CHV1 hypovirus infection on *Cryphonectria parasitica* - effects on pathogenesis and laccase activity

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Bragança

2021

Abstract

Biological control by hypovirulence is an efficient method to control chestnut blight. The presence of *Cryphonectria hypovirus* 1 (CHV1) in *Cryphonectria parasitica* reduces its parasitic growth and sporulation capacity, female fertility, pigmentation, oxalate accumulation and laccase production. Indeed, laccases are involved in lignin degradation, and are also considered as a virulence determinant in *C. parasitica*. The aim of this work was to evaluate the laccase production in both virulent and converted strains and to access the virulence of these strains in vitro and on dormant chestnut stems. Five isolates were converted with two characterized hypovirulent *C. parasitica* isolates (RBB111, SR44.2). To evaluate the virulence of the isolates, dormant chestnut stems were inoculated with the virulent isolates, their converted ones and the hypovirulent isolates. The qualitative evaluation of laccase production was performed using Bavendamm test and RBBR test. For quantitative evaluation of laccase production strains were grown on PDB (Potato Dextrose Broth, 24g/L) and the sample readings taken by spectrophotometry using ABTS. The hypovirulent isolates used in this work has complete ability to convert virulent isolates. The infection area on chestnut stems caused by virulent strains was significantly higher ($P<0.05$) than the infection area caused by converted strains. In the Bavendamm test, the virulent isolates Cast13, Cast26 and VDP11 showed the highest dark area indicator of a higher enzymatic activity while the converted isolates by SR44.2 or RBB111, showed a great reduction of the coloration and this is more observed in the converted isolates by RBB111. For the quantitative assay the specific activity was determined and showed that among all the virulent isolates VDP11 had the highest laccase activity followed by Cast13 and this activity was reduced also in the converted ones especially with RBB111.

Keywords: *Cryphonectria parasitica*, virulence, laccase

Resumo

O controlo biológico por hipovirulência é um método eficiente para controlar o cancro do castanheiro. Em *Cryphonectria parasitica* a presença de *Cryphonectria hypovirus* 1 (CHV1) reduz o seu crescimento, capacidade de esporulação, fertilidade feminina, pigmentação, acumulação de oxalato e produção de lacase. As lacases estão envolvidos na degradação da lignina, e também são consideradas como fatores da virulência em *C. parasitica*. O objetivo deste trabalho foi avaliar a produção de lacase, tanto em estirpes virulentas como convertidas e avaliar a virulência destas estirpes *in vitro* e em ramos dormentes e destacados de castanheiro. Cinco isolados virulentos de *C. parasitica* foram convertidos com dois isolados hipovirulentos anteriormente caracterizados (RBB111, SR44.2). Para avaliar a virulência dos isolados, os ramos dormentes de castanheiro foram inoculados com os isolados virulentos, os convertidos e os isolados hipovirulentos. A avaliação qualitativa da produção de lacase foi efetuada através do teste Bavendamm e do teste RBBR. Para avaliação quantitativa de lacase as estirpes em estudo foram cultivadas em PDB (Potato Dextrose Broth, 24g/L) e as leituras das amostras obtidas por espectrofotometria utilizando ABTS. Os isolados hipovirulentos utilizados neste trabalho evidenciaram total capacidade de converter os isolados virulentos. A área de infeção nos caules de castanheiro causada por estirpes virulentas foi significativamente maior ($P<0,05$) do que a área de infeção causada por estirpes convertidas. No teste de Bavendamm, os isolados virulentos Cast13, Cast26 e VDP11 mostraram o maior indicador de área escura, indicadora de maior actividade enzimática, enquanto que os isolados convertidos por SR44.2 ou RBB111 evidenciaram redução da intensidade de coloração, mais evidente nos isolados convertidos por RBB111. Para o ensaio quantitativo, a actividade enzimática específica foi determinada, evidenciando a maior actividade no isolado virulento VDP11, logo seguido pelo Cast13. Nos isolados convertidos a actividade específica lacásica decresceu, especialmente nos convertidos com RBB111.

Keywords: *Cryphonectria parasitica*, virulência, lacase

Acknowledgement

I would like to express my deep and sincere gratitude to my research supervisors, professor Oucema Souai , EUGÉNIA GOUVEIA and professora LURDES JORGE, for giving me the opportunity to do research and providing invaluable guidance throughout this research .It was a great privilege and honor to work under their guidance .I'm extremely grateful for what they has offered to me .

I would also like to extend my gratitude to Doctor VALENTIM COELHO for the continuous support of my thesis study and research, for his patience, motivation, enthusiasm and caring for the evolution of my thesis.

I am very much thankful to my parents and my sister for their love, understanding, and continuing support to complete this research work.

Last but not least I express my thanks to my friend Khalil Hmida and Hedil Hadded for their support and motivations to complete this thesis successfully.

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Abbreviations

ABTS: Diammonium salt 2, 2′-azino-bis

ANOVA: Analysis of variance test

CABI: Centre for Agricultural Bioscience International

CATs: Conidial anastomosis tubes

cDNA: Complementary deoxyribonucleic acid

CHV1: Mycovirus *Cryphonectria Hypovirus 1*

Cu: Copper

EDTA: Ethylenediaminetetraacetic acid

EPPO: European and Mediterranean Plant Protection Organization

H_2O_2: Hydrogen peroxide

HCl: Hydrochloric acid

Lac: Laccase

Lac3: A tannic acid-inducible laccase

LiP: Lignin Peroxidase

LMW: low molecular weight

MAP: Mitogen-activated protein

$MgSO_4$: Magnesium sulfate.

MM: Minimal medium

Mn^2: Manganese

MnP: Manganese peroxidase

$NaNO_3$: Sodium nitrate

NaOH: Sodium hydroxide

PDA: Potato dextrose agar

PDB: Potato dextrose broth

RBBR: Remazol Brilliant Blue R- $C_{22}H_{16}N_2Na_2O_{11}S_3$

RNA: Ribonucleic acid

Rpm: Revolutions per minute

UV : Ultraviolet

VC : Vegetative compatibility

VCG: Vegetative compatibility group

°**C :** degree Celsius

I. Introduction

The chestnut tree (*Castanea: Fagaceae*) is considered as one of the most remarkable trees due to its economic importance by producing wood and as a fruit producer. Chestnuts belong to the genus *Castanea*, in the Fagaceae family which has 4 major species: American chestnut (*C. dentata* (Marsh) Borkh), European chestnut (*C. sativa* Mill.), Chinese chestnut (*C. mollissima* Blume) and Japanese chestnut (*C. crenata* Siebold & Zucc.) (Rigling and Prospero, 2017).

In the Portuguese mainland, chestnut trees mainly occur in the north. The yield of chestnuts, in an area 38,874 ha, is approximately 34,131 tons (INE, 2019). These chestnuts are recognized in the international markets for their high quality. In 2007 Trás-os-Montes is known as the first chestnut-growing region in Portugal with approximately 85% of the total area of chestnut in the country, followed by Beira Interior with approximately 9% in area and production (Ministério da Agricultura, 2007).

In the last two decades, a decrease of chestnut production was noticed and this was mainly attributed to the occurrence of the pathogenic fungi. Chestnut trees can be affected by different phytopathogens such as *Cryphonectria parasitica* associated with chestnut blight and *Phytophthora cinnamomi* and *Phytophthora cambivora* associated with ink disease (Santos, 2017).

Many measures were taken by the governments to control the spread of the disease, especially the regulation of movement and commercialization, but they were not effective. It was found that *C. parasitica* can be infected naturally by a mycovirus called *Cryphonectria hypovirus* 1 (CHV1), which reduces parasitic growth, sporulation, pigmentation capacity and diminishing the activity of pathogenesis–related enzymes such as laccase, involved in lignin degradation and also considered as a virulence determinant in *C. parasitica* (Chung *et al.*, 2008).

This study aims to understand the effect of *Cryphonectria hypovirus* 1 (CHV1) in pathogenesis and virulence of *C. parasitica* by studying biochemical determinants specially the activity rate of the C. *parasitica* lac3, in virulent, hypovirulent and hypovirulent isogenic converted stains.

II. Literature review

1. Chestnut blight

1.1 History

During the 20th century, *C. parasitica* was accidentally introduced into North America through infected chestnut plants (Griffin, 1986), causing severe ecological and economic problems. In Europe, it was first detected in 1938 in Italy, near Genoa and rapidly spread into the neighboring regions of France, Switzerland and Slovenia (Ringling and Prospero, 2017). Occurred in Portugal for the first time in 1989 in the region of Trás-os-Montes and twelve years were sufficient for chestnut blight to become a widespread disease in northern Portugal (Gouveia *et al.,* 2001)

1.2 Symptoms

The chestnut blight was considered as the most destructive canker disease infecting stems, branches and, eventually, twigs (Rigling and Prospero, 2017). *C. parasitica,* develops rapidly and a typical pale brown mycelial fan is a clear sign of a chestnut blight infection. The most pronounced early symptoms are leaves wilt, turn yellow or brown, and typically remain hanging on the infected dead branches, (so-called flag) (Rigling and Prospero, 2017).

The manifestation of symptoms depending on the virulence of *C. parasitica* strain and the age of the infected tree part: in smaller branches or twigs cankers formed cause the death within a few months and orange to reddish-brown coloration. On thicker branches or stems canker may develop over years before causing mortality and coloration is generally less pronounced (Rigling and Prospero, 2017).

2 .*Cryphonectria parasitica*

2.1 Taxonomy

Cryphonectria parasitica (Murr.) Barr. is a Sordariomycete (Ascomycete) fungus in the family Cryphonectriaceae (Order Diaporthales). Closely related species that can also be found on chestnut include *C. radicalis, C. naterciae and C. japonica*.

2.2 Distribution

C. parasitica is widespread throughout the eastern USA, in China and Japan, where it is native, and in many countries of Europe (Figure 1) that have significant *Castanea* populations (CABI/EPPO 2020). This is explained by the fact that there were several

exchanges of vegetative material (which could be contaminated) between these countries, as well as the fact that the spread is done by fungi sexual spores which can cover large areas. All of these factors contributed to generalize the fungus and to create a worldwide disease.

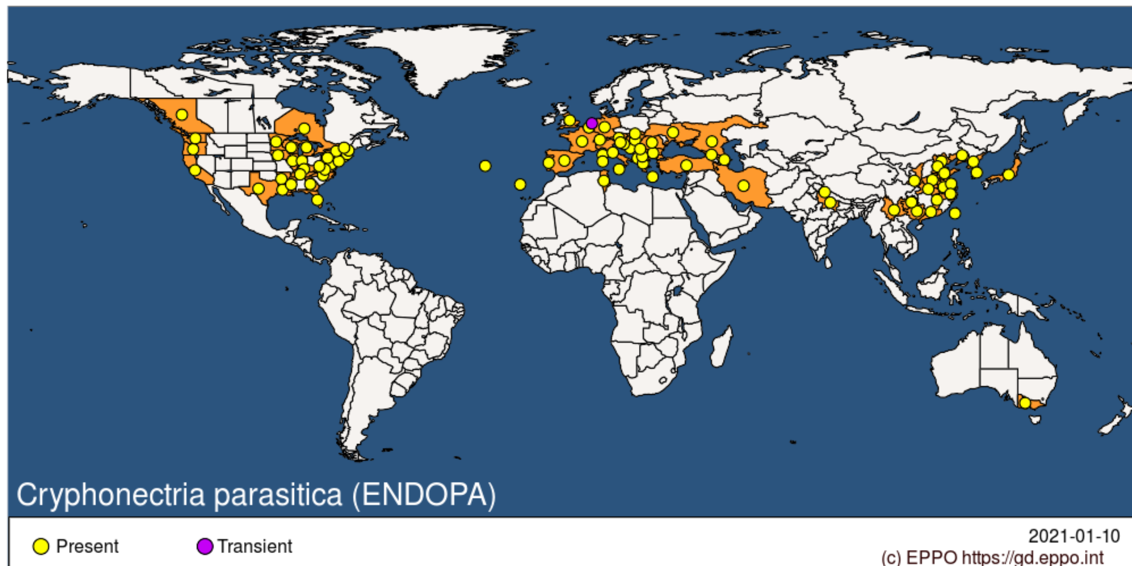


Figure 1 : Distribution of *C. parasitica* in the world (CABI/EPPO, 2020)

2.3 Life cycle

Both asexual and sexual spores are able of causing infection (Prospero *et al*., 2006). The type of reproduction is determined by environmental factors like the nutrition available, temperature, and light (Smith, 2013). The female sexual organ, or protopedthecium, is formed by either mating-type or in response to nitrogen starvation, light and relatively low temperature. The sexual reproduction occurs inside a multicellular fruit body, which is composed of sexual tissue (the hymenium) surrounded by a protective membrane. The sexual life initiates by the differentiation of female organs (ascogonia), which can be fertilized by hyphae or asexual spores. After fertilization, the male gametic nucleus is delivered into the ascogonium, which contains the female gametic nuclei results in the formation of the hymenium (2n). The diploid nucleus undergoes meiosis to give haploid nuclei called ascospores. The ascospores are then released and disseminated in the environment and after germinate, form hyphae and start new mycelia. Asexual reproduction is frequent and involves the production of conidiophores that release haploid conidiospores which are formed by mitosis (male gamete). In *C. parasitica*, mating is controlled by a single mating type (MAT) locus, which contains either the MAT-1 or MAT-2 allele (Marra *et al.,* 2004). After the spores germinate, conidia form lesions and then develop into bark cankers. The conidia are released from the pycnidia, forming long, twisted yellow tendrils.

