Rita Maravilha Leitão Marques

Licenciada em Biologia – Ramo Biologia Celular e Biotecnologia

Transformation of grey poplar to introduce resistance to *Phytophthora* sp.

Dissertação para obtenção do Grau de Mestre em
Biotecnologia para a Sustentabilidade

Orientador: Pedro Fevereiro, Professor Doutor, Faculdade de Ciências da Universidade de Lisboa / Instituto de Tecnologia Química e Biológica

Co-orientador: Susana Araújo, Investigadora Doutorada, Instituto de Tecnologia Química e Biológica

MESTRADO EM
BIOTECNOLOGIA
PARA A
SUSTENTABILIDADE

2020

Rita Maravilha Leitão Marques

Licenciada em Biologia – Ramo Biologia Celular e Biotecnologia

Transformation of grey poplar to introduce resistance to *Phytophthora* sp.

Dissertação para obtenção do Grau de Mestre em
Biotecnologia para a Sustentabilidade

Orientador: Pedro Fevereiro, Professor Doutor, Faculdade de Ciências da Universidade de Lisboa / Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

Co-orientador: Susana Araújo, Investigadora Doutorada, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

Presidente do Júri: Doutora Isabel Alexandra Aguiar de Abreu, Investigadora Principal do Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

Vogais:

- Prof. Doutor Manuel Pedro Salema Fevereiro, Professor Auxiliar c/ agregação da Faculdade de Ciências da Universidade de Lisboa;

- Arguente: Prof. Doutora Ana Margarida Fortes, Professora Auxiliar, Faculdade de Ciências, Universidade de Lisboa

MESTRADO EM BIOTECNOLOGIA PARA A SUSTENTABILIDADE

Novembro de 2020

Transformation of grey poplar to introduce resistance to *Phytophthora* sp.

Acknowledgements/Agradecimentos

I want to start by thanking my advisor Pedro Fevereiro for all the insight, guidance, tools and learning experiences provided to me. Moreover, thank you for introducing and guiding me through the scientific research reality, and for lending me all the resources I required for my work.

I equally want to express my gratitude towards my co-advisor Susana Araújo for all the insight, assets and discussions, and for helping me develop my critical thinking.

A giant word of thanks towards the PlantX lab, which has lent me all the its space and appliances.

I want to thank Maria Belén Colavolpe, which has made the *Phytophthora* spp. inoculation assays possible by kindly providing her insight on the pathogens, and her tools.

A special thanks to Maria Assunção, for having instructed me before, and during my Master's thesis. She was also the one who taught me how to subculture and keep poplar. I knew in my heart I could always count on you.

Thank you, Cláudia Lopes, for helping me so much around the lab and in the greenhouse.

I would also like to acknowledge Jorge Paiva for teaching me all I needed to know in order to successfully extract DNA.

I want to acknowledge Margarida Basaloco, Matilde Sanches and Davide Martins along with my fellow BCV and PlantX colleagues, which have made this work a great experience by being there to help me whenever I needed, and also by keeping me such great company.

Another word of thanks to the Proteome Regulation in Plants lab, in particular to the PI Isabel A. Abreu and the Ph.D. Telma Fernandes for all the support and good advice lent to me.

I'd also like to thank the Plant Functional Genomics lab, in particular Tiago Lourenço, for having lent me his insight regarding Gateway Cloning, and for providing me with the DB3.1 *E. coli* strain and the pDONR P4-P1r plasmid.

I would also like to express my gratitude towards the Plant Gene Regulation lab, and towards André Cordeiro in particular for kindly providing me with the necessary knowledge in RT-PCR, and for lending me the LightCycler 480 II machine from the GPlantS laboratories.

I want to acknowledge ITQB Nova's Research Technician Teresa Baptista da Silva for always being so kind and available to aid me on my work, in particular by providing me competent DH5α *E. coli* cells.

I would like to wish these ITQB Nova people all the best in your endeavors, you truly were an inspiration for me. I wish that one day I will be able to become a great professional like you.

I want to voice a giant word of thanks to the people that kindly revised this manuscript: my advisor Professor Pedro Fevereiro, my co-advisor Susana Araújo, Mónica Louro, Renato Afonso and Dinis Jacob.

No entanto, preciso também de agradecer à minha família e amigos, pessoas que tornaram a conclusão deste ciclo de estudos possível. Sobretudo, quero agradecer à minha mãe e ao meu namorado por todo o apoio que me deram e por terem tido tanta paciência para lidar com o meu entusiasmo ou frustração durante este período.

Um muito obrigada ainda aos meus pais por terem tornado possível frequentar este mestrado e por me facilitarem um bom ambiente de estudo e de pensamento crítico.

Obrigada às minhas três lindas maninhas, de quem eu gosto tanto. Desejo-vos tudo do melhor, vão ser pessoas gigantes.

Agradeço às minhas avós, tios e primos por mostrarem sempre tanto interesse no que estava a desenvolver e por me desafiarem a explicar fenómenos científicos a pessoas de outras áreas.

Obrigada aos meus amigos da Escola Secundária de Camilo Castelo Branco, da Faculdade de Ciências, do Sei Lá, do Nós Jogamos e do VPN (vocês sabem quem são) por me proporcionarem momentos de lazer, boa companhia e bom ambiente quando mais precisava. Vocês são espetaculares, desejo-vos tudo do melhor!

And lastly, thank you, dear reader, for taking the time to read my Master's Thesis.

Resumo

A ***Phytophthora cinnamomi*** é um agente patogénico de plantas ubíquo e extremamente agressivo com uma vasta variedade de **espécies hospedeiras de relevância económica** na silvicultura, agricultura e horticultura. De entre as espécies afetadas pela doença encontra-se o castanheiro europeu, *Castanea sativa*, que constitui uma importante fonte de alimento e de madeira na Europa desde os tempos medievais. Um estudo de genómica e transcriptómica conseguiu identificar vários **genes diferencialmente expressos em variedades de castanheiro resistentes e suscetíveis à doença** provocada pela *P. cinnamomi*. Um dos genes mais promissores do estudo foi o gene *GINKBILOBIN2-like* (*GNK2-like*), cuja proteína, secretada pelas raízes de *Castanea crenata*, contém um **domínio antifúngico** conhecido. O objetivo deste trabalho foi transformar o choupo cinzento (*Populus tremula x alba*), uma espécie-modelo para árvores, com o gene *GNK2-like* sobre-expresso, para **validar a atividade anti-*Phytophthora*** da sua proteína. Para isto, uma estirpe de *Agrobacterium tumefaciens* foi transformada com um vetor que contém a sequência codificante do gene *Cast_GNK2-like* sob controlo de um promotor constitutivo, e foi usada uma transformação mediada por *Agrobacterium* para introduzir este gene no choupo cinzento. Para avaliar os sintomas caraterísticos de infeção da *Phytophthora* no choupo cinzento, os oomycetes *P. cinnamomi* pH107, *P. cinnamomi* DSM 62654 e *Phytophthora cactorum* DSM 62637 foram inoculados junto com a planta, que crescia *in vitro*. O processo de infeção foi seguido morfologicamente, sendo possível identificar sintomas como a **murchidão das folhas**, o **apodrecimento do caule e da raiz** e, por fim, a **morte da planta**. Para avaliar a atividade anti-*Phytophthora* do gene em estudo, as plantas que expressavam o gene *Cast_GNK2-like* foram submetidas a um ensaio *in vitro* semelhante ao anterior. **Foi possível observar algum atraso no desenvolvimento dos sintomas** nas linhas transgénicas de choupo-GNK2, em relação ao choupo não-transformado. No entanto, estas observações não foram tão consistentes como o esperado, pois as diferenças entre choupo não-transformado e choupo-GNK2 surgiram mais no início do ensaio. Este facto que sugere que **um ensaio *ex vitro* e com mais réplicas por genótipo, seria mais fidedigno para tirar conclusões acerca da funcionalidade do gene *Cast_GNK2-like***.

Palavras-chave

Validação funcional, *Populus tremula x alba*, *Phytophthora cinnamomi*, *Phytophthora cactorum*, doença da tinta, gene *GNK2-like*

Abstract

Phytophthora cinnamomi is a widespread, aggressive and destructive plant pathogen with a wide variety of **economically-relevant host species**. One such species is the chestnut tree, *Castanea sativa*, which has provided an important European food and wood source since the medieval times. A previous transcriptomics and genomics study identified **differentially expressed genes on resistant and susceptible chestnut varieties**. One of the most promising genes was the ***GINKBILOBIN2-like* (*GNK2-like*)**, whose protein, secreted by the roots of *Castanea crenata*, has a known **antifungal domain**. This work aimed to **transform grey poplar** (*Populus tremula x alba*), a model for trees, **with an overexpressed *Cast_GNK2-like* gene** in order to **validate the anti-*Phytophthora* activity** of this protein through a heterologous system. In order to achieve this goal, an *Agrobacterium tumefaciens* strain was transformed with a vector containing the *Cast_GNK2-like* coding sequence under the regulation of a constitutive promoter, and *in vitro Agrobacterium*-mediated transformation was performed on grey poplar. The pathogenic oomycetes *P. cinnamomi* pH107, *P. cinnamomi* DSM 62654 and *Phytophthora cactorum* DSM 62637 were inoculated onto grey poplar growing *in vitro* to assess the symptomatology of the disease in poplar. The infection was morphologically followed, identifying symptoms such as **dieback**, **stem rot**, **root rot** and, ultimately, **plant death**. Plants expressing the *GNK2-like* gene were exposed to a similar *in vitro* assay in order to assess the activity of the protein. It was possible to observe a **delay in the development of symptomatology** on some GNK2-Poplar transgenic lines, when compared to the control, wild-type poplar. However, these results were not as consistent as expected, as differences between transformed and non-transformed wild-type were not observed throughout the length of the experiment. This fact suggests that **an *ex vitro* assay with more replicates per genotype is in order to assess more accurately the functionality of the *Cast_GNK2-like* gene**.

Keywords

Functional validation, *Populus tremula x alba*, *Phytophthora cinnamomi*, *Phytophthora cactorum*, ink disease, *GNK2-like* gene

General Contents

List of Abbreviations

bp – base pair

Cp – Crossing Point

CRK – Cysteine-rich kinase

DNA - Deoxyribonucleic acid

DTT – Dithiothreitol

DUF26 – Domain of unknown function 26

EAP – Embryo-abundant protein

gDNA – Genomic DNA

GMO – Genetically Modified Organism

GNK2 – GINKBILOBIN2, a protein from the endosperm of *Ginkgo biloba* seeds

GNK2-like – *GINKBILOBIN2-like* gene

GNK2-Poplar – *Populus tremula x alba* INRA 717-1B4 transformed with the *Cast_GNK2-like* gene

GoI – Gene of Interest

IAA - Indole acetic acid

IBA - Indole butyric acid

INIAV - Instituto Nacional de Investigação Agrária e Veterinária

ITQB – Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

LB - Left Border / Luria-Bertani broth

MAMP - microbial-associated molecular pattern

MCS – Multiple Cloning Site

MS – Murashige and Skoog

NAA - 1-Naphthaleneacetic acid

NCBI – National Center for Biotechnology Information

OD - Optical Density / Absorbance

O/N – Overnight

PCR – Polymerization Chain Reaction

RB – Right Border

RNA – Ribonucleic acid

RT - Room temperature

RT-PCR – Real-Time Polymerization Chain Reaction

T-DNA - The transferred region of the Ti plasmid

Ti – Tumor-inducing

TDZ – Thidiazuron

x *g* – Times gravity

YEB - Yeast Extract Beef Broth

1. Introduction

1.1. The ink disease and Plant Symptoms

The ***Phytophthora*** oomycetes **affect many** different economically **important species in forestry, agriculture and horticulture** (Hausbeck *et al.,* 2004; Hardham, 2005; Dorrance *et al.,* 2009). The most relevant *Phytophthora* fitness components are its latent period, lesion size, sporulation capacity, infection efficiency, overwintering survival, sexual fertility and host range (Goodwin, 1997). Among the most pathogenic species of the genus are: *P. infestans* (Goodwin, 1997); *P. capsici* (Hausbeck *et al.,* 2004); *P. sojae,* (Dorrance *et al.,* 2009); and *P. ramorum,* (Frankel, 2008; **Table 1.1**). More information about which species can be infected by *Phytophthora* spp. can be found on the Pest Management Handbooks from Pacific Northwest (USA) in the Diagnosis and Control of *Phytophthora* diseases. All **these *Phytophthora* species have caused several million dollars' worth of losses** throughout many different countries and continue to present a very serious threat to forestry, agriculture and horticulture (**Table 1.2**).

Table 1.1 – Some of the most problematical species of the *Phytophthora* genus.

Species	Impact	Reference
P. infestans	Caused the Irish and European famine; destroyed Solanaceae supplies in 1840-1846.	Goodwin, 1997
P. capsici	Attacks several important cucurbit and solanaceous vegetables.	Hausbeck *et al*., 2004
P. ramorum	Causes ramorum blight in oak, tanoak and other horticultural plants.	Frankel, 2008
P. sojae	Ravages soybean plantations.	Dorrance *et al*.,2009

Table 1.2 – *Phytophthora*-caused losses (in millions of dollars) throughout different countries.

Losses	Impact	Species	Reference
> US$200	In both crop production and control measures in 1994 due to late blight.	*P. infestans*	Goodwin, 1997
US$134	In up to 25% of 32,356ha of susceptible vegetables in Michigan.	*P. capsici*	Hausbeck *et al*., 2004
AU$1	In Australian pineapple crops (in a single year).	*P. cinnamomi*	Hardham, 2005
US$30	In avocado grooves in California (in a single year).	*P. cinnamomi*	Hardham, 2005
US$5	In pine plantations from Virginia to Mississippi (in a single year).	*P. cinnamomi*	Hardham, 2005
US$15-20	In the Canadian market due to sudden oak death in 2007.	*P. ramorum*	Frankel, 2008

P. cinnamomi is a particularly pathogenic species among the *Phytophthora* genus, **causing the ink, root rot, and dieback plant diseases**. It **affects close to 5000 different host species**, including many economically important genera such as *Castanea* (chestnut), *Quercus*, *Pinus* and *Eucalyptus* spp., and many food crops such as *Persea* (avocado), *Ananas* (pineapple), *Prunus* (peach), *Macadamia*, *Rhododendron* and *Camellia* (Hardham, 2005; Hardham, 2017).

P. cinnamomi is thought to have **originated from Papua New Guinea**, but it is currently widespread all over the globe, with its **presence being confirmed in more than 70 countries** (Goodwin, 1997; Hardham, 2005). Interestingly, even though *P. cinnamomi* is a heterothallic species and both mating types are often present, it **lacks frequent sexual reproduction**, with the exception of its place of origin (Hardham, 2005). Asexual reproduction is known to give rise to less genotypic variation. On the other hand, it was proven that **considerable variation may occur within a single clonal lineage**, **arising only from mitotic recombination** during asexual growth and development (Hardham, 2005). This source of variation allows the species to **adapt to new environmental conditions** and **develop virulence towards new hosts** during asexual growth (Hardham, 2005).

When ***P. cinnamomi*** arrives to an area, it **can kill a large proportion of the plants present**, **leading to drastic changes** in the flora**, as susceptible plants are replaced by resistant species** such as herbaceous, perennials, rushes, sedges or introduced weeds (Hardham, 2005). Currently, it **harms the health of thousands of forests**, with the situation

being particularly problematic in North America and in Australia, where the diseases caused by *Phytophthora* spp. are the number-one plant diseases affecting the countries (Pscheidt, 2008). **Losses caused by ink disease in agriculture, horticulture, and forestry are substantial and may be calculated by considering the expenditure on control measures, the loss of yield and the reduction of product quality and value** (**Table 1.2**; Hausbeck *et al.*, 2004; Hardham, 2005; Frankel, 2008). Harder to calculate is the **impact** of the pathogen in the **plant community structure, floral composition, plant cover and biomass, biodiversity**, the **threat** it poses **to endangered plant species** and the risk it presents on **dependent biota** (Hardham, 2005).

***P. cinnamomi* generally lives saprophytically in the soil**. However, it rapidly takes advantage of favorable conditions, such as **water abundance**, to **produce asexual and mobile zoospores from its sporangia, which will attach themselves to susceptible plants** (**Figure 1.1**, Hardham, 2005). Within 20 to 30 minutes after encystment, **the cysts germinate** and a germ tube emerges from the zoospore's ventral surface (Hardham, 2005). These **tubes** usually grow and **penetrate the root surface** along the anticlinal wall between epidermis cells but may also grow directly through the outer periclinal wall. When this happens, it is possible to verify an appressorium-like swelling of the germ tube (Hardham, 2005). The **root-invasion and colonization processes begin**, and the **hyphae start ramifying throughout the tissues** by absorbing carbohydrates and nutrients from the plant, ultimately **resulting in chlamydospores on the plant surface** two to three days later (Hardham, 2005). The **invaded tissues are destroyed, rotting the root** and **preventing the plant from absorbing nutrients and water** from the soil, resulting in **similar symptoms to those of drought**. As the transport pathway of the plant is cut away, symptoms such as **wilting** and **dieback**, leaf **chlorosis** (yellowing), leaf **necrosis** (browning), premature **leaf fall** and **plant death** will eventually emerge. However, the symptoms that best diagnose this disease are the **rotting of fine and fibrous roots, stem cankers, dieback of young shoots**, and **increased crown transparency** (Hausbeck *et al.,* 2004; Hardham, 2005; Pscheidt, 2008; Cerny *et al.*, 2009; Keča *et al*., 2015; dos Santos, 2017).

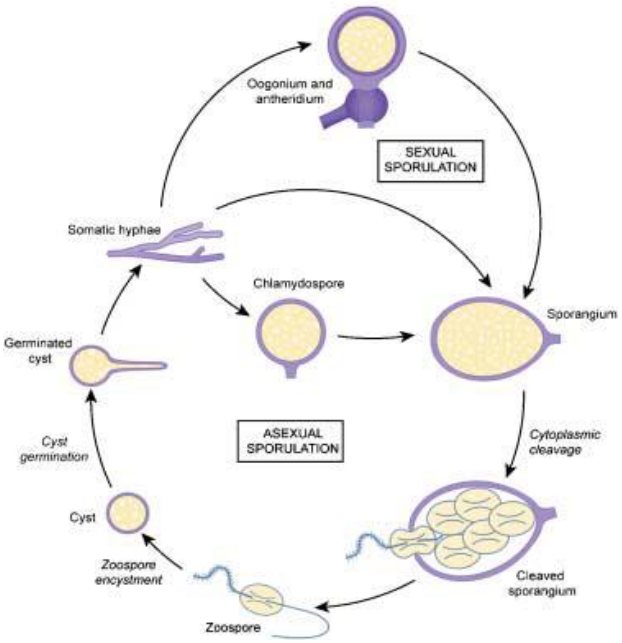

Figure 1.1 - Life Cycle of *P. cinnamomi*. Diagram adapted from Hardham, 2005.

Researchers studying *Phytophthora* spp. concluded that the **incidence, distribution and intensity of its diseases** are primarily affected by **climate and environmental conditions**, rather than exclusively the genotypic and phenotypic variation of the species (Hausbeck *et al.,* 2004; Dorrance *et al.*, 2009; Eggers *et al.*, 2012).) One major factor associated with the **appearance of *P. cinnamomi* epidemics in new areas** is the occurrence of **conditions that facilitate asexual sporulation and zoospore production**, such as **rainfall**, and higher **moisture** and **temperature** (Hardham, 2005; Eggers *et al.*, 2012). Naturally, the **effects of global climate change scenarios will affect the prevalence of the ink disease**. In scenarios in which a 20% increase in rainfall is coupled with three temperature change regimes, it was predicted that not only *P. cinnamomi* diseases in oak will **become more severe** in regions of Europe **where the pathogen is already present**, but also that the **disease would spread** to the north and east

(Hardham, 2005). It is possible for the disease to spread a few hundred kilometers east from the Atlantic coast within the next hundred years (Hardham, 2005).

1.2. The *Phytophthora* genus

The ***Phytophthora*** **genus is characterized by diploid, alga-like oomycete plant pathogens** (Cooke *et al*., 2000). It is taxonomically located on the Chromista kingdom, Oomycota phylum and Pythiales order on the group of heterokont, biflagellate organisms that behave similarly, but are biologically and biochemically distinct from other main fungal groups in Fungi kingdom (Cooke *et al*., 2000). Although the organisms possess fungus-like hyphae and similar nutrient-acquisition strategies, the flagellar apparatus and spindle pore structure differ from true fungi (Hardham, 2005). Other differences relating to fungi include the fact that these oomycetes possess cellulose rather than chitin on their cell wall, the distinct lysine and tryptophan biosynthetic pathways, and the completion of meiosis immediately prior to gametogenesis which leads to a diploid somatic thallus in *Phytophthora* (Hardham, 2005; Mélida *et al*., 2013).

Phytophthora occupies an intermediate position between the saprotrophic, aquatic, zoosporangium-producing genera and the obligate conidial plant pathogens (Cooke *et al*., 2000; Hardham, 2005). The genus comprises **more than 60 species**, most being **soil-borne root pathogens** that are capable of surviving in the soil as oospores (Goodwin, 1997; Cooke *et al*., 2000; Hausbeck *et al.*, 2004). *Phytophthora* species present an extensive **synteny**, and **many key features of pathogenicity are conserved** between species (Hardham, 2005). This is a feature that greatly aids the extrapolation of information from one *Phytophthora* species to another.

In order to distinguish species within the genus, **Waterhouse** (1963) has **divided it into six main groups** that were used as the basis for taxonomic keys, which are still being used for reference today (Hausbeck *et al.*, 2004; Hardham, 2005). However, more recent molecular phylogenetic studies ended up rearranging the monophyletic *Phytophthora* genus in eight clades (Hardham, 2005). According to Cooke *et al*. (2000), species are mainly **discriminated by** the **structure of the sporangium** (nonpapillate, semipapillate or papillate), the **morphology of the antheridium** (amphigynous or paragynous) or **whether they are inbreeding** (homothallic or outbreeding with A1 and A2 sexual incompatibility, or heterothallic with mating types). Approximately half of the 60 species of *Phytophthora* are self-fertile (homothallic), with a single isolate being capable to complete the sexual stage to form oospores (Cooke *et al.*, 2000; Hausbeck *et al.*, 2004). Only 4% of *Phytophthora's* species cannot sexually reproduce to form oospores (Cooke *et al.,* 2000). Additional morphological characteristics such as structural aspects of oogonia and mycelia are also often considered in taxonomic keys (Hardham, 2005). Besides morphological analysis, there are other methods to discriminate in between species. Among these methods are the analysis of electrophoretic patterns of isozymes isolated from cultured samples, antibodies and dipstick assays using chemoattractants (Hardham, 2005). Additionally, it is possible to use molecular markers and species-specific DNA segments to identify the species through PCR-based (Polymerization Chain Reaction) procedures (Ersek *et al*., 1994).

Phytophthora's life cycle is quite complex but remarkably optimized (**Figure 1.1**). A **sexual cycle leads to oospores**, thick-walled environment-resistant structures sporulated by oogonia which can survive from months up to years in the soil, **allowing the near-obligate pathogens to survive outside their hosts** (Goodwin, 1997). Once inside host tissue, **asexual propagules called sporangia** are produced in abundance. In some species, the sporangia are easily detached (caducous) and are **adapted for wide-distance aerial dispersal** (Goodwin, 1997; Cooke *et al.*, 2000). In other species, the **noncaducous sporangia can be spread in water**, releasing biflagellate swimming **zoospores** in response to chilling and soil **moisture**

(Goodwin, 1997; Cooke *et al.,* 2000; Hardham, 2005; Dorrance *et al.,* 2009). These zoospores are chemotactic and actively seek-out and swim-toward suitable hosts (Goodwin, 1997). Within the genus, it was possible to identify two main lifestyles: **some species are soil-inhabiting, root-infecting parasites with noncaducous sporangia which can be spread by water**, while **others rely on caducous papillate sporangia for aerial dispersal and infection over wide distances** (Cooke *et al.*, 2000).

Host penetration by *Phytophthora* **requires** the production and **secretion of degrading plant cell wall enzymes** (Hardham, 2005). Usually, the first line of attack is comprised of enzymes that break down pectin, such as **polygalacturonases**, a family of enzymes that is encoded by more than 20 of *Phytophthora*'s genes (Hardham, 2005). These proteins differ in the number of potential glycosylation sites and in the structure of the N- and C- termini (Hardham, 2005). The cell wall-degrading enzymes are thought to be transported to the surface of hyphae in Golgi-derived apical vesicles (Hardham, 2005). However, these are not the only proteins that promote *Phytophthora* virulence. Another important group of proteins is its **elicitors of host defense responses**, elicitins, 10kDa proteins of about 98 amino acids (Hardham, 2005). Elicitins are sterol carrier proteins that **induce a hypersensitive response** (triggered cell death to prevent pathogen spread throughout plant tissues), tissue necrosis and systemic acquired resistance in tobacco (Hardham, 2005). Sterols are important for oomycete growth and sporulation, but these carrier proteins became known microbe-associated molecular patterns (MAMPs) in plant defense mechanisms due to their conserved nature in many *Phytophthora* and *Pythium* organisms (Derevnina *et al.,* 2016). In particular, *P. cinnamomi* produces alpha and beta-cinnamomin (Hardham, 2005).

Phytophthora species truly are prime examples of pathogens. The **oospores let them survive host-free periods**, the **sporangia allow for** air or water-borne **long-distance dispersal**, while **zoospores detect hosts at short distances** (Goodwin, 1997).

1.3. Current treatments against *Phytophthora*

Since no specific treatment against the pathogen is available, most farmers try to deal with *Phytophthora*'s diseases by **relying on sanitation and management practices**. These practices usually involve the **recognition and management of infected plants**, the **quarantining of infected areas**, **chemical disinfection**, and the **cleaning of material, water and soil** (Frankel, 2008; Hardham, 2005). **Water management is particularly important** since disease incidence and severity are highest in fields with slow drainage or where saturated soil occurs (Hausbeck *et al.,* 2004; Dorrance *et al.*, 2009). Furthermore, Hausbeck (2004) highlighted some important concepts and practices in order to deal with the *Phytophthora* threat: **crop rotation**, the **exclusion of contaminated land and water**, **cultural control** (planting into well drained fields and into raised beds), the use of **fungicides** and **fumigation**, the importance of **genetic resistance or tolerance** towards infection and, finally, the **dissemination of information**.

Since there is a great evolutionary distance between oomycetes, including *Phytophthora,* and true fungi, **active inhibitors of fungi, or fungicides, are often not inhibitors of *Phytophthora*** (Hausbeck *et al.*, 2004; Hardham, 2005). Therefore, **novel approaches are required** to deal with these pathogens. This may prove particularly difficult to do due to the pathogen's ability to survive as chlamydospores on the soil or in roots of symptomless plants.

Back in 1995, O'Neill and Overvoorde performed a study where clearsol (40% Tar acids), formalin (38% formaldehyde), glutaraldehyde 20%, jet 5 (hydrogen peroxide), Opticide H (20% glutaraldehyde + 20% quaternary ammonium compounds), Panacide M (30% dichlorophen), Terspezial (quaternary ammonium compound) and sodium hypochlorite (10-14% available chlorine) were applied on the sand and shown to reduce the average number of dieffenbachia plants developing the disease from *Phytophthora nicotianae*, when compared with untreated sand. Formalin, glutaraldehyde 20%, and Opticide H demonstrated to be particularly effective, preventing plant death (O'Neill and Overvoorde, 1995). However, **none of these treatments were able to successfully prevent root rot** and some even **displayed some degree of phytotoxicity**.

Ten years later, two groups of potent compounds that **successfully inhibited *Phytophthora* growth and plant infection** were dominating the vanguard of the battle against these diseases (Hardham, 2005). The **phenylamides**, such as **metalaxyl**, and **phosphonates** (salts or esters of phosphonic acid, or phosphonic acid salts), like **phosphite**, have been the most effective compounds, and therefore have been widely used over the last 25 years (Hardham, 2005). They are systemic inhibitors, although their mode of action is still unclear. Metalaxyl can be translocated in the xylem and phloem, and phosphite may inhibit pathogen growth directly and stimulate a **plant defense response**, possibly by increasing production of pathogen-derived elicitors or by **decreasing the production of pathogen-derived suppressors of the defense response** (Hardham, 2005). Phosphite also proved to **reduce the production of zoospores** in infected plant material, helping limit the inoculum amplification (Hardham, 2005). Phosphite's effectiveness varies with the particular *Phytophthora* isolate and environmental conditions like phosphorus soil levels (Hardham, 2005). It generally displays **low phytotoxicity** but **some plants have shown foliar damage, reduction of pollen viability and pollen tube growth, higher frequency of abnormal mitotic and meiotic cell divisions**, and a **decrease in root growth** (Hardham, 2005). These facts may propel investigators to **search for more environmentally-friendly** and **targeted ways to deal with** the pathogens of the ***Phytophthora*** genus.

The knowledge of the cellular and molecular basis of pathogen development and pathogenicity is crucial to develop sustainable control measures for *P. cinnamomi*. Fortunately, thanks to next-generation sequencing, researchers can now access the genome of *P. cinnamomi* and obtain more easily its transcriptome. With these tools, researchers can more easily identify potential pathogenicity genes, and determine their roles in plant infection to develop more specific control measures towards these targets (Hardham, 2017).

1.4. Plant Genetic Engineering

Throughout history, plant breeders and growers have propagated plant varieties based on the presence of desirable characteristics through conventional breeding. On the lab, plant biotechnology researchers have been developing more **reliable** and **faster** methods to get such characteristics, namely through **genetic engineering**.

Several strategies are already available to transform plants. Among the most used strategies are the **microprojectile bombardment**, where DNA fragments are inserted randomly into tissue or calli cells, and **direct protoplast transformation**. However, the **most employed method is *Agrobacterium*-mediated transformation**, which is the only strategy of **indirect plant transformation**, as it requires nature's most effective plant genetic engineer as a mediator (Walkerpeach, 1994; Rakoczy-Trojanowska, 2002).

In its natural habitat, *Agrobacterium tumefaciens* **exploits tissue wounds to genetically transform plants by transferring a fraction of its Ti plasmid (T-DNA) into plant cells** (Gelvin,

2000). The bacterium uses the virulence machinery encoded by its Ti plasmid *vir* genes to attach to a plant cell, and insert the T-DNA on the cell genome (Gelvin, 2000). This T-DNA portion carries auxin, cytokinin, and opine-coding genes (Hood *et al.*, 1993; Gelvin, 2000). The ***Agrobacterium*'s ultimate goal is to drive plants to produce a highly-nutritious environment, rich in opines, for the bacteria to grow in**. The **two hormones force plant cells to proliferate exponentially**, making it possible to observe **crown galls** in affected plants, no more than tumours whose morphology greatly varies according to the plant species affected.

Taking advantage of this natural process, **researchers first disarmed the Ti-plasmid of its *vir*, hormone-coding, and opine genes** (Walkerpeach, 1994). Scientists could then **develop plant transformation vectors to place a gene-of-interest** (**GoI**) in the appropriate genetic and microbial context to promote the integration **within the genome of a target plant** (Walkerpeach, 1994). Both the cointegrate and the binary vector systems can accomplish this by **directly linking the GoI to one or more *cis*-acting T-DNA border sequences**, the Left Border, and the Right Border (LB and RB; Walkerpeach, 1994). The **border-GoI segment** must be maintained within the *A. tumefaciens* strain where it can be **acted upon by essential *trans*-acting *vir* gene products** which will **promote the excision and transfer of the T-DNA to the target cell**, as well as its integration on the genome (Walkerpeach, 1994). The ***vir* genes are usually supplied in a disarmed Ti-plasmid** (a plasmid lacking the T-DNA) that **can be either covalently linked to the GoI in cointegrate system vectors or exist as a separate replicon in binary systems** (Walkerpeach, 1994; **Figure 1.2**). Binary systems are currently the most used because of the **great reduction in the size of the plasmid with the T-DNA** (binary vector), which **aids the transformation process and cloning efficiency**. The binary vectors must contain antibiotic marker-selectable genes active in both *Escherichia coli* and *A. tumefaciens* to allow for plasmid propagation and the selection of microbial transformants (**Figure 1.2**). The T-DNA must also contain a plant-functional antibiotic marker to select for plant transformants (**Figure 1.2**). Plants that successfully regenerate in selective media must then be molecularly analysed to ensure the presence of the GoI.

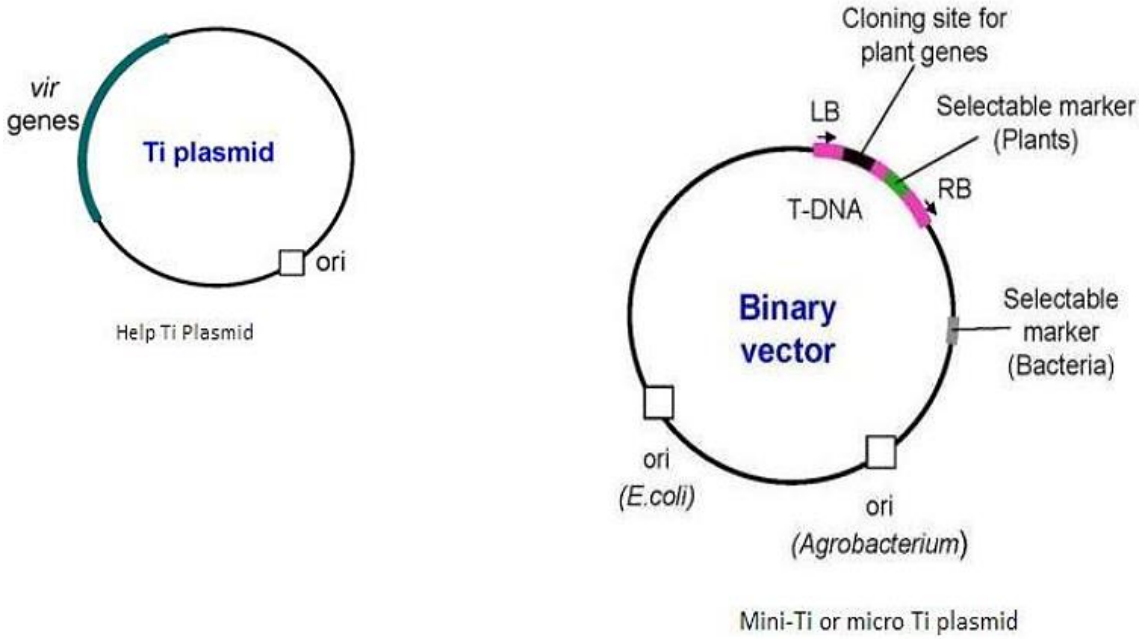

Figure 1.2 – Binary vector system for genetic engineering. This system consists of a pair of autonomously-replicating plasmid vectors. The *vir* genes cassette is contained in a helper plasmid on the *Agrobacterium* strain. The binary vector must contain the cloning site and a plant selection marker between LB and RB *cis*-acting sequences, since this T-DNA region will be the one integrated on the plant genome. Additionally, the binary vector must contain a bacteria selection marker and origins of replication in both *E. coli* and *Agrobacterium* in order to propagate it. Image adapted from https://www.slideshare.net/AAMIRRAINA/agrobacterium-mediated-gene-transfer-in-plants.

1.5. Poplar as a heterologous system

Forest trees are the major form of terrestrial biomass, providing irreplaceable benefits to the environment by **sequestering carbon**, **acting as a water sink**, and by **offering a habitat for wildlife** (Bradshaw *et al.,* 2000). Despite many aspects of tree biology being common to all plants, making it possible to study them in a simple plant model such as *Arabidopsis*, **there are unique characteristics of tree anatomy and physiology that require to be investigated within trees themselves**, thus **a tree model system is required** (Bradshaw *et al.,* 2000; Taylor *et al.,* 2002). Many different tree genera have been suggested to play the role of a model system, such as the *Salix* (willow), *Eucalyptus*, and *Pinus* (pine). Nowadays, the ***Populus L.*** **genus is considered the model organism for forest biotechnology**, **tree molecular biology, tree genomics,** and **tree physiology** (Liu *et al*., 2016). This fact is greatly due to factors such as the **plant's rapid growth**, its **small genome** of 450-550Mbp (Taylor *et al.,* 2002), its **large number of molecular genetic markers**, the **replicability of the experiments** performed on clonal material, and the **availability of protocols for vegetative propagation** in the literature (**Table 1.3**; Constabel *et al.*, 2000; Taylor *et al.*, 2002; Mader *et al.*, 2017). The ease with which *in vitro* cultures could be established allowed *Populus* to be one of the **first woody systems to a undergo genetic transformation**, and for it to be **successful in diverse plant transformation techniques** such as ***Agrobacterium*-mediated transformation**, **direct DNA transfer**, and **electroporation** (Leplé *et al*., 1992; Taylor *et al.*, 2002; Mader *et al.*, 2017). Additionally, poplar biologists have a well-established collaboration, sharing genetic materials, DNA-based markers, and field measurement protocols, founding a solid research network (Bradshaw *et al.*, 2000).

Table 1.3 – *Arabidopsis* vs. *Populus*: a summary of major criteria on which to judge a 'model' plant system. Table adapted from Taylor, 2002.

Criterion	Arabidopsis	*Populus*
Wide genetic diversity	Yes	Yes
Fast growth	Yes	Yes
Short life cycle	Yes	No
Ease of breeding system	Yes	No
Ability to transform	Yes	Yes
Molecular genetic maps	Yes	Yes
Recombinant inbred lines	Yes	No
Genomics	Yes	Yes
Immortal mapping populations	No	Yes
Sequencing data	Yes	On-going

The *Populus L.* genus is part of the *Salicaceae* family, comprising the aspens, poplars, and cottonwoods, and has a wide distribution over the Northern Hemisphere (Douglas, 1989; Bradshaw *et al.,* 2000). The **genus is large**, covering 40 species, **and has a wide spectrum of species** with various heights and shapes, according to local environmental conditions (Douglas, 1989). Poplars are an **important source of timber** and often provide the **sole source of building wood in many rural areas worldwide**, including in Portugal (Douglas, 1989). These trees are dioecious, meaning that the species has male and female individuals, and **wind-pollinated**, producing large amounts of pollen and small cotton-tufted seeds (Douglas, 1989; Bradshaw *et al.*, 2000). These reproductive characteristics allow populations to **spread quickly and invade new sites**. Each poplar pollination may give rise to hundreds of seeds within 4 to 8 weeks (Bradshaw *et al.*, 2000). The seeds germinate in 24 hours and **originate 1 to 2 meters-tall seedlings by the end of the year** (Bradshaw *et al.*, 2000). Additionally, all poplars have the capacity to **reproduce asexually**, sprouting from the root collar, from abscised or broken branches, or from horizontal roots (Douglas, 1989; Bradshaw *et al.*, 2000). This set of capabilities often leads to **clonal poplar stands** that may cover several hectares (Bradshaw *et al.*, 2000).

