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In silico characterization of pathogenicity-related genes in *Phytophthora cinnamomi*

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DEDICATION

To my parents, for your infinite love, support, and unwavering belief in my potential. This achievement is a shared victory, and I dedicate every page to you, in gratitude for the sacrifices you made so that I can stand where I am today.

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First and foremost, I thank Allah for granting me strength, patience, and guidance throughout this journey and for helping me successfully complete this work. Alhamdulillah.

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Finally, I thank my colleagues for their friendship and support during this journey.

SUMMARY

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ABSTRACT

Phytophthora cinnamomi is a highly destructive soil-borne oomycete and one of the world's most invasive plant pathogens. With a host range exceeding 5,000 species, it causes severe root rot and dieback, particularly in *Castanea sativa*, resulting in significant losses in forestry and horticulture. Its broad environmental adaptability makes it an extensive threat to plant health and difficult to manage. This study aimed to characterize molecular factors involved in the pathogenicity of *P. cinnamomi* by identifying molecular pathogenicity-related genes and effectors, and by analyzing their domains, subcellular localization, and 3D structure. Using bioinformatic tools, this work identified and characterized candidate RXLR, CRN, and Polygalacturonase effectors in the genome of *P. cinnamomi*.

In this study, the Polygalacturonase 3D structure was successfully modeled with high confidence and sequence identity with Polygalacturonase I from *Aspergillus niger* and Endo-polygalacturonase from *Phytophthora nicotianae*. These results support its classification within the Glycosyl Hydrolase 28 (GH28) family, characterized by the β-helical fold. Additionally, we identified an RXLR effector sequence of 289 amino acids with 72.43% identity to the Avh240 RXLR protein from *Phytophthora sojae*. Furthermore, analysis with SignalP 6.0 indicated the absence of a classical signal peptide in the *P. cinnamomi* CRN effector, suggesting the possible involvement of alternative secretion or translocation pathways.

Keywords: *Phytophthora cinnamomi,* oomycetes*, Castanea sativa,* Polygalacturonase, CRN, RXLR, bioinformatic tools, SignalP 6.0.

RESUMO

Phytophthora cinnamomi é um oomicete altamente destrutivo de solo e um dos patógenos de plantas mais invasivos do mundo. Com uma gama de hospedeiros superior a 5.000 espécies, causa grave podridão radicular e declínio, particularmente em *Castanea sativa*, resultando em perdas significativas na silvicultura e horticultura. A sua ampla adaptabilidade ambiental torna-o uma ameaça extensiva à saúde das plantas e difícil de gerir. Este estudo teve como objetivo caracterizar os fatores moleculares envolvidos na patogenicidade de *P. cinnamomi*, identificando genes e efetores relacionados à patogenicidade molecular e analisando seus domínios, localização subcelular e estrutura 3D. Utilizando ferramentas bioinformáticas, o trabalho identificou e caracterizou os efetores candidatos de RXLR, CRN e Poligalacturonase no genoma de *P. cinnamomi*.

Neste estudo, a estrutura 3D da Poligalacturonase foi modelada com sucesso, com alta confiança e identidade de sequência com a Poligalacturonase I de *Aspergillus niger* e Endo-poligalacturonase de *Phytophthora nicotianae*. Estes resultados apoiam a sua classificação dentro Glicosil Hidrolases da família 28 (GH28), caracterizada por uma estrutura em hélice-β. Além disso, identificamos uma sequência do efetor RXLR de 289 aminoácidos com 72.43% de identidade com a proteína Avh240 RXLR de *Phytophthora sojae*. Além disso, a análise com SignalP 6.0 indicou a ausência de um péptido de sinal clássico no efetor CRN de *P. cinnamomi*, sugerindo o possível envolvimento de vias alternativas de secreção ou translocação.

Palavras-chave: *Phytophthora cinnamomi,* oomicetes, *Castanea sativa*, Poligalacturonase, CRN, RXLR, ferramentas bioinformáticas, SignalP 6.0.

الملخص

يُعد *Phytophthora cinnamomi* أحد أكثر الأومايسيتات المنقولة عبر التربة تدميرًا، ويُصنف ضمن أكثر مسببات الأمراض النباتية غزوًا على مستوى العالم. وبفضل نطاقه العائلي الواسع الذي يتجاوز 5000 نوع نباتي، فإنه يسبب تعفنًا شديدًا للجذور وظاهرة التدهور التدريجي للأشجار، لا سيما في شجرة *الكستناء* (*Castanea sativa*)، مما يؤدي إلى خسائر كبيرة في قطاعي الغابات والبستنة. كما أن قدرته العالية على التكيف مع مختلف الظروف البيئية تجعله تهديدًا كبيرًا لصحة النباتات، ويصعّب من عملية مكافحته. هدفت هذه الدراسة إلى توصيف العوامل الجزيئية المرتبطة بإمراضية *P. cinnamomi*، وذلك من خلال تحديد الجينات والمؤثرات المرتبطة بالإمراضية، وتحليل مجالاتها الوظيفية، وتحديد مواقعها تحت الخلوية، والتنبؤ ببنيتها ثلاثية الأبعاد. وباستخدام أدوات المعلوماتية الحيوية، تم تحديد وتوصيف مؤثرات مرشحة من عائلات RXLR وCRN وإنزيم Polygalacturonase في جينوم *P. cinnamomi*.

في هذه الدراسة، تم بناء نموذج ثلاثي الأبعاد لإنزيم Polygalacturonase بدرجة عالية من الثقة، مع إظهار نسبة عالية من التشابه في تسلسل الأحماض الأمينية مع إنزيم Polygalacturonase I من *Aspergillus niger* وإنزيم Endo-polygalacturonase من *Phytophthora nicotianae*. وتدعم هذه النتائج تصنيف هذا الإنزيم ضمن عائلة Glycosyl Hydrolase 28 (GH28) التي تتميز ببنية β-helical fold. بالإضافة إلى ذلك، تم التعرف على تسلسل لمؤثر يتكون من 289 حمضًا أمينيًا، ويُظهر نسبة تطابق بلغت 72.43 % مع بروتين Avh240 RXLR من *Phytophthora sojae*. كما أظهر التحليل باستخدام برنامج SignalP 6.0 غياب ببتيد الإشارة التقليدي في مؤثر CRN الخاص بـ *P. cinnamomi* مما يشير إلى احتمال اعتماده على مسارات بديلة للإفراز أو الانتقال داخل العائل.

الكلمات المفتاحية: *Phytophthora cinnamomi*، *الأومايسيتات*، *Castanea sativa*، *إنزيم* Polygalacturonase، مؤثرCRN، مؤثرRXLR، أدوات المعلوماتية الحيوية، SignalP 6.0.

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LIST OF ABBREVIATIONS

Avr: Avirulence (gene/protein)

Ago: Argonaute (protein)

BLAST: Basic Local Alignment Search Tool

Cas9: CRISPR-associated protein 9

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

cDNA: Complementary DNA

DEGs: Differentially Expressed Genes

DNA: Deoxyribonucleic Acid

DSBs: Double-Strand Breaks

Endo-PGs: Endopolygalacturonases

Exo-PGs: Exopolygalacturonases

FASTA: Fast-All (sequence alignment tool)

MAMPs: Microbe-Associated Molecular Patterns

NCBI: National Center for Biotechnology Information

NUC: Nuclease lobe (Cas9 domain)

PAM: Protospacer Adjacent Motif (CRISPR)

PCR: Polymerase Chain Reaction

PTI: PAMP-Triggered Immunity

REC: Recognition lobe (Cas9 domain)

RISC: RNA-induced Silencing Complex

RNA: Ribonucleic Acid

1. INTRODUCTION

Chestnuts have been grown on a large scale for centuries and are considered one of the world's most valuable food crops, due to their great economic value, including Portugal with (187,680 t) and Italy with (43,000 t) as the main producers in Europe (Frangipane, 2024). It has been for centuries a basic food for the survival of the population in many areas of Asia, Southern Europe and most of the countries bordering the Mediterranean Sea (Bounous, 2005). In North Africa, sweet chestnut (*Castanea sativa*) groves are mainly cultivated in the Akfadou mountain range, located in Tizi Ouzou Province, Algeria, where the species represents an important traditional forest crop (Aouali et al., 2025). However, in recent years, chestnut production has declined significantly due to the real threat that comes from fungi such as *Cryphonectria parasitica*, which causes chestnut blight and oomycetes like *P. cinnamomi* that causes ink disease (Fernandes et al., 2022; Martins et al., 2014). in July 2023, *Cryphonectria parasitica* was reported for the first time in Algeria, causing crown dieback, trunk and branch cankers, and tree mortalities in these chestnut groves, making Algeria the second African country to report this devastating pathogen (Aouali et al., 2025).

Phytophthora cinnamomi as a soil-borne oomycete plant pathogen (Kamoun et al., 2015), is considered one of the most invasive species, which has caused irreversible damage to natural ecosystems and horticultural crops (Engelbrecht et al., 2021), having severe impacts on the horticulture, particularly avocado, durian, chestnut, macadamia, peach and pineapple (Hardham & Blackman, 2018). Ink disease caused by *P. cinnamomi* is one of the biggest threats to the *C. sativa* affecting both the development and economic yield of this important crop (Vannini et al., 2001). The infection results in wet rotting of the roots and collar of seedlings and trees, which leads to the death of the plants, or the appearance of new symptoms of the disease including reduced size and chlorotic leaves, thinning of the crown and necrosis in the collar of the tree (De Andrade Lourenço et al., 2020). The pathogen is known to survive in the soil for extended periods and hence its control is highly challenging (Hardham, 2005), because it can produce a wide range of molecular factors; including enzymes that break down cell walls and effectors of host immune responses. In order to develop focused disease control strategies, the current research focused primarily on identification and characterization of these molecular factors (Chen et al., 2022).

1.1. Objectives

The main objective of this work is to perform an in silico characterization of molecular factors (genes and proteins) involved in metabolism and pathogenicity of *Phytophthora cinnamomi*.

To achieve this overall goal, the following specific objectives have been defined to guide the research in a focused and systematic manner:

1. Identify genes and proteins associated with pathogenicity of *Phytophthora cinnamomi.*
2. Perform bioinformatic analyses to identify protein homologs shared between *P. cinnamomi* and other *Phytophthora* species.
3. Characterize the identified molecular factors in terms of functional domains, subcellular localization, and three-dimensional (3D) structure using bioinformatic tools.

2. LITERATURE REVIEW

2.1. Oomycetes

Phytopathogens are quite diverse in terms of evolution and include bacteria, viruses, fungi and oomycetes, among these, oomycetes stand out as a distinct group because of their unique evolutionary lineage. They represent a group of eukaryotic microorganisms commonly referred to as 'fungus-like' organisms; belonging to the kingdom *Stramenopila*, making them more closely related to diatoms, brown algae and golden-brown algae (Judelson, 2017; Kroon et al., 2012; Yin et al., 2020).

Oomycetes share many traits with fungi, such as their nutritional strategies and growth habits. However, they have specific distinguishing properties that separate them from actual fungi. Aside from considerable divergence of DNA sequence, oomycetes vary from fungi in cell structure and physiological, biochemical and genetic properties (Mélida et al., 2013; Van West et al., 2003). Unlike most fungi, oomycetes are diploid, have cell walls made primarily of cellulose and b-glucans instead of chitin, make aseptate hyphae, undergo oogamous reproduction, and produce few secondary metabolites (Fawke et al., 2015). Among the oomycete diversity, the genus *Phytophthora*, the plant destroyer, stands out due to its significant impact on agricultural production and natural ecosystems. Comprising over 100 species (Meng et al., 2014).