2.4 Host range

Chestnuts (*Castanea* spp.), particularly *C. dentata* and *C. mollissima* show resistance but may also become infected (Headland *et al.*, 1976). *C. parasitica* can also attack other species (Table 1) such as *Quercus* spp. *Castanopsis*, *Acer, Rhus typhina* and *Carya ovate.* Within the EPPO region, *Castanea* spp. (especially *C. sativa*) is the main host.

Table 1 : Host Plants and Other Plants Affected(CABI /EPPO, 2020)

Plant name	**Family**
Castanea dentata (American chestnut)	Fagaceae
Castanea sativa (chestnut)	Fagaceae
Eucalyptus	Fagaceae
Malus domestica (apple)	Myrtaceae
Quercus (oaks)	Rosaceae
Quercus coccinea (scarlet oak)	Fagaceae
Quercus frainetto (Hungarian oak)	Fagaceae
Quercus ilex (holm oak)	Fagaceae
Quercus petraea (durmast oak)	Fagaceae
Quercus rubra (northern red oak)	Fagaceae
Quercus stellata (Post oak)	Fagaceae

3. Hypovirulence in chestnut blight fungus

3.1 Pathogenicity and Virulence

Several studies have been carried out in order to understand the difference between pathogenicity and virulence and define the meaning of each. According to Shapiro-Ilan *et al.* (2004), pathogenicity is the quality or state of being pathogenic, the potential ability to produce disease, whereas virulence is the disease producing power of an organism, the degree of pathogenicity within a group or species. Pathogenicity is a qualitative term, whereas virulence is a term that quantifies pathogenicity (Shapiro-Ilan *et al.*, 2004).

3.2 Cryphonectria hypovirus 1 (CHV1)

Cryphonectria hypovirus 1 (CHV1) is a member of the virus family *Hypoviridae*, occurring in Asia and Europe (Peever *et al.,* 1998). Hypoviruses are positive strand RNA viruses, located in the cytoplasm of the fungal host. It is distinguished by its ability to attenuate fungal virulence and alter some development processes like reducing pigment production, suppression of asexual sporulation and loss of female fertility. The use of hypovirulence-associated mycoviruses has been proposed as a new strategy for biological control of fungal diseases (Chiba *et al*., 2009). The success of biological control depends on the integration of multiple factors (Milgroom and Cortesi, 2004). Among the factors influencing the success of biological treatment, we can mention in first place vegetative incompatibility which reduces the horizontal transmission, which is considered as the most important parameter in models of pathogen invasion. This biological control method is very effective when the population structure of the vic genes of the parasitic fungus is known, and the compatible hypovirulent strains are applied (Anagnostakis *et al*., 1986; Heiniger and Rigling, 1994). In Portugal the population of *C. parasitica* is essentially clonal and dominated by vc type EU11, although other vc types, namely EU01, EU02, EU12 and EU66 are also present and sometimes represented by a few or single isolates (Gouveia *et al*., 2016). CHV1 has been successfully used for the control of chestnut blight caused by *C. parasitica* in Europe. Otherwise it has been proved that the success of biological control with hypovirulence depends on hypoviruses spreading through the fungal host population: it should be faster than the fungus reproduction. Finally, a critical factor in chestnut blight epidemiology is whether *C. parasitica* reproduces by ascospores or by conidia. Ascospores are virus-free (Carbone *et al.,* 2004) with a high dispersal capacity compared to that of conidia that maintain genetic barriers by vegetative incompatibility (Milgroom and Cortesi, 2004).

3.3 Mode of transmission

The hypovirus CHV1 has a positive strand RNA genome and, like other fungal virus, can be transmitted mainly horizontally by fungal mycelial anastomosis and vertically only through asexual spores.

The process of vegetative hyphal fusion, or anastomosis, is a fundamental activity for *C. parasitica* that enables the transmission of the viral genome in response to the environment.

The fusion between conidia also occurs and involves the formation and interaction of specialized hyphae called conidial anastomosis tubes (CATs). To understand more the mechanism and the factors influencing the formation some studies (Figure 2) proposed that the CAT formation activates a mitogen-activated protein (MAP) kinase cascade. In fact, a target mutation was produced in the gene encoding the kinase and resulted in the blocking of the formation of CATs (Read *et al*., 2013).

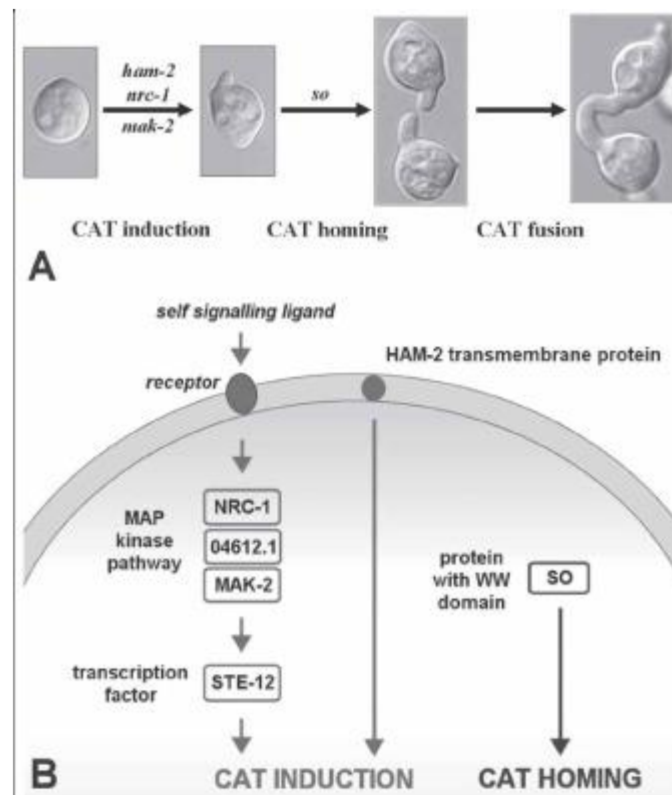


Figure 2 : Conidial anastomosis tube induction and signalling which occurs during CAT induction. A) Mutants blocked in CAT induction. B) Model of the signalling pathways involved in CAT induction (Read *et al.*, 2013)

There are various mechanisms for conidial germination and it varies with conidia producing species. However, in *C. parasitica* some events usually occur: due to the presence of water and air, the conidia rapidly swell (hydrate) and change their surface properties. The nucleus reorganizes and the hyphae begin to grow after a few hours. Among the many genera of plant pathogenic fungi (such as *Magnaporthe, Colletotrichum, Ustilago*), the tip of the hyphae will swell to form the appressorium. At the same time, many metabolic activities, including respiration, RNA and protein synthesis, trehalose breakdown, as well as differences in the composition of the cell wall can be detected (Osherov and Gregory, 2001).

4. Composition of the plant cell walls

Plant cell walls are composed of three main components: cellulose, hemicellulose and lignin. In addition, small amounts of other substances may be found in lignocellulose residues, such as protein and pectin.

Cellulose is a linear biopolymer of glucoses, which are connected by β-1,4-glycosidic bonds and is the main component of all plant materials (Dashtban *et al.*, 2010). Unlike cellulose, hemicellulose is a heterogeneous polymer of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids (Badal, 2000).

Lignin is the most abundant naturally occurring aromatic polymer composed of phenylpropanoid units, known as monolignols or lignin precursors, which are linked together through carbon–carbon and carbon–oxygen bonds with a varying degree of methoxylation. It is predominantly concentrated in the middle lamella and in the primary cell wall providing stiffness to the cell walls and gluing the cell together, allowing the protection of polysaccharides against microbial degradation (Galbe and Zacchi, 2007). By connecting cellulose and hemicellulose, lignin can act as a barrier and prevent enzymes from penetrating the internal lignocellulose structure. As a hydrophobic polymer, lignin also serves as a barrier against water penetration.

5. Characteristics of laccases and their role in fungal phytopathogenesis

Laccases (benzenediol: oxygen oxidoreductases, E.C 1.10.3.2) are multi-copper oxidases. Their active site contains four copper atoms in special oxidation states, which mediate the redox process and are divided into three categories according to their magnetic and spectral properties (Messerschmidt and Huber, 1990). Figure 3 shows the three types of copper coordination found in laccases. According to Chandra and Chowdhary (2014) and Janusz *et al.* (2017):

- Type1 or blue copper center, coordinated with one cysteine, one methionine, and two histidine molecules, evidences a strong electron adsorption at 600 nm (Palmieri *et al.*, 2003) - is the substrate-binding site.
- Type 2 coordinated with two histidines and a water molecule; it is colorless with no absorption in the visible spectrum – it binds to the inducer/inhibitor and oxygen;

- Type 3 coordinated with three histidine, shows a weak UV absorbance at 330 nm (Wong, 2009).

The Type 2 and Type 3 copper atoms form a trinuclear cluster that catalyzes the fixation and reduction of oxygen to water - the O_2 molecule binds to both Type 2 and Type 3 copper active sites (Janusz *et al.*, 2017). The stability of the enzyme is directly affected by the hydrogen bonds and salt bridges existing between copper atoms (Hildén *et al.*, 2009).

Laccases oxidize a vast variety of compounds using O_2 as an electron acceptor, liberating H_2O as the only by-product, without intermediate production of hydrogen peroxide (Morozova *et al.,* 2007). For this they are known as "green catalysts." They can oxidize several aromatic compounds (substituted phenols, amino-phenols, polyphenols, *o*- and *p*-diphenols, polyamines, methoxyphenols, aryl diamines, aromatic amines, and thiols), but also some inorganic compounds such as iodine and ferrocyanide ions (Claus, 2003).

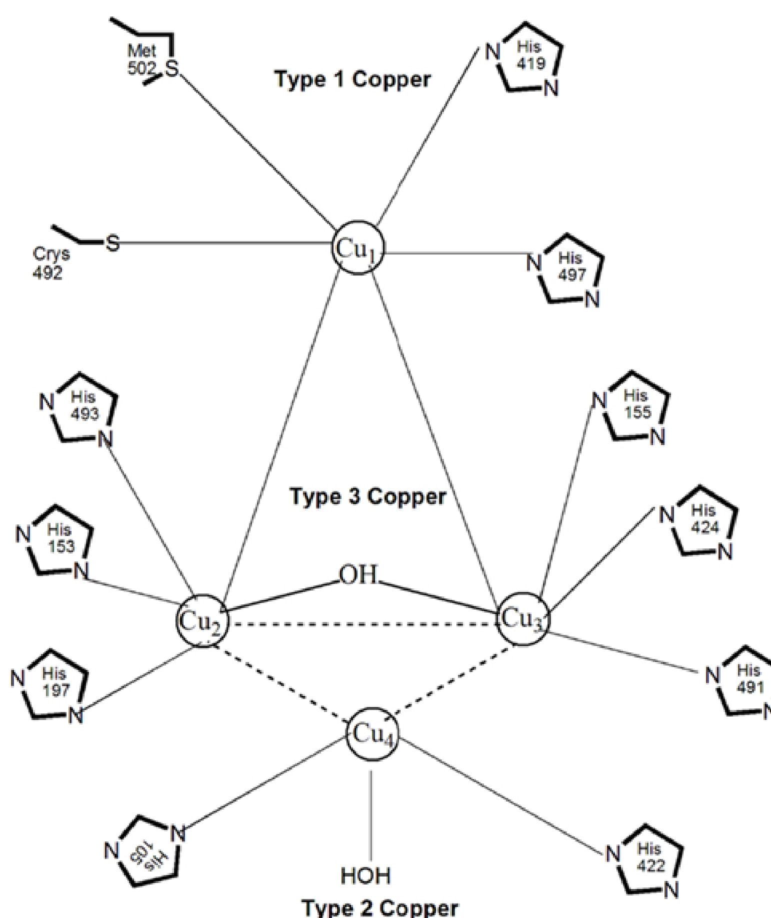


Figure 3 Structure of the copper coordination network of the laccase molecule (adapted from Lew and Bin; 2014).

Nonetheless, the low redox potential of laccases (0.5–0.8 V) hinders them from directly taking part in catalysis of the complex compounds - laccases can only oxidize phenolic lignin structures but not the non-phenolic aromatic structures, which comprises more than 80% of lignin (Wong, 2009). Only a small group of peroxidases secreted by ligninolytic fungi, such as lignin peroxidase, can oxidize the non-phenolic groups of lignin directly (Castillo *et al.,* 1997).

However, in the presence of some low molecular mass mediators (small chemical compounds that are continuously oxidized by the enzyme and then reduced by the substrate; some examples shown in Figure 4), laccases can also attack non-phenolic aromatic compounds with high redox potentials (Kunamneni *et al.,* 2007). The basis of the laccase-mediator concept is that those low molecular weight compounds (LMW), once oxidized by the enzyme to stable radicals, act as redox mediators, oxidizing other compounds that are not substrates of laccase. Because fungal laccases are too large to penetrate the intact wood cell wall, these LMW fungal metabolites, due to their small size, can penetrate the pores of plant cell walls (Srebotnik *et al*., 1988) and target different substrates such as lignin and aromatic compounds which are not directly accessible by laccase - in the presence of LMW, acting as diffusible oxidative agents and electron shuttles for enzymatic systems, substrate range of laccases can be expanded to include the oxidation of non-phenolic lignin, playing a key role in its depolymerizing during the early stages of wood decay (Schmidt, 2007).