On the other hand, and unlike the universal plant model *Arabidopsis*, a model plant such as **poplar has practical commercial applications** (**Table 1.3**; Bradshaw *et al.,* 2000; Taylor *et al.,* 2002). Poplar is currently cultivated worldwide with many different purposes such as the **production of pulp, paper, veneer, excelsior, engineered wood products, lumber,** and **energy** (Bradshaw *et al.*, 2000). Therefore, there is a particular emphasis on the rapid growth of young trees for biomass, with **yield and disease resistance being the top priority for future improvement** and planting of this forest tree (Taylor *et al.*, 2002). Historically, it has also been used in windbreaks and erosion control (Bradshaw *et al.*, 2000). More recently, poplar is gaining more uses, such as being a **phytoremediation tool** of environmental toxins, a **bioindicator** for environmental ozone pollution, and **its rapid growth is even being explored as a means of carbon sequestration** in Europe (Bradshaw *et al.*, 2000).

Among the most popular poplar lines employed in research, is the hybrid grey poplar (*Populus tremula x Populus alba*, also called *Populus canescens*) plant line INRA 717-1B4. This is a clone of a female poplar tree from the Institut National de la Recherche Agronomique, France (Lemoine, 1973). This line has widely been employed as a tree model, and it was transformed for the first time by Leple *et al.* in 1992 (Mader *et al.*, 2017). Furthermore, there are several transgenic poplar lines from this clone that have been transferred to the field and have been tested for commercial applications (Mader *et al.*, 2017).

1.6. *GINKBILOBIN2-like* gene as a strong candidate for resistance against *Phytophthora*

Plants, unlike animals, are sessile organisms that **may not move to escape from danger or to meet better growing conditions**. Therefore, **they developed coping mechanisms against biotic and abiotic stresses**, while maintaining resources for growth and reproduction. In plants, these mechanisms against biotic stress are often translated into innate immunity of individual cells (Miyakawa *et al.*, 2014; Gao *et al.*, 2015). **Pathogen-defense mechanisms** include wide-range **hypersensitive responses** (triggered cell death to prevent pathogen spread throughout plant tissues), the **reinforcement of cell walls**, and the synthesis and secretion of more specific **secondary metabolites like phytoalexins and antifungal proteins** that can be triggered by microbial-associated molecular patterns (MAMPs; Lo & Nicholson, 1998; Miyakawa *et al.,* 2009; Miyakawa *et al.,* 2014; Gao *et al.*, 2015). These antifungal proteins have been discriminated into cyclophilins, defensins, pathogenesis-related proteins, ribosome-inactivating proteins, thaumatins, chitinases, glucanases, miraculins, and proteins and peptides with other structures (Wang and Ng, 2000; Sawano *et al.*, 2007; Miyakawa *et al.,* 2009).

Ginkgo biloba trees have an extensive history of resistance and tolerance to many pathogens, thus being an object of frequent research (Sawano *et al.*, 2007). The **GINKBILOBIN2 protein** (GNK2) was discovered and purified by Sawano *et al.* (2007) and further studied by Miyakawa *et al.* (2009). It **consists of 108 amino acids** and is **present in *Ginkgo biloba* seeds' endosperm, exhibiting antifungal activity** (Sawano *et al.,* 2007; Miyakawa, 2014; Gao *et al.,* 2015; dos Santos, 2017). Sawano *et al.* (2007) found GINKBILOBIN to be particularly effective in inhibiting the growth of *Fusarium oxysporum* (**Figure 1.3**; Miyakawa *et al.,* 2009). Additionally, other reports found it to have potent antifungal activity against *Botrytis cinerea*, *Mycosphaerella arachidicola*, *Rhizoctonia solani, Coprinus comatus*, *Trichoderma reesei*, and *Candida albicans* (Wang and Ng, 2000; Sawano *et al.*, 2007) and it even suppressed the activity of the HIV-1 reverse transcriptase and the proliferation of murine splenocytes (Wang and Ng, 2000).

Although **GNK2 does not show any sequence similarity to other antifungal proteins**, **homologs of this protein are found in all seed plants**, gymnosperms, angiosperms, and in *Selaginella*, having conserved domain structures, peptide motives, and specific cysteine signatures (Sawano *et al.,* 2007; Miyakawa *et al.,* 2009; Miyakawa *et al.,* 2014; Gao *et al.,* 2015). It **displays much more homology** (about 85%) **to Embryo-Abundant Proteins** (**EAPs**) from gymnosperms (**Figure 1.5**; Wang and Ng, 2000; Sawano *et al.,* 2007; Miyakawa *et al.,* 2009). These EAPs are expressed in the late stage of seed maturation and are **involved in protection against environmental and biotic stresses** (Sawano *et al.*, 2007; Miyakawa *et al.*, 2009). EAPs are particularly important plant proteins because seed germination usually occurs in an environment rich in pathogens, and the rupture of the seed coat would allow them to invade the seed storage tissues (Sawano *et al.*, 2007). On this stage of major vulnerability, the antifungal and antibacterial proteins are the ones who provide the necessary protection for the seedling (Sawano *et al.*, 2007).

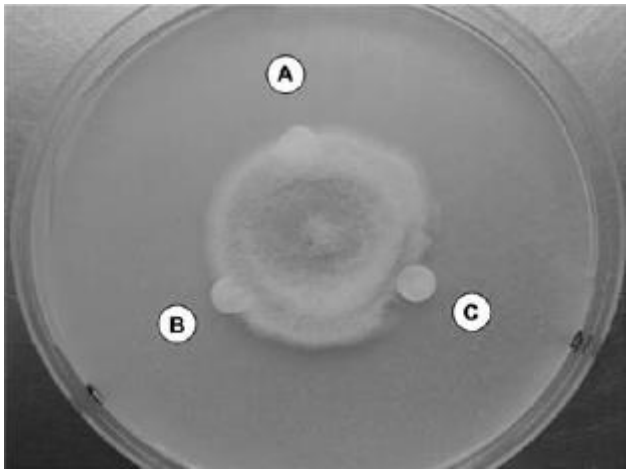

Figure 1.3 – Inhibition of *Fusarium oxysporum* growth by GINKBILOBIN2. (A) Control: 20 ml of 0.1 M sodium phosphate buffer, pH 6.0; (B) GINKBILOBIN2: 20 ml of a 10 mg/ml solution in the same buffer; and (C) GINKBILOBIN2: 20 ml of a 20 mg/ml solution in the same buffer. Figure adapted from Sawano *et al.,* 2007.

A conserved **cysteine-rich motif** called the C-X_8-C-X_2-C motif was found on the amino acid sequence of the GNK2 protein. The motif was part of the domain of unknown function 26 (DUF26; F**igure 1.4**), also known as the **stress-antifungal domain** PF01657. This fact made researchers consider the protein as a **cysteine-rich receptor-like kinase** (**CRK**) (Sawano *et al.*, 2007; Miyakawa *et al.,* 2014; Gao *et al.*, 2015; dos Santos, 2017). **These kinases are major players** in cellular processes and **in the response to biotic and abiotic stresses**, such as **pathogen recognition**, **resistance to bacterial and fungal pathogens**, **hypersensitive response-related cell death**, oxidative stress responses, and **salicylic acid-dependent defense pathways** (Sawano *et al.*, 2007; Miyakawa *et al.,* 2014; Gao *et al.*, 2015). They also take part in important roles such as hormonal response pathways, cell differentiation, plant growth and development, and self-incompatibility (Sawano *et al.*, 2007). In particular, **the *Arabidopsis* CRK with a C-X_8-C-X_2-C motif is induced by pathogen infection**, suggesting a role in **plant defense responses** (Sawano *et al.*, 2007).

Apart from the DUF26 subdomain, identified as subdomain B by Gao (2015), Gao also identified two other subdomains: the **A1**, **responsible for actin-binding**, and the subdomain A2, dispensable for actin-binding. Additionally, Gao (2015) showed that the **26-residues signal peptide** present on the sequence **conveyed efficient secretion of GINKBILOBIN to the outside of the cell**. Gao also evidenced that, in absence of the peptide, **GINKBILOBIN would bind and be co-localized with the actin cytoskeleton**. **The actin-binding subdomain** (A1) **could** also coassemble with actin *in vitro* and **delay the premitotic nuclear positioning of actin, an indicator of its reduced dynamicity** (Gao *et al*., 2015*)*. Binding of a synthetic construct with **the actin-binding subdomain** conjugated with the cell-penetrating peptide BP100 and with rhodamine B as a fluorescent reporter **induced actin-dependent programmed cell death** (Gao *et al.,* 2015). This experiment showed that the antifungal activity of **GINKBILOBIN can** exploit the evolutionary conserved and ancient mechanism to **control cell death via restricting actin dynamics** through its A1 domain.

GNK2 has had its crystal structure disclosed by the work of Miyakawa (2009), revealing a tertiary structure composed of two α-helixes and a five-stranded β-sheet, forming a compact single-domain with an α+β-fold (**Figure 1.4A**). The molecule has a highly basic pI of 9.4 and several acidic residues important for maintaining the helical structure, which is easily lost under acidic conditions as ion pairs and negative charges vanish (Sawano *et al.*, 2007). **The C-terminal half of the protein** (residues 56 to 108) **corresponds to the DUF26, the domain unit in CRKs**, and is **composed of three β-strands** (S3, S4, and S5) **and the second α-helix** (H2) (**Figures 1.4A** and **1.5**). There are three **disulphide bridges between the H2 helix and the β-sheets**, which largely **contribute to the protein's structural stability** (**Figure 1.4B**; Sawano *et al.,* 2007; Miyakawa *et al.,* 2009).

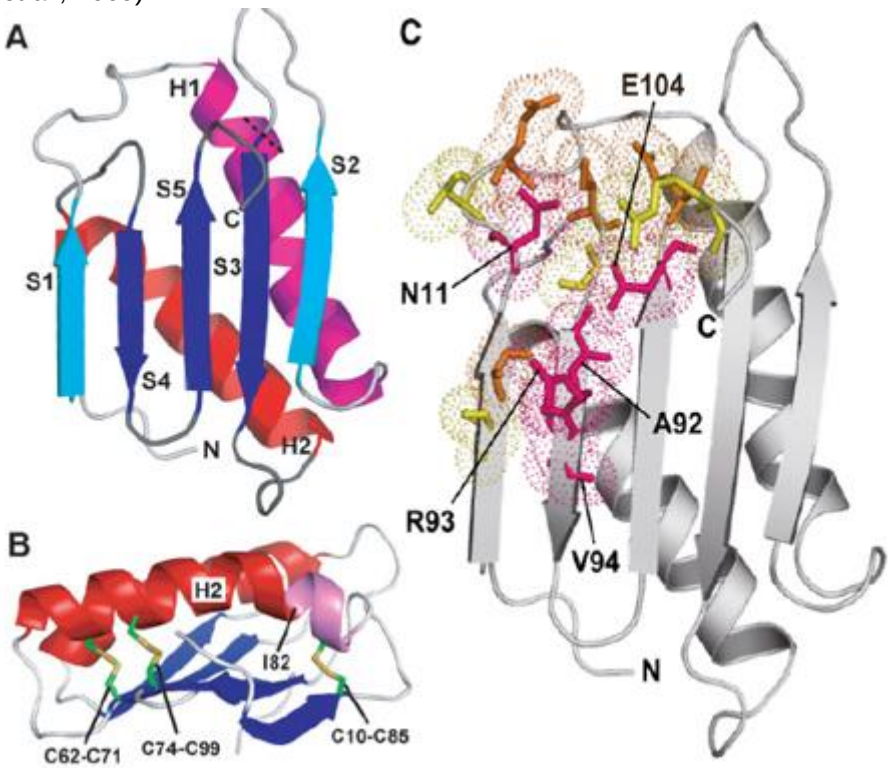

Figure 1.4 – Overall GNK2 structure with three disulphide bridges. (**A**) Ribbon diagram of the GNK2 structure with two α-helices (magenta and red) and a five-stranded antiparallel β-sheet (blue and cyan). The DUF26 region on the GNK2 molecule corresponds to the C-terminal side starting at the broken line, which is colored blue, gray, and red. (**B**) Disulphide bridges between the H2 helix and the β-sheet. The disulphide bridges are shown as stick models with the yellow stick indicating a covalent bond between two sulphurs. The pink region of the H2 helix shows a kink near the disulphide bridge of Cysteine10-Cysteine85; (**C**) The mapping of the residues with CSP (chemical-shift perturbation) of ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) on the GNK2 structure. The residues are represented with stick and dot models. Figures A and B adapted from Miyakawa *et al.*, 2009; Figure C adapted from Miyakawa *et al.*, 2014.

Many pathogenesis-related proteins with antifungal activity initially target the cell wall components of fungi (Miyakawa *et al.,* 2014). Miyakawa's team (2014) went further onto this theme, declaring a **lectin-like function in GNK2**, through proving that it **could directly interact with mannan and** its building block, **mannose**. Lectins are carbohydrate-binding proteins, which are highly specific for sugar groups. They usually have major roles in the recognition of cells, carbohydrates, and proteins, **mediating the attachment of bacteria, viruses, and fungi**. In particular, GNK2 is capable of discriminating the α/β-linkage configuration of the hydroxy group at the C4 position of the monosaccharide through hydrogen bonds formed between asparagine-11, asparagine-93, and glutamate-104, residues considered essential for binding (Miyakawa *et al.,* 2014). **This interaction was directly related to GNK2's ability to inhibit fungal growth**, in particular, *Fusarium* spp., however, it did not allosterically regulate GNK2's function upon binding (Miyakawa *et al.*, 2014).

GNK2 has a single binding site for the monosaccharide with an affinity of 10^{-2} to 10^{-1} toward mannose and **exists as a monomer** in solution (Miyakawa *et al.,* 2014). **Twenty-two proteins were found to be homologous to GNK2** by the DALI server, but **these were not related to any antifungal function** (Miyakawa *et al.*, 2009). These data seemed to point to a **novel fold in this particular antifungal protein** when compared to the conventional ones. On the other hand, **both defensin and GNK2 are cysteine-rich proteins that have an α+β-fold and three to four disulphide bridges** (Miyakawa *et al.,* 2009). Additionally, **defensin has a positively charged surface which contributes to the interaction with negatively charged phospholipids**, and **GNK2 H1 α-helix also displays four arginine residues, granting it the same positively charged surface** (Miyakawa *et al.*, 2009). So, Miyakawa (2009) speculates that **the antifungal activity of GNK2 requires a direct protein-membrane electrostatic interaction between its positively-charged surface and the negatively-charged phospholipids and/or phosphomannan on the fungal cell-surface** (Sawano *et al.*, 2007). This interaction would result in **pore formation** or a more specific interaction with a lipid domain, disrupting the fungal cell (Sawano *et al.*, 2007).

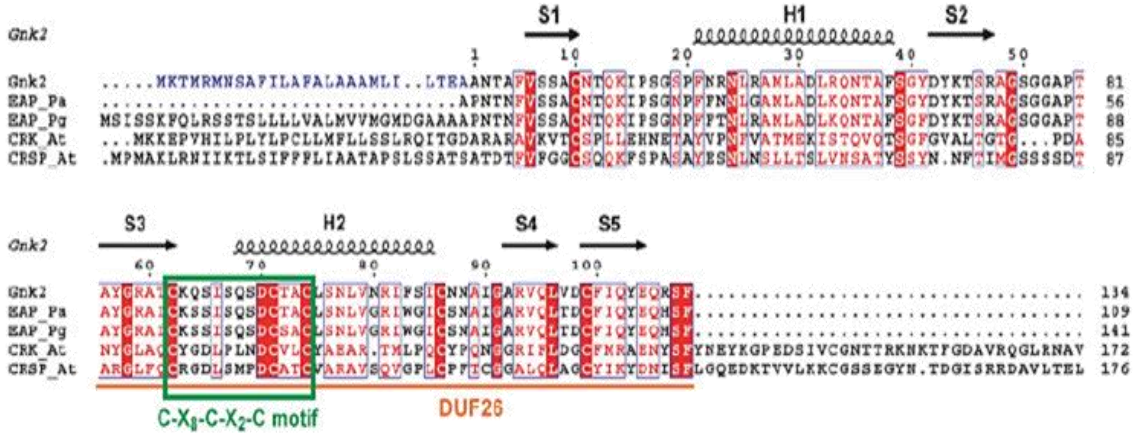

Figure 1.5 – Structure-based sequence alignment of GNK2 and the GNK2-homologous regions of the proteins with the C-X8-C-X2-C motif. Strictly conserved residues are shaded in red, and well-conserved ones are indicated with red letters. The secondary structural elements of GNK2 are depicted at the top of the alignment. The signal-peptide sequence of GNK2 is indicated with blue letters. The green-boxed sequences correspond to the motif, and the DUF26 sequences are underlined in orange. The following sequences are used for comparison: EAP_Pa, EAP from *Picea abies*; EAP_Pg, EAP from *P. glauca*; CRK_At, CRK from *Arabidopsis thaliana* and CRSP_At, CRSP from *A. thaliana*. Diagram adapted from Miyakawa, 2009.

The *C. crenata* ***GNK2-like*** **gene emerged as a strong candidate for resistance against *P. cinnamomi*** in a study performed by Santos *et al*. (2017). This work is comprised of a combined approach of transcriptomics (RNA-Seq) and genomics (Genome-Wide Association Study, GWAS), and aimed to understand the genetic background underlying the *P. cinnamomi* resistance trait in *Castanea* spp, including *C. crenata*, *C. mollissima* and *C. sativa*. *C. sativa* is commonly denominated as the European chestnut and is extremely affected by *P. cinnamomi*, while the Asian *C. crenata* and *C. mollissima*, often show a resistant phenotype (Corredoira *et al.*, 2012; Santos *et al*., 2017). The ***Cast_GNK2-like*** **gene was** ultimately **revealed to be the one that best discriminated between susceptibility and resistance towards *P. cinnamomi* infection**. As such, **functional validation of this gene is in order to understand the viability of its product as a biotechnological solution against *Phytophthora*-caused diseases**. There are two good strategies to perform a functional validation in this context: a gain-of-function, performed by increasing the activity or adding-in a new protein to increase resistance in a susceptible species, and a loss-of-function, performed by knocking-out a gene to decrease susceptibility of this same species. The most appropriate strategy could be the gain-of-function, to overexpress the GoI in *C. sativa*, and expose it to *P. cinnamomi* to check if the presence of the gene alone provided enhanced tolerance to disease. However, although chestnut transformation protocols are available (Corredoira *et al.*, 2012), this is a difficult feat to achieve within the time-spawn of a Master's Thesis, hence the need for a **heterologous system based on poplar**.

1.7. Poplar and *Phytophthora* spp.

The ultimate goal of the thesis was **to perform a functional validation of the Cast_GNK2-like protein** identified by Santos (2017). Ideally, this validation should be performed by transforming a susceptible *Castanea* species with the gene to verify if a phenotype more resistant to *P. cinnamomi* infection could be recovered. However, transforming *Castanea* is much slower than transforming a tree model such as poplar. Although there are no records of *P. cinnamomi* infection available for wild poplar, this tree is one of the many host species that *Phytophthora* spp. may affect. In particular, the **plant can be infected by *Phytophthora cactorum***, which has been **isolated from natural poplar forests** in studies performed by Cerny *et al*. (2009) in the Czech Republic, and Keča *et al*. (2015) in Serbia. As such, in this functional validation, *P. cactorum* will be used, along with *P. cinnamomi*, as an alternative infection agent to assess the anti-*Phytophthora* activity of the *Cast_GNK2-like* gene.

When employing *P. cactorum* and *P. cinnamomi* in infection assays, it is important to understand that these species have distinct lifestyles (Cooke *et al.*, 2000). *P. cactorum* possesses papillate caducous sporangia, it is homothallic and has a paragynous reproductive behavior. It may infect multiple hosts, in particular herbaceous and **woody perennial plants**, and was placed on **Waterhouse group I** (Waterhouse, 1963; Cooke *et al.*, 2000; Hantula *et al.,* 2000). ***P. cactorum*'s caducous sporangia allow for wide-distance dispersal by air currents**. On the other hand, *P. cinnamomi* has nonpapillate sporangia, it is heterothallic, it presents a metaphase ring, and has amphigynous reproductive behavior. This species may also infect multiple hosts, including many **woody perennial plants**. These characteristics placed it on the **Waterhouse group VI** (Waterhouse, 1963; Cooke *et al*., 2000). ***P. cinnamomi* spreads mainly in water** using its zoospores as propagules. Naturally, *P. cactorum*'s symptoms are also slightly different from *P. cinnamomi'*s, causing leather rot of fruits and crown rot in strawberry, apple, and *Betula spp*., and root rot and dieback in rhododendron (Hantula *et al.,* 2000).

1.8. Work Aims

The main objective of this work is to functionally validate the antifungal activity of the *GNK2-like* gene from *C. crenata* using poplar as a heterologous tree system. The two species of *Phytophthora* chosen for this work were *P. cinnamomi* and *P. cactorum*. *P. cinnamomi* was chosen because **the *GINKBILOBIN2-like* gene from *C. crenata* was identified using this pathogen in a previous research work**. However, since there were **no records of *P. cinnamomi* ever infecting wild poplar**, ***P. cactorum* was included**, as it is a natural poplar pathogen (Cerny *et al*., 2009; Keča *et al*., 2015)**.** To achieve the main goal stated above, **four specific objectives were devised:**

(1) To generate transgenic poplar plants constitutively expressing the *GNK2-like* gene from *C. crenata* using *Agrobacterium*-mediated transformation;

(2) To develop an *in vitro* poplar infection assay in order to evaluate the presence or absence of symptomatology of the disease caused by *P. cinnamomi* and *P. cactorum*;

(3) To discover whether *P. cinnamomi* can infect the model tree grey poplar;

(4) To understand if poplar overexpressing *GNK2-like* gene from *C. crenata* present enhanced tolerance/resistance towards ink disease;

(5) To construct a plant transformation binary vector with the *Cast_GNK2-like* gene under the regulation of a root-specific promoter, as *Phytophthora* infection is mainly done via roots of susceptible plant species.

2. Materials and Methods

2.1. EHA105 *Agrobacterium tumefaciens* Transformation with pFHi.GNK2

The main goal of this section was to transfer the gene of interest, the *GNK2-like* gene from *C. crenata*, inside a plant binary vector, into the *A. tumefaciens* EHA105 strain.

pFHi.GNK2 binary vector

The pFHi.GNK2 was the binary vector employed to transfer the *GNK2-like* gene from *C. crenata* to grey poplar and was constructed by Patrícia Fernandes, a Ph.D. student from INIAV (Instituto Nacional de Investigação Agrária e Veterinária; **Figure 2.1**). The vector sequence can be found in the Supplementary Data, **Figure 6.1**. It is considered a plant binary vector because, while it includes a Left and a Right Border delimiting the insert, it does not code for the virulence machinery. The borders are recognized by the virulence machinery of the *A. tumefaciens* strain, allowing the T-DNA to be integrated on the plant genome.

Inducing competence in *E. coli* DH5α (Sambrook *et al*. 1989)

In order to induce competence in *E. coli* DH5α cells, the TB Buffer method based on Sambrook *et al*. (1989) was employed. On the first day, the top of a frozen DH5α glycerol stock was scraped and streaked onto an LB agar plate (media composition is described by the end of each sub-chapter). The plate was left in an inverted position at a 37 ºC incubator overnight (O/N). On the following day, a single colony was picked into 20 mL of liquid LB medium and the falcon tube was left O/N at 37 ºC with 180 rpm of agitation, in order to make a pre-inoculum. On the third day, 100 µL of the pre-inoculum was transferred to 250 mL of SOB medium and the cells were left to proliferate at 24 ºC, with 150 rpm of agitation, until their Optical Density at 600nm (OD_{600nm}) equaled 0.5. The cells were then placed into an ice bath for 10 min and, from this point on, everything that got into contact with them was ice-cooled in order to protect the fragilized cells. The 250 mL of culture was transferred into six falcons (approximately 40 mL for each) and the cells were centrifuged at 520 x *g* for 10 min at 4 ºC. The supernatant was discarded and the pellet was gently resuspended with 10 mL ice-cold 1X TB (10 mM PIPES, 15 mM $CaCl_2$, 55 mM $MnCl_2$, 250 mM KCl). The resuspended pellets of the six falcons were joined into two falcons and the volume was adjusted to 40 mL with TB. The falcons were incubated on ice for 10 min and centrifuged at 520 x *g* for 10 min at 4 ºC to further concentrate the cells. The supernatant was once again removed and each cell pellet was resuspended gently on 10 mL 1X TB. Dimethyl sulfoxide (DMSO) was slowly added to the walls of the falcons to a final concentration of 7% (700 µL per 10 mL). Another incubation step of 5 min on ice followed, and 100 µL aliquots were taken and placed into liquid nitrogen for 1 h. The tubes were stored at -80 ºC.

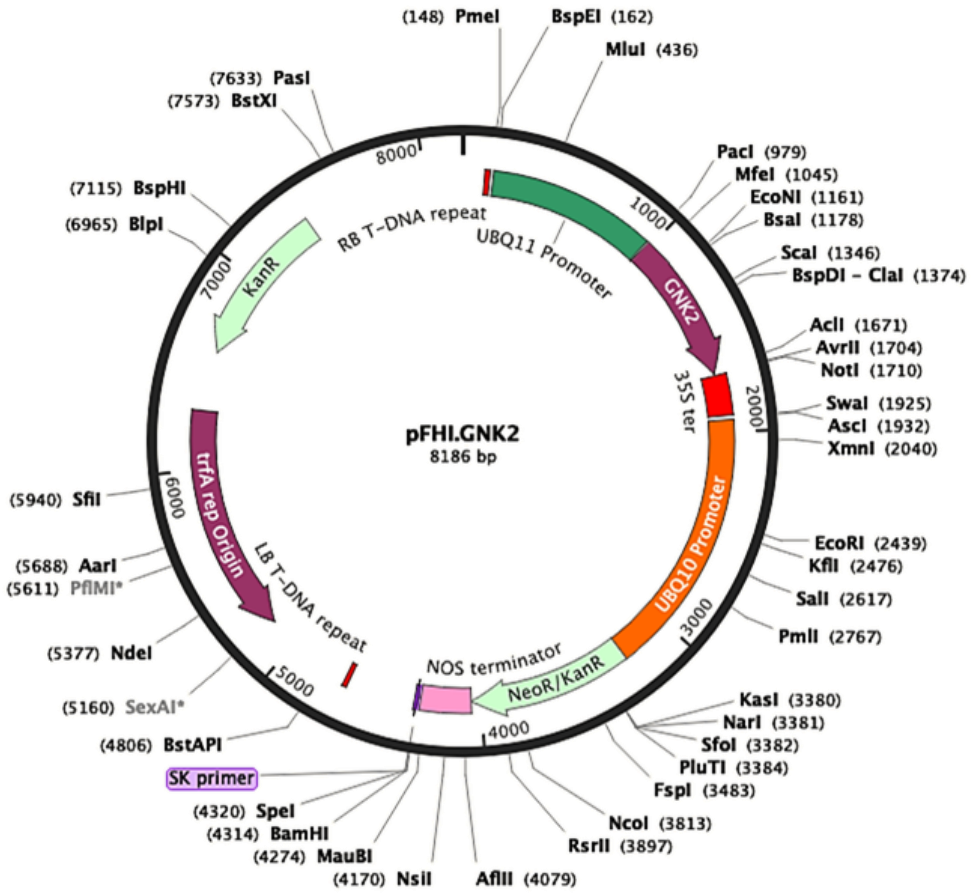

Figure 2.1 - pFHi.GNK2 binary vector. It contains a Left Border (LB) and a Right Border (RB) delimiting the T-DNA segment which will be introduced into plant cells by the *vir* genes machinery of the *A. tumefaciens* strain. The plasmid holds both an origin of replication and a kanamycin selection marker for *E. coli* and *A. tumefaciens*. Inside the T-DNA there is the *Cast_GNK2* gene's cDNA regulated by the constitutive UBQ11 Promoter, along with the resistance marker neomycin phosphotransferase II (*nptII*), which allows the transformed plants to be selected using 25 mL/L of kanamycin. As for selecting transformed bacteria, 50 mL/L were used instead.

Competence validation of the *E. coli* DH5α cells, pUC19 transformation

1 µL of 10 pg/µL pUC19 vector (**Figure 2.2**) was added to 200 µL of competent cells previously thawed on ice. A control vial without plasmid was also used for posterior controls. After gently mixing, the cells, along with the DNA, were incubated on ice for 3 h in order to promote the adsorption of the plasmid to the cell wall of the bacteria. After a heat shock of 42 ºC for 45 s, the cells were ice-cooled again and resuspended in 500 µL of liquid LB medium. Cells were incubated for 1 h at 37 ºC with gentle shaking, to allow the bacteria to recover from the shock and to express the antibiotic marker gene encoded by the plasmid (*AmpR*). Fifty-four microliters were taken out to an Eppendorf tube in order to have a 1:100 dilution to plate. Then, the culture was concentrated by centrifugation (8636 x *g*, 5 min at RT), resuspended in 200 µL LB medium, and plated on LB medium agar supplemented with 100 µg/mL ampicillin, using a glass spreader. A control of viability was done by plating Petri dishes were sealed and incubated O/N in an inverted position at 37 ºC.

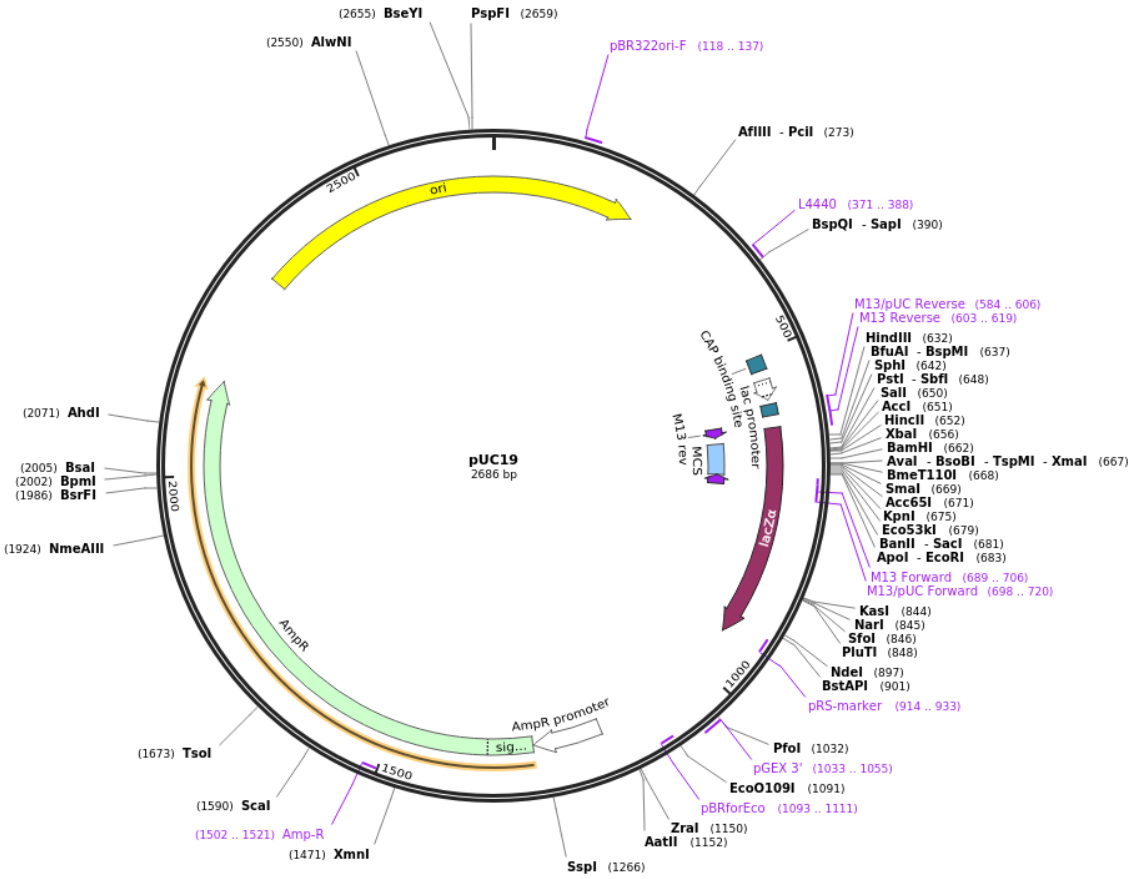

Figure 2.2 - pUC19 vector. This vector contains a replication origin for *E. coli*, which allows it to be propagated, a *AmpR* gene which allows for selection of transformants by using 100 mL/L ampicillin in the media, and a Multiple Cloning Site (MCS) with many unique enzymatic restriction sequences to aid the cloning of DNA fragments. The vector further allows the selection of recombinants by taking advantage of the *lacZ* alpha gene, which allows for blue/white screening in media supplemented with X-gal. X-gal is a colourless analogue of lactose which can be cleaved by the *β*-galactosidase encoded by the *lacZα* gene. When this happens, it dimerizes, forming a blue compound. The recombinants would thus be the white colonies, in which no *β*-galactosidase is being formed due to the insert having interrupted the gene.

E. coli DH5α Transformation using the pFHi.GNK2 plasmid

5 µL of a pFHi.GNK2 aliquot was added to 100 µL of competent cells previously thawed on ice. After gentle mixing, the cells, along with the DNA, were incubated on ice for 3 h in order to promote the adsorption of the plasmid to the cell wall. After a 45-second heat-shock of 42 ºC, the cells were ice-cooled again for 2 min and resuspended in 500 µL of liquid LB medium. The cells incubated for 1 h at 37 ºC with gentle shaking, to allow the bacteria to recover and to express the antibiotic marker gene encoded by the plasmid (*nptII*). Then, the culture was concentrated by a centrifugation step of 5 min at 8636 x *g*, resuspended in 200 µL of the same medium and plated on agar LB agar Petri dishes with appropriate antibiotics (50 µg/mL kanamycin), using a flamed glass spreader. Petri dishes were left at RT, then sealed and incubated O/N in an inverted position at 37 ºC. The resulting colonies are depicted on **Figure 6.6** of the Supplementary Data.

pFHi.GNK2 Plasmid MiniPrep

In order to amplify the amount of the pFHi.GNK2 plasmid, an inoculum was prepared and the DNA was extracted using the GFX Micro Plasmid Prep Kit. One milliliter of an O/N culture was transferred to a 1.5 mL Eppendorf tube. The cells were pelleted by centrifugation at full speed for 30 s, aspiring the supernatant without disturbing the pellet. The cells were resuspended in 150 µL of Solution 1 (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 400 µg/ml RNase I) by vortexing the tubes. One hundred and fifty microliters of Solution 2 (1 M NaOH, 5.3% (w/v) SDS) were added and the tube was inverted 10 to 15 times to help on bacterial lysis. Three hundred microliters of Solution 3 (Buffered solution containing acetate and chaotrope) were added and the tubes were mixed by inverting until a flocculent precipitate appeared. The precipitate was evenly dispersed by gently keeping inverting the tubes. The cell debris were pelleted by centrifugation at full speed for 5 min. Meanwhile, a GFX column (MicroSpin™ columns pre-packed with a glass fiber matrix) was placed on each collection tube, and the supernatant was carefully transferred to it. The tubes were left to incubate 1 min at RT before being centrifuged again at full speed for 30 s. The flow-through from the collection tube was discarded. Four hundred microliters of wash buffer was added to the column, and another centrifugation at full speed for 60 s was performed to remove the buffer. The GFX column was transferred to a fresh microcentrifuge tube and 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) were added on top of the glass fiber matrix of the GFX column. The column was left to incubate at RT for 3 min and a final centrifugation step at full speed for 1 min was performed to recover the purified DNA.

Enzymatic Restriction using BamHI R0136S

A plasmid digestion was made to verify the length of the pFHi.GNK2 plasmid by electrophoresis. The procedure was performed according to a protocol from NEBcloner (New England BioLabs), in which 1 µg of DNA, 5 µL of 10X CutSmart Buffer and 1 µL (0.4 U) of BamHI at 20 U/µL from BioLabs were mixed in an Eppendorf tube. The tube was left to incubate at 37 ºC for 5 h, but 5 to 15 min should have been enough, since this enzyme had the Time-Saver Qualification from New England BioLabs.

Agarose Gel Electrophoresis

In order to visualize the DNA fragments, a 0.7% agarose gel was made by adding 0.35 g of agarose in 50 mL of 0.5X TBE. The solution was microwaved until the agarose dissolved, and the flask was left to cool down at RT until it could be safely grabbed with gloved hands (~50 ºC). Half a microliter of SYBR Safe were added, and the mixture was homogenized and poured onto a gel tray with a 12-wells comb in place. The gel was left to solidify for 30 min before being transferred to a VWR Mini Gel II filled with 400 mL of 0.5X TBE. Five microliters of Loading Buffer was added to 5 µL of plasmid DNA (100-150 ng per well) before loading 10 µL of the samples and 1 µL of the GeneRuler e 1 kb DNA Ladder (250–10,000 bp; SM0318; Fermentas). The gel was left to run at 60 V for 1 h. The DNA migration patterns were visualized inside a UV-based Biorad Gel Doc System w/ Universal Hood II with the aid of the Image Lab software, using the SYBR Safe configuration.