2.2. The Genus *Phytophthora*

The genus *Phytophthora* is composed mainly of plant pathogens. This genus belongs to the oomycete class, also known as "pseudo-fungi", this *Phytophthora* genus is highlighted due to the significant plant diseases that they cause, which represents some of the most economic and cultural losses (De Andrade Lourenço et al., 2020). For many species of *Phytophthora*, the motile zoospores are the main infective agent that initiates plant disease, making them difficult to control in agricultural and forestry situations and even more challenging in natural ecosystems (Hardham & Blackman, 2018). This genus includes many species ranked as the top in plant's pathology according to Kamoun et al., (2015) mentioned in **Table 1**; such as *Phytophthora sojae* which is responsible for soybean root rot, *Phytophthora infestans* responsible for the potato late blight, which caused the Irish Potato Famine in the nineteenth century (De Andrade Lourenço et al., 2022), *Phytophthora capsici* is responsible for root rot, stem and fruit blight, seed rot, and plant wilting in pepper

(*Capsicum annuum*) (Sanogo et al., 2023), *Phytophthora ramorum* that caused sudden oak death in California, *Phytophthora parasitica* or known widely as *Phytophthora nicotianae* that caused black shank of tobacco (R. Wang et al., 2024) and *Phytophthora* root rot of avocado and jarrah dieback of trees in the Jarrah Forest, both caused by *Phytophthora cinnamomi* (Midgley et al., 2022).

Table 1. The top oomycetes in molecular plant pathology (Kamoun et al., 2015).

Rank	Species	Common disease name(s)	Number of papers (2005–2014)
1	*Phytophthora infestans*	Late blight	1230
2	*Phytophthora ramorum*	Sudden oak death: Ramorum disease	378
4	*Phytophthora sojae*	Stem and root rot	276
5	*Phytophthora capsici*	Blight; stem and fruit rot; various others	541
7	*Phytophthora cinnamomi*	Root rot; dieback	315
8	*Phytophthora parasitica*	Root and stem rot; various others	142

2.3. The species *Phytophthora cinnamomi*

Phytophthora cinnamomi, one of the most known devastating plant pathogens. It has a worldwide distribution and a host range approaching 5000 species (Hardham & Blackman, 2018), it is a destructive, widespread, soil-borne pseudo-fungus belonging to the class oomycetes that infects many plant hosts including forest, ornamental and fruit species. *Phytophthora* species are sometimes referred to as "biological bulldozers" for its capacity to destroy natural plant communities across the globe. It has been often associated with the decline of forest species, as well as other woody perennial plant species (Carter, 2004; Martins et al., 2014). Above its food crop hosts, avocado and pineapple (Burgess et al., 2017). Numerous other plant species, notably the *Macadamia* plantations have suffered significant harm as a result of this *Phytophthora* that causes stem lesions, stem cankers, and leaf defoliation, which result in loss of productivity and tree death (Akinsanmi et al., 2016). Beyond its global significance, specific regions have been severely impacted by this pathogen, with notable effects in North America and Europe; as an example, in Eastern U.S. forests, *P. cinnamomi* is primarily associated with root rot (**Figure 1)** in American chestnut (*Castanea dentata*) and little leaf disease in shortleaf pine (*Pinus echinata*) (Sena et al., 2019).

Figure 1. *Castanea* root rot infection: **a.** chestnut seedling infected with *P. cinnamomi* displays dark staining on roots and shoot; **b.** classic symptoms of *P. cinnamomi* infection include chlorotic, wilting leaves and canopy dieback (adapted from The American Chestnut Foundation [TACF], 2021).

In a survey of *Phytophthora* diversity in Portugal's natural and semi-natural forest ecosystems, *P. cinnamomi* was identified as the most common species, accounting for 56% of isolates in forests and 61% in forest nurseries (Horta Jung et al., 2025). Populations of European chestnuts (*Castanea sativa*.) are under severe threat by *Phytophthora cinnamomi*, that causes ink disease (**Figure 2**). According to (STATS FAO, 2025), *C. sativa* manifested a decline in their nut production by 251,549 tons from 1961 to 2015. The decline was associated with the replacement of chestnut forest production areas by other crops, the abandonment of traditional chestnut groves, natural dieback, and the spread of pests and diseases (Yussif, 2025). However, since 2015, chestnut production in Europe has increased due to various factors. Notably, the influence of climate change (Freitas et al., 2021), and Research and breeding efforts (Fernandes et al., 2021).

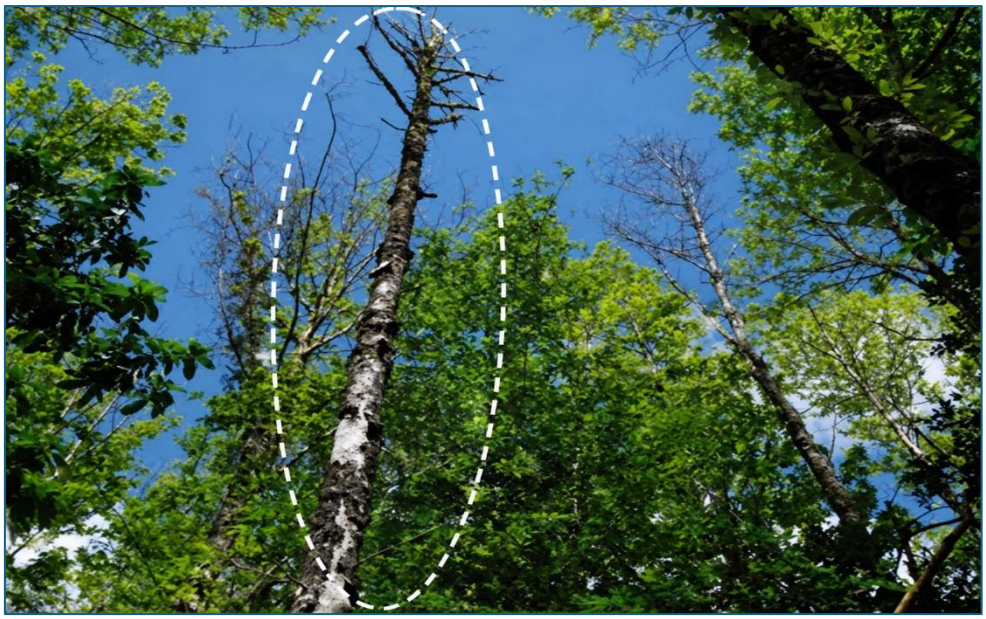


Figure 2. Chlorosis, microphylly, thinning, dieback and mortality of planted chestnut trees caused by *P. cinnamomi* in Chile (Jung et al., 2018).

2.3.1. *Phytophthora cinnamomi* life cycle

P. cinnamomi has two types of reproduction in their life cycle either asexual reproduction via formation of chlamydospores or sexual reproduction through gamete fusion (karyogamy).

2.3.1.1. Asexual sporulation

P. cinnamomi can persist as a saprophyte in the soil for extended periods, capitalizing on favorable conditions to produce abundant asexual, biflagellate zoospores. These motile zoospores are attracted to susceptible infection sites, where they attach and invade the plant. Within a few days, the hyphae spread throughout the tissues of vulnerable plants, forming sporangia on the plant surface and rapidly increasing the disease inoculum. *P. cinnamomi* has been found to survive for up to six years in moist soil, with moisture playing a crucial role in the establishment, spread, and longevity of *P. cinnamomi* diseases. A liquid environment is essential for asexual sporulation, including the formation of sporangia and the release and activity of motile zoospores. In line with this, disease development is particularly enhanced after heavy rainfall and in waterlogged soils (Giachero et al., 2022; Zentmyer & Mircetich, 1966).

2.3.1.2. Sexual sporulation

Under optimal conditions, the encysted zoospores germinate and produce germ tubes that develop into mycelium. The mycelium then differentiates into antheridia and oogonia (**Figure 3**), depending on the mating type and reproductive conditions. After a period of development, the antheridia and oogonia produce the male and female gametes, respectively. The process begins with sexual contact and the penetration of the antheridium precursor by the precursor of oogonium without fusion of cytoplasm between gametes (Gouveia, 2004). The oogonium then passes through an expansion phase, which is made possible by the cytoplasm passing through an oogonium rod that stays open and functional. After this stage is over, a substance that resembles the cell wall is used to seal the oogonium rod. After karyogamy, a diploid oospore is created, which ultimately gives rise to the sporangium, the source of asexual zoospores (Hardham, 2005).

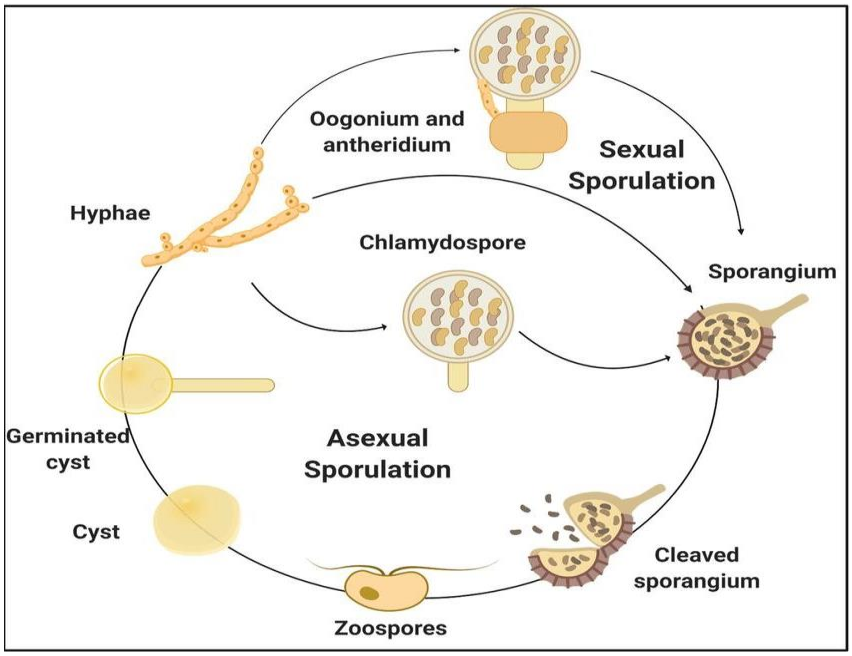


Figure 3. Brief representation of the life cycle of *Phytophthora cinnamomi* including sexual and asexual sporulation (De Andrade Lourenço et al., 2020).

2.4. Plant defense against pathogens

Plants have evolved a multilayered and sophisticated immune system to establish effective resistance to a variety of pathogens (Q. Li et al., 2024). As a physical first line of plant defense, Plants possess a range of preformed defenses that play an important and integral role towards conferring pathogen resistance (Heath, 2000). These defenses consist of

antimicrobial compounds like (phenols, saponins, proteinase inhibitors, and glucosinolates) and structural barriers such as the waxy cuticle on leaves which provide broad-spectrum protection (Dangl & Jones, 2001; Heath, 2000; Osbourn, 1996). While these preformed barriers provide an initial line of protection, plants also activate inducible immune responses upon pathogen recognition to reinforce defense mechanisms.

The plant immune system consists of three layers; a recognition layer, a signal integration layer, and a defense-action layer (**Figure 4**). The recognition layer includes cell surface pattern recognition receptors (PRRs) that can recognize apoplastic effectors, microbe-associated molecular patterns (MAMPs, e.g., flagellin or pathogen cell wall fragments), or damage-associated molecular patterns (DAMPs, e.g., plant cell wall fragments) (Tyler, 2017). The recognition of these patterns activates a cascade leading to PAMP and MAMP-triggered immunity (PTI/MTI) where the signal integration layer takes place (Chisholm et al., 2006), PTI/MTI aims to restrict pathogen colonization through the release of reactive oxygen species (ROS), increasing calcium influx, the expression of defense genes, and the activation of mitogen-activated protein kinases (MAPKs) (Eulgem, 2005).

