharf *et al*., 2014; Thines *et al*., 2006). For example, melanin confers protection against exogenous stresses, contributing to pathogenicity of several human pathogenic fungi (Iyalla, 2017; Scharf *et al*., 2014). Another example is the production of siderophores, which allow the obtainment of iron - an essential nutrient for fungal metabolism - from the host (Iyalla, 2017; Scharf *et al*., 2014). Apart from these examples, research on the role of secondary metabolites during infections is still scarce. More specifically, an emerging research area is directed towards the role of secondary metabolites on human mycoses, with a big emphasis on virulence-related compounds. Many secondary metabolite gene clusters are silent during fungal growth in laboratorial conditions. Hence, more efforts are needed to further investigate the putative role of secondary metabolites in human-fungal interactions.

1.2.1. *Lasiodiplodia hormozganensis* and secondary metabolites

The production of metabolites by *Lasiodiplodia* species varies according to environmental conditions, which was previously suggested to be related to the fungus adaptability to different hosts (Félix *et al*., 2018a; Yan *et al*., 2018). *Lasiodiplodia hormozganensis* genome encodes 52 secondary metabolite gene clusters, including type 1 polyketide synthases (unpublished data). Some secondary metabolites can be virulence factors in host-pathogen interactions (Cramer Jr & Perfect, 2009). Ergo, this highlights the importance of studying the secondary metabolites produced by *L. hormozganensis*, specially concerning potential human opportunistic infections. Furthermore, this fungus is able to produce 3-indolecarboxylic acid, jasmonic acid, metabolites of furanones' class, hydroxymelleins, cyclo-(Trp-Ala), scytalone and botryosphaerialactone A (Félix *et al*., 2018a). All these metabolites have cytotoxic properties towards mammalian cells (Félix *et al*., 2018a), some of which present higher activities at human body temperatures (37 °C). Hence, the metabolites could play important roles in further infections on animals or even humans. In fact, scytalone is a precursor for melanin synthesis, which is an important virulence factor for pathogenesis in both plants and animals by several fungi, as well as human infections by *A. fumigatus* (reviewed by Tsai *et al*., 1999).

Henceforth, there is a lack of knowledge on the production of secondary metabolites by *L. hormozganensis*, specifically with regards to virulence and adaptability of the species to potentially infect humans.

1.3. Isolation of secondary metabolites

The accurate prediction of the process in which all secondary metabolites of a fungus are related to its pathogenicity is still challenging (Bignell *et al.*, 2016). However, studies that involve characterization of secondary metabolites are crucial to comprehend the pathogenesis of fungi. This matter is particularly important when we consider human mycoses that are in need of new therapies for prevention or treatment of the respective infection. Also, the importance is highlighted in rising cross-kingdom infections of phytopathogenic fungi due to changes in environmental conditions. *Lasiodiplodia hormozganensis* (strain CBS339.90 previously misidentified as *L. theobromae*) has been described as an opportunistic pathogen with potential to affect mammals or even humans (Félix *et al.*, 2016; 2018a). Moreover, little information is known regarding this pathogen's potential virulence towards humans. Ergo, efforts focusing on the determination of the role of secondary metabolites produced by this pathogen, specifically related to virulence, are pivotal.

Isolation of compounds from natural extracts is achieved with different method combinations, depending on each sample and the goal of the study. For the partioning of the extract, methods such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) can be used (Ahadi *et al.*, 2011). LLE uses water-immiscible solvents, leading to extraction of the organic phase of the analyte. SPE involves adsorption of the sample on a column (containing a solid adsorbent), washing interfering components and elution with an appropriate solvent. SPE has advantages over LLE, such as time reduction, decreased evaporation volumes, extracts obtained with less residues, low amount of solvent used, higher efficacy in separation, among others (Ahadi *et al.*, 2011). Afterwards, isolation, purification and identification of the compounds are conducted. In this step, HPLC is the most common used technique, however, choice of the method depends on the sample nature and the target products (Dinan *et al.*, 2001). Moreover, the biologically active components of an extract are usually presented in a minor part of the sample. HPLC provides a fast analysis of multicomponent samples with a high resolving power (Martin & Guiochon, 2005). The peaks exhibited by each compound vary according to different parameters on the HPLC system, namely the flow rate, column, detector and the mobile phase (Sasidharam *et al.*, 2011).

1.4. Goal

Some secondary metabolites are involved in fungal pathogenesis. Moreover, *L. hormozganensis* displays cytotoxic activity towards mammalian cells, becoming an important case of study for its potential ability to infect human hosts. Henceforth, in this chapter, the aim consisted on isolation, characterization and identification of secondary metabolites produced by *L. hormozganensis* strain CBS339.90, in order to understand if the compounds played a role on its virulence. For this purpose, large-scale fermentation was conducted, followed by separation of potential active compounds through the use of different techniques, such as HPLC.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Chemicals and media components

a) Water

$H_2O_{deionized}$ (Seradest SD 2000, SERAL - Reinstwasser - System, GmbH, Ransbach-Baumbach) was used to prepare media, solutions and buffers. $H_2O_{Ultra\text{-}filtrated\ ultrapure}$ (Milli-Q® Synthesis, Quantum® EX, Q-Gard® 2, Millipore GmbH, Schwalbach) was used for molecular experiments.

b) Solvents

Acetonitrile (Sigma-Aldrich Chemie GmbH), ethyl acetate (Fisher Chemical, UK), isopropanol (Sigma-Aldrich Chemie GmbH), methanol (Fisher Chemical, UK), DMSO (VWR).

c) Antibiotics

Cycloheximide (Sigma-Aldrich Chemie GmbH), hygromycin B Gold (InvivoGen), streptomycin sulfate (PanReac AppliChem ITW Reagents), antibiotic antimycotic solution (Sigma-Aldrich), penicillin G (Serva, Boehringer Ingelheim).

d) Media components and reagents

Bacto™ Casamino Acids (Becton Dickinson GmbH, Heidelberg), Bacto™ Malt Extract (Becton Dickinson GmbH, Heidelberg), Bacto™ Nutrient Broth (Becton Dickinson GmbH, Heidelberg) Bacto™ Peptone (Becton Dickinson GmbH, Heidelberg), Bacto™ Tryptone (Becton Dickinson GmbH, Heidelberg), Bacto™ Yeast Extract (Becton Dickinson GmbH, Heidelberg), Camptothecin (Alfa Aesar), d-Biotin (Sigma-Aldrich Chemie GmbH), Difco™ Agar Bacteriological

(Becton Dickinson GmbH, Heidelberg), Difco™ Potato Dextrose Broth (Becton Dickinson GmbH), Dulbecco's Modified Eagle Medium (Gibco), Fetal Bovine Serum (Sigma-Aldrich), Glucose (SHS, Gesellschaft für klinische Ernährung, GmbH, Heilbronn), Kartoffel Püree (Edeka, Germany), Maltose (Alfa Aesar, ThermoFisher GmbH, Erlenbachweg), Phosphate Buffered Saline (Gibco), Resazurin (Sigma-Aldrich), Thiamine chloride (Sigma-Aldrich Chemie GmbH), Toluene (Merck), Trypsin EDTA (Corning), H_3BO_3 (Merck KGaA), $CaCl_2.2H_2O$ (Sigma-Aldrich Chemie GmbH), $CoCl_2.6H_2O$ (Riedel-de Haën AG), $CuSO_4.5H_2O$ (Fluka Chemie AG), $FeSO_4.7H_2O$ (Merck), KCl (Sigma-Aldrich Chemie GmbH), KH_2PO_4 (Bernd Kraft, Germany), $MgSO_4.7H_2O$ (Merck), $MnCl_2.4H_2O$ (Merck GmbH), NaCl (Carl Roth GmbH), $NaNO_3$ (VWR Chemicals), $Na_2EDTA.2H_2O$ (AppliChem GmbH), $ZnSO_4.7H_2O$ (Merck).

2.1.2. Equipment

20 L fermenter (Biostat20, B. Braun Biotech, Sartorius), 37 °C incubator (Memmert GmbH), Bath Sonicator (Elma), Analytical HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn), C18 ec Chromabond®-Kartuschen (10000 mg, Macherey-nagel GmbH & Co. KG, Düren), Centrifuge 5415 D (Eppendorf AG, Hamburg), Centrifuge Jouan GR4 22 (Axon), DNA-Concentrator 5301 (Eppendorf GmbH), Forma Orbital Shaker (Thermo Electron Corporation), Freeze dryer (Christ® Gamma 1-20), HPLC-MS system (HPLC/MSD system Series 1260 Infinity, Agilent Technologies, Waldbronn), Mini centrifuge (Axon), Microtiter plate spectrophotometer (Biotek Synergy HT, Portugal), Preparative-HPLC system (1260 Infinity II Preparative LC system, Agilent Technologies, Waldbronn), Rotary Evaporator Laborota 4002-Digital (Heidolph), Speedvac Jouan RC10.22 (Thermo Fisher Scientific), Speedvac RVC 2-33 CD-plus (Christ, Germany), ThermoShaker (Biometra), Vacuum Concentrator Jouan RC 10.22 (Labfish).

2.1.3. Solutions, antibiotics and buffers

a) Solutions

Nitrate Salt Solution (20x)

$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4.7H_2O$	10.4 g
KH_2PO_4	30.4 g

Fill with 1 L of deionized water.

Trace elements solution (pH 6.5)

$CoCl_2.6H_2O$	1.7 g
$CuSO_4.5H_2O$	1.6 g
$FeSO_4.7H_2O$	5 g
H_3BO_3	11 g
$MnCl_2.4H_2O$	5 g
$Na_2EDTA.2H_2O$	50 g
$Na_2MoO_4.2H_2O$	1.5 g
$ZnSO_4.7H_2O$	22 g

Fill with 1 L of deionized water.

Camptothecin solution (1 mg/mL)

Camptothecin	10 mg

Fill with DMSO up to 10 mL of total volume.

b) Antibiotics

Antibiotic solutions were filtered with 0.2 µm pore size sterile filters (Whatman) and stored at -20 °C.

Cycloheximide solution (1 mg/mL)

Cycloheximide	10 mg

Fill with EtOH up to 10 mL of total volume.

Hygromycin solution (100 mg/mL)

Hygromycin B Gold	1 g

Fill with ${}_{UF}H_2O$ up to 10 mL of total volume.

Streptomycin solution (100 mg/mL)

Streptomycin sulfate	1 g

Fill with ${}_{UF}H_2O$ up to 10 mL of total volume.

2.1.4. Reaction kit

Glucose Urine Test Strip, DUS G (DFI).

2.1.5. Culture media

The following media were used in this work, in which the amounts given for each composition are calculated for a total of 1 L of medium. For solid media, 18 g/L of agar was added before autoclaving. All media were autoclaved after preparation, at 121 °C for 20 minutes.

a) Media for *L. theobromae and L. hormozganensis* culture

BAF medium

Maltose	20 g
Glucose	10 g
Peptone	2 g
Yeast extract	0.2 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	1 g
$CaCl_2$ (0.1 M)	5 mL
$FeCl_3$ (10 mg/mL)	1 mL
$ZnSO_4.7H_2O$ (1 mg/mL)	1 mL

Double malt (DM) medium

Malt extract	40 g

Minimal medium (MM, pH 6.5)

Nitrate Salt Solution (20x)	50 mL
Trace elements	1 mL
Glucose	10 g
Thiamin (1 % v/v)	1 mL
Biotin (0.01 %)	250 µL

Minimal medium plus (MM+, pH 6.5)

Nitrate Salt Solution (20x)	50 mL
Trace elements	1 mL
Glucose	10 g
Thiamin (1 % v/v)	1 mL

Biotin (0.01 %)	250 µL
Starch	10 g

Potato Dextrose Agar (PDA) medium

Potato Dextrose Broth	24 g

Yeast malt glucose (YMG) medium (pH 5.5)

Yeast extract	4 g
Glucose	10 g
Malt extract	10 g

b) Media for test organisms’ culture

HA medium (pH 5.5)

Yeast extract	4 g
Glucose	4 g
Malt extract	10 g

NB medium (pH 6.5)

Bacto™ Nutrient Broth	8 g
NaCl	1 g

Malt medium (pH 5.5)

Malt extract	20 g

Complete medium (CM, pH 6.5)

Yeast extract	1 g
Glucose	10 g
Peptone	2 g
Casamino acids	1 g
Trace elements	1 mL
Nitrate Salt Solution (20x)	50 mL

2.1.6. Organisms

2.1.6.1. Fungi

Lasiodiplodia theobromae strain LA-SOL3 was isolated from *Vitis vinifera* in Peru (Rodríguez-Gálvez *et al*., 2015). *Lasiodiplodia hormozganensis* strain CBS339.90 was isolated from a phaeohyphomycotic cyst of a human patient in Jamaica (Alves *et al*., 2008). The strain CBS339.90 was obtained from the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre. Both cultures were maintained on PDA medium at 25 °C.

2.1.6.2. Mammalian and human cell lines

Vero cells (ECACC 88020401, GMK clone)

This cell line was established from the kidney of an adult African green monkey (*Cercopithecus aethiops*). The cells are adherent-elongated epithelial cells that grow in a monolayer.

Vero-B4 cells (ACC 33, Lot 11, DSMZ)

This is a sub-clone cell line that mimics the morphological and growth parameters of the parent cell line (Vero).

Hela S3 cells (ACC 161, DSMZ)

This cell line is a human cancer cell line established from cervical human adenocarcinoma. The cells are adherent and epithelial, growing and forming a monolayer.

A375 cells (ECACC, 88113005)

This human cancer cell line is composed by malignant melanoma cells established from the skin of a 54 years old female human. The cells are adherent and epithelial.

2.1.6.3. Test organisms

Standard organisms (Table 2.1) were used during phytotoxic and antimicrobial assays of 4-ethylaniline (section 2.2.9), as well as antimicrobial assays of crude extracts (section 2.2.4.1).

Table 2.1 | Test organisms for characterization of crude extracts and 4-ethylaniline. Information regarding brand, phylogenetic group, and growth medium and temperature is included.

Taxonomy	Species	Brand	Growth medium	Growth temperature
Gram-negative bacteria	*Enterobacter dissolvens*	LMG 2683	NB medium	27 °C
Gram-positive bacteria	*Bacillus brevis*	ATCC 9999	NB medium	37 °C
	Staphylococcus aureus subsp. aureus	ATCC 11632	NB medium	37 °C
	Micrococcus luteus	ATCC 381	NB medium	37 °C
Fungi	*Botrytis cinerea*	IBWF strain collection, GmbH	Malt medium	27 °C
	Candida albicans	(Robin) Berkhout (ATCC 90028)	HA medium	37 °C
	Magnaporthe grisea	70-15 (IBWF strain collection, GmbH)	CM medium	27 °C
	Mucor miehei	Tü 284	HA medium	37 °C
	Nematospora coryli	ATCC 10647	HA medium	27 °C
	Paecilomyces variotii	ETH 114646	HA medium	37 °C
	Penicillium notatum	IBWF strain collection, GmbH	HA medium	27 °C
Plants	*Lepidium sativum*	Kresse, Dreschflegel	-	28 °C
	Setaria italica	Kolbenhirse, Dreschflegel	-	28 °C

2.2. Methods

2.2.1. Selection of growth medium

In chapter 1, small-scale fermentations of strains LA-SOL3 and CBS339.90 were performed as described in section 2.2.14.1, followed by characterization of all crude extracts (sections 2.2.14.3 – 2.2.14.5). The interpretation of those results was used to select of the ideal growth medium for large-scale fermentation.

2.2.2. Large-scale fermentation

Large-scale fermentation of *L. hormozganensis* strain CBS339.90 was performed in 20 L of BAF medium on a 20 L fermenter equipped with a rotor and an air intake (Biostat20, B. Braun Biotech, Sartorius). Previously, five plugs of the fungus were inoculated, under sterile conditions, into 1 L baffled erlenmeyer flasks with 500 mL of BAF medium, and incubated at room temperature, on an orbital shaker at 120 rpm, for 4 days. First, 20 L of BAF medium were prepared, placed in the fermenter and autoclaved overnight at 121 °C for 60 min. When the medium's temperature was 27 °C, the fermenter was inoculated with the liquid culture previously prepared. A sample (200 mL) was collected daily. Of this sample, 100 mL were used for vacuum filtration, followed by determination of pH and glucose level, and posterior access to mycelium dry weight. Also, 100 mL were used for the separation of culture filtrate and mycelium, extraction of crude extracts [ethyl acetate (1:1) for culture filtrate, and methanol for the mycelium], and subsequent HPLC analysis. The dry crude extracts were previously solved in 500 µL of methanol, followed by centrifugation (Centrifuge 5415 D, Eppendorf AG, Hamburg) at 13200 rpm, for 2 min. Then, 50 µL of each sample were transferred to 0.2 mL HPLC vials. Analytical HPLC was performed with a RP18 separation column (LiChroCART® 125-4, LiChrospher® 100, 125 x 4 mm; 5µm, Merck, Darmstadt). The gradient (Table 2.2) was performed at a flow rate of 1 mL/min, using the eluents $_{UF}H_2O$ with 0.1 % TFA (eluent A) and MeCN (eluent B).

Table 2.2 | Standard gradient for measurement on analytical HPLC.

Time (min)	Eluent A (%)	Eluent B (%)
0	99	1
20	0	100
24	0	100
25	99	1

2.2.3. Extraction of crude extracts

When the carbon source was depleted after 6 days, the fermentation was stopped. Afterwards, vacuum filtration was carried out to separate the culture filtrate from the mycelium. Crude extracts from the culture filtrate were extracted with ethyl acetate (1:1), dried with Na_2SO_4 and evaporated using a rotary evaporator *in vacuo*. After lyophilizing, 15.31 g of the mycelium were submitted to extraction with methanol, filtrated and dried *in vacuo* through the use of the rotary evaporator.

