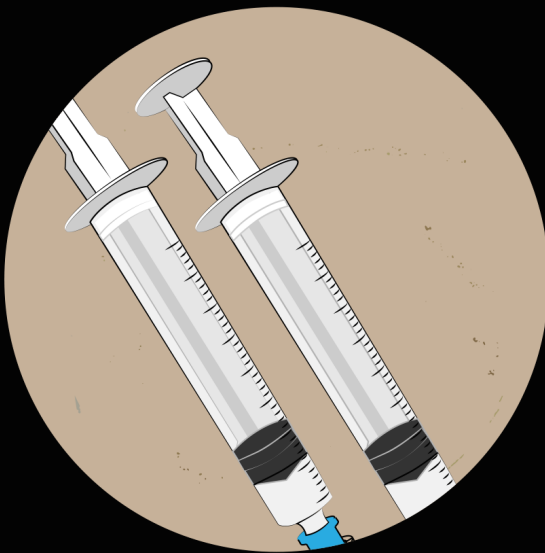
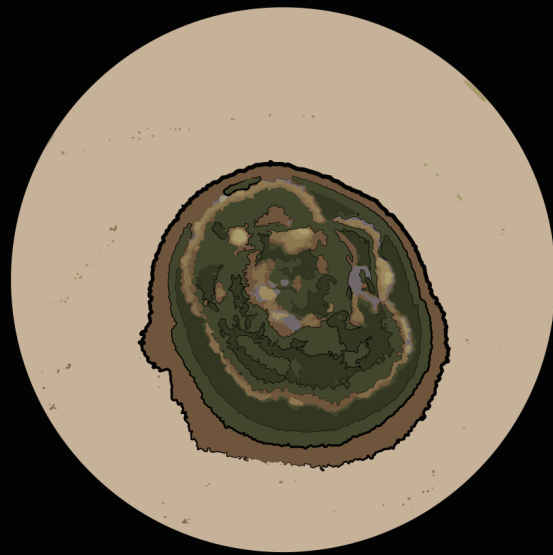
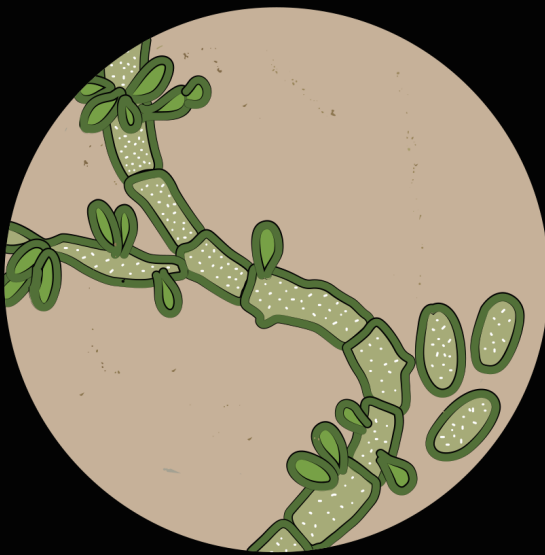


Management of *Gremmeniella abietina* by means of biological control agents and host resistance

