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Molecular Factors in the Metabolism and Pathogenicity of *Phytophthora cinnamomi*:

Characterization of RXLR family genes, *Avr3a*, and *Avr1b*.

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This dissertation include comments and suggestions by the juri

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Dedication

To my dear parents Zoubaier Boughanmi and Azza ben lamine, for the countless sacrifices they have made for me, morally or financially. You have always been my great source of inspiration to move on in life and i will never disappoint you.

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Abbreviations

AA	Amino acid
Ago	Argonaute
BLAST	Basic Local Alignement Search Tool
C.	*Castanea*
$CaCl_2$	Calcium chloride
CWDE	Cell wall degrading enzyme
CMPG1	E 3 ubiquitin ligase
CE	Carbohydrate esterase
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DDJB	DNA Data Bank of Japan
EBI	Uniprot Universal Resource
EMBL	European Molecular Biology Laboratory
FungiDB	The Fungal and *Oomycete* Genomics Resource
GFP	Green fluorescent protein
GH	Glycoside hydrolase
iRNA	RNA interference
Kb	Kilo base pairs
INF1	Infestin trigered cell death
mRNA	Messenger ribonucleic acid
miRNA	Micro RNA
NLPs	Necrosis inducing proteins

NCBI	National center for biotechnology information
ORF	Open Reading Frame
P.	*Phythophthora*
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PVP	Poly-vinyl-pyrolidone
PIR	Protein Information Resource
PROSITE	Database of protein famines and domains
PEG	Poliethylene glycol
PL	Polysaccharide lyase
PCD	Programed cell death
UV light	Ultra violet light
qRT- PCR	Quantitative realtime-Polymerase chain reaction
RNA-seq	Ribonucleic acid suquencing
RXLR	(Arginine-X-leucine-Arginine; X could be any amino acid)
RNAs	RNA degrading enzymes
RISC	RNA- induced silencing complex
Rap1	Repressor activator protein 1
RNP	Ribonucleo protein
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Tm	Melting temperature
TAE	Buffer solution of Tris-Acetate EDTA

Abstract

Phytophthora cinnamomi, soil-borne pseudo fungi from the class of *oomycete*, not only representing a real threat to strategic cultures across the world but also in forestry. This pathogen is able to colonize roots of *Castanea sativa* causing ink disease. For that many studies are established to well understand the mechanism of infection by exogenous molecules secreted during contact with host cells. Those molecules have been identified thanks to the progress in molecular biology using technics like RNA Seq.

The Avr like genes expresses an RXLR protein family (Arginine-X-leucine-Arginine; X could be any amino acid), implicated in the pathogenicity of several pseudo-fungi of the genus *Phytophthora*, especially in *Phytophthora infestans*, *Phytophthora sojae* and *Phytophthora palmivora*. In this work, we isolate sequence, and characterize the *Avr3a* and *Avr1b* homologs in *P. cinnamomi* using molecular biology techniques such as PCR. In addition, we identified, in genomic sequences deposited in the databases, molecular factors involved in the metabolism and pathogenicity of *P. cinnamomi* using bioinformatics tools.

In order to better understand its role in the pathogenicity mechanism, interference RNA silencing cassettes were designed for the genes encoding AVR proteins.

Keywords: RXLR protein family, ink disease, *Avr3a*, RNA interference, *Avr1b*.

Resumo

Phytophthora cinnamomi, é um pseudo fungo do solo da classe dos *oomicetos*, que não só representam uma ameaça real para culturas agrícolas com importância econômica, mas também na silvicultura. Este patógeno é capaz de colonizar as raízes de *Castanea sativa* causando a doença de tinta. Face ao exposto, muitos estudos têm sido realizados com o objectivo de entender bem o mecanismo de infecção por moléculas exógenas secretadas durante o contato com células hospedeiras. Estas moléculas foram identificadas graças ao progresso da biologia molecular usando técnicas como o RNA Seq.

Os genes AVR expressam uma família de proteínas RXLR (Arginina-X-leucina-Arginina; X pode ser qualquer aminoácido), implicada na patogenicidade de vários pseudo-fungos do gênero *Phytophthora*, especialmente em *P. infestans*, *P. sojae* e *P. palmivora*. Neste trabalho, isolamos, sequenciamos e caracterizamos os homólogos *Avr3a* e *Avr1b* em *P. cinnamomi* utilizando técnicas de biologia molecular como PCR. Além disso, identificamos, em sequências genómicas depositadas nas bases de dados, fatores moleculares envolvidos no metabolismo e patogenicidade de *P. cinnamomi* usando ferramentas de bioinformática.

A fim de compreender melhor o seu papel no mecanismo de patogenicidade, foram desenhadas cassetes de silenciamento por ARN de interferência para os genes que codificam as proteínas AVR.

Palavaras-chave: Família de proteínas RXLR, doença da tinta, *Avr3a*, técnica de interferência de RNA, *Avr1b*.

1. Introduction

1.1. The Chestnut tree (*Castanea sativa*)

The chestnut tree is a fruit tree belonging to the genus *Castanea* that together with the genera Fagus and Quercus belongs to the family *Fagaceae*. It is a deciduous tree that can reach 25-45 meters in height, the leaves are simple, their length and wideness vary respectively from10 to 25 cm and from 5 to 8 cm, characterized with a serrated edge. It is a monoecious species that bloom from May to June and the fruits ripen from October to November, commonly known as chestnuts, they are surrounded by a thick and spiny dome. Their growth is quite rapid up to 50-60 years and they live on average 150 years. A population of tame chestnut trees, dedicated to producing chestnuts, is called “souto” and a population dedicated to producing wood is called “castinçal” [1, 2, 3].

C. sativa has different applications depending on the variety, in addition to fruit "chestnuts" and wood, it has an important role in soil ecology, biodiversity, in the balance of ecosystems promoting shelter and food for wildlife. The chestnut is appreciated in the culinary traditions of the Mediterranean, has great organoleptic qualities, a considerable nutritional value, with high starch content, water content, and low levels of fat. The presence of the chestnut enhances the growth of mushrooms and aromatic herbs, contributing to the maintenance of greater biodiversity with ecological and economic interest [4].

C. sativa called also the sweet chestnut, it is a tree species that has attracted particular human attention, perhaps more than any other culture in Europe. The existence of *C. sativa* in Europe comes from such distant times which makes it difficult to trace its original range. Indeed, it is believed to be present since the end of the Mesozoic era, or more certainly since the Cenozoic era, when it expanded [3]. During some historical periods, in various regions of Europe, the cultivation of chestnut became so dominant and indispensable for the survival of mountain populations, in that time *C. sativa* was considered an alternative source of energy during famines where usual sources were not sufficient, therefore some authors do not hesitate to identify these cultures as “chestnut civilizations” [5].

C. sativa is distributed over 25 European countries occupying about two million hectares [5]. This species has a special incidence in Spain, France, Greece, Italy, Portugal, and Turkey. In Italy, the forested area with chestnut trees is one of the largest in Europe [6].

In addition to the European chestnut, which has an economic interest in fruit and wood, the genus *Castanea* also includes other species of high economic interest such as *C. dentata* (American chestnut), nowadays, restricted to the southeast of the North American continent; *C. mollissima* (Chinese chestnut) originating in China and can also be found in North Korea and *C. crenata* (Japanese chestnut), is established in Japan and South Korea as well as others, characterized with low or unknown economic value such as *C. alnifolia*, *C. ashei*, *C. davidii*, *C. floridana*, *C. henry*, *C. ozarkensis*, *C. paucispina*, *C. pumila* and *C. seguinii* (**Figure 1**) [7,8,9].

Figure 1. Species of *Castanea* in the word (Forum florestral da estrutura federativa da floresta Portugesa, 2010) [9].

In Portugal, the chestnut tree occupies mainly the most mountainous part of the interior of the Center and North, which corresponds to the regions of Beira Interior and Trás-os-Montes (Terra Fria Transmontana), located between 600 and 1000 m in height. In Trás-os-Montes there are approximately 25,800 hectares of forest, which represents about half of the total area of Terra Fria. About 85% of the Portuguese nuts are produced here. In Beira Interior, about 5,000 ha of chestnut trees are cultivated; throughout the country, there are chestnut cores as in the North of Alentejo, Algarve, Minho Interior, Azores and Madeira [2, 6, 10].

Until now it was thought that the chestnut tree had been introduced in Portugal by the Romans, surveys carried out on peat bogs in the Serra da Estrela, at 1600 m, finished with discovering a fossil pollen that dates 8000 years. Although it is still necessary to know with greater accuracy the dating of the pollen found, it is possible to consider that the occurrence of chestnut in Portugal is well before the Romans, putting the hypothesis of being a species that has already existed in Portugal since the time of prehistory [11].

C. sativa has been considered one of the most promising crops for centuries. It occupies almost 2 million hectares with a very significant economic contribution in European agriculture [1]. Since the 20th century, the production of chestnut decreased considerably, with losses of around 300 million euros and continues to be seriously threatened until now by pests like the disparate woodworm that attacks wood and the codling moth of chestnuts which damage the fruit. However, the real threat comes from pseudo-fungi belongs to genus *Phytophthora*. *Phytophthora cinnamomi* (most virulent species) and *Phytophthora cambivora*, causing both of them ink disease in addition to the fungus *Cryphonectria parasitica*, responsible for chestnut cancer [3, 8].

1.2. Ink disease

Ink disease is one of the biggest threats to the *C. sativa* affecting both the development and economic yield of this strategic culture. It causes root and collar rot of adult trees and of seedlings in plantations, nurseries, and forests. In addition, Symptoms of the disease include chlorotic leaves reduced in size, immature husks remaining on the tree after falling leaves, and thinning of the crown [12].

Moreover, we notice Flame shaped dark necrosis is evident on the collar of the tree after debarking. During spring and the period corresponding to leaves falling, large roots are mainly infected, they start producing a black exudate that stains the surrounding soil. On young trees with smooth bark, the necrosis can be visible without debarking as depressed, slightly cracked areas at the base of the stem. There is extensive necrosis of the taproot that extends until the lateral roots and up the stem for some centimeters causing stem cankers (Figure 2(d)) [12].

Symptoms of the disease of the ink of the European chestnut (*C. sativa*) were first observed in Spain in 1726. In 1859, the symptoms of the disease were found in the chestnuts in many European countries, including northern Italy, France, and the United States. [5.6]. In

Portugal, the first symptoms of ink disease were described in 1838 and more precisely on the banks of the Lima River [13].

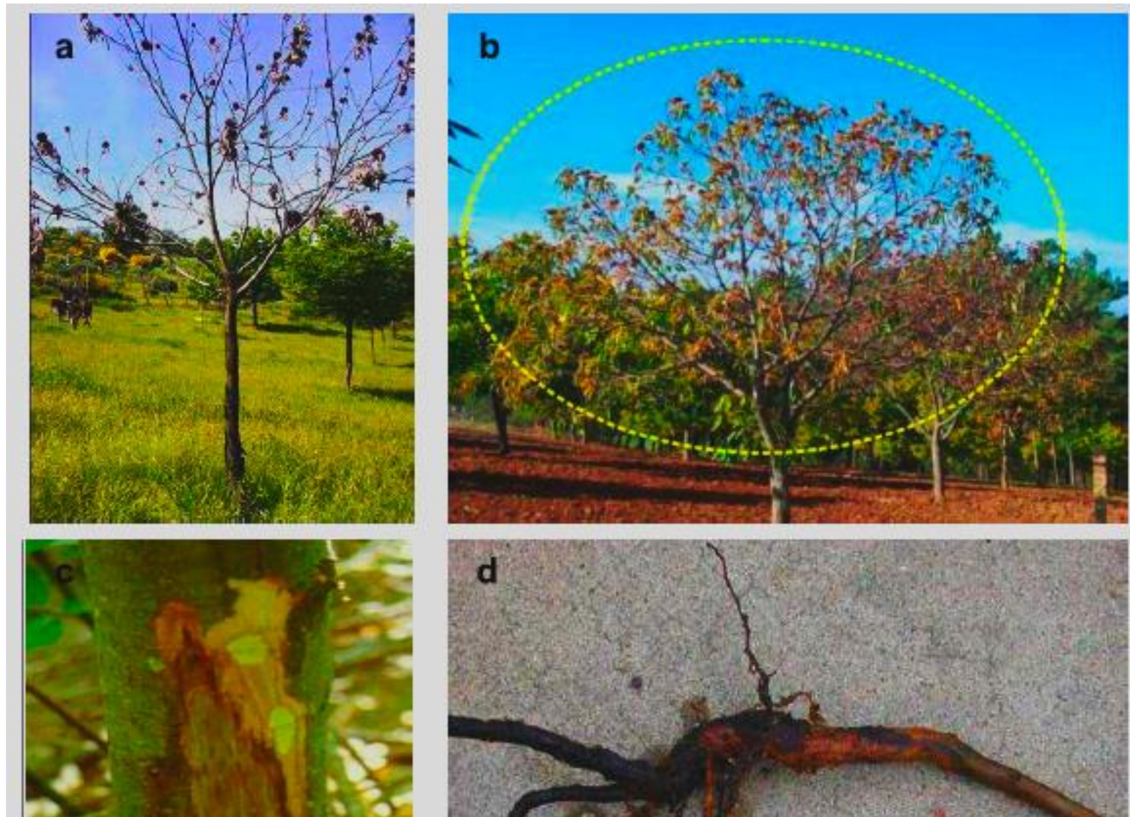


Figure 2. Symptoms of *P. cinnamomi* in chestnut trees at the top (a) and (b), trunk level (c) and root (d) [13].

1.3. Oomycetes

Oomycetes are a group of fungus-like eukaryotes with diverse microorganisms living in marine, freshwater, and terrestrial environments. They can live either saprophytically or parasitically from plants, animals, insects, and various microorganisms. More than 60% of the known species of *Oomycetes* are plant parasites [14].

The main genus of pathogenic *Oomycetes* are *Phytophthora*, *Plasmopara viticola*, *Albugo*, *Bremia*, *Peronospora*, and *Pythium*. Among these genera, species of the genus *Phytophthora* are surely the most destructive pathogens of dicotyledonous plants, such as

potatoes, pepper, tomatoes, soybeans, peas, and alfalfa. Six out of ten most *oomycete* pathogens in molecular plant pathology were from the *Phytophthora* genus [15]. *Oomycetes* are very important pathogens of plants and animals, causing severe and enormous losses in agriculture, being responsible for the death of thousands of trees per year, causing diseases such as potato blight disease caused by *P. infestans* and chestnut ink disease mediated by both *P. cinnamomi* and *P. cambivora* [16].

Since the identification of the *oomycetes*, it was believed to belong to the fungi kingdom due to similarities with "real fungi" in terms of growth allowed by the polarization of the hyphae, vegetative spores adapted to dispersion by wind or water, and infection strategies employed.

Unlike true fungi, this group of pathogen passes most of its life cycle as a diploid, the cell walls are composed of cellulose and β-glucans instead of chitin, it produces biflagellated zoospores possessing a flagellum adorned by tubular hairs that are responsible for forwarding movement [17, 18]. In addition, it does not synthesize sterols, they are resistant to polygenic antibiotics such as pimaricin, but requires sterols to sporulate [19]. Thereby, modern molecular analysis shows that they are more related to heteroontal algae in the Stramenopila Kingdom [20, 14], also called Chromista by Cavalier-Smith in 1986.

Oomycetes, including *Phytophthora*, are at an evolutionary level very far from "true" fungi, although they are part of a vast group of eukaryotic microorganisms and were originally classified as belonging to the Fungi kingdom due to their similarity in growth, morphology, propagation through spores and in the way it infects the host [21, 22, 23]. However, unlike most fungi, *Oomycetes* are organisms that are difficult to manage as they are not affected by most antifungals. The difficulty in controlling these organisms increases the diseases caused by *Phytophthora* since the plants are infected in most cases at the root level where the range of effective treatment is limited [24].

1.3.1. The *Phytophthora* Genus

The genus *Phytophthora* was traditionally classified in the Fungi kingdom, because it is an organism: heterotrophic (can live either saprophytically or parasitically), growth allowed by the polarization of hyphae, vegetative spores adapted to dispersion by wind or water and using plant infection strategies similar to those used by fungi. However, in *Phytophthora*, biological and physiological characteristics have always been recognized that gave it

uniqueness in the context of the kingdom in which it was inserted, and which were successively expanded with further studies [25].

As previously mentioned, unlike other “real” fungi, most of their life cycle is diploid, cell walls are composed of cellulose and ß-glucans instead of chitin, produce biflagellated zoospores, and do not synthesize sterols, so they are resistant to polyenic antibiotics, such as pimaricin, however needing sterols to sporulate [26].

