Infection of *Cryphonectria parasitica* by CHV1 hypovirus - effects on pathogenesis-related peroxidases and hydrolases

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Abstract

The fungus *Cryphonectria parasitica* (Murrill) Barr. is the causal agent of *Castanea sativa* chestnut blight, a disease that causes important economic losses in chestnut orchards along all world. In hypovirulent strains of *C. parasitica*, the hypovirulence is due to the presence of a virus, the *Cryphonectria hypovirus* 1 (CHV1), that attenuates pathogenicity by reducing the sporulation, male fertility, and the enzymatic activity of pathogenesis-related enzymes.

In the northeast region of Portugal, and in other regions of Europe affected by *C. parasitica*, the release of *C. parasitica* hypovirulent strains on chestnut blight affected trees of *C. sativa*, has been used in the field as a biological control.

The objective of this work is to evaluate and compare the effect of hypovirulence by CHV1 on the activity of pathogenesis-related enzymes (hydrolases and peroxidases), in different isolates of *C. parasitica.* For this, several virulent and hypovirulent (donors and converted) isolates were grown in appropriate microbiological media, and some pathogenesis-related enzymes activity was qualitatively and quantitatively evaluated.

Keywords: *Cryphonectria parasitica*, Chestnut Blight, Hypovirulence, *Cryphonectria hypovirus* 1 (CHV1), pathogenesis-related enzymes

Resumo

Cryphonectria parasitica (Murrill) Barr. é o fungo que provoca a doença do cancro no castanheiro (*Castanea sativa*), associada a perdas económicas importantes em plantas de castanheiro em todo o mundo. Nas estirpes hipovirulentas de *C. parasitica*, a hipovirulência a devida à presença de um vírus, o *Cryphonectria hypovirus* 1 (CHV1), que lhes atenua a patogenicidade, causando uma diminuição da esporulação, da fertilidade masculina e de actividade enzimática em enzimas relacionadas com o processo de patogénese.

Na região nordeste de Portugal, e noutras regiões da Europa afectadas por *C. parasitica*, o tratamento por aplicação de estirpes hipovirulentas de *C. parasitica* em árvores de *C. sativa* afectadas pelo cancro tem sido usada no como meio de controlo biológico.

O objectivo deste trabalho é avaliar e comparar o efeito da hipovirulência por CHV1 na actividade de algumas enzimas relacionadas com o processo de patogénese (hidrolases and peroxidases), em diferentes isolados de *C. parasitica.* Para isso, fez-se o crescimento de alguns isolados virulentos e hipovirulentos (dadores e convertidos) em meios microbiológicos adequados, e avaliou-se qualitativamente e quantitivamente a actividade enzimática dessas enzimas associadas ao processo de patogénese em fungos.

Palavras-chave: *Cryphonectria parasitica*, Cancro do castanheiro, Hipovirulência, *Cryphonectria hypovirus* 1 (CHV1), Enzimas relacionadas com patogénese

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Abbreviations

CHV 1: *Cryphonectria hypovirus* 1

MAT: mating locus

CWDE: Cell wall degrading enzymes

LiP: Lignin peroxidase

MnP: Manganese peroxidase

Lac: Laccase

OAH: Oxaloacetate acetylhydrolase

VC: Vegetative compatibility

PDA: Potato Dextrose Agar

PDB: Potato Dextrose Broth

UV: Ultraviolet

I. Introduction

Chestnut trees belong to the large family Fagaceae, which also includes oaks (*Quercus* spp.) and beeches (*Fagus sylvatica*) (**Rigling & Prospero, 2017**). In the genus *Castanea* there are four main species, the American chestnut (*C. dentata*), the European chestnut (*C. sativa* Mill.), the Chinese chestnut *(C. mollissima*) and the Japanese chestnut (*C. crenata*).

Throughout the years, chestnut trees were susceptible to a lot of infecting diseases such as *Phytophthora* root rot, or nut rots. However, the most common disease infecting chestnut trees is the chestnut blight, caused by *Cryphonectria parasitica*.

C. parasitica, originally from Asia, is a non-native fungus that spread to other continents via infected chestnut plants (**Rigling & Prospero, 2017**). This ascomycete infects the bark and cambium of chestnut trees through wounds and causes bark canker, which can lead to death of distal parts after coring of branches or the entire tree trunk (**Heiniger & Rigling, 1994**). Originally spread from Asia in the 20th century,, reaching almost all chestnut growing areas, caused the degeneration of all the chestnut trees along the forward front. (**Krstin et al., 2017**). The impact of the chestnut blight was different on the various host trees, it was noted that there were different susceptibility between host trees species. The American and the European chestnut *(C. dentata and C. sativa)* were the most susceptible compared to the Japanese and the Chinese chestnut. The disease propagated rapidly in these species manifesting its symptoms of necrosis of the bark and increase of mortality of the distal part of the tree (**Heiniger & Rigling, 1994**).

In many regions in Europe symptoms of healing cankers have been noted, characterized by a reddish-orange surface with swelling (**EPPO, 2005**). Accordingly, it appeared to be an early stage of the emergence of the hyper-parasitic virus; *Cryphonectria hypovirus* 1 (CHV1), which originates from Asia (**Feau et al., 2014**). This mycovirus appeared to have the ability to reduce the virulent form of the blight by decreasing the sporulation capacity and the parasitic growth of the pathogen (**Rigling & Prospero, 2017**), thus acting as a biological control of the disease.

The aim of this work is to evaluate how the enzymatic activity that facilitates the invasion of the host tissues by *C. parasitica* are affected by the presence of CHV1

For this purpose, different strains (virulent and hypovirulent) were evaluated quantitatively and qualitatively for different biological tests and cultivation in minimal liquid media, as facilitating materials; we used spectrophotometry UV-Vis Spectrophotometer and colored indicator compounds for determination of different enzymatic activities.

II. Literature Review

In this literature review, the strains of *C. parasitica* (virulent and hypovirulent), the manifestations of chestnut blight, the mode of action of *C. hypovirus 1* (CHV1) and the specificity of the interaction between the infection process and pathogenesis, as well as the associated enzymes such as Manganese Peroxidase (MnP) and Cellulase are discussed in detail.

1.*Cryphonectria parasitica*

1.1 Taxonomy:

C. parasitica (Murr.) Barr. is a Sordariomycete (ascomycete) in the family Cryphonectriaceae (Order Diaporthales). Closely related species that can be found on chestnut trees include *C. radicalis*, *C. naterciae* and *C. japonica*. (**Rigling & Prospero, 2017**)

1.2 Host Species

C. parasitica is most important as a pathogen of *Castanea* and *Quercus* species, though it may be found as saprophyte or a weak pathogen on other species.

Asian chestnut species, *C. mollissima* (Chinese chestnut) and *C. crenata* (Japanese chestnut) are blight resistant, however they can develop severe diseases such as (leaf spot and twig cankers)~~;~~ *C. seguinii* and *C. henryi*, from China, are also hosts; *C. pumila*, from eastern USA, and chinquapin can also be affected directly or indirectly by this fungal infection.

Oaks such as *Q. virginiana* and *Q. stellata* are the only oaks in North America to be seriously affected by *C. parasitica*, and some trees may be killed; some species, like *Q. coccinea* (scarlet oak) can be severely damaged **(Fullbright et al., 1983).**

1.3 Disease symptomology

C. parasitica is a fungal pathogen affecting above-ground tree parts such as stems, branches and twigs; causes bark cankers on trunks, leading to tree death within a short time **(Gouveia et al., 2016)**. Preferentially entering through wounds (cuts, incisions, scars), developing under the bark of the tree, and causing the destruction of the cambium. The first symptoms begin with the appearance of lesions or cankers on the trunk and branches. When the lesion reaches the entire circumference of the trunk or branch, strangulation of the same occurs, so that the sap cannot circulate and the part of the branch above the lesion withers and dries up. In young branches the most obvious symptom is the presence of reddish spots, contrasting with the olive-green color of the normal bark. As the fungus develops subcortically, longitudinal splitting of the bark occurs. Below the infested zone, adventitious fruit bursting may occur, leaving the root system and zone below the canker alive **(Rigling & Prospero, 2018)**. The fungus grows in the cambium and forms yellowish-brownish mycelial fans in the bark, resulting in plant bark cankers. The disease is easily recognized by the dry leaves that remain on the branches **(Gouveia et al., 2010).** (Figure 1a and 1b).

The process of infection with *C. parasitica* begins with the penetration of spores (conidia or ascospores) into chestnut bark through natural openings or wounds **(Hebard et al. 1984; Aguín et al., 2011)**. Later, *C. parasitica* can produce spores (pycnidia and/or peritheca) on infected bark as well as on freshly killed chestnut bark. The formation of wound peridermis is continuously inhibited because the advancement of mycelial lovers kills the host cells via pollutants and enzymes that degrade the cellular wall.

Figure 1) a) Presence of reddish spots on a branch. (Adapted from Gouveia et Moura, 2019). b) Dry leaves on branch. (Adapted from Gouveia et Moura, 2019).

1.4 Life Cycle and reproduction

Ascospores or conidia can initiate infection. *C. parasitica* mating is regulated by a single type of mating locus (MAT) containing either the MAT-1 or the MAT-2 allele **(Marra and Milgroom, 2001)**. Acting as a male gamete, a conidium fertilizes a receptive hypha of another mating type. These conidia are formed by mitosis and are genetically like the mycelium. Asexual spores are generally dispersed throughout insects, birds or by rain **(Rigling and Prospero, 2017**). *C. parasitica* also possesses a mixed mating system that combines self-fertilization and out-crossing that occurs at different frequencies **(Marra et al., 2004)**. Most self-fertile isolates are heterokaryotic, Biparental inbreeding or parasexual recombination via mitotic crossing over produce heterokaryons **(Milgroom et al., 2008).**

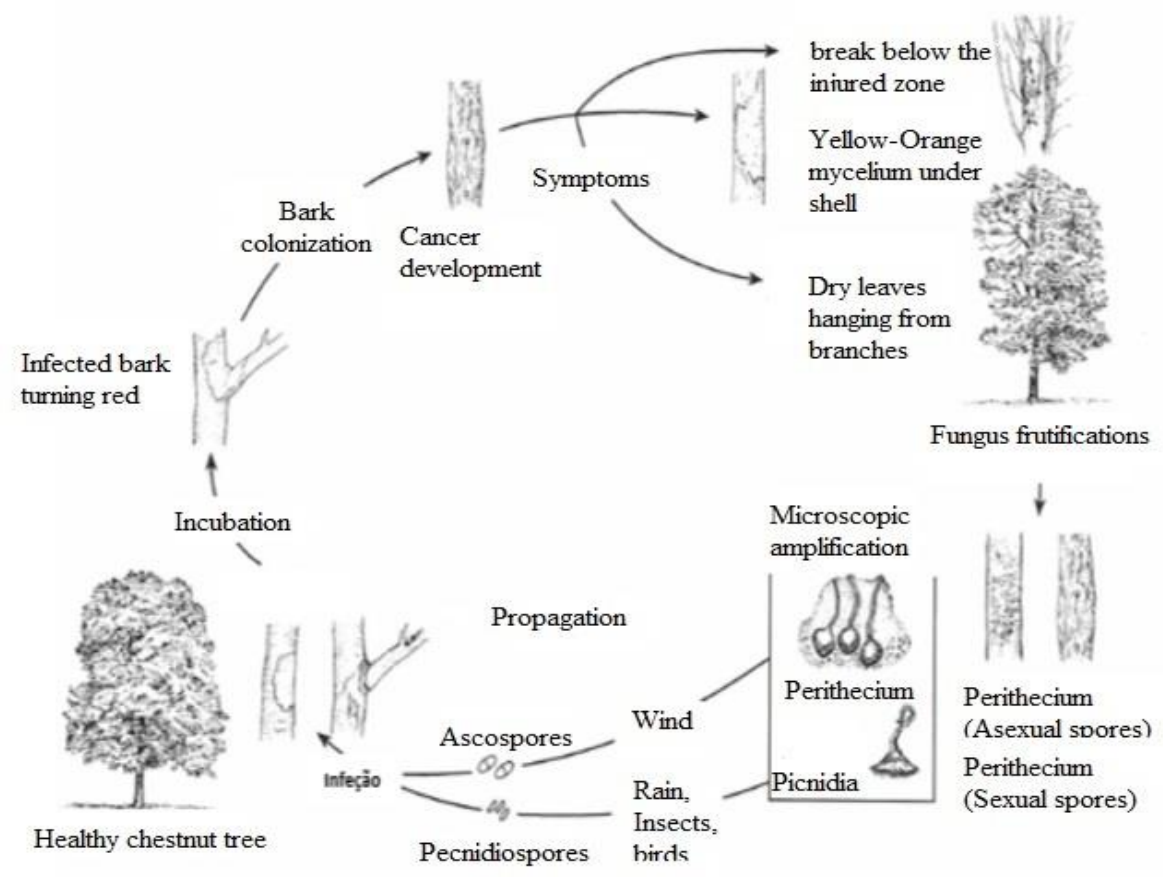


Figure 2) C. parasitica life cycle. (Adapted from Gouveia et al., 2019)

1.5 Origin and dispersion of the disease

Chestnut canker, caused by *C. parasitica*, was first discovered in the United States in 1904 and has spread rapidly throughout the country, destroying six natural habitats of the American chestnut and 3.5 billion chestnut trees in fifty years **(Anagnostakis, 2001)**. *C. parasitica* is a fungus of the natural microflora of Asian chestnut trees *(C. crenata, C. mollissima)* **(Gouveia et al., 2005)**, which are therefore more resistant to the disease due to coexistence and common evolution that allowed Asian trees to acquire a natural tolerance to this pathogen.