The second part of plant innate immunity is known as effector-triggered immunity (ETI) (Jones & Dangl, 2006). ETI is triggered when avirulence (Avr) gene products secreted by pathogens are recognized by plant immune receptors. The recognition of Avr proteins by polymorphic nucleotide binding-leucine rich repeat (NB-LRR) receptor proteins (Monteiro & Nishimura, 2018), results in an incompatible interaction and resistance towards the invading pathogen, where the defense-action layer takes place (Cook et al., 2015; Jones & Dangl, 2006; Tyler, 2017).

Plants are also protected by a mechanism called systemic acquired resistance (SAR), which is induced by pathogens that usually infect leaves or stems of plants (Grant & Lamb, 2006). SAR is effective against a broad range of pathogens and is dependent on several phytohormones including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) (Glazebrook, 2005; Grant & Lamb, 2006; Thomma et al., 2001). SAR is usually characterized by local cell death with the activation of the hypersensitive response (HR) (Durrant & Dong, 2004; Thatcher et al., 2005).

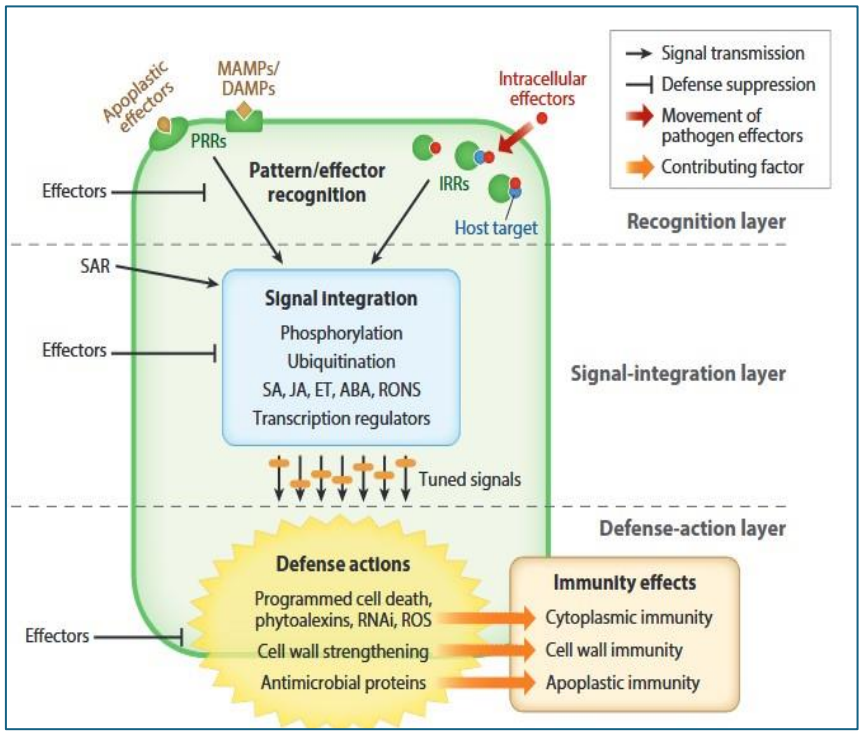


Figure 4. Schematic representation of the layered plant immune system against pathogen infection (Y. Wang et al., 2019).

2.5. Pathogenicity molecular factors of *Phytophthora*

Most *Phytophthora* species are notorious plant pathogens and infect more than 130 known plant species destructive diseases on a variety of agricultural crops (Tyler et al., 2006). *P. cinnamomi* itself cause dieback in almost 5000 plant species (Wilkins et al., 2025). During the early infection stage, it uses numerous virulent effectors to circumvent plant immune responses (Y. Wang et al., 2019). These elicitors can be subdivided into two groups: apoplastic effectors, which include cell wall degrading enzymes (CWDEs), elicitins, and cytoplasmic effectors such as crinklers (CRNs) and RXLRs, which enter the plant cells, and are associated with virulence and host specificity (Armitage et al., 2018; Kamoun et al., 2015).

2.5.1. Apoplastic effectors

The ability to penetrate the basic physical barrier of the plant cell wall is fundamental to successful invasion of plants by pathogens and is facilitated by the secretion of (CWDEs) by the pathogen. These extracellular effectors degrade a wide range of complex and cross-linked polysaccharides and glycoproteins (H. Liu et al., 2005). The plant cell wall is primarily composed of large biopolymers, including cellulose, hemicellulose, lignin, and pectin, which

are broken down during infection by a specific set of CWDEs, such as cellulases, hemicellulases, pectinases, and ligninases (Aro et al., 2005). In *P. cinnamomi*, the genome contains a polysaccharide lyase involved in pectin degradation, with a conserved pectate lyase domain. This highlights its role in the breakdown of plant cell walls and its contribution to pathogenicity (Boughanmi et al., 2022).

Elicitins are classified as apoplastic effectors, meaning that they are secreted into the plant extracellular space. Elicitin proteins induce metabolic or structural changes in host cells that aid pathogen growth and favor the development of the disease. In *P. cinnamomi*, elicitins contain an elicitin domain, that occupies a significant portion of the protein to induce leaf necrosis in infected plants and elicit an incompatible hypersensitive-like reaction (Boughanmi et al., 2022; Hardham & Blackman, 2018).

2.5.2. Cytoplasmic effectors

One of the largest families of effector proteins is defined by the conserved sequence RXLR (Arg-X-Leu-Arg, where X is any amino acid) (Rehmany et al., 2005). RXLR effectors are characterized by three functional domains, including the N-terminal domain that regulates effector protein transport, an RXLR-dEER motif, and a C-terminal domain, crucial for their roles in plant immune interference and target modulation (Bos et al., 2006; Raffaele & Kamoun, 2012; Win et al., 2007). Genomic studies have revealed that the *Phytophthora* genome is rich in genes encoding putative effectors with RXLR motifs, crucial for their translocation from the plant apoplasts into the cytoplasm to modulate immune signaling pathways of the host and disrupt its defense mechanisms (Dou et al., 2008; He et al., 2024). Although there have been some attempts to isolate and study molecular factors of the RXLR family in *P. cinnamomi*, the Avr3a gene has a length of nearly 444 nucleotides, 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. capsici* and was considered as a vector of pathogenicity (Dou, Kale, Wang, Chen, et al., 2008). However, alignment analyses of *P. cinnamomi* Avr3a homologs further indicate that the protein is highly conserved within the genus *Phytophthora*, particularly in the RXLR effector architecture, with the motif consistently identified at positions 44-47. Although the motif is conserved (Arg-X-Leu-Arg), the variable X residue differs among species, with lysine found in *P. infestans*, *P. sojae*, and *P. cinnamomi* (Boughanmi, 2020).

Another major class of cytoplasmic effectors in *Phytophthora* pathogens are the crinklers (CRN, for Crinkling and Necrosis), named due to the response observed when *P. infestans* CRNs were ectopically expressed in plants (Torto et al., 2003). CRN proteins contain a conserved N-terminal domain, which includes the LXLFLAK and HVLVXXP motifs, essential for their translocation into the host cells, followed by a variable C-terminal domain. These effectors were originally identified by their ability to induce crinkling and necrosis in plant tissue, but research has revealed CRNs also function in targeting host factors to suppress plant defenses and play important roles in cell death (Liu et al., 2011; Maximo et al., 2019; Stam et al., 2013; van Damme et al., 2012). The genus *Phytophthora* possesses large multigene families of CRN genes, with 196 in *P. infestans*, 100 in *P. sojae* and 49 in *P. ramorum* and *P. cinnamomi* (Bos et al., 2006; Engelbrecht et al., 2021).

2.6. Genetic transformation strategy and genetic silencing mechanisms

Gene transformation and silencing mechanisms are essential to study the regulatory processes that control gene expression at the transcriptional and post-transcriptional levels. Among the most widely studied approaches, RNA interference (RNAi) and CRISPR/Cas-9 have emerged as powerful tools for gene suppression and genome editing.

2.6.1. RNA interference (RNAi)

RNA interference (RNAi) based gene silencing has become one of the most successful strategies in not only identifying gene function but also in improving agronomical traits of crops by silencing genes of different pathogens (including the *Phytophthora* genus) (Jain et al., 2018). First described by Fire and colleagues (1998) in *Caenorhabditis elegans*. Many studies have shown that the introduction of double-stranded RNA allows the silencing of a gene of interest while sense or antisense RNA introduced separately had at most a modest effect (Fire et al., 1998). RNAi silences genes by degrading their messenger RNA (mRNA), thus preventing protein production. This degradation is carried out by the Argonaut protein (an RNase), which uses a guide RNA to target specific mRNA. The guide RNA directs the Argonaut enzyme to the mRNA of the gene of interest. After binding, the enzyme cleaves the mRNA into two pieces and releases the guide RNA. This prevents translation, effectively silencing the gene (Capriotti et al., 2020; Jahan et al., 2015). RNA interference involves small regulatory RNAs, primarily small interfering RNA (siRNA) and microRNA (miRNA), while

short hairpin RNA (shRNA) is an experimentally designed precursor that mimics endogenous miRNA processing (Sheng et al., 2020). Endogenous precursors (pri-miRNA) are processed in the nucleus by Drosha into pre-miRNA, then exported to the cytoplasm by Exportin 5. In the cytoplasm, double-stranded RNAs are cleaved by Dicer into short segments. These segments bind to Argonaute (Ago) protein, forming the RNA-induced silencing complex (RISC). The short RNA segments act as guides, directing RISC to target specific (mRNA) for degradation by Ago, ultimately silencing the gene (Capriotti et al., 2020; Jahan et al., 2015; Sheng et al., 2020).

RNA interference (RNAi) has been effectively utilized to silence genes in *Phytophthora cinnamomi*, a pathogen responsible for significant plant diseases. For instance, a study by Pascoal-Ferreira et al., (2023) investigated the use of RNAi to silence the necrosis-inducing protein 1 (NPP1) gene in *P. cinnamomi*. The researchers employed hairpin-RNA constructs to target the NPP1 gene, resulting in reduced disease symptoms in infected plants. This approach demonstrated the potential of RNAi as a biological tool for studying molecular factors and managing *P. cinnamomi* infections.

2.6.2. CRISPR/CAS9

The CRISPR/CAS (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system was first identified in bacteria where it serves as an adaptive immune mechanism against viruses. It was developed as a gene editing technology in 2013. Over the subsequent years, it has received extensive attention due to its easy manipulation, high efficiency, and wide application in gene mutation and transcriptional regulation in mammals and plants (X. Liu et al., 2017). CRISPR/Cas system can be divided into Class I (type I, III, and IV) and Class II (type II, V, and VI). The class I systems consist of multi-subunit Cas-protein complexes, while the class II systems utilize a single, multi-domain Cas effector protein. Since the structure of type II CRISPR/Cas9 is relatively simple, it has been well studied and extensively used in genetic engineering (Z. Liu et al., 2020).

CRISPR/Cas9, is a gene-editing technology which involves two essential components; the nuclease Cas9 that can make a double-strand DNA break (DSB) being the most widely used for genomic editing and regulation among the Cas proteins (H. Wang et al., 2016), and a 20-nucleotide RNA molecule called a single guide RNA (sgRNA) that can guide Cas9 to a target DNA sequence via Watson-Crick base pairing (**Figure 5**) (Hsu et al., 2014). Where, Cas9 consists of two regions, called the recognition (REC) lobe and the nuclease

(NUC) lobe. The REC lobe consists of REC1 and REC2 domains responsible for binding guide RNA, whereas the NUC lobe is composed of RuvC, HNH, and protospacer adjacent motif (PAM) interacting domains. The RuvC and HNH domains are used to cut each single-stranded DNA, while PAM interacting domain confers PAM specificity and is responsible for initiating binding to target DNA (Nishimasu et al., 2014). Guide RNA is made up of two parts, CRISPR RNA (crRNA) that specifies the target DNA by pairing with the target sequence, and transactivating CRISPR RNA (tracrRNA) that serves as a binding scaffold for Cas9 nuclease (Mei et al., 2016).