Competence induction in EHA105 *A. tumefaciens* cells

The *A. tumefaciens* strain chosen for this work was the EHA105 from Hood *et al*. (1993). In order to have a stock of *A. tumefaciens*, the streak plate method was performed as shown in

Ruangpan (2004). An *A. tumefaciens* EHA105 glycerol aliquot stored at -80 ºC from a previous competence-induction assay was the source for the inoculation. A flamed loop was dipped into the previously-frozen solution of *A. tumefaciens* and rubbed along the YEB medium, containing 50 mg/L of rifampicin. The Petri dish was turned a quarter turn, the loop was flamed and cooled-down and three parallel lines were drawn from the previous rubbing. This step was repeated until the inoculum was exhausted using a zig-zag pattern. The bacteria were left to grow for 2 days at a 28 ºC chamber.

A single *A. tumefaciens* EHA105 colony from one of the Petri dishes was recovered using a toothpick and placed on a falcon tube with 5 mL of liquid YEB medium supplemented with 50 mg/L rifampicin. The falcon was left O/N at 28 ºC with 180 rpm of agitation, giving rise to a pre-inoculum.

To scale up the bacteria density, 5 mL of the previous culture was used to make the inoculum. The solution was added to 45 mL of YEB medium supplemented with 50 mg/L of rifampicin. The culture was left to grow at 28 ºC with 180 rpm until its Optical Density at 600 nm (OD_{600nm}) reached 0.9, after approximately 3 h and a half. The cells were pelleted by centrifugation at 3000 rpm for 15 min at 5 ºC in a Sigma 4K15 centrifuge. Another centrifugation step of 3500 x *g* for 5 min (5 ºC) was performed to ensure that most of the cells had precipitated. The pellet was gently resuspended in 1 mL of 10 mM $CaCl_2$, previously cooled down on ice. Two hundred and fifty microliters of sterile 80% glycerol was added to protect the cells from freezing and bursting. This mix was distributed, in 100 µL aliquots, into twelve Eppendorf tubes. The now competent cells were frozen in liquid nitrogen and stored at -80 ºC until they were needed.

A. tumefaciens **cell competence Validation**

Several attempts to validate the *A. tumefaciens* cell-competence were performed through freeze-thaw-based transformation (described below) of 100 µL of competent cells with 1 µL of a commercial pUC19 plasmid from the In-Fusion® HD Cloning Kit. However, none of the attempts were successful, as the cells would show resistance against the plasmid's selection marker, ampicillin (not shown). This result was deduced because the negative control consisting of plates of YEB medium supplemented with 50 mg/L rifampicin and 100 mg/L ampicillin with non-transformed *A. tumefaciens* cells would often show signs of bacterial growth (not shown). This fact was verified even though the ampicillin stock was renewed. Therefore, it was decided to transform the cells directly with the plasmid of interest, pFHi.GNK2, as the selection marker for this vector is kanamycin.

Freeze-thaw ***A. tumefaciens*** **transformation**

To transform *A. tumefaciens* cells with the plasmid pFHi.GNK2, a freeze-thaw transformation protocol based on Walkerpeach's work (1994) was employed. Five microliters of a pFHi.GNK2 aqueous solution were used to transform 100 µL of competent cells. The cells incubated for 30 min in ice, being gently mixed from time to time, for the plasmid to adhere to the bacteria surface. The cells were then transferred into liquid nitrogen for 5 min and a heat-shock was applied by transferring them into a 37 ºC water bath for another 5 min. This step allowed the plasmid to permeate through the bacteria cell wall. The cells were transferred to ice for 5 min once again, to avoid the plasmid getting out of the cells.

One milliliter of rich YEB medium was added, and the cells were left to recover for 4 h at 28 ºC with gentle shaking (~160 rpm), allowing the bacteria to recover and express the antibiotic marker gene encoded by the plasmid, *Npt II* (**Figure 2.1**). Meanwhile, YEB medium supplemented

with 50 mg/L rifampicin and 50 mg/L kanamycin was plated on Petri dishes. The cells were pelleted by a full-speed centrifugation for 2 min and resuspended on 100 µL of liquid YEB medium. The suspension was spread onto Petri dishes and the dishes were left to incubate inverted at 28 ºC for 2 days. The resulting colonies are depicted in **Figure 6.6** of the Supplementary Data. It was possible to observe that the amount of *A. tumefaciens* colonies attained is 100 times lower than those obtained for *E. coli*. The efficiency attained by this protocol was of about 20 colony forming units per µg of plasmid.

Validation of the *A. tumefaciens* Transformation by PCR

To verify that a plasmid contains a recombinant sequence, two PCR approaches may be used: 1) to design primers to amplify a known region of the insert or 2) to use universal primers flanking the Multiple Cloning Site (MCS) of the plasmid, such as the M13 primers, amplifying the whole MCS region. In this work, the first approach was used. PCR does not require the plasmid extraction, and may be used directly in colonies resulting from transformation.

In order to perform a 20 µL PCR reaction, 11.24 µL of Milli-Q water, 4 µL of 5X Green GoTaq Flexi Buffer (1X), 2 µL of 25 mM MgCl2 (2.5 mM), 1 µL of 10 mM dNTPs (0.5 mM), 0.3 µL of a 10 µM primer solution (0.15 µM), 0.16 µL of GoTaq G2 Flexi DNA Polymerase at 5 U/L (0.8U) and 1 µL of template DNA (50 ng) were added in this order to 0.2 mL tubes. The tubes were put on a BioRad Thermocycler with the following PCR cycling program: an initial denaturation at 95 ºC for 2 min, followed by 30 cycles of denaturation at 95 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 68 ºC for 1 min; a final extension of 72 ºC for 5 min and a cool-down step at 4 ºC. As for the samples added to each PCR mix, one was from the original pFHi.GNK2 tube, serving as a positive control for the PCR reaction, two of them resulted from the miniprep performed on transformed *E. coli* DH5α cells, and four corresponded to transformed *A. tumefaciens* EHA105 colonies. To prepare the four samples for colony PCR, a sterile toothpick was used to pick a colony. With it, a streak was done on a YEB agar medium plate supplemented with 50 mg/L rifampicin and 50 mg/L kanamycin before scraping the inside a 0.2 mL tube. The pick was thrown inside a 15 mL falcon tube containing 2 mL of YEB medium supplemented with 50 mg/L rifampicin and 50 mg/L kanamycin. The YEB plate was left to incubate for 2 days at 29 ºC and the falcon tubes were left for 2 days at 25 ºC with gentle agitation. Twenty microliters of Milli-Q water were added to the 0.2 mL tubes to dilute the bacterium inoculum before the colony PCR, and 2 µL were added to each colony PCR mix.

An 1% agarose gel electrophoresis was performed as previously described, to visualize the PCR products. One microliter of a ladder solution made of 1 µL of the DNA ladder GeneRuler 100 bp, 1 µL of 6X DNA Loading Dye (1X) and 4 µL of ddH_2O was added to the first well, and 10 µL of each PCR mix were added to each other well.

Bacteria glycerol stock

As it was possible to isolate a single colony harboring the plasmid of interest, it was necessary to store the bacteria. For this purpose, a protocol from Hanahan (1991) was followed. A sterile pick or pipette tip was used to scrape the validated colony and to inoculate 2 µL of selective liquid medium. The culture was left to grow at optimal conditions (an overnight incubation at 37 ºC for *E. coli*, or 2 days at 28 ºC for *A. tumefaciens*) before adding 200 µL of a sterile 80% glycerol solution per 500 µL of culture. The mix was divided into 100 µL aliquots. The glycerol protects the cells from freezing and bursting, allowing them to be stored at -80 ºC.

Media Preparation

The LB medium for *E. coli* was prepared by dissolving a LB broth from Duchefa in water according to the instructions on the package (15.5 grams of powder per Liter). The composition of the medium was 0.5 g/L of sodium chloride, 10 g/L of tryptone and 5 g/L of yeast extract. The pH of the media was around 7.0. Fifteen grams per Liter of microagar was used for solid media flasks.

The YEB medium for *A. tumefaciens* growth was done as described in Wise *et al*., 2006. Five grams per Liter of tryptone, 1 g/L of yeast extract, 5 g/L of nutrient broth, 5 g/L of sucrose and 0.49 g/L of $MgSO_4.7H_2O$ were mixed in with water and the pH corrected to 7.2 using drops of NaOH at a concentration of 0.5 M. Fifteen grams per Liter of agar was deposited on Schott flasks before pouring in the mixture.

All media with sucrose were sterilized by autoclaving for 20 min at 110 ºC before use, while the remaining media were autoclaved for 30 min at 121 ºC, to ensure sterility. Whenever solid media needed supplementation, a flask would be heated in the microwave until the agarose completely liquified, and the flask was left to cool down until it could be safely grabbed (~50 ºC). Only then would the sterile supplements be added inside a flow chamber, and the medium plated.

2.2. Transformation of *Populus tremula x alba*

Plant Material

In this work, the hybrid grey poplar (*Populus tremula x Populus alba*, also called *Populus canescens*) plant line INRA 717-1B4 was used. This is a clone of a female poplar tree from the Institut National de la Recherche Agronomique, France (Lemoine, 1973).

Media Preparation

To prepare the *in vitro* propagation media, ½ Murashige and Skoog broth (MS), 20 g/L sucrose and deionized water were mixed inside an Erlenmeyer. The pH was read using a METTLER TOLEDO pH meter and adjusted using NaOH 1 M until it reached 5.8. Eight grams of agar per liter of solution was added to each Schott flask, before pouring in the content of the Erlenmeyer. The flasks were autoclaved at 110 ºC for 20 min, and the sterile antibiotics and phytohormones were added inside a flow chamber upon use, before the medium solidifies. The supplements used on the media were indole acetic acid (IAA), indole butyric acid (IBA), dithiothreitol (DTT), thidiazuron (TDZ), Naphthaleneacetic acid (NAA), kanamycin (Kan), carbenicillin (Carb) and acetosyringone. Their respective amounts depended on the purpose of the medium, and are depicted in **Table 2.1**.

Table 2.1 – ½ MS-based media composition.

Composition (mg/L)	Micropropagation	Co-Culture	Shoot Regeneration	Shoot Isolation	Propagation w/ Antibiotics
IAA	0.5	-	-	-	0.5
IBA	0.5	-	-	-	0.5
DTT	5	5	5	5	5
TDZ	-	0.1	0.1	0.1	-
NAA	-	0.05	0.05	0.05	-
Kan	-	20	20	20	25
Carb	-	-	500	250	250
Acetosyringone	-	196.2	-	-	-

Poplar Subculture

The Micropropagation medium (**Table 2.1**) was poured into glass flasks and left to gelify. Shoot segments of grey poplar containing two to three nodes (devoid of leaves) were micropropagated in asepsis. The flasks were put inside a 24 ºC plant chamber, with a 12 h photoperiod and 85 µmol m^{-2} s^{-1} of light intensity provided by three fluorescent frosted lights of 230 V, 22 W and 4000 K, at a distance of 30 cm from the flasks. The plants were subcultured once every 4 to 10 weeks, according to their height and developmental stage.

Poplar Acclimation and Genotype Maintenance

In order to acclimate *in vitro* poplar plants to regular humidity values, some healthy-looking 4-8 weeks-old plants were selected. Each plant was uprooted and had its roots washed in clean water before the transfer into Jiffy pots®, which were put on a clean plastic box. It is important to keep the media out of the roots as much as possible because it favors fungi growth, which may kill the plant. The box was filled with a 1 cm layer of clean tap water to ensure a well-watered and wet environment. Then, clear plastic film was used to cover the box, this procedure simultaneously allowed for light to penetrate and prevented humidity to get out. The box was left at 23 ºC, exposed to 120 µmol m^{-2} s^{-1} of light intensity inside a FITOCLIMA 1200 growth chamber from Aralab, with a 12/12 photoperiod. After a week inside the growth chamber, holes were made on the plastic film, to promote plant acclimation to *ex-vitro* conditions. On the third week, the plastic film was further perforated and by the fourth week the film was removed altogether. By this time the plants were ready to be potted onto a soil mixture of 50% “terra de Montemor” commercial soil, 25% vermiculite and 25% peat.

Poplar Transformation

One colony of *A. tumefaciens* strain EHA105 containing the plasmid pFHi.GNK2 was inoculated in 2 mL of liquid YEB medium supplemented with 50 mg/L of rifampicin and 50 mg/L of kanamycin. The culture was left to grow overnight at 27 ºC. The suspension was then centrifuged at 3500 rpm for 10 min in a Sigma 4K15 centrifuge and the cells were resuspended in liquid 0.5X Murashige and Skoog medium with 20 g/L of sucrose and 196.2 mg/L of acetosyringone to an OD_{600nm} of 0.3, being left to activate their virulence mechanisms at 22 ºC for 2 h.

Inside a laminar flow chamber, a scalpel blade was dipped in the suspension at an OD_{600nm} of 0.3, and the excess of bacteria was removed by tapping a sterile glass dish. Leaves from young, well-developed 4 to 6 weeks-old plants were cut perpendicularly to the central vein. The explants were directly placed with the abaxial side down onto Co-culture medium (**Table 2.1**). Each Petri dish contained six cut leaves.

For this experiment, two controls with non-inoculated leaves were made. These leaves were similarly cut with a scalpel, but this time the tool was free from bacterial inoculum. For the positive control, the non-inoculated leaves were always placed on media without any antibiotics. This control allowed to perceive if the hormones from the media were enough to generate regenerant shoots. For the negative control, the non-inoculated leaves were placed on similar media to the inoculated leaves. This control allowed to assess whether the kanamycin provided enough pressure for plant regeneration to be inhibited when the T-DNA was absent from plant cells.

The Co-culture period was accomplished by leaving the dishes in the dark at 24 ºC for 72 h. The explants were then transferred onto Shoot Regeneration medium (**Table 2.1**) and placed in light conditions. A subculture of the explants was performed every week for eight weeks in order to maintain the kanamycin antibiotic pressure. This was an indirect transformation process as shoot regeneration occurred from calli (**Figure 6.7CD**). One-to-two-millimeter shoots emerging from the calli were excised under a binocular microscope, and transferred onto Shoot Isolation, weekly-renovated medium (**Table 2.1**) to directly expose the plantlets to the pressure of the antibiotic. When the plantlets reached a height of one centimeter, they were placed onto regular poplar Micropropagation medium supplemented with 25 mg/L of kanamycin and 250 mg/L of carbenicillin (**Table 2.1**). This medium allowed not only rooting but also a better selection of potential transformants.

The regenerants were named after a coding system of 3 digits: the first digit corresponded to the number of the transformation procedure (1-3), the second digit to the plate from which it was isolated from (1-6) and the third digit to the number of that plant inside that plate (it varied among dishes, but the maximum number of plants excised from a single dish was 8). For example, the 354 plant was the fourth plant recovered from plate number 5 of the third transformation.

RNA Extraction

RNA was obtained mainly from leaf tissue resulting from the subculture of sixteen poplar transformants, and also from non-transformed poplar (original *Populus alba x tremula*), using the Quick-RNA™ MiniPrep Kit from Zymo Research (R1054). The leaves from these poplar plants were mechanically homogenized in liquid nitrogen with the aid of a mortar and pestle. Three hundred microliters of RNA Lysis Buffer were added per 30 mg of the tissue powder. A centrifugation at full speed for 30 s was performed to remove bigger particles. The clear supernatant was transferred to a Spin-Away™ Filter in a collection tube. Another centrifugation was performed and 1 vol of ethanol (95-100%) was added to the flow-through. The mix was transferred to a Zymo-Spin™ IIICG Column in a collection tube and another centrifugation was performed. The flow-through was discarded and 400 µL of RNA Wash Buffer were added to the column. Another centrifugation was performed. Meanwhile, a RNase-free tube with 75 µL of DNA Digestion Buffer and 5 µL of DNase I was mixed by inversion. The content was added directly to the matrix and the column was left to incubate at RT for 15 min. Four hundred microliters of RNA Prep Buffer were added to the column before centrifuging, discarding the flow-through. Seven hundred microliters of RNA Wash Buffer were added to the column before centrifuging once again, discarding the flow-through. Four hundred microliters of RNA Wash Buffer were added and a 2 min centrifugation was performed to ensure the removal of the wash buffer. The column was carefully transferred into an RNase-free tube before adding 50 µL DNase/RNase-free water. A final centrifugation step was performed to elute the RNA, which was stored at -80 ºC.

RNA integrity and gDNA contamination assay

Before proceeding with cDNA synthesis, it was important to check both the integrity and the purity of the extracted RNA to ensure that it was not degraded, and to assess if the RNA did not contain traces of DNA. To check the integrity of the RNA, a 2% agarose gel was prepared and 200 ng of RNA from each regenerant were run for 30 min at 100 V.

In order to ensure that the RNA did not contain traces of genomic DNA (gDNA), a GoTaq PCR was performed on the RNA, as previously described, using primers designed to amplify a 431 bp segment of the *Cast_GNK2-like* cDNA (check Supplementary Data).

cDNA synthesis from Regenerant Poplar RNA

The first step of a two-step Reverse Transcription Polymerization Chain Reaction (RT-PCR) is to generate a cDNA pool from the extracted RNA. For this purpose, the ImProm-II Reverse Transcription System protocol from Promega was followed. For each 20 µL reaction, a 5 µL reaction containing 800 ng of total RNA, 0.5 µg of oligo dT primer and nuclease-free water was prepared and left to incubate at 70 ºC for 5 min. Then, it was quick-chilled to 4 ºC for 5 min and maintained in ice. A negative control for this experiment, without any template RNA, was done to assess if there was any contamination within the reagents.

For each 20 µL reaction, nuclease-free water, 4 µL of ImProm-II™ 5X Reaction Buffer (1X), 2.4 µL of 25 mM MgCl2 (3 mM), 1 µL of 10 mM dNTPs (0.5 mM) and 20 U of Recombinant RNasin® Ribonuclease Inhibitor (1 U/µL) were added and vortexed. One microliter of Improm-II™ Reverse Transcriptase was then added to a final volume of 15 µL. This reverse transcription mix was added to the previous 5 µL with the template and the primer, to make up a final volume of 20 µL.

The reaction tubes were placed in a thermocycler with the following programming: annealing at 25 ºC for 5 min, extension of the first strand at 42 ºC for 60 min, heat-inactivation of the ImProm-II™ Reverse Transcriptase by incubating at 70 ºC for 15 min. The cDNA was ready to be analyzed by PCR or stored frozen.

PCR Amplification of *Cast_GNK2* in Regenerant Poplar cDNA

The cDNA could be amplified by adding the products of the heat-inactivated reverse transcription reaction directly to the PCR mix and proceeding with thermal cycling. A standard GoTaq PCR mix was prepared with the same primers used on the gDNA contamination assay. Two experiment controls were done: one with the pFHi.GNK2 as template and the other was the product of the cDNA synthesis negative control, without any RNA as template. One microliter of cDNA at 40 ng/µL was added to each tube and the reactions were amplified as follows: 2 min at 95 ºC; 30 cycles of 30 s at 95 ºC, 30 s at 55 ºC, 1 min at 68 ºC; 5 min at 72 ºC and an infinite hold at 4 ºC. The PCR products were visualized after in a 1.5% agarose gel electrophoresis.

Real-Time PCR

A mix containing 10 µL 2X PerfeCTa SYBR Green FastMix, 0.6 µL of each 10 mM primer (3rd set of primers in Supplementary Data), and sterile Milli-Q H_2O per reaction was prepared, and 18 µL were poured into each well. Two microliters of template cDNA at 2.5 ng/µL (5 ng) were added to each well, and the 96-well plate was sealed with appropriate sealing film.

This experiment was performed with two technical replicates per sample, along with some controls: **a)** a negative control with Milli-Q H_2O was done to check for contaminations of the mix; **b)** a control with non-transformed poplar was done to check for contamination between the transformed and non-transformed samples; **c)** RNA template was used to assess if the extraction was successful in isolating the RNA from the gDNA; **d)** a positive control with the pFHi.GNK2 plasmid as template was done to ensure that primers amplified the *GNK2-like* gene (by observing if the melting curves coincided with the transformants'); **e)** and some wells received double the amount of template (10 ng) to perceive how the melting curves would be affected by different concentrations of cDNA. No poplar endogenous genes were used in the qPCR because our main objective was to assess the relative expression of the recovered transgenic lines, for us to choose a good subgroup to represent the differential expression throughout the genotypes.

When the plate was ready, it was inserted into a LightCycler® 480 Instrument II from Roche Life Science, where the following program was loaded: a pre-incubation step at 95 ºC for 5 min (initial denaturation) followed by 45 amplification cycles comprising of 10 s at 95 ºC (denaturation), 10 s at 60 ºC (annealing) and 10 s at 72 ºC (amplification); a gradual augment of temperature from 65 ºC to 97 ºC (0.11 ºC/s) to get the primer melting curves, and a final cooling step of 40 ºC for 30 s. When the program was done, the Cps (2nd Derivative Max), and melting curves were extracted from the software and analyzed.

2.3. *In vitro* infection of grey poplar with *P. cinnamomi* (INIAV pH107), *P. cinnamomi* (DSM 62654) and *P. cactorum* (DSM 62637)

The *Phytophthora* strains used for grey poplar infection were the DSM 62637 *P. cactorum*, isolated from *Malus sylvestris* in Germany before 1990 (H. Kröber, also known as IMB 9044); the DSM 62654 *P. cinnamomi*, isolated from *Erica gracilis* in Germany before 1990 (H. Kröber, also known as IMB 11200); and the pH107 *P. cinnamomi* isolated by the Universidade de Trás-os-Montes e Alto Douro in 1990 (UTAD; Duarte, 2015). The oomycetes strains were first propagated on Potato Dextrose Agar (PDA) for 7 days, to verify viability. Afterwards, media plugs containing mycelia were collected into 1.5 mL microcentrifuge tubes containing sterile deionized water and were stored at 4 ºC until needed. Additional 0.5 mm-wide plugs could also be cut directly from the dishes for subculture, or to inoculate poplar *in vitro*.

Preliminary *in vitro* *Phytophthora* Inoculation Assay

Twelve *Populus tremula x alba* plants from a previous transformation with the *GUS* gene growing in Micropropagation medium (**Table 2.1**) were used in a preliminary assay in order to perceive the *Phytophthora* disease severity on poplar growing *in vitro*. The plants used in this preliminary assay were transformed with the *GUS* gene instead of being *wild-type* because this genotype had a higher number of plants on the lab.

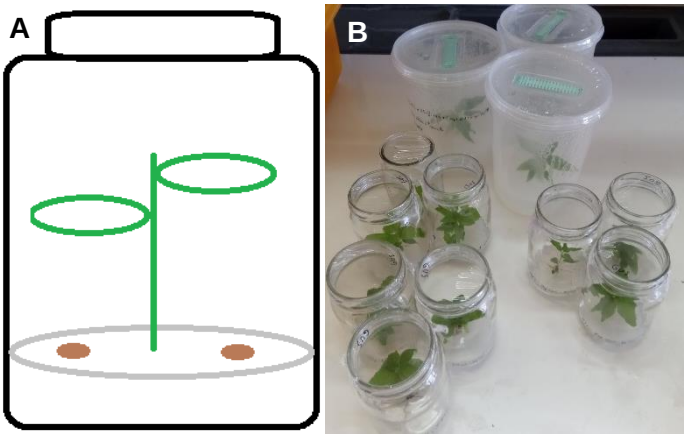

Figure 2.3 - *Populus alba x tremula* infection with *Phytophthora*. (**A**) Scheme representing how *Phytophthora* was inoculated with the *in vitro* grey poplar growing in Micropropagation medium. The brown discs represent the 0.5mm-wide PDA media plugs with *Phytophthora* hyphae grown for 14 days. (**B**) Plants inoculated on the experiment.

Each one of the three *Phytophthora* strains infected three plants. The inoculum consisted of two 0.5 cm-wide plugs from freshly cultivated *Phytophthora* in PDA (Merck), which were placed on opposite sides of the plant as depicted in **Figure 2.3A**. Three more developed plants placed on larger sterile plastic containers were used on the experiment in order to understand if plant development was a deciding factor on the appearance of *Phytophthora* infection symptoms. An additional three plants were used as a control, each being inoculated with a pair of non-inoculated PDA plugs. The *Phytophthora* growth and the plant symptomatology, such as root rot, stem rot, dieback and plant death, were followed throughout 17 days, and photos were taken periodically (Supplementary Data, **Figures 6.9** to **6.11**).

In order to perceive if the *Phytophthora* would grow any different in poplar Micropropagation medium, an assay to record *Phytophthora* growth in its ideal medium, PDA, was done. For this, a media plug with mycelia was cut using a scalpel, and placed onto regular PDA medium. The plates were wrapped in foil and placed onto the poplar plant chamber, being left to grow in the dark at 24 ºC. *Phytophthora* growth was marked on the petri dish every other day using a permanent marker, and its area was calculated using the software Image-Pro Plus 6.

Phytophthora growth in carbenicillin

As the transformants were propagated onto standard poplar Micropropagation medium (**Table 2.1**), some *A. tumefaciens* growth was spotted. To ensure *A. tumefaciens* elimination, 250 ng/µL of carbenicillin were incorporated into the Micropropagation medium of the transformed plants. To check whether the final *in vitro* assay could be done in these conditions, it was important to assess if the *Phytophthora* would grow any different in the presence of carbenicillin. As such, 250 ng/µL carbenicillin was added to microwaved PDA when it had cooled. About 15 µL of the supplemented PDA were spread into each plate. The plates were used to grow the *Phytophthora* strains by cutting a media plug from a freshly-cultivated *Phytophthora* plate. *P. cinnamomi* DSM 62654 and *P. cinnamomi* pH107 grew in the dark at 29 ºC, while *P. cactorum* DSM 62637 grew in the dark at 24 ºC, inside the plant chamber. This temperature discrepancy was due to the fact that *P. cactorum* growth was arrested by the higher temperature.

Final *in vitro Phytophthora* Inoculation Assay

The final assay was performed in a similar way as described on the preliminary assay. However, this time the inoculum plugs were placed at a distance of 2 cm from the stem of the plant, in order to have a higher likelihood of the *Phytophthora* reaching the plants simultaneously. A total of 5 GNK2-Poplar genotypes were used: two with the highest expression of the gene (134 and 212), two with an intermediate expression (341 and 354), and one with the lowest expression of *Cast_GNK2-like* (125). Additionally, a wild-type (Wt) poplar genotype was also included as a control. Three biological replicates were used per genotype. The plants were inoculated with *P. cinnamomi* pH107 and *P. cactorum* DSM 62637, as *P. cinnamomi* DSM 62654 did not display much symptomatology on poplar in the preliminary assay. *Phytophthora* growth and plant symptomatology were followed throughout 21 days (Supplementary Data, **Figures 6.12** to **6.23**).

Leaf *Phytophthora* inoculation in PDA

An assay consisting of a PDA plug inoculum of oomycetes surrounded by six leaves was performed as illustrated in **Figure 2.4**. The leaves were cut and placed onto the PDA at a distance of 1.5 cm of the center of the plate. Six genotypes were used in total: Wt, GNK2-Poplar 212, 134, 341, 354 and 125. Each plate carried six leaves, three from each genotype. A PDA plug of freshly cultivated *P. cinnamomi* pH107 or *P. cactorum* DSM 62637 placed on the center of the plate. *Phytophthora* growth and leaf lesion were followed throughout 14 days for *P. cinnamomi* and 21 days for *P. cactorum* (**Figures 6.24** and **6.25**).

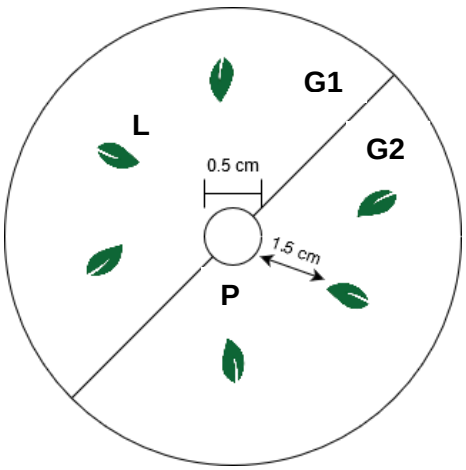

Figure 2.4 - Scheme representing how *Phytophthora* inoculated poplar leaves in a PDA plate. Caption: **L** – Poplar leaf; **P** – *Phytophthora* 0.5cm-wide plug; **G1** and **G2** – Two distinct poplar genotypes.

Media Preparation

The PDA media was prepared by dissolving a PDA mix from Merck in water according to the manufacturer's instructions (39 g/L, pH 5.2). The mix was autoclaved at 110 ºC for 30 min before pouring about 15 mL in each Petri dish. The Micropropagation medium used for poplar is described in **Table 2.1**.

2.4. Vector Construction with a Root-specific promoter

Motive Search on the ET304 Promoter

The main objective of this cloning procedure was to obtain a plant vector with the expression of the *Cast_GNK2-like* gene being controlled by a root-specific promoter. This is an important biological goal in this pathogen-host interaction because *Phytophthora* are mainly soil-borne pathogens, and their infection is primarily done through the host species' roots. As such, it was important to search for promoter analysis work on poplar to find good promoter candidates for this study.

The best candidate was the ET304 promoter found in the Master of Science thesis work of Qian Wu (2004), who identified 12 scattered root motifs within it. The gene under the regulation of this promoter was characterized as a root-specific low-abundance transcript whose expression increases in later stages of adventitious root development. However, the *Populus alba x tremula* genome had yet to be available in NCBI, and as such the promoter ET304 was searched in the 3.0 version of the *Populus trichocarpa* genome.

In order to perceive what length of the ET304 promoter should be included, three tools which identified promoter motives were used: the PLACE (Solovyev *et al.*, 2010), PlantCARE (Lescot *et al.*, 2002) and PlantProm (Ilham *et al*., 2003; Amina *et al.*, 2009). The fragment resulting from this analysis is included on **Figure 6.2** of the Supplementary Data.

Poplar DNA Extraction using Extraction Buffer CTAB 2x

The tissue samples, mainly poplar leaves, were first homogenized in liquid nitrogen using a mortar and nitrogen-frozen tools, with the dust being recovered with the aid of a pipette tip to microcentrifuge tubes. The tubes were stored at -80 ºC until needed. The tubes were frozen in liquid nitrogen before adding 1000 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 10 mM Tris HCl pH 8.0, 1% polyvinylpyrollidone P40, 0.2% β-mercaptoethanol) previously heated to 65 ºC in a water bath. The samples were quickly vortexed and incubated at 65 ºC for 45 min, being vortexed every 10 min. This heat shock step disrupts the cells along with the nuclear membrane, exposing the DNA. The tubes were removed from the bath and left to cool down 1 to 2 min at RT. Five hundred microliters of CIA (chloroform 24:1 isoamyl alcohol) were added to each tube inside the *hotte*, and the mix was homogenized by vortexing and inverting the tubes for 10 to 15 min. A 10 min centrifugation step at full speed was performed and the supernatant was recovered. The last two steps were repeated twice in order to remove impurities. Finally, to precipitate the DNA, which is not soluble in alcohol, ⅔ vol (500 µL) of isopropanol cooled to -20 ºC were added, followed by a centrifugation step at full speed for 30 min at 4 ºC. The supernatant was carefully discarded first into glassware and then into absorbent paper. Two hundred and fifty microliters of 70% ethanol cooled to -20 ºC were added and another centrifugation was performed. The supernatant was completely discarded by inverting the tube into absorbent paper and 45 µL of Milli-Q water was added to the pellet before storing the DNA at -20 ºC.

Gateway Cloning

Gateway is a cloning system based on site-specific recombination which enables the assembly of multiple DNA fragments in a predefined order, orientation and frame register (Karimi *et al.*, 2007). It is a great tool to construct recombinant genes for functional analysis, and does not require any knowledge about restriction enzyme sites to perform the cloning procedure. The recognized DNA sequences are long enough that they will not occur by chance, but short enough not to interfere with the cloned elements (Karimi *et al*., 2007). An *att*B-flanked PCR product can be inserted into an *att*P-containing pDONR plasmid by a BP clonase, generating a pENTR plasmid (entry vector) with the desired sequence flanked by *att*L sites (**Figure 2.5A**). To assemble all the pENTR sequences into a pDEST (destination vector), a LR clonase recognizes the pENTR's *att*L sequences and the pDEST's *att*R sites, recombining the two and generating the final vector (**Figure 2.5B**). This was the chosen strategy to assemble the ET304 promoter and the *Cast_GNK2-like* gene into a plant transformation vector.

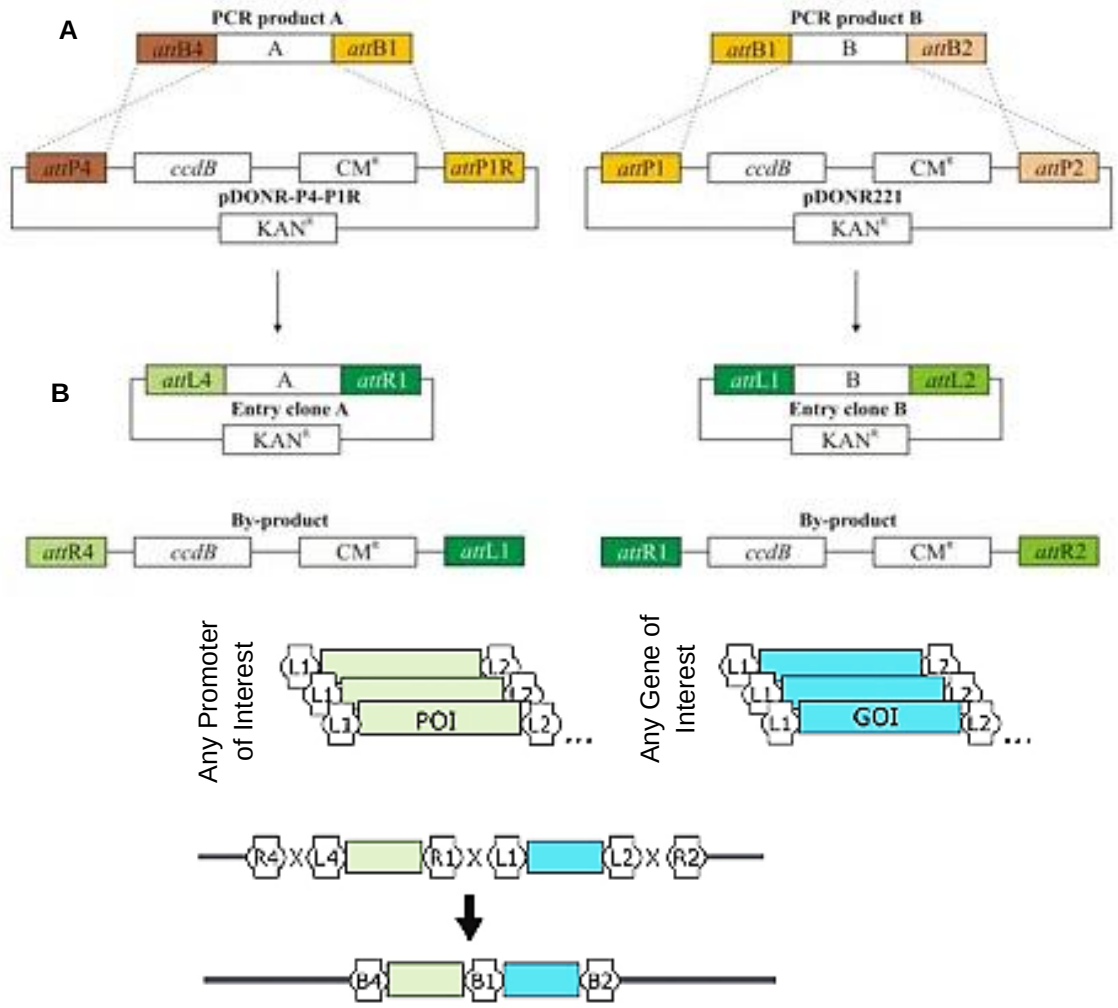

Figure 2.5 - Gateway Cloning of a promoter A and a gene fragment B. (**A**) BP Recombination Reaction. The PCR products "A" and "B" are flanked by *att*B, on the other hand, the pDONR vectors contain the corresponding *att*P vectors. The BP clonase reaction allows for the recombination of these sites, inserting the PCR product on the pDONR vector, generating Entry clones pENTR. (**B**) LR Recombination Reaction. The generated pENTR clones possess *att*L and *att*R sequences. The destination vector possesses corresponding *att*R sequences to those of the pENTRs. The LR clonase will recognize these sites in a similar faction to the BP clonase, and allow them to recombine, originating a final vector. Diagrams present in Magnani *et al.*, 2006 and Karimi *et al.*, 2007.

The Gateway strategy employed consisted in amplifying the ET304 promoter flanked by *att*B4 and *att*B1r sequences from grey poplar DNA and to have it inserted into pDONR-P4-P1r (**Figure 2.6A**). On the other hand, the *Cast_GNK2-like* gene flanked by *att*B1 and *att*B2 sequences was amplified from the provided pFHi.GNK2 plasmid (**Figure 2.1**) and cloned into pDONR221 (**Figure 2.6B**). Once the pENTR-P4-P1r.et304 and the pENTR221.GNK2 were assembled, a LP clonase reaction was to be performed to transfer the two fragments into pK7m24G2, 3, a plasmid which accepts *att*L4 and *att*L2 fragments (**Figure 2.7**).

CloneAmp™ HiFi PCR

In order to get both PCR fragments, one corresponding to the ET304 promoter from *Populus alba x tremula*, and another to the *C. crenata GNK2-like* cDNA for cloning, a PCR CloneAmp™ HiFi procedure was performed according to the manufacturer's instructions. This kit was chosen as a high-fidelity DNA polymerase is required to perform an accurate fragment amplification. The concentrations used on the 25 µL reaction were the following: 1x CloneAmp HiFi PCR Premix, 0.2 µM of primer F and R and less than 100 ng of template DNA. The remaining volume was made up of sterile Milli-Q water. The 30 amplification cycles were done inside a BioRad thermocycler in the following manner: 10 s at 98 ºC, 10 s at 55 ºC, 5 s at 72 ºC, along with an Initial Denaturation step of 2 min at 95 ºC and a Final Extension of 5 min at 72 ºC. The sample was cooled down to 4 ºC when the program finished. A 1% agarose gel was prepared as previously described. It was left to run at 70 V for 1 h.