These fungi-like eukaryotes, taxonomically classified as *Oomycetes*, generate asexual and sexed spores, with characteristics that contribute greatly to their pathogenic success [22].

1.3.2. *Phytophthora cinnamomi* specie

The inadvertent introduction of *P. cinnamomi* into the natural ecosystem has resulted in disastrous facts either on the environment or the biodiversity with its two components flora and fauna. *P. cinnamomi* is classified as one of the most devastating plant pathogens in the world. It has a destructive effect on more than 5000 horticultural and forestry species in the world, and especially on *C. sativa* [27].

This pathogen was described for the first time by Rands when observed first in Sumatra (currently Indonesia) [28]. In 1988, Zentmyer suggests that the species is native to the Southeast Asia and southern Africa, and spread across the Pacific to Latin America in the 18th Century [28]. After a couple of years, Hardam has proved that this phytopathogen originating in the Papua New Guinea region now has a global distribution as showed in **figure 3** [29].

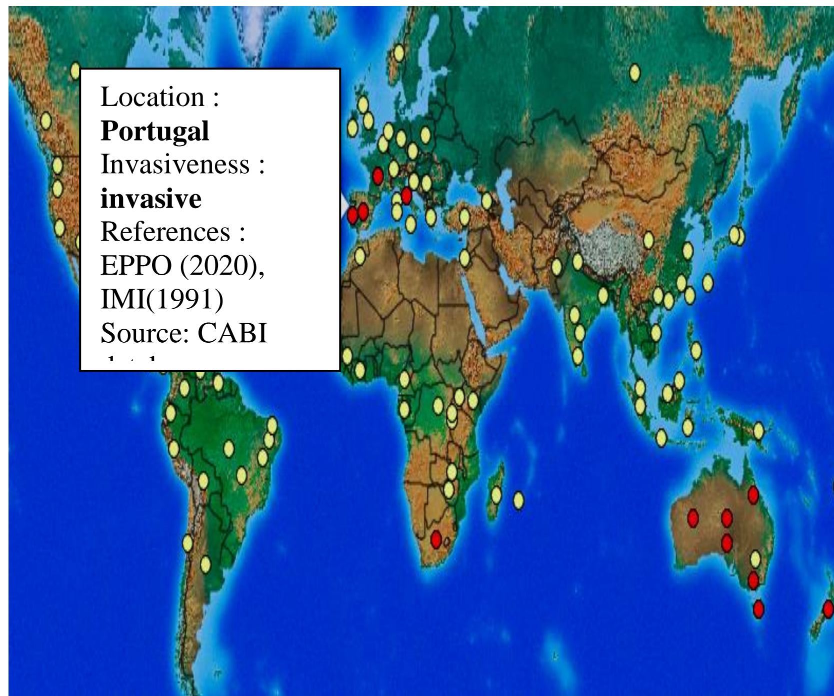


Figure 3. Distribution and pathogenicity state of *P. cinnamomi* in the world. Countries marked with white circles are countries contaminated by the pathogen and those marked with red circles aren't only contaminated but also invaded by *P. cinnamomi* [30].

1.3.2.1. *Phytophthora cinnamomi* life cycle

P. cinnamomi have two types of reproduction in their life cycle either it is asexual reproduction via formation of chlamydospores or sexual reproduction through karyogamy gametes, this variation is strongly related to current climatic conditions.

a) Sexual reproduction

Under optimal conditions, the encysted zoospores begin their germination and transform into mycelium which in turn will transform into antheridium or oogonium depending on the type of mycelium obtained as described in **figure 4**. After a certain time, the antheridium and the oogonium will produce respectively the female gamete and the male gamete. The process begins with sexual contact and the penetration of the antheridium precursor by the precursor of oogonium without fusion of cytoplasm between gametes [25].

After that, the expansion phase of the oogonium takes place due to the flow of cytoplasm through an oogonium rod that remains open and functional. When the expansion phase ends, the oogonium stick is stuck with a material similar to the cell wall. After that, a diploid oospore is formed after karyogamy and gives birth to the sporangium which will be the origin of the sexual zoospores [29] (**figure 4**).

b) Asexual reproduction

In unfavorable living conditions, the mycelium develops into a form of resistance called chlamydospores which is present in all species belonging to the genus *Phytophthora* in general. Chlamydospores are hyaline but they may change to yellowish or slightly brownish marks with time [29]. In ideal conditions, chlamydospores will subsequently form the sporangium giving asexual spores [29] (**figure 4**).

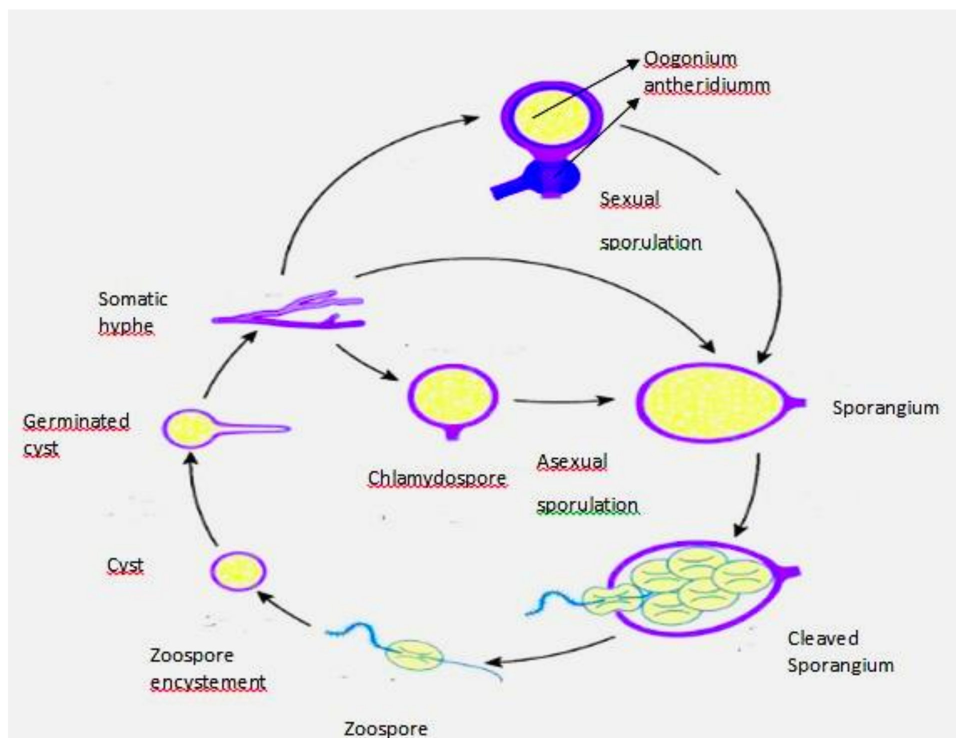


Figure 4. Diagram depicting the life cycle of *P. cinnamomi* [29].

1.3.2.2. Detection and identification of *P. cinnamomi*

Since *P. cinnamomi*'s identification, several tests have been proposed to detect its presence, the tests have evolved from tests based on a simple morphological characterization such as isolation and baiting towards other more sophisticated and innovative allowing detection at the molecular scale such as immunological tests and DNA based tests [31].

a) Isolation

Phytophthora can be detected by placing diseased tissue on selective agar containing antibacterial and antifungal antibiotics allowing specific growth of the genus *Phytophthora* from the tissue and identification by morphological characters [32, 33]. In this kind of experiment, we can see false negative samples, this is explained by the inability of the pathogen to develop outside the tissue [33].

b) Baiting

The baiting is commonly used for the detection of the genus *Phytophthora* in soil. It consists of floating pieces of sensitive tissue on a water suspension of the soil with a high water/soil ratio [34]. The zoospores secreted by *Phytophthora* in the sample infect the baits which, after several days of incubation, are then plated and identified on selective agar as described above. Although this technique has a certain efficiency, it remains not very reliable since the precision of the results depends on many factors. Among these factors, the host species from which the bait tissue is collected, Isolation of *Phytophthora* from infected bait requires considerable time and considerable knowledge of the genus to prepare the selective media [35]. Furthermore, studies using antibody tests [35] have shown that in a significant number (16%) of cases although zoospores were detected in the water the results of the baiting were negative.

c) Immuno-detection

In 1994, Cahill and Hardham developed an immunological dipstick assay for the detection of *P. cinnamomi* zoospores based on the use of a zoospore specific monoclonal antibody. The antibodies are incorporated into a dipstick format. The dipstick also contains zoospore attractants such as phenols, alcohols and amino acids. The assay could detect as few as 40 zoospores /ml [36].

d) DNA detection tests

In recent years, DNA detection test has become the most reliable method for detecting microbial pathogens. DNA has a number of advantages such as high specifity. Unlike proteins, the structure of DNA is not affected by environmental conditions or stage of development, and due to developments in PCR amplification technology, DNA is very easily detected. Virtually all DNA detection assays involve PCR amplification in which a pair of oligonucleotide primers flank a region of interest. The DNA then undergoes 30 to 40 cycles of synthesis resulting in an exponential increase in the number of copies of the flanked region. The amplification products are then detected by gel electrophoresis. PCR detection tests are rapid, sensitive, and highly specific [37]. PCR tests have been developed for many species of *Phytophthora* [31].

1.3.3. Molecular factors responsible for inducing pathogenicity (Elicitors)

Elicitors could be effector molecules, they are secreted by *Phytophthora* during infection to facilitate the onset of the disease upon contact with host cells. After that, these exogenic components are recognized by the host. Subsequently, this recognition is followed by the induction of the defense system - response - in order to limit the extent of the pathogen thereby called elicits or avirulance factors [27].

Elicitors could be proteins, carbohydrates, and lipids. They contribute to the development of the disease by causing metabolic or structural changes in the host cells to aid pathogen growth by interacting with molecules or products of resistant genes in order to inhibit the plant response system or induce an overexpression that leads to anarchic apoptosis [38]. In both cases, the immune response is disrupted and the pathogen grows further. Effectors or elicitors can be divided into two categories of molecules, apoplastic effectors and cell-entering effectors as it is shown in **figure 5**.

1.3.3.1. Apoplastic effectors

P. cinnamomi produces apoplastic effectors that are secreted to the extracellular space of the host, these effectors are mainly cell wall degrading enzymes (CWDE), elicitins, plant enzyme inhibitors, and toxins. For example, secreted enzymes act within the host membrane by causing its degradation [27] (**Figure 5**).

1.3.3.2. cell-entering effectors

Symplastic effectors are capable to pass into the plasma plant membrane with an unknown mechanism and act within the plant cell cytoplasm grouping transglutaminases, Crinkler effectors, NLPs (Nep1-type proteins), and RXLR factors, the class that interests us for this work [27]. (**Figure 5**). The function of the majority of known or putative cytoplasmic effectors is still unclear. Some have been shown to suppress host defense responses, provoke root necrosis or hypersensitive cell death [38, 39].

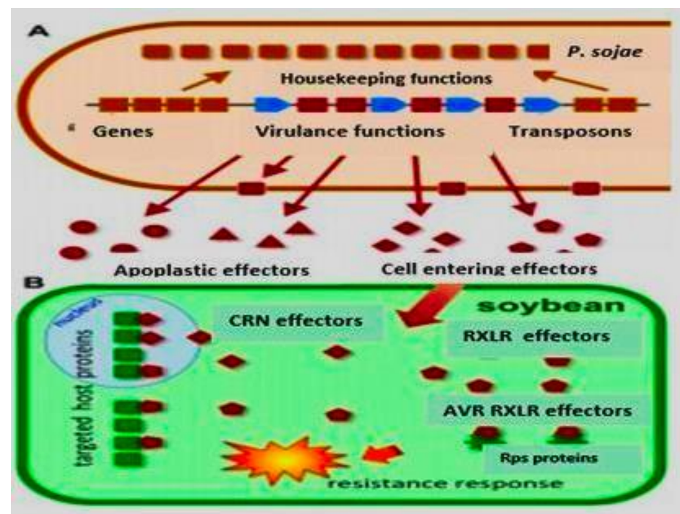


Figure 5. Large diverse families of virulence proteins encoded by the *Phytophthora* genome. (A) The *Phytophthora* genome contains clusters of conserved housekeeping genes (brown) that have conserved orders among *Phytophthora* species, separated by dynamic transported inside the cell. Cell-entering effectors may have targets in the nucleus or cytoplasm and may be detected by resistance proteins (Rps; resistance against *Phytophthora*) [15].

1.4. *Avr3a* gene

During the infection, fungi and *oomycetes* secrete molecular avirulence factors known as elicitors. These factors are responsible for disease arousal in hosts. Elicitors who remain out of host cells are called apoplastic factors such as lytic enzymes and toxins, others act within the cytoplasm and interact with some molecules involved in critical molecular pathways are called cell entering factors [27]. Among these effectors, Avr3a elicitor, encoded by *Avr3a* gene, and believed to be present in the genome of all *Phytophthora* species with a high percentage of homology between ORFs [27, 42].

In the time of infection, *Phytophthora* translocates Avr3a belongs to RXLR family into the cytoplasm of the host, this effector is implicated in suppressing the immune system of the host plant, therefore facilitate the mission to other elicitors to worsen the disease development [40, 41, 42]. Avr3a strongly interacts with E3 ubiquitin ligase CMPG 1 and stabilize it, this molecule is required for programmed cell death mediated by infestin triggered

cell death (INF1) [41, 42]. Thus the immune system of the host is strongly suppressed and the plant is more vulnerable to the infection.

Avr3a effector belongs to the RXLR family, a range of effectors known by their famous motif RXLR-DEER, a motif highly conserved in several effectors from the same family. It was proved that this N-terminal conserved domain allows RXLR -like- effector to translocate from the apoplast to the cytoplasm [43].

A mutation in N-Terminal region of Avr3a from *P. infestans* has deleted the capacity of this effector to link and stabilize E3 ubiquitin ligase CMPG 1 ligase and consequently, the programmed cell-death is no longer inhibited, indicating that *Avr3a* is essential for pathogenicity and a key component for the establishment of the infection [41].

Although there have been some attempts to isolate and study molecular factors of the RXLR family in *P. cinnmomi*, the *Avr3a* gene has a length of nearly 444 nucleotides (nt), is not yet characterized, and remains without a defined sequence in reliable databases such (NCBI, Fungi database, Uniprot…) whereas it is already described in other species of *Phytophthora* such as *P. infestans*, *P. sojae* and *P, caprici* and was considered as a vector of pathogenicity [44].

15. *Avr1b* gene

This gene has been identified in the genome of several pathogenic organisms such as yeasts, fungi and *oomycetes*, its length is around 417 nucleotides with small differences in length and a few nucleotides, especially between species belonging to the same family. In 2008, Dou et al produced transformants of *P. sojae* which contain multiple copies of the *Avr1b-1* gene and produce high levels of Avr1b-1 mRNA [43]. As a result, transformants were observed to kill soybean seedlings slightly faster than native strain P7076 in a hypocotyl inoculation assay. Thus, it has been proven that this gene positively contributes to the virulence of *P. sojae* [43].

The *Avr1b* gene, encoding the Avr1b effector belongs to the same family of RXLR elicitors, it contains two RXLR motifs and one DEER motif, both allowing translocation of the effector within as already described. Once inside the host cell, this effector interacts with the products of resistance genes, specifically the product of the *Rsp16* gene which mediates an immune response (HR) [43]. Furthermore, Avr1b effector suppresses programmed cell death (PCD) triggered by mouse BAX protein [43, 44].

Comparative studies of the Avr1b protein sequences of several pathogens have revealed the identification of 3 conserved regions (w, k, and L) in the C-terminal in addition to the secretory leader and RXLR-DEER domain in the N-terminal [44]. Substitution mutations have been introduced into these 3 regions of the *Avr1b* sequence, consequently, mutant Avr1b proteins were found with reduced or inexistent ability to suppress PCD, and also the avirulence interaction of Avr1b with the Rps1b resistance gene in soybean has been abolished [44].

In spite of its involvement in pathogenicity caused by other species of the genus *Phytophthora*, the *Avr1b* gene remains none studied and described in *P. cinnamomi.*

1.6. Molecular biology technics employed for silencing genes and targeting their products

Biological processes were often difficult to understand because they require in-depth knowledge at the molecular scale such as the identification of molecular factors pathways, their subcellular localization, and functions. In order to study biological processes, researchers around the world have developed several techniques that allow targeting of these factors such as fluorescence detection provided by Green Fluorescent Protein, often abbreviated GFP. In addition, other techniques such as RNA interference are very useful to know a probable function of a gene by silencing it and then notice the difference between a native strain and a transformed strain in order to study its involvement in such a biological process (fundamental metabolism, pathogenicity ...).