The mechanism of CRISPR/Cas9 genome editing can be generally divided into three steps: recognition, cleavage, and repair (SHAO et al., 2016). The designed sgRNA directs Cas9 and recognizes the target sequence in the gene of interest through its 5′crRNA complementary base pair component. The Cas9 nuclease makes double stranded breaks (DSBs) at a site 3 base pair upstream to PAM (Ceasar et al., 2016). Once Cas9 has found a target site with the appropriate PAM, it triggers local DNA melting followed by the formation of RNA-DNA hybrid. Then, the Cas9 protein is activated for DNA cleavage. HNH domain cleaves the complementary strand, while the RuvC domain cleaves the non-complementary strand of target DNA to produce predominantly blunt-ended DSBs (Jiang & Doudna, 2017; Mei et al., 2016).

CRISPR-Cas9 gene editing has proven effective in modifying active genes involved in *Phytophthora* infection (Javed et al., 2021). For example, scientists used CRISPR-Cas9 with four different guide RNAs to knock out the *P. infestans* PMR4 gene, responsible for tomato late blight. The resulting edited tomato lines showed reduced disease symptoms compared to controls, highlighting the precision and potential of this technology for improving plant disease resistance (R. Li et al., 2022).

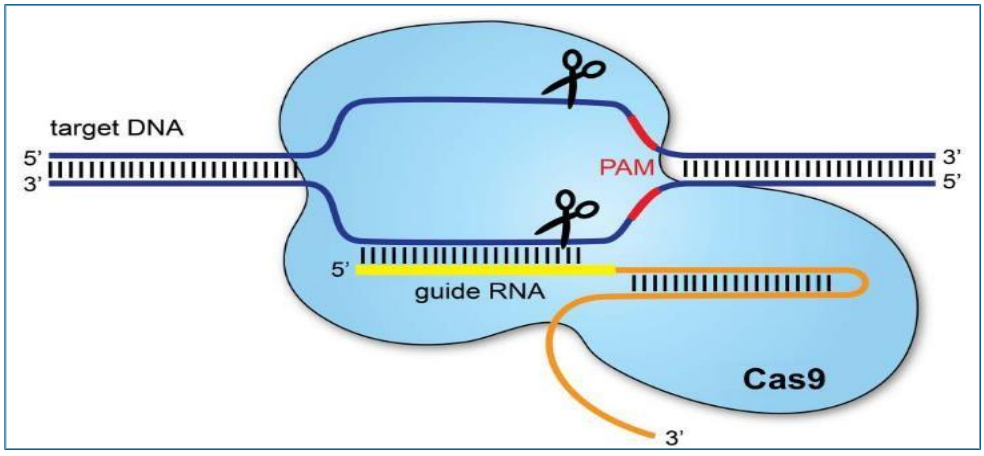


Figure 5. The CRISPR/Cas9 system (Redman et al., 2016).

3. METHODOLOGIES

3.1. The research workflow

In order to identify and characterize pathogenicity-related genes in the *Phytophthora cinnamomi* genome. This study used an in-silico method consisting of four main stages: retrieval of genomic and sequence data, identification of open reading frames (ORFs), protein homology searches and functional annotation, prediction of subcellular localization and three-dimensional protein structures. The work was performed using a variety of well-known bioinformatic tools, all of which are commonly employed in genomic and protein characterization studies and publicly available ensuring the transparency and reproducibility of the workflow.

3.2. Biological information retrieval

For gene research, we used the genome and transcriptome sequence of *Phytophthora cinnamomi*, deposited in the following database:

- NCBI (National Center for Biotechnology Information) at https://www.ncbi.nlm.nih.gov/ .
- EMBL (European Molecular Biology Laboratory) at www.ebi.ac.uk/ena;
- DDBJ (DNA Data Bank of Japan) at www.ddbj.nig.ac.jp/ ;
- FungiDB (The Fungal and Oomycete Genomics Resource) at https://fungidb.org/fungidb/app .

This study used the genome sequence of *Phytophthora cinnamomi* strain GKB4, deposited in NCBI under accession GCF_018691715.1. This genome was selected because it includes annotated genes, which facilitated the identification, characterization, and functional analysis of candidate pathogenicity-related genes. Following the selection of the reference genome, we performed a BLAST search against the complete set of contigs of the *P. cinnamomi* GKB4 genome assembly using reference sequences representing the RXLR, CRN, and Polygalacturonase protein families. For each protein family, the contig showing the strongest alignment, a low E-value, and a sequence identity exceeding 65% was chosen for further characterization, resulting in the selection of contig NW_027150933.1 for RXLR, NW_027150943.1 for CRN, and NW_027150954.1 for Polygalacturonase.

3.3. Open reading frame identification

The selected contigs NW_027150933.1 for RXLR, NW_027150943.1 for CRN, and NW_027150954.1 for Polygalacturonase were analyzed to identify open reading frames (ORFs) regions using NCBI ORF Finder at https://www.ncbi.nlm.nih.gov/orffinder/. For each contig, the ORF showing the strongest similarity to the corresponding reference protein from other *Phytophthora* species, based on a low E-value and a sequence identity greater than 65%, was selected for further analyses. Using these criteria, ORF1 was retained as the most representative coding sequence for each of the three protein families.

3.4. Protein homology encoded by open reading frames

The amino acid sequences encoded by the selected ORFs were subjected to protein homology analysis using the BLAST (smartBlast) and FASTA programs, independently or through the NCBI (National Center for Biotechnology Information). Their use is simple, requiring only the user to paste the sequence under study into the program window and ask the program to establish homology with the sequences from the following protein databases: UniProt Universal Resource (EBI, Swiss Institute of Bioinformatics), Swiss-Prot Protein Knowledgebase (Swiss Institute of Bioinformatics) and PROSITE (Database of Protein Families and Domains). Homologous proteins were identified based on the same previous criteria. Sequence alignments between the *P. cinnamomi* proteins and their corresponding homologs from other *Phytophthora* species were performed to assess the conservation of functional domains, motifs, and biologically relevant residues.

3.5. Prediction of subcellular protein localization

The amino acid sequences encoded by the selected ORFs were analyzed to predict their subcellular localization using several publicly available bioinformatics tools. SignalP v6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/) was used to identify the presence of N-terminal signal peptides and their corresponding cleavage sites. Complementary localization predictions were performed using Cello v2.5 (http://cello.life.nctu.edu.tw/) and EuK-mPLoc v2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) to determine the most probable cellular compartments associated with each protein.

3.6. Bioinformatic characterization of molecular factors

Following the prediction of subcellular localization, the proteins encoded by the selected ORFs were subjected to further bioinformatic characterization. Physicochemical properties were analyzed using ProtPi (https://www.protpi.ch/). Three-dimensional protein structures were predicted using the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/), SWISS-MODEL (https://swissmodel.expasy.org/), AlphaFold (https://alphafold.ebi.ac.uk/), and HHPred (https://toolkit.tuebingen.mpg.de/tools/hhpred). The resulting structural models were visualized and analyzed using PyMOL (https://www.pymol.org/).

3.7. Bibliographic research

The bibliographic research for this study was done using several academic databases to ensure a comprehensive review of the current literature on *Phytophthora* pathogenicity. Primary sources were accessed through NCBI PubMed at (https://pubmed.ncbi.nlm.nih.gov) and ScienceDirect at (https://www.sciencedirect.com). Additionally, Google Scholar (https://scholar.google.com) and ResearchGate (https://www.researchgate.net) were used to track recent citations and access specialized publications on the *P. cinnamomi* genome.

4. RESULTS AND DISCUSSION

4.1 Analysis of pathogenicity related genes in *P. cinnamomi*

The identification of pathogenicity-related proteins in *Phytophthora cinnamomi* was done using the annotated genome of strain GKB4 (NCBI accession: GCF_018691715.1). Comparative sequence analyses revealed 13 proteins sharing significant similarity with previously characterized effectors from other *Phytophthora* species. Based on their mode of action, these homologs were grouped into two categories: apoplastic effectors, which function in the extracellular space surrounding host cells (apoplast), and cytoplasmic effectors, which are translocated into host plant cells. The homologous proteins identified within each category are summarized in **Tables 2** and **3**.

Table 2 provides an overview of the apoplastic effector homologs shared between *P. cinnamomi* and other *Phytophthora* species. Each protein in the table was selected based on specific criteria as described in section **3**: a low E-value, and a sequence similarity exceeding 65% to ensure strong sequence homology and high confidence in the alignment (NCBI BLAST Glossary, 2011). For each of the effectors listed, the table provides the NCBI reference, the specific *Phytophthora* species from which the similar sequence comes, the contig region where the sequence is located, and the product's definition. Proteins identified play a variety of roles and work in different ways, as some of these effectors are quite similar across various *Phytophthora* species.

Table 2. NCBI-deduced homologs of *Phytophthora cinnamomi* GKB4 apoplastic effectors in other *Phytophthora* species.

NCBI ref	Contig region	Product definition	Species
POM57390.1	NW_027150933.1:1..960 (+strand)	Carbohydrate esterase	*Phytophthora megakarya*
KAF4028235.1	NW_027150935.1:1..885 (+strand)	Pectate lyase	*Phytophthora infestans*
XP_009530395.1	NW_027150959.1:1..654 (+strand)	Cutin hydrolase	*Phytophthora sojae*
KUF82063.1	NW_027150959.1:1..654 (+strand)	Cutinase	*Phytophthora nicotianae*
ADN18844.1	NW_027150954.1:1..1419 (+strand)	Polygalacturonase	*Phytophthora capsici*

XP_067750550.1	NW_027150954.1:1..1419 (+strand)	Endopolygalacturonase B	*Phytophthora ramorum*
KAF1772382.1	NW_027151025.1:1..987 (+strand)	Necrosis-inducing protein	*Phytophthora cactorum*
POM77906.1	NW_027150935.1:1..357 (+strand)	Elicitin-like protein	*Phytophthora palmivora*
XP_009536629.1	NW_027150933.1:1..717 (+strand)	Cellulose binding domain-containing protein	*Phytophthora sojae*
XP_009514312.1	NW_027150971.1:139..54 (+strand)	Pectin methyl esterase	*Phytophthora sojae*

Similarly, **Table 3** details the cytoplasmic effector homologs, selected and presented using the same criteria as described for **Table 2**. Otherwise, it shows a limited number of homologous proteins. Specifically, within the CRN and Polygalacturonase family, only a few homologs were identified, demonstrating high sequence similarity and a very low E-value. Contrarily, for the RXLR family only one homolog was found with a 72% sequence similarity in *P. sojae*.

The small number of CRN and RXLR effectors identified in this study may be consistent with previous reports which have identified a high level of sequence diversification in the C-terminal effector domains of RXLR effectors. These domains have shown low sequence conservation, as well as a lack of similarity to other functional proteins. Despite the high level of sequence variation observed in these effectors, various structural components such as the W, Y, and L motifs, have been identified in a large number of RXLR effectors, which have demonstrated the presence of structural conservation in these effectors (Boutemy et al., 2011; Mestre et al., 2016).

Table 3. NCBI-deduced homologs of *Phytophthora cinnamomi* GKB4 cytoplasmic effectors in other *Phytophthora* species.