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BIOLOGICAL CONTROL AGENTS AND HOST RESISTANCE

MANEJO DE *GREMMENIELLA ABIETINA* A TRAVÉS DE AGENTES DE
CONTROL BIOLÓGICO Y RESISTENCIA DEL HOSPEDANTE

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Abstract

Gremmeniella abietina (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karst) v Höhn) is a pathogenic fungus that produces severe damage in coniferous forests and reforestations, causing the death of the trees in Central and Northern Europe, North America and Japan. The pathogen was detected for first time in Spain in 1929 on *Pinus pinaster* (Aiton) and isolated in 1999 on Aleppo pine (*Pinus halepensis* (Mill)) stands in Palencia province. Although no epidemic outbreaks have been registered yet in Spain it causes serious damages to trees affected by the disease. The main symptoms observed consist of dry needles, crown defoliation, branches with some distortion of terminal twigs and eventual death of the trees. The control measures in other countries to prevent or reduce the expansion and incidence of the disease may include some silvicultural practices such as pruning branches or removing dead trees and the application of synthetic fungicides, but mostly as an emergency measure on nurseries. The need of management of the disease, together with the European recommendations of using non-chemical methods, make necessary to provide alternatives to the fight against the pathogen. Therefore, the main objective of the present thesis was to explore the use of alternative methods to control *G. abietina* infections. In order to achieve this objective, we performed four experiments in which we tested the use of several biological control agents (fungal endophytes, their filtrates, mitovirus-infected isolates) and the use of resistant hosts to prevent or reduce the infection.

The first two experiments were made with fungal endophytes and their filtrates because it was previously reported that these organisms were able to reduce or inhibit the growth of several pathogens by different modes of action. The fungal endophytes (first experiment) and their filtrates (second

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experiment) were applied to Aleppo pine seedlings as well as the *G. abietina* isolates were inoculated into the seedlings. Furthermore, to explore the mode of action of these biological control agents, the total phenol content of the plants after the infection was measured as an indicator of the activation of the plant defense system. The content of UV-absorbing compounds of the filtrates was also measured to explore the possible presence of antibiotic substances. At the end of the experiment (6 months after infections) the necrosis produced by the advance of the pathogen was measured in all the seedlings. In order to verify the results, both experiments were repeated 3 weeks after the first inoculation took place. In the third experiment, we tested whether the presence of several mitoviruses (i.e., viruses associated with fungi from the genus *Mitovirus* and sometimes related to a decrease in the fitness of the pathogen) in the isolates of *G. abietina*, was able to modify the behavior and pathogenicity of the fungus both on *in vitro* conditions and on *in vivo* on Aleppo pine seedlings. Lastly, in the fourth experiment, a provenance trial was performed with five different Spanish provenances of Aleppo pine in order to explore if any of them was resistant to the disease. The necrosis length produced by the advance of the pathogen was once again used as response variable. In addition, the concentration of two flavanone compounds as putative resistance indicator of the plants was measured and quantified.

The presence of all the endophytes used in our experiments as well as their filtrates significantly reduce the advance of the *G. abietina* in the seedlings although sometimes the significance depended on the time of inoculation. When using the endophytes as biological control agents, the total phenolic content of the plant did not increase. Therefore, the mechanisms involved in that reduction may be the competition, the parasitism, the production of antibiotic compounds or a combination of them. Furthermore, low-molecular weight phenolic compounds could be detected in some but

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not all filtrates, suggesting that although the antibiosis was likely involved in that reduction, the endophytes probably use several modes of action. The results from the virus experiment showed that mitovirus-infected isolates presented larger mycelial growth than the mitovirus-free ones when at the fungi's optimal growing temperature of 15 °C. In the greenhouse experiment, larger necrosis lengths were observed in the plants inoculated with mitovirus-infected isolates, suggesting that the presence of viruses in *G. abietina* isolates could lead to a hypervirulence of the pathogen. Lastly, the Aleppo pine provenances tested in the fourth experiment presented different levels of susceptibility to the pathogen, in terms of necrosis and visual severity. The amount of naringenin flavanone was significantly different among provenances, suggesting that it is a possible indicator value for the resistance of the provenances.