2.2.4. Characterization of crude extracts

The crude extracts were analyzed via analytical HPLC (as described in section 2.2.2) and tested for antimicrobial activity and cytotoxicity as described in the next subsections. Also, cytotoxic assays were coupled with HPLC-based micro-fractionation (section 2.2.4.2).

2.2.4.1. Agar disk-diffusion assay

The crude extracts were tested for antimicrobial activity against the test organisms (section 2.1.7.5): *Bacillus brevis*, *Micrococcus luteus*, *Enterobacter dissolvens*, *Nematospora coryli*, *Paecilomyces variotii*, and *Mucor miehei*. 50 and 100 µg of each crude extract in methanol were applied to antibiotic assay disks (diam. 6 mm., Macherey-Nagel GmbH), which were placed into the inoculated plates. As positive control, streptomycin (0.5 mg) was used for bacteria and hygromycin (0.5 mg) for fungi. The agar plates were incubated for 24 h, at 37 °C for *Bacillus brevis*, *Micrococcus luteus*, *Paecilomyces variotii* and *Mucor miehei*, or 27 °C for *Enterobacter dissolvens* and *Nematospora coryli*. Inhibition growth zones were measured by diameter.

2.2.4.2. HPLC-based fractionation and cytotoxicity assays

The combination of HPLC-based fractionation with bioassays speeds the process of activity profiling natural product extracts (Järvinen *et al*., 2016). Therefore, in order to discover whether the crude extracts contained active substances, HPLC-based fractionation was combined with cytotoxicity assays on 96-well plates. This procedure was performed with analytical HPLC using a fraction collector (Agilent Technologies, Waldbronn) downstream to the separation column, and the separation parameters described in section 2.2.2. This allowed separation of the sample components, depending on their retention time, into 92 wells of 96-well plates. The plates were dried under vacuum (RVC 2-33 CD Plus, Christ, Germany) and kept at -20 °C until further use.

Morphological observation-based cytotoxicity was evaluated in mammalian (Vero-B4) and human cancer (Hela S3) cell lines, according to Zapf *et al*. (1995), with slight modifications. First, 200 µL of cells ($2x10^4$ cells/mL of Vero-B4 cells; $1.5x10^4$ cells/mL of Hela S3 cells) in supplemented DMEM (with 10 % FBS, 65 µg/mL penicillin G and 100 µg/mL streptomycin) were seeded in 96-well plates. The plates were incubated for 24 h in 5 % CO_2, at 37 °C. The 96-well plates obtained after HPLC-based fractionation were sterilized using 70 % ethanol. After evaporation, 200 µL of supplemented DMEM was added to each well, and the plates were gently agitated for 5 min. Then, cells were exposed to the samples through media replacement, followed by incubation for 72 h, at 37 °C, in 5 % CO_2. Cell viability was evaluated under microscopic observation of cell morphology, comparing with a positive (cells exposed with cycloheximide - 10 µg/mL – and camptothecin – 5 µg/mL) and negative (non-exposed cells) control.

2.2.4.3. Cytotoxicity assay

Cytotoxicity assays were conducted in Vero-B4 and Hela S3 cell lines, as described in section 2.2.4.2, with some modifications. 100 µg of each crude extract in methanol were applied on wells of a 96-well plate. After evaporation, 200 µL of supplemented DMEM was added to each well, and the plate was gently agitated for 5 min. Then, cells were exposed to the samples, in duplicate, through media replacement, followed by incubation for 72 h, at 37 °C, in 5 % CO_2.

2.2.5. Solid phase extraction

SPE was used to prepare samples for preparative HPLC, and consequently for the isolation and purification of secondary metabolites. As the crude extract from the culture filtrate (795 mg) showed previous activity, it was further submitted to solid phase extraction using C18 ec Chromabond®-Kartuschen (10000 mg, Macherey-nagel GmbH & Co. KG, Düren). The sample was previously dissolved in methanol. After preliminary washing steps of the column material with acetonitrile (strongest eluent) and then with $_{UF}H_2O$ (starting eluent), the sample was loaded onto the column. Then, fractionation of the organic components was achieved by elution with 100 % $_{UF}H_2O$ (fraction 1, 278.4 mg), followed by solvent mixtures of increasing hydrophobic strength from MeCN/$_{UF}H_2O$ (fraction 2, 10:90, 173.7 mg) to MeCN/$_{UF}H_2O$ (fraction 3, 20:80, 65.4 mg), MeCN/$_{UF}H_2O$ (fraction 4, 22.5:77.5, 22.2 mg), MeCN/$_{UF}H_2O$ (fraction 5, 25:75, 18.6 mg), MeCN/$_{UF}H_2O$ (fraction 6, 27.5:72.5, 16.6 mg), MeCN/$_{UF}H_2O$ (fraction 7, 30:70, 20.5 mg), MeCN/$_{UF}H_2O$ (fraction 8, 40:60, 54.1 mg), MeCN/$_{UF}H_2O$ (fraction 9, 50:50, 26.2 mg), and, finally, 100 % MeCN (fraction 10, 12.2 mg).

The fractions were dried on the rotary evaporator and reabsorbed in methanol. All fractions were analyzed via analytical HPLC at 10 mg/mL in methanol. The analysis was performed with RP18 separation column (LiChroCART® 125-4, LiChrospher® 100, 125 x 4 mm; 5µm, Merck, Darmstadt), at a flow rate of 1 mL/min, using the eluents $_{UF}H_2O$ with 0.1 % TFA (eluent A) and MeCN (eluent B) [gradient is shown in Table 2.2, section 2.2.2]. Fraction 1 was identified as being composed by polysaccharides. Fractions 2 and 3 were further processed.

2.2.6. HPLC-based fractionation and cytotoxicity assay of fraction 3

HPLC-based fractionation was combined with cytotoxicity assays on 24-well plates. This procedure was performed with analytical HPLC using a fraction collector (Agilent Technologies, Waldbronn) downstream to the separation column. The separation was performed with AG19 column (Zorbax SB-AQ 5µm, 150x4.6mm, Agilent), with a gradient of 12-25 % MeCN in 20 min, and a flow rate of 5.8 mL/min. This allowed separation of the sample components, depending on their retention time, into 20 wells of 24-well plates. The plates were dried under vacuum (RVC 2-33 CD Plus, Christ, Germany) and a total of 6 intermediate compounds (3A, 3B, 3C, 3D, 3E, 3F) were collected with MeCN. After evaporation *in vacuo*, morphological observation-based cytotoxicity of the compounds (100 and 200 µg/mL) was evaluated in Vero-B4 cell line, as described in section 2.2.4.3.

2.2.7. Preparative HPLC

After retrieving fraction 2 (F2) and 3 (F3), preparative HPLC (1260 Infinity II Preparative LC system, Agilent Technologies, Waldbronn) was used for the isolation of active substances in larger amounts. The target substances were chosen according to cytotoxicity results (section 2.2.4.2 for fraction 2; section 2.2.6. for fraction 3). The adequate column and method were previously tested by analytical HPLC and optimized according to the target substances. The runs were performed with AG18 column (Zorbax SBAQ 5 µm, 9.4x250 mm, Agilent), at 22 °C. The samples (170 mg of F2; 56mg of F3) were injected at 500 (F2) and 200 (F3) mg/mL in DMSO, at a flow rate of 6.95 and 5.58 mL/min for fraction 2 and 3, respectively. A MeCN/$_{UF}H_2O$ gradient was used: 4 % MeCN in 15 min (F2); 19 % MeCN in 13 min (F3). A fraction collector was used to separate several substances from the sample, after passage from the detector. The collected fractions (from F2: A – 3.8 mg, B – 2.4 mg, C – 1.2 mg, D – 1.9 mg, E – 36.9 mg, F – 1.8 mg; from F3: G – 1.5 mg, H – 1.5 mg) were absorbed in acetonitrile and toluene, and dried through the use of a rotary vacuum evaporator, until further use.

The purity of each collected fraction was determined via HPLC-MS (HPLC/MSD system Series 1260 Infinity, Agilent Technologies, Waldbronn), at a flow rate of 0.45 mL/min. The analysis was carried out in RP18 column (Superspher 100, 125x2 mm; 4µm, Merck, Darmstadt) at 40 °C. The gradient (Table 2.2, section 2.2.2) was performed at a flow rate of 0.45 mL/min using the eluents $_{UF}H_2O$/$_{UF}H_2O$ with 0.1 % HCOOH (eluent A) and MeCN (eluent B). A database from IBWF, in which both UV/vis and mass spectra of several natural compounds are stored, was also used to identify already known substances.

Regarding fraction 2, given that none of the substances were pure, further purification was required. The ideal method for substance B was optimized by analytical HPLC. Afterwards, purification was achieved via HPLC-based fractionation into a 24-well microplate. The run was performed with a fraction collector and AG18 column (Zorbax SBAQ 5 µm, 9.4x250 mm, Agilent). The sample was inserted at 20 mg/mL in acetonitrile, at a flow rate of 1 mL/min using the eluents $_{UF}H_2O$ with 0.1 % TFA (eluent A) and MeCN (eluent B). A MeCN/$_{UF}H_2O$ gradient was used: 2 % MeCN in 20 min. Two collections were made for the peaks corresponding to retention times of 3.539 min and 4.549 min. After isolation, purity was analyzed via HPLC-MS, and cytotoxicity assays were performed on Vero-B4 cells, as described in section 2.2.4.2, with slight modifications. Seeded cells were exposed to both samples (250 µg/mL) for 72 h, at 37 °C, in 5 % CO_2.

2.2.8. Semi-preparative HPLC

Semi-preparative HPLC allows the isolation of a substance which represents a low amount of the crude extract, too low for separation with a column used in preparative HPLC. The substances obtained in the last section were not pure (i.e. purity < 90 %). Hence, semi-preparative HPLC (Agilent 1100 Series, Agilent technologies, Waldbronn) was used for further isolation of substance E (36.9 mg). The run was performed for 15 min, using 4 % MeCN as the solvent, at a flow rate of 6.95 mL/min. The sample (100 mg/mL in DMSO) was separated using AG18 column (Zorbax SBAQ 5 µm, 9.4x250 mm, Agilent). Finally, the compound (C1) was characterized on the basis of its purity, retention time, UV spectra and molecular weight via HPLC-MS, following the parameters in section 2.2.7. The procedure for recording the mass spectra corresponded to the principle of chemical ionization at atmospheric pressure (APCI). The capillary voltages were 3500 V and 2200 V for positive and negative ionization, respectively. The temperature of the evaporation chamber was 400 °C and the fragment voltage was 140 V. Moreover, the structure was elucidated by the Molecular Biotechnological group of Prof. Dr. Heige B. Bode (Frankfurt, Germany).

2.2.9. Biological characterization of the compound 1 (4-ethylaniline)

2.2.9.1. Antimicrobial assays

Agar disk-diffusion assays were carried out to access antibacterial and antifungal properties of compound 1 (section 2.2.8) [Markwart *et al*., 2019]. Agar plates with the appropriate growth medium were inoculated with the microorganisms (Table 2.1, section 2.1.6.3): *Bacillus brevis*, *Micrococcus luteus*, *Staphylococcus aureus* subsp *aureus*, *Candida albicans*, *Nematospora coryli*, *Paecilomyces variotii, Mucor miehei*, *Penicillium notatum*. Antibiotic assay disks (diam. 6 mm., Macherey-Nagel GmbH) containing 10 or 50 µg of the compound solved in DMSO were applied to the agar plates. All plates were incubated for 24 h, at ideal temperatures (Table 2.1). Inhibition growth zones were measured by diameter. As a control, standard antibiotics, as well as DMSO, were included: streptomycin (50 µg) for bacteria and hygromycin (50 µg) for fungi. Each assay was performed in triplicate.

A spore germination assay was carried out with *Magnaporthe grisea*, as described in Kettering *et al*. (2005). The fungus was grown on CM medium (Talbot *et al*., 1993) in a 16-hour-light

(27 °C) and 8-hour-dark cycle (24 °C). After 10 days, conidia were collected, centrifuged at 1000 g for 10 min, and resuspended in $_{di}H_2O$ or CM medium to achieve a final concentration of $5x10^5$ spores/mL. In 24-well microtiter plates, $2.5x10^4$ conidia in 1 mL of $_{di}H_2O$ or CM medium were exposed to the isolated compound (5, 10, 25 and 50 µg/mL) during 16h, at 28 °C. Germinated spores were counted with an inverted microscope. The assay was performed in triplicate. This approach was adapted for *Botrytis cinerea* (Markwart *et al*., 2019; Schüffler *et al*., 2009), which was previously cultured on the appropriate medium for 3 to 4 weeks (Table 2.1, section 2.1.6.3).

2.2.9.2. Phytotoxicity assays

Phytotoxicity assays towards *Lepidium sativum* and *Setaria italica* were carried out according to Markwart *et al*. (2019). First, 10, 20 and 50 µg (solved in DMSO) of the isolated compound were placed into wells of a 48-well microtiter plate. After solvent evaporation, 12 seeds of each species and 200 µL of $_{di}H_2O$ were added to each well. Consequently, the plates were incubated at 28 °C on a plant humidity chamber, for 5-7 days. All assays were conducted in triplicate. In addition, 5 % H_3PO_4 in water was used as a control.

2.2.9.3. Cytotoxicity assays

Cytotoxic activity by the isolated compound towards human cancer cells was carried out according to Zapf *et al*. (1995), with some modifications. Hela S3 cells were grown in DMEM supplemented with 10 % FBS, 65 µg/mL penicillin G and 100 µg/mL streptomycin sulfate, at 37 °C, in a humidified atmosphere with 5 % CO_2. In a 96-well microtiter plate, $5x10^4$ cells/mL were exposed to the isolated compound (5 and 50 µg/mL). After 72 h, the cell line was observed under the microscope for analysis of cellular proliferation.

Resazurin-based assays were performed as previously described (Duarte *et al*., 2015; Félix *et* al., 2016; 2019b) with slight modifications, in order to evaluate the effect of the isolated compound on Vero and A375 cells' viability. Vero and A375 cell lines were maintained according to Ammerman *et al*. (2008) and ATCC published instructions. After cell adhesion on the 96-well microtiter plate, the cells were exposed to the pure compound (1:1 in DMEM) at different concentrations: 0.01, 0.025, 0.05, 0.1, 0.5 and 1 mg/mL in PBS. For sample solubilizing and preparation, the compound was solved in 4 % methanol (in PBS) and serial dilutions were made with PBS to achieve the final concentrations. After 24 h of incubation at 37 °C, 95 % relative humidity and 5 % CO_2, the medium was removed and replaced with 50 µL of DMEM with 10 % resazurin (0.1 mg/mL in PBS). The microtiter plates were incubated at 37 °C, 95 % relative humidity and 5 % CO_2, for 3 to 4 h. The absorbance was measured at 570 and 600 nm wavelengths in a microtiter plate spectrophotometer. PBS and 4 % methanol (in PBS) were used as controls. Positive and negative controls corresponded to non-exposed cells and resazurin absorbance (empty wells), respectively. The data was expressed as means of 5 technical replicates ± standard error. The statistical analysis was performed with GraphPad Prism v8. Statistical significance of differences between each concentration with the control (4 % methanol) were determined by one-way ANOVA analysis.

3. RESULTS AND DISCUSSION

3.1. Fermentation parameters

Previously, in chapter 1, *L. hormozganensis* CBS339.90 was cultivated in 500 mL of 5 different media. Among these media, BAF was the most promising to induce higher activity and consequently higher production of active compounds by the fungus. For this reason, large-scale fermentation of strain CBS339.90 was conducted to obtain larger amounts of secondary metabolites. This process was monitored every day for pH, glucose and mycelium dry weight (Figure 2.1). The fermentation was ended after 6 days, when glucose was completely depleted and pH level (4.9) was increasing. Both these parameters indicate the growth is on stationary phase, which is the ideal stage for secondary metabolites' production by fungi (Kavanagh, 2011). The glucose level decreased quickly, showing the rapid growth of strain CBS339.90 on a batch submerged fermentation with BAF medium. The biomass of the fungus fluctuated among the process, with the highest value being 6.93 g/L. When fungal biomass is high, the collection of mycelium samples could lead to miscalculations due to obtaining lower quantities than the corresponding in 20 L of media. Therefore, this is a possible explanation for the decreases observed in mycelium dry weight.

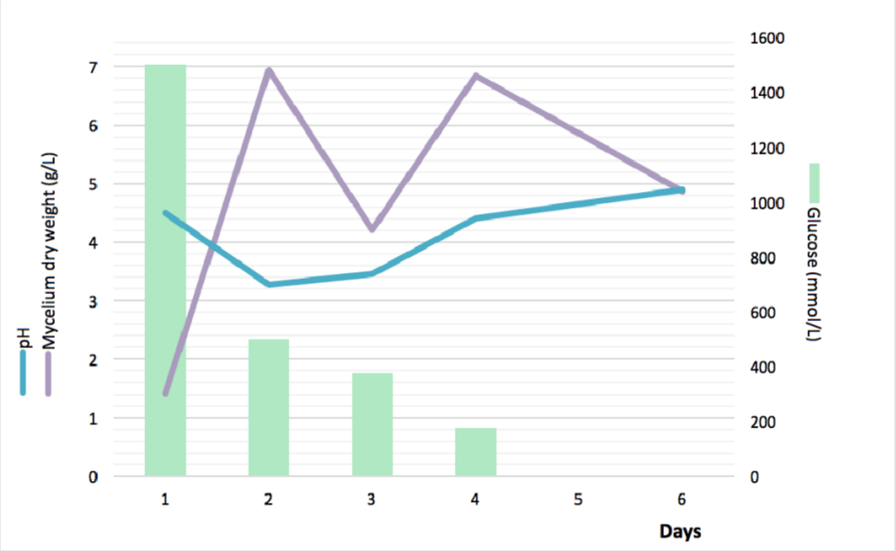


Figure 2.1 | Evaluation of pH, glucose (mmol/L) and mycelium dry weight (g/L) throughout large-scale fermentation of *L. hormozganensis* strain CBS339.90 in BAF medium.