NCBI ref	Contig region	Product definition	Species
XP_067740309.1	NW_027150943.1:1..1284 (+strand)	Crinkler effector protein 1	*Phytophthora ramorum*
E0W4T1.1	NW_027150933.1:1..870 (+strand)	RXLR effector protein Avh240	*Phytophthora sojae*
OWZ15571.1	NW_027150986.1:1..1062 (+strand)	Avr-associated TDF protein	*Phytophthora megakarya*

4.2 Characterization of genes involved in *P. cinnamomi* pathogenicity

Despite extensive research and analysis on the *Phytophthora* genome, there remains limited information available to develop effective treatments aimed at preventing or greatly reducing the impact of ink disease. Previous reviews have mainly focused on genes directly involved in the pathogen's ability to cause disease, such as the NPP1(Necrosis-Inducing Protein1 in *P. cinnamomi*) gene, which has been shown to induce root necrosis in host plants. The GIP (Glucanase Inhibitor Protein) gene, responsible for suppressing the host's defense mechanisms, has also been extensively studied. In addition to that, the family of Glucanases genes, which play key roles in adhesion, penetration, and colonization within host tissues (Götesson et al., 2002; Martins et al., 2014). In this research, we focused on three key effector families: RXLR and CRN, due to the limited number of homologs with other *Phytophthora* species, and Polygalacturonase (PG), since there is still a lack of comprehensive bioinformatic analysis and characterization of this gene in *P. cinnamomi*.

4.3. Characterization of RXLR, CRN, and Polygalacturonase effectors: subcellular localization, and 3D structural analysis

The selected effector proteins RXLR, CRN, and Polygalacturonase were directed to a series of in-silico analyses to predict their potential roles during host pathogen interactions. To begin with, the amino acid sequences of these effectors were processed using tools that were described in section **3** with direct links to each tool for their accessibility. The results obtained from these analyses are summarized in **Table 4**, which presents the main sequence features, homology relationships, and predicted secretion properties of the selected candidate proteins.

Table 4. Summary of the main pathogenicity-related candidate proteins characterized in this study.

Candidate protein	NCBI Accession	Protein length (aa)	Best homolog	Identity (%)	Coverage (%)	E-value	Signal peptide
RXLR	NW_027150933.1	289	Avh240 (*Phytophthora sojae*)	72.43	95	2e-89	Present; predicted cleavage site between aa 23–24

CRN	NW_027150943.1	427	CRN1 (*Phytophthora ramorum*)	80.51	100	0.0	Absent
Polygalacturonase (PG)	NW_027150954.1	472	Endopolygalacturonase B (*Phytophthora ramorum*)	88.45	70	0.0	Present; predicted cleavage site between aa 20–21

4.3.1. Characterization of RXLR effector gene in *P. cinnamomi*

Analysis of the sequence NW_027150933.1 retrieved from the *P. cinnamomi* genome in the NCBI database. The ORF finder tool revealed the presence of four open reading frames (4 ORFs). Among them, ORF1 with a length of 870 nucleotides corresponding to 289 amino acids showed a high coverage of 95% and sequence identity of 72.43% with the RXLR Avh240 effector protein of *Phytophthora sojae* (**Figure 6**).

RXLRs are cytoplasmic effectors with a modular architecture, consisting of an N-terminal signal peptide, a conserved RXLR motif, and a variable C-terminal domain crucial for their roles in plant immune interference as previously described in the literature review (Midgley et al., 2022). Each *Phytophthora* species encodes about 300–700 RXLR effectors (Jiang et al., 2008). Among them, PsAvh240 is a virulence effector that promotes *P. sojae* infection in soybean hairy roots, it associates with a secreted aspartic protease "GmAP1" in the plant plasma membrane and suppresses host immunity by inhibiting the secretion of GmAP1 into the apoplast (Guo et al., 2019).

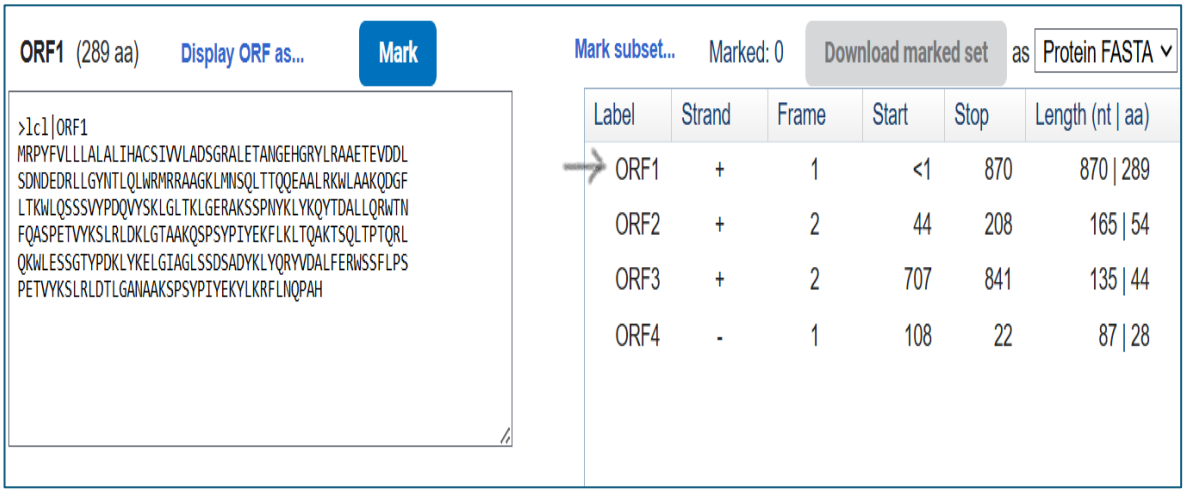


Figure 6. 4 ORFs identified through NCBI ORF finder, where ORF1 encodes a 289 amino acid RXLR protein.

Determining the subcellular localization of infection-related proteins is essential for understanding their functional roles during host colonization and disease progression. Given that the biological activity of RXLR effectors is closely tied to their intracellular destination, the next step of our analysis consisted of predicting the subcellular localization of the protein encoded by ORF1.

Subcellular localization of the ORF1-encoded RXLR effector was predicted using Cello 3.0 and Euk-mPLoc 2.0. According to Cello, the extracellular compartment was the most probable destination for this protein, showing the highest prediction scores among all other locations. Euk-mPLoc shows a similar result, classifying the protein as extracellular as well **(Figure 7)**. Both software tools predict with high accuracy that ORF1 protein is secreted.

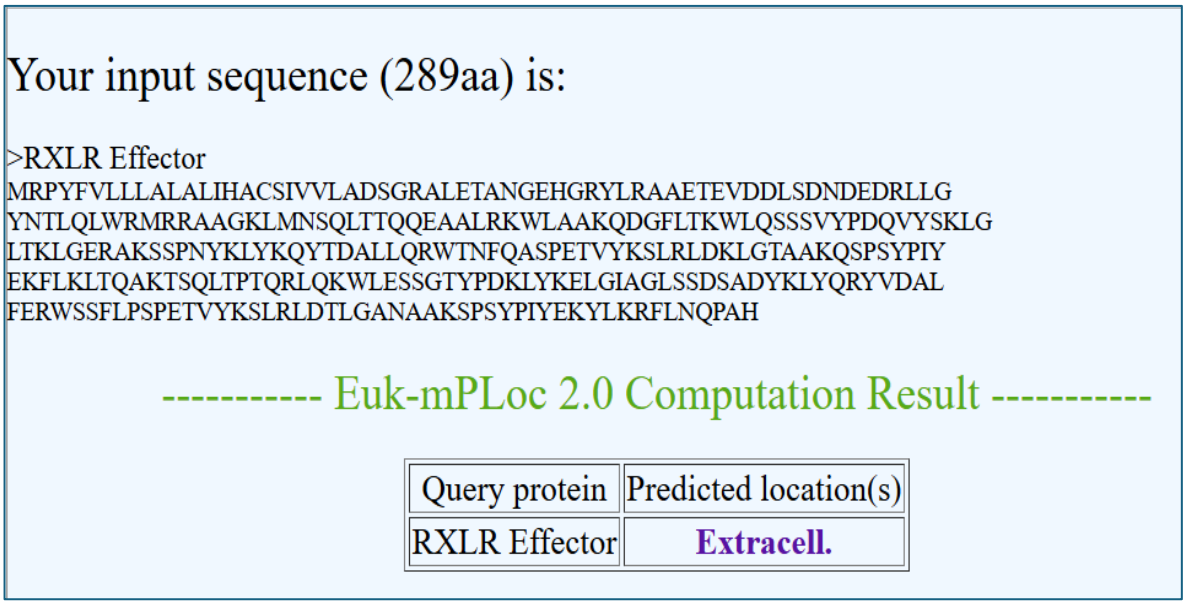


Figure 7. Subcellular localization of ORF1-encoded RXLR effector using Euk-mPLoc 2.0.

To further validate these results, SignalP 6.0 was used to predict the presence of a signal peptide, which requires as the previous programs Cello and Euk-mPLoc 2.0 only the FASTA protein sequence to be copied before starting the analysis with a single command. The analysis revealed a signal peptide cleavage site between amino acids 23 and 24 **(Figure 8)**, confirming the existence of an N-terminal secretion signal. Such peptides direct proteins to the endoplasmic reticulum, after which they are transported through the classical secretion pathway (ER–Golgi–secretory vesicles) toward the cell exterior (Rapoport, 2008). This strongly suggests that the RXLR protein is secreted through the classical secretory pathway.

The extracellular localization predicted by Cello and Euk-mPLoc 2.0 is consistent with the initial secretion of the RXLR effector from the pathogen into the plant's apoplast by the N-terminal signal peptide predicted by SignalP 6.0. Once secreted, the conserved RXLR-dEER motif is then responsible for the translocation of this protein from the plant's apoplast into the cytoplasm, where it exerts its functional role as a cytoplasmic effector (Boughanmi, 2020).

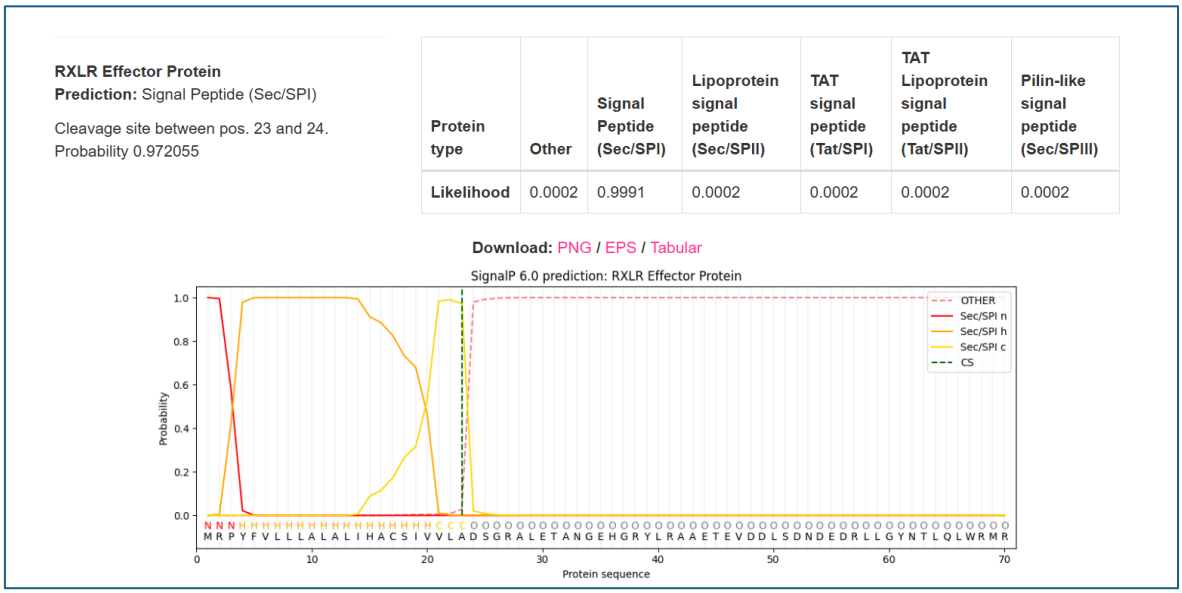


Figure 8. Bioinformatic prediction of the signal peptide in the sequence NW_027150933.1 from *P. cinnamomi* ORF1 RXLR effector using SignalP 6.0.