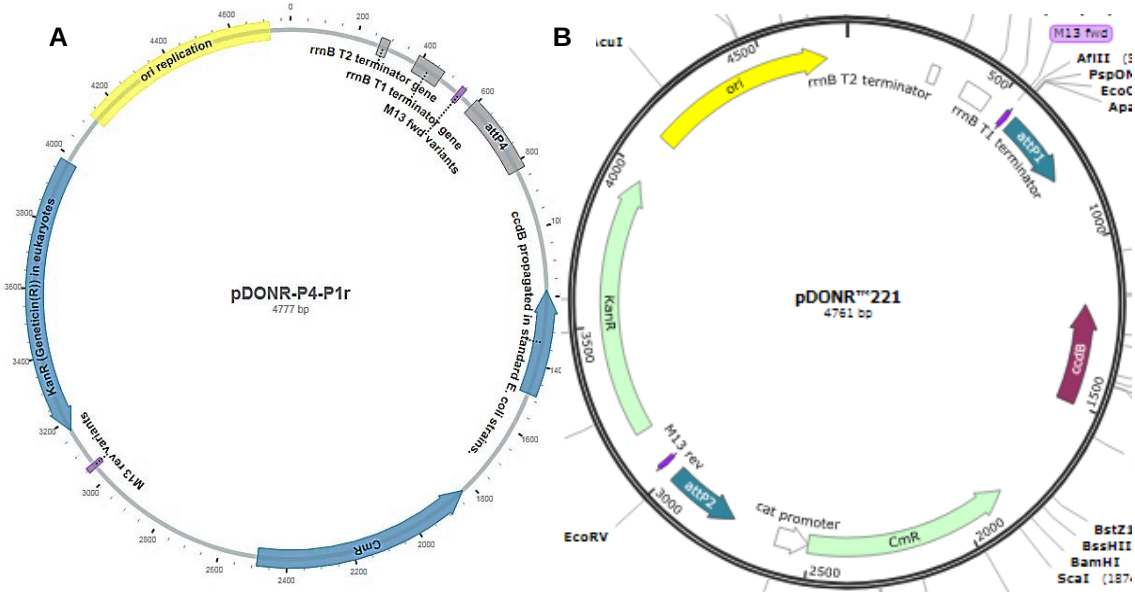

Figure 2.6 - pDONR vectors. (**A**) pDONR-P4-P1r Gateway vector. It harbors an origin of replication in *E. coli*, a kanamycin selection marker, and a *ccdB* cassette with a chloramphenicol selection marker which will be substituted once the BP recombination is performed. This vector allows for a recombination between an *att*B4-*att*B1r PCR fragment and the *att*P4 and *att*P1r plasmid sites. (**B**) pDONR™221 Gateway vector. This vector contains an origin of replication in *E. coli*, a kanamycin selection marker, and a *ccdB* cassette containing a chloramphenicol selection marker which will be substituted once the BP recombination is performed. This vector allows for a recombination between an *att*B1-*att*B2 PCR fragment and the *att*P1 and *att*P2 plasmid sites. More information is available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/gateway_pdonr_vectors.pdf.

DNA Recovery from agarose gel

In order to recover the DNA fragments from the agarose gel, the NZY Gelpure DNA purification from Agarose Gels Kit (MB01101) was used. First, a razor blade was used to cut the agarose under a UV light with a 240 nm intensity, and the slices were transferred to microcentrifuge tubes. The gel was not visualized inside Biorad Gel Doc System w/ Universal Hood II to avoid unnecessary DNA degradation. The amount of cut agarose was weighed and 300 µL of Binding Buffer were added per 100 mg of agarose. The tubes were incubated at 60 ºC for 10 min, being mixed from time to time until the agarose completely dissolved. The mix was loaded to a spin column placed on top of a collection tube and a centrifugation step at 17992 x *g* for 1 min was performed. With the flow through discarded, 500 µL of Wash Buffer were added to the column and a similar centrifugation was performed. Once again, 600 µL of Wash Buffer were added and a third centrifugation was performed. The column was placed onto a clean microcentrifuge tube, and 50 µL of Elution Buffer were added to the center of the column, although this volume should be further reduced up to 20 µL to increase the DNA concentration. After 1 min of incubation at RT, a final centrifugation step was performed to elute the DNA, which was stored at -20 ºC.

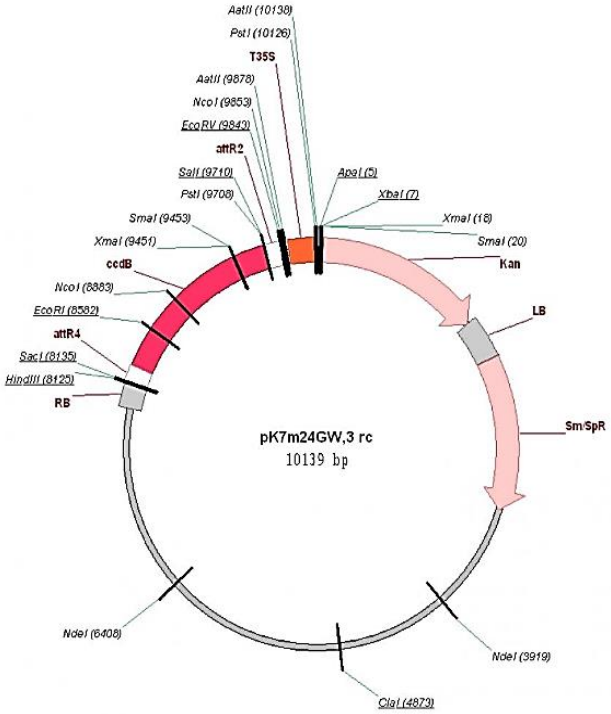

Figure 2.7 - pK7m24GW, 3 Gateway vector. It recombines with *att*L4 and *att*L1 fragments, losing its lethal *ccdB* cassette in the process. Its plant selection marker is kanamycin, while its bacteria selection marker is spectomycin.

PCR Product Concentration and ET304 Sequencing

As the ET304 promoter sequence was unknown in the INRA 717-1B4 grey poplar strain, a sequencing of the recovered DNA was in order. The DNA Clean & Concentrator™-5 Kit from Zymo Research (D4003) was used to improve the quality and concentration of the recovered DNA. To achieve this, five volumes of DNA Binding Buffer were added per volume of DNA sample (100 µL of Binding Buffer were added to 20 µL of DNA) before the mix was transferred to a Zymo-Spin™ Column in a Collection Tube. In order to remove the impurities, a 1 min centrifugation at 14394 x *g* was performed and the flow-through was discarded. Two hundred microliters of DNA Wash Buffer were added to the column and a similar centrifugation was done. This step was repeated two times. The column was finally transferred to a new microcentrifuge tube and 20 µL of Milli-Q water were added directly onto the column matrix, which was left to incubate for 5 min at RT. The DNA was eluted through a similar centrifugation procedure. The amount of DNA was measured through Nanodrop and the concentration was adjusted to 20 ng/µL to send the PCR product alongside the primers for sequencing at STABvida.

Propagating pDONRs using the DB3.1 *E. coli* strain

Gateway vectors are very useful tools that ease the cloning process, yet these vectors contain a lethal gene. The *ccdb* gene produces a lethal product to regular *E. coli* strains, resulting in cell death. However, it is possible to propagate these vectors using strains whose genotype contains the gyrA462 gene, such as DB3.1 *E. coli* (Reece-Hoves *et al.*, 2018).

A protocol described in Reeve-Hoves (2018) was followed for the transformation of *E. coli*. One hundred microliters of competent DB3.1 cells kindly provided by the GPlantS lab at ITQB Nova were thawed on ice. Fifty microliters were transferred to a new pre-chilled 1.5 mL tube and 1 µL of the target DNA at 150 ng/µL, pDONRP4-P1r, was added. The cells were left to incubate for 1 h on ice, gently mixing from time to time, for the plasmid to adhere to the bacteria cell walls. The cells were then exposed to a 45 s heat-shock at 42 ºC, which allowed the plasmid to enter the fragilized cell wall. The cells were put on ice again for 3 min in order to close the cell wall pores and impede the DNA from coming out. In order to aid the cells' recovery and promote the expression of the antibiotic resistance markers of the plasmid, 500 µL of RT LB medium were added and the cells incubated at 37 ºC for 3 h, with 180 rpm of agitation. Then, a centrifugation at 8636 x *g* for 5 min was performed in order to pellet the cells and resuspend them in 100 µL of LB, which were plated on selective LB agar medium (25 µg/mL chloramphenicol and 50 µg/mL kanamycin) using a flamed glass spreader. The Petri dishes were sealed, inverted, and left O/N at 37 ºC. It was possible to attain around 600 colonies with this protocol, corresponding to an efficiency of 4 $x10^3$ colony forming units per µg of DNA.

Generating Gateway Entry Clones: BP reaction, Transformation and Selection of *E. coli*

The *att*B PCR products resulting from the amplification of the ET304 *Populus tremula x alba* promoter and the amplification of the cDNA of the *GNK2-like* gene from *C. crenata* then needed to be incorporated into Gateway pDONR vectors, namely into pDONR221 and pDONR P4-P1r. In order to do this, a 10 µL BP reaction mix containing 50 fmol of the PCR product, 50 fmol of pDONR and 1X Gateway BP Clonase mix in TE Buffer (pH 8.0) was prepared and left to incubate overnight at 25 ºC. One microliter of a 2 µg/µL Proteinase K solution was added and the reaction was left to incubate at 37 ºC for 10 min. The vial could be stored at -20 ºC for up to one week.

The next step of the process was the transformation and selection of competent DH5α *E. coli*. A vial of chemically competent cells per transformation was thawed on ice and 1 µL of the BP recombinase reaction was added by mixing gently without pipetting up and down. One microliter of a 10 pg/µL pUC19 solution was added to an additional vial of DH5α for a competence validation control. The cells were incubated on ice for 30 min before being placed for 35 s on a water bath at 42 ºC. This provided a heat-shock that allowed the plasmid to enter the cells. The vials were placed on ice again for 5 min. Two hundred and fifty microliters of SOC medium was added and the vials incubated for 1 h at 37 ºC, with horizontal shaking (180 rpm) in order to allow the cells to recover and express the antibiotic marker (*KanR*) present on the plasmids. A 1:10 dilution into LB medium (20 µL of culture to 180 µL of LB) of each vial was performed to ensure plates with countable colonies. One hundred microliters from each transformation were spread on a selective LB medium plate using a glass spreader (25 µg/ml kanamycin for the pDONRs and 100 µg/ml ampicillin for the pUC19), and the plates were left to incubate overnight at 37 ºC. With this protocol, it was possible to obtain 51 pUC19 colonies (1.54 $x10^4$ colony forming units per µg of DNA), 3 pENTRP4-P1r.et304 colonies (50 colony forming units per µg of DNA) and 307 pENTR221.GNK2 colonies (5.11 $x10^3$ colony forming units per µg of DNA).

Validation of the presence of ET304 promoter and *Cast_GNK2-like* inserts on *E. coli* using Taq Polymerase Colony PCR

A colony Taq PCR similar to the one used to validate the presence of *Cast_GNK2-like* in *A. tumefaciens* was performed using the same primers employed in the amplification of the inserts. A 1% agarose gel was run for 60 min at 70 V to visualize the PCR products. The *Cast_GNK2-like* gene was confirmed on three colonies but the ET304 fragment was not identified.

NZY Miniprep

In order to get the plasmid from the isolated colonies, 10 mL of an O/N *E. coli* LB culture had its cells pelleted by a 10 min centrifugation at 3500 rpm in a Sigma 4K15 centrifuge. After discarding the supernatant, the pellet was resuspended in 250 µL of Buffer A1 by vortexing. Then, 250 µL Buffer A2 were added to promote cell lysis and the solution was gently mixed by inverting eight times. It was important that this mix would not be left at room temperature for more than 4 min as this could compromise the quality of the extracted plasmid. Three hundred microliters of Buffer A3 were added and the mixture was stirred by inverting the tubes eight times. In order to clarify the lysate, a 10 min centrifugation at 17992 x *g* was performed. The supernatant was loaded in a NZYTech Spin Column on top of a 2 mL collection tube for a DNA binding step. The tubes were centrifuged for 1min at 17992 x *g* and the flow-through was discarded. Five hundred microliters of Buffer AY were added to wash the silica membrane and a centrifugation at 17992 x *g* was performed to remove the buffer. Six hundred microliters of Buffer A4 were added to further wash the membrane and another centrifugation was performed. Finally, to elute the DNA, the column was placed on a clean 1.5 mL microcentrifuge tube and 15 µL of TE were added on top of the matrix. After leaving the matrix to incubate for 1 min, a 1 min 17992 x *g* centrifugation was performed to elute the DNA. Fifteen microliters of TE were added to the matrix once again to further recover the DNA, as it was said that repeating this step could increase yield in 15-20%. The plasmid was quantified using the Nanodrop spectrophotometer and stored at -20 ºC.

Enzymatic Restriction of pDONR P4-P1r and pENTRP4-P1r.ET304

In order to confirm that the plasmid used for cloning was the pDONR P4-P1r (**Figure 2.6A**), a 20 µL enzymatic restriction with PstI and EcoRV was done with nuclease-free water, 2 µL of 10X Buffer (Thermofisher Scientific Buffer O for Fermentas PstI and React®2 for Invitrogen EcoRV), 500 ng of DNA and 1 µL of the Restriction Enzyme. Half a microliter of pDONR P4-P1r and pENTRP4-P1r.ET304, and 1 µL of each Restriction reaction dissolved in 7 µL Milli-Q H_2O was run at 100 V for 30 min in a 1% agarose gel, next to a 1 kb ladder.

Plasmid Concentration for Sanger Sequencing

To sequence the pENTR221.GNK2 and the pENTRP4-P1r.ET304 plasmids, a concentration of 100 ng/µL had to be achieved in a final volume of 15 µL. A Miniprep similar to that of the first chapter was done to purify the plasmids from the colonies, however, the concentration of the DNA was not enough to send it for sequencing. Therefore, the DNA Clean & Concentrator™-5 Kit from Zymo Research (D4003) was used to improve the quality and concentration of the plasmid extraction. To achieve this, two volumes of DNA Binding Buffer were added per volume of DNA sample before the mix was transferred to a Zymo-Spin™ Column in a Collection Tube. A 1 min centrifugation at full speed was performed to remove the impurities and the flow-through was discarded. Two wash steps were done by adding 200 µL of DNA Wash

Buffer to the column and centrifuging for 1 min, discarding the flow-through. Finally, the column was transferred to a new microcentrifuge tube and 10 µL of Milli-Q water were added directly onto the column matrix, which was left to incubate for 2 min at RT. The DNA could then be eluted through a 1 min centrifugation at full speed. The amount of DNA was measured through Nanodrop and the concentration was adjusted to 100 ng/µL to send it for sequencing at STABvida. The resulting sequences were aligned and a consensus sequence was created with the BioEdit Sequence Alignment Editor (**Figure 6.4**, Supplementary Data). The consensus sequence was blasted against the predicted sequence from the *Populus alba x tremula* INRA 717-1B4 genome characterized by Mader *et al*. (2016; **Figures 6.3 and 6.5**, Supplementary Data).

3. Results and Discussion

3.1. Transformation of *A. tumefaciens* with pFHi.GNK2

The first objective of this thesis was to transform poplar with the *Cast_GNK2-like* gene. The first step towards this goal was to clone the cDNA of the *Cast_GNK2-like* gene onto a plasmid. This work was previously done by Patrícia Fernandes, from INIAV, who kindly provided the pFHi.GNK2 plasmid (**Figure 2.1**).

In order to verify if the cloned sequence was the desired one, a search for conserved domains on the *Cast_GNK2-like* cDNA was done using the blastx tool from NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine). The results are depicted in **Figure 3.1**. It was possible to identify two pfam01657 domains, characteristic of CRKs with roles in salt stress responses, and antifungal activity (Marchler-Bauer *et al.*, 2017). This finding was in agreement with what is found for the original GNK2 protein in the literature, as it possesses the DUF26, a stress-antifungal domain characteristic of CRKs (Sawano *et al.*, 2007; Miyakawa *et al.*, 2014; Gao *et al.*, 2015; dos Santos, 2017).

ATGTTGAGCTCAAAATATATTTCTGTCAGCTTTCTATTACTCAGCCTCTCCCTCCATGCAGTCAATTGTGCTGACCC
ATTATACCATTTTTGTTTTAGCCAAGAAAGCTACACTGCCACTAGCCGTTATGGTACAAACTTGAATGGCTTGCTC
AATCTTTTGTCCACCAAAGTTCCTTCAAAAGGGTTTGGTCTCAGCTCGACTGGGCAAGGCCAAGATCGAGCAAAT
GGTTTAGCCCTATGCCGGGGTGATGTCTCAAAAACAAACTGTACGACCTGTGTCATTGATGCAGGCAAAGAGCT
TGGTAATCGTTGTCCTTATAAAAAAGGAGCGATAATTTGGTATGATAACTGTCTTTTGAAGTACTCGAACATTGA
TTTCTTTGGAGAAATCGATAACAAAAACAAGTTCTACATGTGGAACGTCCAAGATGTAGAAAATCCCACTTCATT
CAATCCAAAAGTTAAGGATTTGTTAAGCAGGTTATCTAATAAAGCTTATGCCAATCCAAAATTCTATGCTACCGG
GGACCTAAAGCTTGACTCATCAAGCAAACTATATGGTTTGGCTCAATGCACCAGGGACCTATCAGGTCTTGATTG
TAAGAAGTGTCTTGATACTGCGATTAGTGAACTTCCCAACTGTTGCGATGGAAAACGAGGTGGGCGAGTTGTTG
GTGGCAGTTGTAACGTTAGATATGAACTTTACCCCTTTGTTGATGCCTAG

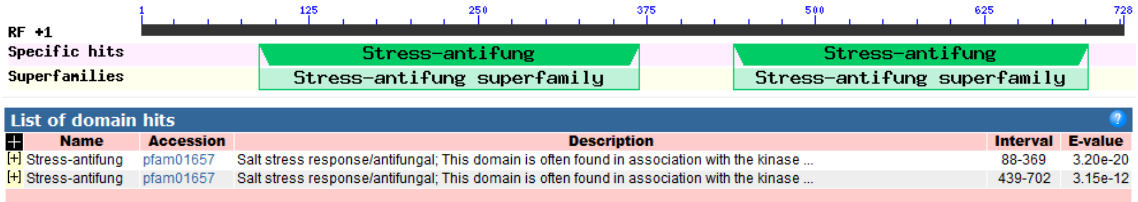

Figure 3.1 – Conserved domains on *Cast_GNK2-like* gene. The red letters represent the two pfam01657 domains found in the *Cast_GNK2-like* cDNA. The pfam01657 domain is often found in association with the kinase domains pfam00069 or pfam07714. It is duplicated in many proteins. It contains six conserved cysteines which are involved in disulphide bridges. The domain has a role in salt stress response and antifungal activity (Marchler-Bauer *et al.*, 2017).

There have been some reports of secreted antifungal agents from poplar calli to protect against a broad spectrum of fungi (Douglas, 1989). In order to search for the existence of a similar gene to *Cast_GNK2-like* in *Populus*, the blastx tool from NCBI was used. The nucleotide sequence of the cDNA of *GNK2-like* gene from *C. crenata* was blasted against the proteome of poplar trees and several candidate proteins with strong correlations were identified. The protein with the top correlation, with an E value of 2^{-98}, 72% identity, 80% positives and a single gap, is from *Populus alba* and does not have any associated functions (TKS08339.1; https://www.ncbi.nlm.nih.gov/protein/1635312966). The second protein identified is a predicted cysteine-rich repeat secretory protein 38-like from *Populus euphratica* (XP_011003220.1, https://www.ncbi.nlm.nih.gov/protein/XP_011003220.1). This finding is in line with the reasoning that ***GNK2-like* sequences are cystein-rich receptor-like kinases** (**CRKs**).

It was necessary to amplify the amount and keep a stock of the plasmid on the lab, and for this purpose, *E. coli* DH5α was transformed. This process consists in competent bacteria taking up foreign DNA. Although it has been reported to occur naturally, it does not occur often in *E. coli* (Chan *et al.*, 2013). By employing the protocol from the **2.1 subchapter** of the methods, it was possible to achieve an efficiency of 6.72 x10^6 colony-forming units per µg of plasmid according to the Bacteria Transformation Efficiency Calculator of science gateway. This efficiency is within the typical yield of optimized versions of the original $CaCl_2$ method, according to Chan *et al*. (2013). Two colonies successfully tested positive for the presence of the gene through PCR (**Figure 3.2**). These colonies were used to make -80 ºC glycerol stocks for the lab.

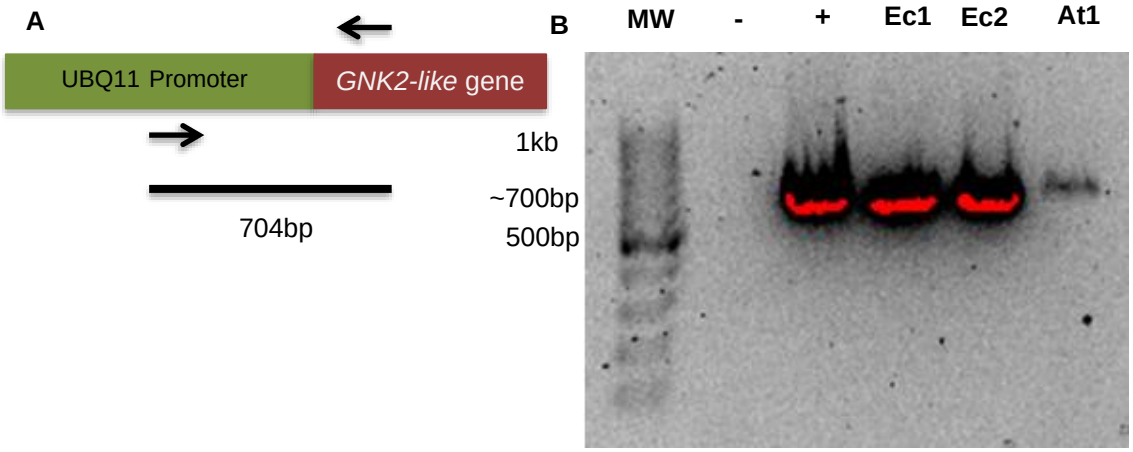

Figure 3.2 - Validation of the presence of the *Cast_GNK2-like* gene through a GoTaq PCR (**A**) Primers used for the amplification of the GNK2 insert; (**B**) 1% agarose Electrophoresis gel ran for 50 min at 80 V. The first lane (**MW**) corresponds to the DNA ladder GeneRuler 100 bp, the second lane (**-**) corresponds to the negative control only with the GoTaq PCR mix and primers, the third lane (**+**) had the purified pFHi.GNK2 plasmid as template, the fourth (**Ec1**) and fifth (**Ec2**) lanes correspond to 2 plasmids purified from 2 different *E. Coli* colonies and the final lane (**At1**) is the product of a colony PCR in a *A. tumefaciens* transformant. *A. tumefaciens* colonies 2 to 4 did not display amplification.

After a successful isolation of the plasmid from *E. coli*, EHA105 *A. tumefaciens* cells were made competent and transformed with the pFHi.GNK2 plasmid through heat-shock. A colony PCR was performed to confirm that one of the four isolated colonies exhibited *Cast_GNK2-like* DNA (**Figure 3.2**). In this process, the efficiency attained was extremely low comparing to the one from *E. coli* (20 colony-forming units per µg of plasmid). The efficiency attained was about 10^4 times less than what the literature reports (Jyothishwaran *et al.*, 2007). This unexpected result might be due to a technical error, to the cells being less viable than what was desirable, or to a poorly optimized protocol. A good alternative way to increase this efficiency would be to use electroporation rather than a freeze-thaw transformation protocol (Hanahan *et al.*, 1991). Regardless, the gene was successfully amplified from one of the selected colonies (**Figure 3.2B At1**), and these transformed *A. tumefaciens* cells will be further used in grey poplar transformation.

During the *A. tumefaciens* competence validation with the pUC19 plasmid, it was quite difficult to get rid of colonies on the negative controls with 100 mg/L ampicillin, as the cells appeared to resist to the antibiotic. This phenomenon is also reported in the literature by Tang *et al*. (2000) where concentrations ranging from 100 to 1000 mg/L were not enough to eliminate *A. tumefaciens* EHA101. This was the reason why the competent *A. tumefaciens* cells were transformed directly with pFHi.GNK2, as the antibiotic marker for this vector was kanamycin.

3.2. Poplar Transformation with the *Cast_GNK2-like* gene

As it was possible to achieve *A. tumefaciens* colonies harboring the construct with the gene of interest, the poplar transformation process was initiated. For this, the bacteria, whose *vir* genes had been activated with acetosyringone, were put in contact with INRA 717-1B4 poplar leaves. This interaction was facilitated through scalpel injuries made on the leaf tissue (**Figure 3.3AB**). The Co-culture period was achieved within three days, after which the leaves were transferred to Shoot Regeneration medium supplemented with carbenicillin and kanamycin (**Table 2.1**). The first is a bacteriostatic which arrested bacterial growth, while the second selected for transformed calli and shoots (**Figure 3.3CD**; de Sousa Araújo *et al*., 2004). Each week, the explants were transferred to fresh Shoot Regeneration medium until enough healthy shoots could be isolated from the leaves, with the aid of an optical magnifier, onto Shoot Isolation medium (**Figure 3.3E**; **Table 2.1**). **Sixteen plantlets were selected for further analysis**, and rooting was promoted in Micropropagation medium supplemented with 25 mg/L kanamycin (**Table 2.1**). **The regenerants recovered were labeled according to the coding system in subchapter 2.2 as 122, 123, 125, 131, 132, 133, 134, 136, 137, 138, 212, 231, 332, 333, 341 and 354**.

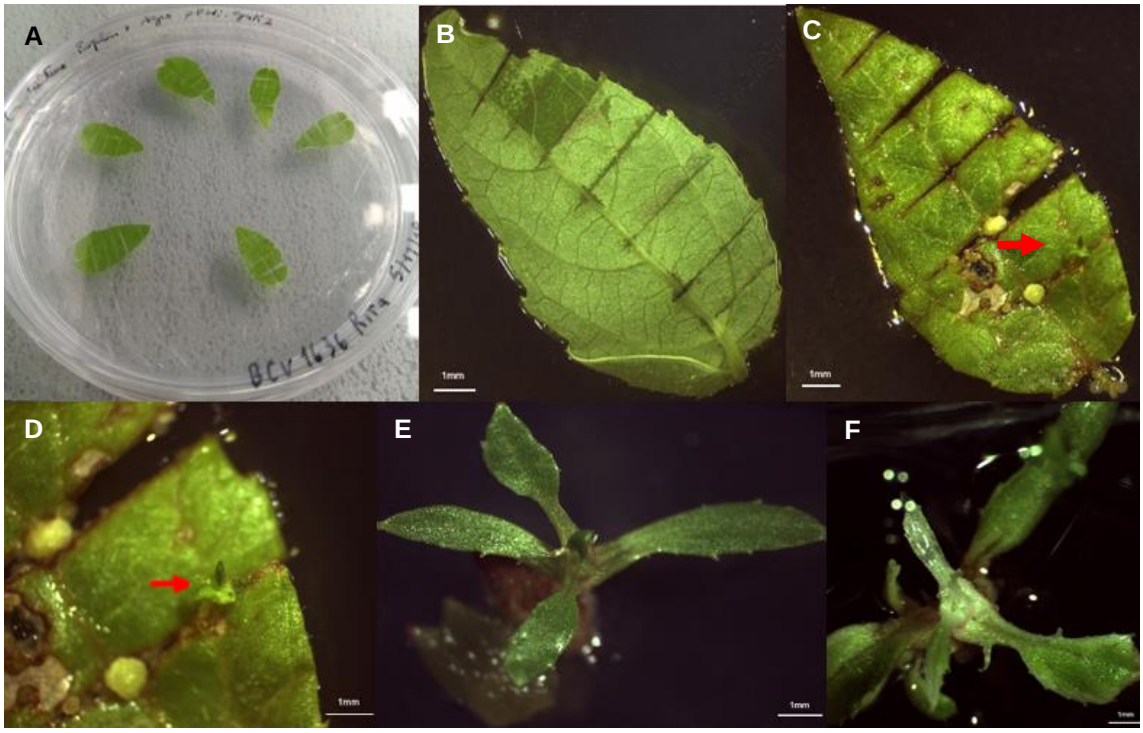

Figure 3.3 - ***Agrobacterium*-mediated Poplar Transformation**. **(A)** Co-culture of *A. tumefaciens* with *Populus tremula x alba* leaves. Six poplar leaves on a Petri dish cut with a scalpel infused with *A. tumefaciens* on top of Co-culture medium. **(B)** Cut poplar leaf 3 days after co-culture initiation at an amplification of 6.3x. It is possible to see *A. tumefaciens* colonies exiting the cut. **(CD)** Poplar leaf from the second transformation, 3 weeks after the co-culture period at an amplification of 6.3x (**C**) and 12.5x (**D**). A differentiated shoot can be observed. **(E)** 5-weeks-old differentiated shoot from the third transformation process. **(F)** False positive shoot turning white due to the kanamycin pressure. This picture was taken on the seventh week after co-culture.

When the selected sixteen plants were ready to be propagated, leaf tissue samples were collected, and RNA was extracted. With the employed protocol, it was possible to achieve concentrations of 210 to 894.5 ng/µL and absorbance ratios (260/280 and 260/230) between 2.00 and 2.10, which suggested that the RNA had a good degree of purity. RNA integrity was analyzed by electrophoresis. The eukaryotic ribosomal RNA bands 28S and 18S were well-pronounced, and it was even possible to observe the organellar 16S (mitochondrial) and 23S (chloroplast) rRNA fragments, in this order, on the gel (**Figure 3.4**; Keren *et al*., 2011). Additionally, the RNA was scanned for genomic DNA through PCR. No amplification was observed for any of the 16 RNA samples, as they did not contain traces of gDNA (**Figure 3.5**). For these reasons, the RNA

was considered to have a good level of integrity and purity, and it could be used as a template for cDNA synthesis. A regular PCR was performed on this cDNA to check for the *GNK2-like* transcript (**Figure 3.6**). **A qPCR was done using the synthesized cDNA as a template to assess the relative expression of the *Cast_GNK2-like* codifying sequence among the different genotypes**. By analyzing the qPCR results depicted in **Figure 3.7**, it was possible to **select five GNK2-Poplar genotypes: two with higher relative expressions (134 and 212), two with an intermediate relative expression (341 and 354), and a lower expression genotype (125).**

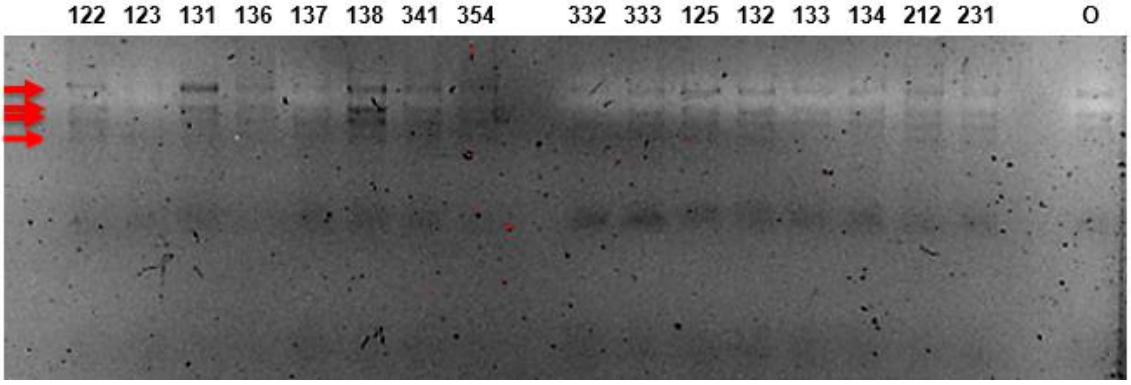

Figure 3.4 - RNA integrity. 2% agarose gel to visualize extracted RNA with variable concentrations. (**O**) Original *Populus alba x tremula,* which never suffered a transformation; (**122-354**) RNA from kanamycin regenerants. The distinct eukaryotic 28S and 18S, and organellar 16S and 23S rRNA bands observed in 138, for instance, are good indicators that the RNA maintained its integrity after its extraction (Keren *et al*., 2011). This gel was run at 100 V for 30 min.

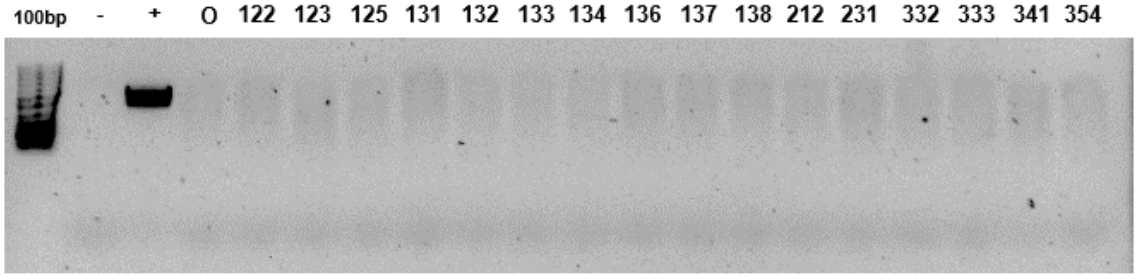

Figure 3.5 - gDNA contamination assay. PCR on *Populus* RNA with primers against the *Cast_GNK2-like* gene. (**100bp**) 100 bp GeneRuler DNA Ladder. (**-**) A negative control without template; (**+**) Positive control (plasmid); (**O**) Original *Populus alba x tremula* which never suffered a transformation; (**122-354**) 25 ng of RNA from kanamycin regenerants. It was possible to visualize a smear corresponding to degraded RNA, as the high temperatures in PCR dismantled its structure. This 0.8% agarose gel was run for 30 min at 100 V.

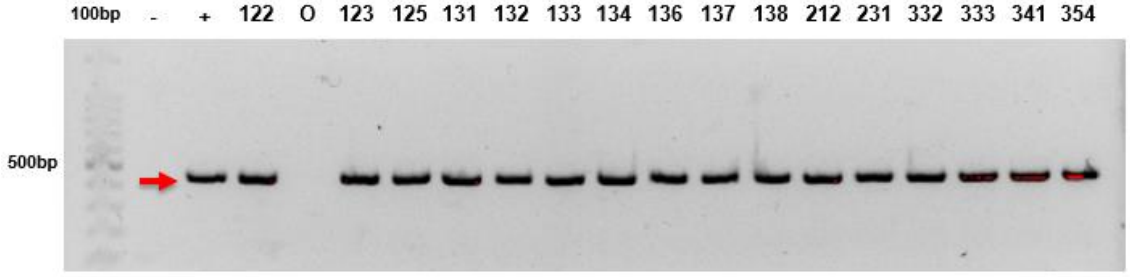

Figure 3.6 - Validation of transformants by Taq PCR. PCR on *Populus* cDNA with primers against the *Cast_GNK2-like* gene, the fragment is expected to have 431 bps. (**100bp**) 100 bp GeneRuler DNA Ladder. (**-**) Negative control of the reverse transcription, without RNA template; (**+**) Positive control, pFHi.GNK2 plasmid as template; (**O**) DNA from the original *Populus alba x tremula* genotype, which never suffered a transformation; (**122-354**) cDNA from kanamycin regenerants. This 1.5% agarose gel was run for 45 min at 100 V.

The three asynchronous *Agrobacterium*-mediated transformation procedures allowed the recovery of many positive poplar regenerants, and **no false positives from those selected for analysis**. This observation might be due to the Shoot Regeneration medium being renovated every week. **By the 78th day, it was possible to individualize a total of 89 shoots, and each plate had an average of 30 regenerated shoots**. Out of these 89 shoots, 19 turned white from

the kanamycin pressure when isolated onto selective medium (**Figure 3.3F**). This evidence shows that **the probability of an isolated shoot to be a false-positive is around 21%**.

As *Cast_GNK2-like* expression was detected (**Figures 3.6 and 3.7**), it is safe to assume that **the codifying sequence was successfully transcribed**. Therefore, the construction with the sequence preceded by the UBQ11 promoter and followed by the 35S terminator engineered by Patrícia Fernandes was recognized by the plant cell machinery. Although the inserted construction was the same, the regenerants displayed variable expression levels (**Figure 3.7**). This **expression variability is dependant on** both **the locus where the construction was inserted**, since transcription is also directed by epigenetic regulation (DNA methylation and histone modifications), **and the number of insert copies in the plant genome**. **GNK2-Poplar genotypes were selected according to this variable expression**, and to the level of development of the plants from each regenerant line, as time was a major limiting factor for this thesis. By the time the infection assay was due, each selected GNK2-Poplar genotype had around four plants, three of which were used for the assay.