3.2. Preliminary screening of biological activity of crude extracts

In total, 153.1 g of mycelium were obtained from the fermentation. After extraction of crude extracts from the culture filtrate (800 mg) and mycelium (280 mg), a preliminary screening was conducted to evaluate biological activity. HPLC chromatograms (Appendix), as well as cytotoxicity and antimicrobial results were obtained. Cytotoxicity results showed that the crude extracts (500 µg/mL) caused a decrease of 100 % on Vero-B4 cells' viability (Figure 2.2). On the other side, only the crude extract from culture filtrate caused effects on cell viability (100 % loss of viability) of Hela S3 cells (Figure 2.2).

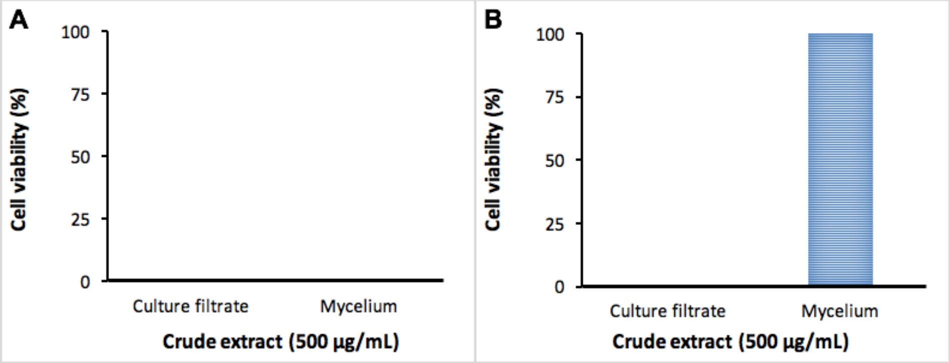


Figure 2.2 | Evaluation of cell viability (%) after 72 h exposure to crude extracts (500 µg/mL) of *L. hormozganensis* CBS339.90 grown in 20 L of BAF medium for 6 days, at 22 °C. The effect of the crude extracts was tested in Vero-B4 (**A**) and Hela S3 (**B**) cells.

In order to further understand which compounds were active towards Vero-B4 and Hela S3 cell lines, HPLC-based fractionation was combined with cytotoxicity assays on 96-well plates as described in section 2.2.4.2. The crude extract from the mycelium showed no effect on the cell lines, contrary to what was seen previously, which could mean the activity seen was due to a mixture of compounds that act synergistically or antagonistically. On the other side, CE_{CF} showed activity towards Hela S3 cells: 50 % loss of cell viability in 2 wells (retention time: 6.010 to 6.252, and 6.258 to 6.502 min, which appeared to represent the same peak). This sample also affected the viability of Vero-B4 cells by 100 % in 3 wells (retention times: 4.758 to 5.002, 5.008 to 5.252, and 5.258 to 5.502 min) and 50 % in 1 well (retention time: 3.508 to 3.752).

In addition, agar-disk diffusion assays were carried out and no effects on microbial growth were observed from the crude extracts (50 and 100 µg/disk) of the fungus (Table 2.3).

Table 2.3 | Agar-disk diffusion assay of crude extracts of *L. hormozganensis* strain CBS339.90 grown in 20 L of BAF medium for 6 days, at 22 °C. Biological activity is represented via diameter (mm) of inhibition halo for each microorganism. 'CF' and 'MY' stand for crude extracts from the culture filtrate and mycelium, respectively.

Sample	Amount		Inhibition diameter zone (± 0.5 mm)					
			Bacillus brevis	*Micrococcus luteus*	*Enterobacter dissolvens*	*Nematospora coryli*	*Paecilomyces variotii*	*Mucor miehei*
CBS339.90	**50 µg/disk**	**CF**	-	-	-	-	-	-
		MY	-	-	-	-	-	-
CBS339.90	**100 µg/disk**	**CF**	-	-	-	-	-	-
		MY	-	-	-	-	-	-
Positive control	Streptomycin		26	33	35			
	Hygromycin					20	24	15

In general, the results showed the crude extract from the culture filtrate of strain CBS339.90 had higher activity than the sample from the mycelium. Therefore, based on the preliminary findings, further procedures were performed with the culture filtrate as a goal to isolate active secondary metabolites.

3.3. Isolation of metabolites

Solid phase extraction was conducted with a hydrophobic column able to retain nonpolar compounds, collecting most organic analytes from aqueous matrices. Upon this, 10 fractions of the culture filtrate were obtained. Each fraction corresponded to a mixture of analytes in different retention time intervals (chromatograms at 230 nm in Appendix). Fraction 1 presented only one peak at 1.504 min, which was considered belonging to polysaccharides class. Further procedures were conducted on Fraction 2 and 3 according to the preliminary findings of biological activity (activity observed at retention times up to 6.502 min).

3.3.1. Isolation of compounds from fraction 2

Fraction 2 was optimized via HPLC to achieve a higher separation of the target analytes (based on cytotoxicity in section 3.2). After preparative HPLC, 6 intermediates were obtained: A (3.8 mg), B (2.4 mg), C (1.2 mg), D (1.9 mg), E (36.9 mg), F (1.8 mg). None of the intermediates were pure (i.e. values > 90 %), although C, E and F had 52, 85, and 81 % of purity, respectively. Further approaches were conducted to obtain pure compounds from intermediates B and E, which corresponded to the active peaks in section 3.2. After purification of B, two compounds were collected (0.5 mg each). However, the amounts were too low for detection by HPLC-MS. Moreover, cytotoxicity of the samples (250 µg/mL) on Vero-B4 cells was absent (data not shown).

After semi-preparative HPLC to purify intermediate E, 27.3 mg of compound (C1) were obtained with 93 % purity. This white color compound was identified as 4-ethylaniline, with an average mass of 121 Da, retention time of 5.769 min, and UV $_{(MeOH)}$ λ_{max} 235 nm (Figure 2.3).

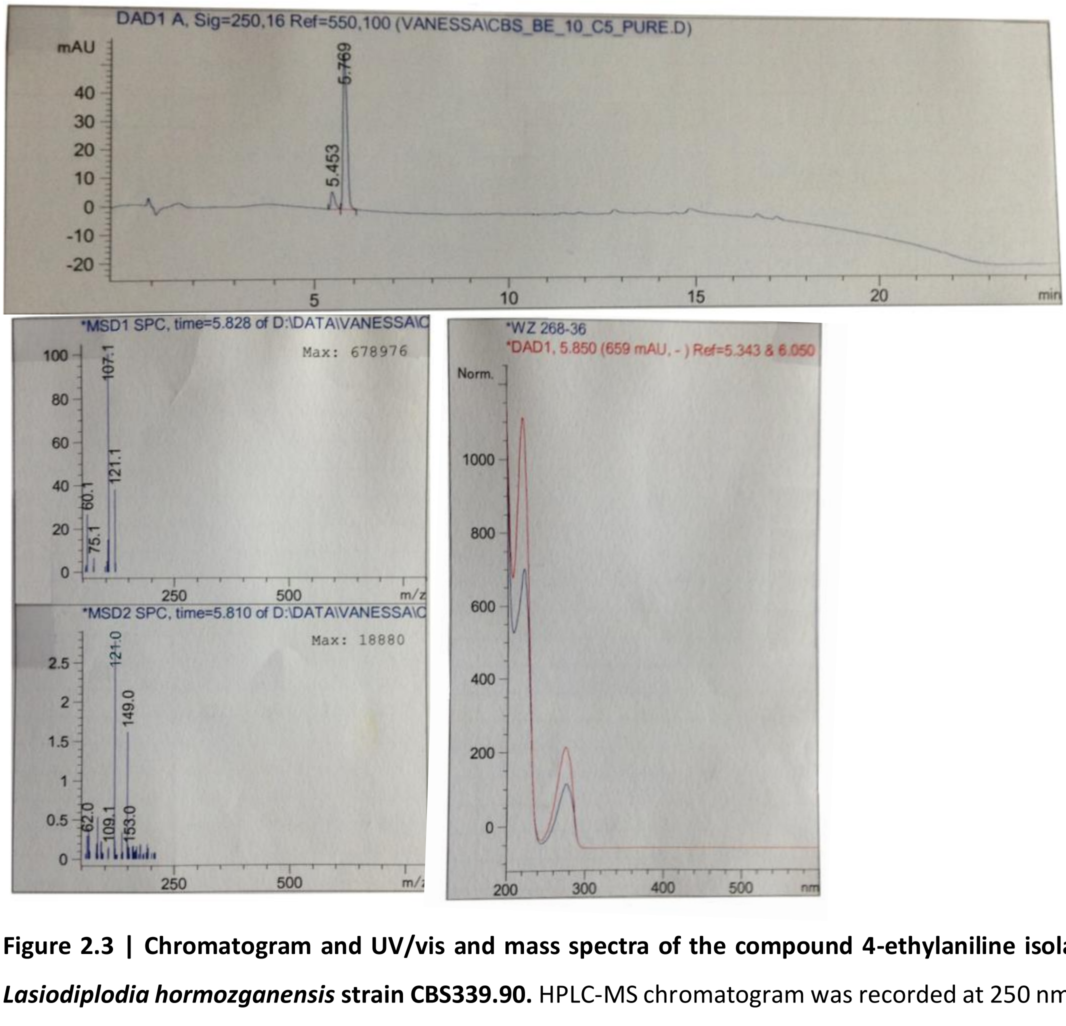


Figure 2.3 | Chromatogram and UV/vis and mass spectra of the compound 4-ethylaniline isolated from *Lasiodiplodia hormozganensis* strain CBS339.90. HPLC-MS chromatogram was recorded at 250 nm. Eluent A was 0.1 % formic acid. The positive ionization is shown in the upper mass spectrum. The negative ionization is shown below. On the right the UV/vis spectrum is presented.

4-ethylaniline is a primary monocyclic aromatic amine, derivative of an aromatic hydrocarbon containing an amino group. Biological assays of 4-ethylaniline showed that this compound had no effect on the tested bacteria, fungi, plants, and Hela S3 cells (Table 2.4). Also, according to the results from cytotoxicity in Vero and A375 cells, no statistical difference was found between the pure compound and the control (Figure 2.4). In fact, *p* value obtained after one-way ANOVA was superior to 0.05 ($p = 0.0717$ for Vero cells; $p = 0.7006$ for A375 cells), thus the differences between the means were not statistically significant.

Several aromatic amines are involved in human carcinogenesis or in the induction of tumors in animals (reviewed by Arora, 2015). The carcinogenic action is due to biotransformation of the compounds (National Research Council, 1981). One of the main processes is *N*-oxidation (e.g. by cytochrome P450 enzymes) which leads to *N*-hydroxylamines products (reviewed by Kim & Guengerich, 2005). These products have been detected in urine of several animals after administration of 4-ethylaniline and other primary aromatic amines, suggesting that *N*-oxidation occured (Damani, 1982). Upon this, *N*-hydroxylamines can be further activated by several enzymes, forming ester derivatives. Acetylation by N-acetyltransferases (NAT) is one of the processes that activates and leads to genotoxic, carcinogenic and reactive aromatic amines (Sim *et al*., 2008; reviewed by Kim & Guengerich, 2005). Moreover, our results show that 4-ethylaniline had no negative effect on the viability of human cancer cell lines, which is in consonance with the information known about this class of compounds.

Some bacteria produce enzymes (e.g. NAT enzymes) that degrade monocyclic aromatic amines, such as anilines, methylanilines, among others, as a way to detoxify the environment (reviewed by Arora, 2015). Furthermore, NAT enzymes are also expressed in humans and several mammals (reviewed by Sim *et al*., 2008). A hypothetical explanation for the absence of activity of 4-ethylaniline towards the test organisms in this study could be related to the expression of NAT enzymes by the organisms. On the other side, unlike other aromatic amines, 4-ethylaniline could be a compound with non-toxic properties. However, the last hypothesis contradicts previous findings that showed toxicity of 4-ethylaniline towards mouse models, quail and birds (National Library of Medicine - TOXNET, 2019). As *L. hormozganensis* is a phytopathogenic fungus with established ability to affect the viability of mammalian cells (Félix *et al*., 2016; 2018a), it is proposed that 4-ethylaniline is not directly related to cytotoxicity.

Aromatic amines are common pollutants in soil, thus, bioremediation strategies through the use of fungi that produce N-acetyltransferases has become an interest research area (Harms *et al*., 2011). In addition, as mentioned in the last paragraph, other organisms are also implicated in detoxifying aromatic amines. Nonetheless, in this work, a fungus is reported for the first time, as a producer of 4-ethylaniline, a compound that belongs to the class of aromatic amines, constituted by several pollutants and carcinogenic compounds. This is the first report concerning this production in *L. hormozganensis*. In this line, further studies could aid to understand the role of 4-ethylaniline, particularly regarding the metabolic pathway in which it is involved. Although this compound exhibited no biological activity, thus appearing not to intervene in virulence by this fungus, future research could be conducted to comprehend if related compounds are involved in virulence by this fungus.

Table 2.4 | Summarized raw data of biological assays of 4-ethylaniline.

Test organisms	Amount of sample	Result
Bacillus brevis	10 µg/disk	-
	50 µg/disk	-
Staphylococcus aureus subsp. aureus	10 µg/disk	-
	50 µg/disk	-
Candida albicans	10 µg/disk	-
	50 µg/disk	-
Mucor miehei	10 µg/disk	-
	50 µg/disk	-
Penicillium notatum	10 µg/disk	-
	50 µg/disk	-
Paecilomyces variotii	10 µg/disk	-
	50 µg/disk	-
***Magnaporthe grisea in* H_20**	5 µg/mL	-
	10 µg/mL	-
	25 µg/mL	-
	50 µg/mL	-
***Magnaporthe grisea in* CM medium**	5 µg/mL	-
	10 µg/mL	-
	25 µg/mL	-
	50 µg/mL	-
Botrytis cinerea	5 µg/mL	-
	10 µg/mL	-
	25 µg/mL	-
	50 µg/mL	-
Setaria italica	10 µg/well	-
	20µg/well	-
	50µg/well	-
Lepidium sativum	10 µg/well	-
	20µg/well	-
	50µg/well	-
Hela S3 cells	5 µg/mL	-
	50 µg/mL	-

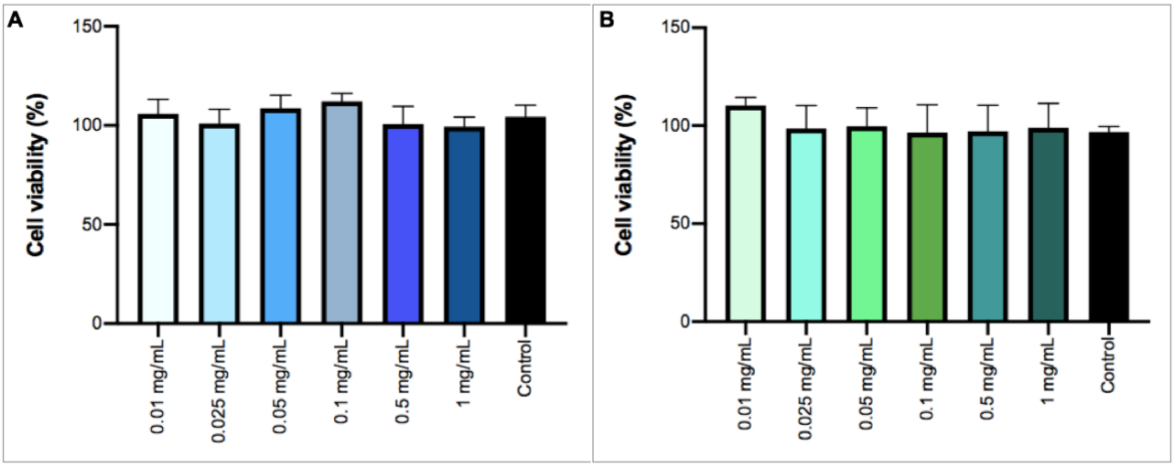


Figure 2.4 | Cytotoxicity of 4-ethylaniline in different concentrations (0.01, 0.025, 0.05, 0.1, 0.5 and 1 mg/mL) in Vero (A) and A375 (B) cells. Data are presented as average ± standard error. One-way ANOVA was used to determine the significance of cytotoxicity of each concentration of the pure metabolite against the control.

3.3.2. Isolation of compounds from fraction 3

Fraction 3 was also optimized via HPLC to achieve a higher separation of the target analytes. After HPLC-based fractionation in 24-well microtiter plates, 6 intermediate compounds were obtained: 3A (1 mg), 3B (0.7 mg), 3C (0.6 mg), 3D (no amount), 3E (0.3 mg), 3F (0.7 mg). None of the intermediates were pure, and cytotoxicity (as described in section 2.2.6) was evaluated. Only intermediate 3F showed to decrease 25 % of total viability in Vero-B4 cells (data not shown). Hence, this preliminary result was used for bioactivity-based isolation of active compounds from fraction 3. Upon this, optimization via HPLC was conducted to promote separation of intermediate 3F. After preparative-HPLC, two compounds were collected (1.5 mg each): compound 2 (C2) and 3 (C3). C3 is unknown (based on comparison with IBWF database) and given its low amount, the structure was still not elucidated. C2 was identified, due to a match with the IBWF database, as orsellinic acid. This metabolite presented an average mass of 167 Da, retention time of 7.624 min, and UV $_{(MeOH)}$ λ_{max} 215 nm (Figure 2.5). Compound 3 is unknown (comparing to IBWF database), with a retention time of 7.560 min and an average mass of 237 Da.

