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Departamento de Biologia

Carina Rafaela Faria da Costa Félix

Lasiodiplodia theobromae: um fungo fitopatogénico que infeta humanos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Dra Ana Cristina Esteves, (investigadora em Pós-doutoramento) do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Dr Artur Alves, (investigador auxiliar do Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro.

“If you compare yourself with others, you may become vain and bitter; for always there will be greater and lesser persons than yourself. Enjoy your achievements as well as your plans. Keep interested in your own career, however humble; it is a real possession in the changing fortunes of time.

(…)

With all its sham, drudgery and broken dreams, it is still a beautiful world.

Be cheerful. Strive to be happy.”

Max Ehrmann

Aos meus pais, que fizeram de mim aquilo que sou hoje.

o júri

presidente

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palavras-chave

Fungo fitopatogénico, crescimento radial, expressão de proteínas, Citotoxicidade.

resumo

Lasiodiplodia theobromae é um fungo fitopatogénico responsável por inúmeras doenças em variadas plantas. Embora este fungo seja tipicamente de regiões tropicais e subtropicais, também ocorre em climas mais frios. *L. theobromae* tem também sido descrito como oportunista em humanos, causando infeções com diferentes níveis de gravidade. Apresenta, assim, uma grande adaptabilidade a diferentes ambientes, sendo capaz de utilizar os seus mecanismos de virulência numa ampla gama de temperaturas.

O objetivo desta investigação é caracterizar o crescimento de dois isolados - um isolado ambiental, CAA019 e um isolado clínico, CBS339.90 - a diferentes temperaturas (temperatura ambiente e temperatura do corpo humano).

Tendo em conta a relevância deste organismo como fitopatogénico assim como a sua crescente importância como oportunista de humanos, este estudo poderá ter uma grande relevância para agricultura, bem como para a saúde humana.

As condições ótimas de cultivo destes isolados foram determinadas: o meio de cultura *Potato Dextrose Agar* como melhor meio para o cultivo e a temperatura de 30ºC como sendo a temperatura ótima de crescimento para ambos os isolados. Verificou-se ainda que a presença de luz continua tem um efeito positivo no crescimento de *L. theobromae* e que o seu crescimento máximo é atingido entre as 96 horas e as 120 horas de incubação.

Verificou-se ainda que ambos os isolados expressam proteínas extracelulares de um modo dependente da temperatura, assim como do isolado.

Por último, foi possível verificar que ambos os isolados produzem moléculas extracelulares com propriedades citotóxicas numa linhagem de células Vero (células de rins de macaco verde africano) verificando-se que ambos os isolados são citotóxicos nestas células. As maiores perdas de viabilidade são atingidas às temperaturas de 25ºC e 30ºC para o isolado ambiental e a 30ºC e 37ºC para o isolado clínico.

keywords phytopathogenic fungi, radial growth, protein expression, cytotoxicity

abstract

Lasiodiplodia theobromae is a phytopathogenic fungus responsible for a countless number of diseases in various plants. Although this fungus is typically from tropical and subtropical regions, it can also occur in colder climates. It has been also described as an opportunist in humans, causing infections of different levels of severity. *L. theobromae* thus presents a great capacity of adaptation to different environments, being able to use its virulence mechanisms in a wide range of temperatures.

The aim of this investigation is to characterize two different isolates – an environmental isolate, CAA019, and a clinical isolate, CBS339.90 – at different temperatures (environmental temperature and human body temperature).

Due to the relevance of this species as a phytopathogenic agent, as well as its growing importance as an opportunist pathogen in humans, this study may reveal itself as being extremely relevant both to agriculture and to human health.

The optimal growth conditions of these isolates have been determined: Potato Dextrose Agar is the best culture medium and the temperature of 30ºC the optimal growth temperature for both isolates. It has also been shown that continuous light has a positive effect in the growth of *L. theobromae* and that this fungus reaches its maximum growth between 96 hours and 120 hours of incubation.

Also, a differential extracellular protein expression has been detected, depending both on the temperature of growth and on the isolate.

Lastly, it was possible to verify that both isolates produce extracellular molecules with cytotoxic properties against a Vero cell line (cells from the kidneys of African Green Monkey), thus concluding that both isolates are cytotoxic for this cells. Lowest values of cell viability have been achieved for the temperatures of 25ºC and 30ºC in the case of the environmental isolate, and for the temperatures of 30ºC and 37ºC in the case of the clinical isolate suggesting that there may be some specificity of the isolate towards its host.

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LIST OF ABBREVIATIONS

2D-PAGE - Two-Dimensional Polyacrylamide Gel Electrophoresis Database

BCA - Bicinchoninic Acid

BSA – Bovine Serum Albumin

CMA – Corn Meal Agar

CWDE – Cell Wall Degrading Enzymes

DMEM - Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic Acid

ECACC - European Collection of *Cell* Cultures

FBS - Fetal Bovine Serum

GH – Glycosyl Hydrolases

GTPase – Guanosine Triphosphatase

ICAT - Isotope-Coded Affinity Tag

KOH – Potassium Hydroxide

LC - Liquid Chromatography

MALDI - Matrix-assisted laser desorption ionisation

OMA – Oatmeal Agar

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

PDB – Potato Dextrose Broth

RPM - Rotations per minute

SDS - Sodium Dodecyl Sulphate

SDS-PAGE - SDS-polyacrylamide gel electrophoresis

TEMED - N,N,N',N'-tetrametiletilenodiamine

TOF - Time-Of-Flight

TRIS - 2-Amino-2-hydroxymethylpropane-1,3-diol

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1. INTRODUCTION

Fungi are an enormous group of eukaryotic organisms with a vast diversity of forms.

Even though most of them are saprophytes, some fungus can only complete their life cycle with the help of a host, thus being plants or animals parasites. There is a limited number that are able to avoid recognition and plant defence responses obtaining nutrients from them; in that case, some can even cause diseases which can lead to the death of the host[1].

1.1 – FILAMENTOUS FUNGI

Fungi are unicellular (yeasts) or multicellular (filamentous) eukaryotic microorganisms. Filamentous forms present tubular cells. Those visible structures exhibit aggregated long, branching threads called hyphae (singular hypha), structured to support spores for reproduction and to promote dissemination. The aerial hyphae grow and branch along the substrate, forming a network termed mycelium, which presents apical growth to proliferate and translocate available nutrients. These hyphae usually present a constant diameter, depending on the environmental factors and the species themselves, ranging from 1 to 30mm or more. Therefore, eucarpic fungi are organisms with no size or age limits, being indeterminate both spatially and temporally[2].

Within the mycelium, some plasticity might be found, as it doesn't present homogeneously, being able to grow, branch, anastomose (fuse), age, die, sporulate, or present different physiological or biochemical characteristics as a result of micro-environmental conditions variations[2, 3].

The plasma membrane (figure 1), part of the fungal cell envelope, is composed by a phospholipid bilayer with globular proteins, which dictates the entry of nutrients and the expulsion of metabolites, representing a selective barrier for the translocation of substances[2, 3].

Concerning to the sterol content, contrary to the plasma membranes of animals and plants, mostly composed by cholesterol and phytosterols, respectively, the membrane of fungal cells contains predominantly ergosterol[4].

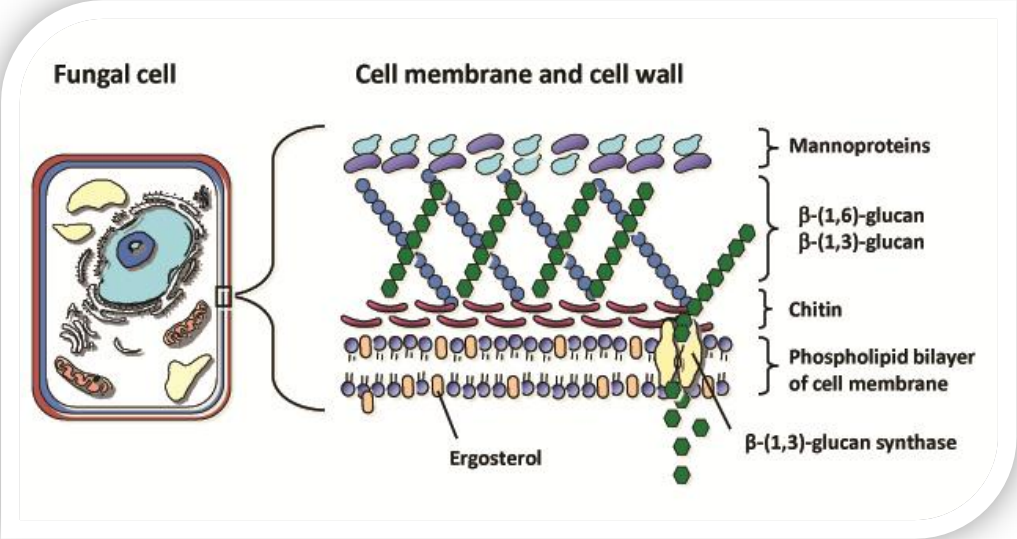


Figure 1: Plasma membrane and cell wall of filamentous fungi. *Adapted from www.doctorfungus.com*[5].

In filamentous fungi, hyphae are often resistant to plasmolysis due to an intimate bound between the cell membrane and wall. Cell walls' composition varies according to taxonomic groups, containing polysaccharides – chitin, glucans, mannoproteins, chitosan, polyglucuronic acid – together with some proteins and glycoproteins[2].

Evidences suggest that the cell wall is not a defined structure, being dynamic and ranging both quantitatively and qualitatively among species, morphotypes and even as a response to environmental stress[2].

Organelles are included in an aqueous plasma, which contains soluble proteins and other macromolecules, as well as low-molecular-weight metabolites, though the hyphae of the older regions of the colony (centre) may lack protoplasm, as it is forwarded and used in the growing tip[2, 4].

Also, other components might be found in fungal cells, including microbodies, ribosomes, proteasomes, lipid particles and a cytoskeletal network, which confers structural stability to the cytoplasm and consists of microtubules and microfilaments; membranous organelles like nucleus, endoplasmic reticulum, mitochondria, Golgi apparatus, secretory vesicles and vacuoles may be found in fungal cells, often forming extended membranous systems[2].

Fungal nutritional needs are relatively simple, as most fungal species would survive in aerobic conditions with glucose, ammonium salts, inorganic ions and a few other growth

factors. From macronutrients, fungi can obtain carbon, nitrogen, oxygen, sulphur, phosphorus, potassium and magnesium; from micronutrients, fungi obtain trace elements, such as copper, calcium, iron, manganese and zinc[3].

As chemo-organotrophs, fungi need fixed forms of organic compounds to obtain carbon and produce energy. Thus, sugars are widely utilized by fungi, and can range from a simple monosaccharide as glucose to complex polysaccharides as starch, cellulose and aromatic hydrocarbons. Generally, fungi can only absorb small and soluble nutrients (monomers or small polymers, as monosaccharaides, amino acids or small peptides). These nutrients entry, as well as the metabolites expulsion, is mainly determined by the selectivity of the cell membrane and its permeability[3].

When fungi are inoculated into a culture medium under optimal environmental conditions, it will develop according to a typical growth curve, which includes three different phases. The lag phase, which is a period of zero population growth, relative to the time necessary for the fungus to adapt to the environment; the exponential phase, in which the adapted fungus doubles logarithmically at a constant, maximum specific growth rate – in this phase, fungal cells undergo primary metabolism, exclusively essential for the healthy growth of the cells; and a stationary phase, correspondent to a period of zero population growth rate, in which the already grown fungus remains constant. After long periods of stationary phase, fungal cells may autolyse. This phase allows the fungus to survive in a nutrient deprivation scenario for long periods of time, as well as in other adverse environmental conditions, such as the presence of toxic metabolites (notably ethanol), low pH, high CO_2 and high temperature. In the stationary phase, fungal cells undergo secondary metabolism, not essential for fungal growth but part of the organism's survival mechanisms[2-4].

1.2 – PHYTOPATHOGENIC FUNGI

Due to the variety of tissues in plants (leaves, flowers, roots, fruits, among others), phytopathogenic fungi have a wide range of infection mechanisms[6]. These fungi have very complex life cycles, as a result of their variable morphologies, with both sexual and asexual reproduction (figure 2), and infective, vegetative and reproductive structures formation[7]. The interaction between the host plant – and its parasite – phytopathogenic fungus - depends on a reciprocal recognition; also, the expression of pathogenicity and virulence factors by the fungus and the defence mechanisms in the plant are key aspects

in the interaction plant-fungus. Pathogenicity of the fungus has been shown to be related to a number of mechanisms and molecules expression, like cell wall degrading enzymes (CWDE), inhibitory proteins and toxin synthesis influenced by enzymes [9, 10].

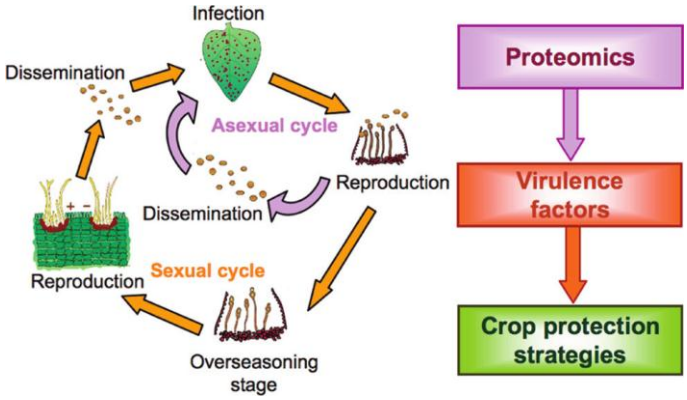


Figure 2: Life cycle of phytopathogenic fungi. *Adapted from Gonzalez-Fernandez et al., 2012*[1].

In various life domains there are representatives of plant pathogens. These microorganisms can cause more than 10,000 different diseases in plants, including in some crops with an elevated value both for food industry or for biofuel production (corn, soybean, alfalfa, wheat, and switch grass)[11]. Nonetheless, plant pathogens can be a great source of new accessory enzymes[11]. Recently, it has been shown that hydrolytic activity can be improved by the addition of certain enzymes (which have few or none activity directly on cellulose), like esterases and family 45 and 61 glycosyl hydrolases (GH), to cellulose, improving biofuel production processes.

Effective saprobes have developed arsenals of CWDE, in order to be able to degrade lignocellulose into sugars for their own nutrition, since this is the most lasting material in plant residues[11].