1.6 Infection process and virulence factors

Histological methods used to study the infection process during canker development showed a formation of mycelial fans that expands intercellularly in the bark and cambium to split up the host cells, they inhibit a wound epidermal formation by killing the host cells in susceptible host trees species by means of toxins and cell wall-degrading enzymes **(Roane et al., 1986).**
In the advancing stages of the infection, *C. parasitica* tend to produce oxalic acid which is a metabolite that has a toxin effect on host cells and enhance cell wall degradation **(Havir & Anagnostakis, 1983).**

1.7 Vegetative Incompatibility (*vic*)

Vegetative incompatibility (*vic*) is a commonly defense mechanism amongst fungi that blocks the transmission of diseases plasmically by preventing the formation of hyphal fusion and cytoplasmic exchange between organisms **(Paoletti, 2016),** thus restricting the horizontal transmission of virulence-attenuating mycoviruses between fungal individuals **(Anagnostakis, 1977).** Currently, identification of six unlinked *vic* loci with two alleles were established **(Cortesi & Milgroom, 1998).** These six di-allelic *vic* loci define 64 *vic* genotypes, which means 64 different *vic* types. Two isolates of *C. parasitica* are vegetatively compatible (meaning they share the same allele at all *vic* loci), when after being co-cultured on the same agar plate, the two cultures combine to form a single culture. Else, if a barrier line is formed

between the two cultures, this suggests an incompatibility and the beginning of a cell death (apoptosis) (Figure 3).

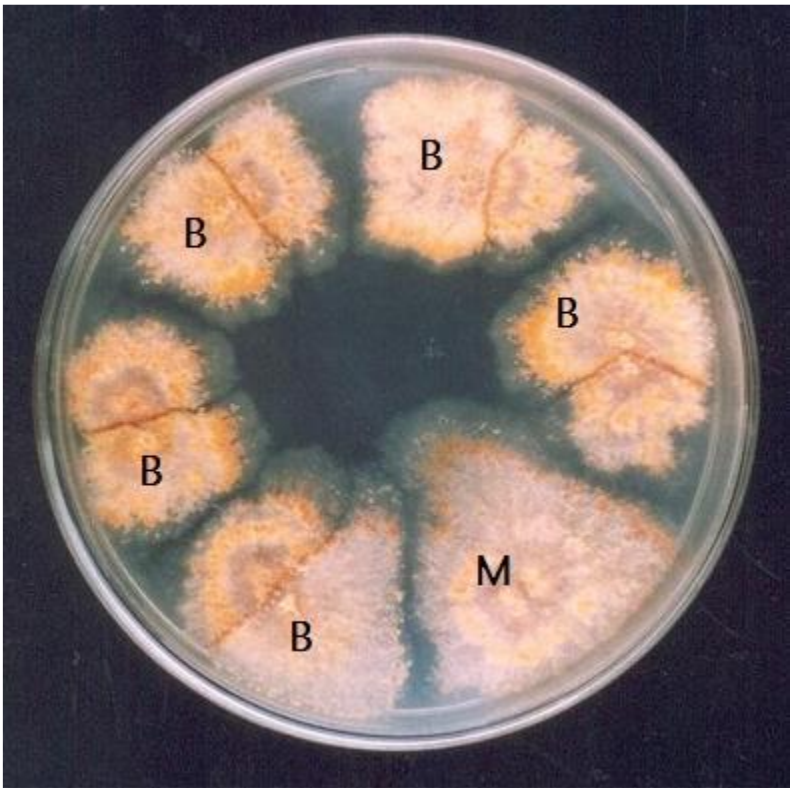


Figure 3) M) Vegetative compatibility B) Vegetative Incompatibility. (Adapted from Rigling & Prospero, 2017).

1.8 Enzymes related to virulence in *Cryphonectria parasitica*

In phytopathogenic fungi belonging to different subphyla of Ascomycetes, like Aspergillus niger and Botrytis cinerea, the main route of oxalic acid production is the hydrolytic cleavage of oxaloacetate catalyzed by oxaloacetate acetyl hydrolase (OAH) (**Han et al., 2007**). OAH activity in each fungal species functions as an important niche adaptation, due to oxalate production, that is known to be associated with fungal pathogenesis. In Sclerotinia sclerotium, another important phytopathogenic fungus, mutants deficient in oxalate synthesis are no longer pathogenic (**Godoy, 2006**). In C. parasitica isolates infected by CHV1, it is known that the production of oxalic acid decreases (**Havir & Anagnostakis, 1983**), which is one of the factors that may be related to attenuation of virulence.

Besides acidification, that initiate ligninocellulose degradation and weaken host tissues, C. parasitica OAH induces crystallization of calcium oxalate **(Chen et al., 2010**). Oxalic acid production triggers calcium and pectin depletion from host cell walls, usually from three to five cell layers in advance of the mycelium (**Dutton and Evans, 1996**). Oxalic acid is also known to be related with MnP activity, in lignin degradation, allowing a Mn (III)-oxalate complex that acts as a diffusible mediator in the oxidation of lignin (Wariishi et al., 1992). Knocking out of *C. parasitica* OAH, affecting oxalic acid production, has confirmed the role of oxalic acid in pathogenesis (**Chen et al., 2010**).

According to **Rigling and Alfen (1991)** and **Kim et al. (1995),** it is believed that the extracellular C. parasitica Lac1, is responsible for the detoxification of tannins in chestnut bark, thus facilitating the host tissues invasion.

Also *C. parasitica* Lac3 another extracellular laccase induced by tannic acid has been related to pathogenesis (**Chung et al., 2008**).

The cellobiohydrolase Chb1 is an exoglucanase codified by *C. parasitica* chb1 was also related to pathogenic activity. Chb1 transcript accumulates in virulent isolates and is suppressed in isogenic hypovirus-infected strain (**Wang and Nuss, 1995**).

2. *Cryphonectria hypovirus* (CHV1)

2.1 Hypovirulence

Attenuating the virulence of a fungal pathogen by a virus is known as hypovirulence **(Zamora et al., 2015**). Transmissible hypovirulence causes an infection that affects the population of the fungus responsible for chestnut cancer. It was observed in several regions of Europe after 1951 and occurred due to infection of *C. parasitica* by *Cryphonetria* hypovirus 1 that attenuates the fungus's virulence **(Anagnostakis et al., 1998; Allemann et al., 1999)**.

Double-stranded RNA hypoviruses reside in the cytoplasm of fungi and reduce pathogen virulence by inhibiting sexual reproduction and reducing mycelial growth and conidia production **(Kolp et al., 2018)**. Hypovirus spread is directly related to vegetative compatibility groups (VC types). Hypovirus transmission occurs horizontally by anastomosis of hyphae into

compatible strains, which causes the virulent strain to lose its pathogenic properties and allows the tree's natural defenses to act. When a virulent strain assumes a hypovirulent character, some morphological characteristics changes. For example, instead of an orange coloration, the colony presents a white one **(Gouveia et al., 2010)**.

Hypovirulent strains are difficult to detect in the field because they cause only superficial cankers that do not damage the tree **(Heiniger & Rigling, 1994)**. Regression of chestnut canker leads to the formation of scar tissue, that resists the progression of the fungus and isolates the infected part, which then dries out and falls off, producing new tissue **(Anagnostakis et al., 1986; Macdonald & Fulbright, 1991)**. The success of hypovirulence depends on the diversity of vegetative compatibility groups present in the chestnut tree and the mode of reproduction of the fungus **(Gouveia et al., 2010).**

2.2 Viral dsRNA, causal agent of Hypovirulence

Similarity in dsRNA size of converted strains has been found in several European countries, such as France **(Day et al., 1977)**, Italy **(Day et al., 1977, Grente and Sauret, 1969)**, Switzerland **(Rigling et al., 1989**), Croatia **(Rigling, 1989)** and Greece **(Xenopoulus, 1988)**. The experiments showed that French and Italian strains were similar **(Hillman et al., 1992, Hostis et al., 1985)**. In addition, experiments with the French hypovirulent strains have been conducted and studied in more detail **(Nuss, 1992).** They indicate the presence of a large dsRNA (L-dsRNA) containing two continuous coding domains (ORFA and ORFB) and several defective interference segments **(Shapira et al., 1991).** The genetic structuring and expression, as well as the technique of L-dsRNA replication clearly indicate a viral origin of the dsRNA **(Shapira et al., 1991; Fahima et al., 1993; Choi et al., 1991)**. This dsRNA is not encapsulated and is bound to vesicles of the fungal membrane **(Hansen et al., 1985, Newhouse et al., 1990).**

2.3 Molecular basis of Hypovirulence

The manifestation of mycelial fans, an important indicator of the fungus infection, is reduced in hypovirulent strains **(Hebard et al., 1984)**. Molecular analysis showed that the dsRNA reduced the accumulation of certain mRNAs and polypeptides, such as metabolic oxalate,

extra- and intracellular laccase **(Rigling et al., 1989)**, cell-surface protein **(Calza, 1993)**, cutinase **(Varley et al. 1992)**, and a putative-mating type pheromone **(Zhang et al., 1993).** Molecular analysis indicates the reduction of pigmentation, sporulation, and laccase induction by a secretion of a specific viral coding domain (ORFA) **(Choi and Nuss, 1992).**

3. Cell wall degrading oxidative enzymes

3.1 Plant cell wall composition

The plants' lignocellulosic substance consists of primary elements, namely cellulose, lignin, hemicellulose, and pectin:

- Cellulose, the most abundant component of plant material, is a linear biopolymer composed of anhydro glucopyranose molecules (glucose) linked by -1,4- glycosidic bonds. The parallel alignment of crystalline structures known as microfibrils results from the coupling of adjacent cellulose chains via hydrogen bonds, hydrophobic interactions, and Van der Waals forces (**Dashtban et al., 2010**).
- • Hemicelluloses are heterogeneous polymers composed of pentoses (such as xylose and arabinose), hexoses (primarily mannose, with less glucose and galactose), and sugar acids. The composition of hemicelluloses varies greatly depending on the plant source (**Badal, 2000**).
- • Lignin, a polymer composed of lignocellulosic residues. It typically contains three aromatic alcohol precursors: coniferyl alcohol, sinapyl, and p-coumaryl (**Wei et al., 2009**).
- Pectin is a carbohydrate polymer of high molecular weight that is present in virtually all plants where it contributes to the cell structure.

3.2 CWDE activity

The Carbohydrate-Active Enzyme (CAZy) database is a knowledge-based resource that focuses on enzymes that build and degrade complex carbohydrates and glycoconjugates.

Degrading enzymes include the glycoside hydrolase (GH) and polysaccharide lyase families. To open a break in the plant cell wall, phytopathogens and saprophytes secrete a wide range of Cell Wall Degrading Enzymes (CWDEs) for the degradation of plant polysaccharide materials, facilitating infection; the most common are glycoside hydrolase (GH) and carbohydrate esterase families (CEs).