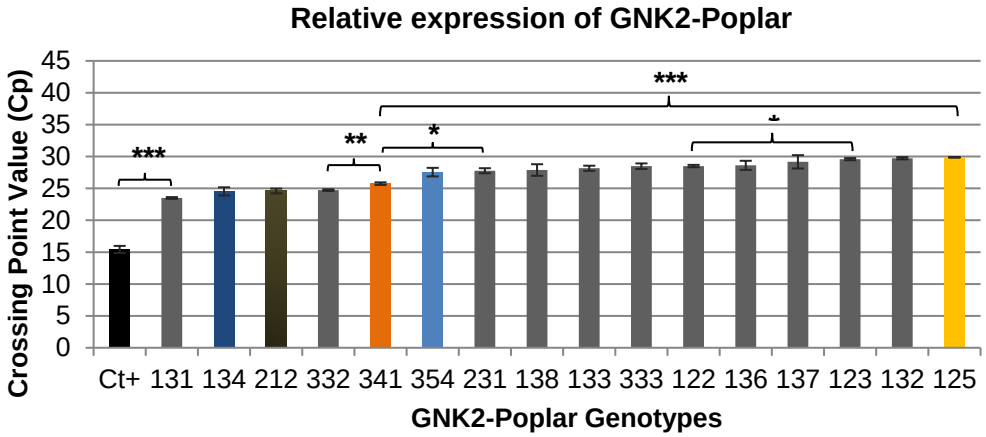

Figure 3.7 - **qPCR results** exhibiting the relative expression of the isolated GNK2-Poplar genotypes. **Ct+** represents the positive control, whose template was 2.5 ng of the original pFHi.GNK2 plasmid. **131-125** represent the 16 GNK2-Poplar genotypes chosen for biomolecular analysis, 5 ng of each cDNA was used as the template. No Cp value was attributed for any of the negative controls consisting of **a)** water; **b)** non-transformed poplar; or **c)** RNA extracted from the transformed poplar. **134** and **212** were chosen as high-expression GNK2-Poplar genotypes, **341** and **354** were chosen as intermediate-expression genotypes and **125** was chosen as a low-expression genotype. The asterisks symbolize statistical significance according with two-tailed t-tests: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$.

During the process of selection of the plants for the final *Phytophthora* infection assay, it was possible to observe that the individuals varied among themselves and the wild-type, and the heights and number of leaves of the plants participating in the assay were registered. No major differences were observed when comparing to the wild-type except for the 341 genotype, which displayed significantly faster growth ($p < 0.01$; **Figure 6.8**, Supplementary Data).

A total of three separate transformation events were performed. On the first two, the **regeneration control consisted of cut leaves** (without the *A. tumefaciens* inoculum) **sitting on media without any antibiotics**. It was observed that the **number of shoots regenerated from this control was consistently less than half of those regenerated from the transformations** (results not shown). As such, on the last transformation procedure, a **control with non-transformed leaves in a Shoot Regeneration medium supplemented with 500 mg/L of carbenicillin** was done (**Table 2.1**). This latter control consistently had **more than double the regenerant plants when compared to the first two controls without carbenicillin**, and would **show regenerant shoots four weeks earlier** (**Figure 6.7**). These findings are most likely due to **carbenicillin intervening in the poplar differentiation process**, a phenomenon documented for other species such as in adventitious roots of *Carica papaya* L. (Yu *et al*., 2001), and cultured tissues of durum wheat, and barley (Yu *et al*., 2008). According to the literature, **carbenicillin is**

similar to auxin-related structures such as 2,4-Dichlorophenoxyacetic acid and NAA, and has plant hormone-like effects on cultured plant tissues, affecting differentiation and somatic embryogenesis in many plant species (Lin *et al.*, 1995; Yu *et al*., 2001). On the other hand, there are even more reports of carbenicillin harming the regeneration of plant tissues in *Arabidopsis*, *Datura*, *Beta vulgaris*, *Delphinium*, *Nicotiana tabacum*, *Picea glauca*, *Solanum tuberosum*, *Vitis* (Nauerby *et al*., 1997), *Nicotiana tabacum* (Lin *et al*., 1995; Nauerby *et al.*, 1997; Silva & Fukai, 2001), *Chrysanthemum* (Silva & Fukai, 2001), and *Medicago truncatula* (de Sousa Araújo *et al*., 2004). One way to explain these contradictory effects of carbenicillin would be that the auxin threshold values for regeneration and embryogenesis vary among plant species.

Keeping cultures *in vitro* can be very laborious, and so the best way to maintain genotypes in a lab is to acclimate them into *ex vitro* conditions. In this way, the greenhouse stock may always be accessed in the event of the loss of a genotype *in vitro*. However, **acclimation is the most difficult process an *in vitro* plant can go through**, **as these plants have no functional stomatal regulation**. Because the humidity in their environment is always rounding 100%, *in vitro* plants may always keep their stomata open, without risking water loss through transpiration. If a plant is physiologically-programmed this way, it will rapidly desiccate in soil. The INRA 717-1B4 poplar strains from the lab were acclimated, with the procedure achieving a **72% success rate in a total of eighteen plants**. The losses were always due to uncontrolled fungi growth from poor root washing and high humidity conditions.

3.3. Preliminary *in vitro* Infection Assay of Poplar with *P. cinnamomi* pH107, *P. cinnamomi* DSM 62654, and *P. cactorum* DSM 62637

Before advancing with the phenotyping of the GNK2-Poplar transformants, a **preliminary assay of exposure of GUS INRA 717-1B4 poplar to the *Phytophthora* strains** was performed. The main goal of this assay was to **assess how *Populus tremula x alba* would respond to the presence of the oomycetes in *in vitro* conditions**. As there were not any publications reporting an interaction between *P. cinnamomi* and grey poplar, a *P. cactorum* strain was also included in the phenotypic analysis. **The pH107 *P. cinnamomi* strain provided by INIAV, the DSM 62637 strain of *P. cactorum*, and the *P. cinnamomi* DSM 62654 strain were used in the inoculation of the poplar**, using three biological replicates per strain.

A *Phytophthora* growth assay in PDA was done simultaneously to assess if the growth patterns of the three *Phytophthora* were similar to those found when the oomycetes grew in the poplar Micropropagation medium. Overall, *P. cinnamomi* DSM 62654 was the fastest grower, followed by *P. cinnamomi* pH107, and then by *P. cactorum* DSM 62637 (**Figure 3.8**).

The *Phytophthora* strains easily spread throughout the ½ MS-based medium (**Figure 3.9A**), and it was possible to observe a much faster depletion of the medium in inoculated plants compared to the non-inoculated controls. Generally, the **first visible symptom of *Phytophthora* infection for these poplar plants *in vitro* was the die back of the leaves**, which are often quite upright in poplar, detected as soon as five days after the infection (**Figure 3.9B**). Around the same time, it was possible to **start observing stem rot** (**Figure 3.9C**). **Root rot was only visible on some superficial roots**, suggesting that *Phytophthora* favored spread throughout the medium surface. The **preferential method of infection in these *in vitro* conditions was hyphae dissemination through the stem**. Yellowness and necrosis of leaves contacting with the hyphae suggested that the **oomycetes could also enter by the leaves**. The **development stage of the plant** (height and leaf number) seemed to slightly **influence the appearance of symptoms**, as **more developed plants showed delayed severe symptoms** when comparing to underdeveloped plants (not shown). Additionally, taller plants could misrepresent the percentage of stem rot, as *Phytophthora* rot progresses at a steady and consistent pace.

In this preliminary assay, **the symptom that best distinguished *Phytophthora* infection was the dieback**. This phenomenon is caused by the **blockage of the water flow through the conductive vessels** by hyphae. As such, **an infected plant may commonly be mistaken for a plant suffering from drought** (Hardham, 2005).

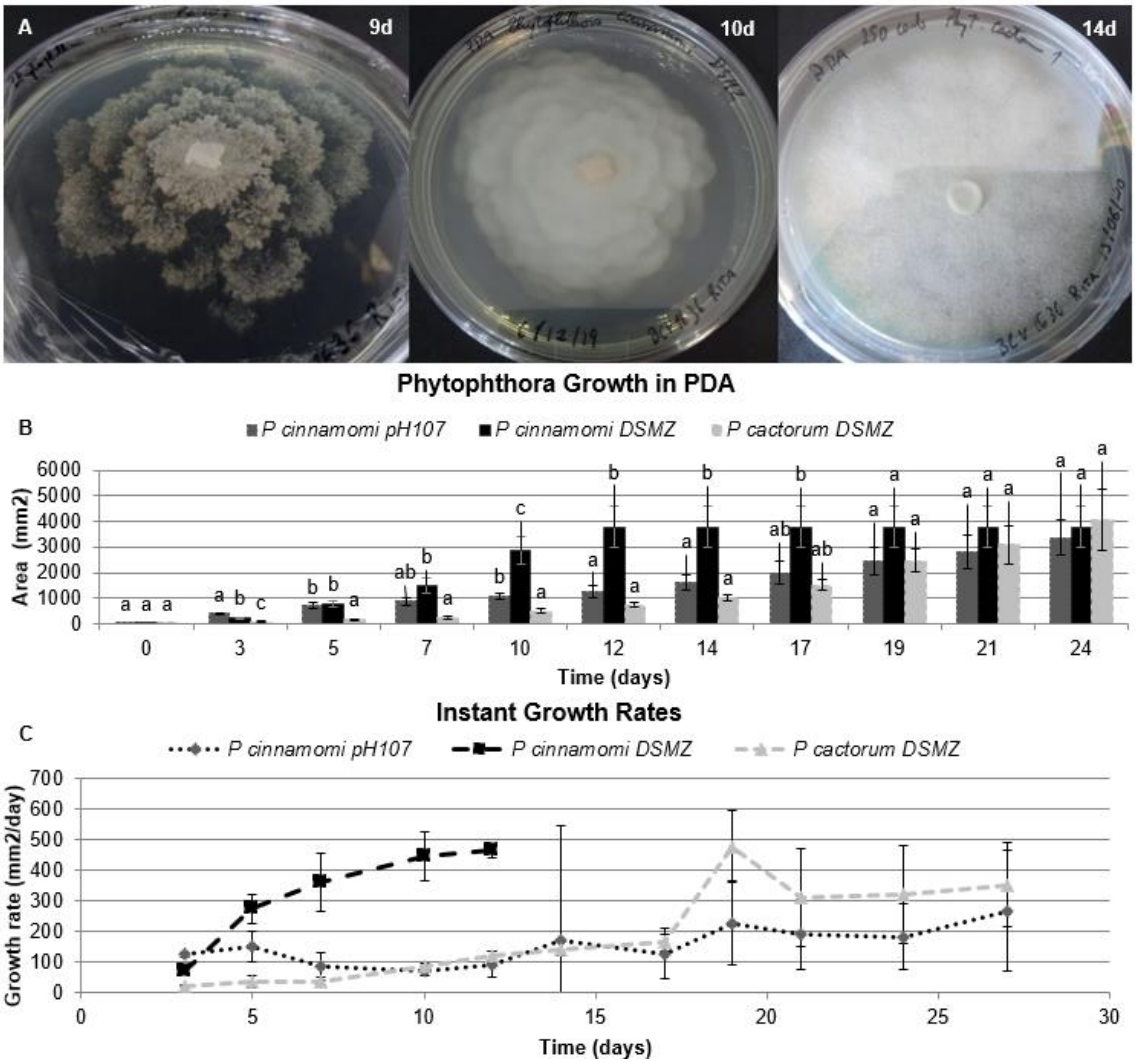

Figure 3.8 - *Phytophthora* growth in PDA. (**A**) *Phytophthora* morphology. From left to right: *P. cinnamomi* pH107 (9 days), *P. cinnamomi* DSM 62654 (10 days) and *P. cactorum* DSM 62637 (14d). (**B**) *P. cinnamomi* pH107, *P. cinnamomi* DSM 62654 and *P. cactorum* DSM 62637 growth in mm^2. The letters represent statistical significance according with a two-tailed t-test: b is different from a when $p < 0.01$; ab symbolizes $p < 0.05$. (**C**) Instant growth rate of the *Phytophthora* represented in mm^2 per day. Total growth rate: *P. cinnamomi* pH107 – 154 mm^2/day, *P. cinnamomi* DSM 62654 – 314 mm^2/day, *P. cactorum* DSM 62637 – 189 mm^2/day.

The *Phytophthora* infection symptoms observed in this preliminary assay coincided, in most cases, with what was expected from the literature (Hardham, 2005). However, because of the characteristics of the infection methodology employed, the **prevalence of root rot was much lower than expected**, comparing to what is reported in nature, as only some superficial roots showed signs of rot. This must have happened because the **thickness of the medium was protecting the roots by hindering the progression of *Phytophthora's* hyphae**. The superficial roots that would not become rotten, would still show much *Phytophthora* growth, becoming "fluffy roots" (**Figure 3.10D**). The appearance of "fluffy roots" might be a good indicator of compatibility between the two organisms. On the other hand, **stem rot was more often visible** than what is

shown in the wild, in the literature. All in all, **since the roots were protected**, ***Phytophthora* found a new pathway of infection**, in this case, **through the stem collar**.

It was possible to observe a faster *Phytophthora* growth when the hyphae came into contact with the stem of the plant, which might indicate that there is some degree of compatibility between the two species, or even that poplar synthesizes chemo-attractants. When the roots would show rot before the stem, the leaf petioles closer to the medium would show necrosis before the stem collar showed any signs of lesion. This evidence could indicate that ***Phytophthora* is capable of traveling upwards through the root and stem vessels**.

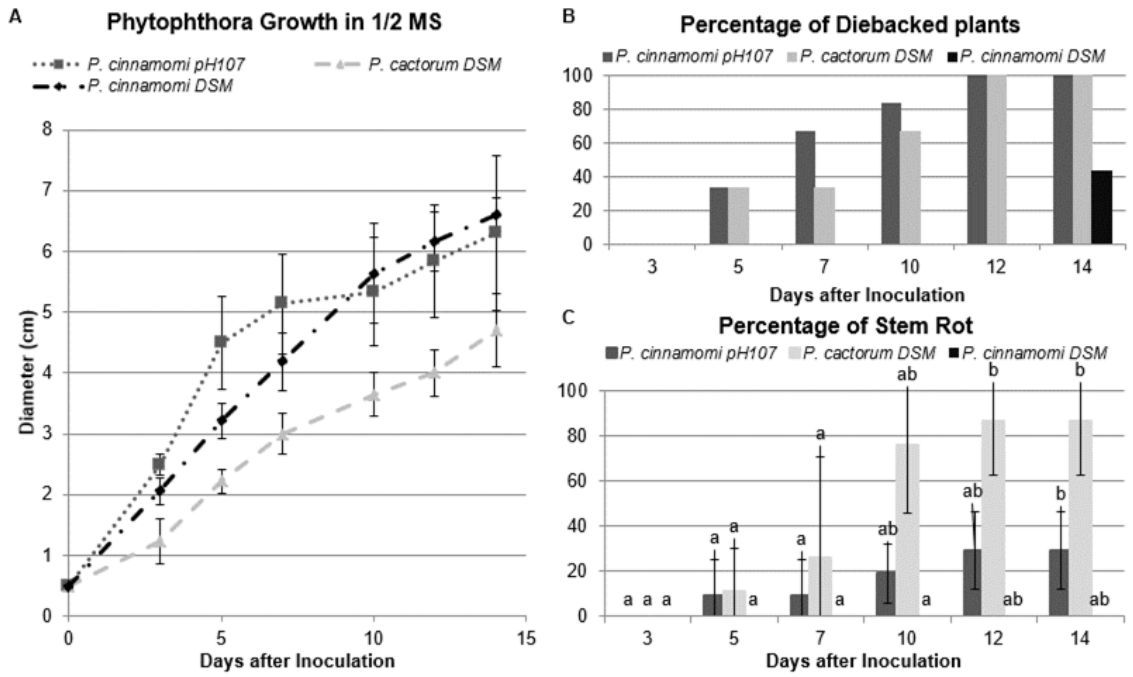

Figure 3.9 - Summary of the observed symptomatology from *Phytophthora in vitro* infection in *Populus tremula x alba* (preliminary assay). (**A**) Rough *Phytophthora* growth in ½ MS-based Micropropagation medium. *P. cactorum* had considerably slower growth than *P. cinnamomi*, which was in accordance with the PDA growth data. (**B**) Percentage of plants exhibiting dieback throughout the experiment. (**C**) Mean percentage of stem rot on the three biological replicates throughout the experiment. The letters represent statistical significance according with a two-tailed t-test: b is different from a when $p < 0.01$; ab symbolizes $p < 0.05$.

Additionally, *Phytophthora* found a different pathway of entry apart from the stem collar. As the large poplar leaves showed dieback symptomatology, they would be directly exposed to *Phytophthora* hyphae, which would infect them, causing chlorosis and posterior necrosis symptoms on the infected leaves. Oftentimes, the necrosis would spread up the leaf to the stem.

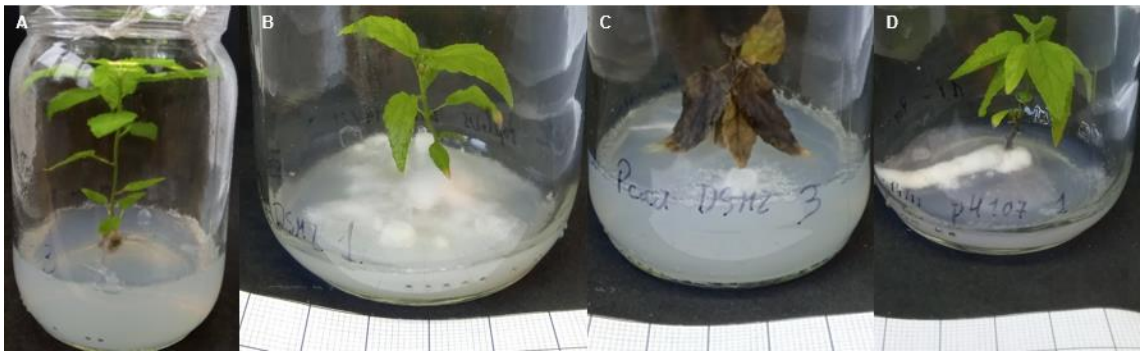

Figure 3.10 - Phenotype of *Populus tremula x alba* infected with *Phytophthora* 14 days after inoculation. (**A**) Control poplar which has never been infected with a pathogen. (**B**) Poplar sapling infected with *P. cinnamomi* DSM 62654. (**C**) Poplar sapling infected with *P. cactorum* DSM 62637. (**D**) Poplar sapling infected with *P. cinnamomi* pH107.

The *P. cinnamomi* strains showed different symptomatology from each other. The **pH107 strain was much more aggressive than the DSM 62654 strain**. This was to be expected because the pH107 strain, isolated from *C. sativa* located in Bornes (Bragança, Portugal; Duarte, 2015), was chosen among several *P. cinnamomi* strains as the most virulent from a previous assay performed by the INIAV (unpublished). Additionally, the **pH107 was the only strain that caused the medium to turn pink around poplar roots**, which might indicate that the **poplar was synthesizing colored substances**, possibly **phytoalexins or anthocyanins**, to fight against the infection.

Out of the three *Phytophthora* strains, ***P. cactorum* seemed to be the most aggressive towards the poplar juveniles** (**Figure 3.9C**). **By day twelve, two of the three inoculated plants had been killed by the pathogen**. This result seems agree with the available data in Cerny *et al*. (2009) and Keča *et al*. (2015), as in these works *P. cactorum* was capable of being isolated from wild poplar forests.

Overall, **this preliminary assay only with GUS-Poplar was very useful towards understanding both the *in vitro* symptoms provoked by *Phytophthora*, and the parameters which needed to be assessed on the final assay**. The three *Phytophthora* strains tested could be **discriminated in terms of growth rate** and **infection severity**. ***P. cinnamomi* DSM 62654** would come into contact with the plant very quickly, as its **growth was the fastest**, but showed very **rare signs of symptomatology** in poplar. Therefore, this pathogen was not included on the final infection assay. ***P. cinnamomi* pH107 would contact the plant relatively fast, and show moderate to severe signs of infection**. ***P. cactorum* had extremely slow growth** but once it contacted the plant, it would **exhibit very prominent signs of infection, and even provoke the death of its host** within the time-spawn of the assay. The differential virulence observed could also be affected by the amount of *in vitro* propagation cycles the strains had to go through, propagated for at least thirty years, as the accurate collection date of the DSM strains is unknown.

The way this *in vitro* assay was designed makes it so that ***Phytophthora* hyphae are the infection agents**, **rather than the *Phytophthora* propagules that usually infect plants in nature**. *P. cinnamomi* commonly infects plants via zoospores, which are mobile water-borne propagules. *P. cactorum*, on the other hand, infects plants by sending out its aerial caducous sporangia. To make an assay that better replicates the conditions felt by the organisms in nature, ***P. cinnamomi* should infect potted plants immersed in water, and *P. cactorum* should be grown in a medium that promotes sporangia formation**, such as the V8 Agar (Guo & Ko, 1993), **and be left inside a sterile ventilated containment alongside with poplar plants**.

3.4. Final *in vitro* Infection Assay of Poplar with *P. cinnamomi* pH107 and *P. cactorum* DSM 62637

The objective of this assay was to **phenotype the responses of the regenerated GNK2-Poplar genotypes**, by including them in an assay similar to the described preliminary *in vitro Phytophthora* infection assay. This assay would provide evidences **towards understanding if the GNK2-like protein from *C. crenata* increased the tolerance against *Phytophthora*-caused symptomatology in poplar**.

To ensure that no *A. tumefaciens* growth surged, the *in vitro* infection needed to be performed in poplar Propagation medium supplemented with 250 mg/L carbenicillin (**Table 2.1**). As such, it was necessary to evaluate how *Phytophthora* growth was affected by the presence of carbenicillin. For this, a PDA assay was performed, and its results are depicted in **Figure 3.11**. As the **presence of carbenicillin did not seem to hinder *Phytophthora* growth**, the final assay could be done in standard poplar Propagation medium supplemented with 250 mg/L of the antibiotic.

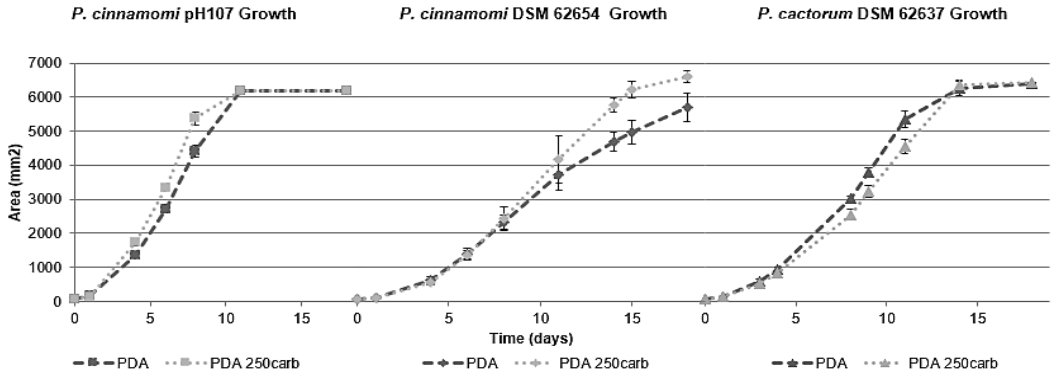

Figure 3.11 - Effect of 250 mg/L carbenicillin (250carb) on *Phytophthora* growth in PDA. (**A**) *P. cinnamomi* pH107 growth. (**B**) *P. cinnamomi* DSM 62654 growth. (**C**) *P. cactorum* DSM 62637 growth. The presence of 250 mg/L of carbenicillin did not seem to hinder any of the *Phytophthora* growth in PDA.

As the DSM 62654 strain of *P. cinnamomi* did not show major symptomatology in the preliminary *Phytophthora in vitro* infection assay (**Figure 3.10BC**), it was decided that it should not be included in the final assay. **This assay was done using the two remaining *Phytophthora* genotypes pH107 and DSM 62637**, **a wild-type genotype of poplar**, **and five GNK2-Poplar genotypes with different relative expression levels**: 212 and 134 displayed higher relative expression; 354 and 341 displayed an intermediate expression; 125 displayed lower relative expression. The higher expression genotypes did not have many individuals, as such, the genotype 212 was used to inoculate *P. cinnamomi* and the genotype 134 was infected with *P. cactorum*.

The preliminary assay allowed us to perceive that it was important to **control the day that the *Phytophthora* contacted the stem of the plant** to attempt to **synchronize the infection** on all plants, and so, in this final assay, the media plug containing the *Phytophthora* inoculum was 2 cm spaced-out from the stem collar. The time point when *Phytophthora* contacted the stem was measured for each individual and the results are depicted in **Figure 3.12**. There were no significant differences between the time point when *Phytophthora* contacted the wild-type poplar and the remaining GNK2-Poplar genotypes. However, **there was a major difference between the day of contact for *P. cinnamomi* and *P. cactorum* (p < 0.001)**, this fact can be attributed to the significant difference in the growth rates of the two oomycetes (**Figure 3.8BC and 3.9A**).

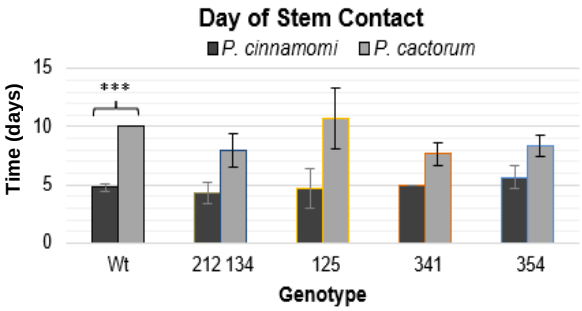

Figure 3.12 - Day of Stem contact: the day when *Phytophthora* would make contact with the stem of the plant during the final *in vitro* inoculation assay. The *Phytophthora* 0.5 cm plug was placed at a 2 cm distance from the plant stem. No statistical significance was observed between the Wt and the GNK2-Poplar genotypes. However, the day of stem contact between *P. cinnamomi* and *P. cactorum* was significant according with a two-tailed t-test (p = 4.59E-04).

This final assay was subdivided into two experiments, as the GNK2-Poplar genotypes were desynchronized in their development. In this final *in vitro* infection assay, each selected GNK2-Poplar genotype had around four plants, three of which were used for the assay. **The first experiment of the assay comprised four 9-weeks-old plants of wild-type poplar, three plants of 354 GNK2-Poplar, and three plants of 341 GNK2-Poplar**. **The second experiment comprised of three 7-weeks-old plants per genotype for wild-type, 212, 134, and 125 poplar**.

The transformed poplar should show less serious or delayed signs of *Phytophthora* symptomatology, at least for *P. cinnamomi* pH107 infection, as this was the strain used to identify *Cast_GNK2-like* as a gene involved in resistance against the ink disease. Furthermore, *Phytophthora* has shown to have an extensive synteny in-between the genus, and as such GNK2-Poplar genotypes are also expected to show higher tolerance to *P. cactorum* DSM 62637 infection (Hardham, 2005).

First Experiment – Wild-type, 354, and 341 GNK2-Poplar

Within this first experiment, few differences between the wild-type and the intermediate-expression genotypes were observed. No differences, in particular, were found for the time point of stem contact, or in the growth of *Phytophthora*, or in the appearance of dieback from *P. cinnamomi* infection (**Figure 3.12**, **3.13**, **3.14A**). For ***P. cinnamomi*, it was possible to observe a major increase of stem rot for the 354 genotype**, when comparing to the wild-type (**Figure 3.14B**). Naturally, this was not an expected result, as we hypothesized that the GNK2-Poplar genotypes should show a higher degree of tolerance when comparing to the wild-type. One way to explain this unexpected result would be that the construction inserted itself within a locus which would have a higher impact in determining the plant response to this particular pathogen. It could also have inserted itself within a growth or development-regulating locus, as this genotype exhibited plants only slightly smaller (**Figure 6.8**, non-significant) than the wild-type.

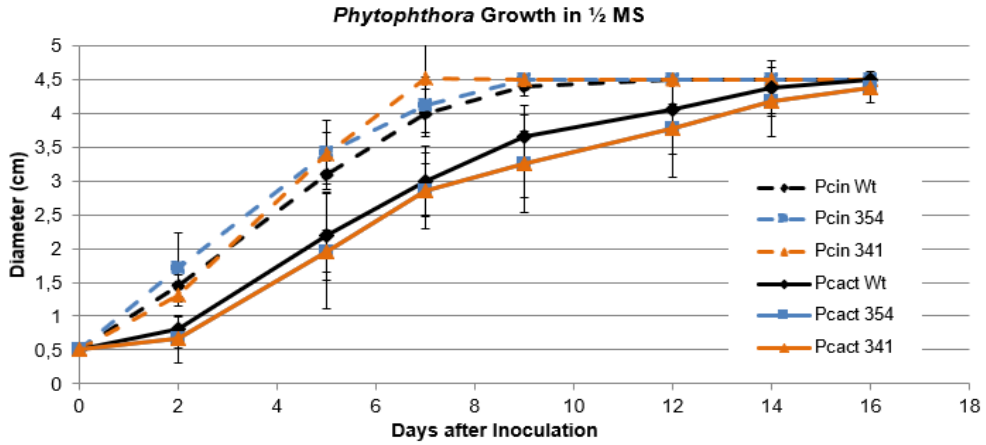

Figure 3.13 – Diameter of the *Phytophthora* growth in ½ MS medium. The maximum diameter was of the glass flasks was 4.5 cm. *P. cactorum* had considerably slower growth than *P. cinnamomi*, which was in agreement with the PDA growth data. Pcin – *P. cinnamomi* pH107, Pcact – *P. cactorum* DSM 62637. **Wt** – wild-type poplar (non-transformed), **354** and **341** – GNK2-Poplar genotypes.

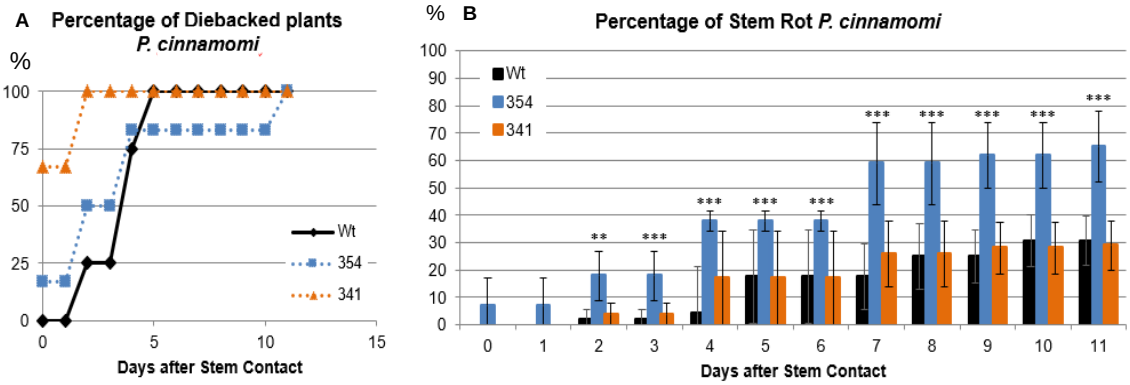

Figure 3.14 - Summary of the symptomatology displayed by grey poplar after *P. cinnamomi* pH107 infection. (**A**) Percentage of plants exhibiting dieback after *P. cinnamomi* contact with the plant stem. (**B**) Mean percentage of stem rot exhibited by the poplar plants after *P. cinnamomi* contact with the stem. The asterisks symbolize statistical significance according with a two-tailed t-test: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$. **Wt** – wild-type poplar (non-transformed), **354** and **341** – GNK2-Poplar genotypes.

As **for *P. cactorum* infection, it was possible to observe a significant delay in the appearance of dieback in the transformed genotypes**, and in 341 GNK2-Poplar in particular (**Figure 3.15A**). Although this assay did not have many replicates per genotype, by day two, 75% of wild-type plants were die backed, while only 33% of transformed plants showed this symptom. Additionally**, a significant delay in the appearance of stem rot** (two days) was registered **for genotype 354** ($p < 0.1$; **Figure 3.15B**). This observation is not consistent with the data attained for *P. cinnamomi* infection, but considering that there are more reports of *P. cactorum* infecting wild poplar than *P. cinnamomi*, this can still be considered an interesting result.

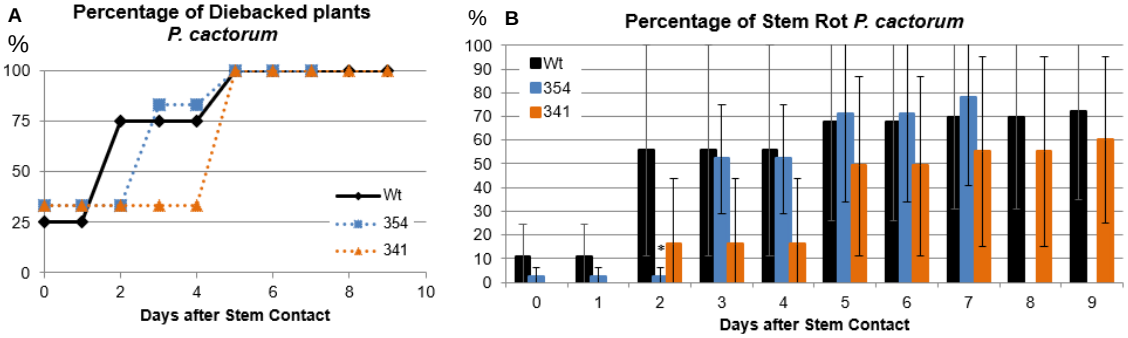

Figure 3.15 - Summary of the symptomatology displayed by grey poplar due to *P. cactorum* DSM 62637 infection. (**A**) Percentage of plants exhibiting dieback after *P. cactorum* contact with the plant stem. (**B**) Mean percentage of stem rot exhibited by the poplar plants after *P. cactorum* contact with the stem. The asterisks symbolize statistical significance according with a two-tailed t-test: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$. **Wt** – wild-type poplar (non-transformed), **354** and **341** – GNK2-Poplar genotypes.

Second Experiment – Wild-type, 212, 134, and 125 GNK2-Poplar

In the second experiment of the final *in vitro* assay, **it was possible to get consistent significantly lower stem rot for *P. cinnamomi* in genotype 125** ($p < 0.05$; **Figure 3.16C**). **This genotype also displayed delayed dieback** in comparison to the wild-type (**Figure 3.16B**). **Genotype 212 also showed a significant delay in the appearance of stem rot for *P. cinnamomi***, comparing to the wild-type ($p < 0.1$; **Figure 3.16C**).

As for *P. cactorum*, there were no significant differences detected in *Phytophthora* growth nor in the observed stem rot, when comparing the GNK2-Poplar genotypes to the wild-type (**Figure 3.17AC**). However, there was a **significant delay in the appearance of dieback for the 125 GNK2-Poplar genotype** (**Figure 3.17B**, day 2 to 5).

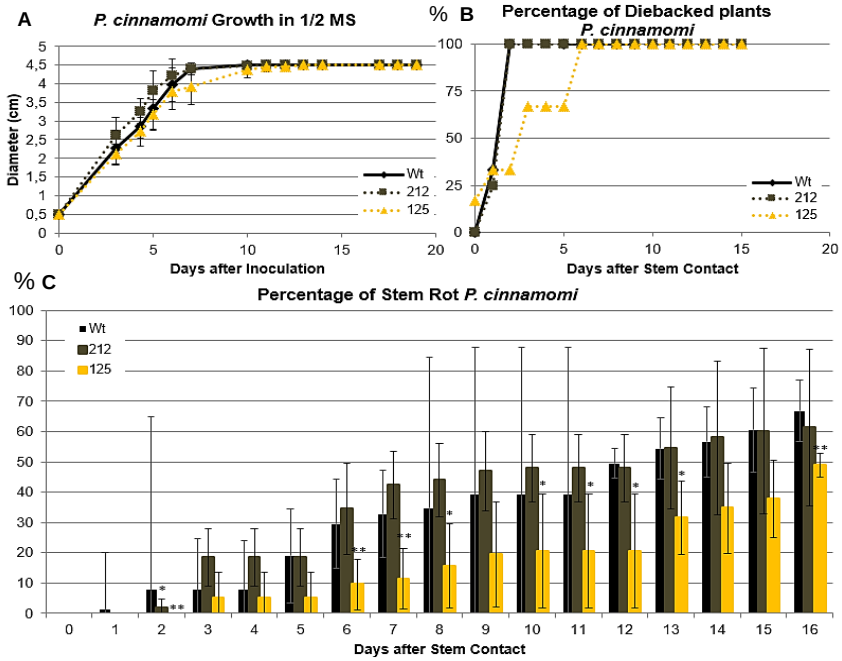

Figure 3.16 - Summary of the Final *in vitro P. cinnamomi* pH107 infection assay with the 212 and 125 GNK2-Poplar genotypes. (**A**) Rough *P. cinnamomi* pH107 growth in ½ MS medium. (**B**) Percentage of plants exhibiting dieback after *P. cinnamomi* contacted the plant stem. (**C**) Mean percentage of stem rot exhibited by the poplar plants after *P. cinnamomi* contacted the stem. The asterisks symbolize statistical significance according with a two-tailed t-test: *, p < 0.1; **, p < 0.05; ***, p < 0.01. **Wt** – wild-type poplar (non-transformed), **212** and **125** – GNK2-Poplar genotypes.

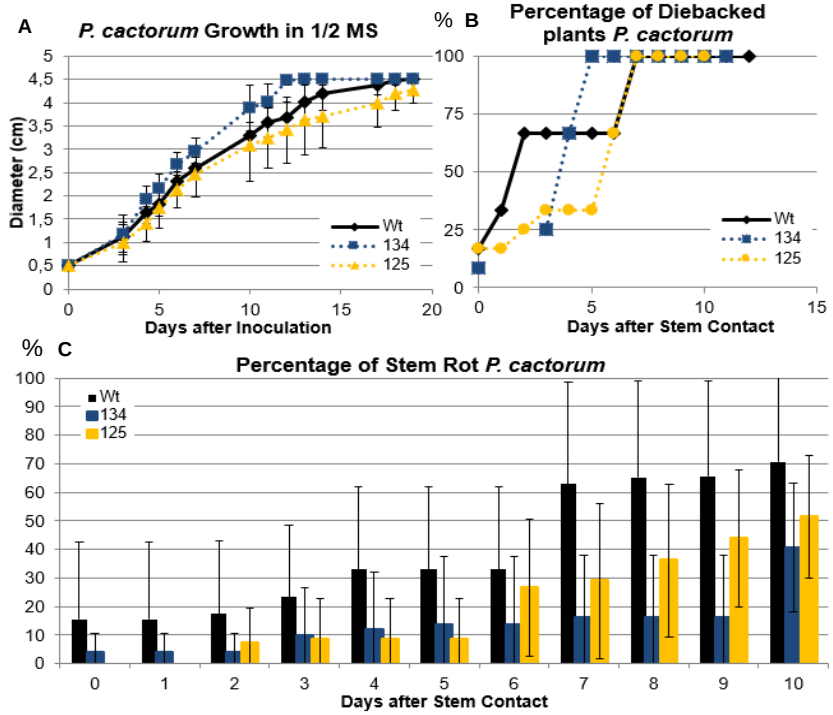

Figure 3.17 - Summary of the Final *in vitro P. cactorum* DSM 62637 infection assay with the 134 and 125 GNK2-Poplar genotypes. (**A**) Rough *P. cactorum* growth in ½ MS medium. (**B**) Percentage of plants exhibiting dieback after *P. cactorum* contacted the plant stem. (**C**) Mean percentage of stem rot exhibited by the poplar plants after *P. cinnamomi* contacted the stem. No statistical significance was attributed to these results when employing a two-tailed t-test. **Wt** – wild-type poplar (non-transformed), **134** and **125** – GNK2-Poplar genotypes.