Resumen

Gremmeniella abietina (Lagerberg) Morelet (anamorfo *Brunchorstia pinea* (P. Karst) v Höhn) es un hongo patógeno que produce daños severos en bosques y plantaciones de coníferas, causando la muerte de árboles en el centro y norte de Europa, en Norteamérica y en Japón. El patógeno fue detectado por primera vez en España en 1929 en árboles de pino marítimo (*Pinus pinaster* (Aiton)) y aislado en 1999 de masas de pino carrasco (*Pinus halepensis* (Mill)) en la provincia de Palencia. A pesar de que aún no se han registrado brotes epidémicos en España, causa problemas serios a los árboles que afecta. Los principales síntomas que produce son acículas secas, defoliación de la copa, distorsión de ramillos terminales y eventualmente la muerte de algunos pies. Las medidas de control en otros países para prevenir o reducir la expansión e incidencia de la enfermedad incluyen prácticas selvícolas como podar ramas o eliminar árboles muertos y la aplicación de fungicidas sintéticos, aunque únicamente como medida de emergencia en viveros. La necesidad de gestión de la enfermedad junto con las recomendaciones europeas de usar métodos que no sean químicos, hacen necesario proveer de alternativas para luchar contra la enfermedad. Por ello, el principal objetivo de la presente tesis fue indagar en el uso de métodos alternativos para el control de las infecciones producidas por *G. abietina*. Para conseguir este objetivo, llevamos a cabo cuatro experimentos en los que se probó el uso de diferentes agentes de control biológico (hongos endófitos, sus filtrados y aislados infectados con virus) así como el uso de hospedantes resistentes.

Los dos primeros experimentos se realizaron con hongos endófitos y sus filtrados, ya que se había comprobado previamente que estos organismos son capaces de reducir o inhibir el crecimiento de diferentes patógenos a

través de varios mecanismos. Los endófitos (primer experimento) y sus filtrados (segundo experimento) se aplicaron a plantas de pino carrasco junto con los aislados de *G. abietina* para producir la infección. Además, para explorar los mecanismos que emplean estos agentes de control biológico para luchar contra el patógeno, se midió la concentración de fenoles totales en las plantas tras la infección, como un indicador de la activación del sistema de defensa de la planta. También se midió el contenido en compuestos de los filtrados para explorar la posible presencia de sustancias antibióticas. Al final del experimento (6 meses después de las inoculaciones) las necrosis producidas por el avance del patógeno se midieron en todas las plantas. Los experimentos se repitieron tres semanas después de la primera inoculación para verificar los resultados. En el tercer experimento, se probó si la presencia de mitovirus (virus del género *Mitovirus* que están asociados con hongos y cuya presencia a veces se relaciona con una disminución de la patogenicidad del mismo) en los aislados de *G. abietina*, era capaz de modificar su comportamiento y patogenicidad del hongo tanto en condiciones *in vitro* como *in vivo* en plantas de pino carrasco. Por último, en el cuarto experimento, se realizó un ensayo de procedencias con cinco procedencias distintas de pino carrasco para estudiar si alguna de ellas era resistente a la enfermedad. La necrosis producida por el avance del patógeno en la planta fue usada como variable respuesta. Además, las concentraciones de dos flavanonas, se midieron como posibles indicadores de la resistencia en plantas a la enfermedad.

La presencia de todos los endófitos usados en nuestros experimentos así como sus extractos redujeron de forma significativa el avance de *G. abietina* en las plantas aunque dependió en ocasiones del momento de la inoculación. Cuando se usaron los endófitos como agentes de control biológico, el contenido en fenoles totales de la planta no aumentó. Por tanto,

Resumen

los mecanismos responsables de esta reducción pudieron ser la competición, el parasitismo, la producción de compuestos antibióticos o una combinación de ellos. Además, algunos compuestos fenólicos, fueron detectados en varios de los filtrados aunque no en todos, sugiriendo que aunque quizá la antibiosis estuviera implicada en esta reducción, los endófitos probablemente empleen varios mecanismos de acción. Los resultados del experimento de virus mostraron que los aislados infectados con mitovirus presentaron un mayor crecimiento micelial que los no infectados a la temperatura óptima de crecimiento del hongo que son 15°C. En el experimento del invernadero se observó que las plantas que habían sido inoculadas con aislados con virus, presentaron mayores necrosis, sugiriendo que la presencia de virus en aislados de *G. abietina* podría estar produciendo una hipervirulencia del patógeno. Por último, las procedencias de pino carrasco evaluadas en el cuarto experimento, mostraron diferencias significativas en su susceptibilidad al patógeno en términos de necrosis y severidad visual. La cantidad de la flavanona naringenina fue diferente entre procedencias, sugiriendo que es un posible indicador de la resistencia de las procedencias.