Since all four copper atoms from the active site are fully oxidized (Cu^{2+}) in the native form of laccases, they are able to decarboxylate, demethylate and demethoxylate phenolic and methoxyphenolic acids in an initial step of lignin biodegradation (d'Acunzo *et al.,* 2002). Camarero *et al.* (1994) suggested that the initial oxidative attack proceeds in the phenolic lignin moiety (<20% of total lignin), resulting in the in the release of LMW compounds, with higher redox potential (>0.9 V), such as the oxidized side chains of phenolic residues, composed by phenolic aldehydes, ketones, and acids, able to act as mediators and to accomplish the oxidation of non-phenolic lignin (Reddy *et al*., 2003).

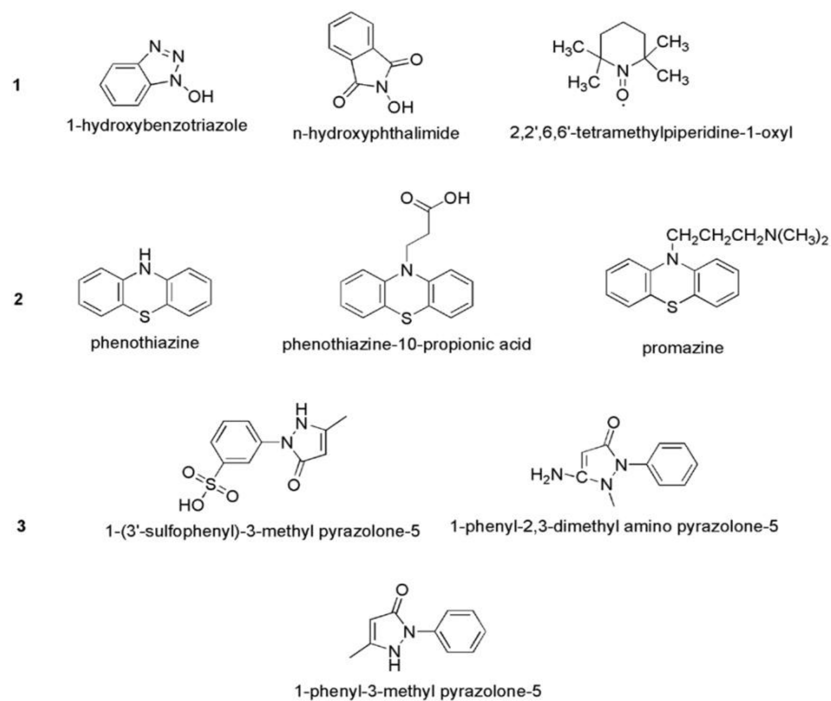


Figure 4 **:** Chemical structures of some synthetic laccase mediators (adapted from Lew and Bin; 2014)

5.1 Factors influencing laccase activity

Laccase production usually occurs in the secondary metabolism of different fungi, and is affected by different aspects, such as fungal species, culture type (stationary or agitated), aeration and time (Brijwani *et al.*, 2010). This can be explained by the fact that metabolism may be controlled by environmental conditions and culture medium composition (Faraco *et al.*, 2002). A study done on *Trametes versicolor*, considered one of the most effective laccase-producing fungus (Jang *et al.*, 2002; Rosales *et al.*, 2007) highlighted copper and ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as the most efficient inducers among the tested inducers.

In order evaluate the effect of copper on laccase production Jang *et al* (2002) tested different concentration of Cu and detected a rapid increase in the laccase activity, especially with the optimal concentration of 0.5mM of Cu. Conversely, 5.0 mM of Cu showed an inhibitory effect on activity. Regarding the ABTS effect, different concentrations of ABTS were tested, and the highest laccase activities were observed between 0.025mM and 0.05 mM ABTS. In order to test the effect of these two inducers on laccase production, it was prepared a media containing 0.5 mM of Cu and 0.05 mM of ABTS, that acted synergistically, showing

a significant induction of laccase production when compared with the utilization of each of the inducers alone.

Although few studies have been conducted that combine multiple inducers, Tong *et al.* (2007) tested the effect of Cu^{2+} and o-toluidine on *Trametes* sp., and obtained induction of laccase activity. Regarding the influence of carbon source, some studies have shown that the specific activity of laccase can be increased according to the choice of carbon source. For example, when fructose is substituted for glucose, the specific activity of laccase increases. In addition, according to Couto and Herrera (2006), studies on *Trametes hirsuta* demonstrated that sequential addition of different carbon sources, such as glucose followed by glycerol, resulted in a higher laccase production rate, compared to cultures supplemented only with glucose or cellulose. Finally, some ions like Ag^{+} and Mn^{+2} have also been reported as modulators of laccase transcription in *Trametes versicolor* (Manubens *et al.,* 2007), but it can exert opposite effects in different fungal species.

6. Role of laccase in pathogenicity and its effect in the virulence of different fungi

Laccase has been shown to be an important virulence factor in many diseases caused by fungi. Among other roles, laccase can protect the fungal pathogen from antibiotic compounds and toxic present in the tissues of the host.

Nun *et al.* (1988) suggested that laccase secreted by *Botrytis cinerea* acted as a detoxifying enzyme to protect the fungus from toxic metabolites. They demonstrated that plants like *Ecballium elaterium*, synthesizing cucurbitacins (a steroide which directly inhibits the synthesis of laccase in *B. cinerea)* is not or only weakly affected by the parasite. To confirm this fact they used EDTA, which causes the suppression of laccase expression (EDTA reduces the availability of Cu^{2}, indirectly reducing laccase activity). Using EDTA for the pretreatment of the fungi to eliminate the presence of laccase, resulted in differences in degree of infection instead of spreading throughout the plant tissues as usual, the pre-treated fungi had no hyphae and the enzymatic activity was hardly observed. They also reported the morphological differences on mycelia: the mycelium had a dark coloration at the untreated tissues but it was not present at the treated tissues, highlighting laccases occur in the first phase of the fungi invasion.

Finally, it has shown that the destruction of plant phytoalexin is directly related to laccase. Phytoalexin is an important factor in plant resistance to phytopathogenic fungi

(Mobius *et al.,* 2009), and the ability of parasites to detoxify these compounds may be an important part of the pathogenic mechanism of the parasites.

7. Application of laccases

Because of their capability to oxidize a wide range of toxic and environmentally problematic substrates, fungal laccases are of particular interest with regard to potential industrial applications, for instance in the textile, food, wood processing, pharmaceutical and chemical industries (Table 2).

The textile industry accounts for two-thirds of the entire dyestuff market and consumes large amounts of water and chemicals to wet-process textiles. The chemical reagents used are resistant and difficult to decolorize due to their synthetic origin. The development of process based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure. In fact nowadays laccases are used to bleach textiles and even to synthesize dyes giving the example of *Lentinus edodes* which displays the greatest decolorization ability both in terms of extent and rapidity.

In the industrial preparation of paper, the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine-based or oxygen-based chemical oxidants. Some environmental benefits are envisaged, and the fact that LMS (low molecule system) can be easily implemented in existing bleaching process is seen as a major advantage, which may lead to a partial replacement of the use chlorine dioxide in pulp mills.

In food industry, laccase can be used in elimination of undesirable phenolics. Keum and Li (2004) used laccases from *Trametes versicolor* and *Pleurotus ostreatus* to degrade hydroxy PCBs (organic chlorine compound).

Table 2 : Applications of laccase (adapted from S.S. Desai and C. Nityanand, 2011)

Application	Organism	Reference
Dye decolorization	*T. hirsuta*	Roriz *et al.* (2006)
	T. hirsuta	Couto and Toca Herrera (2006a, b)
	T. versicolor CCT 4521	Minussi *et al.* (2007)
	Stereum ostrea	Viswanath *et al.* (2008b)
	S. maltophilia AAP56	Dube *et al.* (2008)
	*Trametes sp.*strain SQ01	Yang *et al.* (2009)
	T. villosa	Yamanaka *et al.* (2008)
	T. trogii	Ciullini *et al.* (2008)
	Laetiporus sulphurous and *Coriolus versicolor.*	Mazmanci *et al.* (2009)
Degradation of xenobiotics	*Stropharia rugosoannulata*	Steffen *et al.* (2007)
	Stropharia coronilla	Steffen *et al.* (2007)
	Coriolopsis polyzona	Cabana *et al.* (2007)
	Rigidoporus lignosus	Cambria *et al.* (2008)
Biodegradation and Bioredmediation	*T. versicolor*	Bastos and Magan (2009)
	Cerrena unicolor	D'Souza-Ticlo *et al.* (2009)
	Streptomyces ipomoea CECT 3341	Molina-Guijarro *et al.* (2009)
	White-rot fungi (polyporus)	Zhao *et al.* (2010)
	Ganoderma lucidum Chaaim-001 BCU	Punnapayak *et al.* (2007)
Effluent treatment	*T. versicolor*	Pedroza *et al.* (2007)
	Trametes versicolor	Cordi *et al.* (2007a)
	Lentinula edodes	Cordi *et al.* (2007a)
	Botrytis cinerea	Cordi *et al.* (2007a)
	Trametes trogii	Ellouze *et al.* (2008)
	Lentinus tigrinus	Ellouze *et al.* (2008)
Biosensors	*Cerrena unicolor*	El Kaoutit *et al.* (2008)
	Trametes hirsuta	El Kaoutit *et al.* (2008)

III. Objectives

- To characterize virulent isolates
- To obtain the isogenic hypovirulent isolates through hyphal anastomosis with a compatible CHV isolate
- To evaluate the pathogenic capacity through inoculation in stems
- To check *C. parasitica* laccase 3 activity on plate assays using Bavendamm test
- To evaluate *C. parasitica* laccase activity by spectrophotometry using ABTS

IV. Material and methods

1. *Cryphonectria parasitica* strains

C. parasitica isolates used in this study, listed in the Table 3, were obtained from different orchards of Bragança and Vinhais in the North of Portugal. The RBB111 hypovirulent strain was obtained in 2011 in the Valpaços region and the SR44.2 hypovirulent strain was obtained in 2013 in Sergude (Felgueiras). Hypovirulent strains are characterized by having a white morphology in PDA (Potato Dextrose Agar) culture medium and reduced conidiation. The presence of the hypovirus CHV1 in converted isolates with SR44.2 hypovirulent strain was based on morphological characteristics. All isolates are preserved in the fungal collection of the Instituto Politécnico de Bragança and both characterized hypovirulent isolates are also preserved in the international collection of the Micoteca of the University of Minho.

Table 3 : Strains of *C. parasitica* isolates

Code	VCG	Sites	Virulence
Cast13	EU66	Castrelos (Bragança)	Virulent
Cast26	EU66	Castrelos (Bragança)	Virulent
RBB111	EU11	Rio Bom (Valpaços)	Hypovirulent
SR44.2	EU11	Sergude (Felgueiras)	Hypovirulent
Cast17	EU11	Castrelos (Bragança)	Virulent
Cast07	EU11	Castrelos (Bragança)	Virulent
VDP11	EU11	Vilar de Peregrinos (Vinhais)	Virulent

2. Conversion capacity

Virulent isolates were converted with a characterized hypovirulent of *C. parasitica* CHV1 strain by the method of Rigling *et al.* (1989). To ensure the transmission of CHV1, virus-donor (hypovirulent strain) and virus-recipient isolates (virulent isolate) were paired on PDA medium (Potato Dextrose Agar) (Figure 5). In Petri dishes of 90 mm each combination was done in three replicates to assess the conversion ability. The plates were placed at 25 ± 2ºC in the dark for 14 days followed by 7 days of exposure to natural light to determine whether the hypovirulent strains (RBB111 or SR44.2) has the capacity to transmit the CHV1 hypovirus to virulent strains. After 21 days, the small section of the mycelium of the converted strains were removed and transferred to fresh PDA medium. The white culture morphology was the criterion used for the diagnosis of the converted isolates. The conversion was considered successful when the virulent strain presented the same morphology as the hypovirulent strain and there was no conversion when each strain presented its corresponding morphology. Mycelium of the converted strains was maintained at 6-8 °C at the Instituto Politécnico de Bragança culture collection.

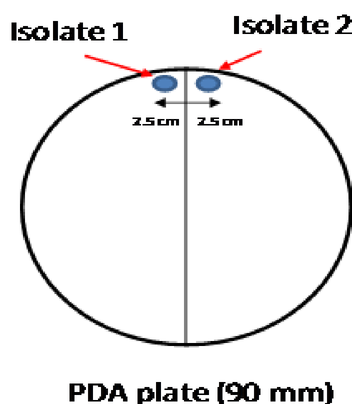


Figure 5: The pairing between *C. parasitica* isolates

3. Growth of *Cryphonectria parasitica* strains *in vitro* (PDA medium)

The isolates were inoculated on PDA Petri dishes and incubated at 25° C in the dark, during one week. The cultures were exposed to daylight at room temperature on the laboratory bench for 5 days and culture morphology was observed. In order to study the growth of isolates measures were taken every 3 days and the area was calculated. Mycelial growth data are presented as mean ± standard deviation (mean ± SD).