Besides being able to remain as saprobes in the remainings of their hosts, thus feeding of their death tissues, plant pathogens have the advantage of having earlier access to nutrients by parasitizing the plants while alive, hence competing with obligate saprobes.

Among plant pathogens there is a great variety of pathogenesis mechanisms, being able to adapt to particular hosts. Some pathogens have the capacity to infect the whole plant, while others infect only specific tissues, like leaves, roots, seeds and floral structures, or even propagative organs. Moreover, most pathogens – including almost all biotrophs and necrotrophs – can only infect one or a few plant species, and some are

even more specific, infecting only a subset of vulnerable genotypes within the same species[11].

Fungal morphology is often adapted to allow infection and growth, according to specific physical and physiological stimulus[12]. Various infection strategies are used, such as the formation of particular structures to promote the penetration[13, 14]. For example, biotrophic and hemibiotrophic fungi invade specific tissues of the host slowly and "silently", before infecting the plant host. Contrary, obligate necrotrophic fungi secrete a cocktail of hydrolytic enzymes to invade the plant's tissues, as intensively as possible, to guarantee success in the colonization[15, 16].

Due to the plant's own defences, it is necessary that the pathogen has the ability to degrade the cell walls rapidly, in order to penetrate the tissues and thus obtain nutrients, being able to grow and reproduce[17]. Concerning the plants defence, there is the production of proteins with inhibiting properties against microbial CWDEs, fact that can potentially stimulate the evolution of plant pathogens into the production of unique enzymes[18]. Among noncommelinoid monocots and dicots, the production of pectin-degrading inhibiting proteins is common, while grasses produce inhibitors of xylan-degrading enzymes[19, 20].

It's very important to understand the pathogenicity mechanisms, in order to allow de development of efficient methods of crop protection, either by genotypical improvement or by the use of fungicides or biological control methodologies[21, 22].

Virulence strategies in fungal plants parasites demand a great attention, due to its impact on plants production and therefore the world's economy[1].

1.2.1 – GENUS *LASIODIPLODIA*

The family Botryosphaeriaceae is taxonomically arranged according to the morphology of the anamorph states frequently found in nature. Nevertheless, the overlapping of morphologies has provoked an increase of the application of DNA sequencing in the definition of species[23].

The genus *Lasiodiplodia* fits in the kingdom Fungi, phylum Ascomycota, Class Dothideomycetes, order Botryosphaeriales and family Botryosphaeriaceae[24] (figure 3).

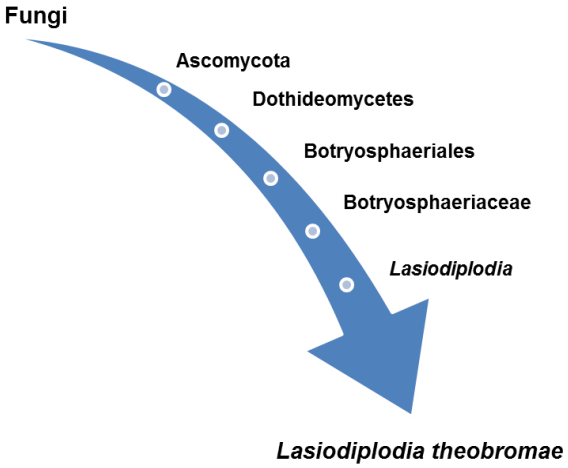


Figure 3: Taxonomy of the genus *Lasiodiplodia.*

There are sixteen recognized species in the genus *Lasiodiplodia* (*L. crassispora*, *L. gonubiensis*, *L. margaritacea*, *L. parva*, *L. plurivora*, *L. pseudotheobromae*, *L. rubropurpurea*, *L. theobromae, L. venezuelensis, L. mahajangana, L. viticola, L. iraniensis, L. missouriana, L. gilanensis, L. citricola and L. hormozganensis*)[25, 26]. The species of Botryosphaeriaceae include both pathogens and saprofites of woody and nonwoody plants. In the past few years, DNA sequence analysis had a significant influence in the taxonomy of the family Botryosphaeriaceae, resulting in the description of several cryptic species[25, 26].

Genera of the family Botryosphaeriaceae with *Diplodia*-like anamorphs, such as *Diplodia*, *Lasiodiplodia* and *Dothiorella*, have a confused taxonomy. Several characteristics have been used in order to distinguish and correctly identify species[27 - 29].

The main features that distinguish this genus from other closely related are the presence of pycnidial paraphyses and longitudinal striations on mature conidia. Conidia are hyaline when young, later becoming medianly 1-euseptate, dark brown, thick-walled, ellipsoid, base truncate, with longitudinal striations. The more recent described species have been separated not only on morphology, but also on the basis of ITS and EF-1α sequence data[27-29].

The use of host association as a factor to distinguish species in this family is now known to be ineffective, since certain Botryosphaeriaceae infect several different unrelated species of plants. Some members are more specialized, infecting only a smaller group of plants, of a specific genus or related genera[23]. This difficulty in identifying members of this family through their morphological characteristics has been an obstacle

to the study of its host association patterns, which is important to understand in order to comprehend the evolutionary process of this group, as well as the adaptations to specific hosts and for pathology/epidemiology studies[23].

1.2.2 – *LASIODIPLODIA THEOBROMAE*

The pathogenic fungus *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. represents the asexual state of *Botryosphaeria rhodina*[30]. It is a phytopathogenic fungus typical from the tropics and subtropics that develops rapidly in moist argillaceous soils. Despite of being able to cause damage between 9ºC and 39ºC, its optimal growth temperature is between 27ºC and 33ºC[31]. Although being widely distributed, it is mostly confined between 40º North and 40º South of the equator.

Lasiodiplodia theobromae has also the ability to colonize healthy tissues without causing any symptoms, and it can even be isolated from plants with no symptoms, which do only appear when the plant is under stress. Thus, *L. theobromae* can be considered as a latent pathogen, capable to induce endophytic infections[32].

Rao *et al*., 1978, have verified that the formation of pycnidia in culture medium is dependent on the induction by light. Honda *et al*., 1978, obtained higher production of pycnidia under wavelengths of 344.5nm and 519nm, and the exudation of conidia only under wavelengths lower than 333nm, being these values near to the ranges of ultraviolet and blue lights[33, 34, 35].

Gupta, 1977 verified that the germination of conidia, at 30ºC, increases with the concentration of sucrose in the medium. Studies from Wang *et al*., 1972, have shown the capacity of this fungus to produce several peptic enzymes, cellulases, proteases and others[33, 36,37].

In oatmeal agar, this fungus grows greyish in tones of grey to black, with great development of cottony aerial mycelium, reverse fuscous black to black. Pycnidia develop on the infected tissues, as leaves, stems and fruits[38].

Pycnidia are immersed and thick-walled, and aggregated in clusters immersed in a stroma frequently up to 5 mm wide, erumpent, often with a distillate papillate ostiole[38].

Conidiophores are hyaline, simple, sometimes septate, rarely branched, cylindrical, arising from the inner layers of cells lining the conidiomatal cavity. Conidia are initially aseptate, hyaline, granulose, subovoid to ellipsoid-oblong, thick-walled, base truncate. Mature conidia are one-septate, cinnamon to fawn, often longitudinally striate,

18-30×10-15 mm. Paraphyses are hyaline, cylindrical, sometimes septate, up to 50 mm long[38].

In order to recognize and characterize populations, DNA molecular markers have been used to follow genes and evidences of speciation of a countless number of pathogenic fungi. These markers have recently been used to analyse genes and genotype flow, his reproductive mode as well as the speciation of a big number of fungi, including *Botryosphaeria* spp. and its anamorphs[39].

A recent study with *L. theobromae*, suggests that the relations between isolates are closer relatively to the type of host each isolate infects, rather than to their geographical proximity[40].

1.2.2.1 – *LASIODIPLODIA THEOBROMAE* HOSTS

Fungi can establish symbiotic, commensal or pathogenic relationships with its hosts, independently of being plants, animals or even humans. Symbioses between plants and fungi are very common – in fact, it is estimated that up to 90% of all higher plants have some kind of symbiosis with fungi. Nonetheless, little changes in the environment can change a symbiotic or commensal relation to a pathogenic phase. For instance, the endophytes and the plant pathogens (organisms that invade the plant causing damage) have extraordinarily similar methods of invasion, which suggests a similarity of attributes related to the adaptation of a fungus to its host[41].

Most of human pathogenic fungi are found in the environment, where they normally live outside the human body, causing sporadic infections. These fungi turn into human pathogens by being stimulated with similar conditions to the human body in what is called an “environmental virulence school”, where fungi adapt to adhere to surfaces, form biofilms, compete with bacteria, obtain all the nutrients and deal with environmental changes in abiotic factors like temperature, pH, osmolarity, among others. Also, they can also contact with warm-blooded animals[42, 43].

One important aspect is the capacity to survive in environments at the human body’s temperature, which clarifies why the variety of successful human pathogenic fungi is low[41].

Another feature of fungi is the absence of motility, since there are no flagella or cilia.

Alternatively, fungi produce spores and grow hyphae to conquer new areas and to expand their mycelium. The variety of morphologies enables functions such as mating, tropism, invasion and dissemination within a host. The growth of fungal cells via hyphal

extension is dependent on several stimuli, like oxygen, sexual pheromones, electric fields, surfaces, among other[41].

For parasite fungi, the adaptation to their favourite niches and the infection of the hosts are dependent on the molecular regulation mechanisms of hyphal orientation, its complex cellular regulation via calcium signalling, GTPase signalling modules and other protein complexes[41].

Whether symbiotic, commensal or pathogenic, the first step in the relationship with the host is the colonization of the niche; after colonization, pathogenic fungi may cause damage, or persist latent asymptomatically[41].

As pH changes results in multiple cellular activities, pH adaptation is more than a central pH response; moreover, pH sensing controls virulence factors not directly related to pH, like adhesion to host cells, tissue invasion, iron acquisition, protease secretion, among others[41].

When compared to bacteria, fungi prefer lower pH values. Human body, though, has a generalized neutral or mild alkaline environment. Despite the fact that fungi can adapt to these environments, it is more likely that the pathogen changes the pH of the micro niche in which it is inserted by secreting metabolites like lactic acid or fatty acids[41].

Fungi also have an enormous flexibility in using different carbon sources, such as glucose, proteins and lipids by the activation of glycolysis, glyconeogenesis and/or the glyoxylate cycle, which allows them to survive long periods of starvation[41].

In order to allow the development of more effective therapeutic methods, it is very important to have detailed knowledge of the *in vivo* metabolism of the pathogen, since the traditional drug screening is based on *in vitro* studies[41].

1.2.2.1.1 – PLANT HOSTS

Hosts of *Lasiodiplodia theobromae* are mostly woody plants, like eucalyptus (*Eucalyptus* spp.) and plantations of different fruit trees, like avocado[45].

In avocado tree, *L. theobromae* causes dieback, which is characterized by necrosis and drought of the affected tissues, and progressive advance from the apex to the thick branches and stem, resulting in the fall of the leaves, emaciation and death[33].

In the case of *Eucalyptus*, it has been shown a high incidence of death among young populations (9-16 months), determined by the presence of cancer and typical constriction in the region of the stalk, caused by this fungus[29].

During inspection performed in February 1992, in Venezuela, to passion fruit plantations (*Passiflora edulis* Sinf. Forma *flavicarpa* Degener), countless branches were found with symptoms of regressive death. Later observations reveal that the disease called "regressive death" is wide spread through Venezuela, causing a slight decrease of the incomes. The affected branches presented white-grey lesions and the dead branches' bark was brittle. The surface had many dark, subepidermal pycnidia. These contained paraphyses, conidiogenous cells and conidia, with typical characteristics of *Lasiodiplodia theobromae*[46].

The largest impact of the disease is located in east Venezuela, where plantations of *Pinus caribaea* have been established. In this case, *L. theobromae* was known for causing distension and disruption of the cell walls and weaken the strength and the hardiness of the wood from *P. caribaea*, thus reducing its value in more than 50%[47].

Many edible tropical fruits, such as guava, banana and mango, can be degraded by the action of this fungus both before and after the harvesting[30].

Mango – the fruit of *Mangifera indica* L. – is a fruit originally from South Asia cultivated for the food industry for years. The production of mango has spread worldwide, into Asia, West Africa, Australia, South America and Mexico[48]. Mango is a very good source of income for Pakistan, as it is one of the top exportable fruits. Despite the good climatic conditions of Pakistan, particularly in Punjab and Sindh provinces, there is low production because of fungal diseases[48]. In *M. indica*, the disease is characterized by twig blight, tip dieback, gummosis and bark splitting. Also, some common symptoms are drying of tip, discoloration and darkening of bark some distance from the tip. In a later phase, it moves downward, affecting bigger branches as well. Finally, the leaves fall and there is exudation of gum in the affected areas. All those symptoms can occur alone or combined in different plantations throughout the world. This fungus also affects the collar region, leading the

plant into decline phase. Every lesion caused by physical trauma or insects can provide an open door for infection by *L. theobromae*[48].

This fungal infection generally has no appropriated treatment, and by the time the fungus affects collar or stem portion, the plant dies within a couple of days[48].

Recently transplanted guava (*Psidium guajava* L.), have been reported with a disease characterized by rot of the stalk in 40% of the seedlings. The observations of the disease in field suggested that the process of infection has its beginning through the grafting point, since the rootstock had no defects[49].

Cassava, yams and sweet potatoes can also be affected before and after harvesting. In these cases, the infection begins through lesions, mostly through the scar from the link between the aerial and terrestrial part of the vegetable[48.]

In the cacao tree, *Lasiodiplodia theobromae* can be found causing the disease called "descendent death". This disease is characterized by the appearance of dark spots in the branches, followed by leaves fall. The branches dry and die, presenting a soft bark that disintegrates and loses itself from the wood, and the internal tissue has necrotic brown lesions. The plant dies and in the affected branches there can be found many pycnidia of the fungus[48].

The gummosis of the cashew tree, caused by *Lasiodiplodia theobromae*, is a disease of growing importance in the semi-arid area of the Brazilian Northeast. The substitution of the crown of the cashew tree turns this disease into a higher threat, since it induces the dissemination of the pathogen[50].

1.2.2.1.2 – HUMAN HOST

Despite the large amount of host plants for this fungus, it has also been associated to a number of cases of human infections, thus behaving as an opportunist in humans. The most common cases are of ocular infection. Nonetheless there are other types of infections, with different levels of seriousness. In this chapter, it is presented some cases of human infections by *Lasiodiplodia theobromae*.