1.6.1. Green Fluorescent Protein (GFP)

The gene GFP was isolated from the jellyfish *Aequoria victoria*. Wild type GFP is composed of 238 amino acids for a molecular mass of approximately 27 kDa. The part responsible for fluorescence is called a chromophore, it is made up of the side chains of glycine, a tyrosine, and a serine [45].

The discovery of GFP and subsequently its use to target molecular factors was considered a revolution in the field of molecular biology. The green fluorescent protein can be used as a reporter gene [46]. Combined with a gene of interest, it makes it possible to follow the expression of the gene in real-time and the localization of the targeted protein in cells under fluorescence microscopy without modifying its usual function [46]. Unlike other

biological markers previously used such as beta-galactosidase , this protein is small and inert, which means that it does not interfere with any biological process. In addition, GFP fluoresces independently of cofactors or exogenous substrates [46, 47, 48]. Also, it requires neither fixation nor destruction of cell organisms to be visualized, hence investigations are getting more precise and credible.

1.6.2. RNA interference (RNAi)

RNA interference is a molecular biology technique which has proved its efficiency in gene silencing; it was recently discovered by Fire et al in 1998 in *Caenorhabditis elegans*. Indeed, experiments have demonstrated that the introduction of double-stranded RNA allows the silence of a gene of interest while sense or antisense RNA introduced separately had at most a modest effect [49].

RNA interference called also RNA mediated gene silencing, consists of preventing the translation of transcriptional products (mRNA) by causing their degradation. This degradation is ensured by making involved an enzyme called Argonaut (RNase) carrying the guide RNA specifically designed to pair with the mRNA of the gene of interest. Once the enzyme / RNA complex binds to the targeted mRNA, the guide RNA is released and the enzyme cuts the mRNA into two fragments and as a result, there is no protein synthesis and the gene becomes silent [50, 51].

RNA interference is mediated by three different types of mediators, small-interfering RNA (siRNA), micro RNA (miRNA), and short hairpin RNA (shRNA) [52]. Although all of them are double-stranded-RNA, their origins differ from one type to another, they can be endogenous or injected artificially into the cytoplasm thus called exogenous. In Vivo, to pass from the nucleus to the cytoplasm, the mediators require some modifications, they are then converted respectively from pri-shRNA or pri-miRNA to pre-shRNA or pre-miRNA by an enzyme called Drosha. After that, they are translocated into the cytoplasm by exportin 5 nuclear transporter. Furthermore, the mechanism of action of gene silencing induced by these mediators is almost the same, once they are in the cytoplasm, they are digested by the type III RNase called Dicer or Dicer-like (Dcl) into short segments. these short segments are directed to binds Argonaute (Ago) protein and form together an RNA- induced silencing complex (RISC), these segments represent therefore ARN guide to allow the complex to target specific

mARN and subsequently degrade it through specific domains of Argonaute enzyme [50, 51, 52].

The first genes to be silenced in fungi using hairpin-iRNA construction were *CAP59* and *ADE2* genes. In 2002, Liu et al have shown that expression of double-stranded RNA corresponding to portions of the coding sequence of both genes results in reduced mRNA levels for those genes, with phenotypic consequences identical to that of gene disruption [53].

In *oomycetes*, RNAi was applied to silence several genes involved in pathogenicity, The RXLR effector Avr3a gene is largely responsible for the virulence of *oomycete P. infestans* causing blight disease was silenced using hairpin RNAi construct. In fact, the vector containing the construct was injected into potato crops cell lines via Agrobacterium tumefaciens. As a result, transgenic potato cultivars were able to reduce the disease, hence the construct of hairpin RNA is demonstrated to induce partial resistance in the host cells [54].

In 2008, Ah-Fong et al have tried to silence *infl* from *P. infestans*, one gene involved in pathogenicity with several approaches. Consequently, a wide variation in effectiveness was reported in each approach. They compared the abilities of sense, antisense, and hairpin transgenes introduced by protoplast, electroporation, and bombardment methods to silence the *infl elicitin* gene in *P. infestans*. Finally, they conclude that a hairpin construct induced silencing three times more often than sense or antisense vectors, and protoplast transformation twice as much as electroporation [55, 56].

2. Objectives

2.1. General objectives

The aim is to develop an updated approach to mitigate *P. cinnamomi* impact on strategic cultures and forestry. This new strategy consists of the identification of molecular factors secreted by the pathogen that leads to ink disease. Moreover, we are looking for understanding the mechanism of infection using combinations of molecular biology technics and bioinformatics tools in order to provide a framework for future research by highlighting potential pathogenicity genes, therefore identifying suitable targets for future control measures.

2.2. Specific objectives

1- Amplification of *Avr3*a and *Avr1b* genes, subsequently their sequencing.

2- In silico characterization and subcellular localization of the identified molecular factors.

3- Cassette design for silencing the cited genes in (1) by RNA Interference technics in *P. cinnamomi*.

4- The establishment of a list that contains genes encoding virulence factors and proteins involved in metabolism from *P. cinnamomi* genome.

3. Materials and methods

The materials and methods part of this work will focus on two parts, a first part consists of the experiences done in the lab (growth of *P. cinnamomi*, DNA extraction, PCR amplification, sequencing genes of interest...), and the second part in the use of the bioinformatics tools to identify some molecular factors of *P. cinnamomi* involved in metabolism and pathogenicity.

3.1. Laboratory work

3.1.1. Biological materials

3.1.1.1 *P. cinnamomi* strains

P. cinnamomi strains were isolated from soil samples in the Trás-os-Montes region (northeast of Portugal), this region is known for chestnut production as described previously and it is affected by ink disease. Strains were then characterized by molecular methods and deposited in the Spanish Type Culture Collection (CECT) with CECT 20919 code.

3.1.1.2 Culture media for *P. cinnamomi* growth

PDA medium

PDA (Potato Dextrose Agar) was prepared by mixing 39 g of commercial PDA powder with 1 L of distilled water, then autoclaved at 120 ° C for 15 min.

Growth of *P. cinnamomi*

The growth of *P. cinnamomi* was carried out in a solid PDA medium in Petri dishes, by first inoculating mycelium taken from the Petri dishes already containing the isolated strains of *P. cinnamomi*. The growth was performed in dark for 5 days at a relatively ambient temperature of 25 ° C.

3.1.2 DNA extraction

After six days of growth, the obtained mycelium of *P. cinnamomi* is sufficient, fresh, and ready to be harvested in order to extract DNA. Although protocols of DNA extraction differ depending on the biological simple, they are all based on the same principle which consists of cell lysis, then deproteinization, and DNA purification using RNAses.

The extraction process firstly consists of mixing a portion of mycelium, 0.2g of glass beads, and CTAB lysis buffer (Cetyltrimethyl ammonium bromide CTAB 3% ,5 M NaCl, 0.5 M EDTA (pH 8.0),1 M Tris-Cl (pH 8.0), Poly-vinyl-pyrrolidone (PVP)1%, β-Mercaptoethanol 0.2%, H2O 24.8%) in an Eppendorf. Then put the tube under vortex for a few minutes in order to weaken the hyphae and induce the release of the DNA mixed with the proteins. After that, the first step of DNA purification which is the deproteinization takes place using isoamyl alcohol to sediment the proteins down the tube after a 10 min centrifugation at maximum speed. After that, DNA is precipitate using isopropanol with ammonium acetate 4M. Finally, DNA filaments were washed twice with 500 µl of ethanol 70% to purify DNA filaments. Subsequently, the DNA pellet is dissolved in 20- 50 µl of ultra-pure water and treated with RNase 30 µg for 5 minutes at 37 ℃ to RNA hydrolysis.

3.1.3. Design primers for amplification *Avr3a* and *Avr1b* genes

Avr3a and *Avr1b* are two genes strongly involved in the pathogenicity of the genus *Phytophthora*. According to the bibliography and bioinformatics comparison tools, these two genes are highly conserved within the genus *Phytophthora* with a percentage of homology that can reach more than 90%. With reference to this homology, we have designed specific primers for these genes for *P. infestans* and *P. sojae* using Fast PCR program (see **table 1**).

The primers were designed using the Fast PCR program, the method is simple and consists of placing the sequence of the gene of interest in the program interface and pressing the command "design primers for PCR". Therefore, the program generates any possible combination of primers, indicating al the parameters that reflect primer's quality like GC%, Tm, primer length, and fragment's size to be amplified with such a primer combination.

The table contains five different combinations of primers including specific and degenerative primers designed to amplify the *Avr3a* and *Avr1b* of interest, each designed primer have its melting temperature (Tm) indicated by the manufacturer " ISOGEN LIFE SCIENCE ".

Table 1. Primers for *Avr3a* and *Avr1b* gene's amplification

Primer	Sequence 5'3'	Tm [°C]
AVR3a F	ATGCGTCTGGCAATTATGCTGTG	60,2
AVR3a R	CTAATATCCAGTGAGCCCCAGGT	62.4
AVR1b F	ATGCGTCTATCTTTTGTGC	52
AVR1b R	TCAGCTCTGATACAGGTGA	54
AVR3a F5	CGCCTCGCTCAAGTTGTGGTCG	65.8
AVR3a R14	TCTTTGGAACAAAGCCGGGTTC	60.3
AVR3aD F	ATGCGTCTRKCWWTTRTGCTKTC	63.8
AVR3aD R	CTAATATCMRSTSWGMYMCAGGT	59.5

3.1.4. PCR amplification of *Avr3a* and *Avr1b*

The thermal cycler (Bio-Rad) was used to amplify the Avr3a gene, with the primers designed as described in Table 3. The PCR cycling conditions were; 94 ℃ / 5 min, followed by 36 cycles of 94 ℃ / 1 min; 59.5 / 1 min; 72 ℃ / 30 s, and ending with 72 ℃ / 5 min.

Each PCR reaction contained 1.6 mM of dNTP, 0.2 mM of each primer, 300 ng of genomic DNA and 1U of Taq DNA polymerase, 10 µl of Promega buffer (5X), 1.5 mM of $Mgcl_2$, 100 µg /ml of BCA (bovine clostridial abomasitis).

Amplification of the ORF of *Avr1b* was performed as mentioned for the *Avr3a* gene. For PCR amplification, annealing temperatures were changed depending on the used primer combination.

3.1.5. Visualization and purification of nucleic acids in agarose gel

The visualization and separation of DNA fragments either genomic DNA or PCR-generated-amplicons were performed by electrophoresis in agarose gel low melting 0.8 % (w/v) in TAE (Tris-Acetate 40 mM, 1 mM of EDTA), with 0.5 µg/ml GreenSafe Premium (NZYtech, Portugal) for 45 min at 55 V.

Once the migration of the nucleic acids on the agarose gel is done, the gel is placed in a transilluminator (ChemiDoc™ XRS+ imaging system (BioRad)) thus allowing clear visualization of the bands of interest, these bands will subsequently be identified by referring to DNA ladders as it is shown in **figure 6**. Consequently, the bands of interest are cut from the agarose gel with a clean razor blade and DNA molecules present in the bands were purified by the GENE CLEAN^{R} KITT II following the manufacturer's instructions.

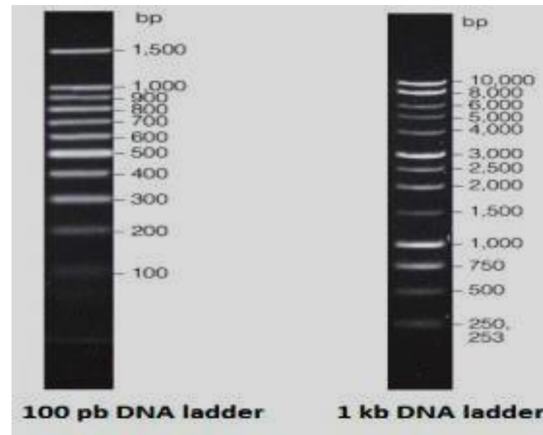


Figure 6. The DNA ladders used to determine the size of bands in agarose gels (Promega).

3.1.6. Quantification and sequencing of nucleic acids

3.1.6.1. Quantification of nucleic acids with spectrophotometry

In molecular biology, quantification of nucleic acids is commonly performed to determine the average concentrations of nucleic acid present in such a sample, as well as their purity. Reactions such as PCR and the Sanger sequencing method often require particular amounts and purity of DNA for optimal performance. After cutting the agarose bands

containing fragments of interest, the DNA was first purified (as described in previous part) and then quantified by spectrophotometry.

The concentration required to perform the Sanger sequencing method is 100 ng (nucleic acid) / 8 µl for bands 1000 bp in length. Knowing that the sizes of the *Avr3a* and *Avr1b* genes are respectively equal to 444 b and 415 bp, the calculations are then made as follows:

100 ng ➔ 1000 bp

x = 44.4 ng ➔ 444 bp (case of *Avr3a*)

y = 41.5 ng ➔ 415 bp (case of *Avr1b*)

The amount of DNA that must be added for a final volume equal to 8ul is determined by the following formula:

Ci * Vi = Cf * Vf

Ci: DNA concentration of the purified band

Vi: The volume of DNA which must be added for final volume equal to 8ul

Cf: it is equal to "x" for the case of Avr3a and equal to "y" for the case of Avr1b.

Vf: equal to 8 ul

Primer concentration =3,2 pmol

After having calculated the amount of DNA that must be added, we mix it with 0.256 ul of one of the primers used to amplify the DNA fragment and subsequently complete with ultra-pure water until the final volume equal to 8ul, hence the simple is ready for sequencing.

3.1.6.2. Sequencing of nucleic acids

The sequencing of the DNA fragments obtained in this work was carried out by the Sanger method using an ABI prism 377 TM automatic sequencer (Department of Microbiology and Genetics, University of Salamanca), which performs the electrophoretic separation and the detection of DNA fragments labeled with fluorescence. Four different fluorescent colors identify the four dideoxynucleotides incorporated in the extension reaction (A, G, T, and C).

3.1.7. Editing of sequencing results

Sequencing results have been provided in extension files (.ab). The program "Bio edit Sequence Alignment Editor" was used to open the extension files (.ab) thus allowing to make corrections in the sequence and to generate standard extension sequences (fasta) which are the files of entry of the open-source BLAST (**Basic Local Alignment Search Tool**) to finds regions of local similarity between sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Once the sequence has been entered, the program compares the sequence with all the genomic sequences belonging to several organisms such as including genus *Phytophthora* deposited universal databases such as (the NCBI database, EMBL, Fungi database…). Therefore, it displays the results of the comparison in order of homology and thus it can be confirmed whether or not the amplified sequence matches the gene of interest. Furthermore, the standard extension sequences (fasta) can be first translated into a protein sequence by "Bio edit Sequence Alignment Editor" and then compared directly with the proteins already described in the NCBI database. Subsequently, sequences with significant homology (>70%) are considered homologs according to bibliographical sources.

3.1.8. Studying the subcellular localization of Avr3a and Avr1b proteins

The prediction of the subcellular localization of proteins after being synthesized in ribosomes can be carried out by bioinformatics tools or by using molecular biology tools such as GFP like markers.

The fluorescent protein GFP is a genetic marker, associated with a gene of interest, allows both the monitoring of the destination of such a protein of interest and also its level of expression in real-time through the quantification of the fluorescence emitted during a biological process. In addition, detection by the fluorescent protein GFP consists of transforming the biological sample (*P. cinnamomi* in our case) with plasmids containing a promoter, the GFP coding sequence, the gene of interest, and a terminator all in a row. Once the organism is transformed, the transcription mechanism generates mRNAs that correspond to the gene of interest associated with the GFP protein. After the translation of the mRNAs in the ribosomes, visualization of the subcellular localization is then possible under microscopy.

In the silico process, there are several publicly available software that use different methods to predict the localization of proteins. Their computational calculus relies on

parameters such as the amino acid composition, N-peptide composition, and Physico-chemical Composition. They require as input only the protein sequence.

The software used for protein localization predictions can be accessed through the URL addresses as follows:

-SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/)

-Cello: (http://cello.life.nctu.edu.tw/)

-LOCtree: (https://rostlab.org/services/loctree2/)

-EuK-mPLoc 2.0: (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/)

- BaCelLo predictor: (http://gpcr2.biocomp.unibo.it/bacello/pred.htm)

In this work, protein subcellular localization was carried out by Cello software, and results were verified with BaCelLo predictor software for more credibility.