4. Growth of *Cryphonectria parasitica* strains in dormant branches of chestnut

Chestnut branches were collected in the nursery at ESAB-IPB open field in the year 2020. Chestnut branches were cut into sections approximately 20 cm long. On both cut sides of the branches, paraffin wax was applied to avoid desiccation. In the middle of each branch a cork borer (3mm diameter) was used to excise a disk of the bark tissues that was inoculated with mycelium of each isolate. The name of the isolate was registered in the branch. Then, the inoculation sites were covered with cotton wool moistened with distilled water and surrounded with parafilm to avoid desiccation. Finally, chestnut branches (three branches from each isolate) were placed on a tray and incubated at 25°C ± 2ºC for 25 days. Inoculated branches were checked for fungal growth after each 5 days. Fungal growth was assessed as the vertical and horizontal expansion of the visible necrotic area. The area of each necrosis was calculated using the mathematical formula for elliptic surfaces.

5. Qualitative evaluation of ligninolytic enzyme production

To assay ligninolytic activity, substrates like ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) - a low molecular weight mediator) and RBBR (Remazol Brilliant Blue R - $C_{22}H_{16}N_2Na_2O_{11}S_3$) were used in Petri plate assays. Tannic acid, a well-known *C. parasitica* laccase 3 inducer (Rigling *et al.*, 1989), was used as a component of the Bavendamm test.

5.1 ABTS ASSAY

In order to assess laccase activity squared Petri plates of 120 x 120 x 15mm were prepared with PDA supplemented with a 0.1% ABTS 1M solution, then inoculated with plugs from fungal strains recovered with sterile glass Pasteur pipettes, and incubated at 25°C in darkness for 7 days. The activity was observed directly on plates based on the intensity of coloration.

5.2 Bavendamm test

This medium contains 1.5% malt extract, 2% agar and 0.5% tannic acid required for induction of *C. parasitica* lac3 activity. For this, a concentrated solution of 5% tannic acid was prepared, autoclaved and added to sterile PDA. The diameter of the browning area in each plate has been calculated

5.3 RBBR test

For detection of ligninolytic activity (not only laccases activity, but also MnP, LiP and VP), mycelia of selected isolates were grown on malt extract agar (MEA) solid medium supplemented with 0.04% RBBR and 600µM $CuSO_4$. For this, a 20% solution of RBBR and a 400mM solution of $CuSO_4$ were prepared and filtered through a 0.2µm filter.

In a previous step, in order to understand the effect of $CuSO_4$ concentration on laccase activity, three different concentrations have been prepared - 0.2 mM, 0.4 mM and 0.6 mM, corresponding to three different modalities. Plates were done in duplicate and as controls two inoculated plates were used without the inducer. The plates were incubated in the dark and the diameter of grown mycelia and the intensity of the halo originated by the isolates were measured each 2 days.

6. Quantitative evaluation of laccase production by spectrophotometry

Five 0.5mm plugs of mycelia from the isolates selected for quantitative laccase evaluation were added to 100mL sterile PDB medium in 250 ml Erlenmeyer flasks and incubated at 25°C and 110rpm during four days. Then, the mycelium was filtered and transferred to a minimal medium with 62.5ml salt solution, 2mg thiamine, 10g glucose in a 1liter. For each 100mL minimal media, three sections of *C. sativa* bark were added, for laccase induction, as shown in Figure 6.

Salt solution composition: 24g/L NH4NO3, 16g/L KH2PO4, 4g/L Na2SO4, 8g/L KCl, 2g/L MgSO4 . 7H2O, 1g/L CaCl2, and 8 mL/L of a trace element solution. Trace elements solution was composed by 60mg/L H3BO3, 140mg/L MnCl2 4H2O, 400mg/mL ZnCl2, 40mg/L Na2MoO4, 100mg/L FeCl3 6H2O, 400mg/L CuSO4 5H2O.

The minimal medium was buffered at pH 4.0, using a Na2HPO4 (0.2M)- citrate (0.1M) buffer (McIlvaine buffer). The Erlenmeyer flasks of 500ml, filled with 200mL minimal medium were autoclaved at 121°C for 20 min and incubated at 110rpm, at 25°C. After 3 days of incubation mycelia were filtered and used to evaluate the amount of dry matter. The supernatants were put in 1.5mL Eppendorf and 50mL Falcon tubes, and stocked at -80°C for further analyses.

Figure 6: Mycelium growth of *C. parasitica* isolates in liquid medium (PDB).

6.1 Determination of dry weight of mycelia

To determine biomass, mycelia were filtered with sterile metallic or glass Buchner filters, adapted to sterile Kitasato flasks, and a vacuum pump. Mycelia obtained were washed with sterile distilled water, blotted between dry paper towels until no excess water emerged, and put onto pieces of pre-weighed aluminum foil. Total mass of mycelium was dried at 60°C until constant weight, to obtain the dry weight for each isolate.

6.2 Spectrophotometer assay

For the spectrophotometric readings the supernatants were recovered from the -80°C freezer, underwent a thawing followed by a centrifugation for 2 min at 13200 rpm to remove mycelium debris. Then, supernatant was transferred to a new Eppendorf tube. For checking the better pH for enzymatic activity two different solutions were prepared with McIlvaine buffer (obtained by mixing adequate volumes of Na_2HPO_4 and citric acid till the wanted pH value) for pH 3.6 and 4.0 and incubated for 5 minutes at 37°C. For the preparation of the blank a volume of 3mL has been prepared containing the 2667 µl of buffer and 333µl of ABTS. For the final optical density reading, a volume of 500 µl of each supernatant was added, and the absorbance value recorded every 5 min for 30 minutes.

7. Statistical analysis

Statistical analyses of mycelia growth and virulence on stems 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).

V. Results

1. Characterization of *Cryphonectria parasitica* isolates

1.1 Conversion of virulent isolates by hypovirulent strain SR44.2

The results of conversion between the *C. parasitica* hypovirulent strains SR44.2 and the *C. parasitica* virulent strains Cast13, Cast26, Cast07, Cast17, VBC02 and VDP11 are shown in Table 4. The pairings show no separation lines between the strains in Cast07, Cast17, VBC02, and VDP11. For the strain Cast13 and Cast26 the presence of the characteristic barrage line is present although the white morphology of the mycelium is evidenced. At the end of 21 days, almost all virulent strains showed corresponding morphology with hypovirulent isolates (white mycelium and reduced/less sporulation) as show in Table 4. The white color morphology of virulent converted strains is due to the transfer of CHV1 through the mycelium of virulent strains during the mycelia growth in plates (Figure 7).

Table 4: Characteristics of conversion between hypovirulent (SR44.2) and virulent (Cast07, Cast13, Cast17, Cast26, VBC02 and VDP11) strains after 21 days.

<table>
<tr><th>Strains</th><th>Plates</th><th>Conversion</th><th>Observations</th></tr>
<tr><td rowspan="3">Cast13</td><td>1</td><td>80%</td><td rowspan="3">• The growth of the two isolates (donor/acceptor) is proportional
• Mycelium less white than the donor</td></tr>
<tr><td>2</td><td>85%</td></tr>
<tr><td>3</td><td>80%</td></tr>
<tr><td rowspan="3">Cast26</td><td>1</td><td>80%</td><td rowspan="3">• The growth of the two isolates is proportional
• Mycelium less developed
• All isolates are white</td></tr>
<tr><td>2</td><td>85%</td></tr>
<tr><td>3</td><td>85%</td></tr>
<tr><td rowspan="3">Cast07</td><td>1</td><td>100%</td><td rowspan="3">• The growth of the two isolates is proportional
• A well-developed white mycelium in the plate majority (2/3)
• Growth is low</td></tr>
<tr><td>2</td><td>100%</td></tr>
<tr><td>3</td><td>100%</td></tr>
<tr><td rowspan="3">Cast17</td><td>1</td><td>70%</td><td rowspan="3">• The growth of the two isolates is proportional
• Well-developed white mycelium
• Growth is low</td></tr>
<tr><td>2</td><td>80%</td></tr>
<tr><td>3</td><td>80%</td></tr>
</table>

VBC02	1	100%	• Normal growth for the donor • Low growth forVBCO2
	2	100%	
	3	100%	
VDP11	1	100%	• Mycelium well developed • Plate homogeneous
	2	100%	
	3	100%	

The strains Cast07, VBC02 and VDP11 had complete conversion. In the other side, the strain Cast13 and Cast26 showed 81.7% and 83.3% respectively, of conversion and the strain Cast17 showed the lowest conversion, of about 77%. On PDA medium the converted strains (Cast07c, Cast13c Cast17c, Cast26c, VBC02c and VDP11c) have white mycelium color and less or no spore's production (Figure 7).

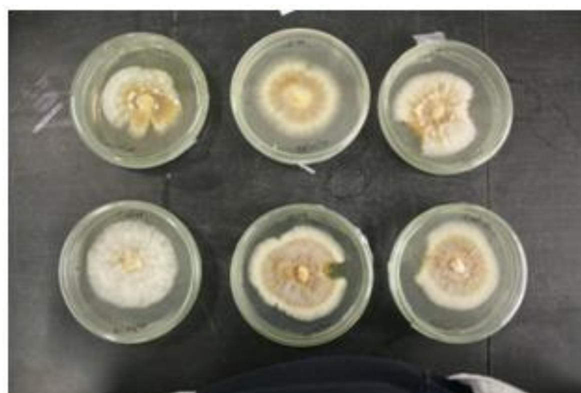


Figure 7: Hypovirulent strain after the conversion (line1 from left to right: VBCO2,Cast17,Cast13; line 2 from left to right : Cast07 , VDP11,Cast26).

Table 5 : Morphological characteristics of the converted strains by the hypovirulent strain SR44.2.

Converted isolates	**Morphology**
Cast 07c	White / no spores
Cast13c	White / no spores
Cast17c	White / orange spores
Cast26c	White / no spores
VBC02c	White / orange area
VDP11c	White / less spores

c- Converted

1.2. Growth evaluation of *Cryphonectria parasitica* strains *in vitro*

The mycelial growth of the strains on PDA was checked in five virulent strains (Cast07, Cast17, Cast26, Cast13 and VDP11), ten converted strains with both donors SR44.2 and RBB111 (Cast07/RBB111, Cast17/RBB111, Cast26/RBB111, Cast13/RBB111 and VDP11/RBB111; Cast07/SR44.2, Cast17/SR44.2, Cast26/SR44.2, Cast13/SR44.2 and VDP11/SR44.2) and in the two hypovirulent strains (SR44.2, RBB111). Resulting mycelium color was between orange and yellow in the virulent strains, and white for the converted ones. The last ones showed no pigmentation, slower growth, and displayed few or no pycnidia or evidence of sporulation. Comparing the growth of the strains with different donors we can observe that converted strains with RBB111 had greater mycelia growth than the SR44.2 converted ones (the highest average mycelia growth was observed for the strain Cast07/RBB111 ($8.38 \pm 0.23cm^2$), with exception for Cast13/SR44.2 which showed the highest growth (as shown in the Table 6). The lowest average mycelia growth was observed for the converted strain Cast26/SR44.2 ($5.63 \pm 0.15cm^2$).

Table 6: Mycelial growth on Petri plates of the strains converted by RBB111 or SR44.2.

	Mycelium growth after 7 days		
	Virulent (cm)	Converted by RBB111 (cm)	Converted by SR44.2 (cm)
Cast 13	6.63±0.42a	8.31 ± 0.10a	8.33 ± 0.15a
Cast 26	6.28 ±1.35a	8.28 ± 0.08b	7.22 ± 0.53a
Cast 07	5.85 ±0.61a	8.38 ± 0.23a	6.92 ± 0.45b
Cast 17	7.23 ± 1.51a	8.33 ± 0.15a	6.83 ± 0.24b
VDP 11	6.5 ± 0.96a	7.73 ± 0.42a	5.63 ± 0.15a

Different letters in the columns means significant differences ($p<0.05$).

In order to better explore the behavior of the isolates, other statistical analyses were carried out, which consisted in the comparison of each virulent isolate with the corresponding converted with RBB111 or SR44.2 (Figure 8).

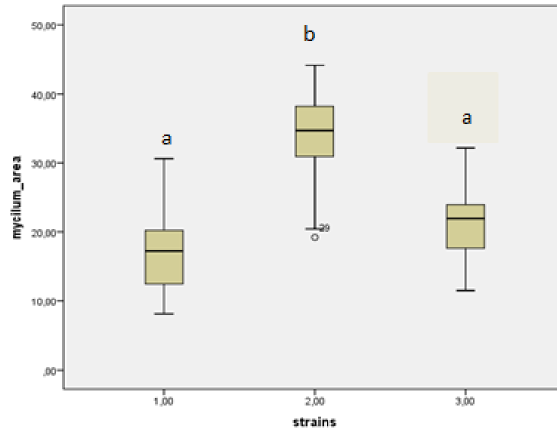


Figure 8: Mycelial growth on each virulent strain and their converted ones. Different letters in the bars means significant differences, (test Tuckey, α<0.05), (1: virulent, 2: converted with RBB111, 3: converted with SR44.2).

The virulent VDP11, Cast07 and Cast13 isolates did not show significant differences when compared with any of the converted ones. For the isolates Cast26 and Cast17 a significant difference was found ($p<0.05$). Cast26 showed a significant difference between the virulent and the isolates converted with RBB111, and in Cast17 the growth is significantly different from the isolates converted with SR44.2. These results are shown for isolates converted by RBB111 in Figure 9.