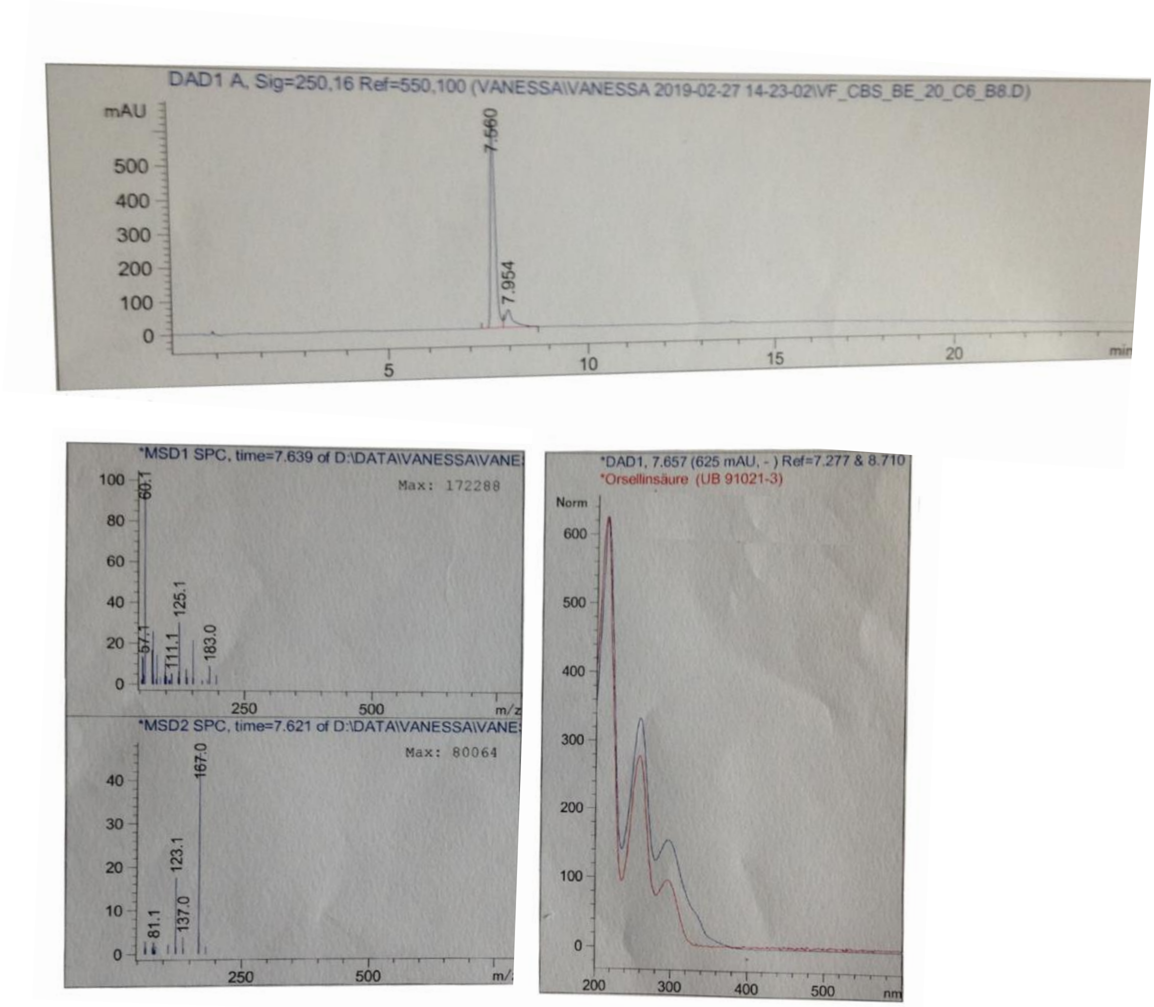


Figure 2.5 | Chromatogram and UV/vis and mass spectra of orsellinic acid isolated from *Lasiodiplodia hormozganensis* strain CBS339.90. HPLC-MS chromatogram was recorded at 250 nm. Eluent A was 0.1 % formic acid. The positive ionization is shown in the upper mass spectrum. The negative ionization is shown below. On the right the UV/vis spectrum is presented.

Orsellinic acid is the simplest acetate-derived phenol, thought to be a product of the polyketide synthases (PKS) pathway, even though the molecular basis of its synthesis is not fully understood (Schroeckh *et al*., 2009; Jørgensen *et al*., 2014). This compound has been previously isolated from fungi, such as *Fusarium graminearum*, *Chaetomium globosum* and *Aspergillus nidulans* (Bashyal *et al*., 2005; Jørgensen *et al*., 2014; Sanchez *et al*., 2010; Schroeckh *et al*., 2009). In addition, this is the first report of *L. hormozganensis* producing orsellinic acid. Nonetheless, in *L. hormozganensis* genome, two genes have been identified (*orsE_O* and *opS1_1*), which could be related to the production of orsellinic acid by this fungus (unpublished data). Gene *orsE_O* encodes for dehydrogenase orsE (or orsellinic acid/F9775 biosynthesis cluster protein E), a participant in the biosynthesis of orsellinic acid. Even though dehydrogenase orsE is involved in secondary metabolism, its function is still unknown (UniProt, 2019). Moreover, *opS1_1* encodes for orsellinic acid synthase (EC 2.3.1), which is involved in the production of orsellinic acid, and consequently oosporein, the latter being a metabolite important for fungal virulence of *Beauveria bassiana* (Feng *et al*., 2015).

Orsellinic acid is a precursor of several fungal metabolites (Schroeckh *et al*., 2009). In literature, several authors demonstrated that the inactivation of orsellinic acid synthases causes absence of production of orsellinic acid by the organisms (Sanchez *et al*., 2010; Feng *et al*., 2015). In this study, a low amount (1.5 mg) of orsellinic acid was isolated from 20 L fermentation, which could be due to transformation of this compound to other derivative metabolites. In turn, these metabolites may be biologically active. Ergo, further studies are required to understand the association of orsellinic acid in *L. hormozganensis* with known produced secondary metabolites, particularly related to virulence in plants and potential mammal or human opportunistic pathogenesis by this fungus.

4. CONCLUSIONS

Large-scale fermentation of *L. hormozganensis* CBS339.90 in BAF medium led to the production of several compounds. Bioactivity-based assays on the culture filtrate of this fungus resulted in the isolation of two known compounds: 4-ethylaniline and orsellinic acid. Both compounds were reported for the first time in *L. hormozganensis*, and 4-ethylaniline was identified for the first time in a fungus. Characterization of 4-ethylaniline showed no biological activity towards bacteria, fungi, plants, human cancer and mammalian cell lines. In this context, this compound seems not to pay a direct role on fungal virulence. In addition, orsellinic acid amount was too low for characterization. Withal, this compound is the precursor of several secondary metabolites which could in turn be active. In conclusion, further studies are needed to comprehend if the fungus produces 4-ethylaniline related compounds, or derivatives of orsellinic acid that may be involved in virulence. Also, orsellinic acid should be further characterized to study its biological activity.

FUTURE PROSPECTS

Lasiodiplodia theobromae LA-SOL3 is a known phytopathogen identified as an opportunist in human infections. Furthermore, the phytopathogen *L. hormozganensis* CBS339.90 affects mammalian cells, leading to an increasing interest on its potential to opportunistically infect humans. Henceforth, further experiments are still required to understand virulence mechanisms of these fungi towards the human host.

Optimization of *Agrobacterium tumefaciens*-mediated transformation protocol in *L. hormozganensis* CBS339.90 and *L. theobromae* LA-SOL3 could be helpful to consequently achieve successful knockout of genes that encode potential virulence-related compounds, specifically related to human infection, such as *ssd1* gene. The present work should be followed by characterization of mutant fungi, and complementation after gene inactivation on fungal genomes, to evaluate if the phenotypic changes between wild-type and mutant fungi are directly due to gene knockout.

One could also conduct research *in vitro* with fungal growth in human environment mimic media, to understand any virulence traits specific for this type of interaction, as well as virulence factors produced by fungi. Moreover, *in vivo* studies should be performed in animal models.

This work could be further completed with large-scale fermentation of *L. theobromae* LA-SOL3 to study the effect of gene inactivation on the secondary metabolism of the fungi (mutant and wild-type), particularly related to virulence attributes.

In addition, research that allows the study of the molecular mechanisms related to fungal virulence are needed, e.g. the study of pathways involved in pathogenicity. In this line, orsellinic acid should be further characterized with different biological assays to access if it has a role on the virulence of *L. hormozganensis* CBS339.90.

REFERENCES

Abdollahzadeh, J., Javadi, A., Goltapeh, E. M., Zare, R., & Phillips, A. J. L. (2010). Phylogeny and morphology of four new species of *Lasiodiplodia* from Iran. *Persoonia: Molecular Phylogeny and Evolution of Fungi*, 25, 1-10.

Ahadi, A., Partoazar, A., Abedi-Khorasgani, M., & Shetab-Boushehri, S. V. (2011). Comparison of liquid-liquid extraction-thin layer chromatography with solid-phase extraction-high-performance thin layer chromatography in detection of urinary morphine. *Journal of Biomedical Research*, 25(5), 362-367.

Al-Sadi, A. M., Al-Wehaibi, A. N., Al-Shariqi, R. M., Al-Hammadi, M. S., Al-Hosni, I. A., Al-Mahmooli, I. H., & Al-Ghaithi, A. G. (2013). Population genetic analysis reveals diversity in *Lasiodiplodia* species infecting date palm, citrus, and mango in oman and the UAE. *Plant Disease*, 97, 1363-1369.

Alam, M. S., Begum, M., Sarkar, M. A., Islam, M. R., & Alam, M. S. (2001). Effect of temperature, light and media on growth, sporulation, formation of pigments and pycnidia of *Botryodiplodia theobromae* Pat. *Pakistan Journal of Biological Sciences*, 4(10), 1224-1227.

Alves, A., Barradas, C., Phillips, A. J. L., & Correia, A. (2013). Diversity of Botryosphaeriaceae species associated with conifers in Portugal. *European Journal of Plant Pathology*, 135, 791-804.

Alves, A., Crous, P. W., Correia, A., & Phillips, A. J. L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity*, 28, 1-13.

Ammerman, N. C., Beier-Sexton, M., & Azad, A. F. (2008). Growth and maintenance of Vero cell lines. *Current Protocols in Microbiology*, A-4E.

Arora, P. K. (2015). Bacterial degradation of monocyclic aromatic amine. *Frontiers in Microbiology*, 6, 820.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6, 71-79.

Baron, C., & Zambryski, P. C. (1996). Plant transformation: A pilus in *Agrobacterium* T-DNA transfer. *Current Biology*, 6(12), 1567-1569.

Bashyal, B. P., Wijeratne, E. M. K., Faeth, S. H., & Gunatilaka, A. A. L. (2005). Globosumones A-C, cytotoxic orsellinic acid esters from the sonoran desert endophytic fungus *Chaetomium globosum*. *Journal of Natural Products*, 68, 724-728.

Bayram, Ö., & Braus, G. H. (2012). Coordination of secondary metabolism and development in fungi: The velvet family of regulatory proteins. *FEMS Microbiology Reviews*, 36(1), 1-24.

Bebber, D. P., Ramotowski, M. A. T., & Gurr, S. J. (2013). Crop pests and pathogens move polewards in a warming world. *Nature Climate Change*, 3, 985-988.

Bignell, E., Cairns, T. C., Throckmorton, K., Nierman, W. C., & Keller, N. P. (2016). Secondary metabolite arsenal of an opportunistic pathogenic fungus. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371, 20160023.

Bleuven, C., & Landry, C. R. (2016). Molecular and cellular bases of adaptation to a changing environment in microorganisms. *Proceedings of the Royal Society B: Biological Sciences*, 283(1841), 20161458.

Braga, R. M., Dourado, M. N., & Araújo, W. L. (2016). Microbial interactions: ecology in a molecular perspective. *Brazilian Journal of Microbiology*, 47S, 86-98.

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nature Reviews Microbiology*, 11, 21-32.

Bullock, W. O., Fernandez, J. M., & Short, J. M. (1987). XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques*, 5(3), 376-379.

Burgess, T. I., Tan, Y. P., Garnas, J., Edwards, J., Scarlett, K. A., Shuttleworth, L. A., … & Jami, F. (2018). Current status of the Botryosphaeriaceae in Australia. *Australasian Plant Pathology*, 48(1), 35-44.

Carlet, C., Tachikart, A., Bellanger, A. P., Aubin, F., Chirouze, C., & Klopfenstein, T. (2019). *Lasiodiplodia theobromae* deep mycosis in a kidney transplant patient. *Médecine et Maladies Infectieuses*, 49, 545-547.

Chagas, M. B. de O., dos Santos, I. P., da Silva, L. C. N., Correia, M. T. dos S., de Araújo, J. M., Cavalcanti, M. da S., & Lima, V. L. de M. (2017). Antimicrobial Activity of Cultivable Endophytic Fungi Associated with *Hancornia Speciosa* Gomes Bark. *The Open Microbiology Journal*, 11, 179-188.

Chen, L., Wang, Q., Chen, H., Sun, G., Liu, H., & Wang, H. (2016a). *Agrobacterium tumefaciens*-mediated transformation of *Botryosphaeria dothidea*. *World Journal of Microbiology and Biotechnology*, 32(7), 106.

Chen, S., Chen, D., Cai, R., Cui, H., Long, Y., Lu, Y., Li, C., & She, Z. (2016b). Cytotoxic and Antibacterial preussomerins from the mangrove endophytic fungus *Lasiodiplodia theobromae* ZJ-HQ1. *Journal of Natural Products*, 79(9), 2397–2402.

Cimmino, A., Scafato, P., Mathieu, V., Ingels, A., D'Amico, W., Pisani, L., … & Evidente, A. (2016). Natural and synthetic furanones with anticancer activity. *Natural Product Communications*, 11(10), 1471–1474.

Correia, K. C., Silva, M. A., de Morais, M. A., Armengol, J., Phillips, A. J. L., Câmara, M. P. S., & Michereff, S. J. (2016). Phylogeny, distribution and pathogenicity of *Lasiodiplodia* species associated with dieback of table grape in the main Brazilian exporting region. *Plant Pathology*, 65, 92-103.

Cramer Jr, R. A., & Perfect, J. R. (2009). CHAPTER 2 - Recent advances in understanding human opportunistic fungal pathogenesis mechanisms. In Clinical Mycology. 2nd ed. Churchill Livingstone: Elias J. A., Michael R. M., Michael A. P. ISBN 9781416056805. Pages 15-31.

Custódio, F. A., Machado, A. R., Soares, D. J., & Pereira, O. L. (2018). *Lasiodiplodia hormozganensis* causing basal stem rot on *Ricinus communis* in Brazil. *Australasian Plant Disease Notes*, 13, 25.

Damani, L. A. (1982). Chapter 7 - Oxidation at Nitrogen Centers. In Metabolic basis of detoxication: metabolism of functional groups. New York: Academic Press. Jakobi, W. B., Bend, J. R., Caldwell, J.. ISBN 9780123800602. Pages 127-149.

de Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., & Beijersbergen (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology*, 16, 839-842.

de Souza, G. G., Pfenning, L. H., de Moura, F., Salgado, M., & Takahashi, J. A. (2013). Isolation, identification and antimicrobial activity of propolis-associated fungi. *Natural Product Research*, 27(18), 1705-1707.

Deshmukh, S. K., Prakash, V., & Ranjan, N. (2018). Marine fungi: A source of potential anticancer compounds. *Frontiers in Microbiology*, 8, 2536.

Desprez-Loustau, M. L., Marçais, B., Nageleisen, L, Piou, D., & Vannini, A. (2006). Interactive effects of drought and pathogens in forest trees. *Annals of Forest Science*, 63(6), 597-612.

Dinan, L., Harmatha, J., & Lafont, R. (2001). Chromatographic procedures for the isolation of plant steroids. *Journal of Chromatography A*, 935(1-2), 105-123.

Dissanayake, A. J., Phillips, A. J. L., Li, X. H., & Hyde, K.D. (2016). Botryosphaeriaceae: Current status of genera and species. *Mycosphere*, 7(7), 1001–1073.

Dong, S., & Wang, Y. (2016). Nudix Effectors: A Common Weapon in the Arsenal of Plant Pathogens. *PLoS Pathogens*, 12(8), e1005704.

dos Santos, A. L. S. (2011). Aspartic proteases of human pathogenic fungi are prospective targets for the generation of novel and effective antifungal inhibitors. *Current Enzyme Inhibition*, 7, 96-118.

Duarte, A. S., Cavaleiro, E., Pereira, C., Merino, S., Esteves, A. C., Duarte, E. P., Tomás, J. M., & Correia, A. C. (2015). *Aeromonas piscicola* AH-3 expresses an extracellular collagenase with cytotoxic properties. *Letters in Applied Microbiology*, 60(3), 288-297.

Elkhayat, E. S., & Goda, A. M. (2017). Antifungal and cytotoxic constituents from the endophytic fungus *Penicillium* sp.. *Bulletin of Faculty of Pharmacy, Cairo University*, 55(1), 85-89.

Félix, C., Duarte, A. S., Vitorino, R., Guerreiro, A. C. L., Domingues, P., Correia, A. C. M., Alves, A., & Esteves, A. C. (2016). Temperature modulates the secretome of the phytopathogenic fungus *Lasiodiplodia theobromae*. *Frontiers in Plant Science*, 7, 1096.