Summary of the Experiment

A **timeline explaining the events observed throughout the experiment** was conceived (**Figure 3.18**) by taking into account the symptomatology observed in these *in vitro* Poplar-*Phytophthora* infection assays. Since, in these conditions, *P. cinnamomi* pH107 and *P. cactorum* displayed similar behavior and symptomatology, it was possible to describe the effects of both *Phytophthora* within a single scheme. The main differences between the two species were that *P. cinnamomi* displayed a considerably higher growth rate (**Figure 3.12**) and that *P. cactorum* was more hazardous, overall displaying more severity in the percentage of stem rot.

In sum, after inoculation, exposed superficial roots would come into contact with hyphae first, possibly showing signs of rot sometime after. A few days later, depending on the *Phytophthora* strain growth rate, the **oomycetes' hyphae would contact the stem collar of the plant**, **initiating the infection process from the bottom towards the apex**. **Dieback usually follows this contact about two days later, preceding stem rot signs**. Meanwhile, the **more developed die backed leaves contact the medium and start to exhibit chlorosis followed by necrosis sometime later**. The **stem rot progresses according to the *Phytophthora* strain's aggressiveness** towards the host, **reaches the apical meristem**, and **the plant eventually dies** (**Figure 3.18**).

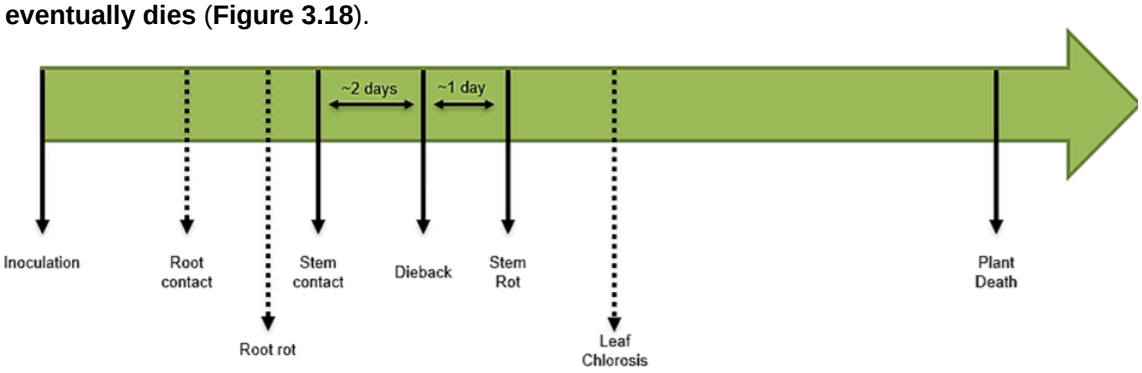

Figure 3.18 - Timeline summarizing the events identified within the *in vitro* poplar infection assays with *Phytophthora*. The dashed arrows represent events that occurred only in certain plant development conditions.

Although some GNK2-Poplar genotypes showed delayed response towards *Phytophthora* infection and mitigated symptoms, **differences between wild-type and GNK2-Poplar were less consistent than expected during this final assay**. Additionally, **the relative amount of *Cast_GNK2-like* expression did not seem to be the deciding factor on whether the genotype acquired enhanced tolerance to *Phytophthora* infection** relating to the wild-type. In fact, **the genotype which showed the most consistent results was the 125**, with the lowest relative expression of the gene. Although quantifying the amount of a transcript provides a good idea of the amount of activity of its respective protein, we must be reminded that **downstream proteomics factors** such as translation efficiency and post-translational modifications also **play major roles in the activity of a gene product**. These factors could be crucial to justify the differential results observed for the phenotypes of the generated transgenic poplar lines.

The results obtained could be an indicator that **the conditions employed in the assay favored *Phytophthora* in this *Phytophthora*-poplar interaction**. **The poplar Micropropagation medium**, in particular, **possessed a very high sucrose amount of 20 g/L, which has likely allowed for enhanced *Phytophthora* proliferation**, comparing to conditions felt in nature. It was not possible to remove this amount of sugar from the medium though, as it is not possible to transfer the poplar roots intact from this medium to a new medium without sucrose. Another important point to address is the developmental stage of these plants. **Subjecting these underdeveloped saplings to a highly aggressive pathogen such as *Phytophthora* might**

not highlight the genotype's potential to deal with the threat. For instance, these young trees do not possess a lignified layer to defend their stems, when comparing to established older trees. The absence of lignin in the plantlets' stem is also the most likely justification for the absence of stem cankers as a symptom in the experiments (Hardham, 2005).

The best strategy to assess GNK2-Poplar tolerance would be one that accurately represented *Phytophthora* infection in nature, with an accurate interaction between the two organisms. For instance, to identify the *Cast_GNK2-like* gene, Santos *et al*. (2017) used a flood assay for *P. cinnamomi* pH107 infection. This way, the zoospores were the main source of infection, instead of hyphae, more accurately mimicking the process of infection that occurs in nature. Additionally, an assay like this would better reflect *Phytophthora*'s life cycle. Typically, *P. cactorum* would infect plants via aerial caduceus sporangia (Cooke *et al.*, 2000; Hantula *et al.,* 2000). As such, *P. cactorum* should be grown in V8 Agar, a medium that promotes sporangia formation (Guo & Ko, 1993), and be left inside a sterile ventilated containment to infect the poplar plants. A good way to further complement these results would be to include histopathological analysis by cutting infected root slices and coloring them with the appropriate staining dyes.

3.5. *In vitro* Infection of Poplar leaves with *P. cinnamomi* pH107 and *P. cactorum* DSM 62637

In this assay, six poplar leaves were placed radially around a 0.5 cm-wide *Phytophthora* inoculum plug in a PDA plate (**Figure 2.4**). In each plate, the leaves belonged to two different genotypes, with **three leaves representing each of the six genotypes** (wild-type poplar, 212, 134, 354, 341, and 125 GNK2-Poplar). *Phytophthora* growth and leaf lesion were followed throughout the assay, and their results are depicted in **Figures 3.19** and **3.20**. The assay was conducted for fourteen days for *P. cinnamomi* and twenty-one days for *P. cactorum*, as the growth rate from these two species differs.

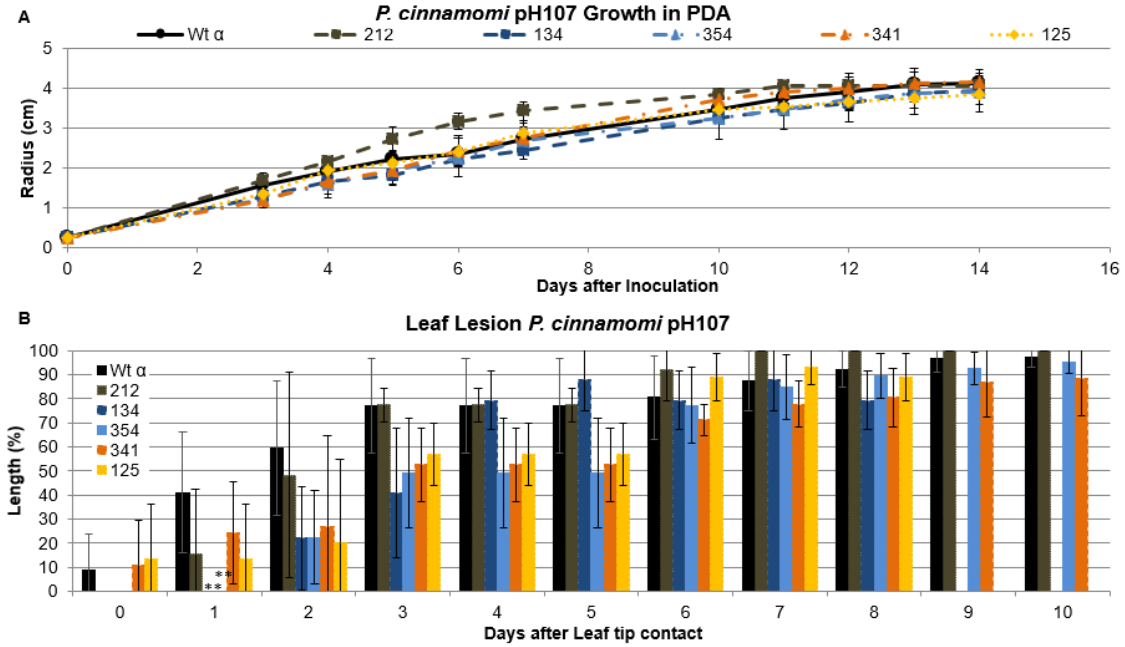

Figure 3.19 - Summary of the Leaf *P. cinnamomi* pH107 infection assay with the five GNK2-Poplar genotypes. (**A**) Rough *P. cinnamomi* growth in PDA. (**B**) Observed leaf lesion, displayed as length percentage, after leaf contact. Both yellowing and necrosis were considered as leaf lesions. The asterisks symbolize statistical significance according with a two-tailed t-test: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$. **Wt α** – wild-type poplar (non-transformed); **212, 134, 354, 341, 125** – GNK2-Poplar genotypes.

No significant differences were observed in the different genotypes for the growth of both *Phytophthora*, except for the 354 GNK2-Poplar genotype subjected to *P. cactorum* inoculation. The oomycetes from this plate showed significantly slower growth ($p < 0.05$) when compared to the wild-type (**Figure 3.20A**). This assay showed that *Phytophthora* hyphae could enter by the plant leaves, making them display chlorosis followed by necrosis sometime later. It was also possible to observe that the leaf lesion progressed mainly through the conductive vessels.

For *P. cinnamomi* pH107**, it was possible to observe a significant delay (two days) in symptomatology for genotypes 134 and 354** comparing to the wild-type ($p < 0.05$; **Figure 3.19B**). There was also a delay in the appearance of stem rot caused by *P. cinnamomi* in the *in vitro* infection assay for GNK2-Poplar genotype 212, a genotype with a similar gene expression to GNK2-Poplar 134 (**Figure 3.16C**). These observations might be indicators that the *Cast_GNK2-like* gene product could offer some degree of tolerance towards *P. cinnamomi* infection.

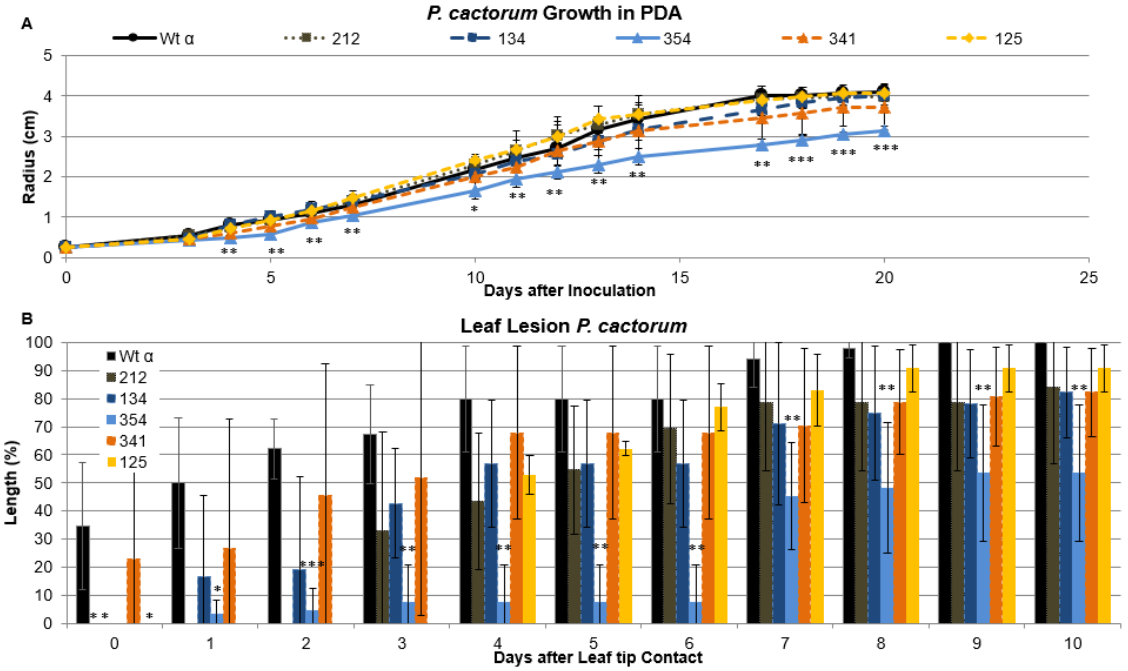

Figure 3.20 - Summary of the Leaf *P. cactorum* DSM 62637 infection assay with the five GNK2-Poplar genotypes. (**A**) Rough *P. cactorum* growth in PDA. (**B**) Observed leaf lesion, displayed as length percentage, after leaf contact. Both yellowing and necrosis were considered as leaf lesions. The asterisks symbolize statistical significance according with a two-tailed t-test: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$. *P. cactorum* growth on the 354 plate was considered statistically different from the 4th day ($p < 0.05$). **Wt α** – wild-type poplar (non-transformed); **212, 134, 354, 341, 125** – GNK2-Poplar genotypes.

GNK2-Poplar genotypes 212, 134, and 125 showed significant delay (one day) in symptomatology for *P. cactorum* when compared to the wild-type ($p < 0.1$; **Figure 3.20B**). **354 was the only GNK2-Poplar genotype that had consistently less *P. cactorum* lesion than the wild-type** ($p < 0.05$), which is in agreement with the symptomatology delay observed in the final *in vitro* assay (**Figure 3.15**). GNK2-Poplar **354 also had significantly lower *P. cactorum* growth** than the wild-type ($p < 0.05$), which might explain the previous observation. **This anomalous growth may be due to the natural variability encountered from *Phytophthora* growth, or due to synthesized pathogen defense-related molecules, potentially the Cast_GNK2-like protein, which contains a signal peptide to be secreted out of the cells** (**Figure 1.5**; Miyakawa, 2009). However, **the most likely explanation for the phenomenon is the first because *P. cactorum* never showed any signs of having its growth hindered by the presence of GNK2-Poplar in the previous assays**. Nevertheless, **GNK2-Poplar genotype 354 has shown some degree tolerance to *P. cactorum***, the most pathogenic wild poplar organism of the two *Phytophthora*, **in both this assay and the *in vitro* assay**.

Some of the GNK2-Poplar leaves would not become completely necrosed within the time-spawn of the assay, although *Phytophthora* was fully grown on the Petri dish. These leaves in particular **displayed red-colored spots on the green tissue, which might indicate activation of pathogen defense mechanisms, such as anthocyanin and phytoalexin accumulation**. Anthocyanins have been proven effective against oxidative stress-induced apoptosis, as these molecules serve as scavengers of free radicals (Fernández-López, 2020). **Phytoalexins** (alexos 'compounds warding off' + phytos 'plant') were actually discovered when *P. infestans* inoculated tuber disks of a resistant potato cultivar (Dixon R., 1986; Harborne, 1993). These are **low-weight, host-synthesized, broad-spectrum antimicrobial compounds triggered by exposure to microorganisms, which can accumulate to sufficient levels to result in limitation of microbial growth within plant cells** (Dixon R., 1986; Harborne, 1993; Lo & Nicholson, 1998).

Ideally, this assay should have been executed with **more leaf replicates per genotype** than three, but it was not possible to increase this number due to the limited amount of GNK2-Poplar material. The lack of consistency attained with the results from this assay came to prove once more that **a more accurately-representative assay of *Phytophthora* infection in nature is in order to understand the implications of *Cast_GNK2-like* presence in tolerance against *Phytophthora* infection**.

3.6. Gateway System Cloning of Promoter ET304 into pDONR P4-P1r and *Cast_GNK2-like* into pDONR221

The goal of this section was to **create a vector with the *Cast_GNK2-like* gene under the regulation of a poplar root-specific promoter**. The promoter chosen for this purpose was the **ET304** discovered on Qian Wu Master's Thesis (2004). The sequence of this promoter was predicted in the v3.0 of *Populus trichocarpa* genome, which was scanned for root-specific regulation sequences using the PLACE (Solovyev *et al.*, 2010), PlantCARE (Lescot *et al*., 2002) and PlantProm (Ilham *et al*., 2003; Amina *et al*., 2009) programs (**Figure 6.2**). The strategy chosen to achieve the cloning goal was the Gateway System, where fragments from the promoter and the gene had to be amplified via PCR with specific extremities for a recombination reaction between their respective entry vectors, and the plant destination vector, to occur (**Figure 2.5**). To clone using this strategy, it was necessary to request for two pDONR vectors: pDONR221 to carry the *Cast_GNK2-like* cDNA with *att*B1 and *att*B2 extremities, and pDONR P4-P1r to carry the ET304 promoter version of the *Populus tremula x alba* INRA 717-1B4 clone with *att*B4 and *att*B1r extremities (**Figure 2.5A**).

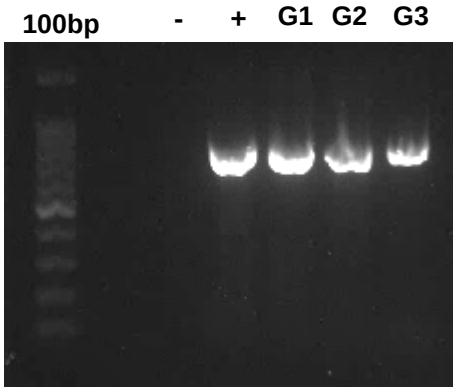

Figure 3.21: Colony PCR of pENTR221.GNK2 *E. coli* colonies: amplification of *GNK2-like att*B-PCR products. (**100bp**) 100 bp DNA Ladder from Promega. (**-**) A negative control with the PCR mix and primers. (**+**) Positive control with pFHi.GNK2 as a template. (**G1** to **G3**) Three kanamycin-resistant *E. coli* colonies displaying amplification of *Cast_GNK2-like*. 1.1% agarose gel ran at 70 V at 55 min.

The strategy proved to be **extremely easy and efficient for cloning the gene**, as it was possible to get more than three hundred potential pENTR221.GNK2 colonies (**Figure 3.21**), however, cloning the ET304 promoter was much more difficult than anticipated. **It was not possible to achieve true positive colonies for pENTRP4-P1r.ET304 presence** (**Figure 3.22**)**, even with several alterations to the protocols**. These alterations comprised of an increase in the equimolar ratio fragment-plasmid to 1:2, 1:3, and 1:5; letting the fragment and the plasmid sit at 40 ºC for 5 min to get rid of secondary structures before the BP reaction; and a slight alteration of the *att*B extremities according to a new reference. Furthermore, an **enzymatic restriction with EcoRV and PstI** was performed to ensure that the vector used on the cloning was pDONR P4-P1r and that the extracted plasmid did not contain the ET304 fragment (**Figure 3.23**). By sequencing the extracted plasmid, we discovered that **not only did the supposed pENTRP4-P1r.ET304 not contain the ET304 promoter, but it also did not contain the *ccdB* cassette**, as about 1 kb was missing from the fragment (**Figure 3.23 E EcoRV, and E PstI**).

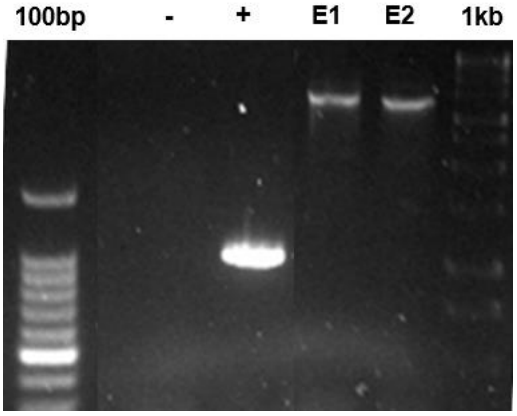

Figure 3.22: CloneAmp HiFi PCR using the extracted pENTRP4-P1r.ET304. The lanes correspond to: (**100bp**) 100 bp DNA ladder from Promega; (**-**) negative control with the PCR mix and primers, but without DNA template; (**+**) positive control with poplar DNA as template showing the ET304 band; (**E1** and **E2**) the template consisted on plasmids extracted from kanamycin-resistant colonies, only the pDONR P4-P1r plasmid band is visible, suggesting an auto-recombination of the plasmid; (**1kb**) 1 kb DNA ladder from Promega. 1% agarose gel ran at 100 V for 5 min, and then at 60 V for 1 h.

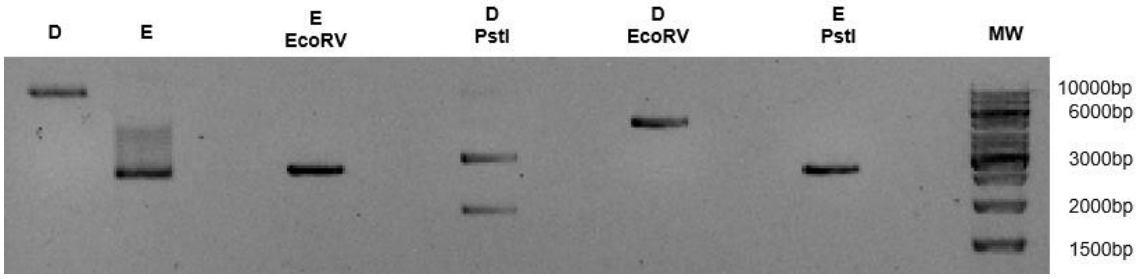

Figure 3.23: Enzymatic Restriction of pDONR P4-P1r and pENTRP4-P1r.ET304 with the EcoRV and PstI enzymes. (**D**) Circularized pDONR P4-P1r plasmid as the template. (**E**) Circularized potential pENTRP4-P1r.ET304 plasmid as the template. **pDONRP4-P1r should have 4777 bp** and **pENTRP4-P1r.ET304 should have about 4000 bp**. **pDONR P4-P1r + PstI should show a 2937 bp, and an 1850 bp band**. According to the gel, **pENTRP4-P1r.ET304 has about 3000 bp**. (**MW**) GeneRuler 1kb DNA ladder. 1% agarose gel ran at 100 V for 30 min.

The *att*B PCR product of the ET304 promoter was sequenced (**Figure 6.4**) and the result was compared with the predicted sequence attained for the INRA 717-1B4 genome (Mader *et al*., 2016; **Figure 6.3**). This comparison resulted in **Figure 6.5**, where it was possible to verify 91% of identities between the two sequences. This observation is a good indicator that the fragment amplified from INRA 717-1B4 was the desired one.

The Gateway System is a reliable cloning strategy that features simple, efficient, and highly specific protocols. Despite this, the strategy was not successful in cloning ET304 into pDONR P4-P1r in the screened conditions, as **the *att*B portions of the ET304 PCR product would not recombine with the *att*P portions of the plasmid**. Instead, **the *att*P portions of the plasmid seemed to recombine within themselves, resulting in a pDONR P4-P1r without its lethal cassette, which made the false-positive transformed cells viable**.

If it were possible to continue to engineer this vector, a good alternative would be to opt for **Cloning by Restriction Enzyme Digestion**. First, it would be required to choose an appropriate plant binary vector and decide on two MCS restriction sites absent in both *Cast_GNK2-like* cDNA and ET304 promoter sequences. Then, the two sequences would be amplified by PCR with primers coupled with the compatible restriction enzyme patterns. The resulting fragments would be cloned directly into the plant binary vector by adding in the appropriate enzymes and their respective buffers into the mix.

4. Conclusions and Future Work

With this work, it was possible to **generate transgenic poplar plants that constitutively expressed the *Cast_GNK2-like* gene**. **These plants**, along with the wild-type, **were exposed to *in vitro* infection assays to evaluate the interaction between *Phytophthora* spp. and the tree saplings**. Although **the transformed GNK2-Poplar showed delayed symptomatology and less severity towards *Phytophthora* infection**, an *in vitro* assay cannot accurately reflect the natural host-pathogen interaction that occurs in nature. As such, it would be interesting to **acclimate and pot the transformant genotypes generated by this work, and perform an *ex vitro* infection assay** to get more accurate results. ITQB NOVA would be a good location for this assay to take place, as assays with transformed plants can be envisaged to be conducted in its GMO-contained greenhouse. A histopathological approach to this assay could also be a good complement to the work. This approach could identify if an eventual tolerance could be attributed to a reduced interaction between host and pathogen, or simply due to inhibited *Phytophthora* growth by the antifungal protein.

This work was successful in providing insight that, although no reports in nature exist, ***P. cinnamomi* could infect the model tree grey poplar in the *in vitro* conditions employed**. As such, it can serve as a warning for future signs of *P. cinnamomi* symptoms in wild poplar.

Although *P. cactorum* caused slightly more severe symptoms, the infection results obtained for *P. cactorum* and *P. cinnamomi* were similar enough to be directly comparable. **All the tested transgenic lines displayed a delay in the appearance of *P. cactorum* symptoms** in at least one of the assays. However, the GNK2-Poplar genotypes that better tolerated one of the two pathogens did not always show enhanced infection tolerance for the other. By having more replicates per genotype, it would be possible to assess if these differences in the infection response were due to the distinct nature of both *Phytophthora*. Overall, we believe that including *P. cactorum* in these assays was a good decision as a safeguard, and to broaden the scope of this functional validation to more than one *Phytophthora* species.

Unfortunately, **it was not possible to obtain a vector with the *Cast_GNK2-like* gene under the regulation of the ET304 root-specific promoter by using the Gateway System** as a Cloning Strategy. Another cloning approach, such as **Cloning by Restriction Enzyme Digestion**, should be attempted in the future to attain this objective.

There is no doubt within the scientific community that, with the current global climate change events, ***P. cinnamomi* will disseminate its disease to new areas, while worsening its effects on currently-affected areas**. **Specific alternative methods must be available to battle against its infection**. One promising candidate for specific treatment against *Phytophthora* could be a natural sprayable solution containing active inhibitors of *Phytophthora* growth. If it were possible to verify the structure, integrity, and effectiveness of the *Cast_GNK2-like* protein product outside the cell, this protein would be a candidate to have in consideration for such a solution. This solution would be coming directly from a plant's natural genome, *C. crenata*, to fight a plant's natural problem, potentially devoid of any phytotoxicity for other plants, contrasting with the currently-applied chemical disinfectants.

5. References

[1] Amina U. Abdulazimova, Nurmamed Sh. Mustafayev, Yagut Yu. Akbarova, Victor V. Solovyev, Jalal A. Aliyev and Ilham A. Shahmuradov (2009) PlantProm DB, Release 2009.02 : plant promoter sequences. Nucleic Acids Res. (submitted).

[2] Bradshaw, H. D., Ceulemans, R., Davis, J., & Stettler, R. (2000). Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. *Journal of Plant Growth Regulation*, *19*(3), 306-313.

[3] Cerny, K., Strnadova, V., Gregorova, B., Holub, V., Tomsovsky, M., Mrazkova, M. and Gabrielova, S. (2009), *Phytophthora cactorum* causing bleeding canker of common beech, horse chestnut, and white poplar in the Czech Republic. Plant Pathology, 58: 394-394. doi:10.1111/j.1365-3059.2008.01970.x

[4] Chan, W. T., Verma, C. S., Lane, D. P., & Gan, S. K. E. (2013). A comparison and optimization of methods and factors affecting the transformation of Escherichia coli. Bioscience reports, 33(6), e00086.

[5] Constabel, C. P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiology*, *124*(1), 285-296.

[6] Corredoira, E., Valladares, S., Allona, I., Aragoncillo, C., Vieitez, A. M., & Ballester, A. (2012). Genetic transformation of European chestnut somatic embryos with a native thaumatin-like protein (CsTL1) gene isolated from Castanea sativa seeds. Tree physiology, 32(11), 1389-1402.

[7] Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., & Brasier, C. M. (2000). A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal genetics and biology*, *30*(1), 17-32.

[8] de Sousa Araújo, S., Duque, A. S. R. L. A., Dos Santos, D. M. M. F., & Fevereiro, M. P. S. (2004). An efficient transformation method to regenerate a high number of transgenic plants using a new embryogenic line of Medicago truncatula cv. Jemalong. Plant cell, tissue and organ culture, 78(2), 123-131.

[9] Derevnina, L., Dagdas, Y. F., De la Concepcion, J. C., Bialas, A., Kellner, R., Petre, B., ... & Aguilera-Galvez, C. (2016). Nine things to know about elicitins. New Phytologist, 212(4), 888-895.

[10] Dixon, R. A. (1986). THE PHYTOALEXIN RESPONSE: ELICITATION, SIGNALLING AND CONTROL OF HOST GENE EXPRESSION. Biological Reviews, 61(3), 239–291. doi:10.1111/j.1469-185x.1986.tb00719.x

[11] Dorrance, A. E., Robertson, A. E., Cianzo, S., Giesler, L. J., Grau, C. R., Draper, M. A., ... & Anderson, T. R. (2009). Integrated management strategies for Phytophthora sojae combining host resistance and seed treatments. *Plant disease*, *93*(9), 875-882.

[12] dos Santos, C. S. P. (2017). Genomic approaches to understand the genetic response to *Phytophthora cinnamomi* Rands in *Castanea spp*. (Doctoral dissertation)

[13] Duarte, A. S. D. S. (2015). Validation of candidate genes for resistance to *Phytophthora cinnamomi* in progenies of controlled crosses between Castanea sativa and Castanea crenata (Doctoral dissertation).

[14] Eggers, J. E., Balci, Y., & MacDonald, W. L. (2012). Variation among *Phytophthora cinnamomi* isolates from oak forest soils in the eastern United States. *Plant disease*, *96*(11), 1608-1614.

[15] Ersek, T., Schoelz, J. E., & English, J. T. (1994). PCR amplification of species-specific DNA sequences can distinguish among Phytophthora species. Applied and Environmental Microbiology, 60(7), 2616-2621.

[16] Fernández-López, J. A., Fernández-Lledó, V., & Angosto, J. M. (2020). New insights into red plant pigments: more than just natural colorants. RSC Advances, 10(41), 24669-24682.

[17] Frankel, S. J. (2008). Sudden oak death and Phytophthora ramorum in the USA: a management challenge. *Australasian Plant Pathology*, *37*(1), 19-25.

[18] Gao, N., Wadhwani, P., Mühlhäuser, P., Liu, Q., Riemann, M., Ulrich, A. S., & Nick, P. (2016). An antifungal protein from Ginkgo biloba binds actin and can trigger cell death. *Protoplasma*, *253*(4), 1159-1174.

[19] Gelvin, S. B. (2000). Agrobacterium and plant genes involved in T-DNA transfer and integration. Annual review of plant biology, 51(1), 223-256.

[20] Goodwin, S. B. (1997). The population genetics of *Phytophthora*. *Phytopathology*, *87*(4), 462-473. Review

[21] Guo, L. Y., & Ko, W. H. (1993). Two widely accessible media for growth and reproduction of Phytophthora and Pythium species. Applied and environmental microbiology, 59(7), 2323-2325.

[22] Hardham, A. R. (2005). *Phytophthora cinnamomi*. *Molecular plant pathology*, *6*(6), 589-604.

[23] Hardham, A. R., & Blackman, L. M. (2018). Phytophthora cinnamomi. Molecular plant pathology, 19(2), 260-285.

[24] Hanahan, D., Jessee, J., & Bloom, F. R. (1991). [4] Plasmid transformation of Escherichia coli and other bacteria. In *Methods in enzymology* (Vol. 204, pp. 63-113). Academic Press.

[25] Hantula, J., Lilja, A., Nuorteva, H., Parikka, P., & Werres, S. (2000). Pathogenicity, morphology and genetic variation of *Phytophthora cactorum* from strawberry, apple, rhododendron, and silver birch. *Mycological Research*, *104*(9), 1062-1068.

[26] Harborne, J. B. (1993). Higher Plant–Lower Plant Interactions: Phytoalexins and Phytotoxins. Introduction to Ecological Biochemistry, 264–297. doi:10.1016/b978-0-08-091858-7.50014-0

[27] Hausbeck, M. K., & Lamour, K. H. (2004). Phytophthora capsici on vegetable crops: research progress and management challenges. *Plant disease*, *88*(12), 1292-1303.

[28] Hood, E.E., Gelvin, S.B., Melchers, L.S. et al. Transgenic Research (1993) 2: 208. https://doi.org/10.1007/BF01977351

[29] Ilham A. Shahmuradov, Alex J. Gammerman, John M. Hancock, Peter M. Bramley and Victor V. Solovyev (2003) PlantProm: a database of plant promoter sequences. Nucleic Acids Res., 31, 114-117.

[30] ImProm-II™ Reverse Transcription System Technical Manual#TM236, Promega Corporation

[31] Jyothishwaran, G., Kotresha, D., Selvaraj, T., Srideshikan, S. M., Rajvanshi, P. K., & Jayabaskaran, C. (2007). A modified freeze–thaw method for efficient transformation of Agrobacterium tumefaciens. Current science, 93(6), 770-772.

[32] Karimi, M., Bleys, A., Vanderhaeghen, R., & Hilson, P. (2007). Building blocks for plant gene assembly. Plant physiology, 145(4), 1183-1191.

[33] Keča N., Milenković I., Keča L. (2015): Mycological complex of poplars in Serbia. J. For. Sci., 61: 169-174.

[34] Keren, I., Abudraham, S., Shaya, F., & Ostersetzer-Biran, O. (2011). An optimized method for the analysis of plant mitochondria RNAs by Northern-blotting. Endocytobiosis & Cell Research, (21).

[35] Leplé JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L (1992) Transgenic poplars: expression of chimeric genes using four different constructs. Plant Cell Rep 11:137-141. http://dx.doi.org/10.1007/BF00232166

[36] Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic acids research, 30(1), 325–327. https://doi.org/10.1093/nar/30.1.325

[37] Lemoine M: Amélioration des peupliers de la section Leuce sur sols hydromorphes. Ph.d. thesis, Universit6 de Nancy I, Nancy, France (1973).

[38] Lin, J. J., Assad-Garcia, N., & Kuo, J. (1995). Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by Agrobacterium tumefaciens cells. Plant Science, 109(2), 171-177.

[39] Liu, X., Wang, Z., Wang, D., & Zhang, J. (2016). Phylogeny of Populus-Salix (Salicaceae) and their relative genera using molecular datasets. Biochemical Systematics and Ecology, 68, 210-215.

[40] Lo, S. C. C., & Nicholson, R. L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls: implications for a compensatory role in the defense response. Plant Physiology, 116(3), 979-989.

[41] Mader, M., Le Paslier, M., Bounon, R., Bérard, A., Rampant, P., Fladung, M., Leplé, J., & Kersten, B. (2016). Whole-genome draft assembly of Populus tremula x P. alba clone INRA 717-1B4, Silvae Genetica, 65(2), 74-79. doi: https://doi.org/10.1515/sg-2016-0019

[42] Magnani, E., Bartling, L., & Hake, S. (2006). From Gateway to MultiSite Gateway in one recombination event. BMC molecular biology, 7(1), 46.

[43] Marchler-Bauer A et al. (2017), *"CDD/SPARCLE: functional classification of proteins via subfamily domain architectures."*, Nucleic Acids Res.45(D)200-3.

[44] Mélida, H., Sandoval-Sierra, J. V., Diéguez-Uribeondo, J., & Bulone, V. (2013). Analyses of extracellular carbohydrates in oomycetes unveil the existence of three different cell wall types. *Eukaryotic cell*, *12*(2), 194–203. https://doi.org/10.1128/EC.00288-12

[45] Miyakawa, T., Miyazono, K. I., Sawano, Y., Hatano, K. I., & Tanokura, M. (2009). Crystal structure of GINKBILOBIN-2 with homology to the extracellular domain of plant cysteine-rich receptor-like kinases. *Proteins: Structure, Function, and Bioinformatics*, *77*(1), 247-251.

[46] Miyakawa, T., Hatano, K. I., Miyauchi, Y., Suwa, Y. I., Sawano, Y., & Tanokura, M. (2014). A secreted protein with plant-specific cysteine-rich motif functions as a mannose-binding lectin that exhibits antifungal activity. *Plant physiology*, *166*(2), 766-778.

[47] Nauerby, B., Billing, K., & Wyndaele, R. (1997). Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of Agrobacterium tumefaciens. Plant Science, 123(1-2), 169-177.

[48] O'Neill, T. M. O., & Overvoorde, J. (1995). Comparison of chemical disinfectants for treatment of a glasshouse sand bed contaminated with *Phytophthora*.

[49] Rakoczy-Trojanowska, M. O. N. I. K. A. (2002). Alternative methods of plant transformation-a short review. *Cellular and molecular biology letters*, *7*(3), 849-858.

[50] Reece-Hoyes, J. S., & Walhout, A. (2018). Propagating Gateway Vectors. Cold Spring Harbor protocols, 2018(1), pdb.prot094920. https://doi.org/10.1101/pdb.prot094920

[51] Ruangpan, L., & Tendencia, E. A. (2004). Bacterial isolation, identification and storage. In *Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment* (pp. 3-11). Aquaculture Department, Southeast Asian Fisheries Development Center.

[52] Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

[53] Santos, C., Duarte, S., Tedesco, S., Fevereiro, P., & Costa, R. L. (2017). Expression profiling of *Castanea* genes during resistant and susceptible interactions with the oomycete pathogen *Phytophthora cinnamomi* reveal possible mechanisms of immunity. *Frontiers in plant science*, *8*, 515.

[54] Sawano, Y., Miyakawa, T., Yamazaki, H., Tanokura, M., & Hatano, K. I. (2007). Purification, characterization, and molecular gene cloning of an antifungal protein from Ginkgo biloba seeds. *Biological chemistry*, *388*(3), 273-280.