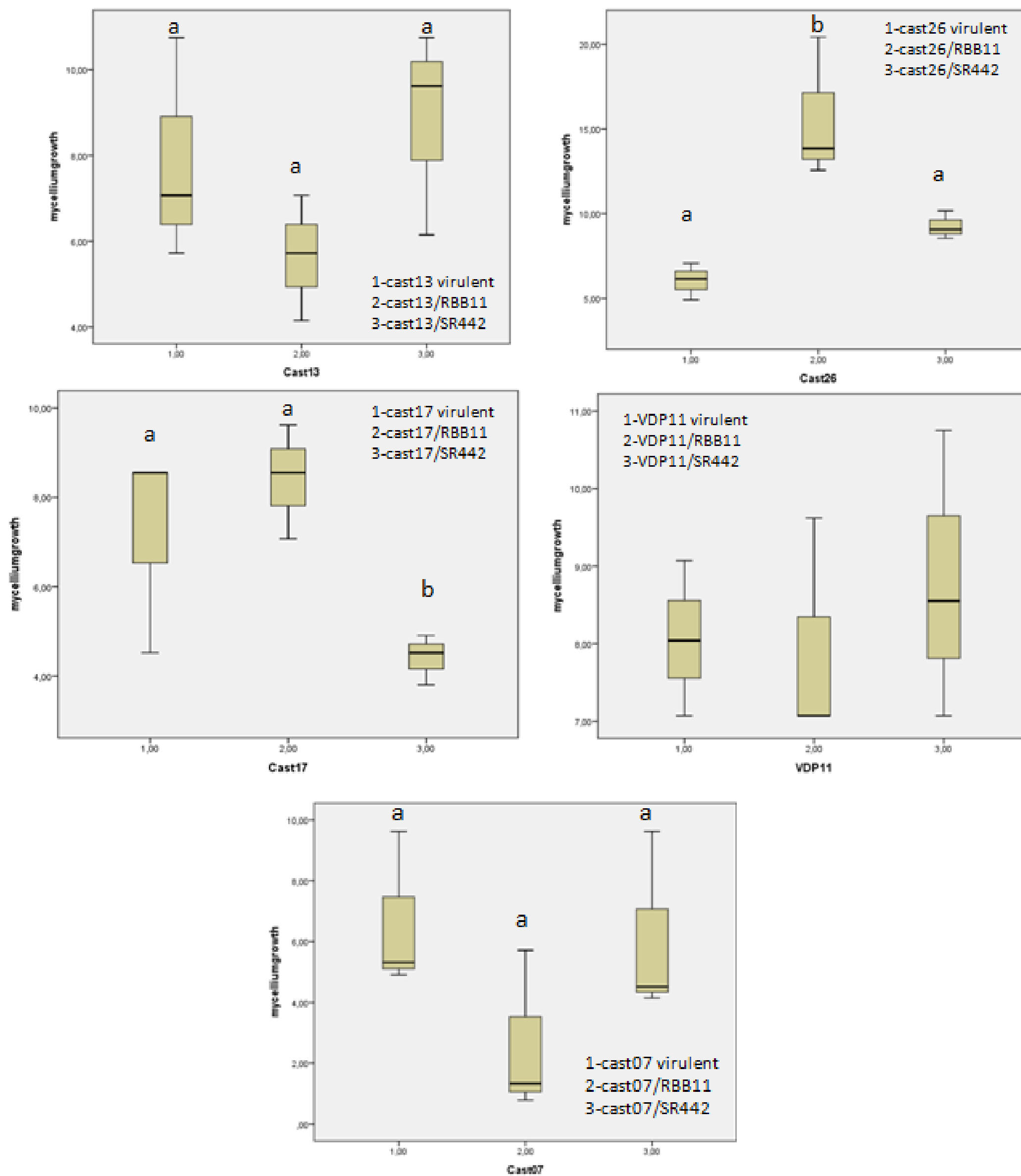


Figure 9: Mycelial growth of virulent and hypovirulent strains. Different letters in the bars in means significant differences, (test Tuckey, α<0.05).

1.3. Growth rate

Daily growth rate was compared between virulent and hypovirulent converted strains (Figure 10), showing that RBB111 converted strains had a slow growth rate with an average of 0.3 cm per day comparing to the virulent ones, which presented an average of 0.4 cm/day. Otherwise those converted with SR44.2 showed an average of 1.1 cm per day, although presenting white mycelium color and no spores production. In general, the converted with SR44.2 isolates, with the exception of the strain Cast17 and Cast13, had a higher daily growth rate comparing with the virulent one. The same happens with the RBB111 converted, being

exceptions Cast07 and Cast17. The virulent strain Cast17 had a higher daily growth rate than their converted ones (Cast17/RBB111 and Cast17/SR44.2).

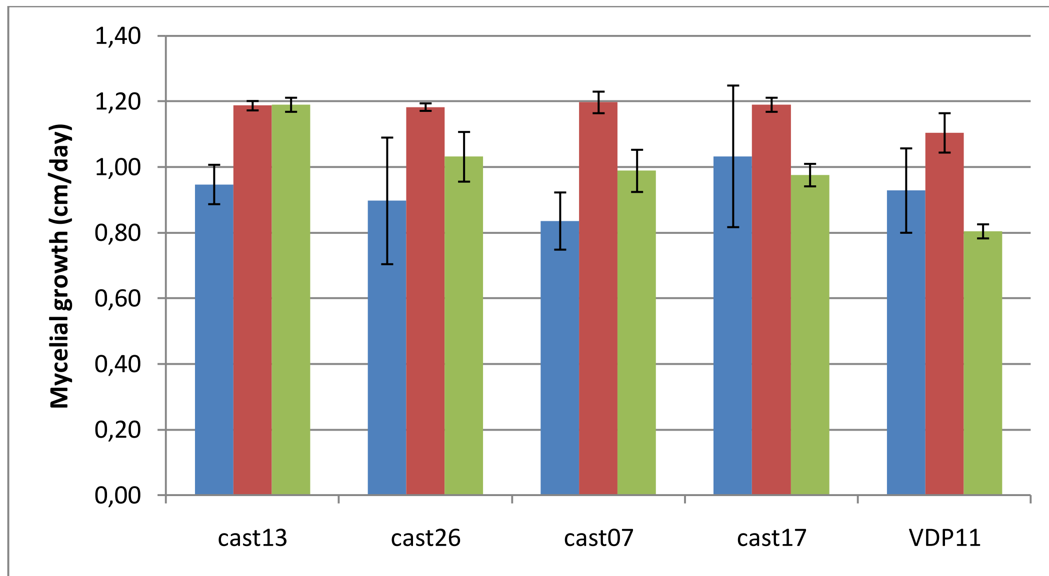


Figure 10 : Daily mycelial growth (cm/day) of the virulent strains (Cast13, Cast26, Cast07, Cast17, VDP11), the converted with RBB111 and SR44.2 donors. (Blue: virulent, Red: converted with RBB111, Green: converted with SR44.2).

2. Evaluation of the virulence of *Cryphonectria parasitica* (virulent and hypovirulent) strains in dormant chestnut branches.

Chestnut tree stems were inoculated with virulent and hypovirulent *C. parasitica* strains (RBB111 and SR44.2) and virulence was assessed as the area of bark tissue having brown, necrotic lesions.

At the end of the experiment the virulent isolates Cast13 produced the largest cankers lesion area with 20.94 cm^2. For the converted strains the largest lesion was obtained for strain Cast26/SR44.2 with 32.80 cm^2. The smallest lesion area on virulent strains was obtained for strain Cast26 with 4.69 cm^2 and for the converted strains was obtained for strain Cast17/SR44.2 with 5.38 cm^2. Chestnut lesions are shown in Figure 11 and measurements are present in Table 7.

Figure 11: Chestnut branches inoculated with plugs of isolates Cast13, VDP11, Cast26, Cast17, Cast07 virulent one converted with SR44.2 and converted with RBB111, twenty-five days post-inoculation

Table 7 : Evolution of canker lesions over time in virulent strains and in strains converted by RBB111 and SR44.2 Different letters in the columns means significant differences ($p<0.05$).

	Day 10			Day15			Day25		
strains	Virulent	Converted byRBB111	Converted bySR44.2	Virulent	Converted byRBB111	Converted bySR44.2	Virulent	Converted byRBB111	Converted bySR44.2
Cast13	1.10±0.33a	1.90± 1.12a	0.44 ±0.22a	1.33±0.3a	3.01± 1.63a	2.96± 1.76a	20.9±2.5a	24.6± 2.48a	8.20 ±1.09b
Cast26	0.7±0.55a	0.36±0.31a	1.96±0.8b	1.20±0.6a	1.15±0.39a	2.92±1.39a	4.96±5.46a	9.16±6.53a	32.80±2.02b
Cast07	0.76±0.22a	0.59±0.31a	0.42±0.34a	1.11±0.4a	1.42±0.25a	1..42±0.25a	10.28±6.18a	6.33±0.98a	16.4±1.21a
Cast17	0.96±0.22a	0.26±0.06b	0.49±0.37a	1.36±0.4a	0.60±0.40a	1.46±0.99a	7.50±2.02a	6.66±4.74a	5.38±6.50a
VDP11	0.78±0.15a	1.23±0.65a	1.43±1.28a	0.81±0.2a	4.04±0.80a	4.23±2.08b	5.80±3.84a	28.41±6.49a	24.72±832a

In order to study the kinetics of the evolution of the lesion caused by the inoculation of the isolate plugs measures was taken every 5 days and curves of the evolution of the lesion area was traced (Figure 12). All isolates showed lesions starting from day 10; this can be explained by the fact that we used dormant branches that delay infection. Also, it was observed a slower evolution of the lesion for the isolates converted by RBB111, compared with the virulent ones. In the case of the RBB111, the converted Cast13 and Cast17, showed a much lower lesion area than the corresponding virulent ones. The same isolates showed the same result with conversion by SR44.2 which give us an idea of the effect of the donor in the virulence process. In contrast other isolates showed opposite behavior, with RBB111 and SR44.2 causing larger lesion areas. The virulent strains VDP11 and Cast26 showed lower lesion areas than its converted ones (VDP11/RBB111; VDP11/SR44.2 and Cast26/RBB111; Cast26/SR44.2) during the observation time.

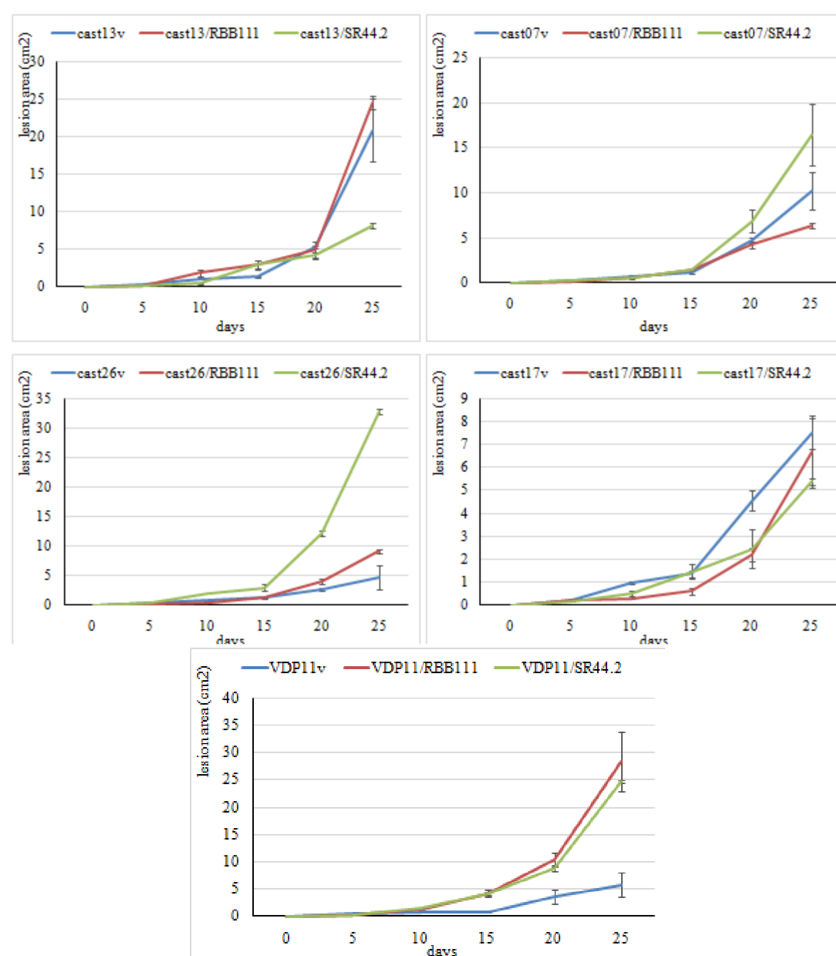


Figure 12 : Evolution of canker lesion over time in virulent strains and in strains converted by RBB111 and SR44.2.

3. Qualitative evaluation of laccase

3.1 RBBR test

In this test, we checked simultaneously the capacity of *C. parasitica* isolates to decolor this anthraquinone dye, and the effect of different $CuSO_4$ concentrations. The results are presented in Table 8 and Figure 13. The positive results with RBBR are observed as a clear discoloration of halo around the isolate as shown in the Figure 13. Most isolates showed higher RBBR discoloration halo when grown in the presence of $CuSO_4$ at 0.6 mM when compared to others concentrations (Table 8) but, at this concentration, Cast26 and Cast13 decolorized the dye without generating a clear halo of discoloration, and Cast07 was even found to be unable to decolorize RBBR. Cast17 and VDP11 evidenced larger halos with higher intensity of media discoloration, that agree with their higher pathogenicity in stems infection.