3.2. Bioinformatics tools used to identify molecular factors of *P. cinnamomi* in metabolism and pathogenicity.

3.2.1. Biological Information Databases

For the research of genes, we used the sequences of the genome and the transcriptome of *Phytophthora cinnamomi*, deposited in the databases:

NCBI (National Center for Biotechnology Information)at https://www.ncbi.nlm.nih.gov/;

EMBL (*European Molecular Biology Laboratory) at www.ebi.ac.uk*/ena

DDJB (DNA Data Bank of Japan) at www.ddbj.nig.ac.jp/

*FungiDB (*The Fungal and *Oomycete* Genomics Resource) at fungidb.org.

The genomic sequence used in this work is the one deposited in NCBI under the following reference: *P. cinnamomi* isolate MP94-48(5,831 rc linear DNA).

3.2.2. Open reading frame search

For the search of open reading frames, in the sequences of the genome and the transcriptome of *P. cinnamomi*, the following programs were used:

- ORF finder *NCBI* at https://www.ncbi.nlm.nih.gov/orffinder/;

- GGENEinfinity at http://www.geneinfinity.org/sms/sms_orffinder.html;

- GGenScript at https://www.genscript.com/sms2/orf_find.html.

3.2.3. Homology of proteins encoded by open reading frames

Detection of protein homology and sequence alignment underlies the prediction of protein structure, function, and evolution. Protein homologies were determined in the BLAST ,Smart BLAST, Clustal Omega ,and Fasta programs independently or through NCBI (National Center for Biotechnology Information). Its use is simple by simply needing to paste the sequence of interest into the program window and ask the program to establish homology with the Protein Databases sequences: UniProt Universal Resource (EBI), Protein Information Resource (PIR, Georgetown University Medical Center (GUMC)), Swiss-Prot Protein Knowledgebase (Swiss Institute of Bioinformatics) and PROSITE (Database of Protein Famines and Domains).

3.2.4. Bioinformatics characterization of molecular factors

After decoding the open reading frame of a gene, a number of bioinformatics tools can be used to characterize the deduced sequence of the protein. A search on the Expasy Proteomics Server website (http://expasy.org/tools) and a nucleotide sequence allow us to identify and characterize proteins, identify motifs, patterns and profiles, infer their stability, cell location or function, predictions of secondary and tertiary structures, look for similar sequences deposited in databases and compare them, and establish phylogenetic relationships. The detection of the physical-chemical characteristics of the proteins was carried out in PROSITE (http://prosite.expasy.org/scanprosite/), in the neural network system of the Pôle BioInformatique Lionnais / Network Protein Sequence Analysis or in the application DiANNA 1.1 (http: //clavius.bc.edu/~clotelab/DiANNA/). Different sites were predicted for post-translational modifications on the Center of Biological Sequence Analysis website (http://www.cbs.dtu.dk/services). The structural predictions were made through the server swiss modelling and the structural representation made through the Pymol program, whose version can be installed in https: //www.pymol.org/.

3.2.5. Molecular docking

Docking of the ligand to the protein was carried out via Autodock Vina performed by the Scripps Research Institute and the results were visualized by Pymol program.
http://vina.scripps.edu/download.html

4. Results and discussion

4.1. DNA extraction of *P. cinnamomi*

P. cinnamomi's genomic DNA was extracted from fresh mycelium filaments grown into Petri dishes containing PDA medium for one 5 days at 25 ° C and in the dark. This DNA is subsequently used as a template to amplify the two genes of interest Avr1b and Avr3a.

After extraction, the extracted DNA was migrated in an agarose gel (1%) to verify the existence, the state of purity and the quality of the DNA. The UV image taken by the transilluminator (ChemiDoc ™ XRS + Imaging System (BioRad)) shows that the DNA is clearly visible in the gel with the absence of degradation and unwanted products like RNA influencing the degree of purity of the DNA. Thus, DNA is ready to be used in PCR reactions.

Figure 7 shows that the distance of migration on the electrophoresis gel that the DNA made compared to the migration of the reference bands of the 1 kb label used (Promega) is small, which makes sense since the size of the genome is several times greater than 1 kb, which explains why the distance migrated is less important and this proves that the sample deposited on the gel is indeed DNA.

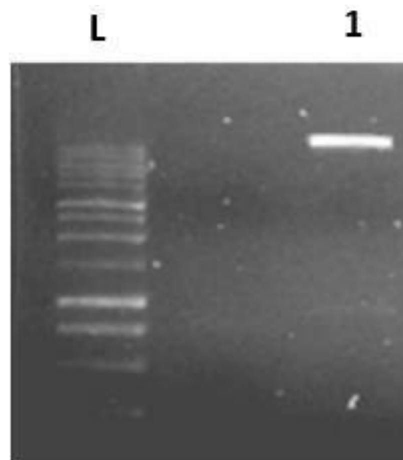


Figure 7. Visualization of *P. cinnamomi* genomic DNA in agaorose gel 1% (w/v); (L) DNA ladder 1kb (promega); (1) genomic DNA of *P. cinnamomi.*

4.2. DNA positive control

Before being used to amplify genes of interest, the extracted DNA was used as a template to amplify a highly conserved region in the genomes of *oomycetes* and fungi using a combination of primers ITS5F 5'GGAAGTAAAAGTCGTAACAAGG 3' and ITS1R 5'GCTGCGTTCTTCATCGATGC 3' described by Ma Jincai et al [88]. This amplified region corresponds to the *ITS 18S rRNA gene* which has a size of 300 bp. this gene serves as a biological marker, it is widely used for the identification of fungi and *oomycetes*.

Figure 8 shows the migration of the PCR products corresponding to the *ITS 18S rRNA* gene on an agarose gel. The size of the product obtained by PCR, have the size of the fragment to be amplified according to the bibliography [88].

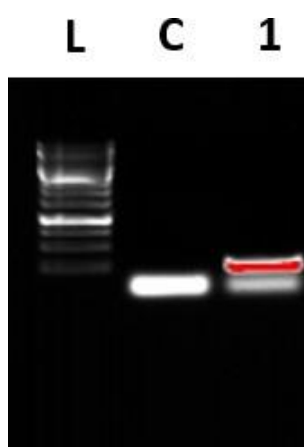


Figure 8. Migration of the amplicon of the *ITS 18S rRNA* gene on an agarose gel; (L) DNA ladder (1kb promega); (C) Control (ITS5F, ITS1R); (1) ITS 18S rRNA

4.3. PCR amplification of *Avr3a*

The Avr3a effector is strongly involved in the pathogenicity of *oomycetes* according to the bibliography, having a slightly variable size depending on the species. The length then varies from 444 bp to *P. infestans* up to 552 bp in *P. sojae* according to data from multiple platforms such as NCBI Database, Uniprot, and Fungi database. Thus, the length of *Avr3a* from *P. cinnamomi* is expected to be close to that of other *Avr3a* belonging to the genus *Phytophthora* due to the very significant similarities between the *Avr3a* genes.

In order to amplify *Avr3a* on *P. cinnamomi*, several combinations of the previously designed primers were used. First, the *Avr3a* gene was amplified using the degenerate primers AVR3aD F 5 'ATGCGTCTRKCWWTTRTGCTKTC 3' and AVR3aD R 5 'CTAATATCMRSTSWGMYMCAGGT 3'. Then another combination of the primers was used but this time specific for the *Avr3a* gene of *P. infestans*, they are the two primers AVR3a F 5 'ATGCGTCTGGCAATTATGCTGTG 3' and AVR3a R5 'CTAATATCCAGTGAGCCCCAGGT 3'.

In the case where PCR reaction was carried out with degenerate primers, migration on electrophoresis gel shows that there is a multitude of bands, each corresponding to a different size. Thus, the band of interest having a size of about 500 bp, is found mixed with several non-specific bands (**figure 9**).

In the case where PCR reaction was carried out with the specific primers AVR3a F and AVR3a R, there is a considerable decrease in the non-specific bands and there are just two bands on the electrophoresis gel (**see figure 9**). The first band at the bottom having a size of about 500 bp since it coincides with the 2nd band of the marker (Promega 1kb), which corresponds to the size of the gene of interest. The other band at the top corresponds to a size almost equal to 750 bp, this band did not disappear after all possible modifications of the PCR conditions, which suggests the hypothesis that this band may correspond to another allele of *Avr3a* (**see figure 9**).

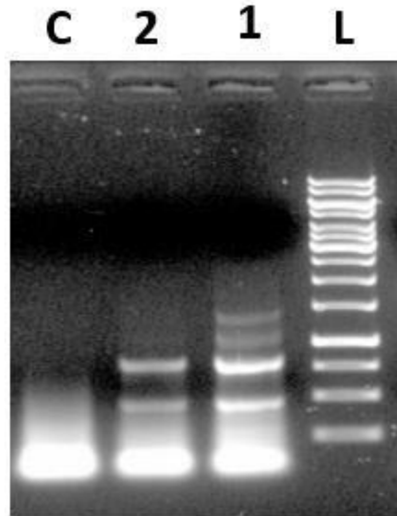


Figure 9. Visualization of *Avr3a* amplification; (L) DNA ladder (1kb promega); (C) Control (AVR3A F , AVR3A R); (1) amplification of *Avr3a* gene performed with degenerative primers; (2) amplification of *Avr3a* gene performed with Specific primers (AVR3A F , AVR3A R).

In addition, two other combinations of the primers were used in order to have a single band, which will be sequenced first and then described as being the gene encoding the Avr3a effector in *P. cinnamomi*.

AVR3a F5 (a forward specific primer for *P. sojae*) 5 'CGCCTCGCTCAAGTTGTGGTCG 3' with AVR3a R (a reverse specific primer for *p. Infestans*) 5'CTAATATCCAGTGAGCCCCAGGT 3 ' were used to perform PCR reaction. As a result, more than a single band was observed after migration on an agarose gel as shown the **figure 10**. Therefore, the band of interest is still mixed with others none specific.

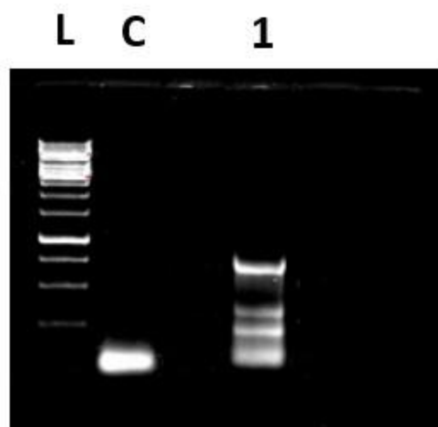


Figure 10. Visualization of *Avr3a* amplification; (L) DNA ladder (1kb promega); (C) Control (AVR3A F, AVR3A R14); (1) amplification of *Avr3a* gene performed with Specific primers. (AVR3A F, AVR3A R14).

Furthermore, a PCR reaction was performed with AVR3a F5 5 ' CGCCTCGCTCAAGTTGTGGTCG 3'and AVR3a R14 5 ' TCTTTGGAACAAAGCCGGGTTC 3' both primers are specific to AVR3a from *P. sojae* were efficient after several PCRs trials to amplify a specific single band that matches AVR3a size. Hence, this band is ready to be isolated, purified, and finally sequenced as shown in **figure 11**.

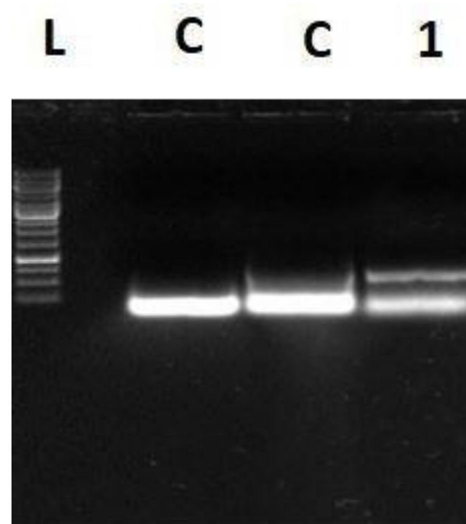


Figure 11. Visualization of *Avr3a* amplification; (L) DNA ladder (1kb promega); (C) Control (AVR3A F5, AVR3A R14); (1) amplification of *Avr3a* gene performed with specific primers (AVR3A F5, AVR3A R14).

All PCR reactions were performed under the same physical and chemical conditions already described in materials and methods, the only parameter to modify is the primer annealing temperature which depends on the combination of primers chosen, and also the number of PCR cycles was sometimes adjusted to minimize the appearance of non-specific and unwanted bands.

4.4. PCR amplification of *Avr1b*

Avr1b is a gene like *Avr3a*, inductor of pathogenicity in *oomycetes*, belonging to the family of RXLR effectors and thought to be involved in ink disease.

Avr1b was amplified using two primers specifically designed from the *Avr1b* gene of *P. sojae*, AVR1b F 5' ATGCGTCTATCTTTTGTGC3' and AVR1b R 5' TCAGCTCTGATACAGGTGA 3'. **Figure 12** shows that after migration of PCR products on an electrophoresis gel, we notice the appearance of two bands, one at the top having a size similar to that of *Avr3a* which may actually correspond to *Avr3a* since the two genes have a large similarity and it is deduced from this case that these primers may amplify the *Avr3a* gene. The other band at the bottom is the one that interests us the most since its size is nearer to *Avr1b* gene size equal to approximately 415 bp.

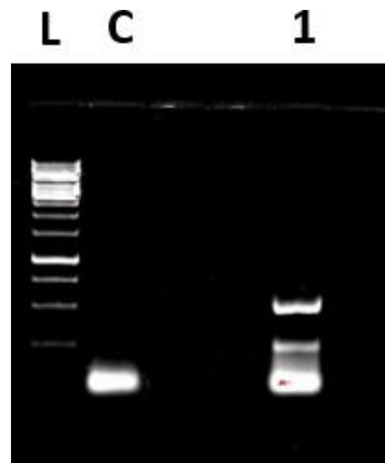


Figure 12. Visualization of *Avr1b* amplification; (L) DNA ladder (1kb promega); (C) Control (AVR1b F, AVR1b R); (1) amplification of gene *Avr1b* carried out with specific primers (AVR1b F, AVR1b R).

4.5. Purification and DNA quantification of the bands of interest.

Once the bands of interest were identified, they are then isolated by cutting the fragment directly from the agarose gel under the UV light emitted by the “BioRad” transilluminator. The cut fragments were immediately placed in an Eppendorf tube and the purification process was performed with Gene Clean Kit according to the manufacturer's instructions. The purified bands are then quantified by spectrophotometry since the success of sequencing by the Sanger method requires a DNA concentration equal to 100ng / µl.1000bp in PCR products.

The following table shows the different concentrations of the samples corresponding to the genes *Avr3a*, *Avr1b* and the marker *ITS 18S rRNA* gene used for the positive control. The final samples delivered for sequencing should have a final volume equal to 8µl containing the concentration of DNA 100ng / µl.1000bp required to perform sequencing by the Sanger method.

The final volume of 8 µl is made up of:

VA: volume of DNA to add after adjustment; VA = VI + 500 µl

VI: the volume of DNA to add in 8ul to have the required concentration

Example of VI calculation for the ITS 18S rRNA gene:

Ci * VI = Cf * VF

100ng ==> 1000bp

x = 30ng ==> 300bp (size of the ITS 18S rRNA gene)

135.579 * VI = 30 * 8 µl

VI = 1,770 µl

VPrimer (µl): 0.256 µl of one of the two primers used to amplify the gene. For the case of the AVR3a gene, the primer used is AVR3a F5, AVR1b F for the samples corresponding to the AVR1b gene, and ITS5F for the sample containing the amplification product of the ITS18 gene.

Ultra-pure water (µl): the volume of water to be completed with up to the final volume equal to 8 µl

VF (final volume) = VA+ VPrimer (µl) + Ultra-pure water (µl)

Table 2. DNA quantification of purified bands and formulation of samples for sequencing.

Simples	Concentration ng/ul	DNA Vi (µl)	DNA VA (µl)	Primer (µl)	Ultra-pure water (µl)	Final volume (µl)
Avr1b (1)	**270.731**	**1.034**	**1.534**	**0.256**	**6.210**	**8.00**
Avr1b (2)	**160.889**	**2.073**	**2.073**	**0.256**	**5.671**	**8.00**
Avr3a (1)	**321.368**	**1.105**	**1.605**	**0.256**	**6.139**	**8.00**
Avr3a (2)	**290.104**	**1.224**	**1.724**	**0.256**	**6.020**	**8.00**
Avr3a (3)	**105.637**	**3.362**	**3.862**	**0.256**	**3.882**	**8.00**
ITS 18	**135.579**	**1.770**	**2.270**	**0.256**	**5.474**	**8.00**

Avr1b (1): Corresponds to the band at the top (see figure), this band was obtained using the primer pair (AVR1b F, AVR1b R).