[55] Solovyev VV, Shahmuradov IA, Salamov AA. (2010) Identification of promoter regions and regulatory sites. *Methods Mol Biol*. 674, 57-83.

[56] Tang, H., Ren, Z., & Krczal, G. (2000). An evaluation of antibiotics for the elimination of Agrobacterium tumefaciens from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. Plant Cell Reports, 19(9), 881-887.

[57] Taylor, G., *Populus*: Arabidopsis for Forestry. Do We Need a Model Tree?, *Annals of Botany*, Volume 90, Issue 6, December 2002, Pages 681–689, https://doi.org/10.1093/aob/mcf255

[58] Silva, J. D., & Fukai, S. (2001). The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and Agrobacterium growth. J. Appl. Hort, 3(1), 3-12.

[59] Walkerpeach C.R., Velten J. (1994) *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. In: Gelvin S.B., Schilperoort R.A. (eds) Plant Molecular Biology Manual. Springer, Dordrecht.

[60] Waterhouse, G. M. (1963). Key to the species of Phytophthora de Bary. *Commonwealth Mycological Institute, Kew, UK, Mycol Papers*, *92*, 22.

[61] Wise, A. A., Liu, Z., & Binns, A. N. (2006). Culture and maintenance of Agrobacterium strains. In *Agrobacterium Protocols* (pp. 3-14). Humana Press.

[62] Yu, T. A., Yeh, S. D., & Yang, J. S. (2001). Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. Bot. Bull. Acad. Sin, 42, 281-286.

[63] Yu, Y., & Wei, Z. M. (2008). Influences of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. *Biologia plantarum, 52*(3), 553-556.

6. Supplementary Data

pFHi.GNK2 Sequence

GATCTGGGGAACCCTGTGGTTGGCATGCACATACAAATGGACGAACGGATAAACCTTTTCACGCCCT
TTTAAATATCCGATTATTCTAATAAACGCTCTTTTCTCTTAGGTTTACCCGCCAATATATCCTGTCaaaca
ctgatagtttaaacTGCCTCAATTCCGGAGGTGTTTCTAGTGTTCAACATGACAAACAAAACCCATCTCTTTCA
GTATATGTCTCTCAGTTGTGCTTAATTCAAATTTCAACTCAGAGAACTTCTTGGCATACTTATCCAGATT
ATCTAATGATCTCATCTAATGGTAATTCAACTTTCAGTATATGTCTCGCAGCAAACTATCTTTACATCAA
ATTTTTAACAACTCAATGCACAAAATACTTTTCCTCAACCTAAAAATAAGGCAATTAGCCAAAAACAACT
TTGCGTGTGAACAACGCGTACACGTCCCTACACATACGTGTCAATTTATAATTGGCTATTGCTTCCAC
GCCTTAGCTTTCTCGTGACCGACCGAGTCGTCCTCGTCTTTTTTGCTTCTATAAATCAAATACCCAAAG
AGCTCTTCTTCTTCACAATTCAGATTCCAATTTTCTCAAACTCTAAAATCAATCTCTCAAATCTCTCAAC
CGTGATCAAGGTAGATTTCTGAGTTCTTATTGTATTTCTTCGATTTGTTTCGTTCGATCGCAATTTAGGC
TCTGTTCTTTGATTTTGATCTCGTTAATCTCTGATCGGAGGCAAATTACATAGTTTCATCGTTAGATCTC
TTCTTATTTCTCGATTAGGGTTCGTATTTTTCGCAGATCTGTTTATTTTCTTGTTGTTTCCTTGTATTTGA
TCCGATTTGTTGAAAGAATTTGTGTGTTCTCGATTATTTACGCTTTGATCTGTGATTTTTATCTAGATTT
GGTGTTAGTTTCTTGTTTGTGCGATCGAATTTGTCGATTAATCTCGGTTTTTCTGATTAACAGAttaattaaA
TGTTGAGCTCAAAATATATTTCTGTCAGCTTTCTATTACTCAGCCTCTCCCTCCATGCAGTCAATTGTG
CTGACCCATTATACCATTTTTGTTTTAGCCAAGAAAGCTACACTGCCACTAGCCGTTATGGTACAAACT
TGAATGGCTTGCTCAATCTTTTGTCCACCAAAGTTCCTTCAAAAGGGTTTGGTCTCAGCTCGACTGGG
CAAGGCCAAGATCGAGCAAATGGTTTAGCCCTATGCCGGGGTGATGTCTCAAAAACAAACTGTACGA
CCTGTGTCATTGATGCAGGCAAAGAGCTTGGTAATCGTTGTCCTTATAAAAAAGGAGCGATAATTTGG
TATGATAACTGTCTTTTGAAGTACTCGAACATTGATTTCTTTGGAGAAATCGATAACAAAAACAAGTTCT
ACATGTGGAACGTCCAAGATGTAGAAAATCCCACTTCATTCAATCCAAAAGTTAAGGATTTGTTAAGCA
GGTTATCTAATAAAGCTTATGCCAATCCAAAATTCTATGCTACCGGGGACCTAAAGCTTGACTCATCAA
GCAAACTATATGGTTTGGCTCAATGCACCAGGGACCTATCAGGTCTTGATTGTAAGAAGTGTCTTGAT
ACTGCGATTAGTGAACTTCCCAACTGTTGCGATGGAAAACGAGGTGGGCGAGTTGTTGGTGGCAGTT
GTAACGTTAGATATGAACTTTACCCCTTTGTTGATGCCTAGGCggccgcCCGGCGCAAAAATCACCAGT
CTCTCTCTACAAATCTATCTCTCTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGA
ATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTT
GTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAATCCatttaaataggcgcgccgtcaACGGATCA
GGATATCCTTGTTTAAGATGTTGAACTCTATGGAGGTTTGTATGAACTGATGATCTAGGACCGGATAA
GTTCCCTTCTTCATAGCGAACTTATTCAAAGAATGTTTTGTGTATCATTCTTGTTACATTGTTATTAATG
AAAAAATATTATTGGTCATTGGACTGAACACGAGTGTTAAATATGGACCAGGCCCCAAATAAGATCCAT
TGATATATGAATTAAATAACAAGAATAAATCGAGTCACCAAACCACTTGCCTTTTTTAACGAGACTTGTT
CACCAACTTGATACAAAAGTCATTATCCTATGCAAATCAATAATCATACAAAAATATCCAATAACACTAA
AAAATTAAAAGAAATGGATAATTTCACAATATGTTATACGATAAAGAAGTTACTTTTCCAAGAAATTCAC
TGATTTTATAAGCCCACTTGCATTAGATAAATGGCAAAAAAAAACAAAAAGGAAAAGAAATAAAGCACG
AAGAATTCTAGAAAATACGAAATACGCTTCAATGCAGTGGGACCCACGGTTCAATTATTGCCAATTTTC
AGCTCCACCGTATATTTAAAAAATAAAACGATAATGCTAAAAAAATATAAATCGTAACGATCGTTAAATC
TCAACGGCTGGATCTTATGACGACCGTTAGAAATTGTGGTTGTCGACGAGTCAGTAATAAACGGCGTC
AAAGTGGTTGCAGCCGGCACACACGAGTCGTGTTTATCAACTCAAAGCACAAATACTTTTCCTCAACC
TAAAAATAAGGCAATTAGCCAAAAACAACTTTGCGTGTAAACAACGCTCAATACACGTGTCATTTTATT
ATTAGCTATTGCTTCACCGCCTTAGCTTTCTCGTGACCTAGTCGTCCTCGTCTTTTCTTCTTCTTCTCT
ATAAAACAATACCCAAAGAGCTCTTCTTCTTCACAATTCAGATTTCAATTTCTCAAAATCTTAAAAACTTT
CTCTCAATTCTCTCTACCGTGATCAAGGTAAATTTCTGTGTTCCTTATTCTCTCAAAATCTTCGATTTTG
TTTTCGTTCGATCCCAATTTCGTATATGTTCTTTGGTTTAGATTCTGTTAATCTTAGATCGAAGACGATT
TTCTGGGTTTGATCGTTAGATATCATCTTAATTCTCGATTAGGGTTTCATAAATATCATCCGATTTGTTC
AAATAATTTGAGTTTTGTCGAATAATTACTCTTCGATTTGTGATTTCTATCTAGATCTGGTGTTAGTTTCT
AGTTTGTGCGATCGAATTTGTCGATTAATCTGAGTTTTTCTGATTAACAGATGATTGAACAAGATGGAT
TGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAA
TCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGA
CCGACCTGTCCGGTGCCCTGAATGAACTCCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGA
CGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGG
GCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGC
TGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACAT
CGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAG
CATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGA
TCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGA
TTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
ATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCG
ATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCtgagggatcgttcaaacatttggcaataaagtttctt
aagattgaatcCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATA

ATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTT
AATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATG
TTACTAGATCGCACGTAGGGGggatccactagttctagagcggcgtgggccatcgccctgatagacggtttttcgccctttgacgttgga
gtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcgggctattcttttgatttataagggattttgccgatttcggaacc
accatcaaacaggattttcgcctgctggggcaaaccagcgtggaccgcttgctgcaactctctcagggccaggcggtgaagggcaatcagctgttgc
ccgtctcactggtgaaaagaaaaaccaccccagtacattaaaaacgtccgcaatgtgttattaagttgtctaagcgtcaatttGTTTACACCACA
ATATATCCTGCCACCAGCCAGCCAACAGCTCCCCGACCGGCAGCTCGGCACAAAATCACCACTCGAT
ACAGGCAGCCCATCAGTCCGGGACGGCGTCAGCGGGAGAGCCGTTGTAAGGCGGCAGACTTTGCTC
ATGTTACCGATGCTATTCGGAAGAACGGCAACTAAGCTGCCGGGTTTGAAACACGGATGATCTCGCG
GAGGGTAGCATGTTGATTGTAACGATGACAGAGCGTTGCTGCCTGTGATCAAATATCATCTCCCTCGC
AGAGATCCGAATTATCAGCCTTCTTATTCATTTCTCGCTTAACCGTGACAGTTGTCTATCGGCAGTTCG
TAGAGCGCGCCGTGCGTCCCGAGCGATACTGAGCGAAGCAAGTGCGTCGAGCAGTGCCCGCTTGTT
CCTGAAATGCCAGTAAAGCGCTGGCTGCTGAACCCCCAGCCGGAACTGACCCCACAAGGCCCTAGC
GTTTGCAATGCACCAGGTCATCATTGACCCAGGCGTGTTCCACCAGGCCGCTGCCTCGCAACTCTTC
GCAGGCTTCGCCGACCTGCTCGCGCCACTTCTTCACGCGGGTGGAATCCGATCCGCACATGAGGCG
GAAGGTTTCCAGCTTGAGCGGGTACGGCTCCCGGTGCGAGCTGAAATAGTCGAACATCCGTCGGGC
CGTCGGCGACAGCTTGCGGTACTTCTCCCATATGAATTTCGTGTAGTGGTCGCCAGCAAACAGCACG
ACGATTTCCTCGTCGATCAGGACCTGGCAACGGGACGTTTTCTTGCCACGGTCCAGGACGCGGAAGC
GGTGCAGCAGCGACACCGATTCCAGGTGCCCAACGCGGTCGGACGTGAAGCCCATCGCCGTCGCCT
GTAGGCGCGACAGGCATTCCTCGGCCTTCGTGTAATACCGGCCATTGATCGACCAGCCCAGGTCCTG
GCAAAGCTCGTAGAACGTGAAGGTGATCGGCTCGCCGATAGGGGTGCGCTTCGCGTACTCCAACAC
CTGCTGCCACACCAGTTCGTCATCGTCGGCCCGCAGCTCGACGCCGGTGTAGGTGATCTTCACGTCC
TTGTTGACGTGGAAAATGACCTTGTTTTGCAGCGCCTCGCGCGGGATTTTCTTGTTGCGCGTGGTGAA
CAGGGCAGAGCGGGCCGTGTCGTTTGGCATCGCTCGCATCGTGTCCGGCCACGGCGCAATATCGAA
CAAGGAAAGCTGCATTTCCTTGATCTGCTGCTTCGTGTGTTTCAGCAACGCGGCCTGCTTGGCCTCG
CTGACCTGTTTTGCCAGGTCCTCGCCGGCGGTTTTTCGCTTCTTGGTCGTCATAGTTCCTCGCGTGTC
GATGGTCATCGACTTCGCCAAACCTGCCGCCTCCTGTTCGAGACGACGCGAACGCTCCACGGCGGC
CGATGGCGCGGGCAGGGCAGGGGGAGCCAGTTGCACGCTGTCGCGCTCGATCTTGGCCGTAGCTT
GCTGGACCATCGAGCCGACGGACTGGAAGGTTTCGCGGGGCGCACGCATGACGGTGCGGCTTGCG
ATGGTTTCGGCATCCTCGGCGGAAAACCCCGCGTCGATCAGTTCTTGCCTGTATGCCTTCCGGTCAA
ACGTCCGATTCATTCACCCTCCTTGCGGGATTGCCCCGACTCACGCCGGGGCAATGTGCCCTTATTC
CTGATTTGACCCGCCTGGTGCCTTGGTGTCCAGATAATCCACCTTATCGGCAATGAAGTCGGTCCCG
TAGACCGTCTGGCCGTCCTTCTCGTACTTGGTATTCCGAATCTTGCCCTGCACGAATACCAGCGACCC
CTTGCCCAAATACTTGCCGTGGGCCTCGGCCTGAGAGCCAAAACACTTGATGCGGAAGAAGTCGGTG
CGCTCCTGCTTGTCGCCGGCATCGTTGCGCCACATCTAGGTACTAAAACAATTCATCCAGTAAAATAT
AATATTTTATTTTCTCCCAATCAGGCTTGATCCCCAGTAAGTCAAAAAATAGCTCGACATACTGTTCTTC
CCCGATATCCTCCCTGATCGACCGGACGCAGAAGGCAATGTCATACCACTTGTCCGCCCTGCCGCTT
CTCCCAAGATCAATAAAGCCACTTACTTTGCCATCTTTCACAAAGATGTTGCTGTCTCCCAGGTCGCC
GTGGGAAAAGACAAGTTCCTCTTCGGGCTTTTCCGTCTTTAAAAAATCATACAGCTCGCGCGGATCTT
TAAATGGAGTGTCTTCTTCCCAGTTTTCGCAATCCACATCGGCCAGATCGTTATTCAGTAAGTAATCCA
ATTCGGCTAAGCGGCTGTCTAAGCTATTCGTATAGGGACAATCCGATATGTCGATGGAGTGAAAGAG
CCTGATGCACTCCGCATACAGCTCGATAATCTTTTCAGGGCTTTGTTCATCTTCATACTCTTCCGAGCA
AAGGACGCCATCGGCCTCACTCATGAGCAGATTGCTCCAGCCATCATGCCGTTCAAAGTGCAGGACC
TTTGGAACAGGCAGCTTTCCTTCCAGCCATAGCATCATGTCCTTTTCCCGTTCCACATCATAGGTGGT
CCCTTTATACCGGCTGTCCGTCATTTTTAAATATAGGTTTTCATTTTCTCCCACCAGCTTATATACCTTA
GCAGGAGACATTCCTTCCGTATCTTTTACGCAGCGGTATTTTTCGATCAGTTTTTTCAATTCCGGTGAT
ATTCTCATTTTAGCCATTTATTATTTCCTTCCTCTTTTCTACAGTATTTAAAGATACCCCAAGAAGCTAAT
TATAACAAGACGAACTCCAATTCACTGTTCCTTGCATTCTAAAACCTTAAATACCAGAAAACAGCTTTTT
CAAAGTTGTTTTCAAAGTTGGCGTATAACATAGTATCGACGGAGCCGATTTTGAAACCACAATTATGGA
CTGCCAGCGCTGCCATTTTTGGGGTGAGGCCGTTCGCGGCCGAGGGGCGCAGCCCCTGGGGGGAT
GGGAGGCCCGCGTTAGCGGGCCGGGAGGGTTCGAGAAGGGGGGGCACCCCCCTTCGGCGTGCGC
GGTCACGCGCACAGGGCGCAGCCCTGGTTAAAAACAAGGTTTATAAATATTGGTTTAAAAGCAGGTTA
AAAGACAGGTTAGCGGTGGCCGAAAAACGGGCGGAAACCCTTGCAAATGCTGGATTTTCTGCCTGTG
GACAGCCCCTCAAATGTCAATAGGTGCGCCCCTCATCTGTCAGCACTCTGCCCCTCAAGTGTCAAGG
ATCGCGCCCCTCATCTGTCAGTAGTCGCGCCCCTCAAGTGTCAATACCGCAGGGCACTTATCCCCAG
GCTTGTCCACATCATCTGTGGGAAACTCGCGTAAAATCAGGCGTTTTCGCCGATTTGCGAGGCTGGC
CAGCTCCACGTCGCCGGCCGAAATCGAGCCTGCCCCTCATCTGTCAACGCCGCGCCGGGTGAGTCG
GCCCCTCAAGTGTCAACGTCCGCCCCTCATCTGTCAGTGAGGGCCAAGTTTTCCGCGAGGTATCCAC
AACGCCGGCG

Figure 6.1 – pFHi.GNK2 sequence (single strand). Color code (in the same order as the sequence): Right border; UBQ11 Promoter; Cast_GNK2; 35S Terminator; UBQ10 Promoter; nptII; NOS Terminator; Left border. See **Figure 2.1**.

ET304 Promoter Fragment

ACTGTTTGTGTAGGTGATGAAAGGATCATACAAAGAATTAATGAAATGGCCATGCAAAGAA
TGTGGATGAGTTGATATGATCATATCTAATTTTGCTTGCTGGCCTCTGCGTTTTTTTTACTG
GATTGATTTCTTCTTTTCATTAATTCTTTCCGGATTTTGGGATTTCTTCAATCGCTGGCGAAC
TTTATCTTTGATATAGTAGTTCTATAGCTTCTTTTTTTTTTTTTTCATATTTTAATATGTTAATA
TATAGTACAATTGATCATTTATTCTTCCTCATACACGTGCAATGAAATAAGTAGTACAATTAA
ACTAATCATTGCTTTGTTTTAATTTTGAGAAGATGATTAAATCAATTGTTAAATAGTAATTAGT
AGCATGATTATATATAGTGTTACACTTGTACTTGTATATTAATAAAATACCCAATTGTCCATT
GAATTGAAAAGTACTCAATTAATGGTTTTATTTTTCTAAGATGAATTACTTCCCTTTTTTTAGG
GTAATTTGCAAAGTATTAATATCAAATTAGATTAACTTTTTTAATTACAAAGAAAAGACTAGAA
CACAAGATCTCTTGAGAAAAAAGAATTTTATTGTCAGTTCAGTTATGTGCTCGTTGACGATA
CTTGATTAATTAATTACTGAGCATTAATTAAGTTTACTTTCTCTAAATTAATCATTTCTGTTCC
AAGGAATCAATTAAATTAATATTGCATAATGAAGGAAAAAAATTTGATAGCATTTTGTTTTAA
TAAGACTAACTTCAAATGCTTTAAAGAATGATGAATATTTATATATCTTGTAGCTTATCTAGTT
AAGAATATTCTGTATCATTCTCATCGAAAGAAAGAAACAAAACCATGTATAAATTAAATTAAG
AAGGGTTAATAATCTCATGGAGCCCAATGATGATATATTCAAGAATAAGTTAATAAAAGATA
CAAATTACATACATTCAAAGACTTGTGCAATATTTTAACAATGAAAGTTGTATTTGTTTATTAC
CATATTGCAGAGACTAGAGA

Figure 6.2 – ET304 Promoter from *Populus trichocarpa* v3.0 genome with predicted Promoter sequences. Root-specific motives are highlighted in brown. The remaining highlights represent regular plant promoter-associated motives.

TGGATTGATTTCTTCTTTTCATT----CTTTCTGGATTTTGGGATTTCTTCAATCGCTGG
CGAACGTTATCTTTGATATAGTAGTTCTATAGCT-CTTTTTTTTTCCCTTCATATTTTAA
TATGTTAATATATAGTACAATTGATCATTTATTCTTTCTCATACACGTGCAATGAAATAA
GCAGTACAATTAAACTAATCATTGTTTTGTTTTAATTTTGAGAAGATGATTAAATCAATT
GTTAAATAGTAATTAGTAGCGTGATTACATATAGTGTTACACTTCTACTTGTATATTAAT
AACATACCCAATCGTCCATTGAATTGAAAAATACTCAATTAATGGGTTTTTTTTTTCTAA
GATGAATTACTTCCCCTTTTTTAGGGTAATTTGCAAAGCATTAATATCAAATCAGATTAA
-TTTTTTTATTACAAAGAAAAGGTTTGAACATGAGATCTCTCGAGAGAAAAAGAATTCTA
TTATCAGTTCAGTTACATGCTCGTTGACGACACTTGATTAACTAATTACTGAG---TAAT
TAAGCTTACCTTCTCTAAATTAATCATTCCTGATTCAAGGAATCAAATAAATTAATATTG
CATAATGAAGGAAAAAACTTTGATAGCATTTTCTTTTCATAATACTAACTTCAAATGCTT
GAAAGAATGATGAATATTTATATATTTTGTAGCTTATCTAGTTAAGAATATTCTGTATCA
TTCTCA-CGAAAGAAAGAAACAAAACCATGCATGTATAAATTAAATTAAGAAGGGTTAAT
AAACTCATGGAGCCCAATGATGATATATTCAAGAATAAGTAAAGAAAAGATACAAATTAC
ATACATTCAAAGACTTGTGCAATATTTTAACAATGAAAGTTGTATTTGTTTATTACCATA
TTGTAGAGACTAGAGA

Figure 6.3 – Predicted ET304 Promoter in *Populus alba x tremula* INRA 717-1B4 (Mader *et al*., 2016).

NNNNNTNNNNNGGGNNNNNNNTTTNNNNAGAAAAGTTGACTGTNNTGTAGGTGATGAAATG
NTCATACAAAGAATTAATGAATGGCCATGCAAAGAATGKGGATTGATTTCTTCTTTYMTTCT
TTCTGGATTTTGAGATTTCTTCAATCGCTGGCGAACTTTATCTTTGATATAGTAGTTCTATAG
CTCTCTTTTTTCCCCTTCATATTTTAATATATTAATATATAGTACAATTGATCATTTATTCTTTC
TCATACACATGTAATGAAATAAGCAGTACAATTAAACTAATCATTGTTTTGTTTTAATTTTGA
GAAGATTAAATCAATTGTTAAATAGTAATTAGTAGCATGATTATATATAGTGTTACACTTCTA
CTTGTGTATTAATAACATACCCAATCGTCCATTGAATTGAAAAATACTCAATTAATGGGTTTT
GTTTTCTAAGATGAATTACTTCCCCTTTTTTAGGGTAATTTGCAAAGCATTAATATCAAATTA
GATTAATTTTTTTATTATAAAGAAAAGGCTTGRACATGAGATCTYTCAAGAAAAAAGAAAAAG
AATTTTATTATCAGTTCAGTTACATGCTCGTTGMCGACACTTAATTAATTAATTASTGAGTAA
TTAAGTTTACCTTCTCTAAATTAATCATTCCTCATTCAAGGAATCAAATAAATTAATATTGCAT
AATGAAGGAAAAAAATTTGATAGCATTTTCTTTTCATAAAACTAACTTCAAATGCTTTAAAGA
ATGATGAATATTTATATATTTTGTAGCTTATCTAGTTAAGAATATTCTGTATCATTCTCACGAA
AGAAAGAAACAAAACTATGTATAAATTAAATTAANGAAGGGWTARTAAWCTCATGGNAGCN
CCAATGATGATATNATTCWAGAATAAGTTAAGNAAAAAATACRAATCTACATACATTCANAW
GACTTGNNNNAGTNNNATTATCNATGCAAAGTNGTATTGTTTAGTACCATATTGCAGAGACT
AGAAKACAAAGTTTGTACNNAANAANNNNNCCCCNNANCCAA

Figure 6.4 – Consensus sequence of the isolated ET304 Promoter from *Populus alba x tremula* INRA 717-1B4 obtained with BioEdit Sequence Alignment Editor. This sequence was obtained using the Optimal Global Pairwise Alignment, with an Alignment score of 1544 and 0.8384321 identities.

Figure 6.5 – BLAST of the Consensus Sequence obtained for the ET304 Promoter against the Predicted ET304 Promoter in *Populus alba x tremula* INRA 717-1B4. The two sequences achieved 91% of identities.

Primers 5'→ 3'

Primers to Validate the presence of *Cast_GNK2-like* in *A. tumefaciens*

F: GCGTACACGTCCCTACACATAC

R: AAGATTGAGCAAGCCATTCAAG

Product Size: 704bp

Primers to search for *Cast_GNK2-like* cDNA before RT-PCR

F: GCCGGGGTGATGTCTCAAAA

R: AACTCGCCCACCTCGTTTTC

Product Size: 431bp

Primers to search for *Cast_GNK2-like* cDNA in RT-PCR

F: TGTCTTGATACTGCGATTAGTGAAC

R: CCCACCTCGTTTTCCATC

Product Size: 57bp

Primers to clone *Cast_GNK2-like* from the plasmid pFHi.GNK2 into pDONR221

F: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAATGTTGAGCTCAAAATATATTTCTG

B1 sequence

R: GGGGACCACTTTGTACAAGAAAGCTGGGTGCCGCCTAGGCATCAACAAAGGGGTAAA

B2rev sequence

Product Size: 792bp

Primers to clone the ET304 promoter variant from *Populus tremula x alba* into pDONR P4-P1r

F: GGGGACAACTTTGTATAGAAAAGTTGACTGTTTGTGTAGGTGATGAAAGG

B4 sequence

R: GGGGACTGCTTTTTTGTACAAACTTGTCTCTAGTCTCTGCAATATGGTAA

B1rev sequence

Product Size: 1083bp

Bacterial Transformation with pFHi.GNK2

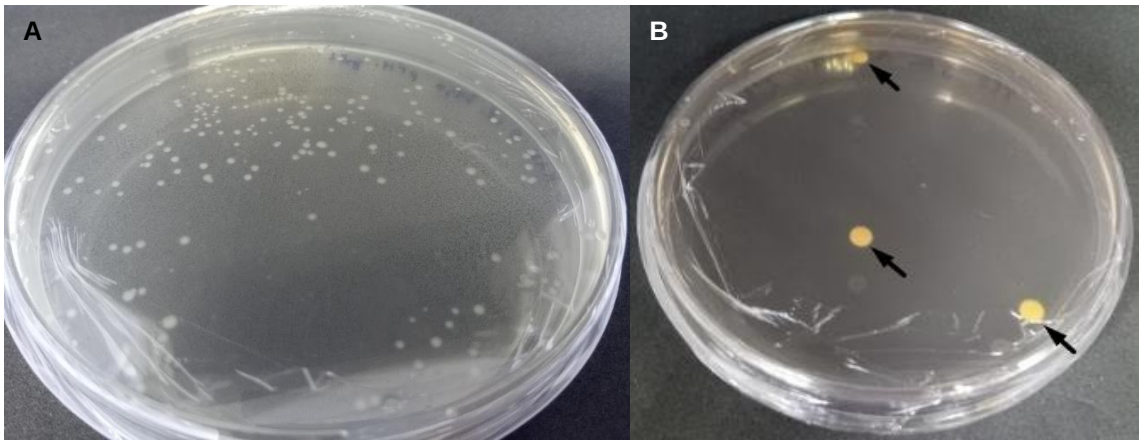

Figure 6.6 - *Agrobacterium* Transformation with the pFHi.GNK2 plasmid. **(A)** DH5α *E. coli* transformants containing the pFHi.GNK2 plasmid growing in LB medium supplemented with 50 mL/L kanamycin. **(B)** EHA105 *A. tumefaciens* transformants containing the pFHi.GNK2 plasmid growing in YEB medium supplemented with 50 mL/L rifampicin and 50 mL/L kanamycin.

***Agrobacterium*-mediated Poplar Transformation**

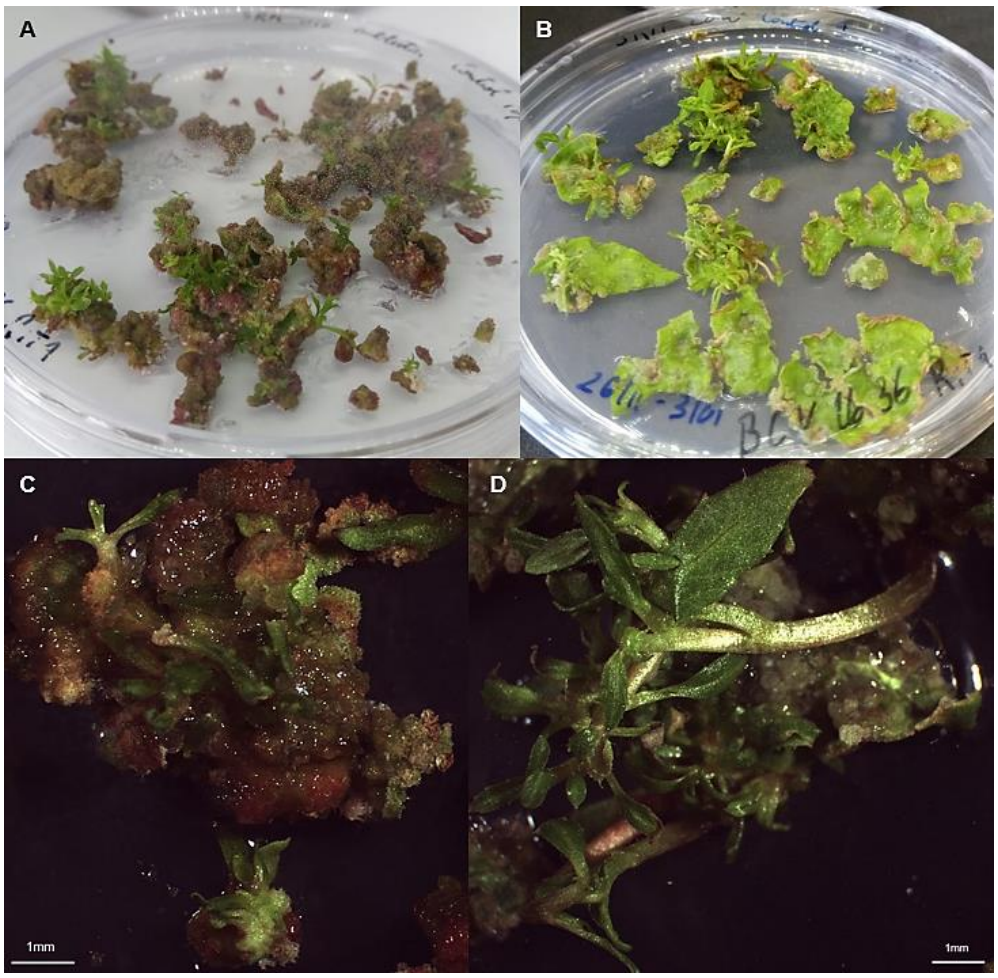

Figure 6.7 - Regenerants from grey poplar transformation positive controls. (**A**) Petri dish of the 10th week after co-culture. The medium does not contain carbenicillin. (**B**) Petri dish of the 6th week after co-culture. The medium contains 500 mg/L carbenicillin. (**C**) Eight-week-old shoots from medium without antibiotics. (**D**) Six weeks-old shoots from medium with 500 mg/L carbenicillin. Photos **C** and **D** were taken with an amplification of 12.5X.

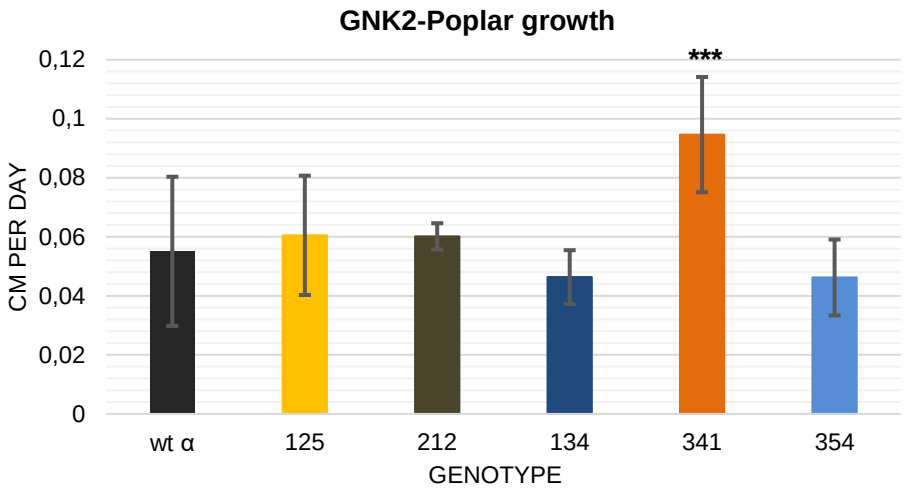

Figure 6.8 - Growth rate of the poplar plants used for the final *in vitro Phytophthora* infection assay. GNK2-Poplar genotype 341 had considerably higher growth rate than the wild-type (wt α). The asterisks symbolize statistical significance according with a two-tailed t-test: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$.

Preliminary *in vitro* Infection Assay in GUS-Poplar

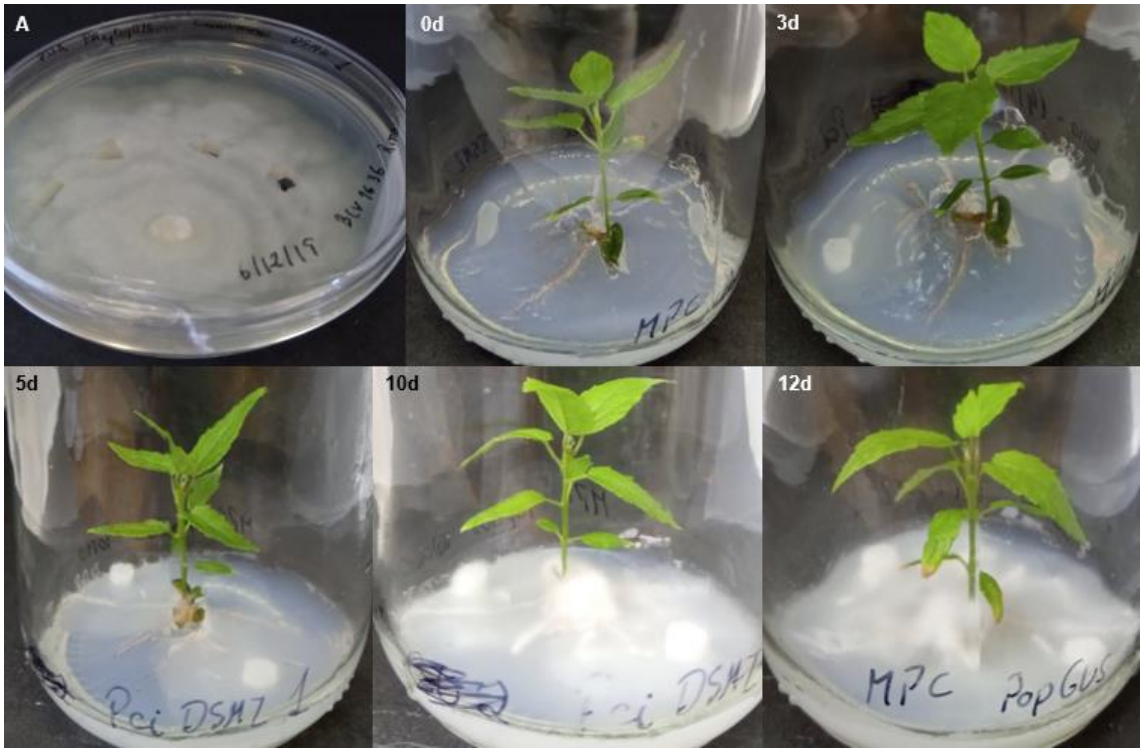

Figure 6.9 – *P. cinnamomi* (DSM 62654) *in vitro* infection of GUS *Populus tremula x alba*. (**A**) 14-days-old *P. cinnamomi* growing in PDA from which the inoculum was excised. (**0**-**12d**) Photos of a single inoculated plant throughout 12 days. *Phytophthora* growth can be observed in ½ MS medium but major symptomatology could not be detected.

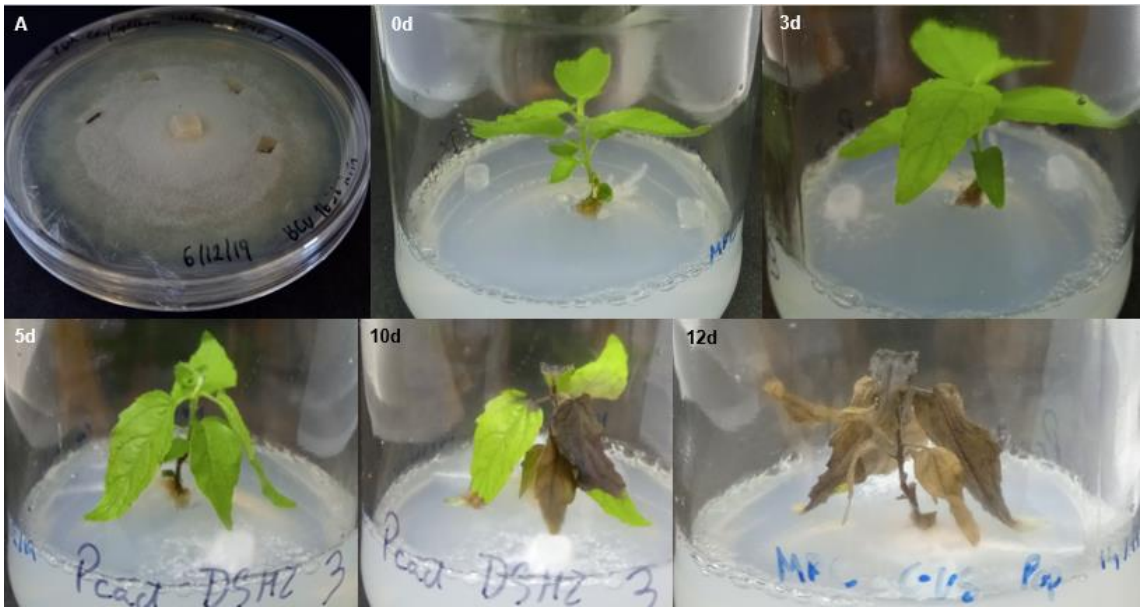

Figure 6.10 – *P. cactorum* (DSM 62637) *in vitro* infection of GUS *Populus tremula x alba*. (**A**) 14-days-old *P. cactorum* growing in PDA from which the inoculum was excised. (**0**-**12d**) Photos of a single inoculated plant throughout 12 days. *Phytophthora* growth can be observed in ½ MS medium. When the hyphae reached the poplar's stem collar, dieback and stem rot began to be visible. By the 12th day, the plant was considered dead, as all the tissue exhibited necrosis.