While subcellular localization provides information on the cellular compartment in which the RXLR effector is active, the analysis of its 3D structure is essential for understanding how its conformation interacts with plant proteins and contributes to its function during infection.

The three-dimensional structure of the ORF1-encoded RXLR effector was predicted using the Phyre2 server, which identified the RXLR effector Avh240 from *Phytophthora sojae* (PDB ID: 6J8L, Chain A) as the optimal structural template presented by an alpha-helical fold, with a confidence score of 99.9%. Based on this template, 41% of the sequence was modeled with very high confidence, while up to 78% of the protein could be predicted at confidence levels exceeding 90% when multiple templates were considered (Figure 9). Structural characterization using SWISS-MODEL supported the Phyre2 results by identifying the same Avh240 RXLR effector from *P. sojae* as the best homologous template, with a sequence identity of 72.43% and an increased structural coverage of 64.36%. Although both software tools supported the structural similarity of the *P. cinnamomi* RXLR

to Avh240, approximately 36% of the sequence remained structurally uncovered. This is common among RXLR effectors, particularly within the C-terminal region, which is known to be highly variable and often lacks structural conservation (Boutemy et al., 2011; Mestre et al., 2016). As this region is associated with host-target interactions, the unmodeled residues may represent variable regions that contribute to the specificity of the *P. cinnamomi* RXLR effector and its adaptation to host targets, including the modulation of host defense responses.

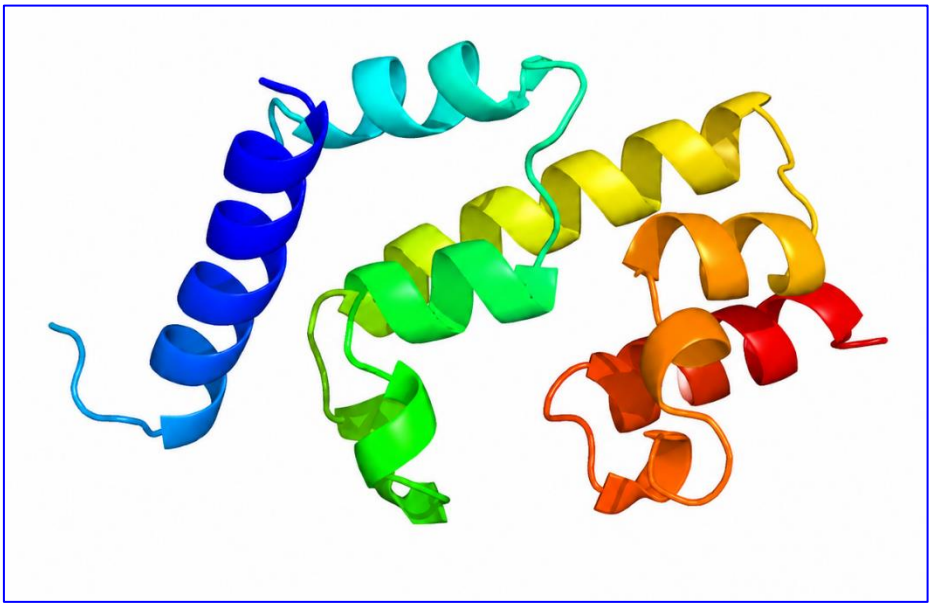


Figure 9. Predicted 3D structure of the *Phytophthora cinnamomi* RXLR effector based on the P. sojae Avh240 template (15.91 KDa in Protein Data Bank) with 137 amino acid length.

Previous studies have shown that RXLR effectors from *Phytophthora* species possess a conserved RXLR motif (Arg-X-Leu-Arg), and share a biological role that interferes with the plant's immunity (Dou et al., 2008; He et al., 2024). Further support for the relationship between the ORF1-encoded RXLR effector and Avh240 was obtained through sequence analyses. SignalP 6.0 predicted the presence of an N-terminal signal peptide in both proteins (**Figure 10**), with an identical cleavage site located between amino acids 23 and 24, indicating conservation of the N-terminal signal peptide region (1-23). The Clustal Omega alignment of the two sequences further showed that the RXLR motif is positioned at the same location in both proteins (residues 38–41). In the *P. cinnamomi* protein, the motif is represented by RYLR, where in Avh240 from *P. sojae,* it occurs as RHLR, with the variable X position occupied by

tyrosine (Y) and histidine (H) (**Figure 10**), respectively. Avh240 is a well characterized RXLR effector known to suppress host immunity by interfering with protein secretion (Guo et al., 2019). These findings suggest that the *P. cinnamomi* RXLR effector may play a similar role in host immune suppression, targeting host secretory pathways or defense-related proteins during infection.

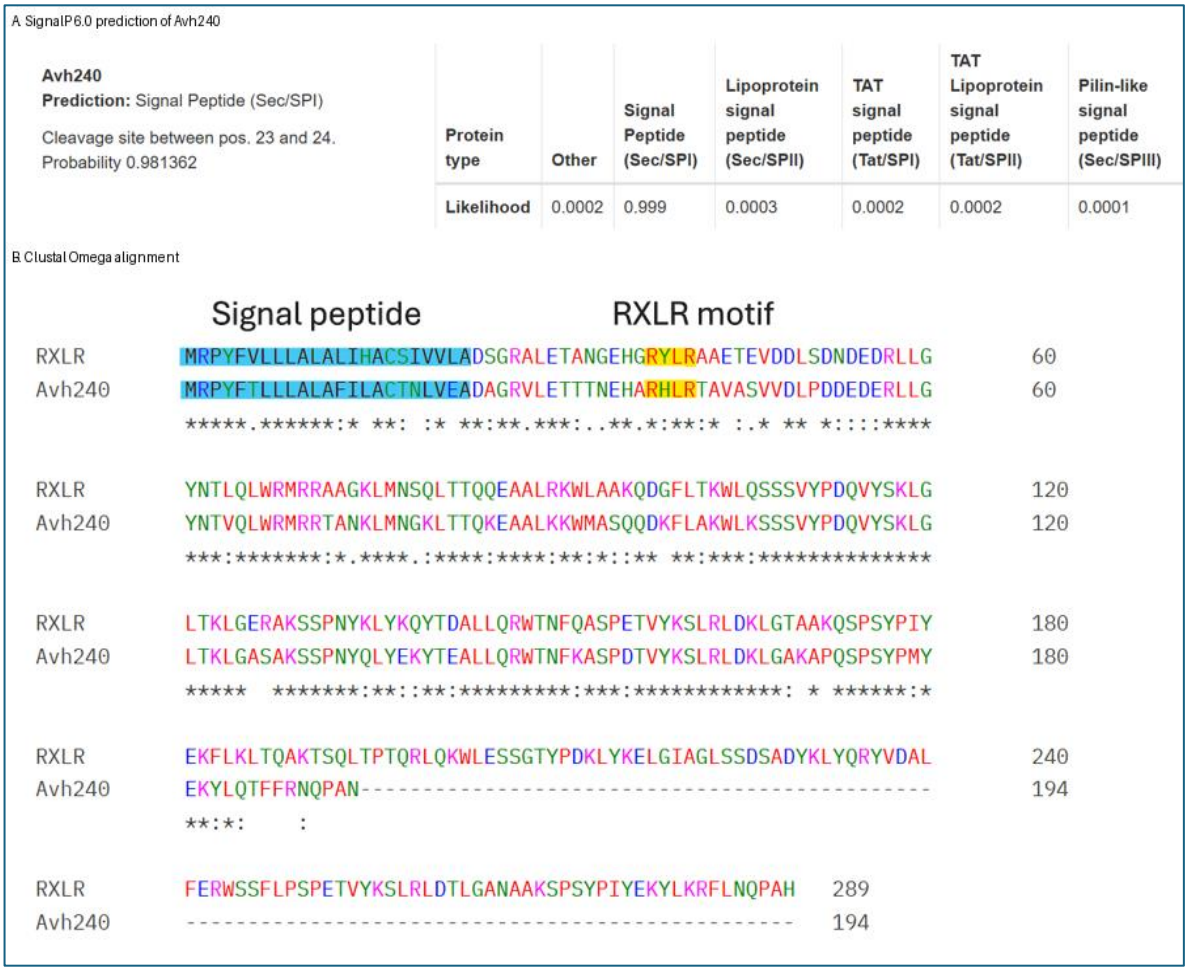


Figure 10. SignalP 6.0 prediction of the Avh240 effector from *Phytophthora sojae* **(A)** and Clustal Omega alignment of Avh240 with the ORF1-encoded RXLR effector from *Phytophthora cinnamomi* **(B)**.

4.3.2. Characterization of CRN effector gene in *P. cinnamomi*

Within contig 10 (NCBI accession NW_027150943.1). Among 7 ORFs, ORF1 encoding a protein of 1284 nucleotides corresponding to 427 amino acids was identified and showed homology to the CRN1 protein from *Phytophthora ramorum*. Blast analysis revealed a high sequence identity of 80.51%, along with complete query coverage (100%) and an E-value of 0.0 of this sequence, indicating a highly significant alignment and strong evolutionary conservation between the two proteins.

CRN effectors are recognized as conserved intracellular proteins broadly distributed among oomycetes. These effectors often accumulate in the host nucleus, where they contribute to the modulation of host cellular responses during infection. At the structural level, as previously reported in the literature review, CRN proteins are characterized by a conserved N-terminal domain coupled with C-terminal domains to determine their specific functional activities (Amaro et al., 2017). They were first identified in *Phytophthora infestans* because of their ability to induce crinkling and necrosis in plant tissues (Torto et al., 2003).

Midgley et al., (2024)'s transcriptomic analyses of *Phytophthora cinnamomi* CRN effectors have demonstrated that crinkler proteins are differentially expressed during infection and are associated with the modulation of host programmed cell death. Certain CRNs are upregulated during the transition to necrotrophy and cluster with known cell death inducing effectors from other *Phytophthora* species, whereas others are expressed at earlier stages and are associated with cell death suppression.

To predict the presence of a signal peptide in the ORF1 CRN protein, the SignalP 6.0 tool was used, which has shown that this identified CRN protein lacks a classical secretion signal **(Figure 11)**. With the absence of these signal peptides, the protein would be localized in the pathogen's cytoplasm. Therefore, this in silico prediction is not consistent with the protein's biological function; to trigger the crinkling and necrosis, the effector must be secreted and translocated into the host cytoplasm, which is supported by Schornack et al., (2010), who demonstrated that most CRNs require nuclear accumulation to induce plant cell death. Previous studies in *P. infestans* have shown that cytoplasmic effector Pi04314 was secreted through non-conventional pathways independent of the classical ER–Golgi system, as its secretion was not inhibited by brefeldin A (BFA), an inhibitor of conventional secretion. This effector was shown to accumulate in host nuclei following haustorium-mediated translocation into plant cells. In this study, *P. infestans* haustoria have been identified as specialized sites for the delivery of both apoplastic and cytoplasmic effectors during infection through distinct secretion pathways (Wang et al., 2017). These findings suggest that the CRN protein likely employs an unconventional secretion mechanism or uses a non-canonical translocation motif not recognized by the SignalP 6.0 algorithm.