Avr1b (2): Corresponds to the band at the bottom (see figure), this band was obtained using the primer pair (AVR1b F, AVR1b R).

Avr3a (1): Corresponds to the single band observed (see figure), this band was obtained using the primer pair (AVR3a F5, AVR3a R14).

Avr3a (2): Corresponds to the band observed at the top (see figure), this band was obtained using the primer pair (AVR3a F, AVR3a R).

Avr3a (3): Corresponds to the band observed at the bottom (see figure), this band was obtained using the primer pair (AVR3a F, AVR3a R).

4.6. Characterization of Avr3a effector

Avr3a is one of the most interesting genes for the study of infection mediated by *P. cinnamomi*. It belongs to the RXLR family, varying in length from about 444 bp in *P. infestans* to 552 bp in *P. sojae*. This gene codes for the Avr3a effector composed of approximately 145 amino acids, the number of amino acids varying little depending on the species. This protein is like any protein of the RXLR family, characterized by the famous RXLR motif (arg-x-leucine-arg) that allow the translocation of the effector towards host cell's cytoplasm.

Sequencing of the PCR products corresponding to the *Avr3a* gene was carried out as described in section **2.1.4**. The nucleotide sequence obtained was then translated into a protein sequence using the BioEdit sequence alignment software. Next, the protein sequence was aligned using the Clustal Omega (a multiple sequence alignment program), with the other Avr3a of other *Phytophthora* species such as *P. sojae*, *P. palmivora.var palmivora* and *P. infestans.*

Figure 13 shows that the results of alignment of potential Avr3a protein of *P. cinnamomi* with the Avr3a homologs of the above-mentioned species are highly significant, these homologies are further confirmed by the Smart Blast tool of the NCBI as shown in **figure 14**. In fact, potential Avr3a of *P. cinnamomi* has a homology equal to 71.57% with Avr3a of *P. palmivora.var palmivora* while it is a homolog to Avr3a of *P. infestans* with a percentage which varies from 94.17% to 100% depending on the isolate.

The alignment of the Avr3a revealed that this protein is highly conserved in the genus *Phythophthora.* **Figure 13** shows the three domains described in the bibliography (N-Terminal Signal peptide, RXLR, C-Terminal effector peptide). The figure shows that the RXLR reference motif was found in positions 44 to 47, which agrees with the bibliographic data [31]. as previously mentioned, the RXLR motif (Arginine-X-leucine-Arginine; X could be any amino acid) is a highly conserved motif in this type of effector, composed by 4 amino acids of which 3 are fixed and one which differs from one species to another. The X of the RXLR of the *P. infestans's* Avr3a, *P. sojae's* Avr3a, and the *P. cinnamomi* Avr3a corresponds to the amino acid lysine while the X of the RXLR of *P. palmivora.var palmivora*'s Avr3a corresponds to the amino acid Phenylalanine.

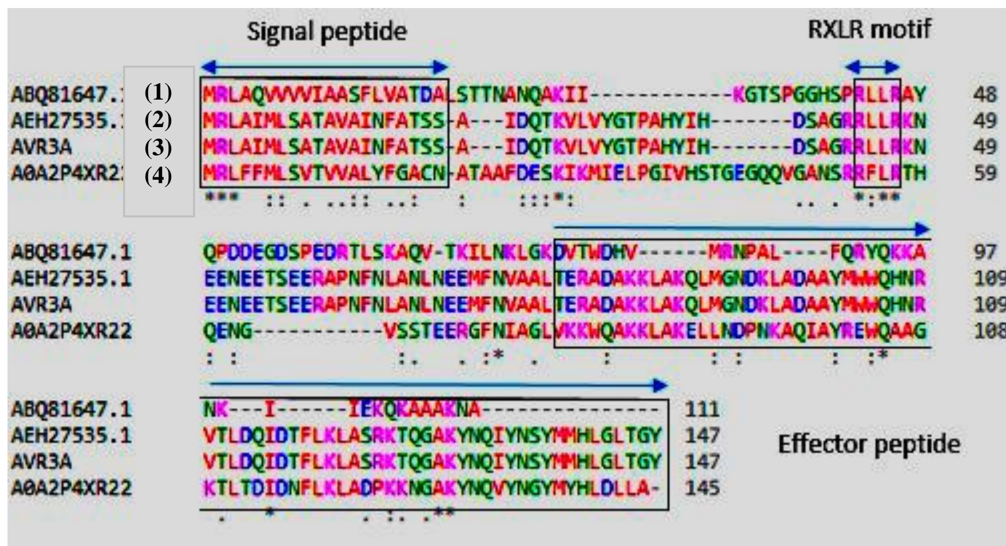


Figure 13. Alignment of the protein sequences of the Avr3a effector where (2) designates the sequence of the Avr3a of *P. infestans*, (4) designates the sequence of the Avr3a of *P. palmivora.var palmivora*, (1) designates the sequence of *P. sojae* Avr3a and (3) correspond to the identified *P. cinnamomi* Avr3a. The signal peptide, the RXLR motif and the effector peptide are consecutively the domains conserved in the family of RXLR type effector.

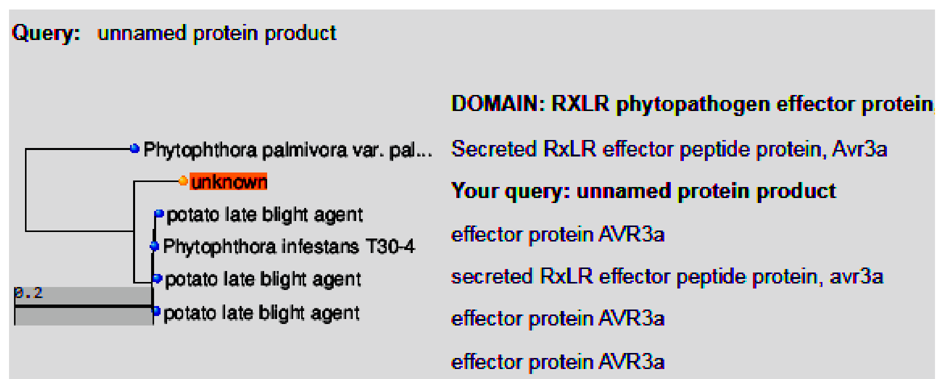


Figure 14. Phylogenetic tree of Avr3a proteins from *P. cinnamomi* (unknown in the figure) with different species of the genus *Phytophthora*.

The fact that the protein of this pathogen has an RXLR motif (**Figure 13**), gives it the ability to pass from the apoplast to the cytoplasm through a molecular interaction between the host receptors and the constant protein sequence of the RXLR -DEER [48](**Figure 15**). Inside, the effector part of this exogenous protein will lead to the induction of a cascade of reactions that favors the suppression of the defense response, and therefore the establishment of the disease [27].

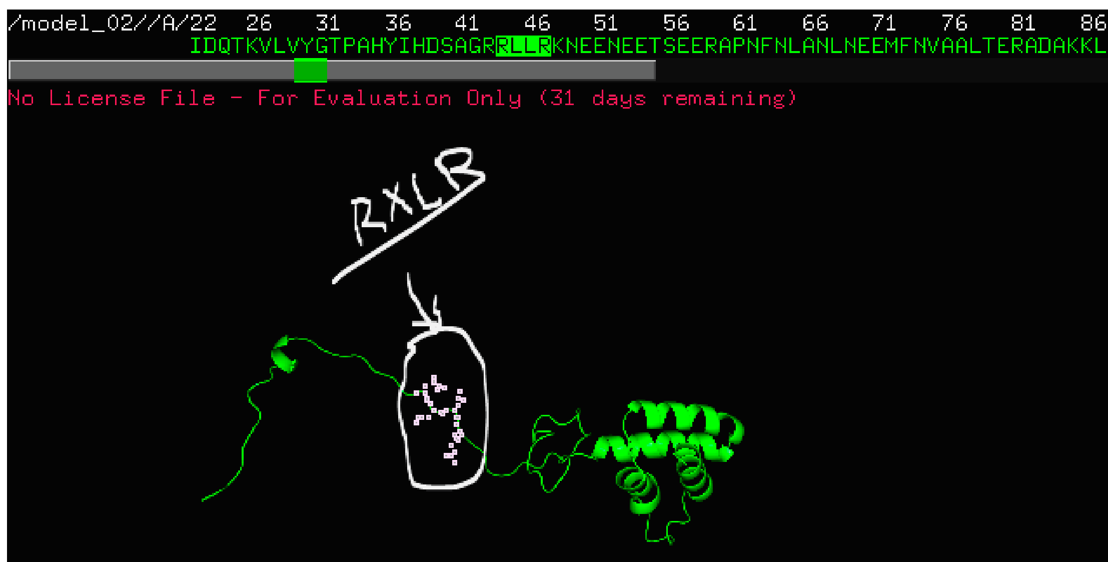


Figure 15. 3D structure of Avr3a protein of *P. infestans* and exact location of RXLR motif. (This result was determined by the Pymol visualization program).

4.7. Characterization of Avr1b effector

As already shown, amplification of the *Avr1b* gene resulted in two different PCR products (see **figure 12**), the classic isolation which consists of cutting the band of interest directly from the agarose gel under UV light is then no longer effective to isolate in the case where we have more than one band. An attempt at sequencing was made from the cut fragment directly yielded insignificant results and the two sequences were found to be mixed during sequencing by the Sanger method, which made their identification impossible.

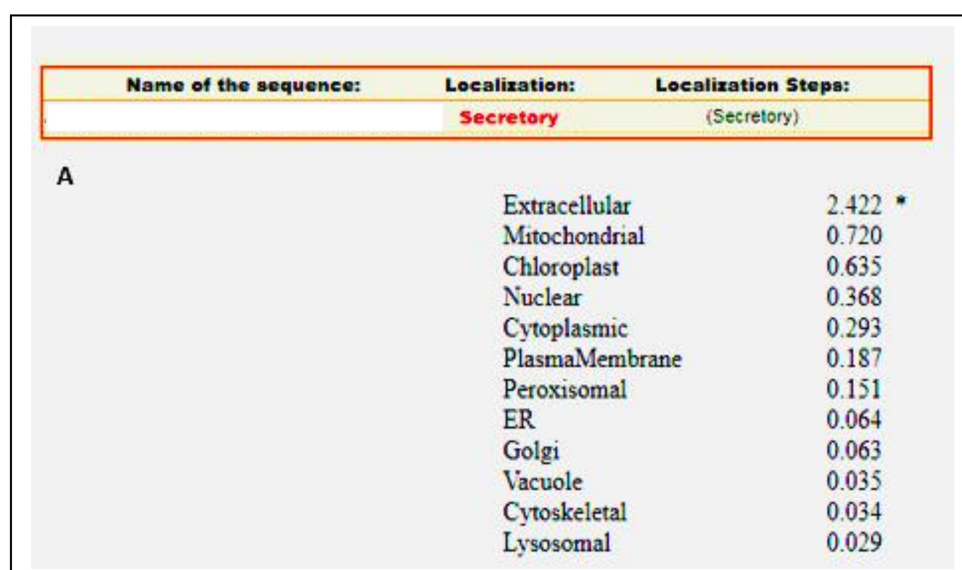
The two fragments must then be cloned each in a vector in order to be sequenced (this process is ongoing).

4.8. Subcellular localization of *P. cinnamomi*'s effector Avr3a

Upon contact with host cells, *P. cinnamomi* secretes effectors that mediate the disease. These effector molecules must be destined for the extracellular medium in order to execute their probable functions.

To determine the subcellular localization of these effectors, various software described in **3.5** are publicly available and have been used to predict the location of the proteins we characterize.

The subcellular prediction of the Avr3a effector was first made with the Cello 3.0 software and then confirmed with the BaCelLo software. The use of these two programs is very simple, and only requires the fasta protein sequence as input into the program interface, then click the "Run" command. The prediction was made from the identified sequence of the Avr3a effector of *P. cinnamomi*. The two programs show that the localization of the protein is indeed extracellular (see **figure 16**), which is in accordance with the function of the protein involved in the pathogenicity process. Also, the results are consistent with what has been mentioned in the bibliography.



Name of the sequence:	Localization:	Localization Steps:
	Secretory	(Secretory)

A

Extracellular	2.422 *
Mitochondrial	0.720
Chloroplast	0.635
Nuclear	0.368
Cytoplasmic	0.293
PlasmaMembrane	0.187
Peroxisomal	0.151
ER	0.064
Golgi	0.063
Vacuole	0.035
Cytoskeletal	0.034
Lysosomal	0.029

Figure 16. Subcellular localization of Avr3a effector. A; prediction using CELLO 3.0 software. B; prediction using BaCelLo software.

49. Design of a genetic construct for silencing *Avr3a* and *Avr1b* genes by RNA interference

To construct an efficient conventional silencing cassette that targets the corresponding mRNA of the Avr3a gene and Avr1b, the following measures should be taken into consideration:

• The potential construction sequence should not have a perfect match of more than 16 nucleotides with other *P. cinnamomi* genes (verified by BLAST search at http://www.ncbi.nlm.nih.gov/BLAST/);

• The construct must not have internal repeats or palindromes and must include low to medium G / C content [57].

• It is recommended to use a fragment of the silencing cassette between 300 and 1000 bp in length as an adequate size to maximize the silencing efficiency obtained [58].

The sense and antisense sequences can be produced by performing two separate PCR reactions, followed by the PCR products bound by T4 DNA ligase. Until now, there are no reliable programs that allow design cassettes to silence genes with RNAi, so we chose to build the cassettes to silence *Avr3a* and *Avr1b* genes manually.

4.9.1. Silencing construct for *Avr3a*

For the *Avr3a* gene, as shown in **Figure 17**, we place 444 bp of the "ORF" in the sense and antisense direction (in red) between an intron (in green) that will serve as a "loop" for the formation of double-stranded RNA after the transcription. Once in the cytoplasm, this Ds RNA will be processed and then serve as RNA guide for specific enzymes which after bind and degrade target mRNA, subsequently hide gene expression (see part **1.5.1** for further details).

```
ATGCGTCTGGCAATTATGCTGTCTGCTACGGCTGTCGCCATAAACTTTGCAACCAGCAGTGCAATC
GACCAAACCAAGGTCCTGGTGTATGGGACGCCAGCTCACTACATACACGATTCAGCCGGCAGAAG
ACTTCTTCGCAAGAACGAAGAGAATGAAGAAACGTCTGAGGAGCGAGCCCCAAATTTCAATTTGG
GAGAAGGGTAAGCCAAGACATTTGACTTCCACAAAAGTAAGAGTACTCACGCACCACGCTGGCAC
CCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGG
AGGTGGCAACGCCAATCAGTACGCAGACCGTTAATACGACAGACGATGCCGACAGCGGTATTTG
AAA CGTTGGTCGTCACGTTAGCTGGTTTGGTTCCAGGACCACATACCCTGCGGTCGAGTGATG
TATGTGCTAAGTCGGCCGTCTTCTGAAGAAGCGTTCTTGCTTCCTTACTTCTTTGCAGACTCCTC
GCTCGGGGTTTAAAGTTAAACC
```

Figure 17. Silencing Cassette the *Avr3a* gene of *P. cinnamomi* with RNA interference. Sense and antisense (in red), intron in the middle (in green).

4.9.2. Silencing construct for *Avr1b*

For the *Avr1b* gene, the same procedure as that used for Avr3a was followed to design the silencing cassette, it suffices to separate the two nucleotide sequences (sense and antisense) by an intron which promotes the formation of double-stranded RNA. Thus, 415 bp of *Avr1b* was placed in the sense and antisense direction (in red) with an intron (in green) in between, as shown in the **figure 18**.

ATGCGTCCATCTTTTGTGCTTTCTCTTGTCGTGGCCATTGGCTACGTCGTGACCTGCAACGCAACT
GAGTACTCCGACGAAACCAATATCGCCATGGTGGAATCTCCAGATCTCGTCCGTCGCTCGCTCA
GGAACGGCGACATTGCCGGTGGAAGATTTCTTCGAGCGCATGAAGAGGACGATGCGGGGGAGC
AGAGAAGGGTAAGCCAAGACATTTGACTTCCACAAAAGTAAGAGTACTCACGCACCACGCTGG
CACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAG
ACTGGAGGTGGCAACGCCAATCAGTACGCAGGTAGAAAACACGAAAGAGAACAGCACCGGTAA
CCGATGCAGCACTGGACGTTGCGTTGACTCATGAGGCTGCTTTGGTTATAGCGGTACCACCTTAG
AGGTCTAGAGCAGGCAGCGAGCGAGTCCGTTCCGCTGTAACGGCCACCTTCTAAAGAAGCTCGC
GTACTTCTCCTGCTACGCCCCCTCGT

Figure 18. Silencing Cassette the *Avr1b* gene of *P. cinnamomi* with RNA interference. Sense and antisense (in red), intron in the middle (in green).