Fungal infection in the eyes is common. Up to one-third of all traumatic infectious keratitis may be caused by filamentous fungi, and is associated to trauma in the cornea with vegetable matter[51]. In the tropical regions, it is common among male agricultural workers. Fungal keratitis in the tropics seems to be caused by a large and diverse number of genera, including *Lasiodiplodia theobromae*[52].

In India, a rare case of fungal keratitis was described. The patient – a 32-year-old woman with a history of vegetative trauma – had keratitis in the left eye; a microbiological analysis of a sample of corneal scraping tested positive for hyphae with aseptate branching filaments and black pigmented colonies in vitro. PCR allowed the identification of DNA 100% homologous with *Lasiodiplodia theobromae*[53].

Phaeohyphomycosis is a fungal disease characterized by the occurrence of dark walled, melanised fungal filaments in human tissues[54].

A 50 year old woman who stumbled in an outdoor wooden staircase in Jamaica, presented injury in the right leg, of which a biopsy have shown septate, melanised fungal filamentous growing into the woman's tissues. Microbiological culture presented as a nonsporulating melanised mycelium. The isolate was susceptible to cycloheximide a benomyl, although it grew at 37ºC. After 16 weeks of cultivation *in vitro*, on modified Leonian's agar at 25ºC, the isolate developed pycnidia which allowed the identification of the fungus as *Lasiodiplodia theobromae*. *L. theobromae* is, on rare cases, capable to infect humans causing subcutaneous phaeophomycosis, which is usually treatable by debridement of the affected area[54].

A man with 45 years old presented severe sepsis after 14 days of a cadaveric-liver transplantation. He was diagnosed in July 2006 with a hepatitis B virus-related hepatocellular carcinoma, and transplanted in September 2006 in mainland china, which was complicated by portal vein thrombosis and liver failure. Chest radiography showed air space over both lungs, Computed Tomography scanning of the abdomen showed portal vein thrombosis and ascites and the analysis of a direct KOH smear of the bronchoalveolar presented with numerous leukocytes and septate hyphae. Eleven hours after the operation, the patient died. Cultures of two bronchoalveolar lavages (from before and after the operation) didn't grow any bacteria, but a dematiaceous mould yielded after 3 days of incubation at 37ºC. Further examination showed pycnidia with an obvious neck and conidiogenous cells and paraphyses – sterile filaments amongst conidia – covering the internal wall. Finally, the fungus was identified as *Lasiodiplodia theobromae*[55].

Another case of subcutaneous infection appeared in Melbourne, when a Cambodian patient presented with a subcutaneous abscess on the right buttock. A biopsy showed septate fungal hyphae. The formation of pycnidia and of typical conidia allowed the identification of the fungus as *Lasiodiplodia theobromae*, and the abscess was treated by drainage and debridement[56].

In 2010, a 30-year-old woman presented with a case of fungal sinusitis, with intermittent bleeding and nasal discharge from the left side during a week. Also, the

patient had headaches mostly on the left side, as well as intermittent heaviness for two months. Radiological and mycological evidence supported the diagnosis, and the patient was treated with endoscopic surgery and antifungal treatment.

The cause of the infection remains unknown, since other cases of fungal infection by *L. theobromae* are believed to be the result of direct inoculation in the affected area (eyes, skin, soft tissues), making rhinosinusitis caused by this pathogen exceptional.

In general, the treatment for fungal sinusitis is surgery and antifungal agents, mostly in invasive types of the disease. This patient completely recovered, and further examination by nasal endoscopy revealed completely healed mucosa and bilateral patent ostia[57].

1.3 – FUNGAL PROTEOMICS

The presence of fungi in our life is very significant: we eat fungi directly or as a part of the food-making processes; we use fungi in white biotechnology; in ecosystems, they play an important role as mutualistic symbionts, parasites and as primary decomposers of organic matter. Also, fungi can affect negatively our lives, as they may act as plant pathogens – causing damage in crops worldwide – as well as human pathogens, causing infections with different levels of severity but potentially lethal[58].

Due to the emergent information resultant from genome sequencing projects, a new experimental approach is growing in biology known as proteomic analysis, which consists of the study of the proteins expressed by a determined genome, the proteome.

The evolution of this experimental approach results of the increasing development, integration and automation of a variety of techniques and equipment that are able to separate, identify, quantify and characterize proteins, and also relate that information with functional information using bioinformatics tools[59].

For the past 5 years, research in fungal proteomics has progressed extraordinarily. This evolution has been promoted by the need to fully understand pathogenesis mechanisms of fungi against immunocompromised humans and plants, as well as the interest in their enzymes (e.g. for biofuel production). Therefore, proteomics has become an important component of all large-scale ‘omics’ and other approaches to study the complexity of fungal biochemistry, since it has a great capacity to obtain information on protein identity, localization, posttranslational modification and the accuracy of *in silico* gene model prediction in fungi[60].

The sequencing of the genome of an organism is, by itself, a first step which is, however, very informative as it allows the development of plenty of other studies. Nonetheless, the analysis of the genome alone doesn't allow obtain information about the levels of protein and genetic expression, or about the characteristics of the expressed proteins, such as its lifetime, its subcellular localization, eventual posttranslational modifications, protein-protein interactions and protein-DNA interactions, their structure and their biological function[59].

A significant effort has been directed to the techniques of quantitative proteomics, in order to enable the clarification of the regulation mechanisms in global genetic expression. This analysis complements the monitoring of the global genetic expression at the mRNA level, since it is not capable to evaluate the regulation mechanisms post transcription and posttranlation[59].

The identification of the proteins whose expression has been significantly changed allows obtaining clues about their possible involvement in the biological process in study.

The analysis of the modifications in the proteins after translation is also of extreme relevance, since it can determine its traffic, function and its activity level, which is highly important for the comprehension of the complex regulation webs associated to cell responses to environmental stress stimuli[59].

There are several solutions for protein identification in an complex sample, but mainly the workflow (described in a very simplistic way) follows 3 basic steps: protein separation, digestion with a known enzyme and peptide mass determination (which allows proteins identification).

Several different methods are used: the individual protein identification followed by SDS-PAGE or 2D-PAGE or 'shotgun' proteomics, where total protein digests are analysed by tandem LC-MS, resulting in complete protein data sets[60].

Nowadays, there is a large variety of solutions in terms of equipment, for the determination of the masses of peptides obtained by enzymatic hydrolysis. Among these solutions, the most common is the Mass Spectrometer of the type MALDI-TOF which is composed by an ionizer, MALDI (matrix-assisted laser desorption/ionization), an analyser, TOF (time-of-flight), and a detector that registers the time of flight of the various ionized peptides. As the smaller ions fly with higher velocity, the ratio m/z may be calculated using the registered times of flight, after calibration of the instrument, thus allowing to determine the mass of each peptide[59].

More recently, methods of *in vivo* and *in vitro* have been developed, in order to determine, by mass spectrometry, the relative concentration of the protein in study in two biological samples, thanks to the isotopic differences in molecular weight.

While the *in vivo* labelling is made through the inclusion of stable isotopes in the culture media, allowing their incorporation, the in vitro marking is made with protein extracts through several steps of chemical modification, being isotope-coded affinity tag (ICAT) one of the most utilized. Associated with this method of quantitative proteomics is liquid chromatography (LC), which can be multidimensional, allowing a more effective fractionation of complex protein samples[59].

The absence of information concerning fungal proteome's characterisation and the relative abundances of proteins mean that most of the investigation made in this area is still focused on the optimization of the protein extraction process. Also, much effort has been made in the cataloguing of the mycelial and organellar proteomes, as well as the secreted proteins (secretome) among a range of fungal species[60].

A curious fact about fungal proteomic research is the discovery of both "predicted proteins" and "hypothetical proteins", occurring in all fungal protein data sets of Ascomycete and Basidiomycete investigations. Therefore, the classification of the protein must obviously be upgraded to that of "unknown function protein (UFP)", since it is now known to exist. The attribution of functions to the discovered UFPs is now a big challenge in fungal proteomics research[60].

1.4 – Cell Culture and Cytotoxicity Assays

Generally, cell lines will suffer a limited number of doublings, generating therefore a limited number of subcultures – these cell lines are named finite cell lines. This limit occurs when the cells stop growing because of senescence, which is determined by a number of specific factors of the cell's cycle regulation.

When a cell line is sub-cultured, it will grow again until reaching the same cell density it had before the subculture, which is no more than plotting a growth curve from a sample of the cell line taken at specific intervals along the growth cycle. This shows that cells can stop growing, entering a period of latency after reseeding – the *lag period*.

This period can last from only a few hours up to 48h, generally around 12-24h. In this period the cells can recover from trypsinization, reconstruct their cytoskeleton, secrete matrix to aid attachment, and spread out on the substrate, enabling the re-entrance in the

cell life cycle properly[61]. In mammalian cells culture, trypsin has been used for dislodging cells in primary cell cultures to remove single cells from entire tissues and organs. It has also been used to detach cells from either static or carrier surfaces, in order to obtain a sample to subcultivate. Trypsin hydrolyses peptide bonds of pepsin-digested peptides, process commonly named trypsin proteolysis or trypsinization. This process is a well-established procedure in tissue culture[62].

Afterwards, a phase of exponential growth – *log phase* – happens, in which the cell population doubles over a specific period (the doubling time), which is characteristic of each cell line (figure 4).

When all the substrate is occupied and the cell population is crowded, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. Subsequently, the cells enter the *plateau* or *stationary phase*, in which there is almost no culture growth[61].

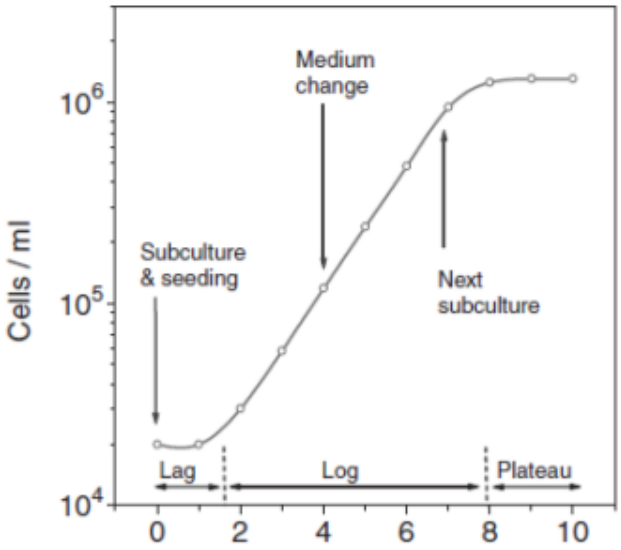


Figure 4: Growth curve. Increase in cell number on a log scale plotted against days from subculture. Defines the lag, log (exponential), and plateau phases, and when culture should be fed and subcultured after the indicated seeding time. *Adapted from Freshney, 2006*[61].

In this phase, some cells may differentiate, while others simply stop into G0, yet retaining viability. Thus, although cells may be subcultured from plateau, it is preferable to

do it before they reach this phase, in the top end of the log phase, in which the growth fraction is higher and the recovery time (lag period) shorter[61].

Vero cells – a cell line derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in the 1960s - are a very common mammalian continuous cell line used in research. This cell line has been commonly used in virology assays, as well as in other applications in the propagation and study of intracellular parasites and bacteria and evaluation of the effects of chemicals, toxins and other substances on mammalian cells[63].

Vero Cells have been tested and shown to be free of several adventitious agents (for which there are already available tests).

Alamar Blue is a water-soluble dye that has been used for in vitro cell's viability assays. It allows a continuous monitoring of the cell culture, once it is very stable and, more importantly, nontoxic. Mainly for this reason, the Alamar Blue assay has been considered a superior test in comparison to other classical cytotoxicity tests such as the MTT test[55].

When it is added to a cell culture, the oxidized form of the Alamar Blue enters the cell and is reduced by mitochondrial enzyme activity, by the acceptance of electron from NADPH, FADH, FMNH, NADH as well as from the cytochromes. This redox reaction results in an alteration of the culture medium's color, which turns from indigo blue to fluorescent pink, allowing a colorimetric or fluorometric measurement and a determination of cell's viability[65].

Vero cells are easily available, grow fast and require no rigor culture conditions. Furthermore, their potential for free oncogenic properties and the fact that this cells are not presenting any threat to human health when used as a substrate for biological production, make them one of the most cell line utilized for toxicity assays[63].

1.5 - OBJECTIVES

The main purpose of this work was to analyse the differential protein expression of two isolates of *Lasidiplodia theobromae*, an environmental isolate and a clinical isolate, subjected to stress conditions.

In order to accomplish this purpose, temperature was selected as main stressor: 25ºC and human body temperature, 37ºC, were selected as the temperatures of interest due to their relation to infection of plants and humans, respectively.

Thus, the hypothesis of this study was to verify if the differential protein expression of L. theobromae, at 25ºC and 37ºC, is dependent on the origin of the isolate.

2. MATERIALS AND METHODS

In this chapter, the materials and methods utilised in this work, are described.

2.1 - ISOLATES

Two different isolates from the fungus *Lasiodiplodia theobromae* were used: an environmental isolate (CAA019), from the collection of Dr. Artur Alves (University of Aveiro) and a clinical isolate (CBS339.90), from the CBS culture collection.

2.2 - CULTURE MEDIA

Along the process, several culture media were used, of which compositions are described below, for a final volume of 1L. After the preparation of the media, these were autoclaved for a period of 20 minutes, at 121ºC.

Potato Dextrose Agar (PDA)

Potato Dextrose Agar (PDA)
39g PDA (Merck)

Potato Dextrose Broth (PDB)

Potato Dextrose Broth (PDB)
39g PDA

Potato Dextrose Broth was prepared using PDA medium, which was then filtered (after solubilisation) to remove the agar.

Corn Meal Agar (CMA)

Corn Meal Agar (CMA)
17g CMA (Difco)

Oatmeal Agar (OMA)

Oatmeal Agar (OMA)
20g oatmeal flakes
15g Agar (Merck)

Czapeck-Dox agar

Czapeck
30g Sucrose
3g $NaNO_3$
1g K_2HPO_4
0.5g $MgSO_4.7H_2O$
0.5g KCl
0.01g $FeSO_4.7H_2O$
20g Agar

Skim Milk

Skim Milk
10g Skim Milk (Molico)
5g Malt Extract (Merck)
15g Agar

Skim milk was prepared previously: 10mL of a 1g/mL solution of skim milk was autoclaved (15min at 121 ºC) and added to 990mL of Malt extract agar medium previously sterilized.