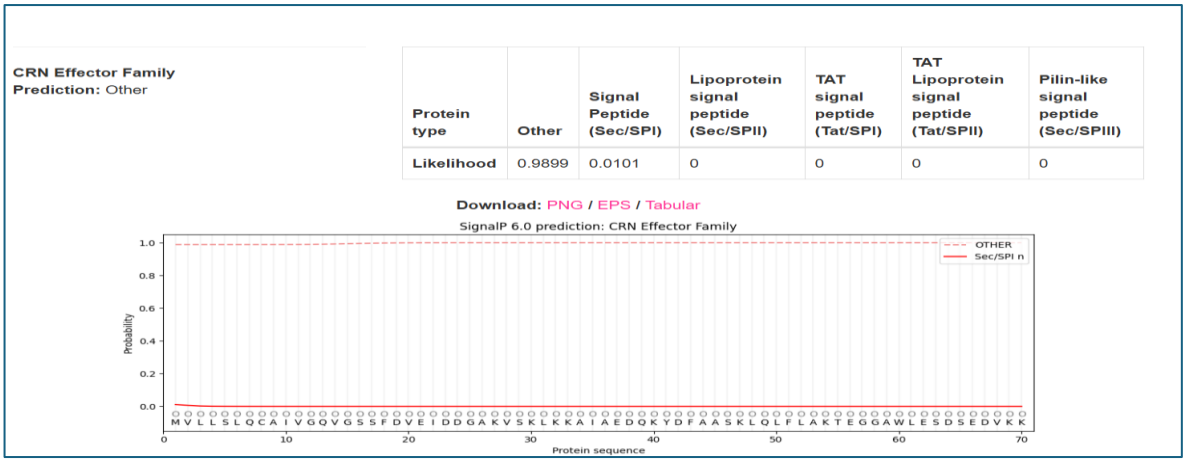
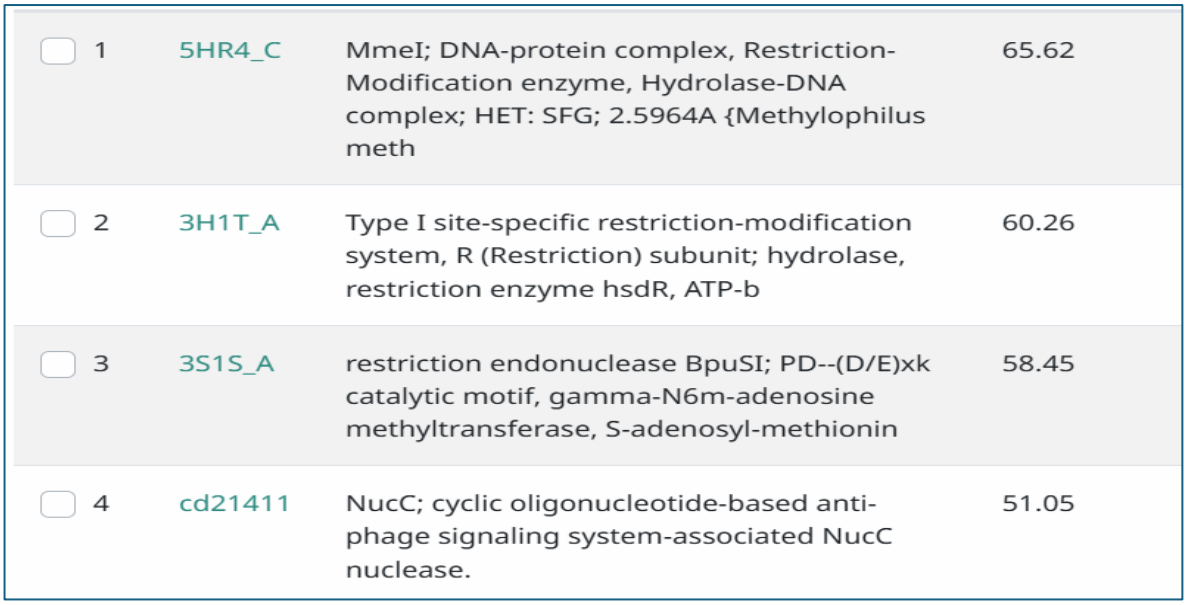


Figure 11. Subcellular localization and secretion signal analysis of *P. cinnamomi* CRN protein using SignalP 6.0.

The structural study of the *Phytophthora cinnamomi* CRN protein via the Phyre2 server indicated homology to the NucC protein from *Vibrio metoecus* RC341 (PDB ID: 6UXF, Chain A), that is displayed in a 3D ribbon diagram with a different alpha-helix and beta-sheet configuration **(Figure 13)**. Despite the sequence coverage was limited to 18% (73 residues), the fold was predicted with a confidence level of 91.7%. NucC is a characterized DNA-binding endonuclease associated with bacterial defense systems, such as the cyclic oligonucleotide-based antiphage signaling system (CBASS), where it functions as a homohexameric ring to degrade DNA (Lau et al., 2020). Given the limited coverage, the similarity likely reflects conservation of protein fold rather than a direct evolutionary relationship. This interpretation is supported by HHPred analysis, which recovered several proteins involved in DNA binding and nuclease activity, including NucC, MmeI, HsdR, and BpuSI, with moderate confidence and limited alignment coverage **(Figure 12)**. The presence of nucleic-acid-associated proteins among the top structural matches suggests that this region of the CRN protein may share structural features involved in DNA interactions. These observations are consistent with the findings of Ramirez-Garcés et al., (2016), who reported conserved HNH-like endonuclease motifs associated with DNA targeting and the induction of host DNA damage responses in CRN effectors from several oomycete species, including *Phytophthora sojae*, *P. parasitica*, *P. infestans*, and *Aphanomyces euteiches*.



1	5HR4_C	MmeI; DNA-protein complex, Restriction-Modification enzyme, Hydrolase-DNA complex; HET: SFG; 2.5964A {Methylophilus meth	65.62
2	3H1T_A	Type I site-specific restriction-modification system, R (Restriction) subunit; hydrolase, restriction enzyme hsdR, ATP-b	60.26
3	3S1S_A	restriction endonuclease BpuSI; PD--(D/E)xk catalytic motif, gamma-N6m-adenosine methyltransferase, S-adenosyl-methionin	58.45
4	cd21411	NucC; cyclic oligonucleotide-based anti-phage signaling system-associated NucC nuclease.	51.05

Figure 12. HHPred analysis of the *Phytophthora cinnamomi* CRN effector revealing structural similarity to DNA-binding and nuclease-associated proteins.

Since the Phyre2 analysis covered only 18% of the sequence, the protein was remodeled using SWISS-MODEL in order to obtain a more comprehensive structural prediction, which was highly successful, giving a top model that shared over 83.06% sequence identity and 99% coverage with an AlphaFold v2.0 template of a *Phytophthora ramorum* CRN N-terminal domain (ID: H3G7B2) **(Figure 13)**. This degree of conservation in the N-terminus is a characteristic of CRN family across the *Phytophthora* species, as the region typically contains the conserved "LFLAK" and "HVLVXXP" motifs required for translocation into the host cell and for nuclear localization (Amaro et al., 2017). Together, these results suggest a 3D structure that covers an N-terminal delivery system and a variable C-terminal domain that may act as a DNA-interacting molecule.

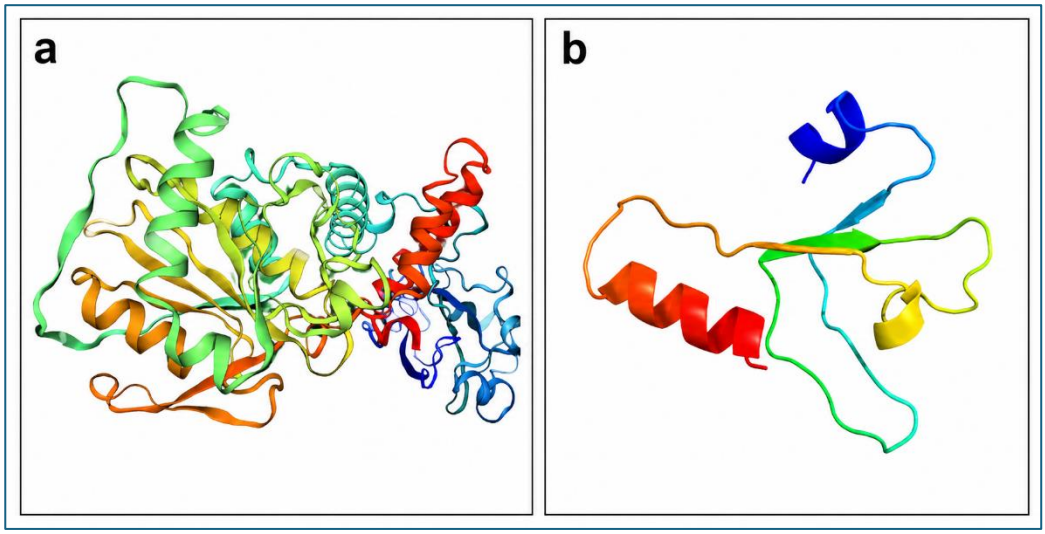


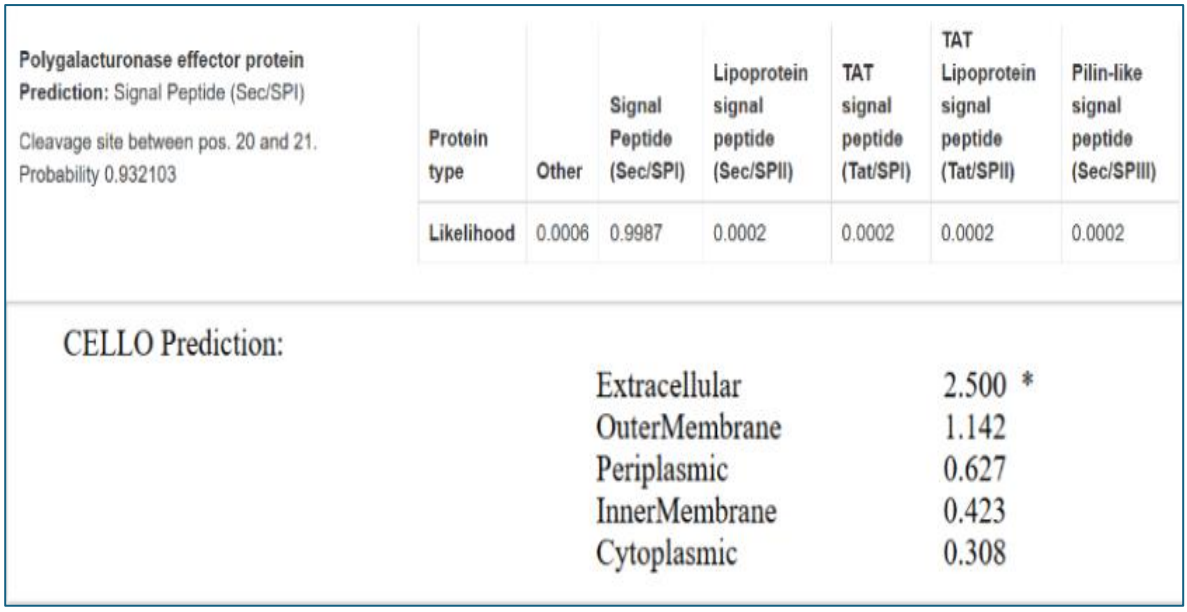
Figure 13. Predicted 3D structure of the *Phytophthora cinnamomi* CRN effector: **a.** based on *Phytophthora ramorum* CRN N-terminal domain using SWISS-MODEL with 83% sequence identity; **b.** based on the NucC protein of *Vibrio metoecus* RC341 template (28.07 KDa in Protein Data Bank) using the Phyre2 server program.