3.2.1 Cellulolytic Activity

Cellulose is one of the major cell wall components in most plants. Cellulases catalyze the degradation of the ß-1,4- glycosidic bonds in cellulose. They have been divided into three types: endoglucanases, exoglucanases, and ß-glucosidases. Endoglucanases randomly hydrolyze internal glycosidic linkages, resulting in a rapid decrease in polymer length and a gradual increase in reducing sugar concentration. Exoglucanases hydrolyze cellulose chains by the removal of cellobiose from either the reducing or nonreducing ends, resulting in the rapid release of reducing sugars but little change in polymer length. Endoglucanases and exoglycanases act synergistically on cellulose to produce cellobiose, which is then cleaved by ß-glucosidase to glucose (**Beguin and Aubert, 1994**). The importance of cellulases to the pathogenicity of plant-pathogenic fungi is elusive. A few cellulases of plant-pathogenic fungi have been shown to be involved in pathogenicity (**Eshel et al., 2002; Muller et al., 1997**).

3.2.2 Hemicellulolytic Activity

Hemicellulose is a highly heterogeneous, branched polysaccharide composed of pentoses (including xylose and arabinose), hexoses (including mannose, glucose, and galactose), and acidic sugars (including galacturonic and glucuronic acid) (**Zhao et al., 2012**). Many hemicellulolytic enzymes are necessary for the successful degradation of hemicellulose due to its high complexity. Hemicellulose is predominantly hydrolyzed by glycosyl-hydrolases, unlike cellulose.

3.2.3 Pectinolytic activity

Pectin is an α-(1,4)-polysaccharide branched acid consisting predominantly of D-galacturonic acid and of varying amounts of other sugars, including L-rhamnose, L-arabinose, D-galactose, and D-xylose. Pectin is degraded by microbial glycosyl-hydrolases (i.e., endo-polygalacturonases and exo-polygalacturonases) and lyases (i.e., pectate lyases).

3.3 Oxidative enzymes

These enzymes can be classified as diphenol oxidases, such as Laccase (EC 1.10.3.2), or heme-peroxidases such as Manganese Peroxidase (EC 1.11.1.13),Lignin Peroxidase (EC 1.11.1.14) and Versatile Peroxidase (EC 1.11.1.16) **(Martinez et al., 2005)**.

3.3.1 Laccase (Lac) (EC 1.10.3.2)

A wide range of Enzyme uses are related to fungal growth and development, while others are related to interactions with the host plant (**Patel et al.,2020**).

Laccases is ρ-diphenol oxidases (systematic name – benzenediol: oxygen oxidoreductases), also known as blue oxidases, present in almost all fungi (**Kaczmarek et al., 2017**).

They are mainly dimeric or tetrameric glycoproteins, mostly occurring as isoenzymes, ranging from 50kDa to 100 kDa. Laccases are multicopper oxidases (MCOs) acting as oxidoreductases by reducing the molecular oxygen to two water molecules. They can oxidize organic and inorganic compounds, such as mono-, di, poly-. amino and methoxyphenols. It has been proposed that the major function of laccase in fungus interactions with the host plant is the enzymatic decomposition of plant-produced toxic metabolites that are hazardous to fungus **(Bolobova et al., 2002**). Laccase neutralizes the toxicity of these low molecular weight compounds in plants, rendering them non-toxic to fungal hyphae. Laccases are related to *C. parasitica* pathogenicity.

3.3.2 Manganese Peroxidase (MnP) (EC 1.11.1.13)

Manganese peroxidase belongs to the family of oxidoreductases, more specifically those that act on peroxide as acceptors (peroxidases) and is an extracellular heme protein that catalyzes the H_2O_2-dependent oxidation of lignin derivative-based polymers. MnP is a specific enzyme that can oxidize Mn^{2+} to $Mn^{3+,}$ which diffuses from the enzyme surface and in turn oxidizes the phenolic substrate, including lignin model compounds and some organic pollutants **(Xu et al., 2017**). In nature, MnP catalyzes the depolymerization of plant lignin as a component of the ligninolytic enzyme complex. Thus, it is one of the most abundant enzymes for lignin degradation and has great potential applications in the field of agriculture for the degradation of lignin, etc.

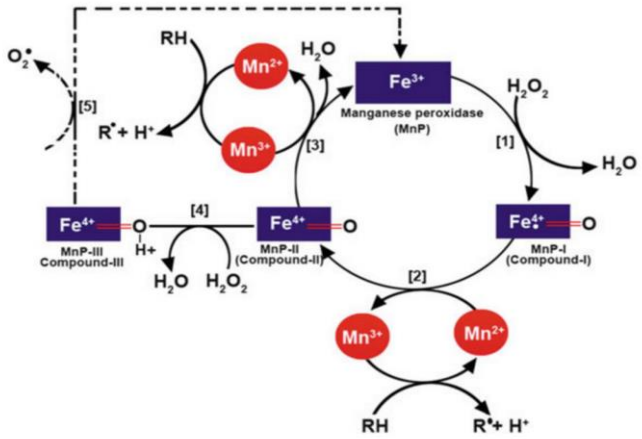


Figure 4) Catalytic cycle of manganese peroxidase
(adapted from Chandra et al., 2017)

3.3.3 Lignin Peroxidase (LiP) (EC 1.11.1.14)

LiP can attack lignin polymers. LiPs are known to oxidize various phenolic aromatic compounds and several non-phenolic lignin model compounds **(Wong, 2009**). The global structure of LiPs consists of eight major and eight minor alpha-helices with minimal β elements and is divided into two domains that form an active core cavity consisting of a single ferric ion heme-chelating **(Choinowski et al., 1999).** LiPs contain two glycosylation sites, two Ca^{2+} binding sites and four disulfide bridges, all stabilizing the three-dimensional structure of this enzyme. The native enzyme is oxidized by H_2O_2 and creates an intermediate compound that exists as a radical cation of ferry oxo porphyrin [Fe(IV)=O.+] (Figure 7). Via the electron donor substrate, the enzyme is subjected to two single-electron reduction steps, such as veratryl alcohol (VA), leading to the transient formation of compound II [Fe (IV)=O] and VA radical cation (VA.+) (**Romero et al., 2019**). The second VA molecule is further oxidized by compound II, simultaneously returning to its native stage to begin a new LiP catalytic cycle **(Wong, 2009).**

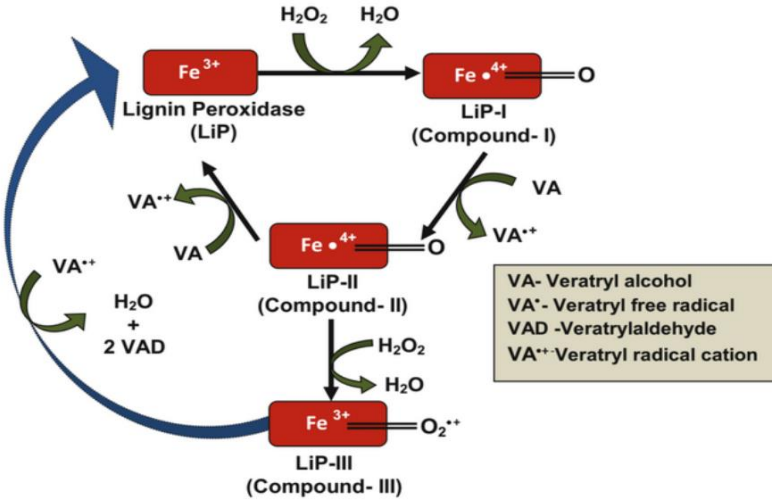


Figure 5) Catalytic cycle of lignin peroxidase. (Adapted from Chandra et al., 2017)

3.3.4 Versatile Peroxidase (VP) (EC 1.11.1.16)

Versatile peroxidases have a molecular hybrid architecture combining different substrate binding and oxidation sites linked to the heme pocket (similar to those in manganese peroxidases and lignin peroxidases). The Mn 3+ during VP (and MnP) activities act as a diffusible oxidant on phenolic lignin and free phenols, but also promotes lipid peroxidation reactions, necessary for decomposition of recalcitrant compounds (**Kapich et al., 2005**). The site of manganese binding in Versatile peroxidases, contains three residues of acidic amino acids (two Glu and one Asp) and heme-propionate and is very similar to that of Manganese peroxidases.

VPs also have an exposed tryptophanyl radical on the enzyme surface, as found in LiPs, acting as second active site, responsible for the oxidation of high redox potencial model substrates like Veratryl alcohol, the natural substrate of LiPs, or the dye Reactive Black 5 (RB5) (**Perez-Boada et al., 2005**), that LiP can only oxidize in the presence of VA acting as redox mediator (**Heinfling et al., 1988**). This site is responsible for the direct oxidation of molecules with high redox potentials, as well as those that LiP can only oxidize in the presence of redox mediators (**Heinfling et al., 1988**). Although VP was about ten times less effective than lignin peroxidase

in oxidizing Veratryl alcohol (**Tien et al., 1986**), both phenolic and nonphenolic b-O-4 lignin model dimers can be degraded by VPs. Among basidiomycetes, VPs have the most catalytic versatility, degrading compounds that other peroxidases cannot oxidize directly. In this way, VPs are able to oxidize a variety of (high and low redox potential) substrates including Mn 2+ , phenolic and non-phenolic lignin dimers, α-keto-γ-thiomethylbutyric acid (KTBA), veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols and Hydroquinones (**Perez-Boada et al., 2005**).

All the cell wall degrading enzymes previously referred, and OAH contribute synergistically to defusing the plant's cell wall structure, facilitating the penetration of fungi in host tissues. The process, for basidiomycete white-rot fungi is illustrated in Figure 8.

Extracellular oxidases, such as glyoxal oxidase (GLOX, a copper-radical) (**Kersten and Kirk, 1987**) and aryl-alcohol oxidase (AAO, a flavoprotein) (**Guillén and Grans, 1994**), provide the hydrogen peroxide required by peroxidases. The Mn 2+ needed for Mn-dependent peroxidases is taken from plants bark, rich in magnesium.

3.4 Oxaloacetate acetyl hydrolase (OAH) (EC 3.7.1.1)

OAH is a catalyst for the hydrolysis of oxaloacetate to oxalic acid and acetate. It belongs to the phosphoenolpyruvate mutase (PEPM)/isocitrate lyase (ICL) family by acting on α-oxocarboxylate substrates to cleave C-C or P-C bonds.

OAH function is to acidify host tissues and sequester calcium from host cell walls. The formation of calcium oxalate crystals weakens the cell walls, allowing polygalacturonate to cause more rapid degradation in a synergistic reaction. In the degradation of lignocellulose by wood-destroying basidiomycetes, oxalate plays a unique role as a low molecular weight agent that initiates degradation. In addition, oxalate serves as a potential electron donor for the reduction catalyzed by lignin peroxidase and manganese chelates into white rot fungi, allowing the dissolution of Mn3+ from the manganese enzyme complex and thus stimulating extracellular manganese peroxidase activity.