Félix, C., Libório, S., Nunes, M., Félix, R., Duarte, A. S., Alves, A., & Esteves, A. C. (2018b). *Lasiodiplodia theobromae* as a producer of biotechnologically relevant enzymes. *International Journal of Molecular Sciences*, 19(2), pii: E29.

Félix, C., Meneses, R., Gonçalves, M. F. M., Tilleman, L., Duarte, A. S., Jorrín-Novo, J. V., Van de Peer, Y., Deforce, D., Van Nieuwerburgh, F., Esteves, A. C., & Alves, A. (2019a). A multi-omics analysis of the grapevine pathogen *Lasiodiplodia theobromae* reveals that temperature affects the expression of virulence- and pathogenicity-related genes. *Scientific Reports,* 9, 13144.

Félix, C., Salvatore, M. M., DellaGreca, M., Ferreira, V., Duarte, A. S., Salvatore, F., Naviglio, D., Alves, A., Esteves, A. C., & Andolfi, A. (2019b). Secondary metabolites produced by grapevine strains of *Lasiodiplodia theobromae* grown at two different temperatures. *Mycologia*, 111(3), 466-476.

Félix, C., Salvatore, M. M., DellaGreca, M., Meneses, R., Duarte, A. S., Salvatore, F., Naviglio, D., Gallo, M., Jorrín-Novo, J. V., Alves, A., Andolfi, A., & Esteves, A. C. (2018a). Production of toxic metabolites by two strains of *Lasiodiplodia theobromae*, isolated from a coconut tree and a human patient. *Mycologia*, 110(4), 642-653.

Feng, P., Shang, Y., Cen, K., & Wang, C. (2015). Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity. *Proceedings of the National Academy of Sciences of the United States of America*, 112(36), 11365-11370.

Fiedler, S., & Wirth, R. (1988). Transformation of bacteria with plasmid DNA by electroporation. *Analytical Biochemistry*, 170, 38-44.

Fingrut, O., & Flescher, E. (2002). Plant stress hormones suppress the proliferation and induce apoptosis in human cancer cells. *Leukemia*, 16(4), 608–616.

Fisher, M. C., Hawkins, N. J., Sanglard, D., & Gurr, S. J. (2018). Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*, 360(6390), 739-742.

Frandsen, R. J. N., Frandsen, M., & Giese, H. (2012). Targeted gene replacement in fungal pathogens via *Agrobacterium tumefaciens*-mediated transformation. *Methods in Molecular Biology*, 835, 17-45.

Gank, K. D., Yeaman, M. R., Kojima, S., Yount, N. Y., Park, H., Edwards, J. E., … & Fu, Y. (2008). SSD1 is integral to host defense peptide resistance in *Candida albicans*. *Eukaryotic Cell*, 7(8), 1318–1327.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6, 343–345.

Gonzales, M. F., Brooks, T., Pukatzki, S. U., & Provenzano, D. (2013). Rapid protocol for preparation of electrocompetent *Escherichia coli* and *Vibrio cholerae*. *Journal of Visualized Experiments*, 80, 50684.

Gu, H. J., Kim, Y. J., Lee, H. J., Dong, S. H., Kim, S. W., Huh, H. J., & Ki, C. S. (2016). invasive fungal sinusitis by *Lasiodiplodia theobromae* in a patient with aplastic anemia: An Extremely Rare Case Report and Literature Review. *Mycopathologia*, 181(11-12), 901-908.

Hahismoto, S., Yazawa, S., Asao, T., Faried, A., Nishimura, T., Tsuboi, K., … & Kuwano, H. (2008). Novel sugar-cholestanols as anticancer agents against peritoneal dissemination of tumor cells. *Glycoconjugate Journal*, 25, 531–544.

Handayani, D., Rasyid, W., Rustini, Zainudin, E. N., & Hertiani, T. (2018). Cytotoxic activity screening of fungal extracts derived from the West Sumatran marine sponge *Haliclona fascigera* to several human cell lines: Hela, WiDr, T47D and Vero. *Journal of Applied Pharmaceutical Science*, 8(1), 55-58.

Harms, H., Schlosser, D., & Wick, L. Y. (2011). Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. *Nature Reviews Microbiology*, 9(3), 177-92.

He, L., Feng, J., Lu, S., Chen, Z., Chen, C., He, Y., Yi, X., & Xi, L. (2017). Genetic transformation of fungi. *International Journal of Developmental Biology*, 61, 375-381.

Heatley, N. G. (1944). A method for the assay of penicillin. *Biochemical Journal*, 38(1), 61-65.

Hooykaas, P. J. J., van Heusden, G. P. H., Niu, X., Roushan, M. R., Soltani, J., Zhang, X., & van der Zaal, B. J. (2018). *Agrobacterium*-mediated transformation of yeast and fungi. *Current Topics in Microbiology and Immunology*, 418, 349-374.

Hube, B. (2009). Fungal adaptation to the host environment. *Current Opinion in Microbiology*, 12, 347-349.

Idnurm, A., Bailey, A. M., Cairns, T. C., Elliott, C. E., Foster, G. D., Ianiri, G., & Jeon, J. (2017). A silver bullet in a golden age of functional genomics: the impact of *Agrobacterium*-mediated transformation of fungi. *Fungal Biology and Biotechnology*, 4(6), 1-28.

Iyalla, C. (2017). A review of the virulence factors of pathogenic fungi. *African Journal of Clinical and Experimental Microbiology*, 18(1), 53-58.

Järvinen, P., Nybond, S., Marcourt, L., Queiroz, E. F., Wolfender, J., Mettälä, A., Karp, M., Vuorela, H., Vuorela, P., Hatakka, A., & Tammela, P. (2016). Cell-based bioreporter assay coupled to HPLC micro-fractionation in the evaluation of antimicrobial properties of the basidiomycete fungus *Pycnoporus cinnabarinus*. *Pharmaceutical Biology*, 54(6), 1108-1115.

Jin, B., Jiang, F., Xu, F., & Ding, Z. (2011). An antitumor activity endophytic fungus A33 isolated from *Viscum Coloratum* of Chinese. *2011 International Conference on Remote Sensing, Environment and Transportation Engineering, RSETE 2011 - Proceedings*, 7368–7371.

Jørgensen, S. H., Frandsen, R. J. N., Nielsen, K. F., Lysøe, E., Sondergaard, T. E., Wimmer, R., Giese, H., & Sørensen, J. L. (2014). *Fusarium graminearum* PKS14 is involved in orsellinic acid and orcinol synthesis. *Fungal Genetics and Biology*, 70, 24-31.

Jung, S. I., Finkel, J. S., Solis, N. V., Chaili, S., Mitchell, A. P., Yeaman, M. R., & Filler, S. G. (2013). Bcr1 functions downstream of *ssd1* to mediate antimicrobial peptide resistance in *Candida albicans*. *Eukaryotic Cell*, 12(3), 411-419.

Kavanagh, K. (2011). CHAPTER 4 - Fungal Fermentation Systems and Products. In Fungi: Biology and Applications. Chihcester: Wiley-Blackwell. ISBN 978-0-470-97710-1. Pages 89-112.

Kettering, M., Valdivia, C., Sterner, O., Anke, H., & Thines, E. (2005). Heptemerones A-G, seven novel diterpenoids from *Coprinus heptemerus*: Producing organism, fermentation, isolation and biological activities. *Journal of Antibiotics*, 58(6), 390-396.

Kim, D., & Guengerich, F. P. (2005). Cytochrome P450 activation of arylamines and heterocyclic amines. *Annual Review of Pharmacology and Toxicology*, 45, 27-49.

Lazo, G. R., Stein, P. A., & Ludwig, R. A. (1991). A DNA transformation–competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology*, 9(10), 963-967.

Lehrnbecher, T., Frank, C., Engels, K., Kriener, S., Groll, A. H., & Schwabe, D. (2010). Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *Journal of Infection*, 61(3), 259-265.

Li, D., Tang, Y., Lin, J., & Cai, W. (2017). Methods for genetic transformation of filamentous fungi. *Microbial Cell Factories*, 16, 168.

Li, G. Q., Arnold, R. J., Liu, F. F., Li, J. Q., & Chen, S. F. (2015). Identification and Pathogenicity of *Lasiodiplodia* Species from *Eucalyptus urophylla* × *grandis*, *Polyscias balfouriana* and *Bougainvillea spectabilis* in Southern China. *Journal of Phytopathology*, 163(11–12), 956–967.

Li, M. Z., & Elledge, S. J. (2007). Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC. *Nature Methods*, 4, 251-256.

Lind, A. L., Smith, T. D., Saterlee, T., Calvo, A. M., & Rokas, A. (2016). Regulation of secondary metabolism by the velvet complex is temperature-responsive in *Aspergillus*. *G3: Genes, Genomes, Genetics*, 6, 4023-4033.

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V., & Brakhage, A. A. (2016). Regulation and Role of Fungal Secondary Metabolites. *Annual Review of Genetics*, 50, 371-392.

Mahmood, S., Rehman, S., & Hasnain, S. (2015). Cross-kingdom pathogenicity across plants and human beings. *Journal of Bacteriology & Parasitology*, 6, 4.

Markwart, J. C., Battig, A., Zimmermann, L., Wagner, M., Fischer, J., Schartel, B., & Wurm, F. R. (2019). Systematically Controlled Decomposition Mechanism in Phosphorus Flame Retardants by Precise Molecular Architecture: P–O vs P–N. *ACS Applied Polymer Materials*, 1(5), 1118-1128.

Martin, M., & Guiochon, G. (2005). Effects of high pressure in liquid chromatography. *Journal of Chromatography A*, 1090(1-2), 16-38.

Maslen, M. M., Collis, T., & Stuart, R. (1996). *Lasiodiplodia theobromae* isolated from a subcutaneous abscess in a Cambodian immigrant to Australia. *Journal of Medical and Veterinary Mycology*, 34, 279-283.

Michielse, C. B., Hooykaas, P. J. J., van den Hondel, C. A. M. J. J., & Ram, A. F. J. (2005). *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Current Genetics*, 48, 1-17.

Mohan, M., Shalin, S. C., Kothari, A., Rico, J. C., Caradine, K., & Burgess, M. (2016). *Lasiodiplodia* species fungal osteomyelitis in a multiple myeloma patient. *Transplant Infectious Disease*, 18(5), 761-764.

Monod, M., Capoccia, S., Léchenne, B., Zaugg, C., Holdom, M., & Jousson, O. (2002). Secreted proteases from pathogenic fungi. *International Journal of Medical Microbiology*, 292(5-6), 405-419.

Moron, L. S., Lim, Y., & dela Cruz, T. E. E. (2018). Antimicrobial activities of crude culture extracts from mangrove fungal endophytes collected in Luzon Island, Philippines. *Philippine Science Letters*, 11(Supplement).

Mullins, E. D., Chen, X., Romaine, P., Raina, R., Geiser, D. M., & Kang, S. (2001). *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. *Phytopathology*, 91(2), 173-180.

Muniz, C. R., Da Silva, G. F., Souza, M. T., Freire, F. C. O., Kema, G. H. J., & Guedes, M. I. F. (2014). *Agrobacterium tumefaciens*-mediated transformation of *Lasiodiplodia theobromae,* the causal agent of gummosis in cashew nut plants. *Genetics and Molecular Research*, 13(2), 2906-2913.

National Library of Medicine – TOXNET (2019). 4-ethylaniline. Available: https://chem.nlm.nih.gov/chemidplus/rn/589-16-2. [Accessed at 22 October 2019].

National Research Council (1981). Aromatic amines: an assessment of the biological and environmental effects. Washington, DC : The National Academies Press. Pages 40-59.

Olivera, I. E., Fins, K. C., Rodriguez, S. A., Abiff, S. K., Tartar, J. L., & Tartar, A. (2016). Glycoside hydrolases family 20 (GH20) represent putative virulence factors that are shared by animal pathogenic oomycetes, but are absent in phytopathogens. *BMC Microbiology*, 16, 232.

Orlandelli, R. C., Alberto, R. N., Almeida, T. T., Azevedo, J. L., & Pamphile, J. A. (2012). *In vitro* antibacterial activity of crude extracts produced by endophytic fungi isolated from *Piper hispidum* sw. *Journal of Applied Pharmaceutical Science*, 2(10), 137-141.

Pandi, M., Manikandan, R., & Muthumary, J. (2010). Anticancer activity of fungal taxol derived from *Botryodiplodia theobromae* Pat., an endophytic fungus, against 7, 12 dimethyl benz(a)anthracene (DMBA)-induced mammary gland carcinogenesis in Sprague dawley rats. *Biomedicine and Pharmacotherapy*, 64, 48–53.

Papacostas, L. J., Henderson, A., Choong, K., & Sowden, D. (2015). An unusual skin lesion caused by *Lasiodiplodia theobromae*. *Medical Mycology Case Reports*, 8, 44-46.

Parker, I. M., & Gilbert, G. S. (2004). The evolutionary ecology of novel plant-pathogen interactions. *Annual Review of Ecology, Evolution, and Systematics*, 35, 675-700.

Phillips, A. J. L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M. J., Groenewald, J. Z., & Crous, P. W. (2013). The Botryosphaeriaceae: Genera and species known from culture. *Studies in Mycology*, 76, 51-167.

Purwandari, U. (2018). Metabolites of *Botryodiplodia theobromae* for therapeutic agent and food industry. *International Food Research Journal*, 25(3), 884–889.

Rho, H. S., Kang, S., & Lee, Y. H. (2001). *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. *Molecules and Cells*, 12(3), 407-411.

Rodríguez-Gálvez, E., Maldonado, E., & Alves, A. (2015). Identification and pathogenicity of *Lasiodiplodia theobromae* causing dieback of table grapes in Peru. *European Journal of Plant Pathology*, 141(3), 477-489.

Rotem, R., Heyfets, A., Fingrut, O., Blickstein, D., Shaklai, M., & Flescher, E. (2005). Jasmonates: Novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Research*, 65(5), 1984–1993.

Saha, A., Mandal, P., Dasgupta, S., & Saha, D. (2008). Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. *Journal of Environmental Biology*, 29(3), 407-410.

Saha, S., Sengupta, J., Banerjee, D., & Khetan, A. (2012). *Lasiodiplodia theobromae* Keratitis: A Case Report and Review of Literature. *Mycopathologia*, 174(4), 335-339.

Sakalidis, M. L., Slippers, B., Wingfield, B. D., Hardy, G. E. S. J., & Burgess, T. I. (2013). The challenge of understanding the origin, pathways and extent of fungal invasions: Global populations of the *Neofusicoccum parvum-N. ribis* species complex. *Diversity and Distributions*, 19(8), 873–883.

Sanchez, J. F., Chiang, Y., Szewczyk, E., Davidson, A. D., Ahuja, M., Oakley, C. E., … Wang, C. C. C. (2010). Molecular genetic analysis of the orsellinic acid/F9775 gene cluster of *Aspergillus nidulans*. *Molecular BioSystems*, 6(3), 587-593.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1), 1-10.

Schaller, M., Borelli, C., Korting, H. C., & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses*, 48(6), 365-377.

Scharf, D. H., Heinekamp, T., & Brakhage, A. A. (2014). Human and plant fungal pathogens: the role of secondary metabolites. *PLoS Pathogens*, 10(1), e1003859.

Schoettler, S., Bascope, M., Sterner, O., & Anke, T. (2006). Isolation and characterization of two verrucarins from *Myrothecium roridum*. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, 61(5-6), 309-314.

Schroeckh, V., Scherlach, K., Nützmann, H., Shelest, E., Schmidt-Heck, W., Schuemann, J., … Brakhage, A. A. (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences of the United States of America*, 106(84), 14558-14563.

Schüffler, A., Liermann, J. C., Kolshorn, H., Opatz, T., & Anke, H. (2009). Isolation, structure elucidation, and biological evaluation of the unusual heterodimer chrysoxanthone from the ascomycete IBWF11-95A. *Tetrahedron Letters*, 50, 4813–4815.

Sim, E., Walters, K., & Boukouvala, S. (2008). Arylamine N-acetyltransferases: From structure to function. *Drug Metabolism Reviews*, 40, 479-510.

Slippers, B., & Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews*, 21, 90-106.

Slippers, B., Boissin, E., Phillips, A. J. L., Groenewald, J. Z., Lombard, L., Wingfield, M. J., … Crous, P. W. (2013). Phylogenetic lineages in the Botryosphaeriales: A systematic and evolutionary framework. *Studies in Mycology*, 76, 31-49.

Sørensen, L. Q., Lysøe, E., Larsen, J. E., Khorsand-Jamal, P., Nielsen, K. F., & Frandsen, R. J. N. (2014). Genetic transformation of *Fusarium avenaceum* by *Agrobacterium tumefaciens* mediated transformation and the development of a USER-Brick vector construction system. *BMC Molecular Biology*, 15(15).

Steil, D., Schepers, C. L., Pohlentz, G., Legros, N., Runde, J., Humpf, H. U., Karch, H., & Müthing, J. (2015). Shiga toxin glycosphingolipid receptors of Vero-B4 kidney epithelial cells and their membrane microdomain lipid environment. *Journal of Lipid Research*, 56, 2322-2336.

Summerbell, R. C., Krajden, S., Levine, R., & Fuksa, M. (2004). Subcutaneous phaeohyphomycosis caused by *Lasiodiplodia theobromae* and successfully treated surgically. *Medical Mycology*, 42, 543-547.

Sweigard, J. A., Chumley, F., Carroll, A., Farrall, L., & Valent, B. (1997). A series of vectors for fungal transformation. *Fungal Genetics Reports*, 44, 19.

Talbot, N. J., Ebbole, D. J., & Hamer, J. E. (1993). Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *The Plant Cell*, 5, 1575-1590.