2.3 - RADIAL GROWTH

Fungal growth was evaluated based on the development of the mycelium in solid media during the incubation period, and the radius of mycelium was measured. All assays were carried out in triplicate – protocol in section 6.1.

2.4 - BIOMASS GROWTH

Fungal development was determined by cultivation in liquid medium and determination of mycelium's dry weight – protocol in section 6.2.

2.5 - PROTEOLYTIC EXTRACELLULAR ACTIVITY DETECTION – SKIM MILK

The use of culture media containing skim milk to grow fungi, allows the evaluation of the presence of extracellular proteolytic activity. Thus, if there is secretion of proteases, there's the conversion of casein into soluble nitrogenous compounds, appearing a transparent halo around the mycelium[66] – protocol in section 6.3.

2.6 - LYOPHILISATION

The extracellular samples for SDS-PAGE were lyophilized.

Lyophilisation is a process which extracts the water from the samples, so that the samples remain stable and are easier to store at room temperature. This technique is carried out using the principle of sublimation.

The process of lyophilisation consists of freezing the samples so that the water becomes ice and under a vacuum, sublimating the ice directly into water vapour. Once the ice is sublimated, the samples are freeze-dried and can be remove from lyophiliser (Snijders Scientific)[67].

The lyophilisation occurs at, approximately, -50 ºC and 0.13mbar.

2.7 - PROTEIN QUANTIFICATION - BCA METHOD

The BCA method (BCA Protein Assay Reagent Kit, Pierce) allows the determination of the protein concentration in a sample, through colourimetry with bicinchoninic acid. This method consists of the reduction of Cu^{2+} into Cu^{+} and the colorimetric detection of the Cu^{+}, by the use of a reagent which contains bicinchoninic acid. In the reaction occurs the creation of a purple product, with absorbance at 562nm, increasing the intensity of colour with the amount of protein[68].

Protein concentration was determined according to the manufacturer instructions, with slight alterations as described below – protocol in section 6.4.

2.8 - PROTEIN QUANTIFICATION - 2D QUANT KIT

The 2-D Quant Kit (GE Healthcare) is designed for the accurate determination of protein concentration in samples containing reagents such as detergents, reductants, chaotropes and carrier ampholytes which are incompatible with other protein assays.

Initially, the kit reagents induce the precipitation of proteins, thus separating them from the supernatant in which there are the reagents capable to solubilize them, though susceptible to interfere in the quantification. Afterwards, the proteins are ressuspended in a copper solution, which will bind specifically to the proteins, allowing evaluating the amount of copper that didn't react.

Therefore, the proteins concentration is inversely proportional to the density of the solution's colour.

Protein concentration was determined according to the manufacturer instructions, with slight alterations as described below – protocol in section 6.5.

2.9 - ZYMOGRAPHY

Zymography is an electrophoretic technique that allows to determining the number and the apparent molecular weight the proteases in a sample, since an appropriate substrate, such as gelatine, is incorporated in the gel. To supply a uniformly scattered substrate, gelatine is included in the polyacrylamide standard gel solution, which is then spilled and polymerized. The samples are denatured with SDS and then separated by electrophoresis. Afterwards, the proteases are then renatured inside the gel by substitution of the SDS for a non-ionic detergent, such as Triton X-100. The gel is then incubated in an appropriate reaction buffer, allowing the renatured proteases to digest the protein substrate in the zone of the gel in which they stopped, after electrophoresis. This digestion zones are visualized by gel coloration with a dye, Coomassie Brilliant Blue, resulting in the appearance of bright digested zones in contrast with a blue background[69].

Polyacrylamide gels were elaborated according to Laemmi, 1970, as described below[70] – protocol in section 6.6.

2.10 - SDS-PAGE

Electrophoretic separation of proteins in denaturing conditions (SDS-PAGE) is a method which uses a discontinuous polyacrylamide.

SDS is an anionic detergent that links itself to hydrophobic amino acids and this way, the proteins accumulates a negative charge approximately constant in all its length. Thus, the polyacrylamide gels have the capacity to stop larger molecules of migrating as fast as smaller ones. In this way, is possible to achieve a separation based on molecule size[71] – protocol in section 6.7.

The polyacrylamide gels were made according to the protocol described by Laemmli, 1970[70] and the utilized system was the Mini-Protean® 3 Cell, from Bio-Rad, linked to an Electric Potential Difference generator, PowerPac 300 from Bio-Rad.

2.11 - GEL ANALYSIS

Gel analysis were performed after staining the proteins and scanned on a GS-800 Calibrated Densitometer (Bio-Rad). Quantity One (Bio-Rad) was the software used to estimate the molecular mass of proteins and their optical densities. The apparent molecular weight of the proteins was determined using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standard, from Bio-Rad).

2.12 - EVALUATION OF CYTOTOXICITY BY THE METHOD OF RESAZURIN

A Vero cell line from the kidneys of the African green monkey (*Cercopithecus aethiops*) was used to resazurin-based cytotoxicity assay. Its maintenance was made in culture chambers at 37ºC, with 5% CO_2 in DMEM media supplemented with 10% of FBS and 2 mM of glutamine. The culture medium was previously filtered and heated up to 37ºC. *In vitro*, the adherent cell lines grow until they cover the entire available surface or until they reach the limit of nutrients in the medium. Thus, the cells line must be subcultured in order to prevent the culture's death, either by lack of nutrients or by lack of space. For the subculture, proteases are used, like trypsin, which break the links between cells, leaving them in suspension in the bottle[63]. All the procedure was performed under aseptic conditions. Afterwards, 100µL Vero cells suspended in DMEM supplemented with 10 % FBS were distributed into a 96-well tissue culture plate ($2x10^4$ cells per well) and incubated for 24h (± 80 % confluent monolayer) at 37 ºC in 5 % CO_2 atmosphere.

When it's desired to know the rate of viability of the culture, is used an aliquot for counting the cell suspension diluted in a solution of trypan blue. Trypan blue is dye exclusion and only comes into permeabilized cells, death cells. After entering the cell, trypan blue stays in the core, remaining blue – protocol in section 6.8.1.

The resazurin, also known as Alamar Blue, is a redox indicator, which presents colorimetric and fluorescent alterations according to the metabolic activity's response. The viable cells are able to reduce this compound, occurring a change of colour from blue (resazurin) to reddish pink (resofurin)[72].

The use of resazurin is not toxic for the cells, thus allowing obtaining results with no cell death. These are achieved by the reading of the absorbance at 570nm (wavelength for resofurin) and at 600 nm (wavelength for resazurin)[73] – protocol in section 6.8.2.
Each sample was tested in two independent experiments performed in triplicate.

3. RESULTS AND DISCUSSION

3.1 – GROWTH CHARACTERISATION: EFFECT OF ENVIRONMENTAL PARAMETERS

3.1.1 – Agitation

The effect of agitation in the growth of *Lasiodiplodia theobromae* isolates was analysed after a period of 4 and 8 days of incubation, corresponding to two different stages in the development of *L. theobromae*: 4 days growth corresponding to the development peak and 8 days growth corresponding to mycelium degeneration (figure 5). The isolates were incubated at the temperature of 30 ºC, in the absence of light, under agitation (120rpm) or without agitation, in PDB medium.

The assays were made in triplicate and data is presented as average ± standard error.

After both periods of incubation, the mycelium were collected and dried at 50 ºC (as described in materials and methods), in order to obtain dry biomass weight. The results indicate that an agitation of 120rpm does not induce significantly effects to *L. theobromae* growth, since the values are very similar to those of the incubation without agitation ($p>0.05$) (figure 5).

The growth of this fungus under agitation results in mycelium fragmentation and increases the complexity of mycelium removal process. Once the fungus is under constant stirring, the hyphal growth will not be uniform as it turns out to promote the mycelium fragmentation. However, this makes the mycelium to be in permanent contact with the medium, contrary to what happens when the mycelium is continuous. Agitation of the culture also promotes gas exchanges between the medium and the air, increasing oxygenation promoting a higher enzymatic activity[74]. On the other hand, an excessive agitation is known to damage fungal mycelia, leading to cell destruction, thus lowering the enzyme production[75].

Given the results obtained, agitation was not considered favourable for fungi growth/manipulation: there was no significant increase in biomass production and the process mycelium removal from the medium for further processing became more complex.

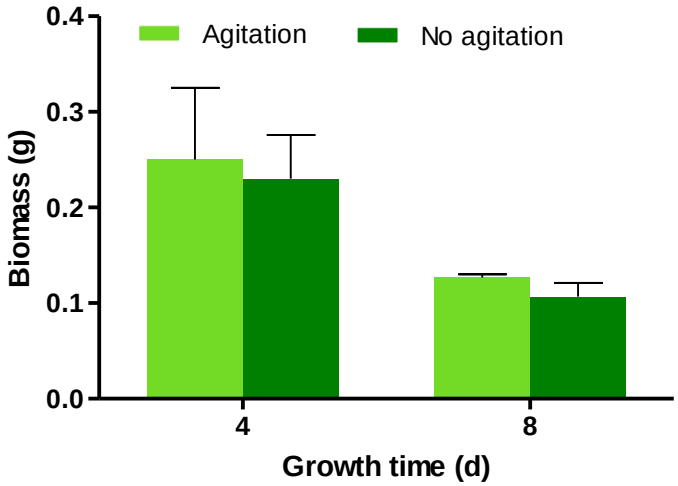


Figure 5: Effect of agitation on *L. theobromae* (CAA019) growth. Mycelium's dry weight was determined after growth at 30 ºC under 120rpm agitation and without agitation, for 4 and 8 days. Data is presented as average ± standard error. A t-test was used to determine the statistical significance of the presence and the absence of agitation.

3.1.2 – Temperature

Figures 6A and 6B represent the radial growth, after 48h of incubation of the environmental and clinical isolates, in 4 different culture media at 8 temperatures. The assays were made in triplicate and data is presented as average ± standard error.

L. theobromae has a great capacity to adapt to different environments, as one can infer by its worldwide distribution. This suggests that its growth is possible under a wide range of temperatures.

As a typical species from the tropics and subtropics, it has an optimal growth temperature between 27 ºC and 33 ºC, even though it is able to grow in temperatures between 9 ºC and 39 ºC[31].

The results indicate that both isolates are not able to grow at 5 ºC and at 40 ºC. Furthermore, maximum growth is reached at 30 ºC, when *L. theobromae* inoculation is made in PDA medium.

In opposition to PDA medium which was shown to be the best medium for the cultivation of both the isolates, Czapeck medium – the only medium with a defined composition – showed to be the least adequate to the growth of *L. theobromae*, being

associated to the lowest values of radial growth and to the absence of growth, in the case of CAA019, at 15 ºC and 20 ºC. Fungi cannot fix nitrogen and need to be supplied with nitrogen-containing compounds, either in inorganic form such as ammonium salts, or in organic form such as amino acids[2]. The lowest values of radial growth obtained with Czapeck medium could be related with an inadequate nitrogen source to *L. theobromae*. Thus, both isolates behave accordingly to their typical tropical and subtropical preferential distributions, when exposed to temperatures between 5 ºC and 40 ºC. Also, they do not grow with temperatures below 9 ºC and above 39 ºC.

Media generally contain a source of carbon, nitrogen and vitamins and glucose (dextrose) is the most widely utilizable carbon source. A wide variety of culture media is used for the isolation of different fungi groups, having different effects over their growth, colony morphology, pigmentation and sporulation[76]. All fungi require specific elements for their growth and reproduction. Of the four culture media tested, only Czapeck medium has a defined composition, being the poorest in carbohydrate concentration. In contrast, PDA medium is the medium with higher values of carbohydrates, followed by CMA and OMA media, both of which contain the hardest carbohydrates to digest[2, 77].

The results obtained suggest that both isolates have a better growth when inoculated in culture medium with higher concentration of dextrose, which, in this case, is PDA. Also, they have more difficulties to grow when the culture medium is less complete or when the carbon source is more complex[2]. The effect of culture media and temperatures were considered extremely significant.

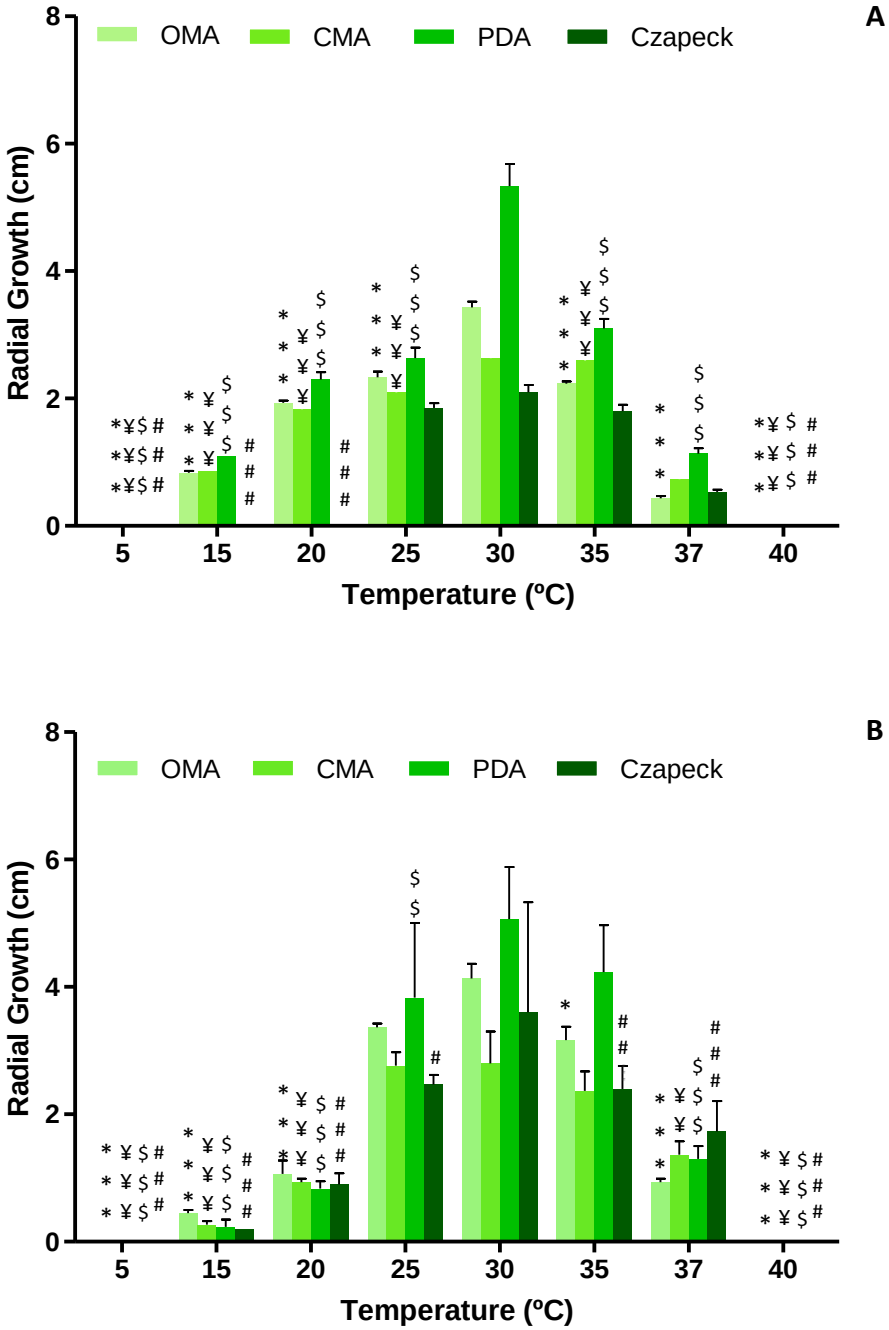


Figure 6: Effect of temperature on the growth of *L. theobromae*. Radial growth of *L. theobromae*, CAA019 (A) and CBS339.90 (B), grown at different temperatures and in different culture media was determined after 48 hours of incubation. Data is presented as average ± standard error. Two-way ANOVA, followed by a Bonferroni multiple comparison test, was used to determine the statistical significance of different temperatures compared to 30ºC (*p<0.05, **p<0.01 and ***p<0.001, for OMA; $^{¥}$p<0.05, $^{¥¥}$p<0.01 and $^{¥¥¥}$p<0.001, for CMA; $^{\$}$p<0.05, $^{\$\$}$p<0.01 and $^{\$\$\$}$p<0.001, for PDA; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001, for czapeck).