Table 8 : RBBR test using different concentrations of CuSO4

Strains / $CuSO_4$ concentration	Control	0.2mM	0.4 mM	0.6 mM
Cast 13	-	+++	++	+++
Cast 26	-	+	++	++
Cast 07	+	+	+	+
Cast 17	+	++	++	+++
VDP11	-	+	+	++

Dye discoloration in RBBR medium: + to +++ refer to increasing discoloration

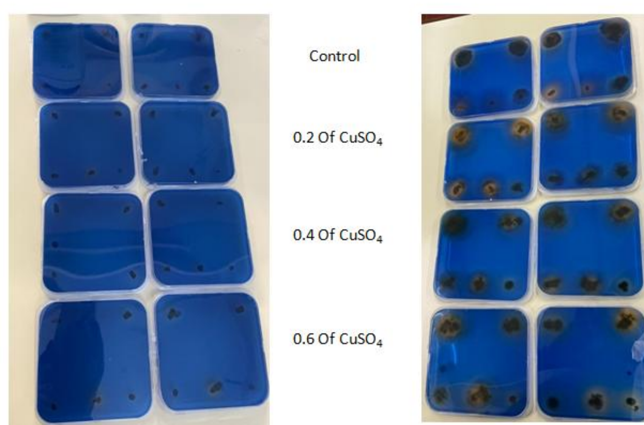
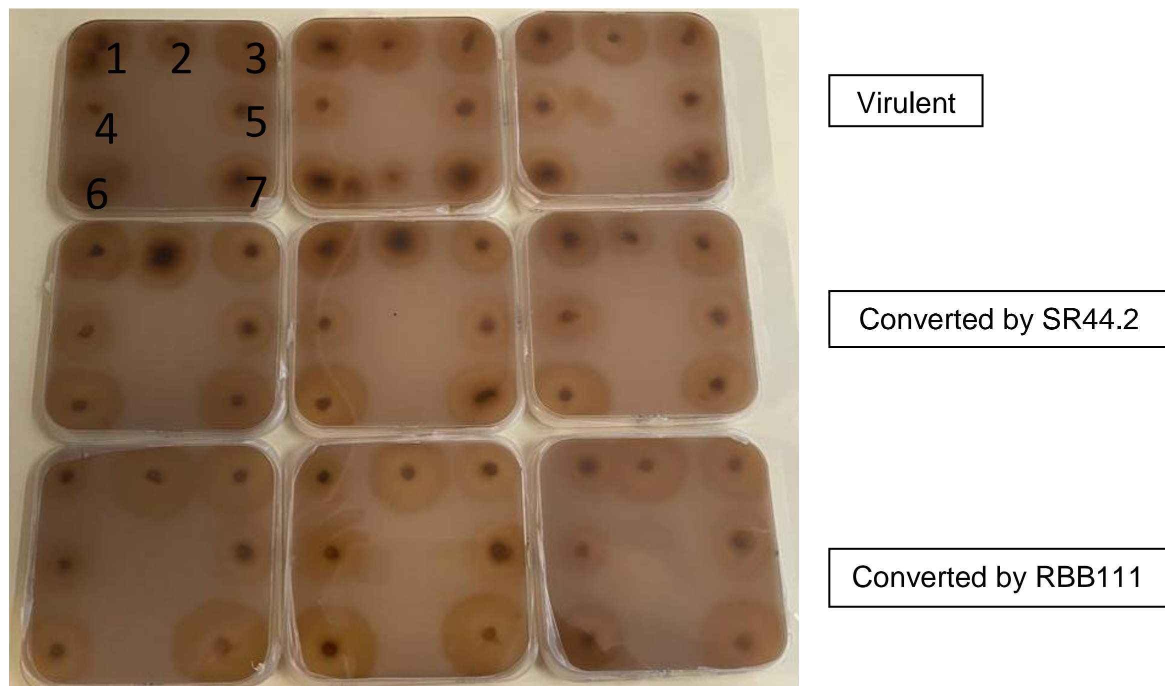


Figure 13: Evolution of the enzymatic reaction in the presence of RBBR as a function of inducer.

3.2 Bavendamm Test

We assessed phenol oxidase (laccase) activity applying the Bavendamm test specific for *C. parasitica* lac3 activity evaluation with five virulent isolates, but also with the corresponding converted isolates, obtained using SR44.2 or RBB111 as donors. As shown in Figure 14, the virulent isolates showed a strong color reaction especially Cast26, Cast13 and VDP11. Otherwise the area of dark brown coloring, indicative of phenol oxidase activity, was greatly reduced comparing the virulent strains with those converted SR44.2 with the exception of Cast17/SR44.2. The dark brown area was even smaller in the isolates converted with RBB111. These results indicate that *C. parasitica* lac3 activity was greatly hampered by the introduction of the virus, suggesting *C. parasitica* lac3 activity play a role in the virulence, and highlighting CHV1 donnors as agents for *C. sativa* canker biocontrol.



1 : VDP11 , 2 : Cast17 , 3 : Cast07 ,4 : SR44.2 ,5 : RBB111, 6 : Cast26 ,7 : Cast 13

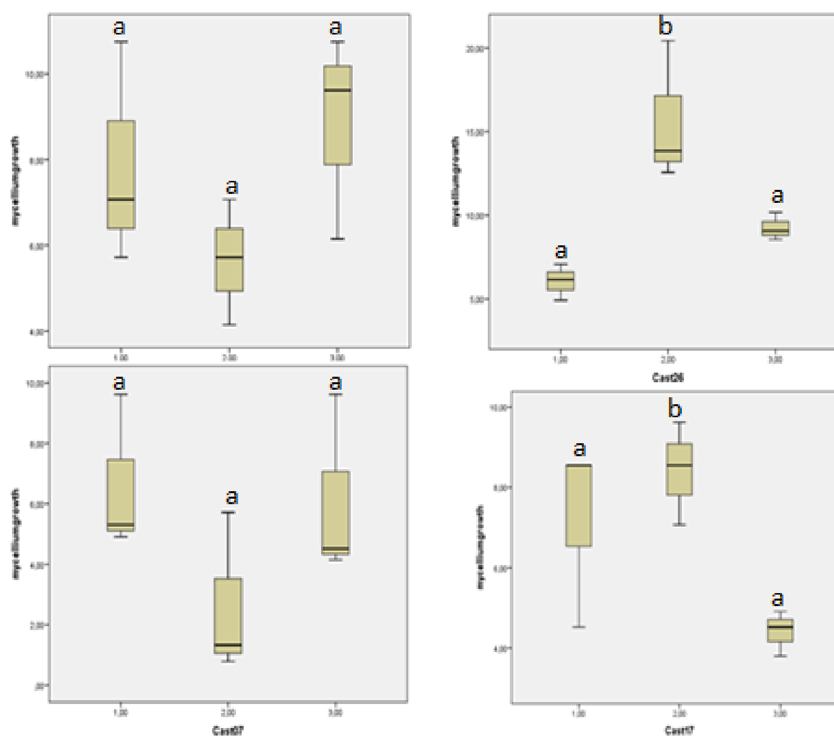
Figure 14: Five isolates tested for Bavendamm test after 4 days

The dark areas produced during the reactions were measured and statistically treated (Table 8 and Figure15). Cast26 and Cast17 isolates showed significantly different ($\alpha < 0.05$) coloring reactions comparing the virulent strains with the converted with RBB111, which expressed the lowest reactions. Otherwise the strains Cast13, Cast07 and VDP11 didn't show any significant differences comparing the virulent with the converted strains.

Table 9 Evaluation of the dark area during growth of different fungal strains

Strains	Virulent	RBB111 converted	SR44.2 converted
Cast13	+++	-	+
Cast26	+++	-	-
Cast07	++	-	-
Cast17	+	-	+++
VDP11	++	-	+

Bavendamm test: + to +++ refer to increasing color reaction obtained in the test



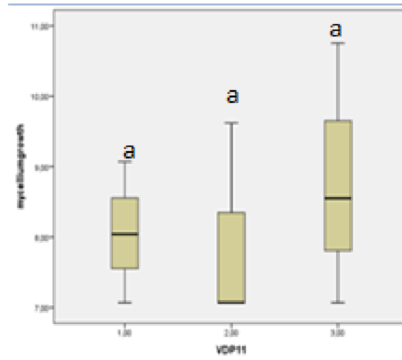


Figure 15 : Diagram of browning area evaluation in Bavendamm test (1: virulent 2: converted with RBB111; 3: converted with SR44.2). Different letters in the bars means significant differences, (α<0.05).

4. Quantitative evaluation of lacase production

Following the qualitative assay, the isolates that showed a significant potential for laccase enzyme activity were selected for the quantitative evaluation, which was conducted by measuring optical density over time and in the presence of ABTS as a substrate. This study was made using 4 virulent isolates (VDP11, Cast17, Cast13, Cast26), 4 isolates converted by SR44.2 (VDP11c, Cast17c, Cast13c, Cast26c) and 1 isolate converted by RBB111 (VDP11c).

It has been observed that the virulent isolates VDP11 and Cast17 showed the highest enzymatic activity rates, and the corresponding isolates converted by RBB111 or SR44.2 presented lower activity rate especially the converted with RBB111 (Figure 16). This decrease in enzymatic activity proves the conversion of isolates as well as it can explain the decrease in their virulence at the level of chestnut branches.

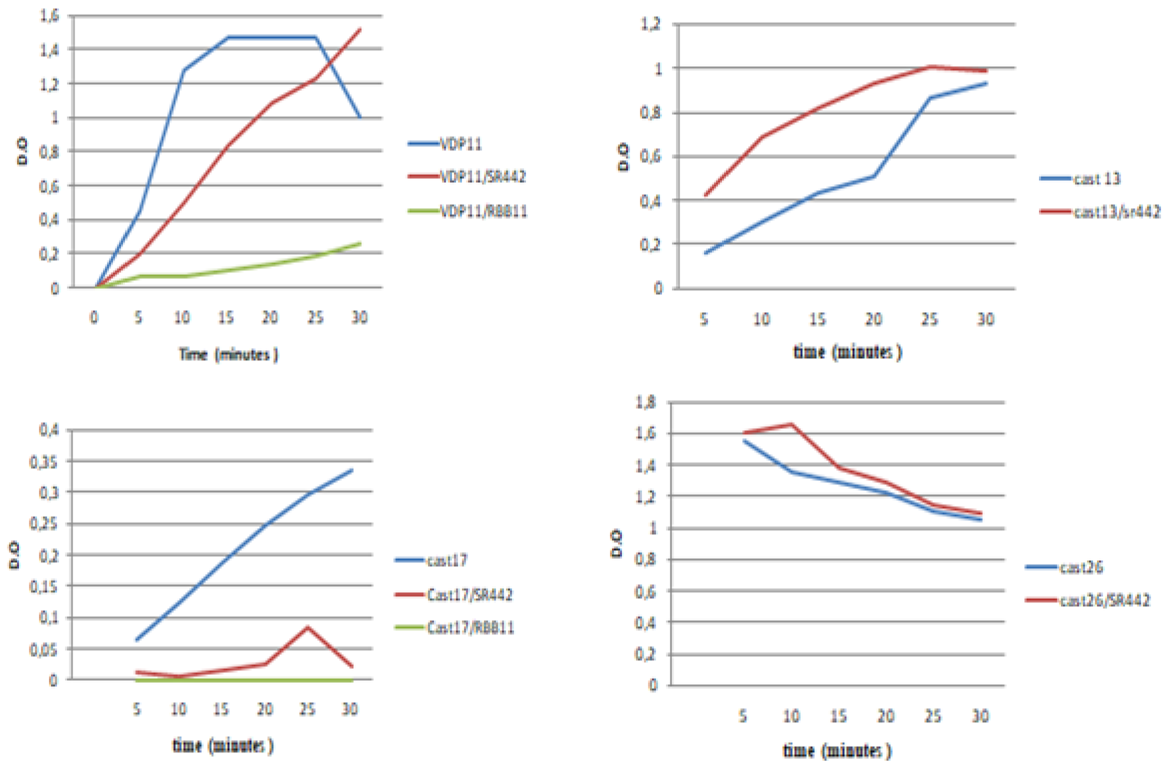


Figure 16: Curve of the evolution of the optical density of laccase in the presence of ABTS as substrate

Cast13 or Cast26 have shown different results that consisted of higher enzymatic activity in converted strains comparing with virulent ones. This can be explained by the vegetative compatibility that did not allow the complete transformation of Cast13 and Cast26 isolates (both from the EU66 VCG type) by the SR44.2 donor, from the EU11 VCG type), seeming that the conversion was carried out at the morphological level, but not at the physiological one.

To investigate further those results an extra step was conducted. For this we chose to calculate the higher slope in each graph as an indicator of higher enzyme activity.