Tanaka, A., Christensen, M. J., Takemoto, D., Park, P., & Scott, B. (2006). Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *The Plant Cell*, 18, 1052-1066.

Tawfike, A. F., Romli, M., Clements, C., Abbott, G., Young, L., Schumacher, M., … & Edrada-Ebel, RA. (2019). Isolation of anticancer and anti-trypanosome secondary metabolites from the endophytic fungus *Aspergillus flocculus* via bioactivity guided isolation and MS based metabolomics. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1106-1107, 71-83.

Templeton, S. P., Rivera, A., Hube, B., & Jacobsen, I. D. (2018). Editorial: Immunity to human fungal pathogens: mechanisms of host recognition, protection, pathology, and fungal interference. *Frontiers in Immunology*, 9, 2337.

Thines E., Aguirre J., Foster A. J., & Deising H. B. (2006). Genetics of phytopathology: Secondary metabolites as virulence determinants of fungal plant pathogens. In Progress in Botany, vol 67. Springer, Berlin, Heidelberg: Esser K., Lüttge U., Beyschlag W., Murata J. ISBN 9783540279983. Pages 134-161.

Thirumurugan, D., Cholarajan, A., Raja, S. S. S., & Vijayakumar, R. (2018). CHAPTER 1 - An introductory chapter: secondary metabolites. In Secondary Metabolites – Sources and Applications. IntechOpen. Rijeka: Vijayakumar R., Raja S. S. S.. ISBN 9781789236439. Pages 3-21.

Tsai, H., Wheeler, M. H., Chang, Y. C., & Kwon-Chung, K. J. (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *Journal of Bacteriology*, 181(20), 6469-6477.

Tsukada, K., Takahashi, K., & Nabeta, K. (2010). Biosynthesis of jasmonic acid in a plant pathogenic fungus, *Lasiodiplodia theobromae*. *Phytochemistry*, 71(17-18), 2019-2023.

Tzima, A. K., Paplomatas, E. J., Schoina, C., Domazakis, E., Kang, S., & Goodwin, P. H. (2014). Successful *Agrobacterium* mediated transformation of *Thielaviopsis basicola* by optimizing multiple conditions. *Fungal Biology*, 118(8), 675-682.

UniProt (2019). Q5AUW6 (ORSE_EMENI). Available: https://www.uniprot.org/uniprot/Q5AUW6. [Accessed at 25 October 2019].

Úrbez-Torres, J. R. (2011). The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea*, 50(Supplement), S5−S45.

Utermark, J., & Karlovsky, P. (2008). Genetic transformation of filamentous fungi by *Agrobacterium tumefaciens*. *Protocol Exchange*, 83.

Valayil, J. M., Kuriakose, G. C., & Jayabaskaran, C. (2015). Modulating the biosynthesis of a bioactive steroidal saponin, cholestanol glucoside by *Lasiodiplodia theobromae* using abiotic stress factors. *International Journal of Pharmacy and Pharmaceutical Sciences*, 7(7), 114–117.

van Baarlen, P., Van Belkum, A., Summerbell, R. C., Crous, P. W., & Thomma, B. P. H. J. (2007). Molecular mechanisms of pathogenicity: How do pathogenic microorganisms develop cross-kingdom host jumps? *FEMS Microbiology Reviews*, 31, 239-277.

van Burik, J.-A. H., & Magee, P. T. (2001). Aspects of fungal pathogenesis in humans. *Annual Review of Microbiology*, 55, 743-772.

van De Wouw, A. P., & Howlett, B. J. (2011). Fungal pathogenicity genes in the age of "omics." *Molecular Plant Pathology*, 12(5), 507-514.

Vanam, H. P., Ather, M., Madhura, K. S., & Rudramurthy, S. M. (2019). First report of *Lasiodiplodia pseudotheobromae* keratitis susceptible to voriconazole in an Indian mango grower. *Access Microbiology*, 1(6).

Woo, P. C. Y., Lau, S. K. P., Ngan, A. H. Y., Tse, H., Tung, E. T. K., & Yuen, K. Y. (2008). *Lasiodiplodia theobromae* pneumonia in a liver transplant recipient. *Journal of Clinical Microbiology*, 46(1), 380-384.

Yan, J. Y., Zhao, W. S., Chen, Z., Xing, Q. K., Zhang, W., Chethana, K. W. T., … & Li, X. H. (2018). Comparative genome and transcriptome analyses reveal adaptations to opportunistic infections in woody plant degrading pathogens of Botryosphaeriaceae. *DNA Research*, 25(1), 87-102.

Yan, L., Xu, R., Zhou, Y., Gong, Y., Dai, S., Liu, H., & Bian, Y. (2019). Effects of medium composition and genetic background on *Agrobacterium*-mediated transformation efficiency of *Lentinula edodes*. *Genes*, 10(6), 467.

Yasumura, Y., Kawakita, Y., Simizu, B., & Terasima, T. (1988). Studies on SV40 in tissue culture: preliminary step for cancer research *in vitro.* In VERO cells: origin, properties and biomedical applications. Chiba University, Japan Department of Microbiology School of Medicine. Simizu, B., Terasima, T.. Pages 1-19.

Zapf, S., Hossfeld, M., Anke, H., Velten, R., & Steglich, W. (1995). Darlucins A and B, New Isocyanide Antibiotics from *Sphaerellopsis filum* (*Darluca filum*). *The Journal of Antibiotics*, 48(1), 36-41.

Zhang, W., Liu, A., Zhang, X., Yan, H., Liu, R., Pang, Q., Huang, J., Li, X., & Yan, J. (2014). Construction and evaluation of a transformant library of *Lasiodiplodia theobromae* generated through restriction enzyme-mediated integration. *Phytopathologia Mediterranea*, 53(3), 451-458.

Zhao, X., Mehrabi, R., & Xu, J. (2007). Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryotic Cell*, 6(10), 1701-1714.

APPENDIX

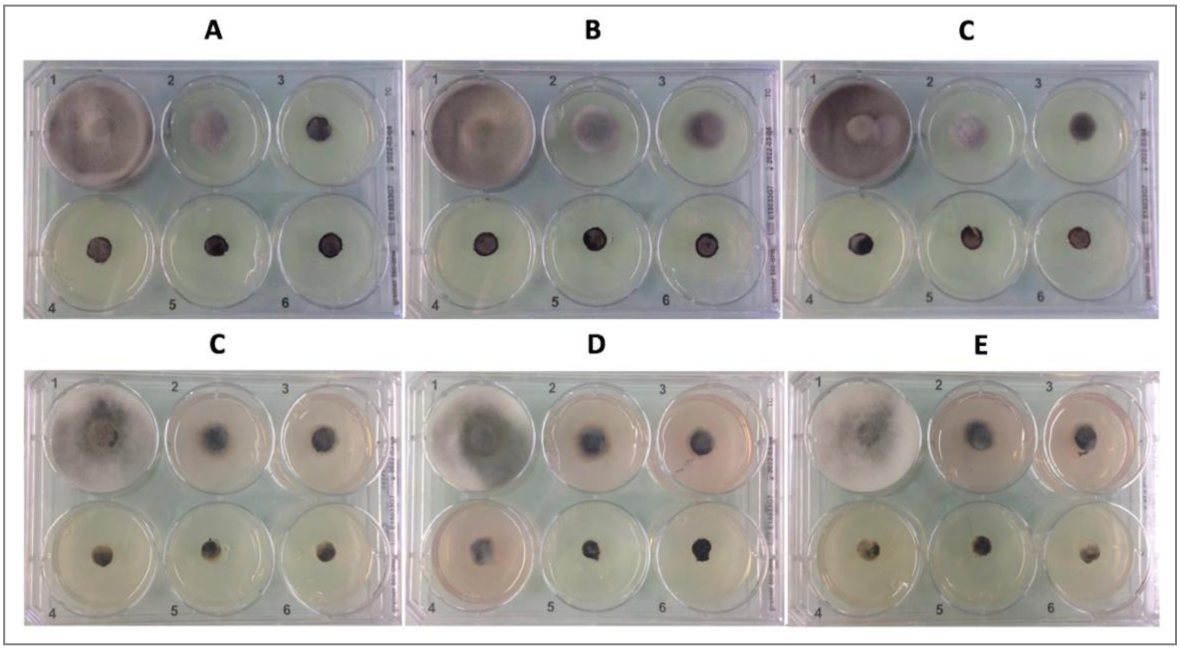
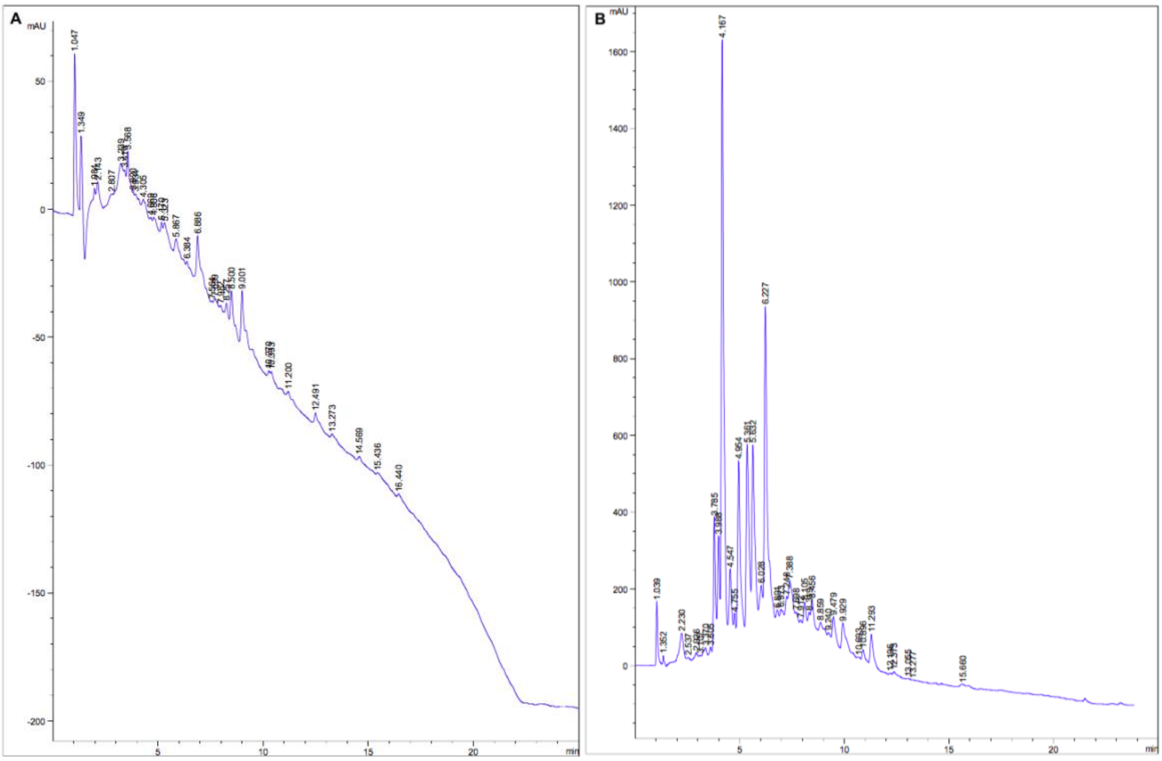


Figure S1 | Sensibility test of *Lasiodiplodia hormozganensis* strain CBS339.90 (**A**, **B**, **C**) and *Lasiodiplodia theobromae* strain LA-SOL3 (**D**, **E**, **F**) for hygromycin: 0 mg/L – well 1; 10 mg/L – well 2; 20 mg/L – well 3; 50 mg/L – well 4; 75 mg/L – well 5; 100 mg/L – well 6.



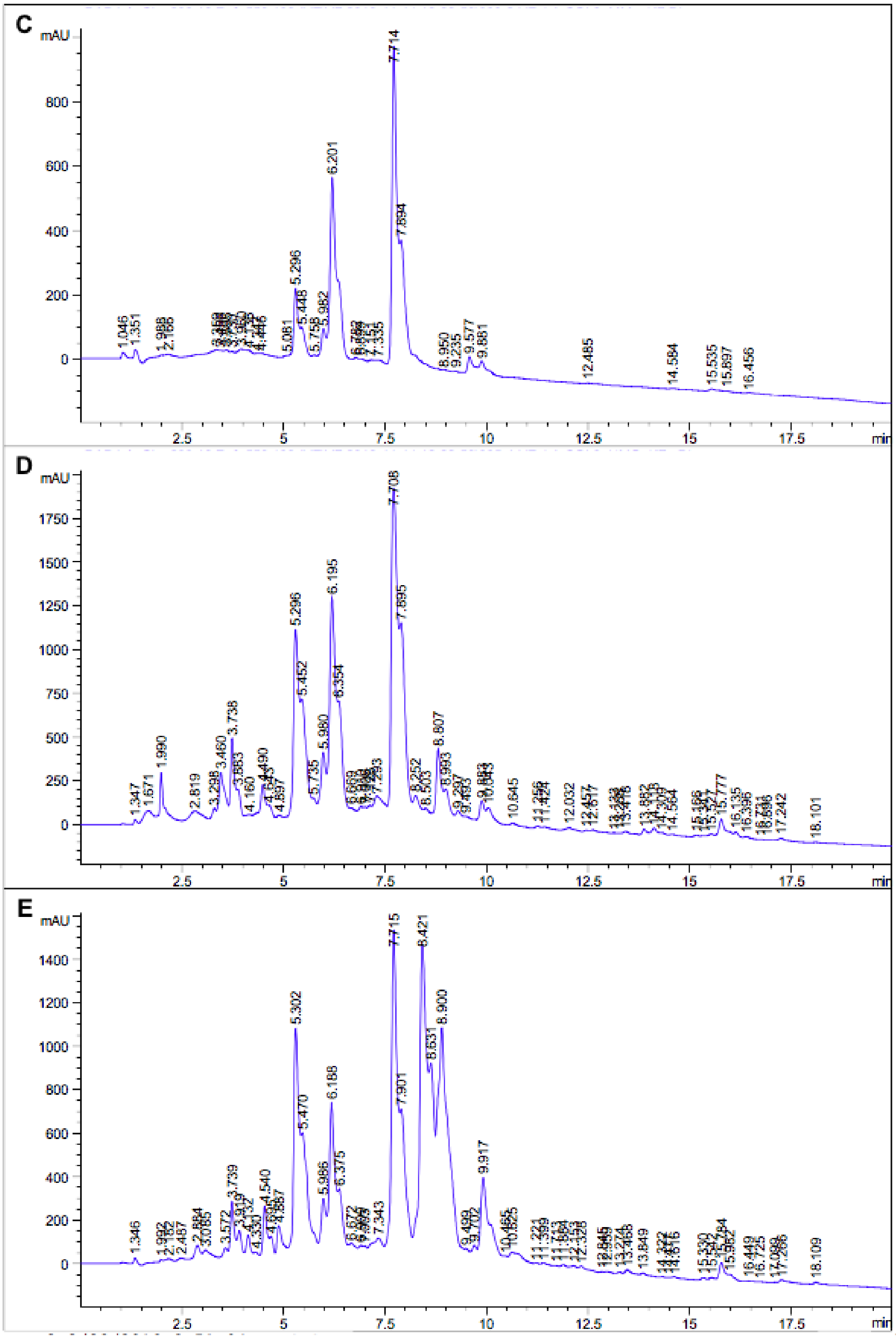


Figure S2 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate of *L. theobromae* LA-SOL3 grown in 500 mL of MM (**A**), DM medium (**B**), MM+ (**C**), YMG medium (**D**), and BAF medium (**E**). Retention time is represented.