4.10. Identification *P. cinnamomi's* molecular factors involved in metabolism and pathogenicity.

In this part, the genomic sequence deposited in NCBI under the following reference: *P. cinnamomi* isolate MP94-48(5,831 rc linear DNA) was used to identify each cited molecular factor. Firstly, Contigs from this assembly were analysed via the ORF finder tool in order to find out the ORFs from these random sequences (contigs). Secondly, we performed a smart blast with the identified ORFs to find homologs of molecular factors already described in other pseudo-fungi, such as *P. sojae, P. infestans,* with known pathways and functions. These molecular factors are then divided into two classes, molecular factors involved in pathogenicity and others implicated in fundamental metabolism (see **Table 3** and **Table 4**).

This classification was based on bibliography, conserved domains, and subcellular location of molecular factors of interest using BaCelLo predictor and Cello tools. Proteins which have extracellular locations are considered to be involved in pathogenicity, those which do not have extracellular locations (nuclear, cytoplasmic, and mitochondrial) are considered to be involved in fundamental metabolism. This classification was in agreement with what has been shown in the bibliography.

In **Table 3** and **4**, each cited protein is codified by a specific ORF, which was identified as described in the previous part with its contig number and the name of the homolog organism. Proteins are described in terms of function and subcellular location as mentioned above. Each protein is defined by its NCBI gene bank accession.

We made a comparison of the proteins deduced from the obtained open reading phases, in order to find homologous proteins characterized in other living organisms and deposited in the Uniprot and Swiss-Prot protein databases. In both sources, most of the open reading phases encode proteins homologous to metabolic and pathogenic proteins of different species of the genus *Phytophthora*, as expected, especially *P. infestans*, *P. sojae*, *P. nicotianea* and *P. parasitica*. There were also a large number of open reading frames that coded for hypothetical proteins and/or with unknown functions.

4.10.1. Characterisation of *P. cinnamomi's* molecular factors involved in metabolism

A bioinformatic analysis of the *Phytophthora* genomic sequences allowed us to identify several genes involved in metabolism, the most important of which code for proteins belonging to several families, namely kinases, ligases, polymerases, transferases, and methylases (see table 3). Metabolism is the set of thousands of chemical reactions of anabolism and catabolism by proteins which are in turn coded for by specific genes. The good functioning of the mechanisms will allow survival and development and consequently enhance the virulent power of the pathogen.

Table 3. *Phytophthora cinnamomi*, molecular factors involved in metabolism.

Contig	Protein length	Homolog organism	Name of protein	Localization	Function	Accession
1	137aa	*Phytophthora megakarya*	Polycomb protein	Nuclear	Direct Assembly of transcriptionaly repressed chromatin [59].	GenBank: OWZ24764.1
	132aa	*Phytophthora cactorum*				GenBank: KAF1784310.1
2	113aa	*Phytophthora Cactorum*	Nucleotide-binding alpha-beta plait domain	Nuclear	RNA binding [60].	GenBank: KAF1773404.1
	166aa	*Phytophthora megakarya*	P21-activated protein kinase-interacting protein	Nuclear and mainly cytoplasmic	Regulate cell shape and polarity [61].	GenBank: OWZ18359.1
	121aa	*Phytophthora megakarya*	Glucose repression regulatory protein TUP1	Nuclear	Greatly implicated in glucose repression and affect mating type [62].	GenBank: OWZ07049.1
	241aa	*Phytophthora cactorum*	Frag1/DRAM/Sfk1	Plasma membrane	Induces macro-autophagy as an effector of p53-mediated death [63].	GenBank: KAF1793710.1
3	177aa	*Phytophthora infestans*	Rap1 Myb domain		critical for maintaining normal telomere length and structure [64].	GenBank: KAF4146400.1
102	791aa	*Phytophthora megakarya*	Clavaminate synthase	Cytoplasmic	conversion of proclavaminic acid to clavaminic acid [65].	GenBank: OWZ22087.1

Characterisation of *TUP 1* gene in *P. cinnamomi*

In contig 2 (LGSJ01000002.1 sequence in NCBI), an ORF with a length of 121aa (366nt) has been identified as a homolog of *TUP 1* (glucose repression regulatory protein). It has a high percentage of homology equal to 83.19% with TUP1 of *P. megakarya* according to smart blast; also this isolated ORF has a very significant coverage up to 93% with the same gene of *P. megakarya*. *TUP 1* is a gene involved in the repression of glucose for a multitude of genes and also influences the type of mating, and an induced mutation of this gene has reduced the degree of repression of glucose considerably, changed mating type, and disrupted some other pathways [66]. *TUP 1* affecting the reproduction of the species, can be considered as one of the ways to inhibit the pathogen. The analysis of the sequence revealed 2 conserved domains, WD40 (accession: cl29593) and DUF2205 (accession: cl10911), as seen in **Figure 19.**

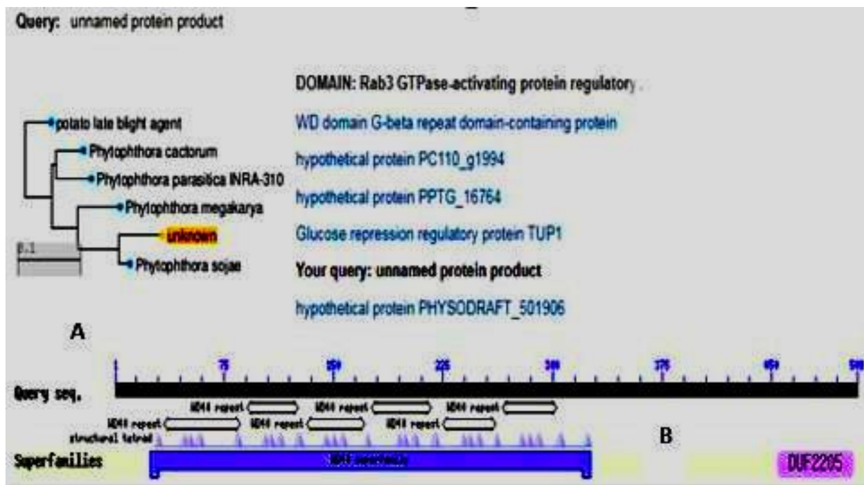


Figure 19. (a) Phylogenetic tree of TUP 1 protein from *Phytophthora cinnamomi* (unknown in the figure) with different species of the *Phytophthora* genus. (b) Conserved domains identified in the query of interest, WD40 domain, found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing, and cytoskeleton assembly. Also, DUF2205 has a highly conserved 100 residue region, which is likely to be a coiled-coil structure. The exact function is unknown.

Characterisation of *Rap1* gene in *Phytophthora cinnamomi*

Analysis of the sequence LGSJ01000003.1 from *P. cinnamomi* taken from NCBI, with the ORF research platform, as described in the Methodologies section, revealed an ORF of length equal to 366 nucleotides / 121aa, which has a significant coverage and homology of 98% and 65.28 %, respectively, with myb Rap 1 domain of *P. infestans*. Rap1 (repressor activator protein 1) was first discovered in budding yeast, *Saccharomyces cerevisiae,* as a positive transcriptional regulator of multiple growth-related genes, such as ribosomal protein genes [67]. Other studies have identified Rap1 as the main repeat binding protein for double-stranded telomeres in *S. cerevisiae* and necessary for maintaining the length and structural integrity of telomeres [68, 69]. Indeed, telomeres are specialized nucleoprotein structures that preserve the integrity of eukaryotic chromosomal terminations by protecting them from fusion

and recombination by initiating their replication. In most organisms, telomeric DNA is made up of short repetitive sequences characterized by an abundant presence of G residues on the strand containing the 3 'end. These repeat sequences are maintained by a ribonucleoprotein (RNP) known as telomerase, acting as an unusual reverse transcriptase [70, 71]. Telomeric binding proteins and telomerase are both essential for preserving the integrity of telomeres through multiple cell divisions, which in turn are essential for supporting genome stability and extend the lifespan of cell. The Myb domain binding to the DNA of *C. albicans* Rap1 is sufficient to suppress most of the aberrations of the telomeres observed in the null mutant. In addition, in a recent analysis, Rap1 was recognized as a key component of the coupling-type silencer and has been shown to be essential for transcriptional silence [72- 73, 74].

Characterisation of gene coding for clavaminate synthase in *Phytophthora cinnamomi*

Clavaminate synthase is an enzyme involved in metabolism, the gene coding for this protein has been identified in the following reference sequence LGSJ01000102.1 of the genome of *P. cinnamomi* deposited in the NCBI platform using the ORF finder tool. The identified ORF has a length of 2376 nucleotides which corresponded to 792 amino acids.
This fragment has a very significant percentage of query cover and homology with the pseudo fungus of the same genus, *P. megakarya,* having percentages of 100% and 79.90%, respectively. It is an enzyme that has industrial utility and more specifically in the pharmaceutical field. It catalyzes the reaction of the transformation of proclavaminic acid into clavaminic acid [75]. This molecule has proven synergistic effects when combined with beta-lactam antibiotics, such as amoxicillin. This combination is used only in patients suspected of infections with beta-lactamase-producing bacteria.

Amoxicillin is a beta-lactam antibiotic that disrupts the synthesis of the bacterial cell wall by binding to penicillin-binding proteins present inside the bacterial cell wall, thereby preventing the synthesis of the peptidoglycan layer in the cell membrane [76]. This disruption of cell wall synthesis results in cell lysis and bacterial death. In some cases, the bacterial species produce the beta-lactamase enzyme, which can inactivate beta-lactam drugs by hydrolyzing the beta-lactam bond in the antibiotic compound, leading to drug resistance. In this case, clavulanic acid at its beta-lactam bond binds to the active site of beta-lactamase and inactivates the enzyme, thereby improving the antibacterial effect of beta-lactam antibiotics.

4.10.2. Characterisation of genes involved in mechanisms of pathogenicity of *Phytophthora cinnamomi*

In this section, we will shed light on the molecular factors thought to directly trigger an infection in a host cell (see **table 4**). Thus, all the genes cited code for extracellular proteins according to the bioinformatics tools described in the methodologies. The function of each protein will be discussed by referring to the function of its counterpart already described in other living organisms.

Table 4. *Phytophthora cinnamomi*, molecular factors involved in pathogenicity.

Contig	ORF's length	Homolog organism	Name of protein	Localization	Function	Accession
102	406	*Phytophthora megakarya*	Murein transglycosylase	Extracellular	Involved in the biogenesis of the cell wall, required for growth, invading ecological niches, and counteracting the host immune response [77].	GenBank: OWZ01054.1
81	135aa	*Phytophthora sojae*	Elicitin	Extracellular	Elicitin proteins produce metabolic or structural changes in host cells that aid pathogen growth and favour the development of the disease [27].	GenBank: XP_00952494 0.1
	135aa	*Phytophthora ramorum*	Elicitin-like protein RAL11D	Extracellular		GenBank: ABB55948.1
101	478aa	*Phytophthora megakarya*	Transglutaminase elicitor, partial	Extracellular	Involved in the establishment of *Phytophthora* disease during early phases [27].	GenBank: OWY99932.1
	478aa	*Phytophthora palmivora var. palmivora*	Transglutaminase elicitor-like protein	Extracellular		GenBank: POM62262.1
95	118aa	*Phytophthora palmivora var. Palmivora*	Polysaccharide lyase, partial	Extracellular	Enzyme involved in plant polysaccharide and pectin degradations [78].	GenBank: POM79686.1
108	115aa	*Phytophthora palmivora var. palmivora*	Zinc ion binding protein	Extracellular	Sequester zinc from host cells and tissues [79].	GenBank: POM7200 1.1

Characterisation of *murein tranglycosylase* gene in *Phytophthora cinnamomi*

Analysis of the sequence LGSJ01000102.1 from *P. cinnamomi* available in the NCBI, with the ORF finder tool revealed an ORF of length equal to 1221 nucleotides / 406aa, which has a significant coverage and homology equal to 99% and 83.74 %, respectively, with a gene coding for murein transglutaminase from *P. megakarya*..

The fungal cell wall is an essential structure that maintains cell form and protects fungi against environmental critical conditions. Glycosyltransferases, glycoside hydrolases, and transglycosylases are involved in the biogenesis of the cell wall, responsible for growth, invading ecological niches, and counteracting the host immune response. Murein transglycosylase is a lytic enzyme that belongs to the class of autolysin that cleaves the cell wall heteropolymer peptidoglycan (murein) to facilitate its biosynthesis and regeneration [80]. Moreover, the protein function described is in agreement with the extracellular localization deduced, since this enzyme is involved in the maintenance of cell structure and growth. In *Aspergillus fumigatus*, a family of five Crh transglycosylases was detected. Indeed, *in vitro* biochemical assays and localization studies demonstrated that detected enzymes are specifically transglycosylases for both chitin-glucan and chitin-chitin cell wall linkages forming a three-dimensional network mesh required to strengthen cell wall and ensure its integrity. Furthermore, crh genes aren't only dispensable for cell viability when ensuring cellular turnover but also renders cells sensitive to cell wall interfering compounds [81]. This makes this gene a target to be taken into consideration in order to inhibit or disrupt pathogen growth.

Characterisation of *polysaccharide lyase* gene in *Phytophthora cinnamomi*

Polysaccharide lyase is an enzyme involved in plant polysaccharide and pectin degradation. The gene coding for this protein has been identified in the following reference sequence LGSJ01000095.1 of the genome of *P. cinnamomi* deposited in the NCBI platform using the ORF finder tool, and the identified ORF has a length of 357 nucleotides, which correspond to 118 amino acids.

This fragment has a very significant percentage of query cover and homology with the pseudo fungus of the same genus *P. palmivora var. palmivora,* having percentages of 100% and 91.53%, respectively. Fungal enzymes involved in plant polysaccharide degradation are assigned to at least 35 glycoside hydrolase (GH) families, three carbohydrate esterase (CE) families, and six polysaccharide lyase (PL) families [82, 83]. Thereby, the identified query coding for polysaccharide lyase has the power to degrade pectin backbones when combined with glycoside hydrolases [83, 84]. In addition to that, the analysis of this protein has shown the conservation of specific domain pectate lyase (accession: pfam03211) responsible for pectin degradation belonging to the pectate lyase superfamily according to sequence analysis of conserved domains carried out with NCBI.

Characterisation of the gene coding for the zinc ion binding protein in *Phytophthora cinnamomi*

Analysis of the sequence LGSJ01000108.1 from *P. cinnamomi* available in the NCBI, with the ORF finder tool, revealed an ORF of length equal to 348 nucleotides / 115aa, which has a significant coverage and homology respectively equal to 80% and 55.9 %, with a gene coding for a zincophore-like protein from *P. palmivora var. palmivora*.

Zinc is fundamental for all domains of life, as it composes the catalytic and structural center of a large array of proteins. It is a ubiquitous metal in all life forms, as it is a structural component of almost 10% of eukaryotic proteins. In fact, zinc-depleting conditions are known to reduce fungal growth and evidence suggests that host cells employ sequestration of zinc to inhibit fungal development [85]. In order to overcome this defence system, pathogenic fungi activate the expression of several systems to enhance the uptake of zinc, through secretion of zincophores, which are proteins able to chelate zinc. Therefore, silencing this zinc uptake system can be seen as an innovative way to inhibit *P. cinnamomi* (see **figure 20 and 21**).

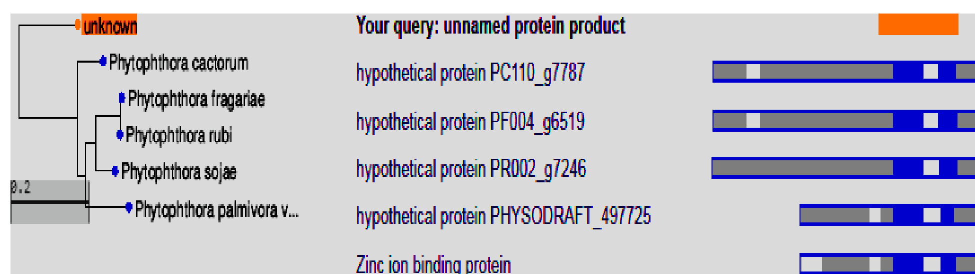


Figure 20. Phylogenetic tree of zincophore protein from *Phytophthora cinnamomi* (unknown in the figure) with different species of the *Phytophthora* genus.