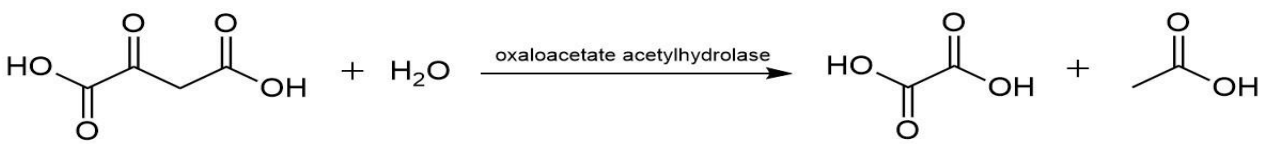
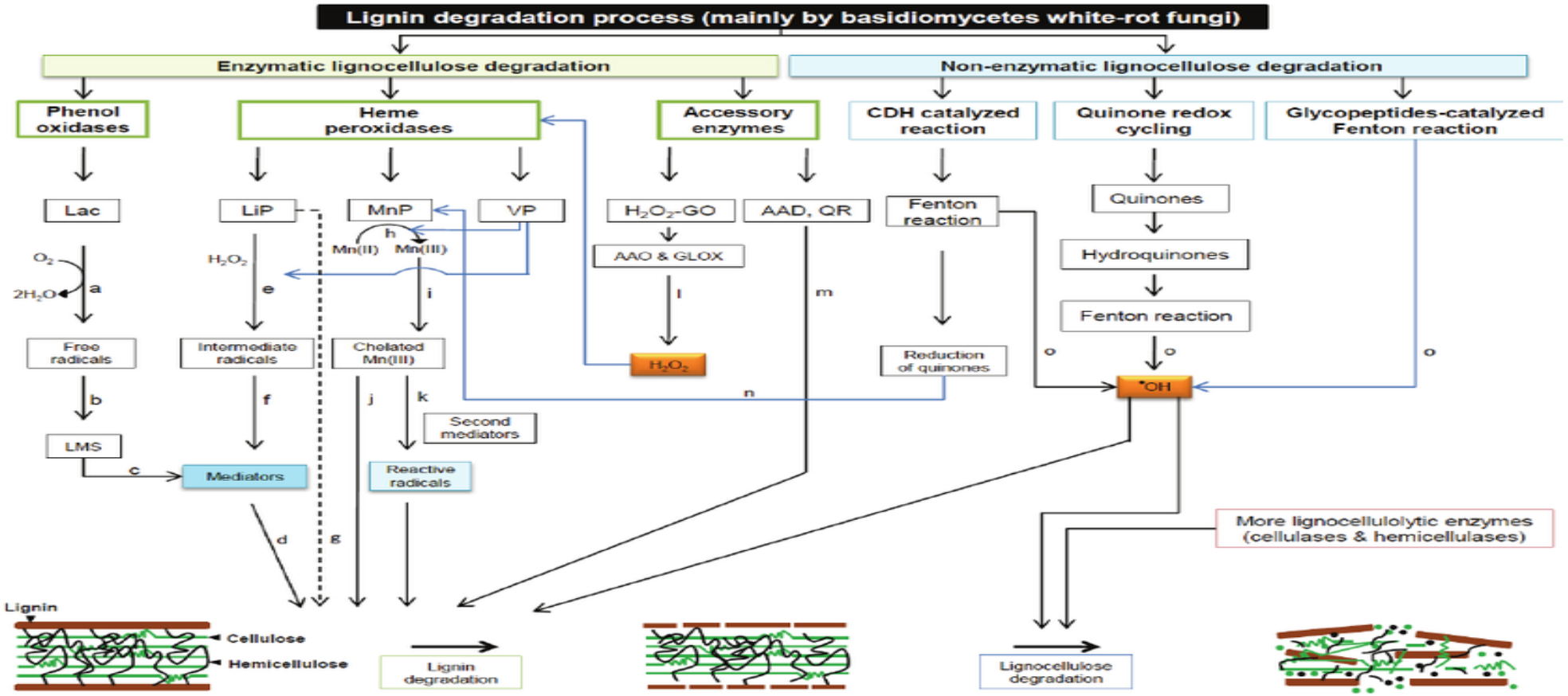


Figure 6) OAH catalyzed reaction



Lac: laccase, LMS: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidases, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and $^{\cdot}OH$: free hydroxyl radicals.

Figure 7) Schematic diagram of lignin degradation by basidiomycetes white-rot fungi: the major steps and enzymes involved (adapted from Dashtban et al., 2010).

III. Objectives

1. Growth of wild type and hypovirulent strains of C. parasitica
2. Evaluate the production of oxalic acid, lignin peroxidase and manganese peroxidase in virulent and hypovirulent strains of *C. parasitica*
3. Assess the activity of hydrolases and peroxidases quantitatively and qualitatively.

IV. Materials and Methods

1. *Cryphonectria parasitica* isolates characterization

C. parasitica isolates used throughout this study, provided by Dr. Eugénia Gouveia, were gathered from various Bragança and Vinhais orchards in northern Portugal. The SR442 hypovirulent strain was discovered in Sergude in 2013 while the RBB111 hypovirulent strain was discovered there in 2011. In PDA (Potato Dextrose Agar) growing media, hypovirulent strains exhibit a whitish morphology and decreased conidiation. Based on morphological traits, the presence of the hypovirus CHV1 in converted isolates with the SR44.2 hypovirulent strain was determined. All the isolates are kept in the Instituto Politécnico de Bragança's fungal collection, while both of the defined hypovirulent isolates are kept in the Micoteca of the University of Minho's global collection. They belong to two different vegetative compatibility groups (EU 11 and EU 66), and are listed in Table 1:

Table 1) *C. parasitica* Isolates (Virulent and Hypovirulent)

Code	VCG	Site	Virulence
Carragosa 02	EU 11	Carragosa (Bragança)	Virulent
Curopos 15	EU 11	Curopos (Vinhais)	Virulent
Cast 13	EU 11	Castrelos (Bragança)	Virulent
Cast 17	EU 66	Castrelos (Bragança)	Virulent
Cast 26	EU 11	Castrelos (Bragança)	Virulent
VDP 11	EU 66	Vilar de Peregrinos (Vinhais)	Virulent
SR442	EU 11	Sergude (Felgueiras)	Hypovirulent
RBB111	EU 11	Rio Bom (Valpaços)	Hypovirulent

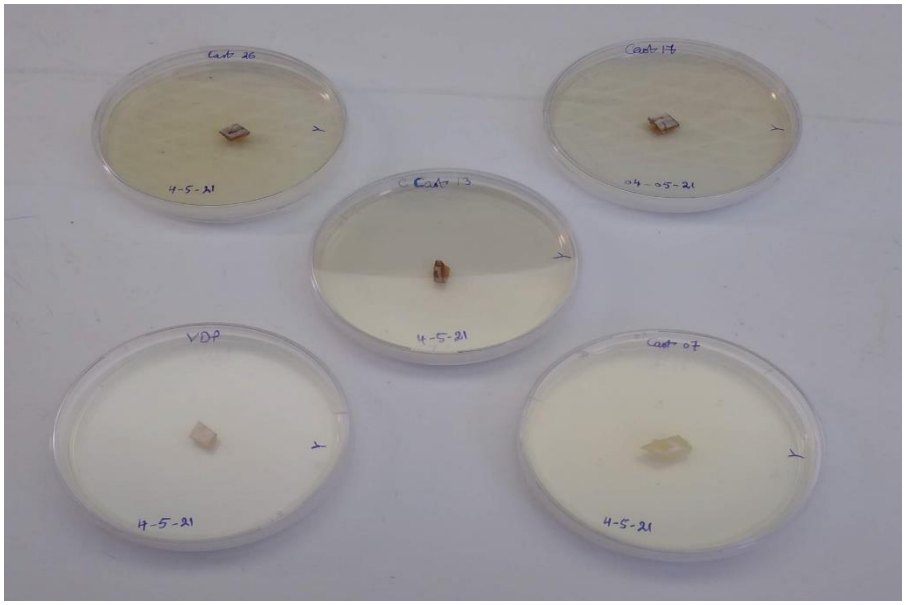


Figure 8) *C. parasitica* isolates on Petri plates

1.1 Conditions for growth and maintenance of the microorganisms

The growth of the *C. parasitica* isolates was carried out in solid medium (on Petri dishes) or in liquid medium (inside Erlenmeyer flasks). Routine incubation temperatures were 25ºC, for one week in the dark. Cultures in liquid medium were grown under conditions of agitation at 110 rpm in an orbital incubator (S1600C, Stuart ®). The inoculation at liquid media was carried out introducing four plugs of 5mm diameter per 100 mL medium. All media were prepared in double-distilled water (ddH_2O), and autoclave sterilized for 15 minutes at 121˚C and 1 atm.

The maintenance of the isolates in collection was carried out through periodic inoculation (from 10 to 20 days) in solid media, and subsequent incubation at 25ºC. Additionally, to keep the strains viable for long periods of time, the isolates were kept in sterile ddH_2O under temperature of 5ºC.

1.2 Culture media used for growth and maintenance

For growth and maintenance, Oxoid® Potato Dextrose Agar (PDA, 39g/L) and Potato Dextrose Broth (24g/L) were used.

1.3 Liquid culture media used for OAH activity determination

For OAH activity determination, each strain is grown in 100 mL sterile PDB in 250 mL Erlenmeyer flasks, supplemented with 2mM $MnSO_4$.

1.4 Liquid culture media used for ligninolytic induction

The isolates used in the ligninolytic enzymatic assays were submitted to a first stage of growth in PDB for four days, then the mycelia were filtered using a Buchner filter adapted to a Kitasato device coupled to a vacuum pump, washed with ddH_2O and transferred to a minimal medium, in order to induce ligninolytic activity.

By milliliter, the induction medium contained a 62.5mL salt solution, 2mg thiamine, and 10g glucose.

Salt solution contained: 24g/L NH_4NO_3, 16g/L KH_2PO_4, 4g/L Na_2SO_4, 8g/L KCl, 2g/L $MgSO_4$. $7H_2O$, 1g/L $CaCl_2$, and 8mL/L of a trace elements solution. Trace elements solution was composed of: 60mg/L H_3BO_3, 140mg/L $MnCl_2 \cdot 4H_2O$, 400mg/mL Z_nCl_2, 40mg/L Na_2MoO_4, 100mg/L $FeCl_3 \cdot 6H_2O$, 400mg/L $CuSO_4 \cdot 5H_2O$.

Figure 9) Prepared Medium for Mycelial growth

A Na_2HPO_4 (0.2M)- citrate (0.1M) buffer (**McIlvaine, 1921**) was used to buffer the induction medium at pH 4. By isolate, two 500mL Erlenmeyer flask filled with 200mL sterile induction medium and at which six small pieces of autoclaved C. sativa stems were previously introduced, were inoculated with the transferred mycelium, and incubated at 25°C at 110rpm.

Figure 10) Orbital incubation of Erlenmeyer flasks

After three days of incubation, the mycelium was filtered, weighed for ulterior dry weight determination, and stored at -80°C for molecular techniques. The supernatants were put into tubes (in 50mL Falcons and in 1.5mL Eppendorf tubes) and stored at -80°C for further analysis.

1.5 Solid culture media used for ligninolytic and cellulolytic activity evaluation

PDA with 25mg/L Azur B added was used to test peroxidases activity in Petri plates. For medium decolorization evaluation, an uninoculated plate was used as a control.

For cellulase activity evaluation, a medium containing 0.5% carboxymethylcellulose (CMC) from Sigma, 2.0 % agar, and 0.1% yeast extract was produced, inoculated with the selected isolates, and left to grow during four days at 25ºC in darkness. Then, the plates were flooded with a 0.1 % Congo Red solution for 45 minutes, the stain was poured off, and they were de-stained with a 1M solution of NaCl for 15 minutes **(Sazci et al., 1986).**

2. Quantitative evaluation of enzymatic activity

2.1 Oxaloacetate acetylhydrolase (OAH) activity

The oxalic acid production from *C. parasitica* isolates due to OAH activity was evaluated as described by Xu and Zhang (2000). The method is based on the catalytic action of oxalic acid on the oxidation of the Bromophenol Blue (BB) by dichromate, in dilute sulfuric acid medium. Several calibration curves were assayed, to check the most suitable range of sample volumes to use in the reaction. For this, an oxalic acid (1.0 mg/mL) stock solution was prepared by dissolving oxalic acid in water.

The working standard solutions (with the concentrations 0.1 µg/mL, 0.3 µg/mL, 0.5 µg/mL, 0.7 µg/mL, 0.8 µg/mL, 1.0 µg/mL, 3.0 µg/mL, 5.0 µg/mL, 7.0 µg/mL and 8.0 µg/mL) were obtained by adequate dilutions of the stock solution. All reagents used in the reaction were prepared in double-distilled water and were of analytical grade.

Figure 11) Different concentrations of Oxalic acid (0.1 to 8.0 µg/mL)

The 20mL test tubes reactions were initiated in the order of oxalic acid (standard or sample), BPB ($2.5x10^{-5}$M), sulfuric acid ($3.6x10^{-2}$M), potassium dichromate ($3.2x10^{-3}$M) and ddH_2O to complete the final volume.