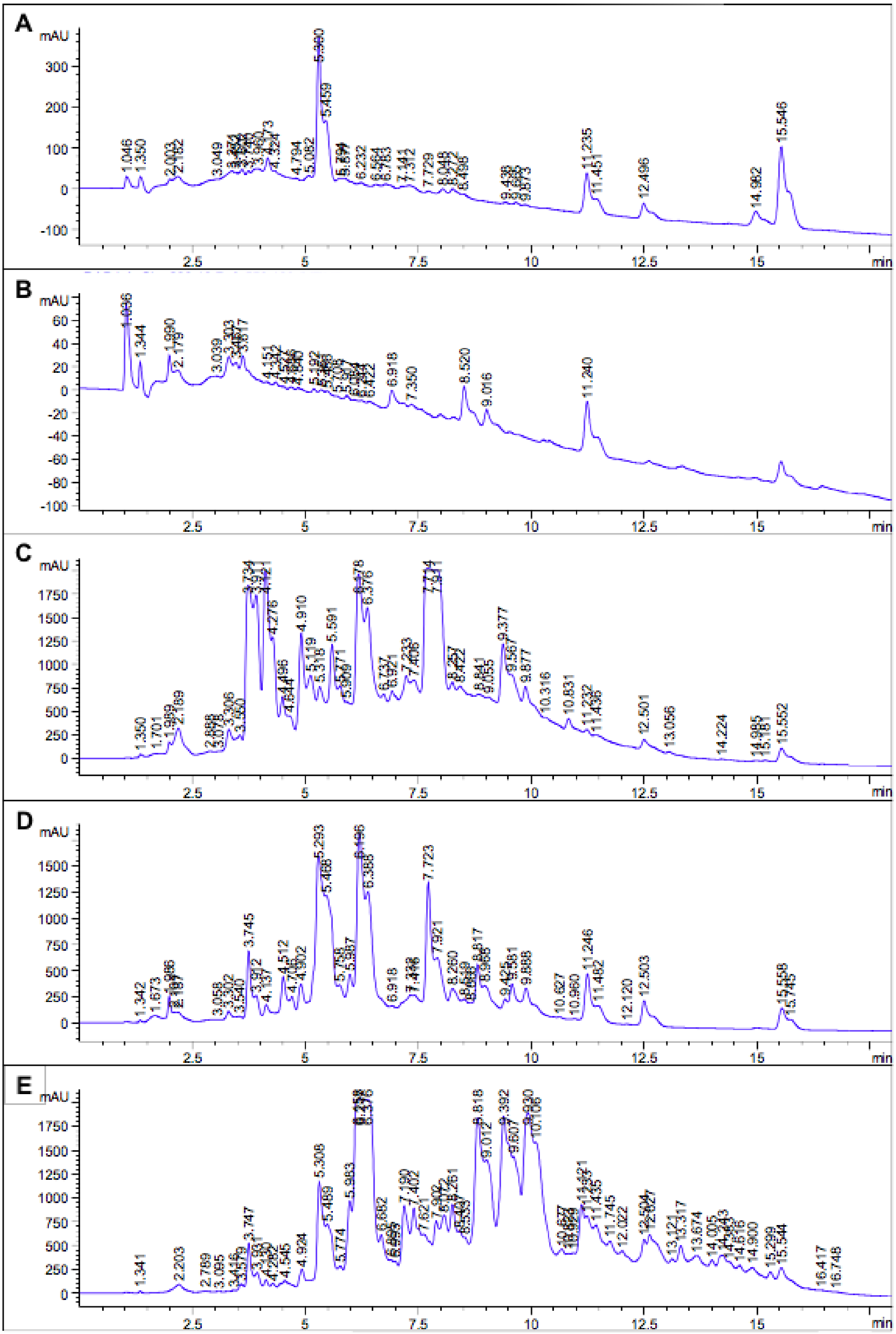
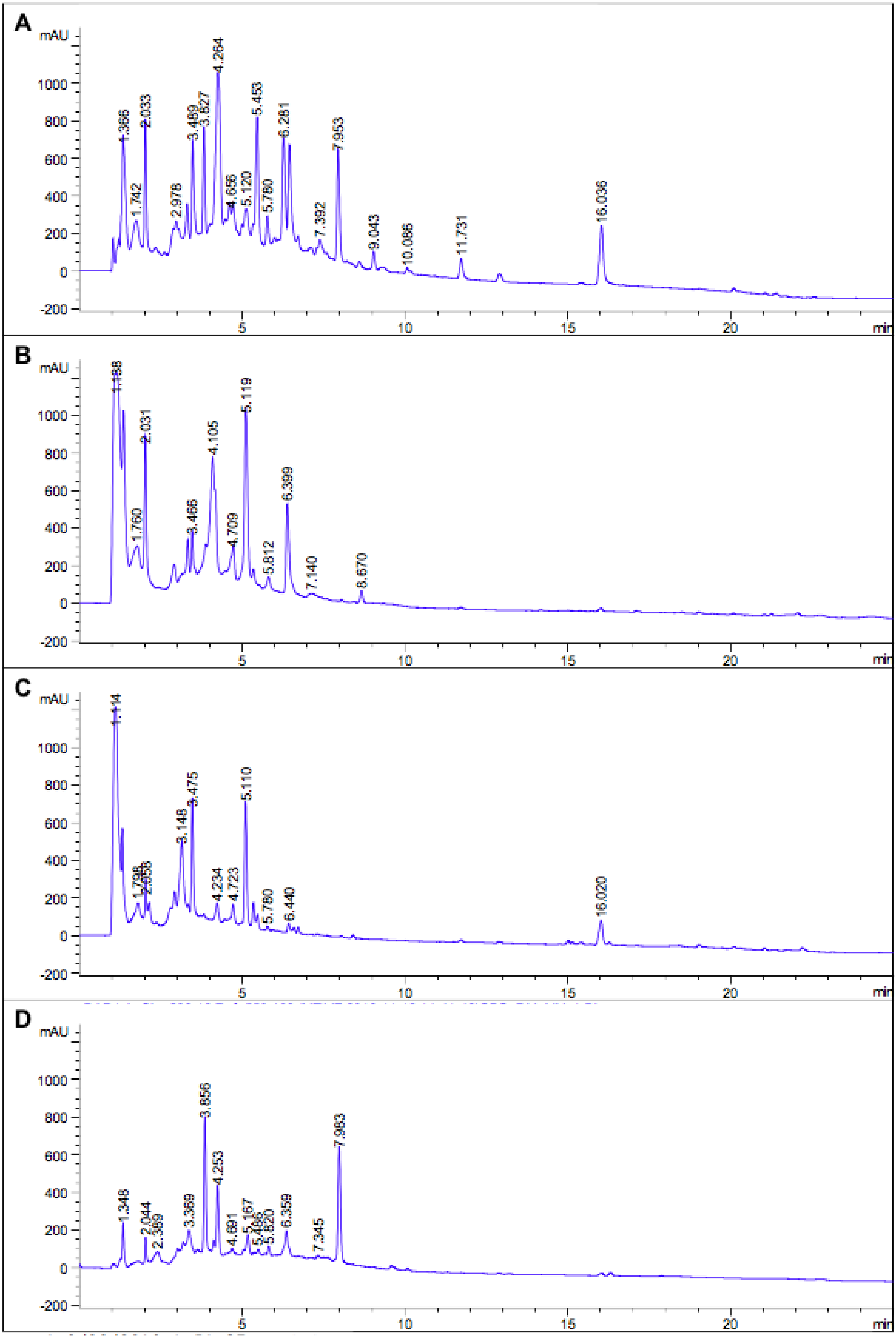


Figure S3 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate of *L. hormozganensis* CBS339.90 grown in 500 mL of MM+ (**A**), MM (**B**), DM medium (**C**), YMG medium (**D**), and BAF medium (**E**). Retention time is represented.


A
mAU
1000
800
600
400
200
0
-200
1.366
1.742
2.033
2.978
3.469
3.827
4.264
4.656
5.120
5.453
5.780
6.281
7.392
7.953
9.043
10.086
11.731
16.036
5
10
15
20
min
B
mAU
1000
800
600
400
200
0
-200
1.188
1.760
2.031
3.466
4.105
4.709
5.119
5.812
6.399
7.140
8.670
5
10
15
20
min
C
mAU
1000
800
600
400
200
0
-200
1.114
1.798
2.058
3.148
3.475
4.234
4.723
5.110
5.780
6.440
16.020
5
10
15
20
min
D
mAU
1000
800
600
400
200
0
-200
1.348
2.044
2.389
3.369
3.856
4.253
4.691
5.157
5.486
5.820
6.359
7.345
7.963
5
10
15
20
min

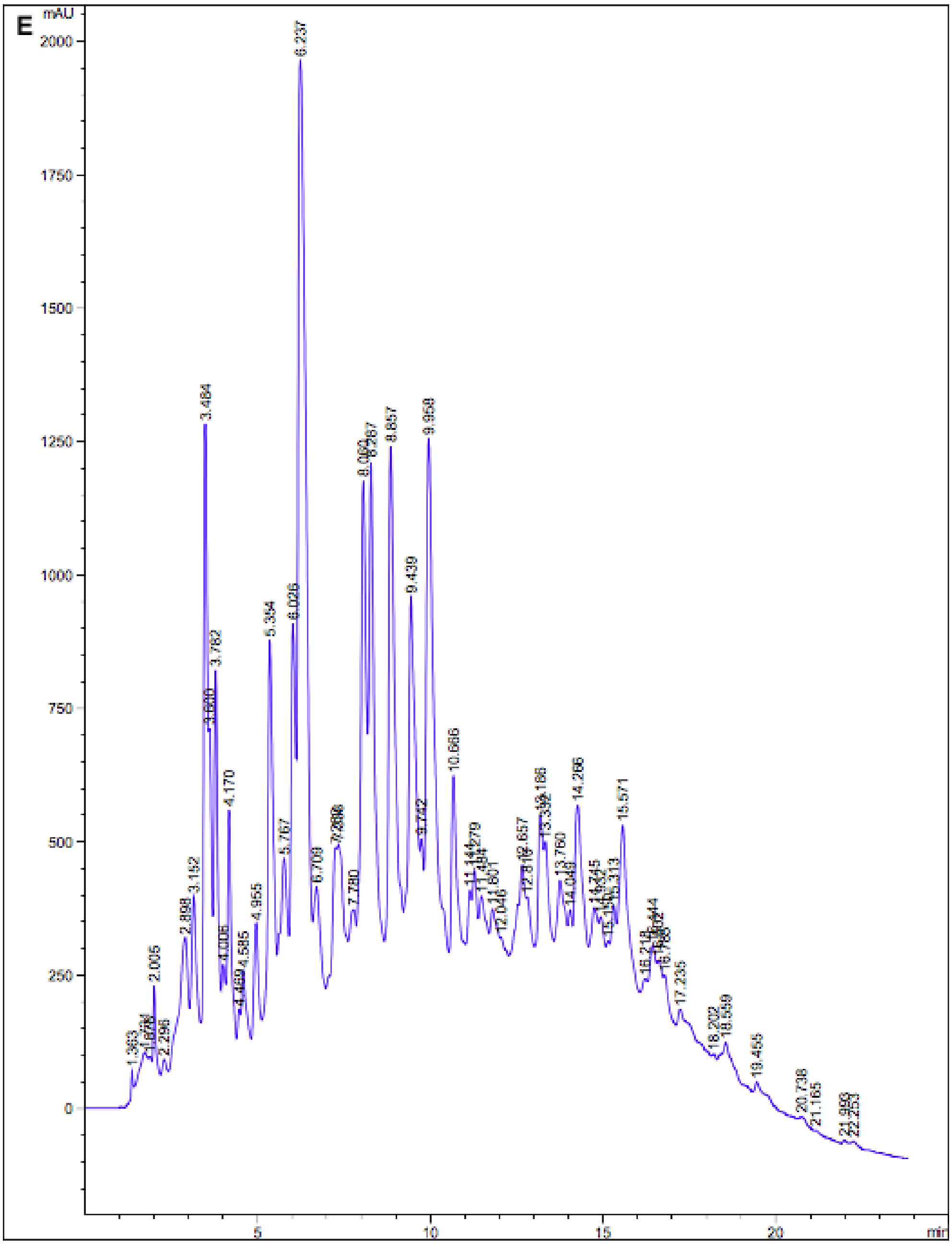
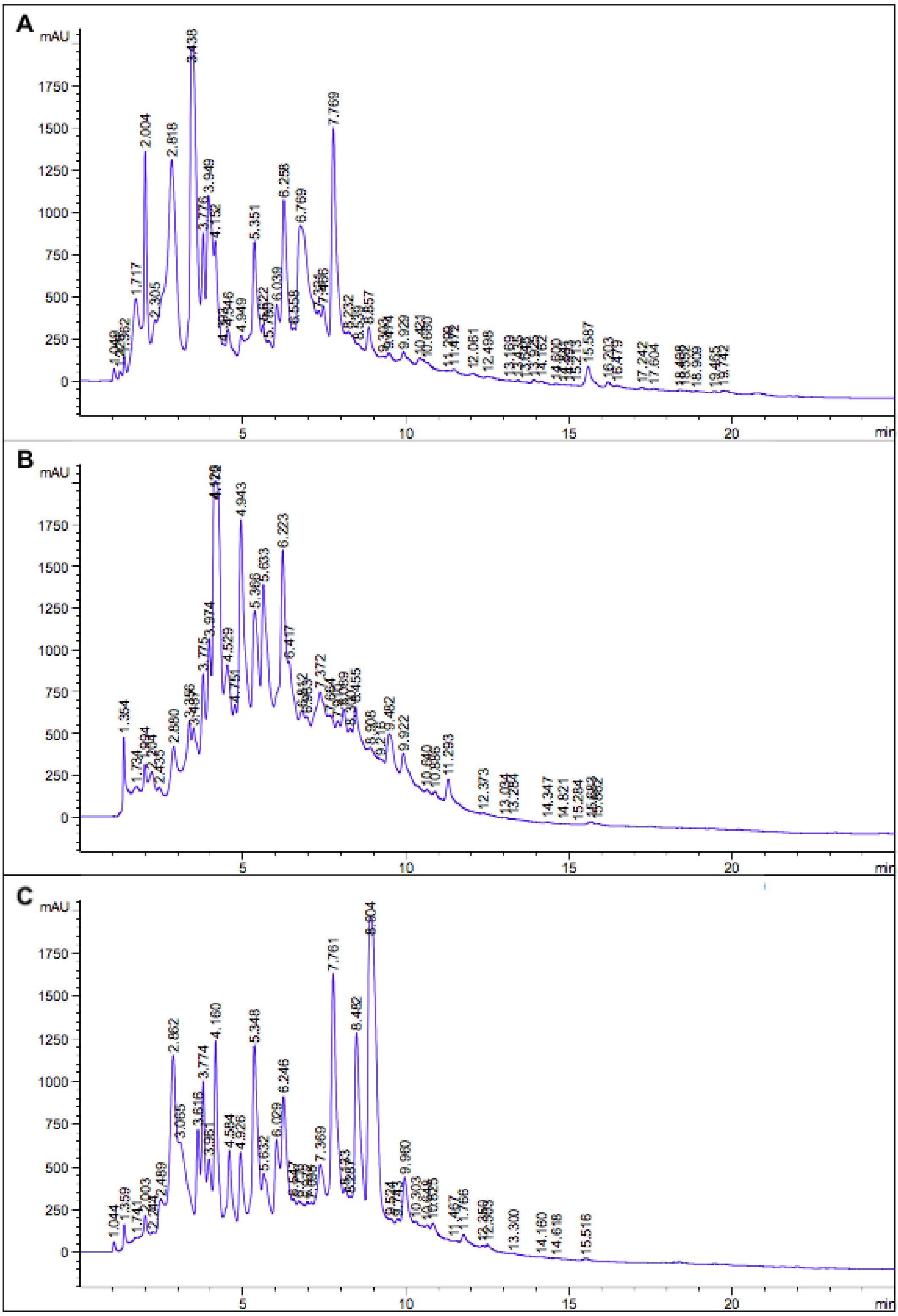


Figure S4 | HPLC chromatograms (230 nm) of crude extracts from mycelia of *L. hormozganensis* CBS339.90 grown in 500 mL of YMG medium (**A**), MM (**B**), MM+ (**C**), DM medium (**D**), and BAF medium (**E**). Retention time is represented.


A
mAU
1750
1500
1250
1000
750
500
250
0
3.438
2.004
2.818
3.949
3.776
4.152
1.717
2.305
5.351
6.258
6.769
7.769
6.039
8.857
9.929
15.587
5
10
15
20
min
B
mAU
4.129
4.943
6.223
5.633
5.366
3.974
3.775
4.529
6.417
7.372
8.455
9.482
9.922
11.293
1.354
2.880
12.373
14.347
10
15
20
min
C
mAU
8.904
7.761
8.482
2.862
4.160
5.348
3.774
3.616
6.246
3.065
6.029
7.369
9.960
2.489
1.359
1.044
11.766
13.300
14.160
14.618
15.516
5
10
15
20
min

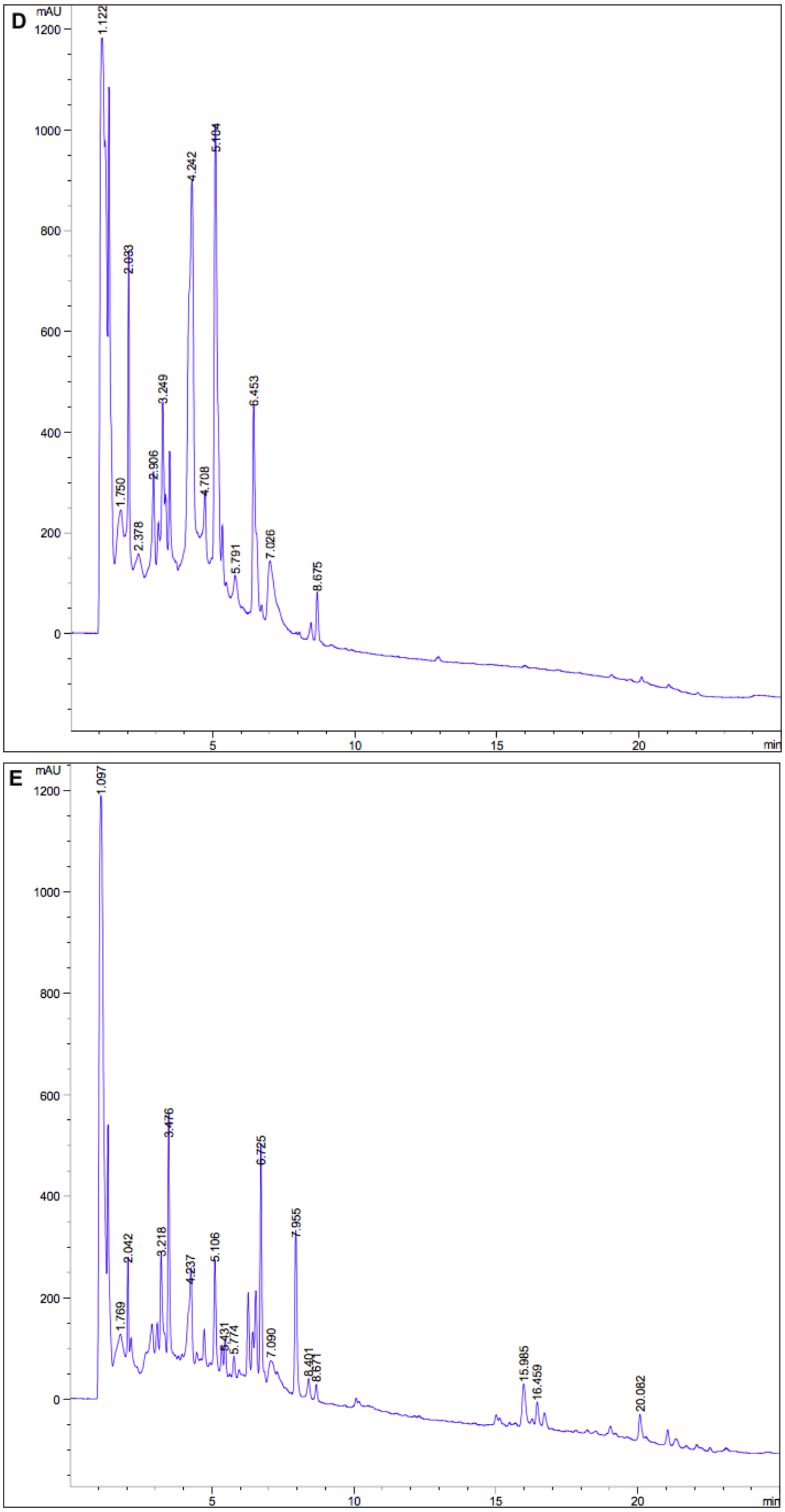


Figure S5 | HPLC chromatograms (230 nm) of crude extracts from mycelia of *L. theobromae* LA-SOL3 grown in 500 mL of YMG medium (**A**), DM medium (**B**), BAF medium (**C**), MM (**D**), and MM+ (**E**). Retention time is represented.