Applying the Lambert-Beer law, we calculate the enzymatic activity, defined as the amount of enzyme transforming 1 µmol of substrate per minute and liter, which is calculated by the following formula: $U/L = (\Delta E \times Vt)/(\varepsilon \times d \times Vs)$, taken from Holme and Peck (1996), where :

- ΔE being the change in extinction of light [min-1] at 420 nm,
- ε being the molar absorption coefficient of ABTS [M-1 cm-1]
- d being the layer thickness in the cell that the light has to pass [cm]
- Vt is the total volume

- and Vs is the volume of the enzyme stock solution

The results obtained are in Table10.

Table 10 : Calculation of laccase activity during the higher interval of time

Isolates	ε (M cm^{-1})	Interval of time (min)	Slope (min^{-1})	The enzymatic activity (U/L)
Cast13	36000	20-25	0.07	13.6
Cast26	36000	5-10	0.04	7.78
Cast17	36000	5-25	0.011	2.14
VDP11	36000	5-10	0.16	31.1
Cast13/SR44.2	36000	10-25	0.02	3.89
Cast26/SR44.2	36000	5-10	0.011	2.14
Cast17/SR44.2	36000	20-25	0.011	2.14
VDP11/SR44.2	36000	5-20	0.05	9.72
Cast17/RBB111	36000	5-20	0	0
VDP11/RBB111	36000	5-25	0.006	1.17

In order to determine the specific activity of laccase, the total amount of protein in each isolate supernatant was calculated using a Bradford assay calibration curve with BSA, using an O.D of 595nm (Appendix 12 and Appendix 13). The results obtained are in Table11.

Table 11: Specific laccase activity

Strains	Laccase activity (U/L)	Total protein (µg)	Specific laccase activity (U/L.µg)
Cast13	13.6	5	2.72
VDP11	31.1	1,63	19.07
Cast17/RBB111	0	1,00	0
VDP11/RBB111	1.17	2,17	0.54

Specific laccase activity (U/L.µg) = Laccase activity (U/L)/ Total protein (µg)

The virulent isolates showing the highest specific activity were VDP11 with 19.07 U/L.µg, followed by Cast13 with 2.72 U/L.µg. Comparing between the virulent isolates and the corresponding converted ones; we observed that this activity was thirty times reduced in the converted isolate VDP11/RBB111 with a 0.54 U/L/µg (Table 11).

In other isolates as we could not detect the protein content using the BSA standard curve, we could not calculate their specific activity.

VI. Discussion

Conversion capability

According to obtained results there is complete conversion in strains VBC02, Cast07 and VDP11, this means that hypovirulent isolate used in this work (SR44.2) has complete ability to convert these isolates with effective hypovirus transmission and this transformation, was firstly observed by color change from orange to white, indicative of successful hypovirus transmission.

On the other hand, the transformation of the isolates Cast13, Cast26, Cast17 was partial and this can be observed on the morphology of the mycelium that didn't appear white in all the plate. At the end of the plate, opposite the contact zone, the mycelium of virulent isolates preserves its orange coloration probably due to the slower growth of the fungi SR44.2. Concerning isolates converted with RBB111, utilized in this work, were recovered following the same procedure by Gadoum, 2020.

The white culture morphology of hypovirus infected strains enables the visual assessment of hypovirus transmission (Dutech *et al*., 2012) and in this work; the white color and the absence or reduced presence of spores was considered to be the factor that determined the success of the conversion capacity.

Mycelial growth of the strains on PDA

According to obtained results of mycelial growth of the strains on PDA, the highest average mycelial growth was observed for the converted strain Cast26 by SR44.2. The lowest average mycelial growth was observed on converted strain Cast07 with RBB111.

In general, the analysis of two donors (RBB111 - CHV1 Italian type and SR44.2 – CHV1 French type) showed a different phenotypic trait. For example, SR44.2 grows more rapidly on solid synthetic medium than corresponding virus-free isogenic strains, while RBB111 grows more slowly. RBB111 forms small, superficial cankers on chestnut tissue and produces little asexual spores either on synthetic medium or on chestnut stems. In contrast, SR44.2 is only moderately hypovirulent. Aggressive canker expansion early after inoculation eventually slows or ceases, concomitant with heavy callus formation at the canker margins and the same results was proved by a study made by Peever *et al*. (2000)

Other studies showed that converted strains with RBB111 had greater mycelia growth than the converted strains with SR44.2. The growth of *C. parasitica* isolates (converted or

virus–free isolates) *in vitro* could not be correlated with canker growth or callus formation on tree (Sotirovski *et al.*, 2011), however the test on PDA give some indication about which strain could have a potentially strong hypovirulent effect (Krstin *et al.*, 2016). The orange pigmentation was observed in some converted strains. However, the pigmentation was not a typical bright yellow color, but rather a diffused pale grayish brown as the culture continued to grow.

Growth of *C. parasitica* strains in chestnut stems

The strains were inoculated in chestnut branches, to characterize their degree of virulence. Chestnut shoots are composed of lignin, hemicellulose and cellulose (Glushkova *et al.*, 2010) therefore the lesions made by virulent strains, which have higher laccase activity, were more important than the damage made by strains that contains dsRNA, which suffer from decrease in laccase activity, corroborating the data of Chung et al. (2008). This study showed that the infection area caused by almost all the converted strains by SR44.2 or RBB111 (Cast07, Cast17, Cast26,) is lower than the one caused by the virulent strains (Cast07, Cast17, Cast26,), with exception for Cast13 and VDP11. The smaller lesion area caused by converted (hypovirus-infected) strains on dormant chestnut branches was also observed by Rigling *et al.* (2018).

Although the converted isolates caused smaller lesion size than the virulent ones, the converted strains did not all have the same growth on the branches. The difference between isolates has been observed in different studies on chestnut branches, either actively growing or dormant branches (Krstin *et al.*, 2016, Melzer *et al.,* 2009).

Consequently, infection area may be one factor related with *C. parasitica* pathogenicity in *C. sativa.* Moreover, environmental conditions strongly influence plant diseases. Therefore, climate changes can also be considered drivers of disease outbreaks (Sturrock *et al.*, 2011). The success of hypovirulence is determined by the ability of CHV1 to infect a large proportion of a *C. parasitica* population (Dutech *et al.*, 2010)

Qualitative assays

Five different fungal strains and their converted ones with RBB111 and SR44.2 were screened on PDA media containing colored indicators, like RBBR and tannic acid, related with ligninolytic activity. In RBBR test it was also assayed the influence of CuSO4 concentration in the activity of ligninolytic enzymes production. The tannic acid present in

Bavendamm test acted like a *C. parasitica* lac3 inducer. These compounds and indicators were more reactive with the virulent strains of *C. parasitica* than with hypovirulent ones, putting in evidence the reduction in phenol oxidase and ligninolytic activities after the transfer of the dsRNA into virulent strains. This difference was higher in Bavendamm test with the strains Cast26, Cast13 and VDP11 when comparing the virulent strains with the converted with RBB111 and SR44.2, as it has been reported by Rigling *et al*. (1989). Nonetheless, the growth rate of virulent and hypovirulent strains was not affected the same way in the Bavendamm test, proving that dsRNA affects phenol oxidase activities but not general viability of the strains.

Quantitative assays

Laccase plays a role in the infection process and participates in the degradation of lignin, pathogenesis, formation of fruiting bodies and pigmentation (Rigling and Prospero, 2017).

The results obtained in this study show that the virulent strains of *C. parasitica* always produced more laccase than both hypovirulent isogenic isolates and characterized donor isolates. In fact, the production of laccase in virulent isolates varied from 2.14 µg/mL to 31.1 µg/mL, while in the isogenic hypovirulent strains varied between 0 µg/mL and 9.72 µg/mL. This behavior of the reduction of the laccase and lignin peroxidase in the isolates affected by dsRNA was proved by many studies before (Rigling *et al*., 1989; Rigling and Alfen, 1993; Chung *et al.,* 2008).

VII. Conclusion

The conversion of five wild strains of *C. parasitica:* Cast26, Cast13, Cast07, Cast17 and VDP11 by the hypovirulent characterized donor strains RBB111 and SR44.2, resulted in different phenotypic traits such as the white color of the converted isolates, the reduction of sporulation and the slower growth rate on solid medium. After the conversion of the five isolates by SR44.2 and RBB111, the evaluation of virulent pathogenic capacity on inoculated young branches of *C. sativa* showed that damages on chestnut branches made by the converted isolates were smaller than the ones done by virulent strains, and this was more evident with the isolates converted by RBB111. Results of the Bavendamm test showed a reduction in the dark coloration of the converted isolates, especially with the converted by RBB111, showing that converted isolates had a reduced laccase 3 activity, as already referred by some authors. It was also confirmed reduction in laccase specific activity of the converted isolates.

However, not always a higher laccase specific activity is related to the development of larger stems lesions area. These differences may be due to the physiological condition of the branches (dormant or active growing), or to variations in the composition of the culture medium between the two assays (with or without inducer).

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Appendix

Appendix 1: Saprophytic capacity of the virulent isolates

		Growth after 3 days			Growth after 7 days		
		Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
EU66	Cast13	5	4,4	17,27	6,4	6,2	31,1488
		4,5	3,9	13,77675	7,2	7	39,564
		5,9	5	23,1575	7	6	32,97
	Cast26	4,2	3,6	11,8692	6,5	6	30,615
		3,4	3,2	8,5408	5,5	4,4	18,997
		6	5,3	24,963	7,9	7,4	45,8911
EU11	Cast07	4,5	3,7	13,07025	6,1	4,5	21,54825
		3,7	2,8	8,1326	6,5	6,5	33,16625
		5	4,5	17,6625	6	5,5	25,905
	Cast17	4,6	3,2	11,5552	6	5	23,55
		5	4,8	18,84	7,9	7,9	48,99185
		6,5	6	30,615	8,3	8,3	54,07865
	VDP11	5	4,7	18,4475	6,5	5,2	26,533
		4,8	3,6	13,5648	6,5	5,6	28,574
		5,5	5	21,5875	7,7	7,5	45,33375

Appendix 2: Saprophytic capacity of the converted isolates by RBB111

		Growth after 3 days			Growth after 7 days		
		Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
EU66	Cast13	7,5	7,5	44,15625	8,2	8,2	52,7834
		7	7,2	39,564	8,4	8,3	54,7302
		7,2	6,5	36,738	8,5	8,3	55,38175
	Cast26	6,6	6,2	32,1222	8,3	8,1	52,77555
		6,5	6,5	33,16625	8,5	8,2	54,7145
		6	5,5	25,905	8,5	8,1	54,04725
EU11	Cast07	7,5	7,5	44,15625	8,6	8,6	58,0586
		7,3	6,9	39,54045	8,5	8,3	55,38175
		7,2	6,5	36,738	8,3	8	52,124
	Cast17	6,7	6,6	34,7127	8,5	8,5	56,71625
		6,3	6,2	30,6621	8,4	8	52,752
		7	6,7	36,8165	8,4	8,2	54,0708
	VDP11	5,2	5	20,41	7,3	7,2	41,2596
		5,1	4,8	19,2168	8	7,9	49,612
		6,3	6,3	31,15665	8,2	7,8	50,2086

Appendix 3: Saprophytic capacity of the converted isolates by SR44.2

		Growth after 3 days			Growth after 7 days		
		Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
EU66	Cast13	6,6	6,5	33,6765	8,3	8,3	54,07865
		6,9	6,9	37,37385	8,2	8,2	52,7834
		6,5	6,3	32,14575	8,5	8,5	56,71625
	Cast26	5,2	4,9	20,0018	6,9	6,6	35,7489
		5	3,9	15,3075	7,7	6,5	39,28925
		5,5	5	21,5875	8,2	7,4	47,6338
EU11	Cast07	5,5	5,4	23,3145	7,3	7	40,1135
		5,6	5,6	24,6176	7,3	7,1	40,68655
		5,5	5,4	23,3145	7,3	5,5	31,51775
	Cast17	5,6	5	21,98	7	6,5	35,7175
		5,1	5,1	20,41785	7,3	6,9	39,54045
		5,4	5,2	22,0428	6,9	6,4	34,6656
	VDP11	4,2	3,5	11,5395	5,9	5,3	24,54695
		4,1	4	12,874	6,1	5,5	26,33675
		4,4	3,5	12,089	5,9	5,1	23,62065

Appendix 4: Saprophyte capacity of the hypovirulent strains

	Growth after 3 days			Growth after 7 days		
	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
SR44.2	3,3	3,2	8,2896	6	5,5	25,905
	3,7	3,2	9,2944	6	5,9	27,789
RBB111	3	2,3	5,4165	4,5	4	14,13
	3,2	2,7	6,7824	5	4,1	16,0925