After vortexing, the tubes were placed in a water bath at 60ºC for 10 minutes. After, the reactions were quenched by adding 1.0 mL sodium hydroxide solution 2.0M, as described by Xu and Zhang (2000). The tubes were vortexed and allowed to cool, then the absorbance values of standards (or samples), **Ac**, were read at $\lambda=600nm$ in a spectrophotometer (NanocolorUV/VIS, Macherey- Nagel). The absorbance value, **Au,** for the blank solution of the non-catalytic reaction (without oxalic acid) was obtained at the same way. The difference value (**Δ**A) between Au and Ac was determined, and the **log (Au/Ac)** value was calculated to determine oxalic acid concentration.

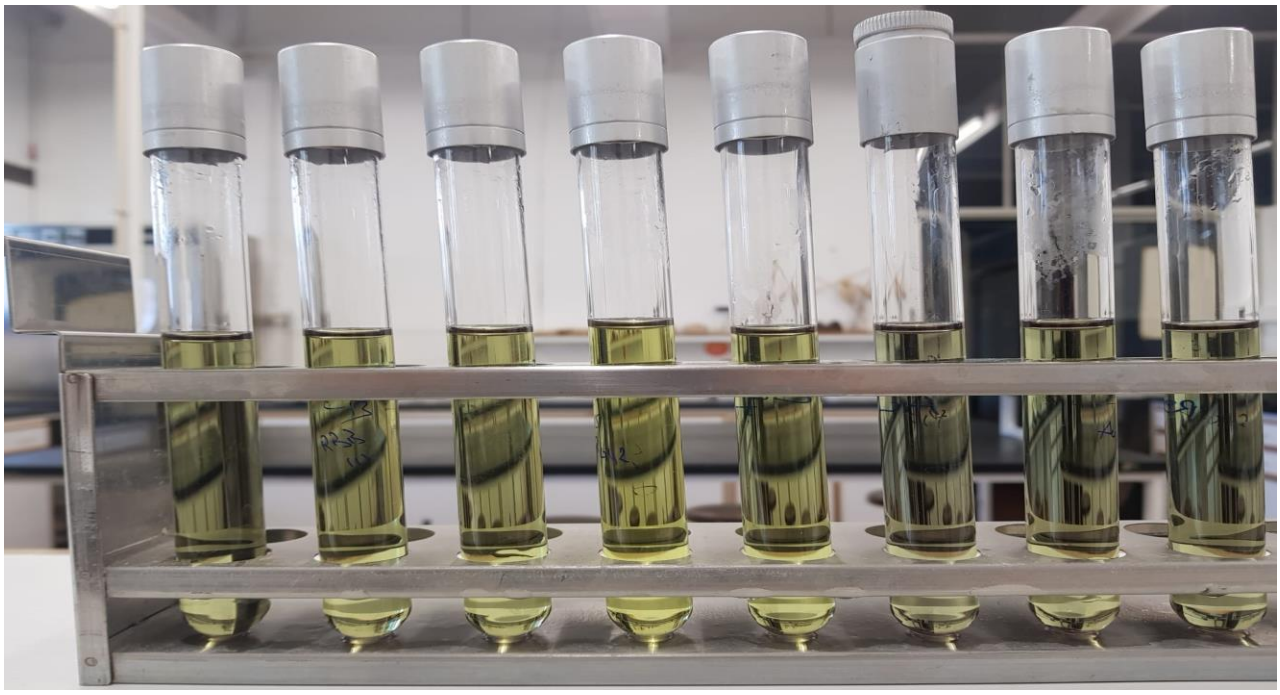


Figure 12) Reaction quenched with NaOH.

2.2 Manganese peroxidase (MnP) activity assay

Manganese peroxidase activity is determined by the oxidation of phenol red at 610 nm. The assay mixture included 0.4 mL of culture filtrate,0.2mL of 0.5M malonate buffer (pH 4.5), 0.2mL of 0.25M sodium lactate,0.2mL of 0.1% phenol red, 0.4mL of 0.5% BSA,0.1mL of 2.0 mM $MnSO_4$ and 10mM H_2O_2. The changes in absorbance of the reaction mixture were monitored at 610 nm (ε = 22,000 M-1 Cm-1) for 5 min. Then to stop the reaction it was used 2.0M of NaOH and read at 610nm (Kawahara et al., 1984).

2.3 Lignin peroxidase (LiP) activity assay

Lignin peroxidase (LiP) activity is determined by monitoring the oxidation of Azure B dye; The oxidation of azure B dye is used to determine activity.0.25mL of 250 mM sodium tartrate

buffer (pH 3.0), 50µL of 0.160 mM Azure B, 500 µL of culture filtrate, and 1.35mL of 2.0 mM H2O2 in the reaction mixture. The reaction was initiated with H_2O_2, and after a 5-minute incubation, we read at 651 nm. An O.D. drop of 0.1 units per minute per ml of culture filtrate has been used to represent one unit of enzyme activity (Narkhede, 2014)

3. Qualitative evaluation of enzymatic activity

3.1 Peroxidase medium

Decolorization of the dye Azure-B by fungi has been positively correlated with production of lignin peroxidase and Mn dependent peroxidase. For peroxidases evaluation, it was used PDA with 25mg/l Azur B added. After sterilization it was aseptically transferred into rectangular Petri dishes, inoculated, and incubated at 25°C in darkness.

3.2 Cellulase medium

Carboxymethylcellulose (CMC) is a substrate for endoglucanase and so can be used as a test for endoglucanase and B-glucosidase activity. This assay is a good indicator of cellulolytic ability since endoglucanase, is generally produced in larger titres by fungi than cellobiohydrolase. The medium was prepared with 0.5% of carboxymethyl cellulose (CMC) and 1.6% agar for the growing of nine isolates. Four days after inoculation and incubation at 25°C, the plates were flooded with a 0.1% solution of Congo Red for 45min, then the stain was poured off, and they were distained with a 1M solution of NaCl for 15min.

4. Determination of isolates' dry weight:

Mycelia were filtered using sterile metallic or glass Buchner filters, adapted to sterile Kitasato flasks, and a vacuum pump to determine biomass. Mycelia were rinsed in sterile distilled water, wiped between dry paper towels until no excess water remained, and placed on pieces of aluminum foil that had been pre-weighed. To obtain the dry weight for each isolate, the total quantity of mycelium was dried at 60°C until it reached a consistent weight. According to the formula:

$W_f = W_1 - W_0$

W_f = dry weight (mg)

W_1 = weight of final harvested mycelia after drying upon the aluminum foil (mg)

W_0 = initial weight of aluminum foil empty (mg)

5. Statistical analysis:

Statistical analyses of mycelia growth and halo expansion were performed using unidirectional analysis of variance (One-way ANOVA) followed by a post hoc test of LSD- and Tuckey with the software Statistica 12 (StatSoft Inc.,Tulsa, OK) to determine cellulosic activity.

V. Results and Discussion

1) Growth of wild type and hypovirulent strains of *C. parasitica* in different enzymatic activity induction media

The growth of the isolates tested throughout this study was being carried out in solid media with Potato Dextrose Agar (39g/L). As a result, hypovirulent strains expressed a white morphology and decreased conidiation while virulent strains expressed orange pigmentation when being grown on Petri plates such as shown in Figure 13.

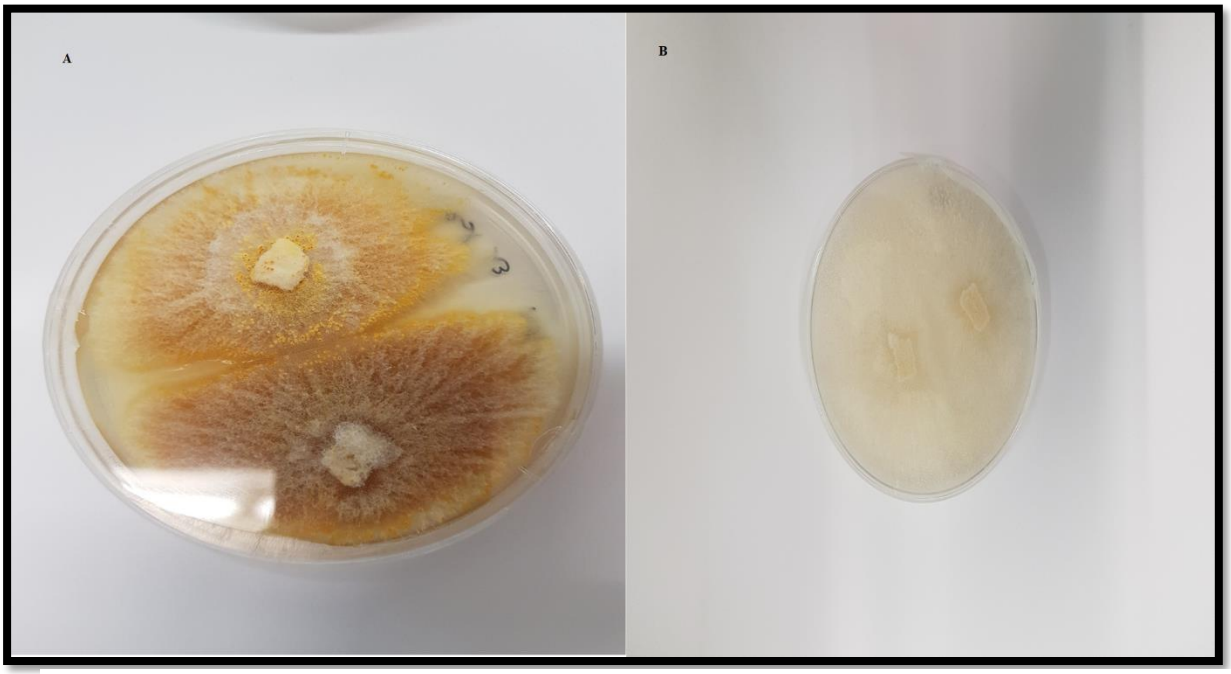


Figure 13) A) virulent strain. B) Hypovirulent strain

2 Qualitative and Quantitative evaluation of ligninolytic enzymes production

2.1 Qualitative evaluation of ligninolytic enzymes

C. parasitica isolates were tested on PDA media containing colored indicator compounds that allowed the production of ligninolytic enzymes to be visually detected. Azure B and Carboxylmethyl cellulose (CMC) were the compounds that have been used as indicators of ligninolytic and cellulolytic enzymes. The results of the qualitative plate assays, represented in

Figures 14 (for cellulase) and 15 (for peroxidases), and described in Table 2, show the differences in cellulase and peroxidase production for the nine different strains.

In the cellulase test, hypovirulent and isogenic converted strains showed a cellulolytic activity higher than the virulent ones. These virulent strains presented a good microbial growth rate, even though didn't appear to manifest a high cellulolytic activity.

A positive Azure B test result is a colorless halo surrounding the microbial growth, confirming the production of lignin and manganese peroxidase. It occurs with simultaneously blue coloring of the mycelium. In the Azure B assay, virulent strains (VCB02, Cast26 and Cast17) achieved a higher mycelial growth and better decolorization of the dye than the converted and the hypovirulent ones (RBB111, Ser05 and SR442), confirming the production of lignin and manganese peroxidase activity.

In cellulase assays, almost all of the hypovirulent converted strains evidenced higher ratio halo/growth than the isogenic virulent.Some converted strains, like Cast13 SR442 didn't have mycelial growth from the inoculum disk, but produced a cellulose degradation halo, otherwise Cast13 RBB111 showed mycelial growth but no visible halo.

Statistical analysis was performed using unidirectional analysis of variance (One-way ANOVA) and post hoc test of LSD and Tuckey, to compare the mycelial growth and halo expansion in plate cellulase assays. For that, the registered values of mycelial growth, halo, and ratio halo/mycelial growth, presented in Appendix I were taken, and the results shown in Figure 14 and 15.