Chapter 1 Introduction

1.1. Introduction to fungi

Fungi were once considered to be plants and thus were in the realm of the botanists; however they are nowadays classified as a separate kingdom. Their principal characteristics are: (i) they are heterotrophic organisms, i.e. they cannot fix carbon; (ii) they lack chlorophyll; (iii) they typically grow as filaments, called hyphae which form the mycelium; (iv) the fungal cell walls are composed of glucans and chitin and unlike plants they do not normally contain cellulose-rich cell walls (except some fungal-like organisms); (v) they have haploid or diploid nuclei and (vi) they reproduce both sexually and asexually by means of spores (Deacon, 2006; Gould 2007). Furthermore, fungi can be dikaryons, which means that compatible cell-types fuse cytoplasm and the two nuclei of different cells cohabit. If there are multinucleate cells (with genetically different nuclei) then the cell is heterokaryon. The sexual state of a fungus is known as the teleomorph while the asexual state as the anamorph. The fungi have a metabolism of eukaryotic organisms and produce fungal metabolites. Primary metabolites are the intermediates or end products of the common metabolic pathways which are essential for the normal cellular functions of fungi. Secondary metabolites include a diverse range of compounds formed by specific pathways that are not essential for growth (Deacon, 2006).

The classification of fungi has changed over the years as more information becomes known about them. Their taxonomy has been based classically on sexual and asexual spore morphology and other characteristics such as hyphal and colony features. In the early 1990s, the molecular techniques such as the analysis of PCR-amplified ribosomal RNA genes

revolutionized the traditional taxonomy. Based on recent molecular phylogenetic analyses, as well as on other morphological characteristics, a new phylogenetic classification was proposed in 2007 as a result of efforts among researchers, mycologists and other scientists working on fungal taxonomy. This new classification proposed seven phyla called: *Microsporidia*, *Chytridiomycota*, *Blastocladiomycota*, *Neocallimastigomycota*, *Glomeromycota*, *Ascomycota*, and *Basidiomycota* (Hibbett et al., 2007).

According to how the fungi obtain the organic nutrients they need for growth, they can be classified as symbionts, parasites, and saprophytes (Deacon, 2006). The symbionts form associations with other organisms (Martin and Schwab, 2013). The parasites obtain the nutrients from other living organisms and can be biotrophs or necrotrophs. They are called pathogens if they produce a disease. The biotrophs (or obligate parasites) feed from living host cells without killing them but making use of the host's reserves of nutrients. Typically, biotrophs are host-specific fungi and produce infection structures and specialized organs for nutrient uptake and metabolite exchange, e.g. haustoria (Deacon, 2006). The other types of parasites, the necrotrophs, kill the host tissues, usually by producing toxins and/or enzymes and obtain nutrients from dead cells and from structural C sources and they may also feed on tree defense compounds (Oliva et al., 2014). Another group of organisms called hemibiotrophs can function as biotrophs and necrotrophs during their life cycle. Finally, the saprotrophs (or saprophytes) are organisms that use nonliving organic matter as a source of food. Saprophytes have an important role in the forest because they recycle carbon by decomposing cellulose and lignin residues by means of degrading enzymes. Most plant pathogenic fungi are versatile organisms that can live as saprophytes or

parasites depending on the food reservoirs available. They are called facultative saprophytes and are very difficult to control (Manion, 1981; Deacon, 2006; Gould, 2007).

The fungi can maintain different relationships with other fungi (Table 1.1). Mutualism is a symbiotic interaction between different species that is mutually beneficial (e.g. mycorrhizas). Commensalism is a class of relationships between two organisms where one organism benefits from the other without affecting it. In antagonistic interactions, one species benefits at the expense of another (e.g. biological control agents may be antagonistic to pathogens). Neutralism describes the relationship between two species that interact but do not affect each other. Amensalism is an interaction where an organism inflicts harm to another organism without any costs or benefits received by the other. Competition is a mutually detrimental interaction between individuals, populations or species (Deacon, 2006).