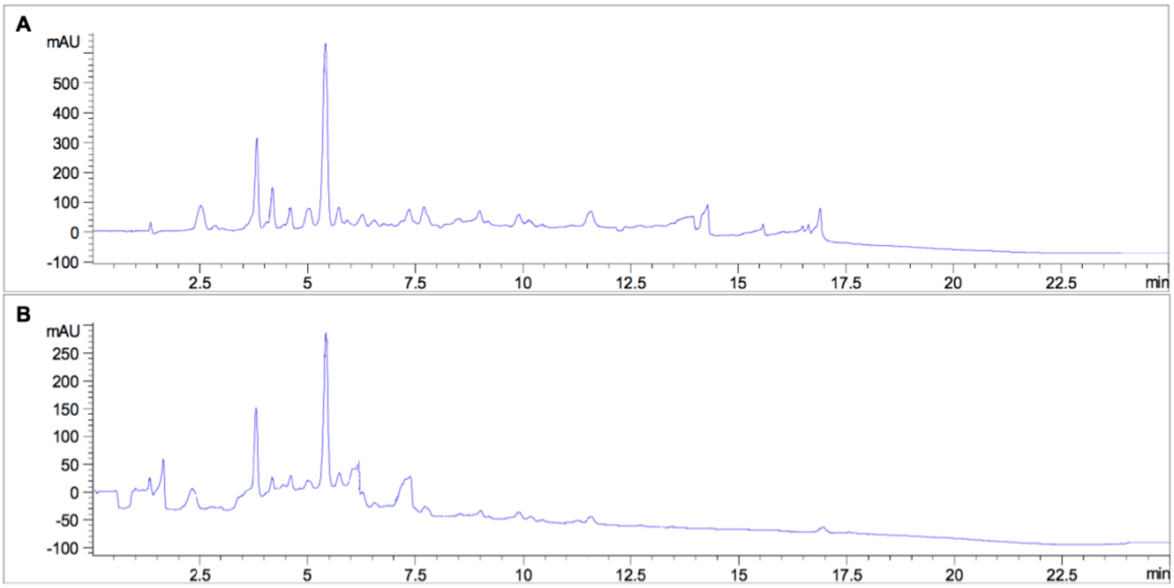
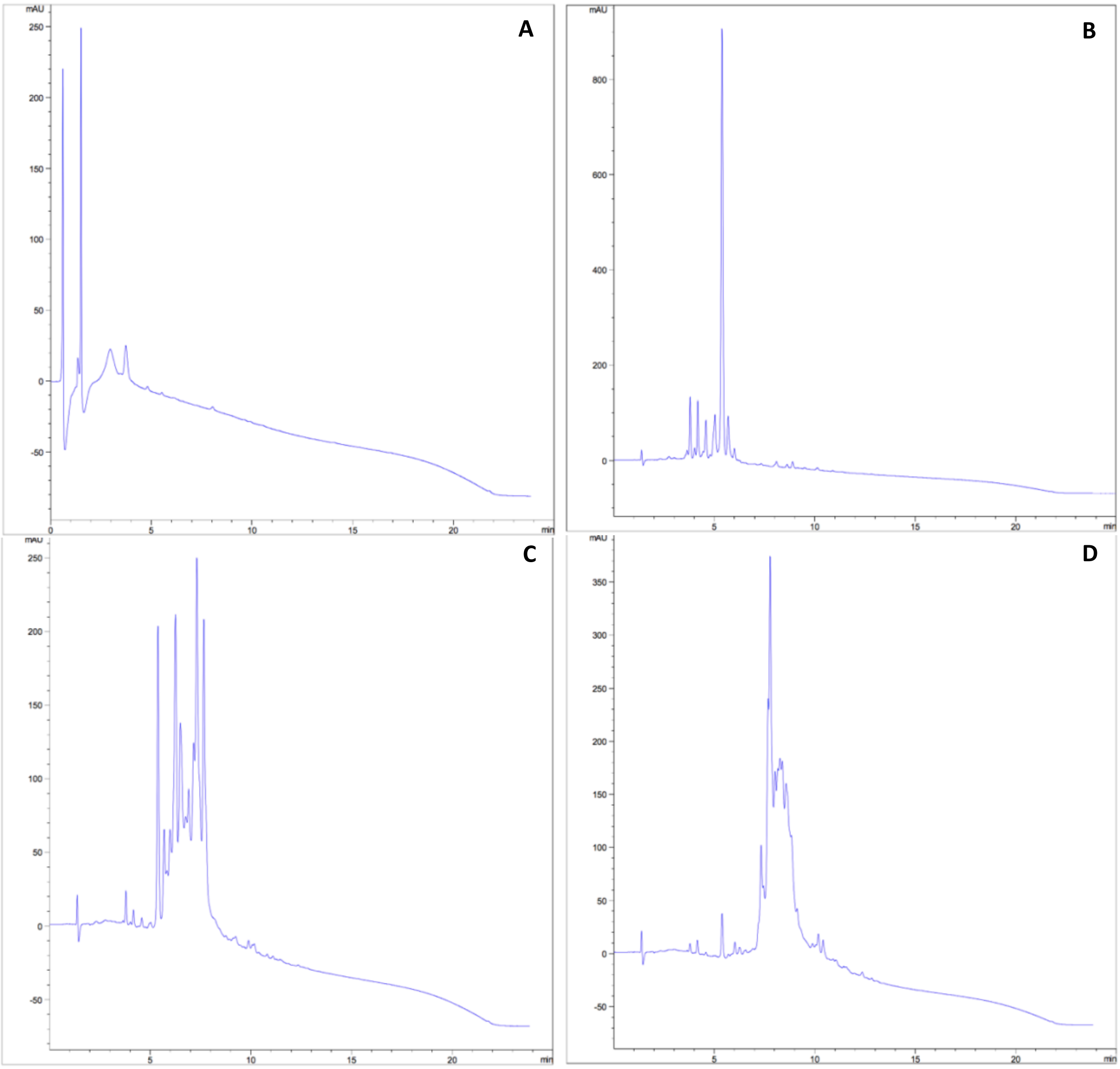


Figure S6 | HPLC chromatograms (230 nm) of crude extracts from culture filtrate (**A**) and mycelium (**B**) of strain CBS339.90 obtained after large-scale fermentation.



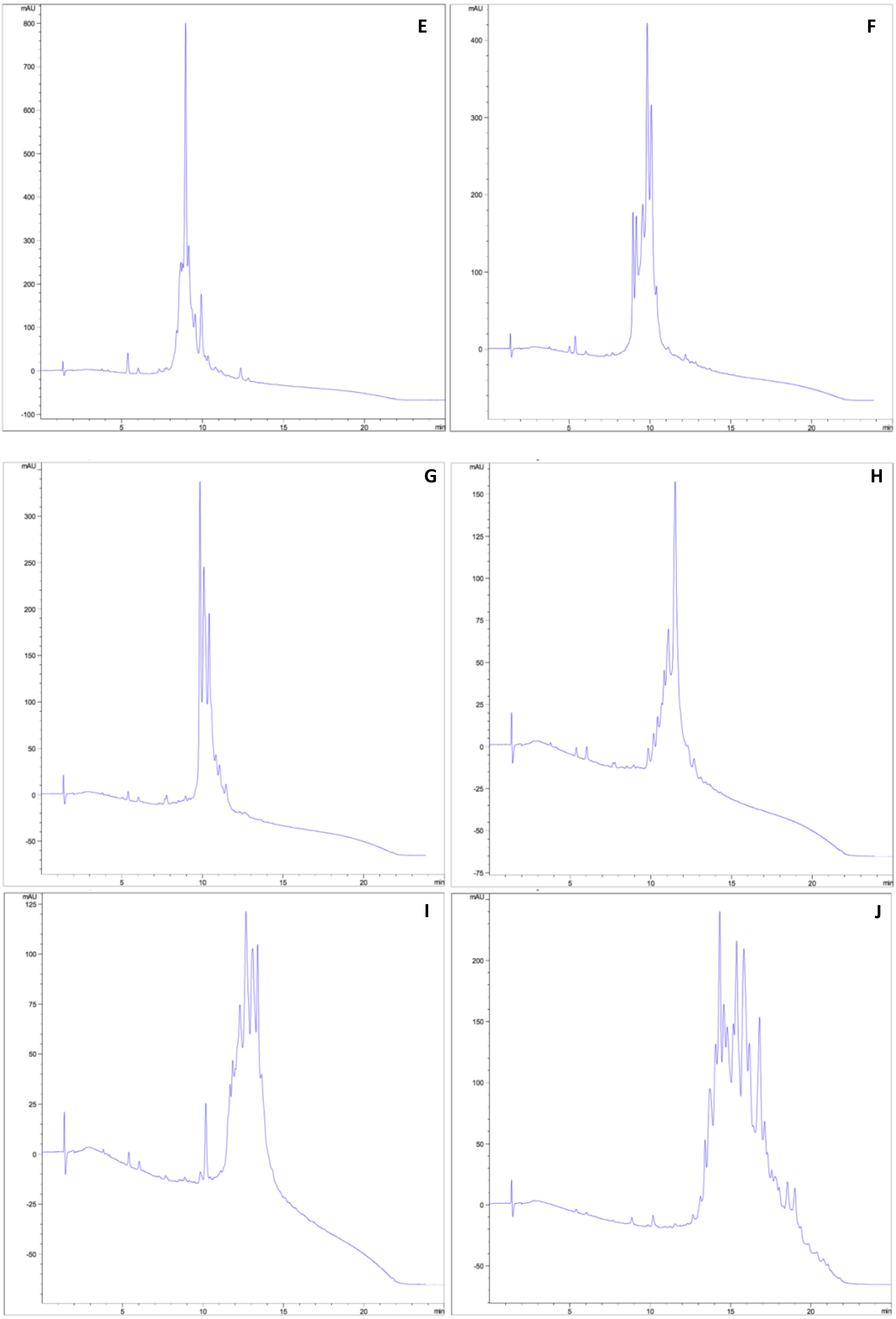


Figure S7 | HPLC chromatograms (230 nm) of fractions from culture filtrate of *L. hormozganensis* CBS339.90. Fraction 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 are represented in (**A**), (**B**), (**C**), (**D**), (**E**), (**F**), (**G**), (**H**), (**I**), (**J**), respectively.

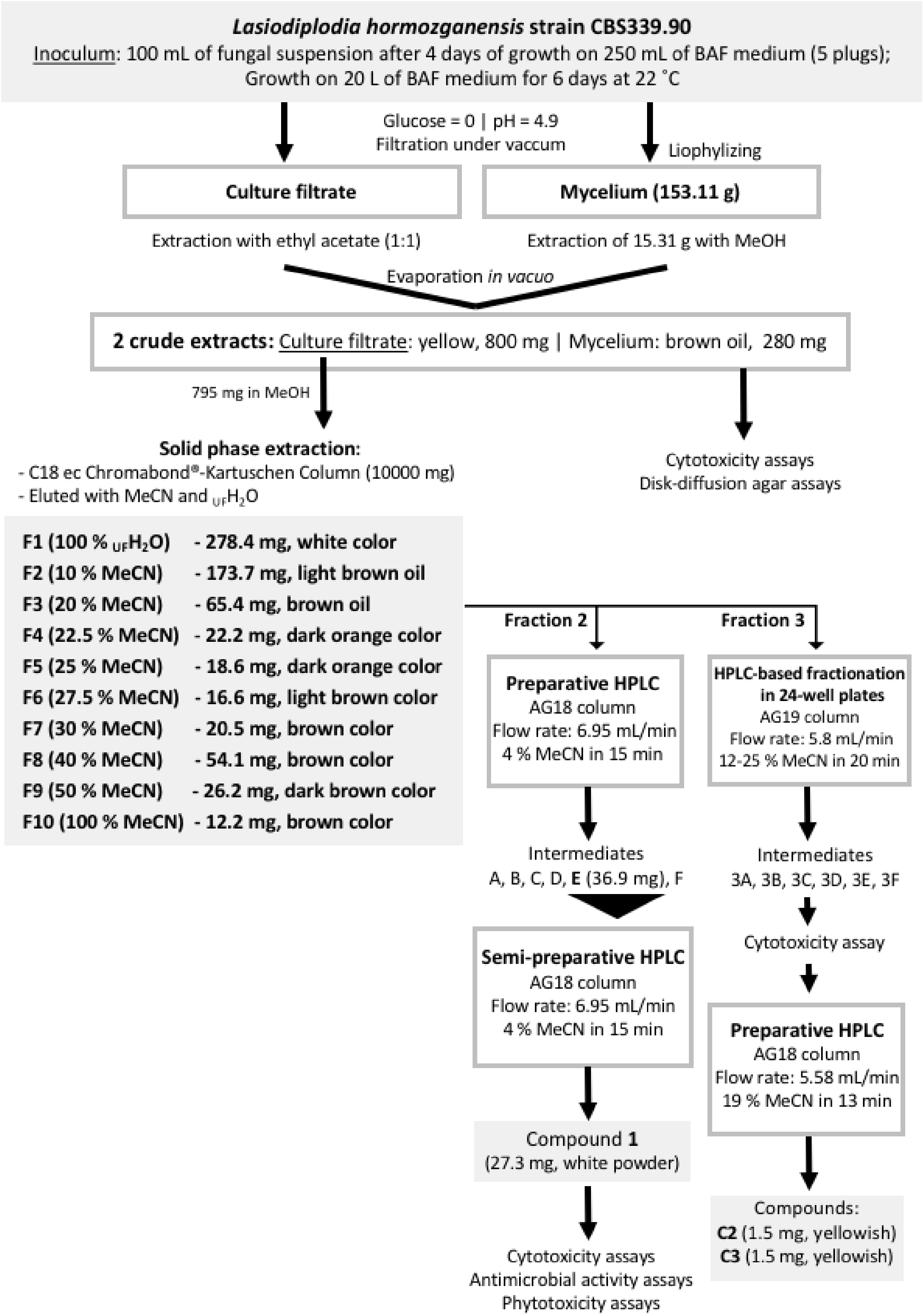

Lasiodiplodia hormozganensis strain CBS339.90
Inoculum: 100 mL of fungal suspension after 4 days of growth on 250 mL of BAF medium (5 plugs);
Growth on 20 L of BAF medium for 6 days at 22 °C
Glucose = 0 | pH = 4.9
Filtration under vaccum
Liophylizing
Culture filtrate
Mycelium (153.11 g)
Extraction with ethyl acetate (1:1)
Extraction of 15.31 g with MeOH
Evaporation *in vacuo*
2 crude extracts: Culture filtrate: yellow, 800 mg | Mycelium: brown oil, 280 mg
795 mg in MeOH
Solid phase extraction:
- C18 ec Chromabond®-Kartuschen Column (10000 mg)
- Eluted with MeCN and $_{UF}H_2O$
Cytotoxicity assays
Disk-diffusion agar assays
F1 (100 % $_{UF}H_2O$) - 278.4 mg, white color
F2 (10 % MeCN) - 173.7 mg, light brown oil
F3 (20 % MeCN) - 65.4 mg, brown oil
F4 (22.5 % MeCN) - 22.2 mg, dark orange color
F5 (25 % MeCN) - 18.6 mg, dark orange color
F6 (27.5 % MeCN) - 16.6 mg, light brown color
F7 (30 % MeCN) - 20.5 mg, brown color
F8 (40 % MeCN) - 54.1 mg, brown color
F9 (50 % MeCN) - 26.2 mg, dark brown color
F10 (100 % MeCN) - 12.2 mg, brown color
Fraction 2
Fraction 3
Preparative HPLC
AG18 column
Flow rate: 6.95 mL/min
4 % MeCN in 15 min
HPLC-based fractionation in 24-well plates
AG19 column
Flow rate: 5.8 mL/min
12-25 % MeCN in 20 min
Intermediates
A, B, C, D, E (36.9 mg), F
Intermediates
3A, 3B, 3C, 3D, 3E, 3F
Cytotoxicity assay
Semi-preparative HPLC
AG18 column
Flow rate: 6.95 mL/min
4 % MeCN in 15 min
Preparative HPLC
AG18 column
Flow rate: 5.58 mL/min
19 % MeCN in 13 min
Compound 1
(27.3 mg, white powder)
Compounds:
C2 (1.5 mg, yellowish)
C3 (1.5 mg, yellowish)
Cytotoxicity assays
Antimicrobial activity assays
Phytotoxicity assays

Figure S8 | Scheme of isolation of metabolites from crude extracts of *Lasiodiplodia hormozganensis* strain CBS339.90.