Appendix 5: Pathogenic capacity of the virulent isolates in dormant stems

		Growth after 5 days			Growth after 10 days			Growth after 15 day			Growth after 20 days			Growth after 25 days		
		Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
EU66	Cast13	0,8	0,6	0,38	1,3	1,1	1,12	1,7	0,8	1,07	2,5	2,5	4,91	7,4	6,1	35,43
		1,2	0,5	0,47	1,5	1,2	1,41	2	1,1	1,73	3	2,2	5,18	5	3,3	12,95
		0,6	0,4	0,19	1,2	0,8	0,75	1,4	1,1	1,21	3,2	2,3	5,78	4,6	4	14,44
	Cast26	0,3	0,3	0,07	0,8	0,5	0,31	1,1	0,6	0,52	3,3	1,8	4,66	5	2,8	10,99
		0,9	0,5	0,35	1	0,6	0,47	1,6	1,4	1,76	1,6	1,4	1,76	1,6	1,4	1,76
		1,5	0,4	0,47	1,3	1,3	1,33	1,3	1,3	1,33	1,3	1,3	1,33	1,3	1,3	1,33
EU11	Cast07	0,8	0,6	0,38	1,4	0,9	0,99	0,7	1,7	0,93	4	1,8	5,65	5,2	3,4	13,88
		0,5	0,4	0,16	0,6	0,5	0,24	1,8	0,6	0,85	3,8	1,3	3,88	5,5	3,2	13,82
		0,8	0,4	0,25	1,5	0,9	1,06	2,2	0,9	1,55	2,4	2,4	4,52	2	2	3,14
	Cast17	0,4	0,4	0,13	1,2	0,9	0,85	2,5	0,9	1,77	3	1,3	3,06	3,8	2,2	6,56
		1,3	0,4	0,41	1,4	1,1	1,21	2,1	0,6	0,99	4	1,8	5,65	5	2,5	9,81
		0,5	0,4	0,16	1,3	0,8	0,82	1,2	1,4	1,32	2,5	2,5	4,91	4,1	1,9	6,12
	VDP11	0,4	0,3	0,09	1,2	0,9	0,85	1,3	0,8	0,82	1,8	1,2	1,70	1,1	1,1	0,95
		1,3	1	1,02	1,4	0,8	0,88	1,6	0,8	1,00	4	2,5	7,85	5,6	3,1	13,63
		0,4	0,3	0,09	1,1	0,7	0,60	1,1	0,7	0,60	1,5	1,1	1,30	2	1,8	2,83

Appendix 6: Pathogenic capacity by RBB111 converted isolates in dormant stems

		Growth after 5 days			Growth after 10 days			Growth after 15 day			Growth after 20 days			Growth after 25 days		
		Big diameter (cm)	Small diameter	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Area (cm²)
EU66	Cast13	0,4	0,3	0,09	2,1	2,0	3,30	2,7	1,6	3,39	4,2	1,5	4,95	6,0	5,8	27,32
		0,3	0,3	0,07	2,4	0,6	1,13	3,0	1,9	4,47	1,5	1,5	1,77	6,0	5,1	24,02
		0,5	0,4	0,16	1,8	0,9	1,27	1,8	0,9	1,27	5,0	2,1	8,24	5,5	5,2	22,45
	Cast26	0,6	0,4	0,19	0,7	0,5	0,27	2,5	0,6	1,18	3,2	2,8	7,03	4,0	2,8	8,79
		0,4	0,4	0,13	0,8	0,4	0,25	1,2	0,8	0,75	1,9	1,1	1,64	4,7	4,3	15,86
		0,8	0,6	0,38	1,0	0,7	0,55	1,4	1,4	1,54	2,7	1,5	3,18	3,0	1,2	2,83
EU11	Cast07	0,5	0,3	0,12	0,8	0,7	0,44	1,7	0,9	1,20	3,1	1,4	3,41	3,2	2,1	5,28
		0,6	0,4	0,19	1,1	1,1	0,95	2,7	0,8	1,70	3,5	2,0	5,50	4,6	1,8	6,50
		0,5	0,2	0,08	0,7	0,7	0,38	2,5	0,7	1,37	4,0	1,2	3,77	4,0	2,3	7,22
	Cast17	0,4	0,4	0,13	0,8	0,5	0,31	1,4	0,9	0,99	2,4	1,5	2,83	3,6	3,2	9,04
		0,6	0,5	0,24	0,7	0,5	0,27	1,3	0,6	0,61	2,5	1,3	2,55	4,0	3,1	9,73
		0,5	0,5	0,20	0,5	0,5	0,20	0,5	0,5	0,20	1,4	1,1	1,21	1,4	1,1	1,21
	VDP11	0,3	0,3	0,07	1,3	0,6	0,61	3,8	1,1	3,28	6,5	2,8	14,29	5,7	2,3	10,29
		0,5	0,4	0,16	1,5	1,0	1,18	3,6	1,4	3,96	5,4	2,3	9,75	7,0	5,9	32,42
		0,8	0,4	0,25	2,2	1,1	1,90	2,7	2,3	4,87	4,5	2,0	7,07	8,6	6,3	42,53

Appendix 7: Pathogenic capacity by SR44.2 converted isolates in dormant stems

		Growth after 5 days			Growth after 10 days			Growth after 15 day			Growth after 20 days			Growth after 25 days		
		Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Small diameter (cm)	Big diameter (cm)	Small diameter (cm)	Area (cm²)	Big diameter (cm)	Area (cm²)
EU66	Cast13	0,3	0,3	0,07	0,5	0,5	0,20	3	1,9	4,47	3	2,5	5,89	3,8	2,4	7,16
		0,3	0,3	0,07	0,8	0,8	0,50	1,2	1,1	1,04	2,2	1,8	3,11	4,4	2,7	9,33
		0,4	0,3	0,09	1	0,8	0,63	2,7	1,6	3,39	2,2	2,1	3,63	4,7	2,2	8,12
	Cast26	0,5	0,3	0,12	2	1,8	2,83	3,2	1,8	4,52	5,8	3,2	14,57	8	7,9	49,61
		1,2	0,8	0,75	2,2	1	1,73	2,4	1,2	2,26	6	4	18,84	8	6,3	39,56
		0,8	0,3	0,19	2,1	0,8	1,32	2,3	1,1	1,99	2,7	1,4	2,97	5,6	2,1	9,23
EU11	Cast07	0,5	0,5	0,20	0,7	0,7	0,38	1,7	0,9	1,20	4,7	1,6	5,90	4	2,8	8,79
		0,7	0,6	0,33	1,4	0,7	0,77	2,7	0,8	1,70	4,4	3,2	11,05	6,5	5,5	28,06
		0,4	0,3	0,09	0,4	0,3	0,09	2,5	0,7	1,37	2,4	1,9	3,58	5	3,2	12,56
	Cast17	0,3	0,3	0,07	0,3	0,3	0,07	1,6	0,8	1,00	1,7	0,9	1,20	2,5	1,3	2,55
		0,7	0,4	0,22	1	0,8	0,63	2,2	1,5	2,59	3,8	1,8	5,37	5,1	3,2	12,81
		0,5	0,4	0,16	1,1	0,9	0,78	1,1	0,9	0,78	1,1	0,9	0,78	1,1	0,9	0,78
	VDP11	0,9	0,7	0,49	1,7	1	1,33	4,5	1,8	6,36	6,2	3,2	15,57	8,2	4,3	27,68
		0,4	0,3	0,09	0,5	0,5	0,20	3,5	0,8	2,20	4	1,3	4,08	6,3	6,3	31,16
		0,5	0,4	0,16	2,5	1,4	2,75	3,3	1,6	4,14	5,3	1,6	6,66	6,1	3,2	15,32

Appendix 8: Bavendamm test assay of the virulent isolates.

		After 2 days			After 4 days		
		Mycelium growth (cm²)	Dark area (cm²)	Ratio	Mycelium growth (cm²)	Dark area (cm²)	Ratio
EU66	Cast13	1,6	1,7	0,94	2,7	3,3	0,82
		1,5	1,4	1,07	3,7	4,3	0,86
		1,5	1,4	1,07	3	3,2	0,94
	Cast26	1,4	1,5	0,93	2,5	3,1	0,81
		1,2	1,6	0,75	2,8	3	0,93
		1,4	1,6	0,88	3	3,1	0,97
EU11	Cast07	1,5	1,8	0,83	2,6	3,5	0,74
		1,4	1,6	0,88	2,5	3,2	0,78
		1,2	1,3	0,92	3,5	4	0,88
	Cast17	0,2	0,6	0,33	2,4	2,4	1,00
		1,6	1,8	0,89	3,3	3,5	0,94
		1,9	2	0,95	3,3	3,7	0,89
	VDP11	2,5	2,7	0,93	3,4	3,6	0,94
		2,1	2,5	0,84	3,2	3,5	0,91
		1,5	1,8	0,83	3	4	0,75

Appendix 9: Bavendamm Test assay second culture of the converted isolates by RBB111.

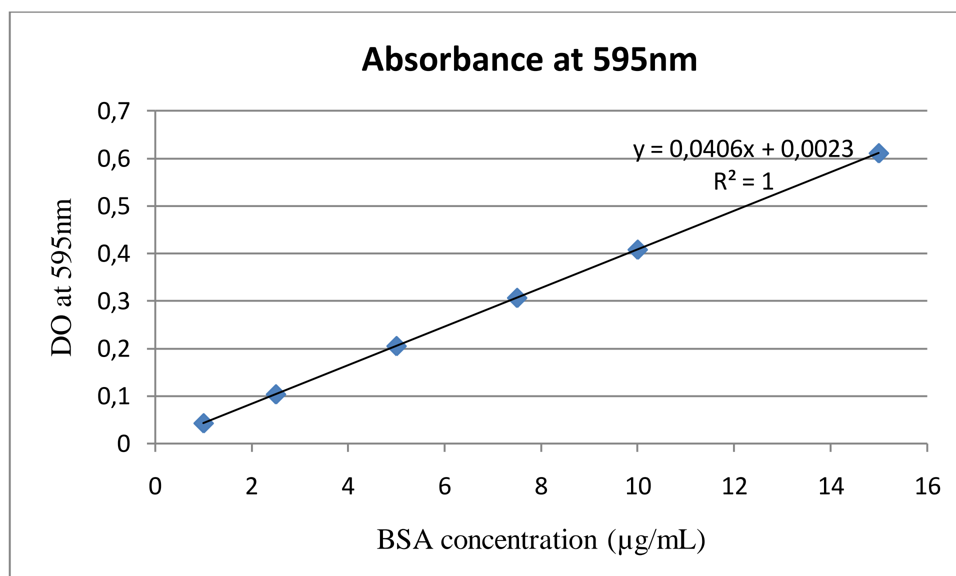
		After 2 days			After 4 days		
		Mycelium growth (cm²)	Dark area (cm²)	Ratio	Mycelium growth (cm²)	Dark area (cm²)	Ratio
EU66	Cast13	0,9	1,2	0,75	2,7	3,2	0,84
		0,6	1	0,60	2,3	3,3	0,70
		1,4	1,6	0,88	3	4,5	0,67
	Cast26	1,3	1,4	0,93	4,2	4,1	1,02
		1,2	1,6	0,75	4	4,2	0,95
		1,7	1,6	1,06	5,1	4,8	1,06
EU11	Cast07	0	0,2	0,00	1	1,7	0,59
		0,7	1,2	0,58	2,7	2,4	1,13
		0	0,9	0,00	1,3	1,7	0,76
	Cast17	1,6	1,7	0,94	3	4,4	0,68
		1,8	1,9	0,95	3,3	4,1	0,80
		1,6	1,8	0,89	3,5	4,3	0,81
	VDP11	1,3	1,5	0,87	3	3,2	0,94
		1,2	1,3	0,92	3,5	3,7	0,95
		1	0	0	3	3,4	0,88

Appendix 10: Bavendamm test assay of the converted isolates by SR44.2.

		After 2 days			After 4 days		
		Mycelium growth (cm²)	Dark area (cm²)	Ratio	Mycelium growth (cm²)	Dark area (cm²)	Ratio
EU66	Cast13	1,5	1,7	0,88	2,8	3,9	0,72
		1,7	1,8	0,94	3,5	3,7	0,95
		1,2	1,9	0,63	3,7	4	0,93
	Cast26	1,7	1,8	0,94	3,3	3,3	1,00
		1	1,2	0,83	3,4	3,6	0,94
		1,9	2,1	0,90	3,6	3,7	0,97
EU11	Cast07	1,2	1,5	0,80	2,3	3,5	0,66
		1,6	1,7	0,94	2,4	3	0,80
		1,6	1,8	0,89	3,5	3,5	1,00
	Cast17	1,1	1,4	0,79	2,5	2,5	1,00
		1,6	1,8	0,89	2,4	3	0,80
		0	0	0	2,2	3,8	0,58
	VDP11	1,2	1,6	0,75	3,3	3,4	0,97
		1,5	1,9	0,79	3	3,4	0,88
		1,1	1,3	0,85	3,7	3,8	0,97

Appendix 11: Absorbance at 420 nm for laccase activity during the time

Time (min) / Strains	0	5	10	15	20	25	30
Cast 13	0	0,164	0,304	0,432	0,509	0,862	0,928
VDP11	0	0,455	1,282	1,476	1,477	1,473	1
VDP11/SR44.2	0	0,197	0,504	0,835	1,085	1,229	1,517
VDP11/RBB111	0	0,067	0,067	0,102	0,142	0,188	0,257
Cast17/SR44.2	0	0,013	0,007	0,016	0,026	0,084	0,0208
Cast17	0	0,064	0,124	0,188	0,249	0,297	0,337
Cast13/SR44.2	0	0,421	0,689	0,823	0,932	1,011	0,984
Cast07/RBB111	0	0,726	0,975	1,09	1,713	1,111	1,281
Cast26	0	1,556	1,357	1,294	1,219	1,101	1,059
Cast26/SR44.2	0	1,606	1,663	1,388	1,287	1,143	1,097



Appendix 12: Standard curve for protein analysis using BSA as standard

Appendix 13 : Calculation of total protein concentration.

BSA concentration (µg/mL)	1	2.5	5	7.5	10	15
OD at 595nm	0.0429	0.1038	0.2053	0.3068	0.4083	0.6113