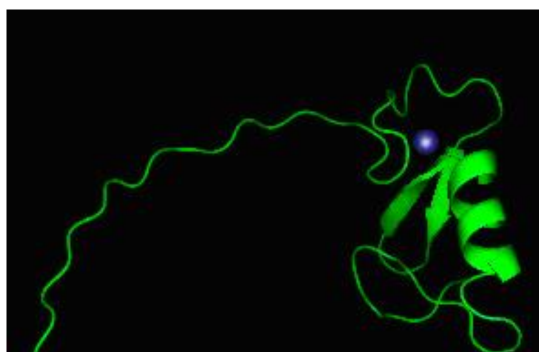


Figure 21. Molecular docking of zincophore protein with its ligand, which is zinc.

Characterisation of a gene coding for elicitor protein in *Phytophthora cinnamomi*

Analysis of the sequence LGSJ01000081.1 from *P. cinnamomi* genome available in the NCBI, with the ORF finder tool, revealed an ORF of length equal to 408 nucleotides / 135aa, which has significant homology with elicitin proteins from *P. sojae* and *P. rammourm,* equal to 75.56% and 68.22 %, respectively.

During infection, *P. cinnamomi* secretes a diverse range of effector molecules in order to infect the plant. The intended function of these effectors is to facilitate the establishment of disease during the infection. From the plant's point of view, the goal is to recognize the effectors and induce a defence response that will inhibit or mitigate the development of the disease. When an intended effector is recognized by the plant and elicits a defence response, it is termed an avirulence factor or elicitor. Elicitins are classified as apoplastic effectors, meaning that they are secreted into the plant extracellular space. In fact, elicitin proteins produce metabolic or structural changes in host cells that aid pathogen growth and favour the development of the disease [27].

The analysis of conserved domains in this identified homolog with NCBI conserved domain option has revealed the presence of elicitin domain (Accession: pfam00964). This domain occupies a significant part of the protein in terms of length, from position 21 to position 113, and is believed to induce leaf necrosis in infected plants and elicit an incompatible hypersensitive-like reaction.

Characterisation of a gene coding for transglutaminase elicitor-like protein in *Phytophthora cinnamomi*

As in other’s cases described, analysis of the sequence LGSJ01000101.1 from *P. cinnamomi* genome available in the NCBI, with the ORF finder tool, revealed an ORF of length equal to 1437 nucleotides / 478 aa, which has significant homology with transglutaminase elicitin like-protein from *P. megakarya* and *Phytophthora palmivora var. palmivora* equal to 59.86% and 54.61%, respectively (Fig 22 (a)).

Transglutaminases are omnipresent in multiple *Phytophthora* species and catalyze an acyl transfer reaction that provides peptide bonds with more resistance to proteolytic degradation. They are expressed during early infection, suggesting that they may function during the establishment of *Phytophthora* disease [86].The analysis of conserved domains in this identified homolog with NCBI conserved domain option has revealed the presence of the TGase_elicitor domain (Accession: cl25039). This domain occupies a significant part of the

protein in terms of length, from position 147 to position 475, and is believed to trigger infections in early stages (Fig 22 (b)).

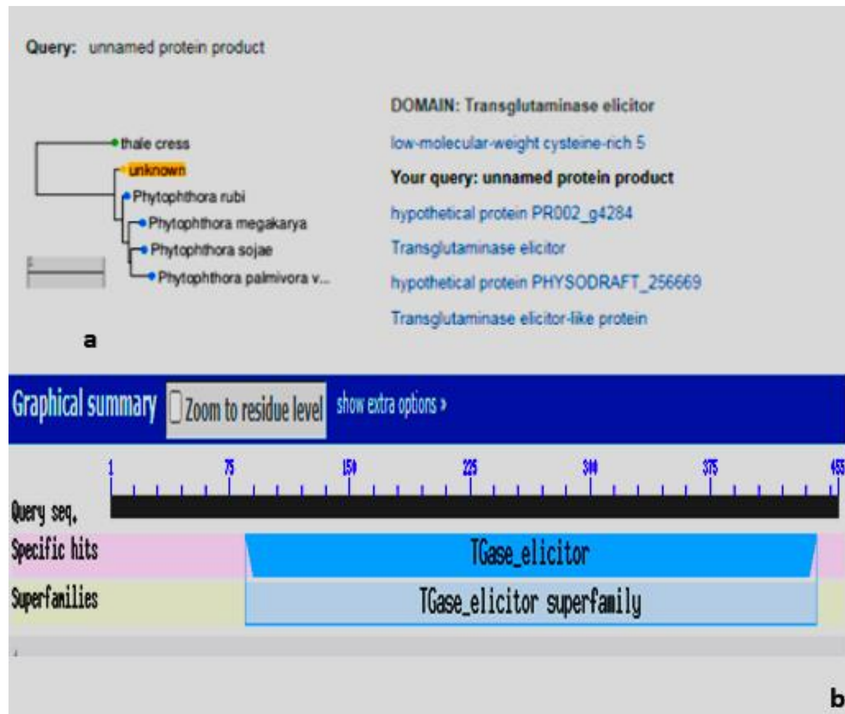


Figure 22. (a) Phylogenetic tree of transglutaminase elicitor protein from *Phytophthora cinnamomi* (unknown in the figure) with different species of the *Phytophthora* genus. (b) TGase_elicitor domain occupies a significant length in this query of *Phytophthora cinnamomi* according to the tool "Identify conserved

5. Final considerations

In this work, we isolate the two genes, *Avr3a* and *Avr1b*, both strongly linked to the pathogenicity of the genus *Phytophthora* according to various bibliographic sources. Also, a complementary list of homologs of molecular factors involved in metabolism and pathogenicity has been identified using bioinformatics tools. All these identified molecular factors can be used to design silencing cassettes following the same principle used for the design of the silencing cassettes of *Avr3a* and *Avr1b* in order to study gene expression. Therefore, using the results from this work, especially the cassettes designed, we can silence the studied genes, locate their products (proteins) and quantify the transcripts in situations of infection of the host, according to what is described in the following sections.

5.1. Transformation of *P. cinnamomi* with the silencing cassettes of *Avr3a* and *Avr1b* genes.

Following this work, we can transform *P. cinnamomi* in order to silence the genes of interest *Avr3a* and *Avr1b* using the RNA interference technique. For this, the previously designed cassettes must be incorporated into vectors such as pHAMT35H and pTH210 used by Horta et al to silence the genes encoding the elicitins in the same pathogen *P. cinnamomi*. These vectors have promoter and termination sequences of the *ham34* gene (*Bremia lactucae*) and of the hygromycin resistant gene which will serve as a selection marker to identify positive transformants of *P. cinnamomi* (Horta et al., 2008).

To achieve a stable transformation of *P. cinnamomi* with interfering RNA, the protoplast method could be performed to deliver recombinant vectors with treatment with CaCl2 / polyethylene glycol (PEG), after the cultivation of *P. cinnamomi* must be done on selective medium growth that contains hygromycin to separate transformed strains of P. *cinnamomi* from other non-transformed ones. Thus, the transformed strains can grow normally while for the others not having received the gene of resistance to hygromycin, the growth is limited. (Horta et al., 2008).

5.2. Subcellular localization of Avr3a and Avr1b effectors using pTOR-eGFP vector

As described in the previous parts, the potential subcellular localization was carried out using bioinformatic tools. In order to confirm the results obtained, the *Avr1b* and *Avr3a* ORFs could be cloned into pTOR-eGFP type vectors and then delivered to *P. cinnamomi* using protoplasts. Thus, we are able to confirm the subcellular localization under fluorescence microscopy since the proteins of interest will be translated coupled to the fluorescent protein GFP.

5.3. Quantification of Avr3a and Avr1b transcripts during infection using quantitative real-time PCR (qRT-PCR)

qRT-PCR is an extremely sensitive method, which allows the quantification of the expression of genes of interest responsible for the pathogenicity during the early stages of infection when the biomass of the pathogen in the host is very low and difficult to be detected by traditional methods.

First, it is necessary to carry out extraction of RNA from *P. cinnamomi*, subsequently, this RNA must be converted into cDNA by RT-PCR and then quantified using the qRT-PCR technique to determine the level of expression of the *Avr3a* genes and *Avr1b* in both transformed and native strains. Finally, we can establish a correlation between the level of pathogenicity and the level of expression of the genes of interest based on the quantification by qRT-PCR in the two types strains (native and transformed) and the phenotypic observation of the host (*C. sativa*).

6. Conclusions

Phytophthora cinnamomi, one of the most harmful organisms affecting both plants of economic interest, such as the European chestnut as well as other forest plants. Since its detection in Europe has caused serious damage to *C. sativa*, which is considered important in European agriculture. Thus, the current situation requires researchers around the world to conduct in-depth research on this pathogen by studying its genome and the molecular factors employed in pathogenicity.

In this work an attempt has been made to list molecular factors that already studied, using a combination of bioinformatics tools and molecular biology technics, thus genes and proteins critical to metabolism and others involved in pathogenicity have been described.

Since the number of chemical products with an inhibitory effect on *P. cinnamomi* is much reduced, research on a molecular scale can be an effective alternative that can direct us towards other means of inhibition, such as the disruption of metabolism or pathogenicity vectors of the pathogen, which will automatically reduce its virulent power. For that purpose, some technics of gene silencing such as RNA interference could be employed.

In this context and from our work, we can present the following conclusions:

First - Although the sequences of the genome and transcriptome of *P. cinnamomi* are available in databases, they are poorly studied compared to several investigations performed in *P. infestans* and *P. sojae*.

Second - The number of molecular factors linked to the pathogenicity of *P. cinnamomi*, studied so far, is very limited, with glucanases and polygalacturonases standing out as the most studied factors while other factors such as RXLR type effectors remain less studied.

Third - In this work, we isolate and characterize the *Avr3a* and *Avr1b* genes.

Fourth - the research of ORFs from the genomic sequence of *P. cinnamomi* using bioinformatics tools allows us to identify several molecular factors involved in pathogenicity and metabolism.

Fifth- The secretory localization prediction of the proteins Avr1b and Avr3a performed with bioinformatics tools was in accordance with bibliography.

Sixth - To analyze the expression of the studied genes and more accurately deduce their role in the pathogenic mechanism, interference RNA silencing cassettes were designed that can be used to transform *P. cinnamomi*.

7. References

[1] Corredoira E, Montenegro D, San-José M C, Vieitez A Ballester A (2004) Agrobacterium-mediated transformation of European chestnut embryogenic cultures. Plant Cell Rep. 23: 311–318. https://doi.org/10.1007/s00299-004-0804-0

[2] Serrazina S (2004). Isolamento e caracterização de genes de resistência à doença a tinta em Castanea sativa Mill. Universidade de Lisboa, Faculdade de Ciências.Grau de Doutor em Biologia.

[3] Monteiro M L, Patrício M S. (2007) Conservação, regeneração e exploração do castanheiro. In Silva, Joaquim Sande (ed.) Do castanheiro ao teixo: as outras espécies florestais Lisboa: Público, Fundação Luso-Americana para o Desenvolvimento. p. 51-78.
ISBN 978-989-619-102-3

[4] Borges O,Gonçalves B, de Carvalho J L S, Correia P, Silva A P (2008)Nutritional quality of chestnut (*Castanea sativa Mill.*) cultivars from Portugal. Food Chemistry.106:976-984. https://doi.org/10.1016/j.foodchem.2007.07.011

[5] Choupina A, Branco J (2020) In Silico Characterization of the Phytopathogenic Efector, avr3a, from *Phytophthora cinnamomi*. Journal of Basic & Applied Sciences.16:20-30 DOI:10.29169/1927-5129.2020.16.03

[6] Gomes-Laranjo, J.; Peixoto, F.; Ferreira-Cardoso, J. (2009). Castanheiros, Técnicas e Práticas. 1ª ed. Pulido Consulting – Industria Criativa & Universidade de Trás-osMontes e Alto Douro.

[7] Vieitez E, Madriñán M, Madriñán F (1996). El Castaño,1.ª Ed.. Léon. Edition Edilesa. Cit. por Carvalho, J. (2014). Métodos de luta alternativos contra a doença da tinta do castanheiro. Instituto Superior de Agronomia, Universidade de Lisboa. Master thesis.

[8] Costa, R.; Bragança, H. & Machado, H. (2011). Os últimos 75 anos de investigação para o melhoramento do castanheiro em Portugal. Agrorrural – Contributos Científicos, 915-926.

[9] Fórum Florestal (2010). Estrutura Federativa da Floresta Portuguesa. Estudo Económico de Desenvolvimento da Fileira da Castanha.

https://projects.iniav.pt/NewCastRootstocks/images/Relatorio-Global-Castanha.pdf

[10] Choupina A, Estevinho L, Martins I (2014b).Scientifically advanced solutions for chestnut ink disease. Appl Microbiol Biotechnol. 98: 3905-3909.

DOI: 10.1007/s00253-014-5654-2

[11] Van Den Brink L, Janssen C(1985). The effect of human activities during cultural phases on the development of montane vegetation in the Serra da Estrela, Portugal. Review of Palaeobotany and Palynology. 44: 193-215.

https://doi.org/10.1016/0034-6667(85)90016-8

[12] Carvalho, J. (2014). Métodos de luta alternativos contra a doença da tinta do castanheiro. Instituto Superior de Agronomia, Universidade de Lisboa. Tese de Mestrado

[13] Crandall S (1950) The distribution and significance of the chestnut root rot *Phytophthoras*, *P. cinnamomi* and *P. Cambivora* . Plant Dis. 34: 194-196

http://forestphytophthoras.org

[14] Thines M, Kamoun S (2010) *Oomycete*-plant coevolution: Recent advances and future prospects. Curr Opin Plant Biol. 4:427-33.

DOI: 10.1016/j.pbi.2010.04.001.

[15] Kamoun, S, Furzer O, Jones, JDG, Judelson HS, Ali GS, Dalio RJD, Roy SG, Schena L, Zambounis A, Panabières F, Cahill D, Ruocco M, Figueiredo A, Chen XR, Hulvey J, Stam R , Lamour K, Gijzen M, Tyler BM, Grünwald NJ, Mukhtar MS, Tomé DFA, Tör M Van Den Ackerveken G, McDowell J, Daayf F , Fry WE, Lindqvist-Kreuze H, Meijer HJG, Petre B, Ristaino J, Yoshida K, Birch PRJ, Govers F. (2015) The Top 10 *oomycete* pathogens in molecular plant pathology. Mol. Plant Pathol.
16:413-434
https://doi.org/10.1111/mpp.12190

[16] Santos L, Martins I, Maia V, Domínguez A, Choupina A. (2016) Genes found in partial sequencing of *Phytophthora cinnamomi* genome. Revista de Ciências Agrárias. 39: 431-436
DOI: 10.19084/RCA16083

[17] Beakes GW, Glockling SL, Sekimoto S (2012) The evolutionary phylogeny of the *oomycete* "fungi". Protoplasma. 249:3-19
DOI: 10.1007/s00709-011-0269-2

[18] Cahill DM, Cope M, Hardham AR (1996) Thrust reversal by tubular mastigonemes: immunological evidence for a role of mastigonemes in forward motion of zoospores of *Phytophthora cinnamomi*. Protoplasma. 194:18-28
https://doi.org/10.1007/BF01273164

[19] Erwin D, Ribeiro O. *Phytophthora* diseases worldwide. St. Paul, Minnesota, USA: APS Press (1996).

[20] Kamoun S (2003) Molecular genetics of pathogenic *oomycetes*. Eukaryot Cell. 2:191-199.
DOI: 10.1128/ec.2.2.191-199.2003

[21] Latijnhouwers M, Wit P, Govers F (2003). *Oomycetes* and fungi: similar weaponry to attack plants. Trends in microbiology. 11: 462-469
https://doi.org/10.1016/j.tim.2003.08.002

[22] Judelson H, Blanco F (2005). The spores of *Phytophthora*: weapons of the plant destroyer. Nature Reviews Microbiology.3: 47-58.
https://doi.org/10.1038/nrmicro1064

[23] Govers F, Gijzen M (2006) P*hytophthora* genomics: The plant destroyers` genome decoded. Molecular plant-mibrobe interactions.19:1295-1301.

DOI: 10.1094/MPMI-19-1295

[24] Tyler B, Tripathi S, Aerts A, Bensasson D, Dehal P, Dubchak I (2009) Genome sequences of two *Phytophthora* species responsible for Sudden Oak Death and Soybean Root Rot provide novel insights into their evolutionary origins and mechanisms of pathogenesis. Lawrence Berkeley National Laboratory.