3.1.3 – Light

The effect of light on the radial growth of *L. theobromae* [CAA019 isolate (figure 7A) and the CBS339.90 (figure 7B)] was assayed at 25 ºC, 30 ºC and 37 ºC, in PDA medium. The assays were made in triplicate and data is presented as average ± standard error as described in material and methods chapter.

In the case of the environmental isolate, CAA019, it is possible to verify that at 25 ºC and 30 ºC the radial growth is very similar both in the presence and the absence of continuous light. Nevertheless, at 37 ºC, the presence of light induces a significantly higher growth ($p<0.001$). Considering the clinical isolate, no growth differences were detected at 25 ºC, while at 30 and 37 ºC growth was superior in the presence of continuous light ($p<0.05$).

The maximum radial growth was, thus, obtained when *L. theobromae* was incubated in the presence of continuous light, achieving around 5 cm of growth in the case of CBS339.90 at 30ºC and about 3 cm in the case of CAA019, for the temperatures of 25 ºC and 30 ºC.

Light is the fundamental energy source for life on earth and as such is a major environmental signal for organisms from all kingdoms of life. In the fungal kingdom, light can regulate growth, the direction of growth, asexual and sexual reproduction, and pigment formation, all of which are important aspects for the survival and dissemination of fungal species. These processes have negative implications to many aspects of human life, as the uncontrolled proliferation of fungi can lead to devastating plant disease, mold, and human disease[78].

Light has long been linked to disease. However, the mechanisms behind many of these observations are not well understood. Recently, a direct link has been established between specific protein photosensors and the ability to cause disease in both pathogenic bacteria and fungi. Thus, certain pathogens require these photosensors for full virulence[79].

Photosensory proteins are defined as those that regulate a signal transduction pathway. As other non-sensory proteins use the same cofactors found in photoreceptors, these proteins can be affected by light and elicit a response to light[78]

A role for photoperception is likely to emerge as a common theme in microbial pathogenesis. Sequencing projects reveal that fungal genomes encode putative photosensory proteins of the rhodopsin, phytochrome, cryptochrome, and LOV domain classes. A fungal LOV domain photosensor, first identified and named WHITE COLLAR 1 in the non-pathogen *Neurospora crassa*, is present throughout most of the kingdom,

suggesting an ancient origin. In all species examined, WC-1 physically interacts with a second protein, WC-2, that contains a zinc finger DNA-binding domain such that the complex can act as a light-sensitive transcription factor[79].

It is in agreement with the obtained results, which had, globally, a higher radial growth in the presence of continuous light.

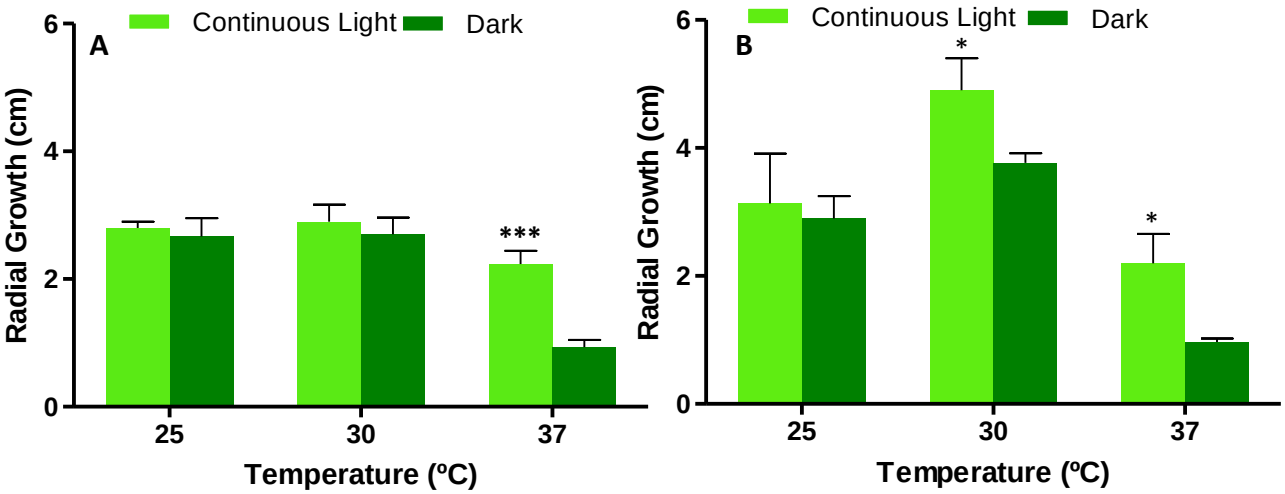


Figure 7: Influence of continuous light in the radial growth in both isolates, CAA019 (A) and CBS339.90 (B), after 48 hours of incubation. Data is presented as average ± standard error. Two-way ANOVA, followed by a Bonferroni multiple comparison test, was used to determine the statistical significance of the presence of continuous light on the growth of *Lasiodiplodia theobromae* within the same temperature (*p<0.05, **p<0.01 and ***p<0.001).

3.1.4 – Incubation Time

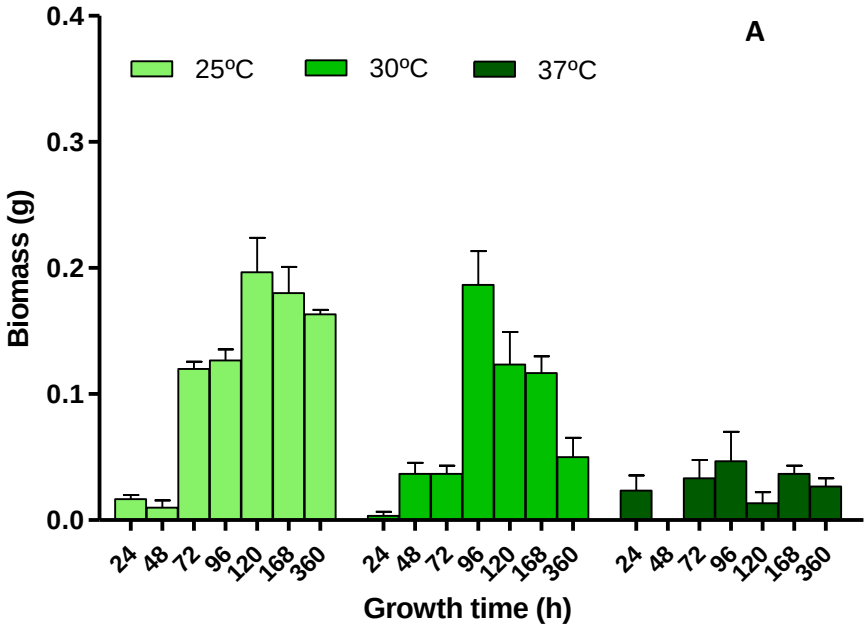
In order to better understand the growth of *L. theobromae*, the growth curve based on the biomass of CAA019 and CBS339.90 for 15 days was determined (figure 8).

The filaments or the hyphae are branched and divided into segments by transverse walls or septa. The growth of the hypha is restricted to its tip which grows linearly by the apical addition of new cell wall material. For this to happen, it is necessary the presence of

both nutrients and space. When space and nutrients are limiting factors, such as in laboratory trials, the mycelium enters in degeneration until the fungus dies[79].

When fungi are inoculated into a culture medium under optimal conditions, it will develop according to a typical growth curve, which includes the lag phase, which is a period of zero population growth, relative to the time necessary for the fungus to adapt to the environment; the exponential phase, in which the adapted fungus doubles logarithmically at a constant and a stationary phase, correspondent to a period of zero population growth rate, in which the already grown fungus remains constant. After the stationary phase, fungal cells may autolyse[2, 3].

After drying the mycelium, it was possible to determine that, in the case of CAA019, there is an increase of biomass until 5 days of incubation at 25 ºC and until 4 days of incubation for the temperatures of 30 ºC and 37 ºC. After this period of time, the fungus starts to degenerate and there is loss of biomass with increasing incubation periods. The maximum growth was also obtained at the temperatures of 25 ºC and 30 ºC. For the clinical isolate the growth pattern was similar, although the biomass values were found to be higher than those of the environmental one. Thus, it is possible to verify that both isolates complete the growth curve along a 360 hours period.



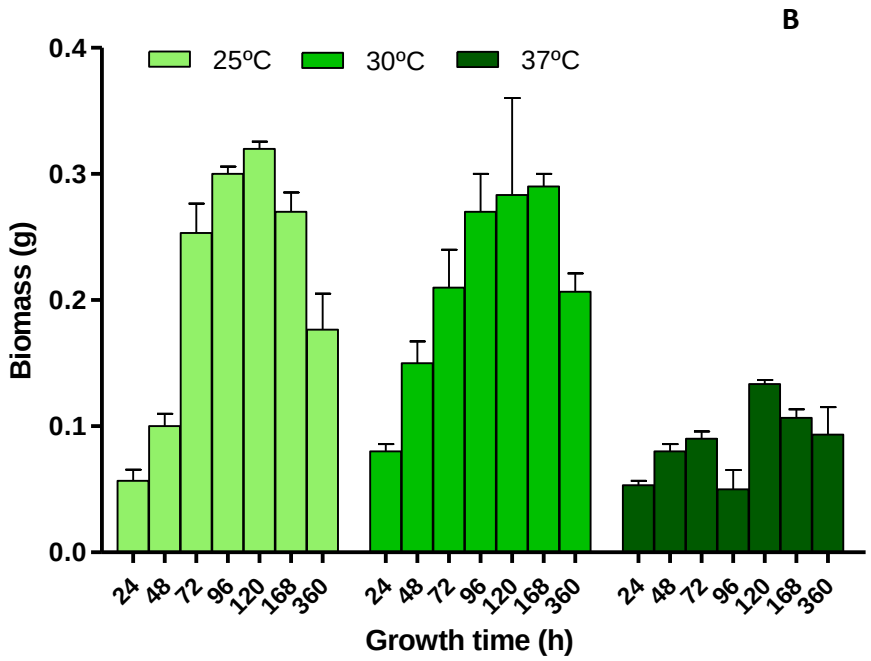


Figure 8: Effect of time on the growth of *L. theobromae*. Dry biomass of *L. theobromae*, CAA019 (A) and CBS339.90 (B) grown at different temperatures and in different periods of incubation. Data is presented as average ± standard error.

3.2 – SECRETOME CHARACTERISATION

The extracellular proteome of *L. theobromae* was characterized in terms of total protein content as well as in terms of proteolytic activity as described below.

3.2.1 – Extracellular Proteolytic Activity detection

Extracellular proteolytic activity at 25 ºC, 30 ºC and 37 ºC was determined by plate assay as described in materials and methods (figure 9). Both isolates were shown to exhibit extracellular proteolytic activity at all the temperatures investigated. This detection was made based in the radius of the halos formed by the degradation of caseins, and showed higher extracellular activity at 30 ºC ($p<0.01$) for the clinical isolate. At 25ºC and 37ºC, the extracellular activity of both isolates were similar ($p>0.05$). Pathogenicity of fungi has been shown to be related to a number of mechanisms and molecules expression, like

cell wall degrading enzymes (CWDE), inhibitory proteins and toxin synthesis influenced by enzymes[8, 9, 10].

Since both isolates are pathogenic fungi, it is expected for them to produce extracellular proteolytic activity. Extracellular enzymes are the most common mechanisms used by fungi to invade the host, causing sometimes an infection. The presence of higher proteolytic activity in the clinical isolate, may be related to the fact that this isolate is an opportunistic pathogen of humans. Is this case, the proliferation of the fungus is related with the matrix of host: human body, with higher content of proteins, in contrast to the plants, which have higher content of cellulose[11].

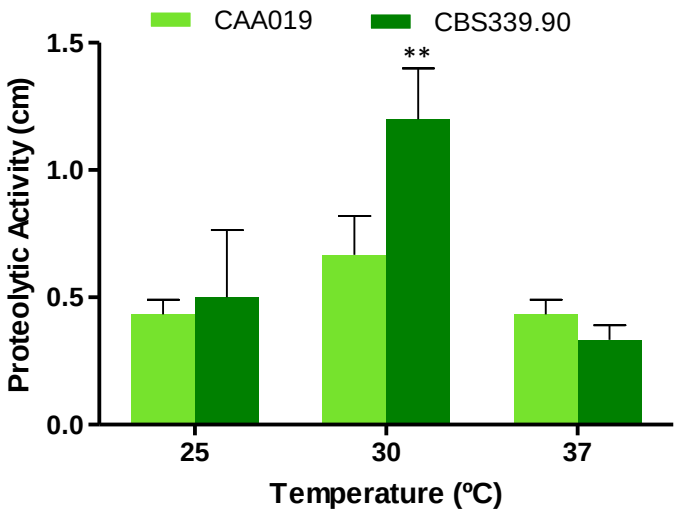


Figure 9: Proteolytic activity of both isolates of *L. theobromae* after a 24 h incubation period. Data is presented as average ± standard error. Two-way ANOVA, followed by a Bonferroni multiple comparison test, was used to determine the statistical significance of proteolytic activity within the same temperature (*p<0.05, **p<0.01 and ***p<0.001).