Table 1.1: Types of species interactions among fungi.

	Positive	Neutral	Negative
Positive	Mutualism	Commensalism	Antagonism
Neutral		Neutralism	Amensalism
Negative			Competition

1.2. Forest Diseases

A plant disease is a deviation from the essential functions of a tree caused by either pathogenic microorganisms or an adverse environmental factor (Manion, 1981; Agrios, 1997). The plant diseases are the result of the natural interactions among the host, the pathogen and the environment.

Changes in any of these factors could alter this balance and produce the disease (Hansen and Lewis, 1997). Forest pathology is the branch of plant pathology that deals with diseases of woody plants growing in natural forests, plantations and in urban environments (Manion, 1981). The forest diseases may be due to biotic or abiotic agents or a combination of factors (declines). Within the biotic factors, the main diseases are produced by fungi, bacteria, viruses and nematodes although the fungi are the most destructive agents of disease in the forest (Manion, 1981; Tainter and Baker, 1996). Furthermore, the planting of tree species in non-native sites may lead to significant disease problems involving indigenous pathogens either as a consequence of greater pathogen pressure in the new planting site or due to poor adaptation to the site and increased stress (Ennos, 2014).

In every disease, a series of events occur in succession that leads to the development and perpetuation of the disease and the pathogen. This is called the disease cycle and includes inoculation, penetration, infection, invasion, dissemination and survival (Agrios, 1997). To achieve this, a pathogen employs several physical and chemical methods. Inoculation is the coming into contact of a pathogen with a plant, which then initiates infection through the inoculum or propagule (any material that is used for the purpose of propagating an organism to the next stage in their life cycle, e.g. spores, parts of mycelium, sclerotium, a rhizomorph...etc.). Once the spores are in contact with any surface of the plant, they germinate and produce a germ tube, the first part of mycelium, which will penetrate the surface. The penetration can be achieved by direct penetration or by indirect through natural openings or wounds (Agrios, 1997; Windham and Windham, 2007). Penetration of pathogens into parenchymatous tissues is brought about by the breakdown of the cell walls due to sets of enzymes secreted by the pathogen.

However, colonization can occur without breakdown of parenchymatous tissue for pathogens that are exclusively vascular. The infection is the process by which pathogens establish contact with the susceptible cells or tissues of the host and procure nutrients from them. During infection, pathogens release in the host a number of biologically active substances such as (i) enzymes that degrade the components of the cell walls or affect components of the protoplast; (ii) toxins, that act directly on protoplast components and interfere with the permeability of its membranes; (iii) growth regulators, that exert a hormonal effect on the cells and either decrease or increase their activity causing an imbalance in the hormonal system and (iv) polysaccharides, that interfere with the translocation of water in the vascular diseases. Host responses to infection are known as symptoms, but others remain latent until the conditions of the host or the environment become more favorable (Agrios, 1997; Windham and Windham, 2007). After the infection stage the invasion of the plant tissues includes the growth and reproduction of the pathogen. Afterwards, the dissemination of the spores takes place, for the most part carried out by wind, rain-splashes, insects, animals or humans. Lastly, the pathogens survive in perennial plants during seasons, regardless of whether the plants are growing or are dormant or they can overwinter or oversummer in infected plant debris or in the soil (Agrios, 1997).

Once the pathogen has infected a tree, the host physiology is altered in various ways (Agrios, 1997) although physiological modifications in a host are mostly dependent on the plant organ that is infected (seeds, flower, branches, foliage, stem, roots). There is an alteration in the photosynthesis, because of the reduction of the foliar surface, the inhibition of the metabolism and the decreased water flow. The pathogens also modify the movement of the water and the nutrients within the plant due to the alteration

of the absorption by the roots and the increase of the transpiration. The respiration rate is also modified because the pathogen combusts the sugars and the carbon reservoirs of the plant. Furthermore, there is a change in cell membrane permeability, a loss of electrolytes, produced by the stimulation of the pathogen of certain membrane-bound enzymes, such as ATPase, which are involved in the pumping of H⁺ and K⁺ out through the cell membrane (Agrios, 1997; Oliva et al., 2014).