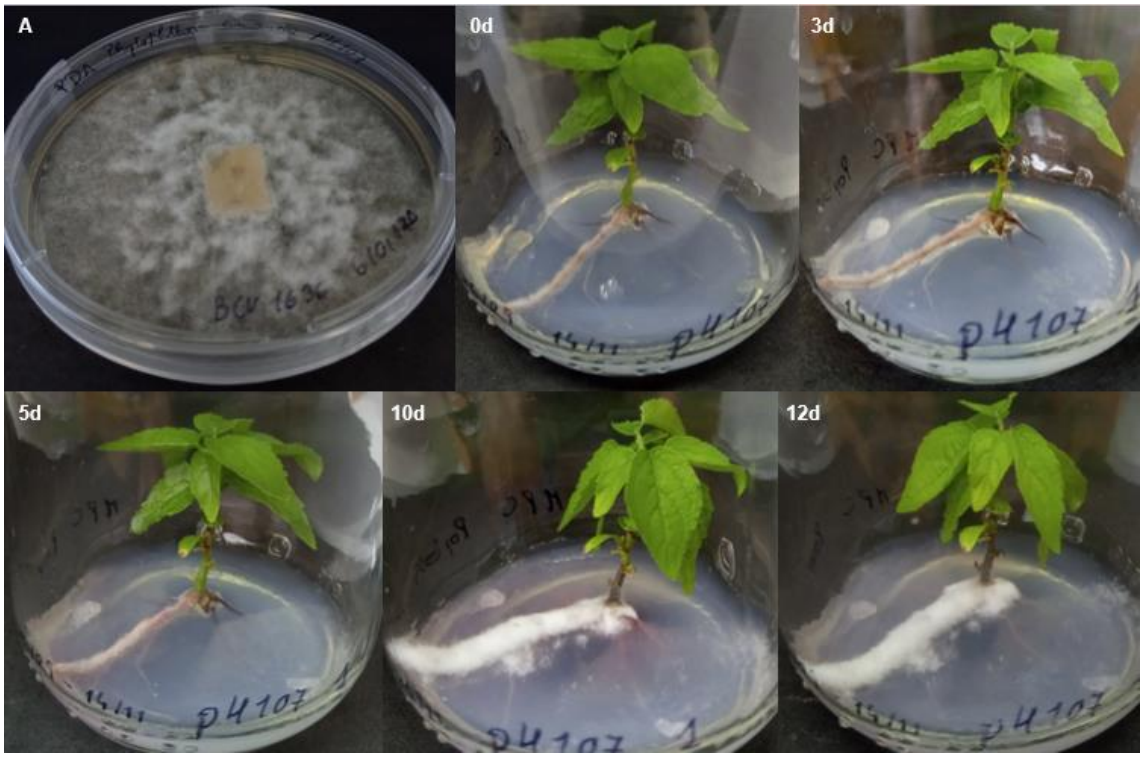

Figure 6.11 – *P. cinnamomi* pH107 *in vitro* infection of GUS *Populus tremula x alba*. (**A**) 14-days-old *P. cinnamomi* pH107 growing in PDA from which the inoculum was excised. (**0**-**12d**) Photos of a single inoculated plant throughout 12 days. *Phytophthora* growth can be observed in ½ MS medium. *Phytophthora* hyphae showed some affinity towards the exposed root from this plant, but it was not possible to verify root rot. When the hyphae reached the stem collar, the plant started to exhibit dieback and stem rot symptoms.

Final *in vitro* Infection Assay (First Experiment – Wild type, 341 and 354 GNK2-Poplar)

Figure 6.12 – *P. cinnamomi* pH107 *in vitro* infection in wild-type *Populus tremula x alba* (first experiment of the final assay). (**1-15d**) Photos of a single inoculated plant throughout 15 days.

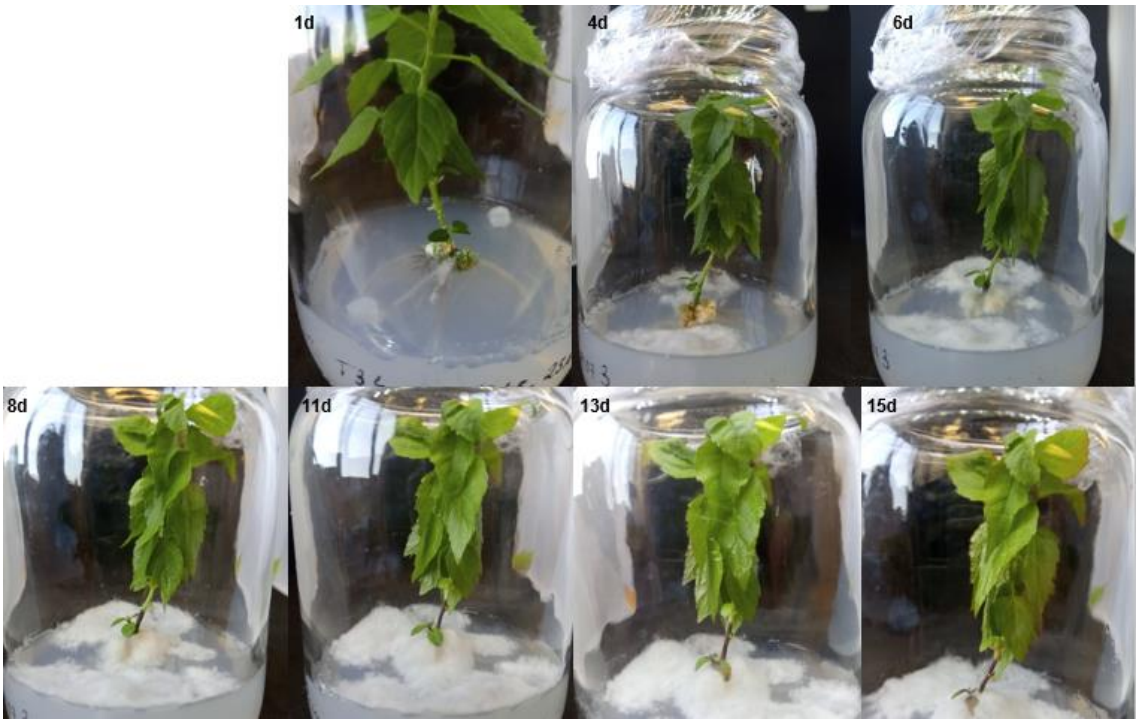

Figure 6.13 – *P. cinnamomi* pH107 *in vitro* infection in 341 GNK2-Poplar. (**1-15d**) Photos of a single inoculated plant throughout 15 days.

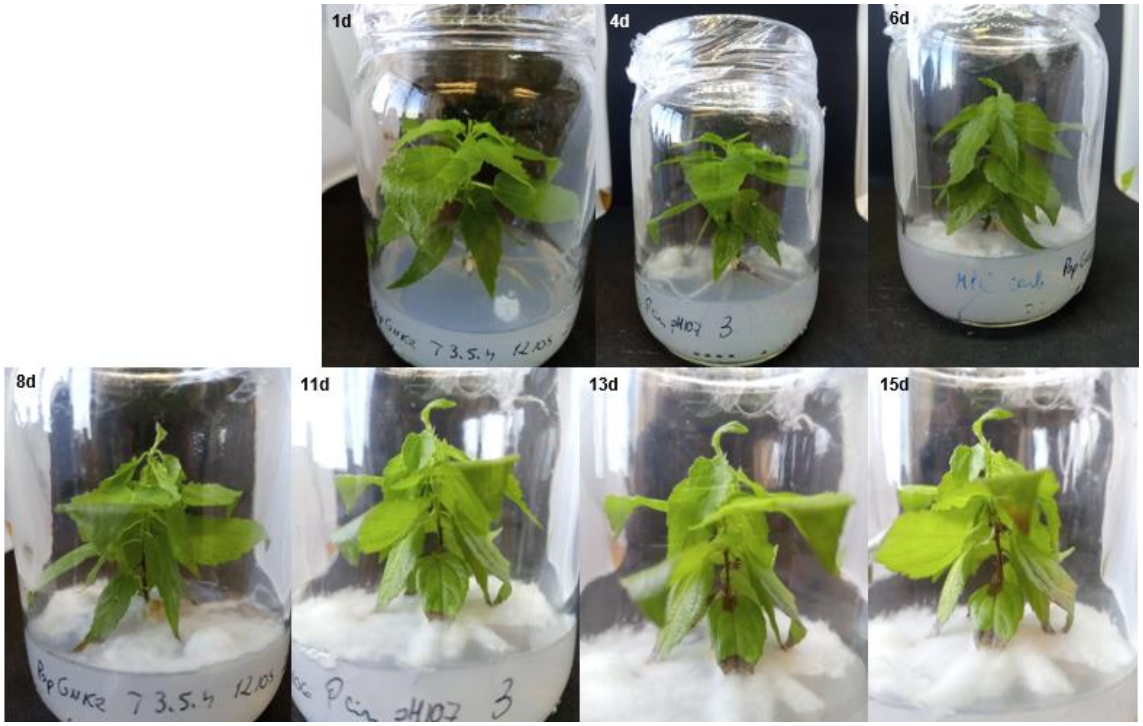

Figure 6.14 – *P. cinnamomi* pH107 *in vitro* infection in 354 GNK2-Poplar. (**1-15d**) Photos of a single inoculated plant throughout 15 days.

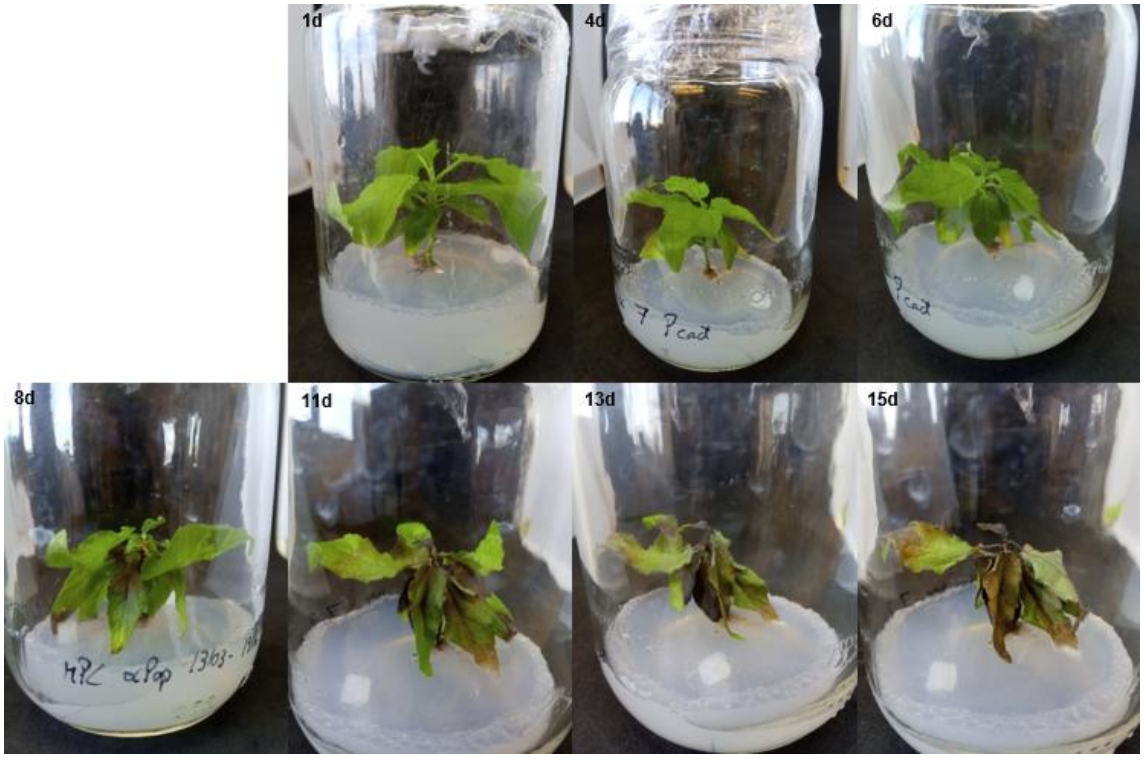

Figure 6.15 – *P. cactorum* DSM 62637 *in vitro* infection in wild-type *Populus tremula x alba* (first experiment of the final assay). (**1-15d**) Photos of a single inoculated plant throughout 15 days.

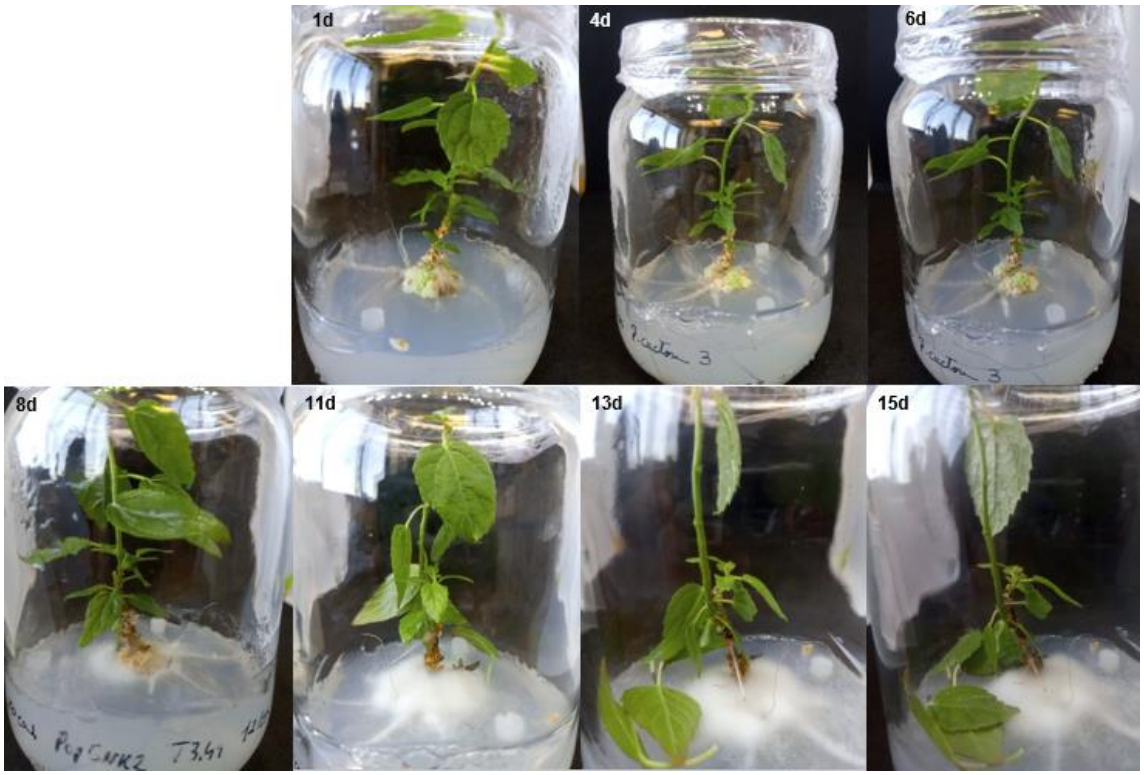

Figure 6.16 – *P. cactorum* DSM 62637 *in vitro* infection in 341 GNK2-Poplar (first experiment of the final assay). (**1-15d**) Photos of a single inoculated plant throughout 15 days.

Figure 6.17 – *P. cactorum* DSM *in vitro* infection in 354 GNK2-Poplar. (**1-15d**) Photos of a single inoculated plant throughout 15 days.

Final *in vitro* Infection Assay (Second Experiment – Wild-type, 212, 134 and 125 GNK2-Poplar)

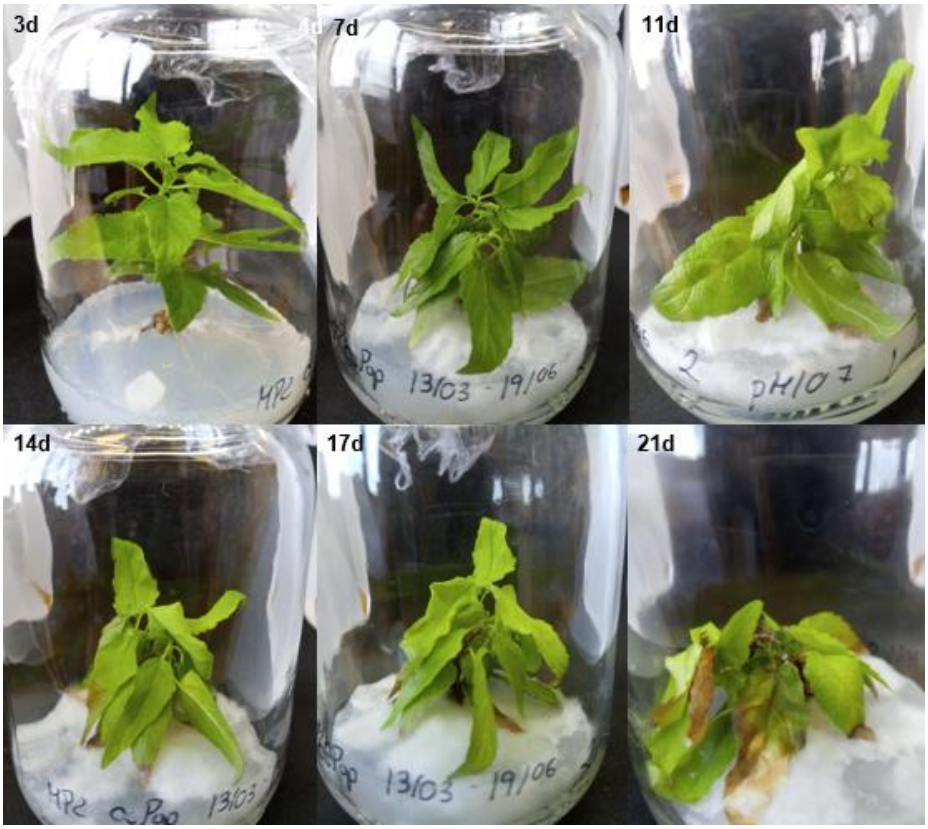

Figure 6.18 – *P. cinnamomi* pH107 *in vitro* infection in wild-type *Populus tremula x alba* (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

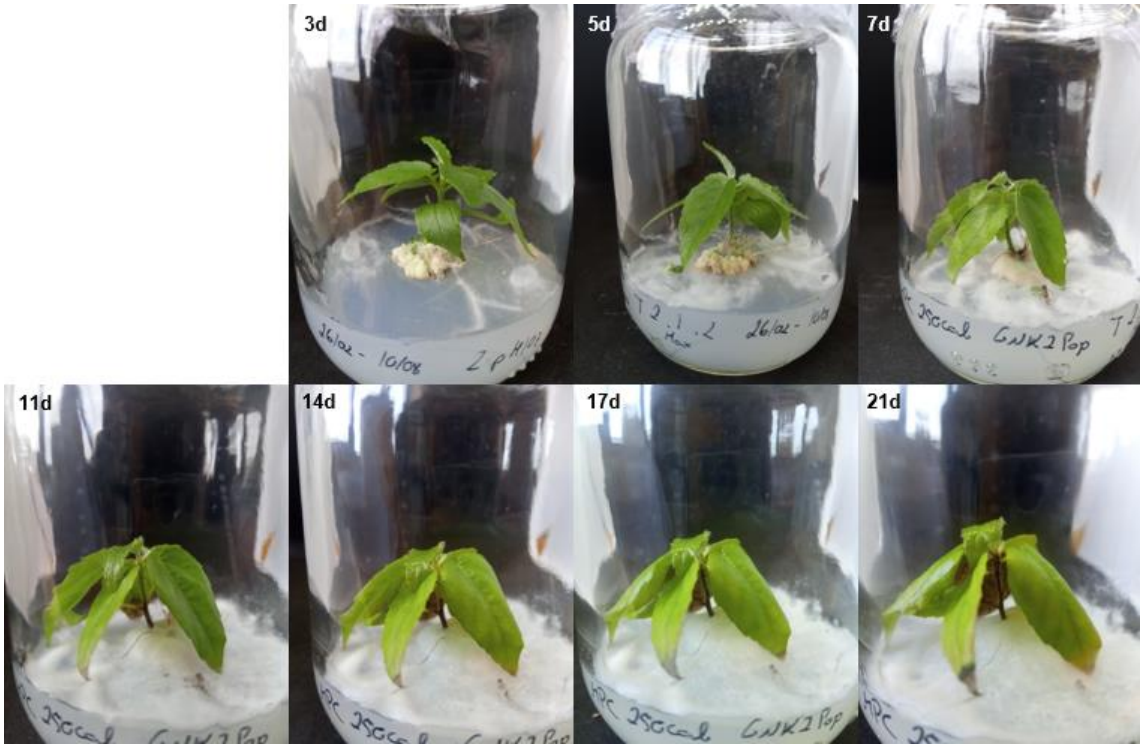

Figure 6.19 *P. cinnamomi* pH107 *in vitro* infection in 212 GNK2-Poplar (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

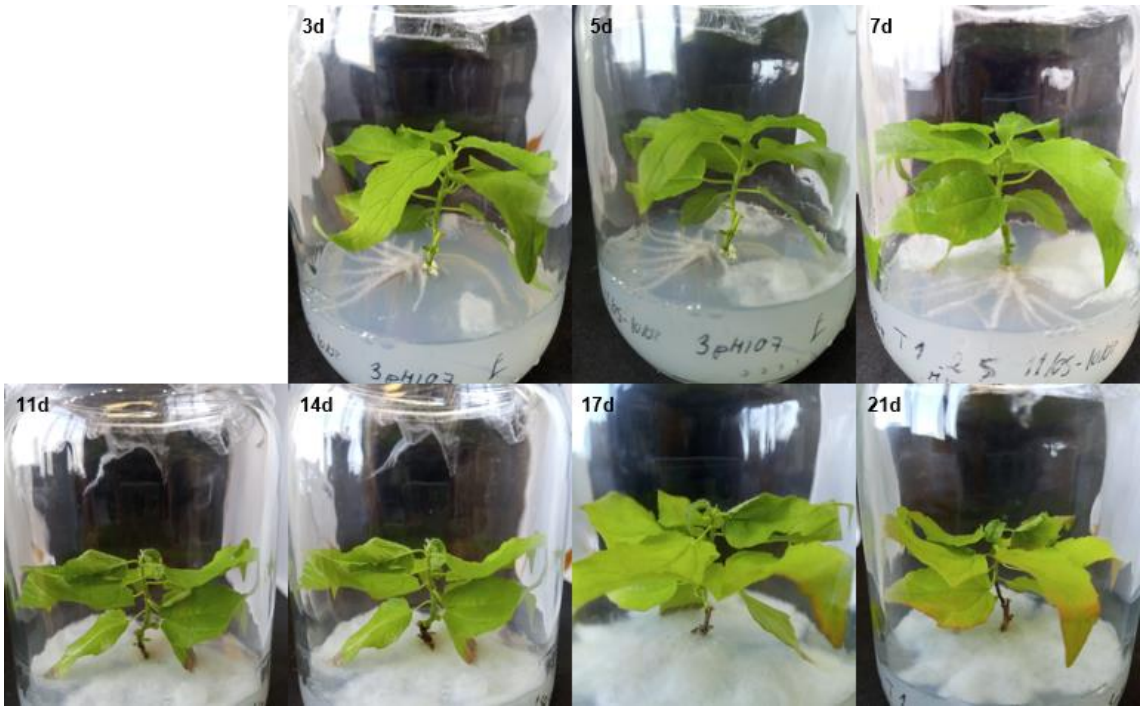

Figure 6.20 – *P. cinnamomi* pH107 *in vitro* infection in 125 GNK2-Poplar (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

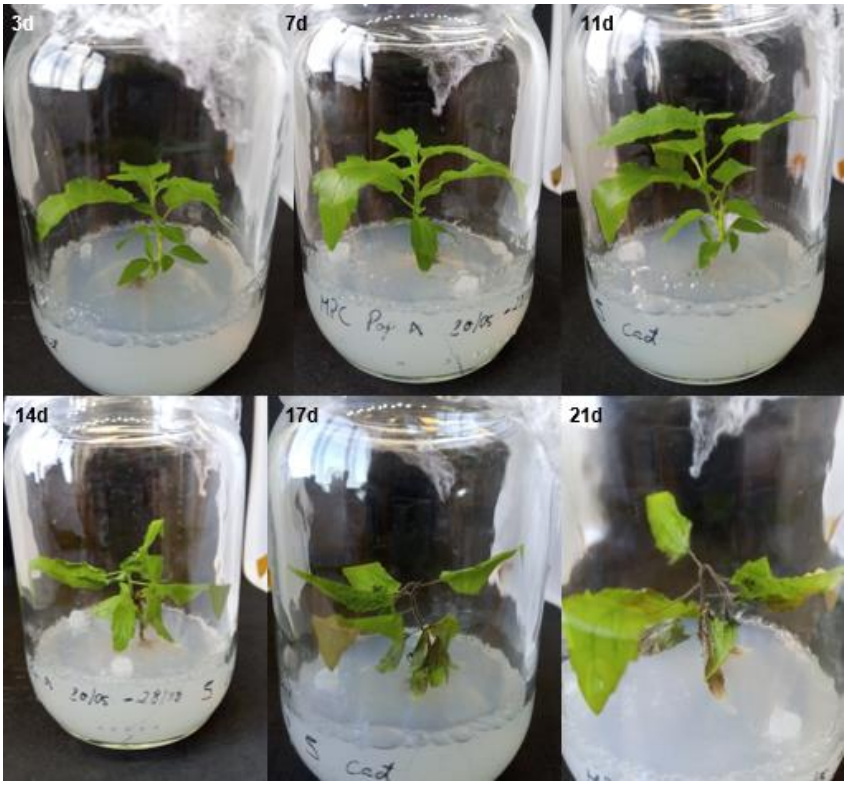

Figure 6.21 – *P. cactorum* DSM 62637 *in vitro* infection in wild-type *Populus tremula x alba* (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

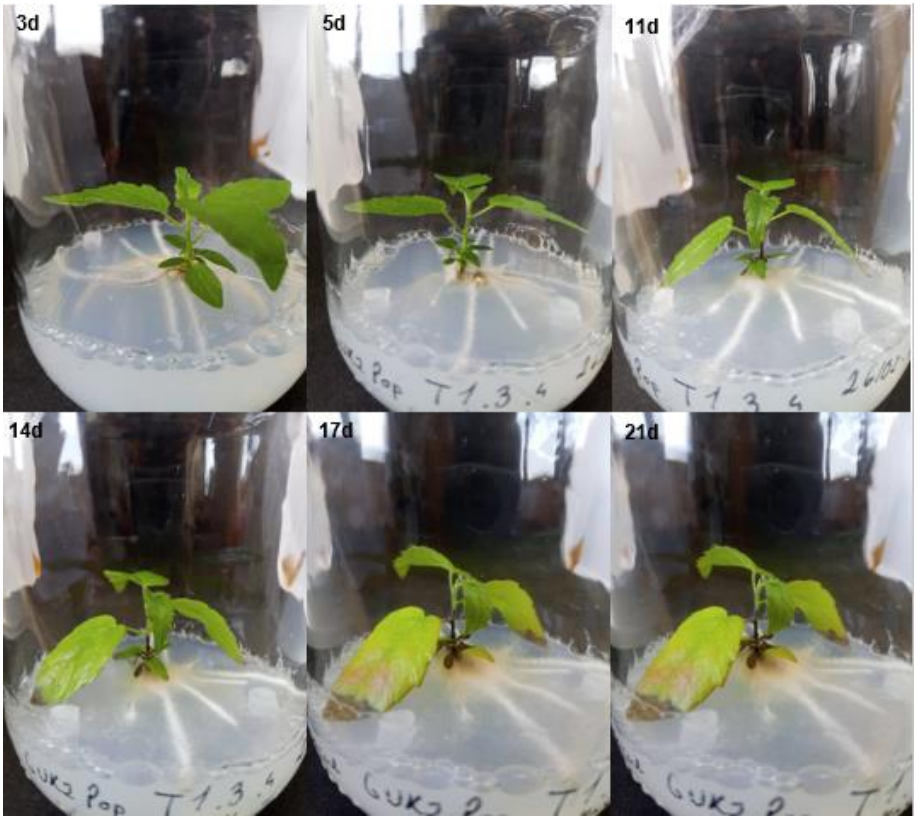

Figure 6.22 – *P. cactorum* DSM 62637 *in vitro* infection in 134 GNK2-Poplar (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

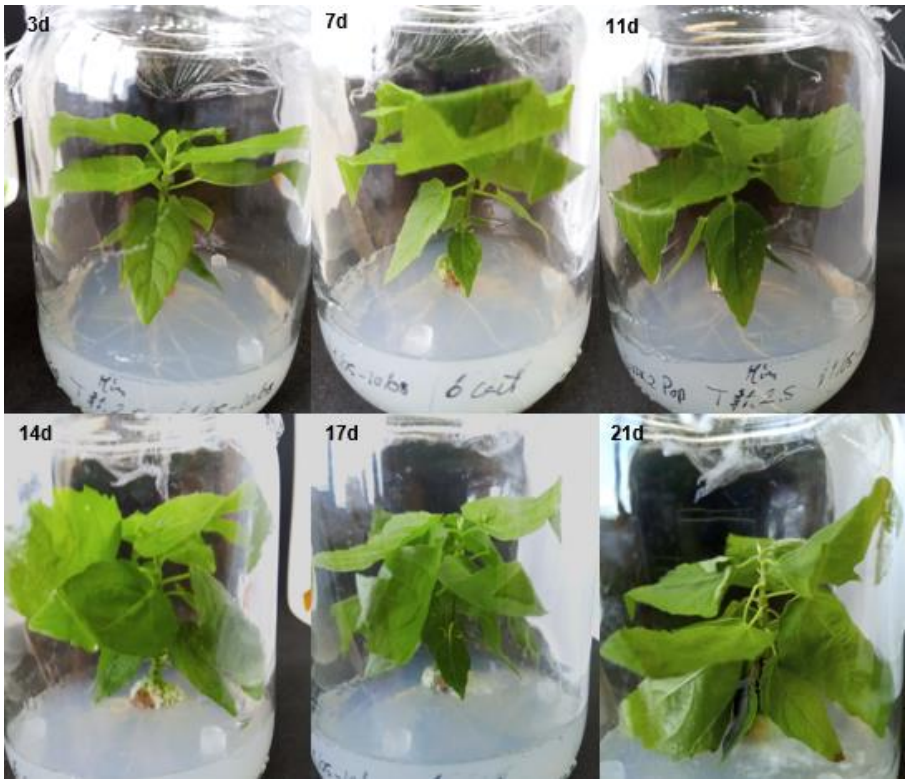

Figure 6.23 – *P. cactorum* DSM 62637 *in vitro* infection in 125 GNK2-Poplar (second experiment of the final assay). (**3-21d**) Photos of a single inoculated plant throughout 21 days.

Leaf Infection Assay – Wild type, 212, 341, 354, 134 and 125 GNK2-Poplar

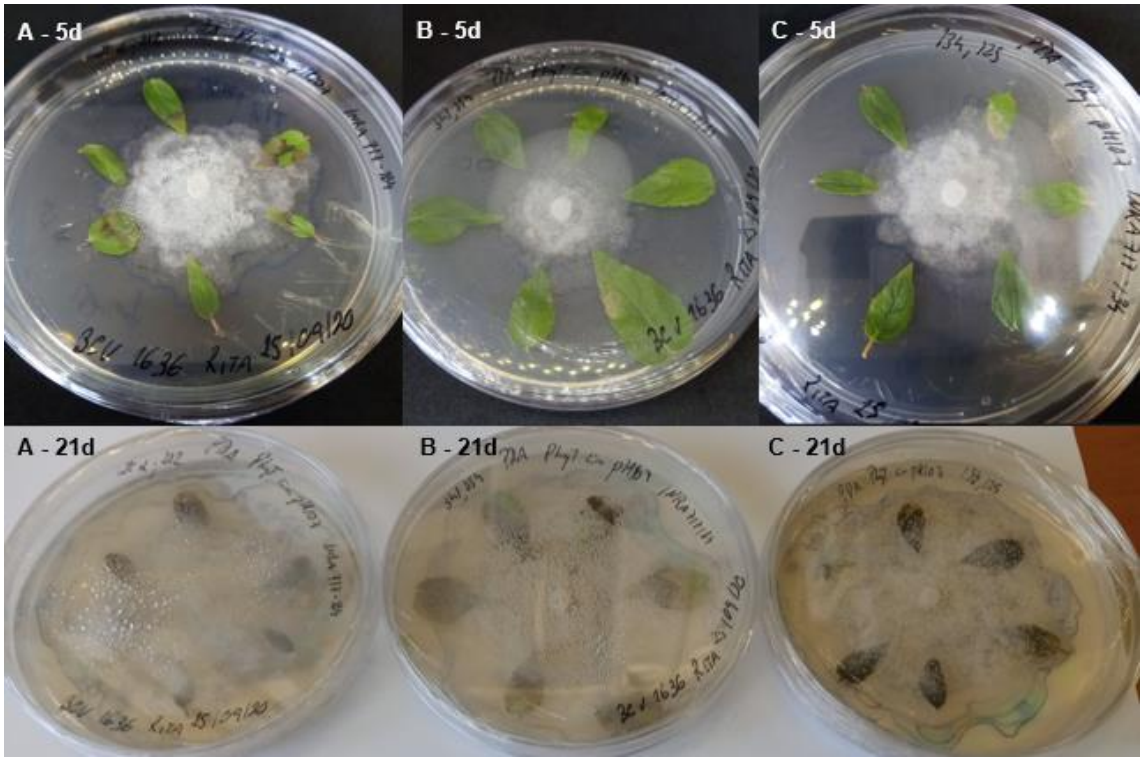

Figure 6.24 - *In vitro* Infection of Poplar leaves with *P. cinnamomi* pH107. (**A**) Plate with leaves from wild-type and 212 GNK2-Poplar genotypes. (**B**) Plate with leaves from 341 and 354 GNK2-Poplar genotypes. (**C**) Plate with leaves from 134 and 125 GNK2-Poplar genotypes. (**5d**) Photo taken five days after inoculation. (**21d**) Photo taken on the 21st day of infection.

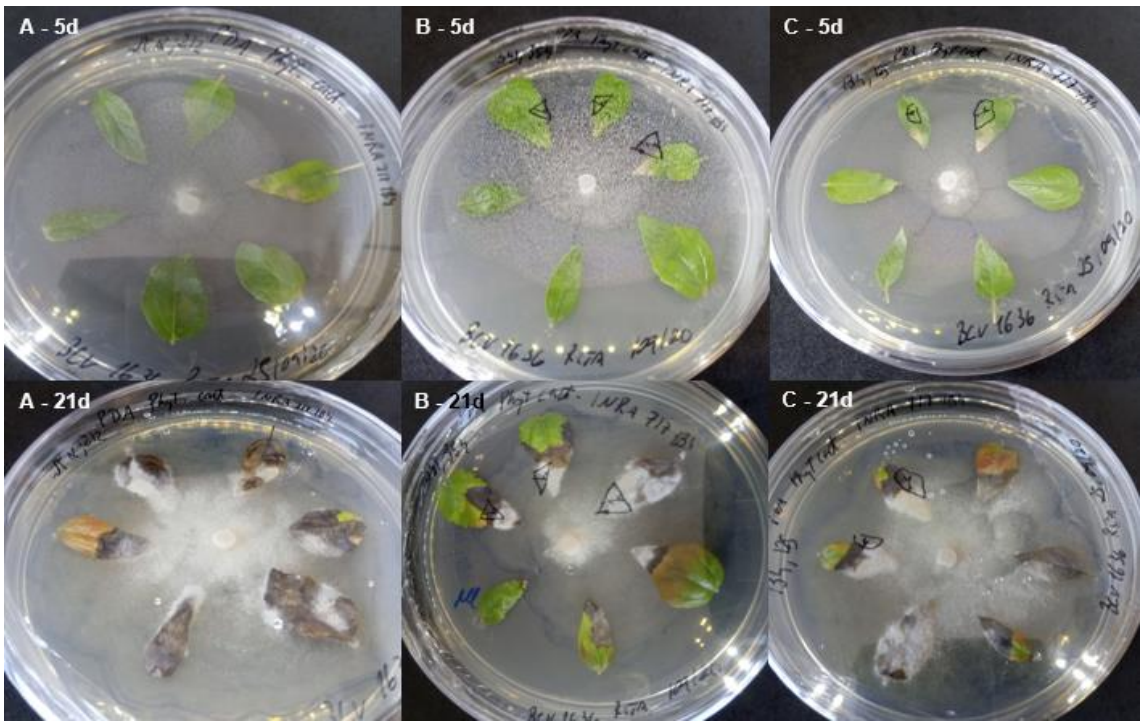

Figure 6.25 - *In vitro* Infection of Poplar leaves with *P. cactorum* DSM 62637. (**A**) Plate with leaves from wild-type and 212 GNK2-Poplar genotypes. (**B**) Plate with leaves from 341 and 354 GNK2-Poplar genotypes. (**C**) Plate with leaves from 134 and 125 GNK2-Poplar genotypes. (**5d**) Photo taken five days after inoculation. (**21d**) Photo taken on the 21st day of infection.