3.2.2 – Zymography

Secretory fungal proteases play an important part in the metabolism of producing fungi. The specific osmotrophic type of nutrition of fungi makes the secretion of hydrolytic enzymes vital for their nutrition, since these extracellular enzymes turn macromolecular compounds occurring in the medium suitable for nutrition. Indeed, extracellular proteolytic

enzymes are likely to be necessary for penetration of fungi into host tissues and, hence, may play an important role in various forms of pathogenesis[80].

In order to characterize the extracellular proteolytic proteome, gelatine zymographies of the extracellular media of both isolates grown at 25 ºC and 37 ºC were carried out. For this characterisation, 25 ºC and 37 ºC, human body temperature, were the selected temperatures due its influence on infections in plants and humans, respectively.

Figures 10 and 11 correspond to the zymographies performed for the environmental isolate at 25 ºC and 37 ºC, respectively, and show that there is the expression of proteolytic enzymes, mostly enzymes with a low apparent molecular weight. It is possible to verify that at the two temperatures there is a differential expression of proteases. It should be stressed that at 37 ºC there seems to emerge the expression of an enzyme (after 12 days of incubation, but more significant after 24 days of incubation), which is absent at 25 ºC.

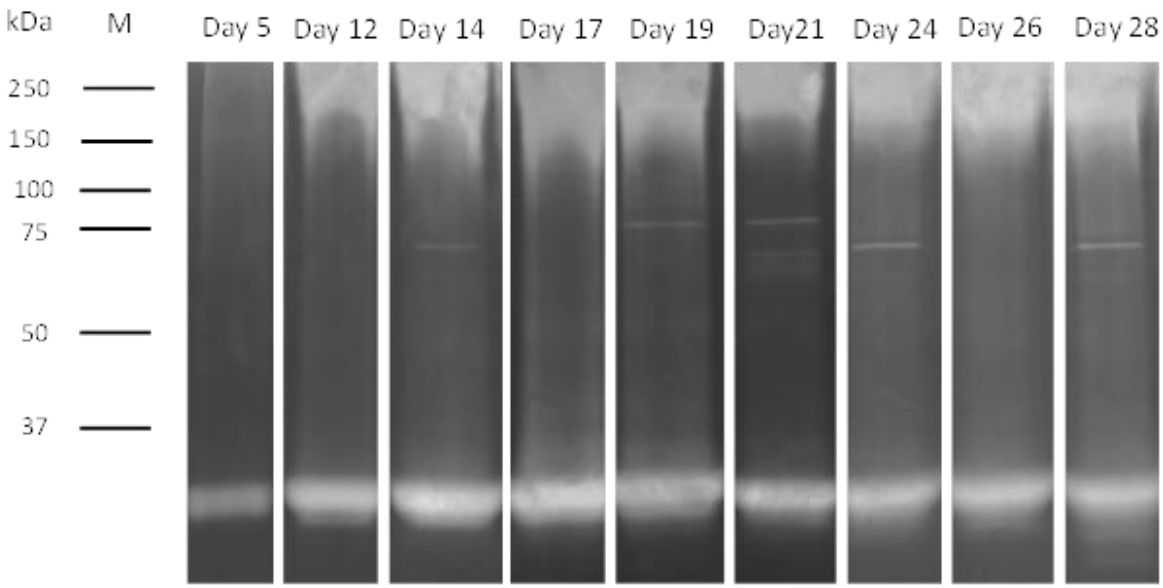


Figure 10: Extracellular proteolytic activities of *L. theobromae* (CAA019) grown in PDA, as described in material and methods, detected by zymography, using gelatine as substrate. *L. theobromae* was grown for 28 days at 25 ºC. Gel is representative of three analyses.

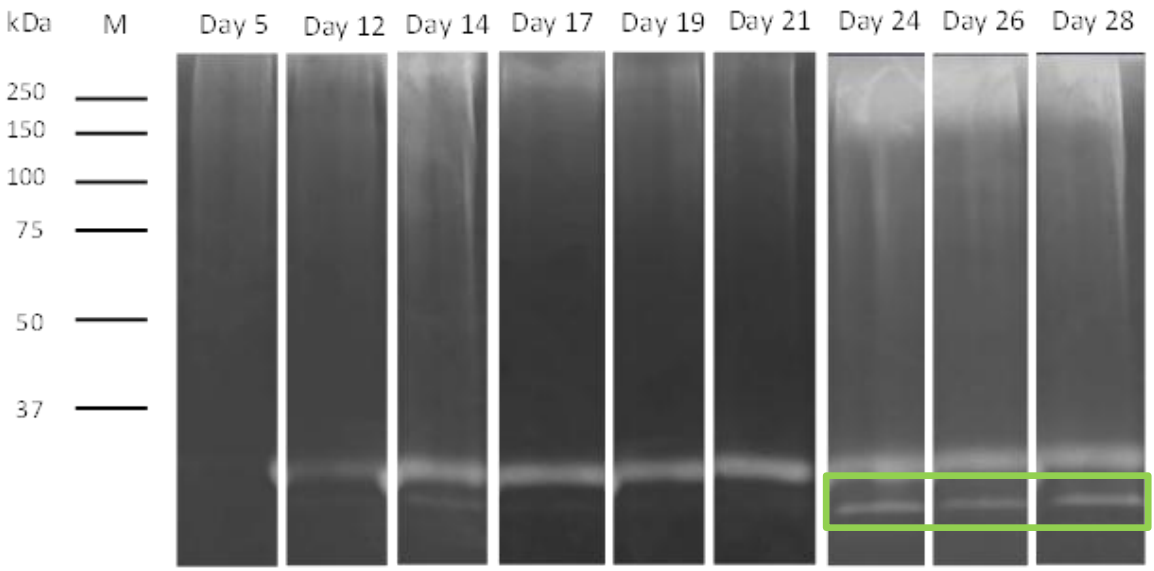


Figure 11: Extracellular proteolytic activities of *L. theobromae* (CAA019) grown in PDA, as described in material and methods, detected by zymography, using gelatine as substrate. *L. theobromae* was grown for 28 days at 37 ºC. Gel is representative of three analyses.

The analysis of the extracellular proteases of the clinical isolate (figures 12 and 13) were performed in the same conditions of those of the environmental isolate. Data show that this isolate, express several extracellular proteolytic enzymes and that temperature does not seem to play a significant role. It should be stressed that at both temperatures there seems to emerge the expression of an enzyme, more significant after 24 days of incubation.

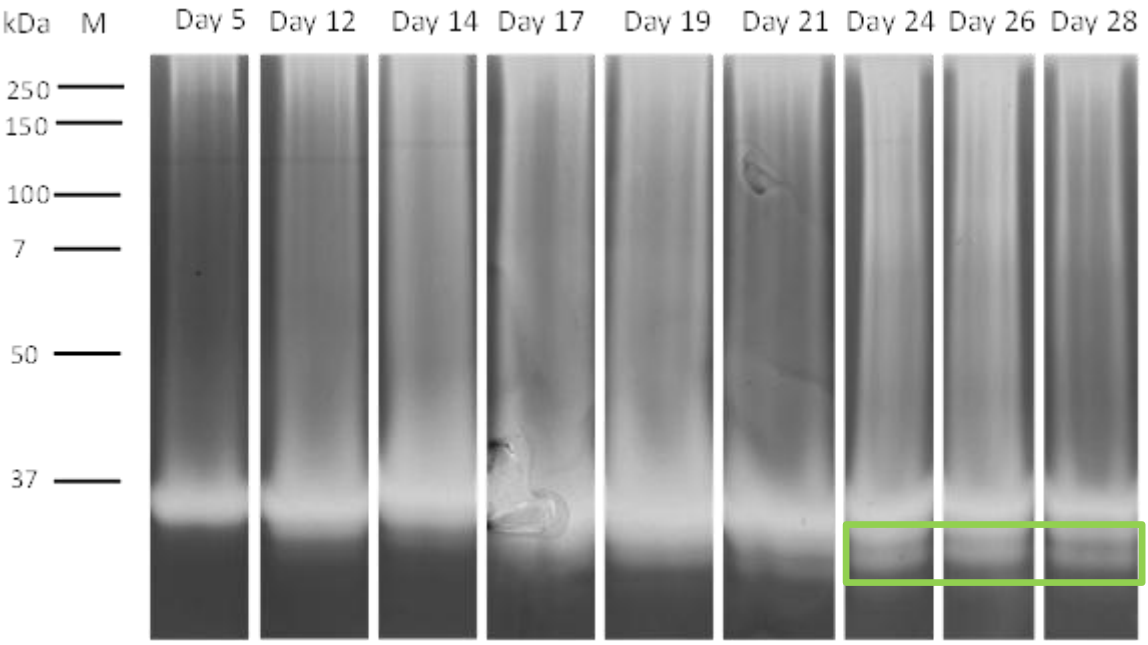


Figure 12: Extracellular proteolytic activities of *L. theobromae* (CBS339.90) grown in PDA, as described in material and methods, detected by zymography, using gelatine as substrate. *L. theobromae* was grown for 28 days at 25 ºC. Gel is representative of three analyses.

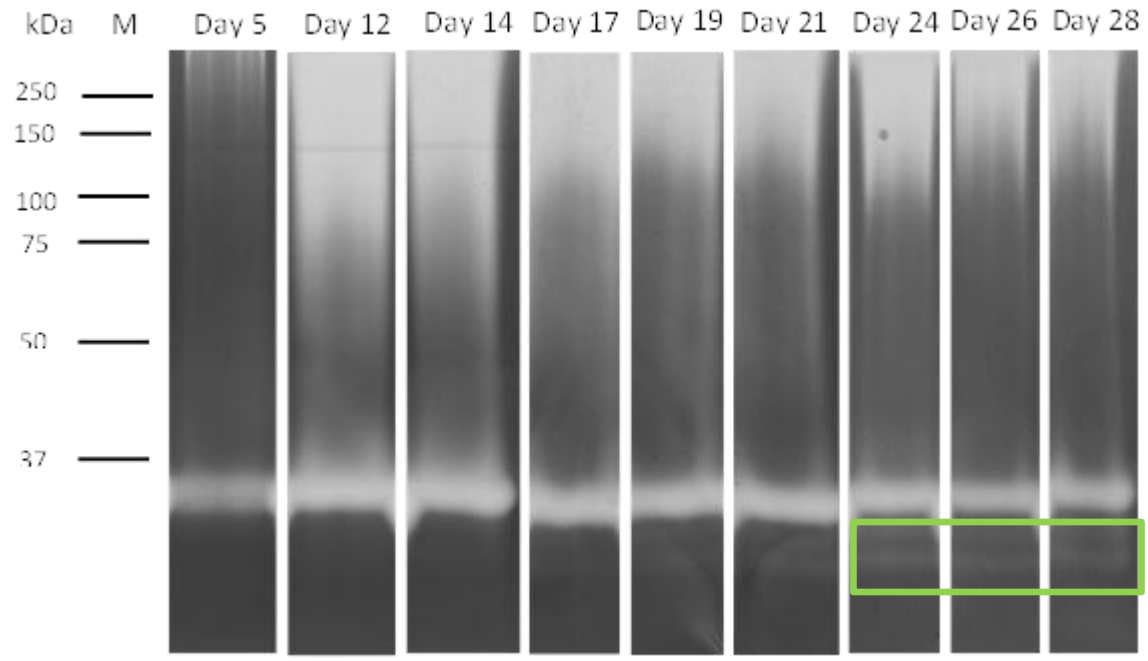


Figure 13: Extracellular proteolytic activities of *L. theobromae* (CBS339.90) grown in PDA, as described in material and methods, detected by zymography, using gelatine as substrate. *L. theobromae* was grown for 28 days at 37 ºC. Gel is representative of three analyses.

It is thus possible to verify a differential proteolytic enzymes expression between the two isolates, and also between the 25 ºC and the 37 ºC for each isolate.

Dunnaevskii *et al*., 2006 found that the proteases of the saprotrophic and phytopathogenic fungi differ in physicochemical properties and that some proteases are only detected on phytopathogenic fungi. Generally, these proteases emerge after the detection of the others proteases. This observation was explained by the specificity of different proteases.

Primarily, the expression of proteases less specific to the type of cleaved bonds occurs, attacking a range of peptide bonds, disrupting the cell wall of the host. Thus, is possible that the late appearance of the others proteases is due to its specific role in phytopathogenesis, which shows up after the cell wall of the host has been disrupted by other hydrolytic enzymes[80].

3.2.3 – SDS-PAGE

The plant-fungus interaction depends on mutual recognition. Considering phytopathogenic fungi this interaction depends on the expression of pathogenicity and virulence factors by the fungus, while in the plant it depends on the existence of passive, preformed, or inducible defence mechanisms. A number of fungal mechanisms and molecules have been shown to contribute to fungal pathogenicity or virulence, including cell wall degrading proteins, inhibitory proteins and enzymes involved in the toxin synthesis[1].

The secretome of both isolates from *L. theobromae* grown at 25 ºC, 30 ºC and 37 ºC (Figure 14) was analysed by SDS-PAGE.

Quantity One (Bio-Rad) was the software used to estimate the molecular mass of proteins and their optical densities. The apparent molecular weight of the proteins was determined using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standard, from Bio-Rad).