1.2.1. The pathogen of study: *Gremmeniella abietina* (Lagerb.) Morelet.

The pathogen *G. abietina* is an ascomycete fungus (anamorph *Brunchorstia pinea* (P. Karst) v Höhn) that has been described as being responsible for the destruction of many plantations and natural forests all over the Northern Hemisphere such as Central and Northern Europe, North America and Japan (Dorworth, 1979; Kaitera and Jalkanen, 1992). The pathogen mainly attacks tree species from the genus *Abies*, *Larix*, *Picea* and *Pinus*. The fungus causes the death of shoots, cankers and bark damage that can lead to the death of the trees, although the extent of the damage varies within the hosts and the environmental conditions (Butin, 1995). In Spain, *G. abietina* was detected for the first time in 1929 on *Pinus pinaster* stands in the northern part of the country (Martínez, 1933). However, the asexual stage of the fungus was isolated in 1999 on *P. halepensis* stands causing dried up needles, terminal twig distortion, defoliation and the death of some trees (Santamaría et al., 2003).

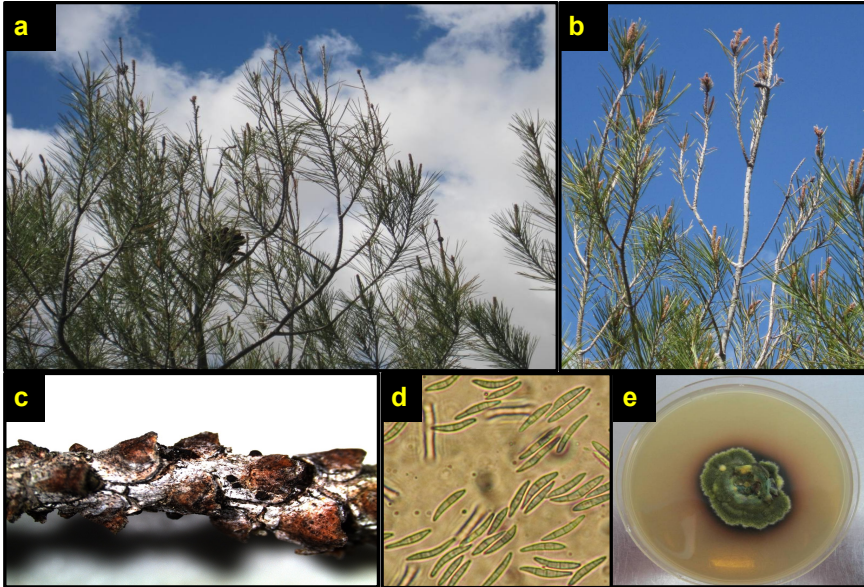


Figure 1.1: *Gremmeniella abietina* symptoms in Spanish stands: **a** dry needles, crown defoliation; **b** Terminal twig distortion; **c** Asexual fruiting bodies; **d** Conidia; **e** Mycelium in pure culture.

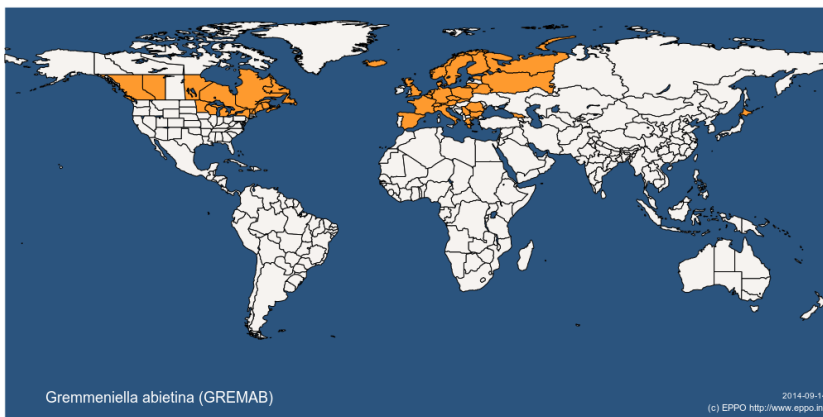


Figure 1.2: Distribution of *G. abietina* worldwide (Source: www.eppo.int, 2014).