4.3.3. Characterization of Polygalacturonase (PG) effector gene in *P. cinnamomi*

The cell wall is the first barrier that plant cells use to oppose the attack of pathogens. However, most of the phytopathogenic microorganisms such as *P. cinnamomi* succeed in penetrating this physical barrier by the secretion of a variety of cell wall degrading enzymes (CWDEs). Some of the very first CWDEs secreted by the pathogen during infection are those that break down the pectin component of the plant cell wall (Blackman et al., 2014; Hardham, 2005). Among these CWDEs, Polygalacturonases (PGs), which are pectinases that cleave the α-1,4 linkage between two galacturonic acid residues. Two types of PGs can be distinguished by their mechanism of action. EndoPGs (EC 3.2.1.15) act by the random cleavage of the homogalacturonan backbone of pectin internally, releasing oligogalacturonides (OGAs), and exoPGs (EC 3.2.1.67) attack pectin from the non-reducing end to release monomers (Götesson et al., 2002; Mallick et al., 2025). An Endopolygalacturonase B gene was identified in contig 21 of the *P. cinnamomi* sequence (NCBI: NW_027150954.1) in this research. Six distinct open reading frames (6 ORFs) were found by a bioinformatic analysis with the ORF finder tool. Notably, ORF1, which consists of 1419 nucleotides and encodes a protein of 472 amino acids, showed 88.45% sequence identity and 70% query coverage with the Endopolygalacturonase B effector protein from *Phytophthora ramorum.*

Polygalacturonases (PGs) are considered pathogenicity factors in some pathosystems. However, the exact role of PGs in virulence is not fully understood and is likely to differ depending on the pathosystem being studied (Torto et al., 2002). Beyond their role in pathogenesis, PGs often function as elicitors that are recognized by membrane bound receptors in plant cells that either bind directly to PGs or to the oligogalacturonides (OGs) released from homogalacturonan by PG activity (Nakamura & Iwai, 2019). In addition, these degradation products, including OGAs, serve as a source of nutrients for the pathogen, enabling successful colonization of the host plant (Esquerré-Tugayé et al., 2000; Götesson et al., 2002). The study of Miyambo et al., (2022) identified 37 putative Polygalacturonases in *P. cinnamomi*, where the majority of the PG genes including PcPG22 and PcPG19 were up-regulated during the early stages of plant infection, which reflects the biotrophic phase where *P. cinnamomi* must limit the number of active PGs to suppress host defense and obtain nutrients from living plant tissue, whereas only one PG (pcpg5) was induced during the necrotrophic phase of infection to acquire carbon and energy through the extensive degradation of plant tissue, leading to the eventual death of the host plant (Andrew et al., 2012; Glazebrook, 2005; Sprockett et al., 2011).

The subcellular localization of the Polygalacturonase protein encoded by the *P. cinnamomi* sequence NW_027150954.1 deposited in the NCBI database was predicted using Cello and Euk-mPLoc 2.0. Both tools showed that the protein is predominantly localized in the extracellular compartment. This prediction was supported by SignalP 6.0 analysis, which identified a signal peptide with a predicted cleavage site between amino acids 20 and 21 **(Figure 14)** and a high probability score (0.93), which is in accordance with the function of Polygalacturonases as secreted enzymes that degrade pectin in the plant cell wall as described previously.



Polygalacturonase effector protein
Prediction: Signal Peptide (Sec/SPI)

Cleavage site between pos. 20 and 21.
Probability 0.932103

Protein type	Other	Signal Peptide (Sec/SPI)	Lipoprotein signal peptide (Sec/SPII)	TAT signal peptide (Tat/SPI)	TAT Lipoprotein signal peptide (Tat/SPII)	Pilin-like signal peptide (Sec/SPIII)
Likelihood	0.0006	0.9987	0.0002	0.0002	0.0002	0.0002

CELLO Prediction:

Extracellular	2.500 *
OuterMembrane	1.142
Periplasmic	0.627
InnerMembrane	0.423
Cytoplasmic	0.308

Figure 14. Subcellular localization and secretion signal analysis of *P. cinnamomi* Polygalacturonase using Cello v.2.5 and SignalP 6.0.

The 3D structure analysis of the PG protein was predicted using both the Phyre2 and SWISS-MODEL servers to obtain a reliable structural model. The Phyre2 analysis identified Polygalacturonase I from *Aspergillus niger* (PDB ID: 1NHC, chain D) as the best structural template, with a confidence score of 100% and a coverage of 69%. Similarly, SWISS-MODEL generated a structural model based on an Endo-polygalacturonase template from *Phytophthora nicotianae*, showing a high sequence identity of 82.58% and a 90% coverage.

The homology between the *P. cinnamomi* PG and the *A. niger* Polygalacturonase I **(Figure 15)** is supported by the computational analysis of Noorbatcha et al., (2011), who characterized the *A. niger* Polygalacturonase and defined the right-handed parallel β-helix as the fundamental fold of this protein family comprising 10 complete turns. According to Abbott & Boraston, (2007), this β-helical fold contains an active-site cleft that is open at both the N and C-termini. This characteristic is consistent with the classification of PGs into the Glycosyl Hydrolase 28 (GH28) protein family, in which the three aspartate residues in the cleft are essential for the catalytic reaction and are functionally conserved between endo- and exoPGs. In addition, the study of Miyambo et al., (2022) provided more support to this structural model, which indicated that most of the *P. cinnamomi* PGs contain conserved regions, where the Histidine (H) residue from S/GHG motif (Serine/Glycine-Histidine-Glycine) is responsible for maintaining ionization in the catalytic site of PGs, and the three

conserved Aspartate (D) residues from the N-terminal and QDD (Glutamine-Aspartate-Aspartate) domains serve as the primary active site component in PGs.

In homology modeling, sequence identity above 50% is considered to produce highly accurate models, so 82.58% places this prediction in the high-accuracy range (Xiang, 2006). Despite the high identity and coverage given by SWISS-MODEL, it left approximately 10% of the *P. cinnamomi* sequence uncovered, suggesting it corresponds to terminal extensions, or disordered/flexible regions. These regions may also include species-specific structural features that are not present in the *P. nicotianae* template. Together, these findings suggest that this *P. cinnamomi* PG may follow a β-helix GH28 architecture with endo-activity that cleaves the internal α-(1,4) bonds of homogalacturonan backbone of pectin.

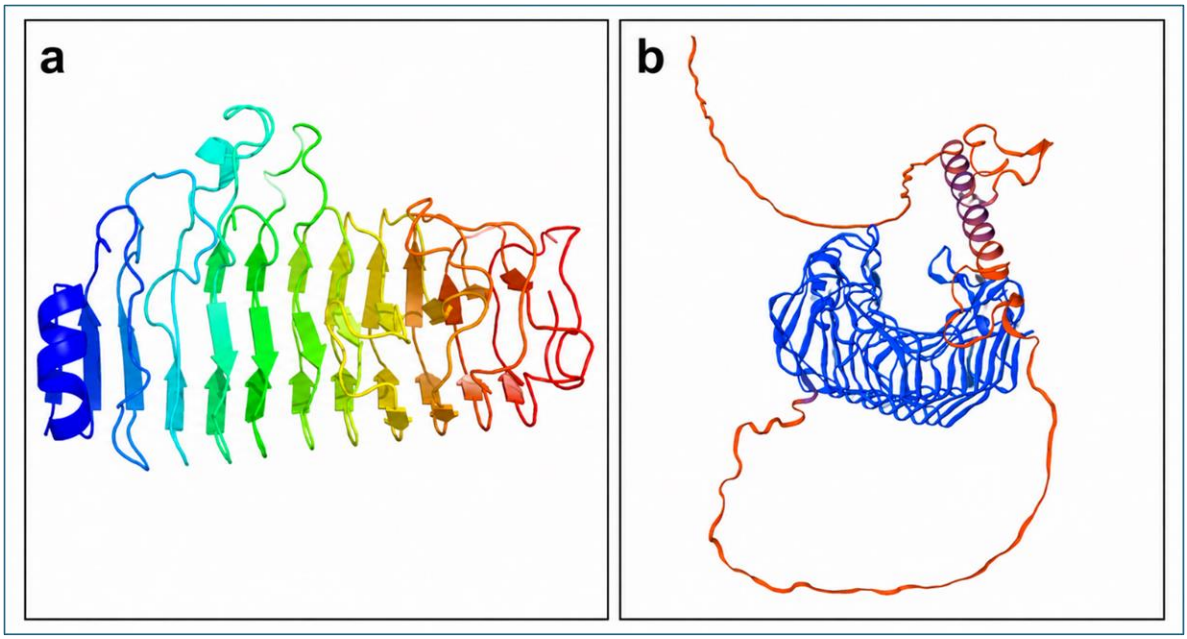


Figure 15. Predicted 3D structure of the *P. cinnamomi* Polygalacturonase effector: **a.** based on Polygalacturonase I from *Aspergillus niger* (216.08 KDa in Protein Data Bank) using the Phyre2 server program; **b.** based on *Phytophthora nicotianae* Endo-Polygalacturonase with 82.58% sequence identity using SWISS-MODEL.

5. CONCLUSION

The soil-borne oomycete *Phytophthora cinnamomi* is a major threat to global agriculture and biodiversity. Known as the "biological bulldozer", It has a severely impacted European chestnut production, leading to a reduction of around 251,549 tons between 1961and 2015. Identifying the specific molecular factors and mechanisms that contribute to its pathogenicity allows researchers to develop effective disease management strategies, create resistant plant varieties and optimize treatment protocols to control the spread of this devastating pathogen.

Although this in-silico study highlighted several challenges, including limited availability of the annotated *P. cinnamomi* genes in the NCBI database, the number of characterized cytoplasmic effectors such as RXLR and CRN and the insufficient sequence coverage obtained using the Phyre2 server. These challenges were overcome to reach the following conclusions:

First, structural characterization of the ORF1 encoded RXLR effector in *P. cinnamomi*, which was successfully achieved through a high-confidence homology with the *P. sojae* Avh240 protein using the Phyre2 server, and giving a 72% sequence identity and a 64% structural coverage via SWISS-MODEL.

Second, subcellular analysis using the Cello program predicted an extracellular destination for both the RXLR effector and Polygalacturonase, identifying them as secreted proteins.

Third, analysis with SignalP 6.0 revealed the absence of a signal peptide in the *P. cinnamomi* CRN effector. Although this does not exclude its function as a CRN effector, it suggests the possibility of non-classical secretion or translocation mechanisms that require experimental validation.

Fourth, the 3D structural modeling of *P. cinnamomi* Polygalacturonase provided a high-confidence prediction based on the template from *A. niger*, characterized by a right-handed parallel β-helix fold, this structure is validated by 90% coverage using Swiss model.

Overall, the findings of this study represent in-silico predictions based on bioinformatic analyses and structural modeling. These predictions provide specific molecular targets for future experimental validation and contribute to a better understanding of the molecular mechanisms underlying *P. cinnamomi* pathogenicity.

For future perspectives, the subcellular prediction of *P. cinnamomi* CRN effector should be validated experimentally by generating Green Fluorescent Protein (GFP) fusions and using live-cell fluorescence microscopy to confirm its subcellular localization. Therefore, Brefeldin A (BFA) treatment could be used to determine whether the effector relies on the conventional ER–Golgi secretion pathway or use a non-conventional secretion mechanism.

Additionally, the use of Co-immunoprecipitation (Co-IP) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) could be used to identify the chestnut proteins targeted by the RXLR effector. RNAi and CRISPR/Cas9 could also be used to silence or edit the Polygalacturonase gene in *P. cinnamomi* to evaluate its contribution to virulence and host colonization in chestnut tissues. In parallel, site-directed mutagenesis of the three conserved PG catalytic aspartate residues located in the NTD and QDD domains could be performed to determine whether these residues constitute the catalytic site of the *P. cinnamomi* Polygalacturonase involved in chestnut infection.

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