Table 2) Cellulase and Azure B effect on strains

Table 2) Cellulase and Azure B effect on strains **Strains**	**Cellulase**		**Azure B**	
Growth	**Halo (mm)**	**Growth (mm)**	**Halo (mm)**	**Growth (mm)**
Curopos 15	+++	**0**	-	-
Curopos 15 SR442	+++	**1**	-	-
CP 33 PAR	++	**0**	-	-
RBB111	+	**2**	+++	**3**
SR442	-	-	+++	**3**
Ser05	++	**3**	+++	**4**
Cast 17	-	-	+++	**3**
Cast 26	+++	**0**	+++	**4**
Cast 13 SR442	+	**4**	-	-
Cast 13	**C**	-	-	-
Cast 13 RBB111	+++	**1**	-	-
VBC02	-	-	+++	**3**

Cellulase plates: 1-2 => small ø halo (1-3 mm), 3-4 => big ø halo (4-6 mm); Azure B plates: + to +++ indicates increase in decolorization.

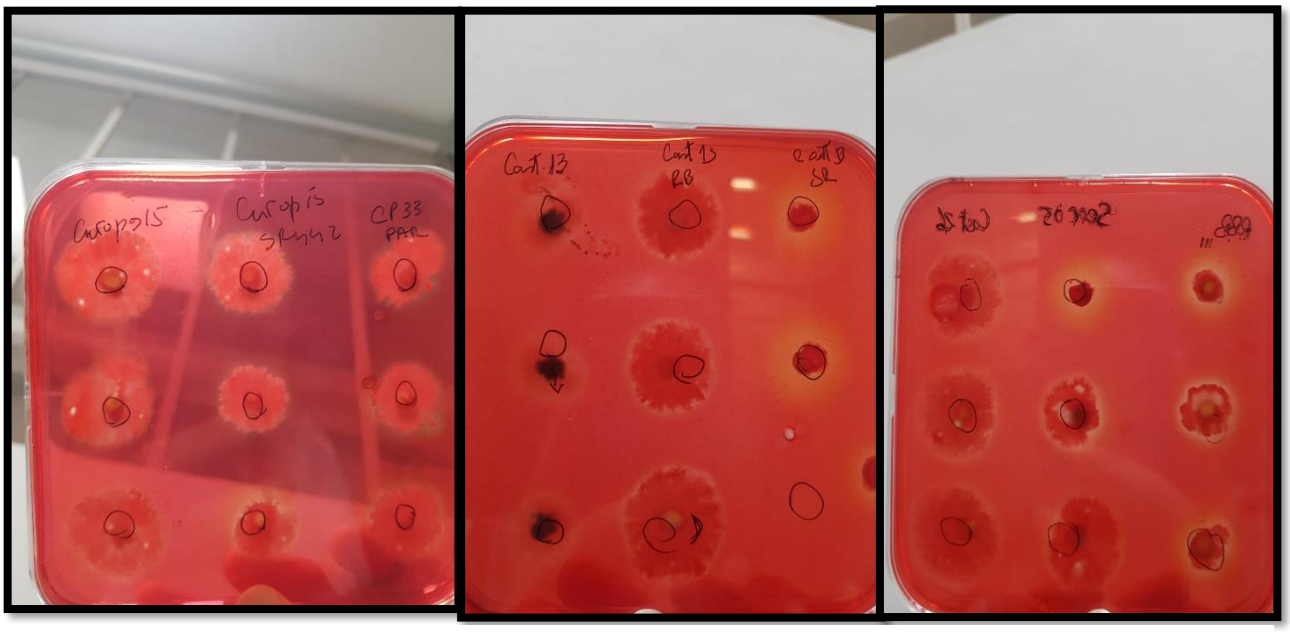


Figure 14) Reaction of strains to Cellulase assay.

For the two different Cast13 isogenic isolates, Cast13 RBB111 and Cast13 SR442 the ratio halo/growth were significantly different ($p < 0.01$).

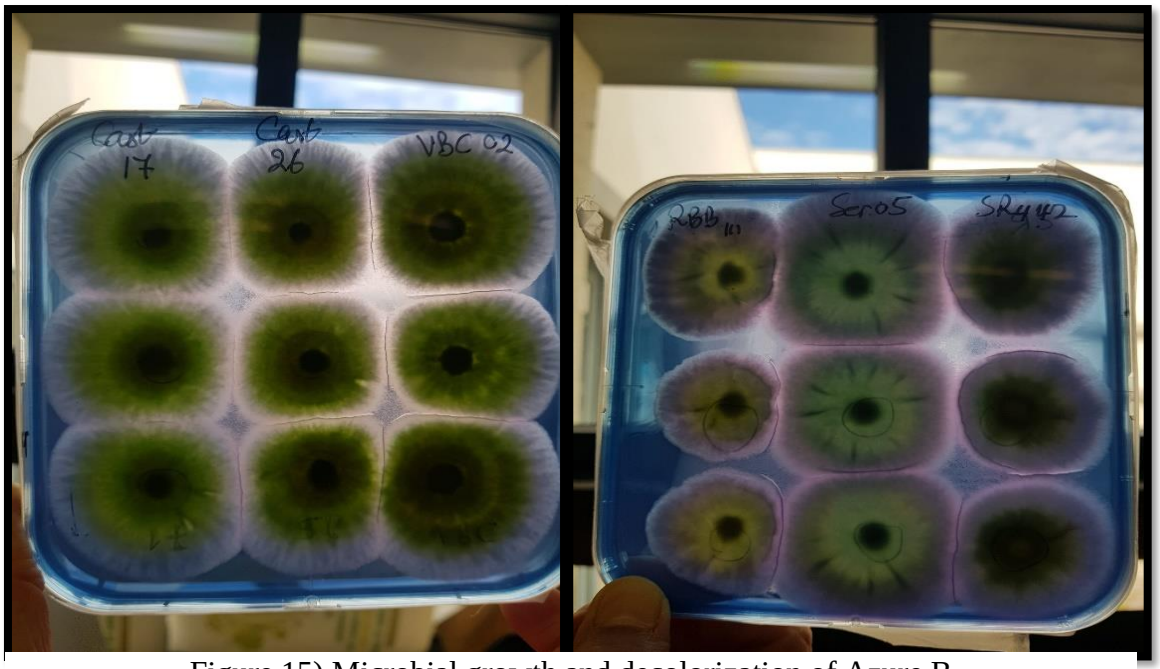


Figure 15) Microbial growth and decolorization of Azure B

Nevertheless, Cast13 SR442 who displayed no mycelial growth, presented significant differences towards all the virulent isolates Curopos15, CP33 Par and Cast26, all the converted Curopos15 SR442, but not with Cast13 RBB111.

Cast26 and Curopos15 are equal in halo reaction, but different from CP33 Par ($p<0.05$).

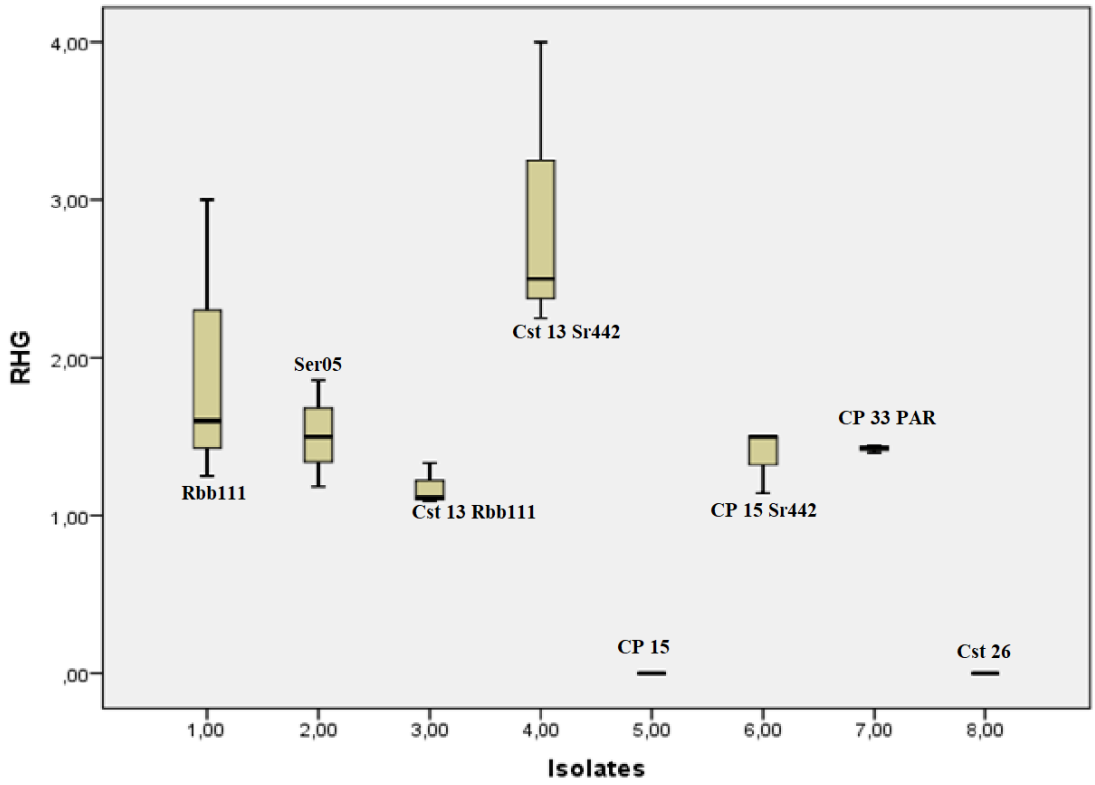


Figure 16) Diagram of cellulolytic activity of strains

The fact that the converted strains always presented higher cellulolytic activity in CMC is an unexpected result, attending previous studies about the effect of CHV1 in cellulase activity (where the virulent isolates always showed higher enzymatic activity then the converted ones, or the donors), the fact that a slightly different growth media, and a different CMC source were used, can explain the different results between experiments. But Nuskern et al. (2021), in a study with laccases, related CHV1-converted strains with increased activity upon CHV1-infection, also influenced by culture conditions or fungal/viral genotypes. Also, Savino et al. (2021) have reported a *C. parasitica* hypovirulent strain, with higher laccase and esterase activities (-10-fold and 2- fold, respectively) than the other strains assayed, all virulent, including its virus-free isogenic.

2.2 Quantitative evaluation of ligninolytic enzymes

2.2.1 Evaluation of Oxaloacetate acetyl hydrolase (OAH) activity

a) Calibration curve

Figure 18 depicts the calibration curve plot, which shows a linear relationship between log (Au/Ac) and the oxalic acid concentration ranging from 0.1 to 8.0 g/mL, as given by the equation log (Au/Ac) = 0.0142C + 0.0026 (R= 0.9964), where C is the oxalic acid concentration in µg/mL.

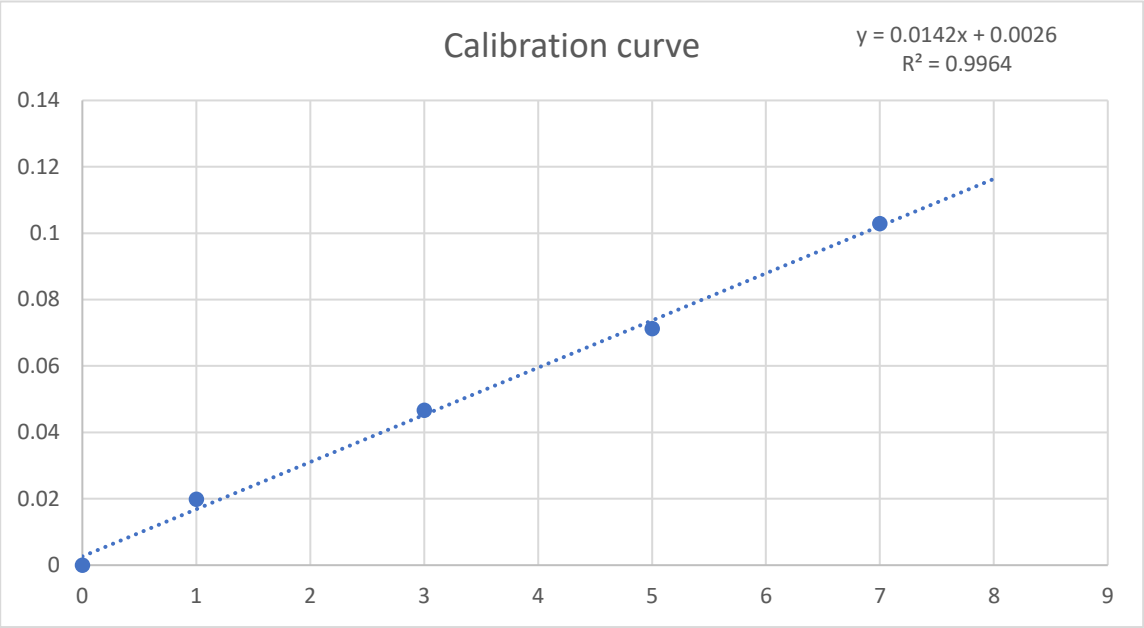


Figure 17) Calibration curve for oxalic acid determination. Au – absorbance value for the blank solution of the non-catalytic reaction; Ac – absorbance values of the oxalic acid standards; λ = 600 nm

This calibration curve was used to understand the instrumental response to oxalic acid, and to predict the concentration of oxalic acid in a *C. parasitica* isolates. It was created by first preparing a set of standards solutions from 0.1µg/mL to 8.0 µg/mL and based on the establishment of the calibration curve, the determination of oxalic acid production in samples were obtained.

b) Production of Oxalic acid:

Table 3 summarizes the oxalic acid concentrations found in *C. parasitica* isolate supernatants. Oxalic acid concentrations were higher in virulent strains (9,7µg/mL for Cast26; 6,8µg/mL for

Curopos15 and 7,7µg/mL for Carragosa02) compared to their converted strains who presented lower values in oxalic acid production, as should be expected.