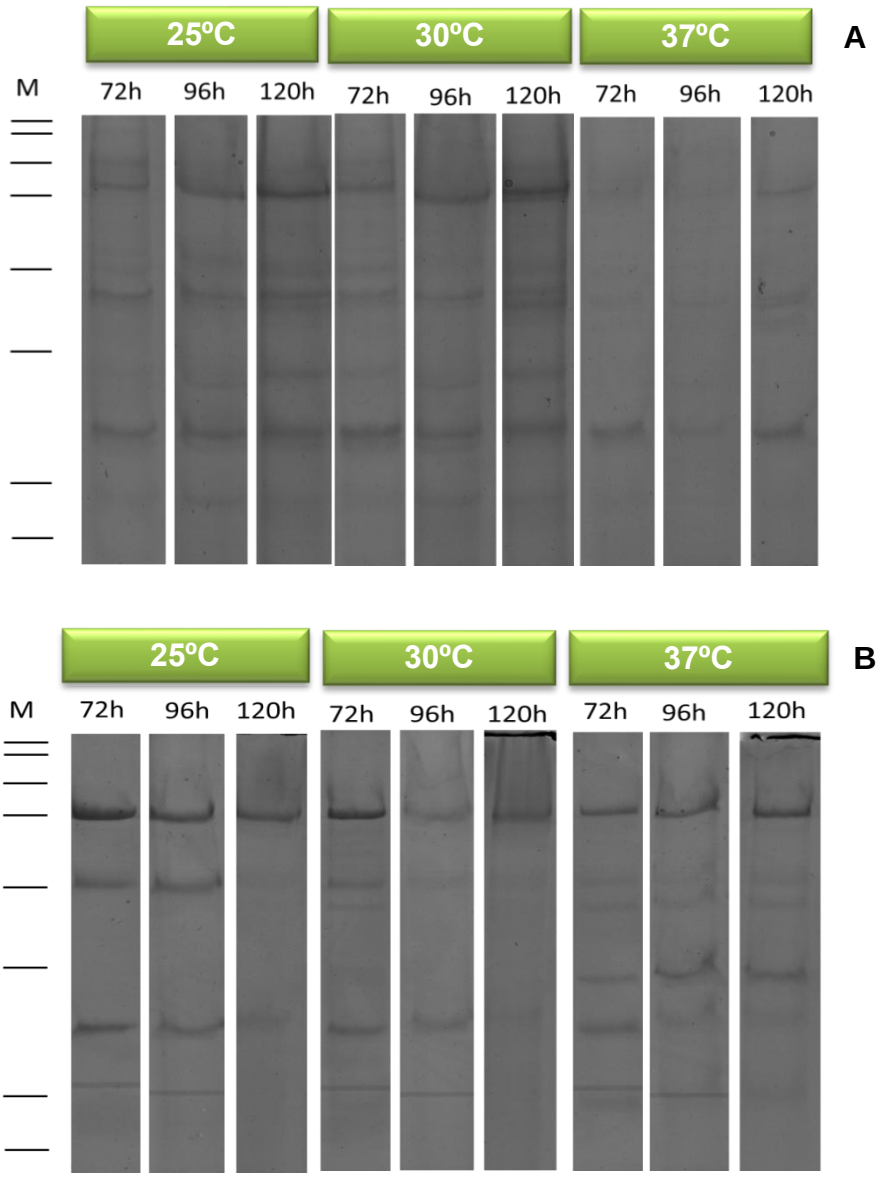


Figure 14: SDS-PAGE of extracellular samples of CAA019 (A) and CBS339.90 (B) along 120 hours of incubation, at 25 ºC, 30 ºC and 37 ºC. M - Marker. Gel is representative of three analyses.

Table I presents the molecular weight of proteins present in the environmental isolate after 72h, 96h and 120h of incubation.

A total of 12 proteins were found; 4 of them were present in all conditions. Proteins with apparent molecular weights of 136.2, 112.5, 83.1, 70.6 and 49.5 kDa were only present at 25 ºC. Proteins with 54.4 and 31.6 kDa were only detected at 30 ºC and 37 ºC. A protein with an apparent molecular weight of 90.7 kDa was only expressed at 37 ºC.

Table I: Apparent molecular weights of extracellular proteins produced by isolate CAA019, grown for 120h, detected by SDS-PAGE.

CAA019	**25 ºC**			**30 ºC**			**37 ºC**		
Molecular weight (kDa)	72h	96h	120h	72h	96h	120h	72h	96h	120h
136.2	+	+	-	-	-	-	-	-	-
112.5	+	+	+	-	-	-	-	-	-
90.7	-	-	-	-	-	-	+	+	-
83.1	+	+	+	-	-	-	-	-	-
78.2	+	+	+	+	+	+	+	+	+
70.6	+	+	+	-	-	-	-	-	-
54.4	-	-	-	+	+	-	+	+	-
49.5	+	+	-	-	-	-	-	-	-
45	+	+	+	+	+	+	+	+	+
37.2	+	+	+	+	+	+	+	+	+
31.6	-	-	-	+	+	+	+	+	+
27.3	+	+	+	+	+	+	+	+	+

Table II presents the apparent molecular weights of extracellular proteins produced by the clinical isolate after 72h, 96h and 120h of incubation.

A total of 11 proteins were detected; from these only one was present in all conditions. Proteins with apparent molecular weights of 54.1 and 25.7, kDa were expressed at 25 ºC and 30 ºC and with 71.4 and 37.6 kDa only at 25 ºC. Two proteins with apparent molecular weights of 102.7 and 44.9 kDa were only expressed at 37 ºC.

Table II: Apparent molecular weights of extracellular proteins produced by isolate CBS339.90, grown for 120h, detected by SDS-PAGE.

CBS339.90	**25 ºC**			**30 ºC**			**37 ºC**		
Molecular weight (kDa)	72h	96h	120h	72h	96h	120h	72h	96h	120h
102.7	-	-	-	-	-	-	+	+	+
78.1	+	+	+	+	+	+	+	+	+
71.4	-	-	+	-	-	-	-	-	-
54.1	+	+	+	+	+	+	-	-	-
49.5	-	-	+	-	-	-	-	-	-
48.6	-	+	+	-	-	+	+	+	+
44.9	-	-	-	-	-	-	+	+	+
37.6	-	-	+	-	-	-	-	-	-
36.9	-	+	+	-	-	+	+	+	+
31.8	+	+	+	+	+	+	+	+	+
25.7	-	+	+	-	-	+	-	-	-

The results suggest that for the environmental isolate, CAA019, there is the loss of expressed proteins, when the fungus is grown at 37 ºC, as well as the decrease of the intensity of the proteins present (table I, figure 14A). Contrariwise, the clinical isolate seems to over express extracellular proteins, seen by an increase of band intensity (figure 14B). The isolates apparently express extracellular proteins with different molecular weights, indicating that in similar conditions, these isolates have distinct protein profiles and therefore different interactions with the environment where they are inserted.

The capacity to infect humans by *L. theobromae*, that has been recently described[57], may be related with the climate changes that certainly have impact on the dynamic of host/pathogen interactions[41].

Being proteomics a tool to identify unknown mechanisms underlying environmental alterations, analysis the secretome of these two isolates becomes relevant to understand the differential expression of proteins under different conditions in an attempt to relate them with fitness and pathogenicity mechanisms.

3.2.4 - Cytotoxicity

Once CBS339.90 was isolated from an infection site in humans, it was relevant to verify if both isolates have the ability to induce cytophatic effects on animal cells, and if this ability is related with growth temperature of the fungus.

In order to analyse the effect of the extracellular metabolites produced by *L. theobromae* in Vero cells, extracellular extracts of the isolates CAA019 e CBS339.90 incubated at the temperatures of 25 ºC, 30 ºC and 37 ºC were used in cytotoxicity assays. For each extract, four doses were used [1:1, 1:4, 1:8 and 1:16 (v:v)], in which the initial amount of extract were normalized to the mycelium dry weight.

The extracts that induced cytopathic effect at least up to 1:16 dilution in 50 % or more cells were recorded as a cytotoxic as previously described[81, 82].

For the temperature of 25 ºC (figure 15-A), a cytotoxic effect was detected after 96 hours of incubation of the isolate CAA019. For the clinical isolate, cytotoxic effect was detected mainly after a period of incubation of 120 hours (figure 15-B).

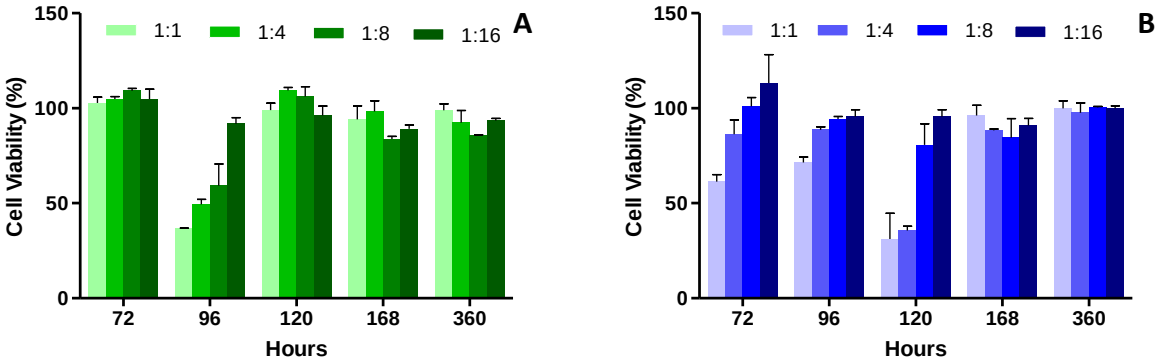


Figure 15: Evaluation of verotoxicity: extracellular fraction of *L. theobromae*, CAA019 (A) and CBS339.90 (B), along 360 hours of incubation at 25 ºC. Data is presented as mean ± standard error of two independent experiments performed in triplicate.

An alteration of temperature of incubation of *L. theobromae* for 30ºC didn't induce any major alterations of the profile obtained for the isolate grown at 25 ºC. However, the clinical isolate seems to induce a higher Vero cytotoxicity at this temperature, inducing a decrease of cell viability after 72 h, 96 h, 120 h and 168 h of fungus growth. The highest cytotoxicity effects were detected after 120 h and 168 h of growth, reaching values of approximately 90% of cell mortality.

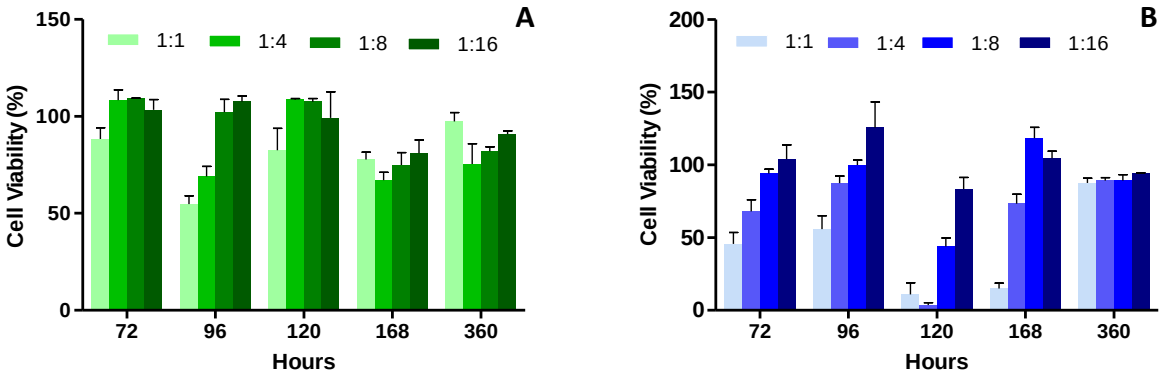


Figure 16: Evaluation of verotoxicity: extracellular fraction of *L. theobromae*, CAA019 (A) and CBS339.90 (B), along 360 hours of incubation at 30 ºC. Data is presented as mean ± standard error of two independent experiments performed in triplicate.

Inversely, the cytotoxicity of the environmental isolate secretome grown at 37 ºC was not significant (figure 17A). Cell viability was close to 100% for every incubation periods,

suggesting that this isolate, at 37ºC, doesn´t produce any cytotoxic compounds for these cells.

For the clinical isolate (figure 17B) the opposite occurs: there is production of cytotoxic metabolites after 96h growth. After 120 hours of growth, the viability loss is most accentuated, reaching values of cell viability of 10%.

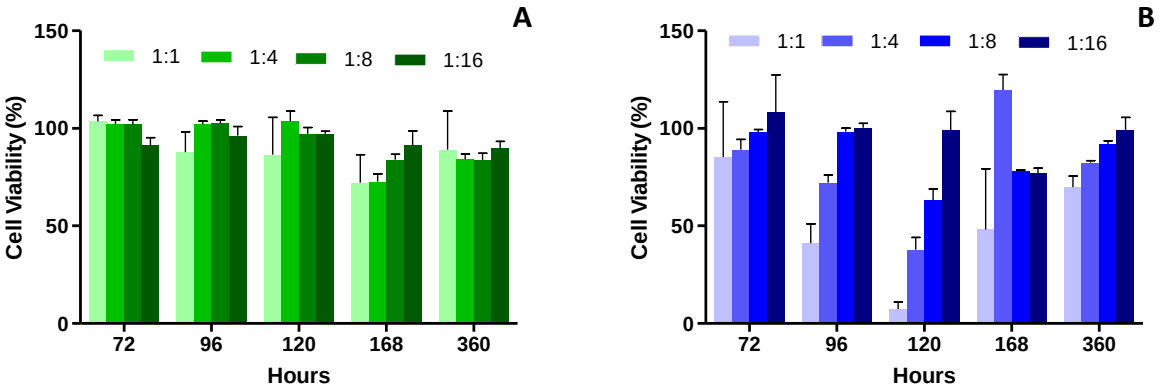


Figure 17: Evaluation of verotoxicity: extracellular fraction of *L. theobromae*, CAA019 (A) and CBS339.90 (B), along 360 hours of incubation at 37 ºC. Data is presented as mean ± standard error of two independent experiments performed in triplicate.

Once *L. theobromae* is a phytopathogenic fungi that may also be an opportunistic pathogen of humans, the temperatures at which they infect their host (approximately 25 ºC and 37 ºC) are of paramount importance. Results obtained show that, globally, the environmental isolate has cytotoxicity only for the temperatures of 25 ºC and 30 ºC. In the other hand, the clinical isolate has cytotoxicity for the three temperatures, but with higher effects at 30 ºC and 37 ºC. The results suggest that different isolates express different molecules. Environmental parameters, such as temperature, have effect on the expression of secretome, which are distinct between the isolates.

4. CONCLUSIONS

This study allowed attaining several conclusions, concerning growth and extracellular protein expression of two isolates of *Lasiodiplodia theobromae*, isolated from different hosts (from a plant host, CAA019, and from human host, CBS339.90).

Concerning the growth characterisation of these isolates it was possible to conclude that from the several culture media studied, PDA medium showed to be the best cultivation medium for *L. theobromae*.