[25] Gouveia M (2004) Molecular methods in the identification, characterization, and detectionof *Phytophthora cambivora* (Petri) Buisman and *Phytophthora cinnamomi* Rands associated with chestnut ink disease. UTAD. Villa Real. Doctoral thesis in Agronomic Sciences / Plant Protection.

[26] Erwin D, Ribeiro O. (1996) *Phytophthora* diseases worldwide. .Department of Plant Pathology, University of California, Riverside, USA.

[27] Hardham A R, Blackman L M (2019) *Phytophthora cinnamomi*. Molecular Plant Pathology. 19:260-285
doi: 10.1111/mpp.12568.

[28] Zentmeyer G A (1988) Origin and distribution of four species of *Phytophthora*. Transactions of the British Mycological Society. 91:367-378
https://doi.org/10.1016/S0007-1536(88)80111-6

[29] Hardham A R (2005) *Phytophthora cinnamomi*. Molecular Plant Pathology. 6:589-604
DOI: 10.1111/J.1364-3703.2005.00308.X

[30] EPPO global database (2020). Distribution of *P. cinnamomi* in the word.

https://www.cabi.org/isc/datasheet/40957#todistributionDatabaseTable

[31] O'Brien P A, Williams N, Hardy G E St J (2009) Detecting *Phytophthora*. Critical Reviews in Microbiology. 35:169-181

https://doi.org/10.1080/10408410902831518

[32] Kox L F F, van Brouwershaven I R, van de Vossenberg B T L H, van den Beld H E, Bonants P J M, de Gruyter J (2007) Diagnostic values and utility of immunological, morphological, and molecular methods for in planta detection of *Phytophthora ramorum*. The American Phytopathological Society (APS). 97:1119-1129
doi/10.1094/PHYTO-97-9-1119

[33] Hüberli D, Tommerup I C, Hardy G E St J (2000) False-negative isolations or absence of lesions may cause mis-diagnosis of diseased plants infected with *Phytophthora cinnamomi*. Australasian Plant Pathology. 29:164-169
https://doi.org/10.1071/AP00029

[34] Marks G C, Kassaby F Y (1974) Detection of *Phytophthora cinnamomi* in Soils. Australian Forestry. 36:198-203
https://doi.org/10.1080/00049158.1972.10675585

[35] Wilson, B A, Aberton J, Cahill D M (2000) Relationships between site factors and distribution of *Phytophthora cinnamomi* in the Eastern Otway Ranges. Victoria Australian Journal of Botany. 48:247-260

[36] Cahill D M, Hardham A R (1994) Exploitation of zoospore taxis in the development of a novel dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* Phytopathology. The American Phytopathological Society.
84:193-200
DOI: 10.1094/Phyto-84-193.

[37] Vincelli P, Tisserat N (2008) Nucleic acid based pathogen detection in applied plant pathology Plant Disease. Plant Disease. 92:660-669
DOI: 10.1094/PDIS-92-5-0660

[38] Du Y, Mohamed H M, Birch P R J, Bouwmeester K, Govers F (2015) *Phytophthora infestans* RXLR Effector AVR1 Interacts with Exocyst Component Sec5 to Manipulate Plant Immunity. Plant Physiology. 169:1975-1990
DOI: https://doi.org/10.1104/pp.15.01169

[39] Chen X R, Li Y P, Li Q Y, Xing Y P, Liu B B, Tong Y H, Xu J Y (2015) SCR96, a small cysteine-rich secretory protein of *Phytophthora cactorum*, can trigger cell death in the *Solanaceae* and is important for pathogenicity and oxidative stress tolerance. Molecular Plant Pathology. 17:577-587

DOI: 10.1111/mpp.12303

[40] Chaparro-Garcia A, Schwizer S, Sklenar J, Yoshida K, Petre B, Bos JI, Schornack S, Jones AM, Bozkurt TO, Kamoun S (2015) *Phytophthora infestans* RXLR-WY Effector AVR3a Associates with Dynamin-Related Protein 2 Required for Endocytosis of the Plant Pattern Recognition Receptor FLS2. PLoS One. 10:9 e0137071

DOI: 10.1371/journal.pone.0137071

[41] Bos J I, Armstrong M R, Gilroy E M, Boevink P C, Hein I, Taylor R M, Zhendong T, Engelhardt S, Vetukuri R R, Harrower B, Dixelius C, Bryan G, Sadanandom A, Whisson SC,

Kamoun S, Birch PR (2010) *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. Proc Natl Acad Sci U S A. 21: 9909–9914.

doi: 10.1073/pnas.0914408107

[42] Yaeno T, Li H, Chaparro-Garcia A, Schornack S, Koshiba S, Watanabe S, Kigawa T, Kamoun S, Shirasu K (2011) Phosphatidylinositol monophosphate-binding interface in the *oomycete* RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. Proc Natl Acad Sci U S A. 35:14682-14687.

doi: 10.1073/pnas.1106002108.

[43] Dou D, Kale SD, Wang X, Jiang R H Y, Bruce NA, Arredondo F D, Zhang X, Tyler B M (2008b) RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. Plant Cell. 20:1930-1947
doi: 10.1105/tpc.107.056093

[44] Dou D, Kale SD, Wang X, Chen Y, Wang Q, Wang X, Jiang RH, Arredondo FD, Anderson RG, Thakur PB, McDowell JM, Wang Y, Tyler BM (2008) Conserved C-terminal motifs required for avirulence and suppression of cell death by *Phytophthora sojae* effector Avr1b. Plant Cell. 20:1118-1133.

DOI: 10.1105/tpc.107.057067.

[45] Cody C W, Prasher D C, Westler W M, Prendergast F G, Ward W W. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. Biochemistry. 32:1212-1218

DOI: 10.1021/bi00056a003. PMID: 8448132.

[46] Soboleski M R, Oaks J, Halford W P (2005) Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. FASEB J. 19:440-442.

DOI: 10.1096/fj.04-3180fje.

[47] Chalfie M (2009) GFP: Lighting up life. Proc Natl Acad Sci U S A. 106:10073-10080.

DOI: 10.1073/pnas.0904061106.

[48] Ward W W, Cody C W, Hart R C, Cormier M J (1980) Spectrophotometric identity of the energy-transfer chromophores in renilla and aequorea green-fluorescent proteins.Photochemistry And Photobiology. 31: 611-615.

[49] Fire A, Xu S, Montgomery M.K, Kostas S.A, Driver S.E, Mello C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391: 806-811.

[50] Capriotti L, Baraldi E, Mezzetti B, Limera C, Sabbadini S (2020) Biotechnological Approaches: Gene Overexpression, Gene Silencing, and Genome Editing to Control Fungal and *Oomycete* Diseases in Grapevine. Int J Mol Sci. 2:e25890.

DOI: 10.3390/ijms21165701.

[51] Sultana N. Jahan, Anna K. M. Åsman, Pádraic Corcoran, Johan Fogelqvist, Ramesh R. Vetukuri, Dixelius C (2015) Plant-mediated gene silencing restricts growth of the potato late blight pathogen *Phytophthora infestans*, Journal of Experimental Botany. 66:2785–2794, https://doi.org/10.1093/jxb/erv094

[52] Sheng P, Flood K A, Xie M (2020) Short Hairpin RNAs for Strand-Specific Small Interfering RNA Production. Frontiers in Bioengineering and Biotechnology.

https://doi.org/10.3389/fbioe.2020.00940

[53] Liu H, Cottrell T, Pierini L, Goldman W, Doering T (2002) RNA interference in the pathogenic fungus *Cryptoccoccus neoformans*. Genetics. 160: 463-470.

[54] Sanju S, Siddappa S, Thakur A, Shukla P K, Srivastava N, Pattanayak D, Sharma S, Singh B P (2015) Host-mediated gene silencing of a single effector gene from the potato pathogen *Phytophthora infestans* imparts partial resistance to late blight disease. Funct Integr Genomics. 15:697-706.

DOI: 10.1007/s10142-015-0446-z.

[55] Ah-Fong A, Bormann-Chung C, Judelson H (2008). Optimization of transgene mediated silencing in *Phytophthora infestans* and its association with small interfering RNAs. Fungal Genet Biol. 45: 1197-1205.

https://doi.org/10.1016/j.fgb.2008.05.009

[56] Van West P, Kamoun S, van 't Klooster J W, Govers F (1999) Internuclear Gene Silencing *in Phytophthora infestans*. Molecular Cell. 3:339-348

DOI: https://doi.org/10.1016/S10972765(00)80461X.

[57] Cheng T, Chang W (2007) Construction of simple and efficient DNA vector-based short hairpin RNA expression systems for specific gene silencing in mammalian cells. Methods In Molecular Biology. 408: 223-241.

[58] Wesley S, Helliwell C, Wang M, Waterhouse P (2004) Posttranscriptional gene silencing in plant. Methods in Molecular Biology. 265: 117-129.

[59] Zachary A Lewis (2017) Polycomb Group Systems in Fungi: New Models for Understanding Polycomb Repressive Complex 2. Trends in genetics. 33:220-231
DOI: 10.1016/j.tig.2017.01.006.

[60] Kielkopf C L, Lucke S, Green MR (2004) U2AF homology motifs: protein recognition in the RRM world. Genes & Development. 34:23-24
DOI: 10.1101/gad.1206204.

[61] Zhao ZS, Manser Ed (2012) PAK family kinases physiological roles and regulation. Cellular logistics. 1:59-68
DOI: 10.4161/cl.21912.

[62] Williams FE, Varanasi U, Trumbly RJ (1991) The CYC8 and TUP1 Proteins Involved in Glucose Repression in *Saccharomyces Cerevisiae* Are Associated in a Protein Complex. Molecular and cellular biology. 11:3307-3316
DOI: 10.1128/mcb.11.6.3307.

[63] Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison P R, Gasco M, Garrone O, Crook T, Ryan KM (2006) DRAM, a p53-induced Modulator of Autophagy, Is Critical for Apoptosis. Cell. 126:121-134
DOI: 10.1016/j.cell.2006.05.034.

[64] Yu E Y, Yen W F, Steinberg-Neifach O, Lue N F (2009) Rap1 in *Candida Albicans*: An Unusual Structural Organization and a Critical Function in Suppressing Telomere Recombination. Molecular and cellular biology. 30:1254-1268
DOI: 10.1128/MCB.00986-09

[65] Salowe S P, Marsh E N, Townsend C A (1990) Purification and Characterization of Clavaminate Synthase From *Streptomyces Clavuligerus*: An Unusual Oxidative Enzyme in Natural Product Biosynthesis. Biochemistry. 29:6499-6508
DOI: 10.1021/bi00479a023.

[66] Williams.FE, Varanasi.U, Trumbly.RJ (1991) The CYC8 and TUP1 Proteins Involved in Glucose Repression in *Saccharomyces Cerevisiae* Are Associated in a Protein Complex. Molecular and cellular biology. 11:3307-3316
DOI: 10.1128/mcb.11.6.3307.

[67] Huet J, Cottrelle P, Cool M, Vignais ML, Thiel, Marck C, Buhler JM, Sentenac A, Fromageot P (1985) A general upstream binding factor for genes of the yeast translational apparatus. EMBO J. 4:3539-3547
https://doi.org/10.1002/j.1460-2075.1985.tb04114.x

[68] Bechard LH, Butuner BD, Peterson GJ, McRae W, Topcu Z, McEachern MJ (2000) Mutant Telomeric Repeats in Yeast Can Disrupt the Negative Regulation of Recombination-Mediated Telomere Maintenance and Create an Alternative Lengthening of Telomeres-Like Phenotype. Molecular and Cellular Biology. 29:626-639
DOI: 10.1128/MCB.00423-08

[69] Conrad MN, Wright JH, Wolf AJ, Zakian VA (1990) RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell. 63:739-750
DOI: 10.1016/0092-8674(90)90140-a

[70] Autexier C, Lue NF (2006) The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 75:493-517.
doi:10.1146/annurev.biochem.75.103004.142412.

[71] Collins k (2006) The biogenesis and regulation of telomerase holoenzymes. Nat Rev Mol Cell Biol. 7:484-494
doi: 10.1038/nrm1961

[72] Buchman AR, Lue NF, Kornberg RD (1988) Connections between transcriptional activators, silencers, and telomeres as revealed by functional analysis of a yeast DNA-binding protein. Molecular and Cellular Biology. 8:5086-5099
https://doi.org/10.1128/MCB.8.12.5086

[73] Shore D (1994) RAP1: a protean regulator in yeast. Trends Genet. 10:408-412
DOI: 10.1016/0168-9525(94)90058-2

[74] Shore D, Nasmyth K (1987) Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell. 51:721-732
doi: 10.1016/0092-8674(87)90095-x.

[75] Salowe S P, Marsh E N, Townsend C A (1990) Purification and Characterization of Clavaminate Synthase From Streptomyces Clavuligerus: An Unusual Oxidative Enzyme in Natural Product Biosynthesis. Biochemistry. 29:6499-6508
DOI: 10.1021/bi00479a023.

[76] Evans J, Hannoodee M, Wittler M. Amoxicillin Clavulanate. [Updated 2020 Apr 20]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538164/

[77] Salowe S P, Marsh E N, Townsend C A (1990) Purification and Characterization of Clavaminate Synthase From *Streptomyces Clavuligerus*: An Unusual Oxidative Enzyme in Natural Product Biosynthesis. Biochemistry. 29:6499-6508
DOI: 10.1021/bi00479a023.

[78] Den Brink J V, De Vries R P (2011) Fungal enzyme sets for plant polysaccharide degradation. Applied Microbiology and Biotechnology. 91:1477–1492
doi: 10.1007/s00253-011-3473-2.

[79] Staats C C, Kmetzsch L, Schrank A, Vainstein M H (2013) Fungal zinc metabolism and its connections to virulence. Frontiers in Cellular and Infection Microbiology. 3: 65
doi: 10.3389/fcimb.2013.00065.

[80] Blackburn N T, Clarke A J (2000) Identification of Four Families of Peptidoglycan Lytic Transglycosylases. Journal of molecular evolution. 52:78-84
DOI: 10.1007/s002390010136.

[81] Fang W, Sanz A B, Bartual S G, Wang B, Ferenbach A T, Farkaš V, Guerrero R H, Arroyo J, Van Aalten D M F (2019) Mechanisms of redundancy and specificity of the *Aspergillus fumigatus* Crh transglycosylases. Nature Communications.
https://doi.org/10.1038/s41467-019-09674-0

[82] Battaglia E, Benoit I, van den Brink J, Wiebenga A, Coutinho PM, Henrissat B, de Vries RP (2011) Carbohydrate-active enzymes from the zygomycete fungus Rhizopus oryzae: a highly specialized approach to carbohydrate degradation depicted at genome level. BMC Genomics. 12:38
DOI: 10.1186/1471-2164-12-38

[83] Coutinho PM, Andersen MR, Kolenova K, vanKuyk PA, Benoit I, Gruben BS, Trejo-Aguilar B, Visser H, van Solingen P, Pakula T, Seiboth B, Battaglia E, Aguilar-Osorio G, de Jong JF, Ohm RA, Aguilar M, Henrissat B, Nielsen J, Stalbrand H, de Vries RP (2009) Post-genomic insights into the plant polysaccharide degradation potential of *Aspergillus nidulans* and comparison to *Aspergillus niger* and *Aspergillus oryzae.* Fungal Genet Biol. 46:S161-S169
doi: 10.1016/j.fgb.2008.07.020.

[84] Martens-Uzunova ES, Schaap PJ (2009) Assessment of the pectin degrading enzyme network of *Aspergillus niger* by functional genomics. Fungal Genet Biol. 46:S170-S179
DOI: 10.1016/j.fgb.2008.07.021

[85] Lulloff SJ, Hahn BL, Sohnle PG (2004) Fungal susceptibility to zinc deprivation. Journal of Laboratory and Clinical Medicine. 144:208-214
https://doi.org/10.1016/j.lab.2004.07.007

[86] Martins IM, Matos M, Costa R, Silva F, Pascoal A, Estevinho LM, Choupina A(2014) Transglutaminases: recent achievements and new sources. Applied Microbiology and Biotechnology. 98:6957-6964.
DOI: 10.1007/s00253-014-5894-1

[87] Horta M, Sousa N, Coelho A, Neves D, Cravador A (2008) In vitro and in vivo quantification of elicitin expression in *Phytophthora cinnamomi*. Physiological and Molecular Plant Pathology. 73: 48-57.

[88] Jincai Ma, Sumiya N, Ziming Hn, Guannan H, Huiru L, Rui Z, Liyue Z, Jiafen L (2019) The Variation of the Soil Bacterial and Fungal Community Is Linked to Land Use Types in Northeast China. Sustainability. 11: 3286.

https://doi.org/10.3390/su11123286