Table 3) Oxalic acid production of *C. parasitica* strains

Strains	**Log (Au/Ac)**	**Oxalic acid production**
Carragosa02	0.11189	7.696 µg/mL
Carragosa02 SR442	0.06808	4.611 µg/mL
Curopos15	0.09929	6.809 µg/mL
CP 33	0.10937	7.519 µg/mL
Cast 26	0.1431	9.7µg/mL
Cast26 SR442	0.03443	2.24µg/mL
Cast26 Rbb111	0.0486	6.48µg/mL

The responses of the virulent strains appear to differ depending on the hypovirulent isolate used in the conversion (for example, Cast26 RBB111 shows 34% reduction in the content of oxalic acid present in the supernatant when compared to Cast26. However, Cast26 SR442 presents 67% reduction). Nevertheless, there were not enough double-paired data points between virulent and converted strains to allow for more in-depth comparisons. In practice, the repeated assays registered different values each time which consumed many virulent isolates and time.

It seems that in the protocol used for Oxalic acid evaluation PDB acts as a buffer that resists to pH variation, leading many times to variations in the values obtained for sample replicates, rendering the results inconclusive. Nevertheless, the OA determination methodology proposed by Xu and Zhang (2000) showed to be effective when samples in aqueous solution, like oxalic 33 acid standards, are used, but it didn't work using samples in PDB media. So, in the future different microbiological media should be assessed, to choose one that will provide reliable values

2.2.2 Evaluation of Peroxidases activity assay

For peroxidase activity, we selected different strains on which we followed the protocols described by Casciello et al. (2017) and Ruiz-Dueñas (2001), respectively, with some modifications, results obtained are illustrated in figure.

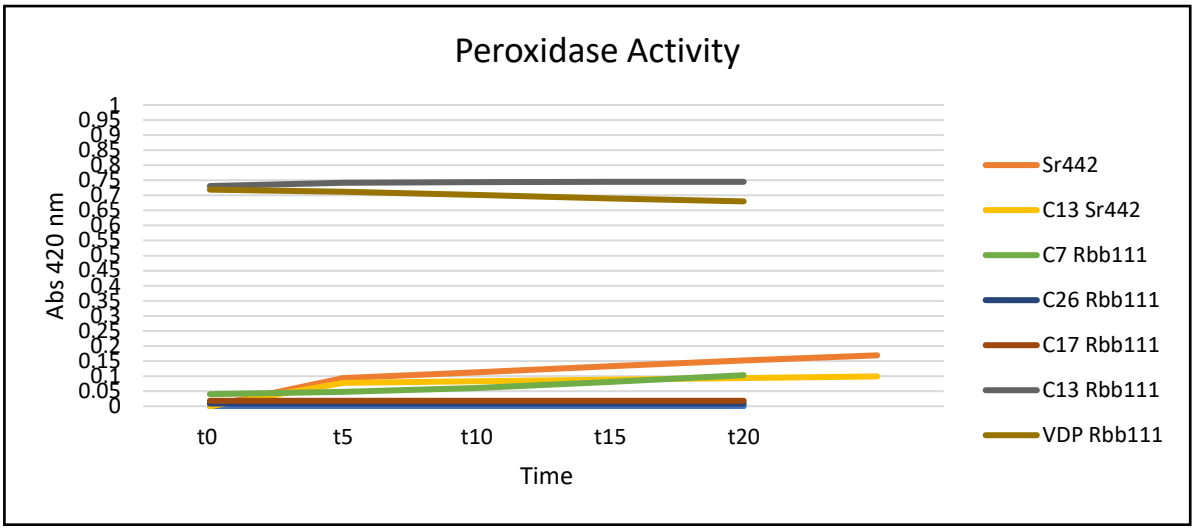


Figure 18) Curve of the evolution of strains with Peroxidase activity

Two isolates (Cast13 Rbb111 and VDP Rbb111) showed high activity absorbance at 420 nm which reflected the possibility of Laccase existence in the strains, as ABTS is also a common substrate for laccases activity determination (**Arora and Sharma, 2010**), all the components of the reaction at the same concentrations were mixed, with exception of H_2O_2 and $MnSO_4$ (both absent), and pre-incubated for 10 minutes at 25ºC, before reading the absorbance values, during a 20-minute interval. The increase in absorbance caused by laccase activity was then

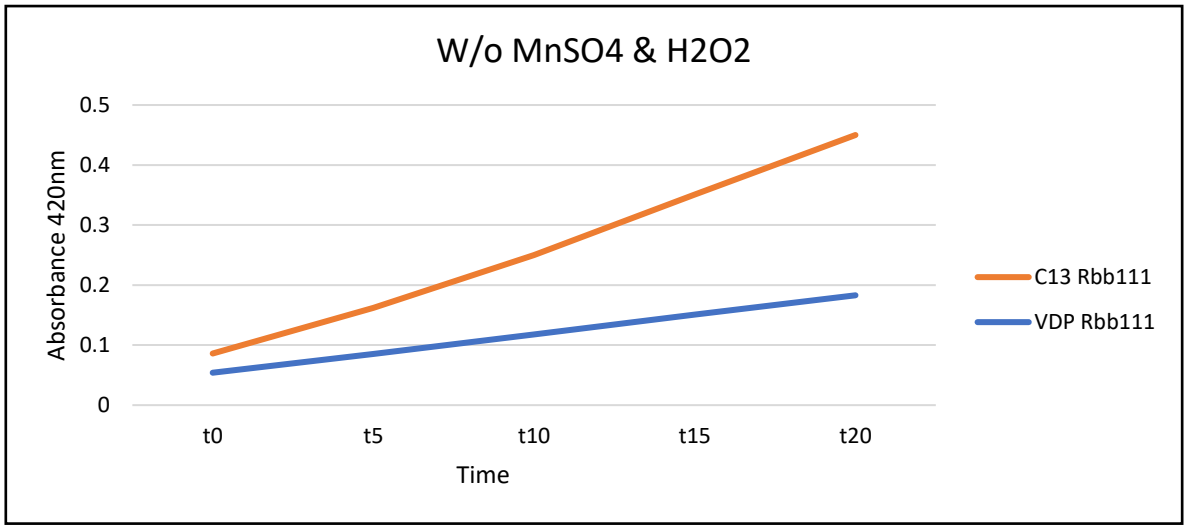


Figure 19) Change in absorbance without H2O2 and MnSO4

subtracted from the values of peroxidases activity as shown in figure

Applying the Lambert-Beer law, we calculate the enzymatic activity (in units, U or international units, IU), defined as the amount of enzyme transforming 1 µmol of substrate per

minute and liter, which is calculated by the following formula, taken from Holme and Peck (1996):

$$\mathbf{U\ (\mu mole/L.min) = (\Delta E \times Vt) / (\varepsilon \times d \times Vs) \times 10^6}$$

where:

ΔE being the change in extinction of light per minute at 420 nm,

ε being the molar absorption coefficient of ABTS (36,000 M -1 cm -1)

d being the light pass of the cell (cm)

Vt is the total volume of the reaction (mL)

Vs is the volume of the enzyme solution (mL)

Table 4) Calculation of Peroxidase activity during an interval of time

Isolates	**ε (M cm-1)**	**Interval of Time**	**Slope (min $^{-1}$)**	**Enzymatic Activity:U (µmole/L.min)**
VDP Rbb111	36000	0-20	0.00615	1.71
Cast13 Rbb111	36000	0-20	0.0105	2.91
Sr442	36000	0-20	0.0038	1.06
Cast13 Sr442	36000	0-20	0.00105	0.292
CP33 Sr442	36000	0-20	0.00025	0
Rbb111	36000	0-20	0	0
Cast07 Rbb111	36000	0-20	0.00315	0.875

It looks like converted isolates with Rbb111 showed higher enzymatic activity than those converted with Sr442 (Cast13 Rbb111 with 2.91 compared to 0.292 to Cast13 Sr442) or Sr442 with 1.06 to 0 for Rbb111.

2.2.3 Manganese Peroxidase and Lignin Peroxidase activity assays

In the LiP assays there was no detection of activity in the tested isolates, as seen in Figure 17.

For determination of manganese peroxidase activity, we followed the protocol described by Kuwahara et al., 1984, which consisted of the oxidation of phenol red, which was monitored at 432nm, and at 610nm with NaOH for stopping the reaction. strains showed unstable values each time they were put to assessment which disabled us from interpreting the results

Figure 20) Absence of LiP activity

2.2.4 Evaluation of Peroxidases activity assay

For peroxidase activity, we assayed different strains following the protocols described by Casciello et al. (2017) and Ruiz-Dueñas (2001), respectively, with some modifications.

The results obtained for Mn-dependent peroxidase activity are show in Figure 18 and Table 4.

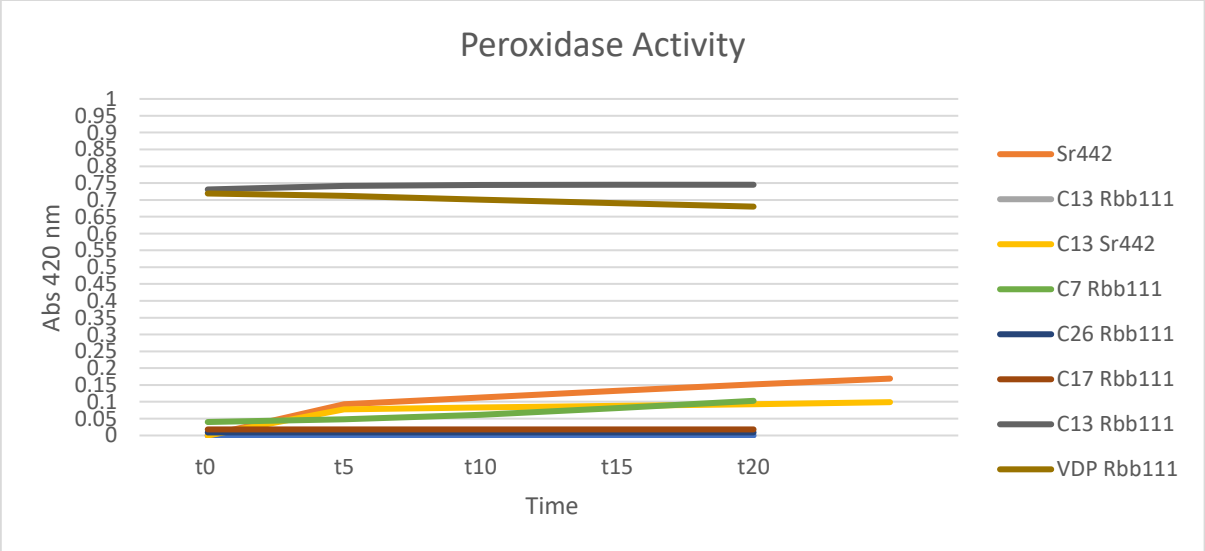


Figure 21) Curve of the evolution of strains with Mn dependent peroxidase activity.