Furthermore, data suggest that both isolates are able to grow in a wide range of temperatures, being 25 ºC – 35 ºC the range where a highest development was registered.

The presence of continuous light during *L. theobromae* development seems to favour the radial growth of *L. theobromae*, mostly at the temperature of 37 ºC, with growth rates of fungi deprived of light nearly half of those obtained for the continuous light growth.

It is noteworthy that fungal biomass does not increase continuously during growth: after a maximum mycelium growth between 96 hours and 120 hours, fungus starts degenerating with biomass loss.

The different growth rates detected were accompanied by differential expression of extracellular proteins. Both isolates express extracellular proteases at 25 ºC, 30 ºC and 37 ºC. It was possible to detect several proteases in both isolates but, there were different protein expressions in similar growth conditions of CAA019 and CBS339.90.

Data showed that temperature induces the expression of different proteases by both isolates.

The extracellular proteome of *L. theobromae* grown at 25 ºC, 30 ºC and 37 ºC for 120 hours of incubation shows that there is a differential protein expression that is time and temperature dependent. In the case of the environmental isolate, there seems to be a higher protein expression for the temperatures of 25 ºC and 30 ºC. Contrary, the clinical isolate presented a higher intensity of the bands for the temperature of 37 ºC, suggesting that at this temperature there is a higher protein expression. This evidence might be related to the fact that environmental changes on the habitat of this fungus may promote different expression of proteins for their adaptation. A higher protein secretion by the clinical isolate at 37ºC, opposed to the environmental isolate, suggests an adaptation of these isolates to their host's environment.

The environmental isolate secretome is cytopathic mostly when the fungus is grown at 25 ºC. At 30 ºC and 37 ºC, the extracellular extracts of this fungus do not induce damages to Vero cells.

However, the clinical isolate presented cytotoxicity during most of the study period, as well as for the three temperatures, mostly at 30 ºC and 37 ºC. After a period of 120 hours, at 37 ºC cells viability reached its minimum – 10%. Thus, the results show that the clinical isolate is more toxic for this cells line than the environmental isolate, being able to induce cell viability losses of around 90%, which, again, in accordance with our previous suggestion that there seems to be an adaptation of each isolate to its host.

5. FUTURE PROSPECTS

In order to try to relate the protein differential expression under different stress conditions with the virulence mechanisms of *Lasiodiplodia theobromae* it is essential to identify the proteins involved in these processes. With this information it will be possible to relate the activity of determined secreted proteases with the presence of certain proteins in the secretome and relate them with fungal virulence as well as to fungal fitness.

The characterisation of intracellular proteins under the same conditions of the characterisation of secretome can also offer relevant information about the behaviour of fungus.

Virulence assays, using a plant host, could be the next step to provide results closer to reality, being possible to make a comparison with the results obtained so far.

Lasiodiplodia theobromae is a phytopathogenic fungus responsible for a countless number of diseases in various plants, having and enormous impact on plant production worldwide. It has also been described as an opportunist in humans. Thus, knowledge of the pathogenic cycle and virulence factors is crucial for the design of effective crop protection strategies and effective treatments to humans' infections[1, 52].

Due to the relevance of this species as a phytopathogenic, as well as its increasing relevance as an opportunist pathogen in humans, this study may reveal as being extremely relevant both to agriculture and to human health.

6. ANNEXES

ANNEX I

6.1 - Radial Growth

1 – Plate the appropriated solid culture medium in Petri dishes (90mm Ø).

2 – Inoculate a plug with approximately 8mm of an actively growing fungal culture at 1 cm from the border of the Petri dish.

3 – Incubate the inoculated dishes under the desired conditions.

4 – Measure the radium (cm) of the mycelium developed along the period of incubation.

6.2 - Biomass Growth

1 – Add 50mL of PDB culture medium to 250mL sterilized erlenmeyers.

2 – Inoculate two plugs with approximately 8mm of a fungal culture with 72 hours of incubation in each erlenmeyer with containing culture medium.

3 – Incubate at the desired conditions.

4 – Weight filter paper for each sample.

5 – After the period of incubation, filter the culture media.

6 – Dry the mycelium at 50ºC for 48 hours.

7 - Weight the filter paper with the dry mycelia; determine the weight of dry mycelium by difference.

6.3- Proteolytic Extracellular Activity Detection – Skim Milk

1 – Plate solid culture medium (Skim Milk) in Petri dishes (90mm Ø).

2 – Inoculate a plug of approximately 8mm of an actively growing fungal culture in the centre of the Petri dish.

3 – Incubate the inoculated dishes at the desired conditions.

4 – Measure the radius (cm) of halos after a 24 hours period of incubation.

6.4- Protein quantification - BCA method

Microplate Procedure

1 – Prepare the solutions for the standard curve:

Microtube	Volume of Diluent (µL)	BSA Volume	BSA final concentration (µg/mL)
A	0	150µL of Stock	2000
B	62.5	187.5µL of Stock	1500
C	162.5	162.5µL of Stock	1000
D	87.5	87.5µL of dilution B	750
E	162.5	162.5µL of dilution C	500
F	162.5	162.5µL of dilution E	250
G	162.5	162.5µL of dilution F	125
H	200	50µL of dilution G	25
I	200	0	0 (Blank)

2 – Prepare the BCA Working Reagent (200µL/sample replicate), mixing 50 parts of reagent A with 1 part of reagent B.

3 – Pipette 25µL of each BSA standard solution into a multiwell of 96 wells and 25µL of diluent to build the point zero of the standard curve.
4 – Pipette the respective volume of the samples, making up with diluent to 25µL.
5 – Add 200µL of BCA Working Reagent and agitate the plate for approximately 30 seconds.
6 – Cover the plate and incubate it at 37ºC for 30 minutes, letting it cool to environmental temperature afterwards.
7 – Read the absorbance at or near 562 nm on a microtiter spectrophotometer.
8 – Elaborate the standard curve for posterior determination of the concentration of proteins for every sample.

Microtube Test Procedure

1 – Prepare the solutions for the standard curve:

Microtube	**Diluent Volume (µL)**	**BSA Volume**	**BSA final concentration (µg/mL)**
A	350	50µL of Stock	250
B	200	200µL of dilution A	125
C	225	150µL of dilution B	50
D	200	200µL of dilution C	25
E	200	50µL of dilution D	5
F	200	0	0 (Blank)

2 – Prepare the BCA Working Reagent (1mL/sample replicate), mixing 50 parts of reagent A with 1 part of reagent B.

3 – Pipette 50µL of each BSA standard solution and unknown sample replicate into an appropriately labelled test tube.
4 – Add 1mL of BCA Working Reagent to each tube and mix well.
5 – Cover the plate and incubate it at 60ºC for 30 minutes, letting it cool to environmental temperature afterwards.
7 – Read the absorbance at or near 562 nm on a spectrophotometer, using a cuvette filled only with water to calibrate the instrument.
8 – Elaborate the standard curve for posterior determination of the concentration of proteins for every sample.

6.5- Protein quantification - 2D Quant Kit

1 - Prepare the standard curve:

Tube	Volume of 2mg/mL BSA standard solution(µL)	Protein Quantity (µg)
1	0	0
2	5	10
3	10	20
4	15	30
5	20	40
6	30	60

2 - Prepare the reaction solution Working Colour Reagent, (1mL/sample replicate), mixing 100 parts of reagent A with 1 part of reagent B.

1 - Homogenize the samples by inversion, distributing 5-50µL of each sample in 2ml tubes.

2 - Add 500µL of precipitant to each tube (samples + patterns). Homogenize in the vortex and incubate 2-3 minutes at the environmental temperature.

3 - Add 500µL of co-precipitant to each tube and homogenize quickly in the vortex.

4 - Centrifuge at 16000×g for 5 minutes, at 23 ºC.

5 - Decant immediately the supernatant, avoiding the dispersion of the pellet.

6 - Recentrifuge the tubes to discard the remaining supernatant, removing it with a micropipette.

7 - Add 100µL of copper solution and 400µL of ultrapure water to each tube. Homogenize in the vortex to dissolve the proteins precipitate.

8 - Add 1mL of Working Colour Reagent to each tube, homogenizing immediately by inversion.

9 - Incubate at the environmental temperature for 15-20 minutes.

10 - Read the absorbance at 480nm in disposable *cuvettes* within 40 minutes after the addition of the working solution.

11 - Elaborate the standard curve for posterior determination of the concentration of proteins for every sample.

6.6 - Zymography

Preparation of the polyacrylamide gels.

1 – Set up the glasses, using ultrapure water to verify if there are no leaks in the system.

Running Gel: 10%	
Ultrapure H_2O	1.67mL
Tris 1.5M pH 8.8	1.68mL
Gelatine 1%	250µL
SDS 10%	100µL
Acrylamide: Bisacrylamide 40%	1.25mL
Ammonium Persulfate 10%	50µL
TEMED	5µL

Stacking Gel: 4%	
Ultrapure H_2O	1.68mL
Tris 0.625M pH 6.8	625µL
SDS 10%	50µL
Acrylamide: Bisacrylamide 40%	244 µL
Ammonium Persulfate 10%	25µL
TEMED	1.25µL

2 – After homogenizing the separation gel, apply 4 mL to the glass plates;

3 - Covering the surface of the gel with isopropanol 50% (v/v) to avoid the contact between the gel with the atmospheric oxygen. Allow the gel to polymerize for at least 30 minutes;

4 – Once the polymerization is finished, pour the isopropanol and fill the remaining space with the concentration gel;

5 – Insert the comb carefully, avoiding the development of bubbles in the gel. Allow the gel to polymerize for at least 30 min;

6 – Put the gel in the electrophoretic chamber, filling it with running buffer.

Running the gels

7 – Add sample buffer to each sample (1:1 v/v), allow it to incubate for 10 minutes at room temperature;

8 – Add 2.5 µL of molecular marker (Precision plus protein standards, Bio-Rad) and 25 µL of sample in the wells;

9 – Start the electrophoretic run for 2 hours at 120V, at 4 ºC

10 – After electrophoresis, submerge the gel in Triton X-100 (0.25%) at room temperature for 30 minutes, in order to remove all the present SDS.

11 – Incubate the gel in reaction buffer for 14 hours, with agitation.

12 – Incubate the gel for about 90 minutes in a staining solution (Coomassie Brilliant Blue R-250).

13 – Destain the gel by using a destaining solution, until digested areas become visible.

14 – Scan the gel with a densitometer.

Running Buffer
Tris-Bicine 100mM
SDS 0.1%
Ultrapure H_2O

Sample Buffer
Tris 1.5M pH 8.8
SDS 10% (m/v)
Glycerol 20%
Ultrapure H_2O

Renaturation Buffer
Tris 50mM pH8.8
NaCl 5mM
$CaCl_2$ 10mM
$ZnCl_2$ 1µM
Ultrapure H_2O

Staining Solution
Coomassie Brilliant Blue 0.25% (w/v)
Acetic Acid 10% (v/v)
Ethanol 50% (v/v)
Ultrapure H_2O

Destaining Solution
Ethanol 25% (v/v)
Acetic Acid 5% (v/v)
Ultrapure H_2O

6.7- SDS-PAGE

Preparation of the polyacrylamide gels

1 – Set up the glasses, using ultrapure water to verify if there are no leaks in the system.

Running Gel: 15%	
Ultrapure H_2O	1.70mL
Tris 1.5M pH 8.8	1.675mL
SDS 10%	100µL
Acrylamide: Bisacrylamide 40%	1.50mL
Ammonium Persulfate 10%	50µL
TEMED	2.5µL

Stacking Gel: 4%	
Ultrapure H_2O	1.63mL
Tris 0.625M pH 6.8	625µL
SDS 10%	50µL
Acrylamide: Bisacrylamide 40%	244µL
Ammonium Persulfate 10%	25µL
TEMED	1.25µL

2 – After homogenizing the separation gel, apply 4.5mL to the glass plate;

3 - Covering the surface with isopropanol 50% (v/v) to avoid the contact between the gel with the atmospheric oxygen. Allow the gel to polymerize for at least 30 minutes;

4 – Once the polymerization is finished, pour the isopropanol and fill the remaining space with the concentration gel. Allow the gel to polymerize for at least 30 minutes;

5 – Insert the comb carefully, avoiding the development of bubbles in the gel.

6 – Put the gel in the electrophoretic chamber, filling it with running buffer.

Running the gels

7 – To denature the samples, add denaturing solution to the proteins solution, in a 1:1 ratio (v/v) and 2% of β-mercaptoethanol.

8 – Heat the samples to 100ºC, for 5 minutes, letting it cool at the environmental temperature afterwards.

8 – Add 2.5 µL of molecular marker (Precision plus protein standards, Bio-Rad) and 20 µL of sample in the wells using a Hamilton syringe.
9 – Start the electrophoretic run for 3 hours at 120V, at 4ºC.
10 – After electrophoresis, incubate the gel for about 90 minutes in a staining solution, Coomassie Brilliant Blue R-250.
13 – Destain the gel using a destaining solution, until the strains become visible.
14 – Scan the gel with a densitometer.

6.8- Evaluation of Cytotoxicity by the method of Resazurin

6.8.1 – Viable Cell Counts

1 - Remove about 100-200µL of cell suspension to a microtube.
2 - Add an equal volume of trypan blue solution and homogenize.
3 - Place a coverslip over the hematocytometer counting chamber.
4 - Add 5-10µL of cell suspension in the counting chamber and observe in the microscope with a 20x amplification.
5 - Count the number of viable cells (bright cells) and non-viable cells (blue cells). To obtain a higher precision of this numbers, several fields should be count.
6 - Calculate the concentration of the percentage of viable cells and non-viable.

6.8.2 - Resazurin-based Cytotoxicity Assay

1 – Add 50µL of successive dilutions of the samples (1:1; 1:4; 1:8; 1:16), all in triplicate.

2 – Use, also in triplicate, empty wells, wells only with cells, wells with physiological serum and wells with the fungus culture media, to serve as control.

3 – Incubate for 24 hours at 37ºC.

4 – After the period of 24 hours, remove the medium by aspiration and add 50µL of DMEM with 10% resazurin (0.1mg/mL).

5 – Incubate at 37ºC in 5% CO_2 until the resazurin's reduction reaction.

6 – Use a microtiter plate spectrophotometer (Infinite 200, Tecan i-control) to read the absorbance at the wavelengths of 570nm and 600nm.

7 – Calculate the cell viability.

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