The results obtained demonstrated that there is in fact Mn-dependent peroxidase activity within almost all converted strains such as Cast 13 RBB111, Cast13 SR442 and others. Cast13 SR442

showed more effective reduction of Mn-dependent peroxidase activity than Cast13 RBB111, with 0.389U/L and 2.91U/L respectively.

Table 5) Enzymatic activity of Mn-dependent peroxidases activity

Isolates	**ε (M cm-1)**	**Interval of Time (min)**	**Slope (min -1)**	**Enzymatic Activity U (µmole/L.min)**
VDP RBB111	36000	0-20	0.00615	1.71
Cast13 RBB111	36000	0-20	0.0105	2.91
Cast07 RBB111	36000	0-20	0.00315	0.875
RBB111	36000	0-20	0	0
SR442	36000	0-20	0.0038	1.06
Cast13 SR442	36000	0-20	0.0014	0.389
CP33 SR442	36000	0-20	0.00025	0
Curopos15 SR442	36000	0-20	0.0181	5.02

When testing the Mn-independent peroxidase activity by excluding MnSO4, we obtained the results in Figure 19 and Table 5.

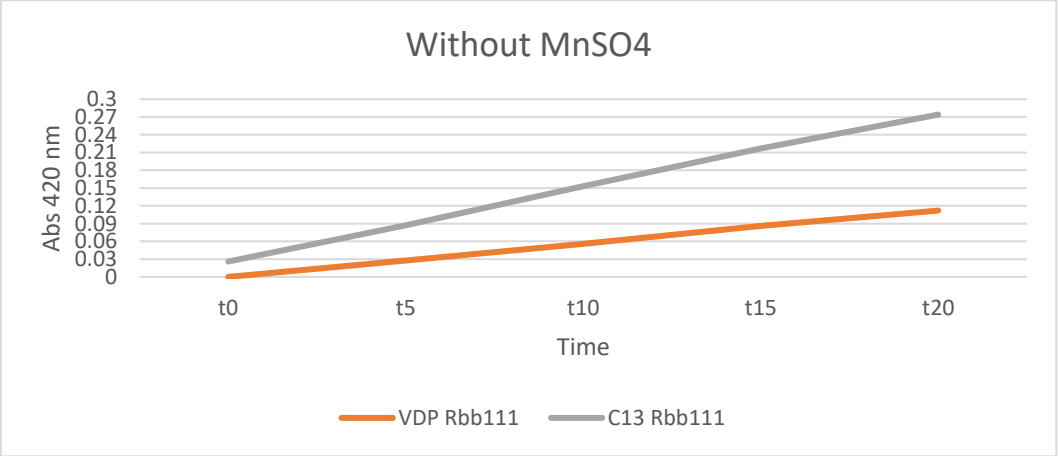


Figure 22) Mn independent peroxidase activity

Table 6) Calculation of Mn Independent Peroxidase activity

Isolates	**ε (M cm-1)**	**Interval of Time (min)**	**Slope (min -1)**	**Enzymatic Activity (U/L)**
VDP RBB111	36000	0-20	0.0056	1.544
Cast13 RBB111	36000	0-20	0.0124	3.44

In the Mn-independent peroxidase assay the converted isolates Cast13 RBB111 presented also higher activity than VDP RBB111 (3.44 U/L approximately the double of VDP RBB111).

When excluding from the reaction both MnSO4 and H2O2 we obtained the Laccase activity values, presented in Table 6. Even here, Cast13 RBB111 had higher Laccase activity with 3.26U/L against 1.79U/L of VDP RBB111.

Table 7) Calculation of Laccase activity

Isolates	**ε (M cm-1)**	**Interval of Time (min)**	**Slope (min -1)**	**Enzymatic Activity (U/L)**
VDP RBB111	36000	0-20	0.00645	1.792
Cast13 RBB111	36000	0-20	0.01175	3.264

VI. Conclusion

- The growth of wild type and hypovirulent strains on Petri plates was successful in all the media tested.
- The oxalic acid production of Cast26 RBB111 showed a reduction of 34% when compared with Cast26, as for Cast26 SR442 that presented 67% reduction, indicating that conversion with SR442 was more effective in reduction of OAH activity.

- For cellulase activity the two different Cast13 isogenic isolates, Cast13 RBB111 and Cast13 SR442 showed significantly different ratios halo/growth ($p < 0.01$). In the virulent isolates, Cast26 and Curopos15 are equal in halo reaction, but different from CP33 Par ($p<0.05$).

- In the LiP and MnP assays there was no detection of activity in the tested isolates.

- For all Mn peroxidase assays, the converted isolate Cast13 RBB111 presented always higher activity than VDP RBB111.

- Cast13 SR442 showed to be more effective in reduction of Mn-dependent peroxidase activity than Cast13 RBB111, with 0.389U/L and 2.91U/L respectively.

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VIII. Appendix

Table 8) Differences between strains. 1.00) Rbb111. 2.00) Ser05. 3.00) Cst13 Rbb111. 4.00) Cst13 Sr442. 5.00) CP 15. 6.00) CP15 Sr442. 7.00) CP33 Par. 8.00) Cst 26

Dependent Variable:VAR00002

						95% Confidence Interval	
	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	1,00	2,00	,43701	,40089	,950	-,9509	1,8250
		3,00	,77155	,40089	,555	-,6164	2,1595
		4,00	-,96667	,40089	,299	-2,3546	,4213
		5,00	1,95000*	,40089	,003	,5620	3,3380
		6,00	,56905	,40089	,836	-,8189	1,9570
		7,00	,52566	,40089	,882	-,8623	1,9136
		8,00	1,95000*	,40089	,003	,5620	3,3380
	2,00	1,00	-,43701	,40089	,950	-1,8250	,9509
		3,00	,33454	,40089	,988	-1,0534	1,7225
		4,00	-1,40368*	,40089	,046	-2,7916	-,0157
		5,00	1,51299*	,40089	,028	,1250	2,9009
		6,00	,13203	,40089	1,000	-1,2559	1,5200
		7,00	,08865	,40089	1,000	-1,2993	1,4766
		8,00	1,51299*	,40089	,028	,1250	2,9009
	3,00	1,00	-,77155	,40089	,555	-2,1595	,6164
		2,00	-,33454	,40089	,988	-1,7225	1,0534
		4,00	-1,73822*	,40089	,009	-3,1262	-,3503
		5,00	1,17845	,40089	,129	-,2095	2,5664
		6,00	-,20250	,40089	,999	-1,5905	1,1855
		7,00	-,24589	,40089	,998	-1,6338	1,1421
		8,00	1,17845	,40089	,129	-,2095	2,5664
	4,00	1,00	,96667	,40089	,299	-,4213	2,3546
		2,00	1,40368*	,40089	,046	,0157	2,7916
		3,00	1,73822*	,40089	,009	,3503	3,1262
		5,00	2,91667*	,40089	,000	1,5287	4,3046
		6,00	1,53571*	,40089	,025	,1478	2,9237
		7,00	1,49233*	,40089	,031	,1044	2,8803
		8,00	2,91667*	,40089	,000	1,5287	4,3046
	5,00	1,00	-1,95000*	,40089	,003	-3,3380	-,5620
		2,00	-1,51299*	,40089	,028	-2,9009	-,1250
		3,00	-1,17845	,40089	,129	-2,5664	,2095
		4,00	-2,91667*	,40089	,000	-4,3046	-1,5287
		6,00	-1,38095	,40089	,052	-2,7689	,0070
		7,00	-1,42434*	,40089	,042	-2,8123	-,0364
		8,00	,00000	,40089	1,000	-1,3880	1,3880
	6,00	1,00	-,56905	,40089	,836	-1,9570	,8189
		2,00	-,13203	,40089	1,000	-1,5200	1,2559
		3,00	,20250	,40089	,999	-1,1855	1,5905
		4,00	-1,53571*	,40089	,025	-2,9237	-,1478
		5,00	1,38095	,40089	,052	-,0070	2,7689
		7,00	-,04339	,40089	1,000	-1,4313	1,3446
		8,00	1,38095	,40089	,052	-,0070	2,7689
	7,00	1,00	-,52566	,40089	,882	-1,9136	,8623
		2,00	-,08865	,40089	1,000	-1,4766	1,2993
		3,00	,24589	,40089	,998	-1,1421	1,6338
		4,00	-1,49233*	,40089	,031	-2,8803	-,1044
		5,00	1,42434*	,40089	,042	,0364	2,8123
		6,00	,04339	,40089	1,000	-1,3446	1,4313
		8,00	1,42434*	,40089	,042	,0364	2,8123
	8,00	1,00	-1,95000*	,40089	,003	-3,3380	-,5620
		2,00	-1,51299*	,40089	,028	-2,9009	-,1250
		3,00	-1,17845	,40089	,129	-2,5664	,2095
		4,00	-2,91667*	,40089	,000	-4,3046	-1,5287
		5,00	,00000	,40089	1,000	-1,3880	1,3880
		6,00	-1,38095	,40089	,052	-2,7689	,0070
		7,00	-1,42434*	,40089	,042	-2,8123	-,0364
LSD	1,00	2,00	,43701	,40089	,292	-,4128	1,2869
		3,00	,77155	,40089	,072	-,0783	1,6214
		4,00	-,96667*	,40089	,028	-1,8165	-,1168
		5,00	1,95000*	,40089	,000	1,1001	2,7999
		6,00	,56905	,40089	,175	-,2808	1,4189
		7,00	,52566	,40089	,208	-,3242	1,3755
		8,00	1,95000*	,40089	,000	1,1001	2,7999
	2,00	1,00	-,43701	,40089	,292	-1,2869	,4128
		3,00	,33454	,40089	,416	-,5153	1,1844
		4,00	-1,40368*	,40089	,003	-2,2535	-,5538
		5,00	1,51299*	,40089	,002	,6631	2,3628
		6,00	,13203	,40089	,746	-,7178	,9819
		7,00	,08865	,40089	,828	-,7612	,9385
		8,00	1,51299*	,40089	,002	,6631	2,3628
	3,00	1,00	-,77155	,40089	,072	-1,6214	,0783
		2,00	-,33454	,40089	,416	-1,1844	,5153
		4,00	-1,73822*	,40089	,001	-2,5881	-,8884
		5,00	1,17845*	,40089	,010	,3286	2,0283
		6,00	-,20250	,40089	,620	-1,0524	,6474
		7,00	-,24589	,40089	,548	-1,0957	,6040
		8,00	1,17845*	,40089	,010	,3286	2,0283
	4,00	1,00	,96667*	,40089	,028	,1168	1,8165
		2,00	1,40368*	,40089	,003	,5538	2,2535
		3,00	1,73822*	,40089	,001	,8884	2,5881
		5,00	2,91667*	,40089	,000	2,0668	3,7665
		6,00	1,53571*	,40089	,001	,6859	2,3856
		7,00	1,49233*	,40089	,002	,6425	2,3422
		8,00	2,91667*	,40089	,000	2,0668	3,7665
	5,00	1,00	-1,95000*	,40089	,000	-2,7999	-1,1001
		2,00	-1,51299*	,40089	,002	-2,3628	-,6631
		3,00	-1,17845*	,40089	,010	-2,0283	-,3286
		4,00	-2,91667*	,40089	,000	-3,7665	-2,0668
		6,00	-1,38095*	,40089	,003	-2,2308	-,5311
		7,00	-1,42434*	,40089	,003	-2,2742	-,5745
		8,00	,00000	,40089	1,000	-,8499	,8499
	6,00	1,00	-,56905	,40089	,175	-1,4189	,2808
		2,00	-,13203	,40089	,746	-,9819	,7178
		3,00	,20250	,40089	,620	-,6474	1,0524
		4,00	-1,53571*	,40089	,001	-2,3856	-,6859
		5,00	1,38095*	,40089	,003	,5311	2,2308
		7,00	-,04339	,40089	,915	-,8932	,8065
		8,00	1,38095*	,40089	,003	,5311	2,2308
	7,00	1,00	-,52566	,40089	,208	-1,3755	,3242
		2,00	-,08865	,40089	,828	-,9385	,7612

*. The mean difference is significant at the 0.05 level.