The taxonomy of *G. abietina* is complex and sometimes confusing. This fungus was divided into two varieties *G. abietina* var *abietina* affecting mainly pines and *G. abietina* var. *balsamea* affecting spruces and firs (Petrini

et al.,1989). Within *G. abietina* var *abietina* three races on the basis of serology were distinguished; European, North American and Asian (Dorworth and Krywienczyk, 1975). However, this classification does not have any taxonomic standing and after several years of genetic analysis it has been suggested that the races might be regarded as separate species (Uotila et al., 2000; Laflamme, 2012). Within the European race three biotypes have been determined based on the length of spores, number of septa, disease symptoms, and molecular markers: biotype A (LTT, large tree type), biotype B (STT, small tree type) and alpine biotype (Uotila, 1983; Hamelin et al., 1996; Hellgren and Hogberg, 1995; Kaitera and Jalkanen, 1996; Hantula and Muller, 1997). Spanish *G. abietina* is currently recognized as part of the European race (Santamaría et al., 2005) and has recently been related to biotype A, although it has a unique genotype. It is genetically highly differentiated from any other *G. abietina* population in Europe with a probable A-type origin (Botella et al., 2010).

The fungus needs two years to complete its life cycle (Hellgren and Barklund, 1992). The infection normally takes place during the spring, when the buds are developing. During the winter the fungus invades the tissues taking advantage of the dormancy of the tree. Then, in the upcoming spring, the first symptoms start to become visible: resin exudation in the buds and necrotic tissue as a result of the advance of the pathogen (Phillips and Burdekin, 1992). Later on, the asexual fruiting bodies of the fungus, the pycnidia, come out and their spores, the conidia, are dispersed by splashing water (Butin, 1995). During the following spring, one year later, some buds do not sprout or grow deformed and the needles dry up, producing the defoliation of the crown and the terminal twig distortion. Death of lower branches and the yellowing of needle bases on infected branches have also

reported to be caused by the fungus (Manion, 1981). The presence of cankers in the twigs and/or in the bark is also frequent although it depends on the race and biotype (North American Race; and sometimes European Race biotype A). As a result of the defoliation and the cankers, some trees can die, especially if the attack is severe. The sexual fruiting bodies, the apothecia, emerge the following spring, two years after the infection (Tainter and Baker, 1996), but some races or biotypes have been described to have only asexual reproduction.

The pathogen *G. abietina* is a necrotroph that colonizes the host through stomata (Ylimartimo et al., 1997) and then has to break down the cell walls in order to continue with the infection. To achieve this objective, the fungus has an extracellular sheath which contains chemical compounds such as chitin, galactose, proteins, lipids and polygalacturonic acids (Benhamou and Ouellette, 1987 a, b). Furthermore, to help the fungus to degrade the cellulose during the shoot invasion, it produces enzymes like cellulase, exoglucanases, xylanase and polygalacturonase (Petäistö et al., 1992, 1994; Petäistö and Kajander, 1993; Petäistö and Lappi, 1996; Simard et al., 2013) which seem essential during this process.

1.2.2. The host: Aleppo pine (*Pinus halepensis* Mill.)

Aleppo pine is a species naturally present in almost all Mediterranean countries with an estimated area of 3 million ha (Figure 1.3). Its distribution is mainly coastal, however in Morocco and Spain it also grows in the interior part of the country. Its height can reach 15-20 m in good environmental situations and sometimes grows over 30 m with a trunk diameter up to 60 cm. On young individuals the bark is grey before becoming brown and deeply fissured. The crown is cone-shaped and transparent because the needles soon fall down, usually by the second year (Ruiz de la Torre, 1979).

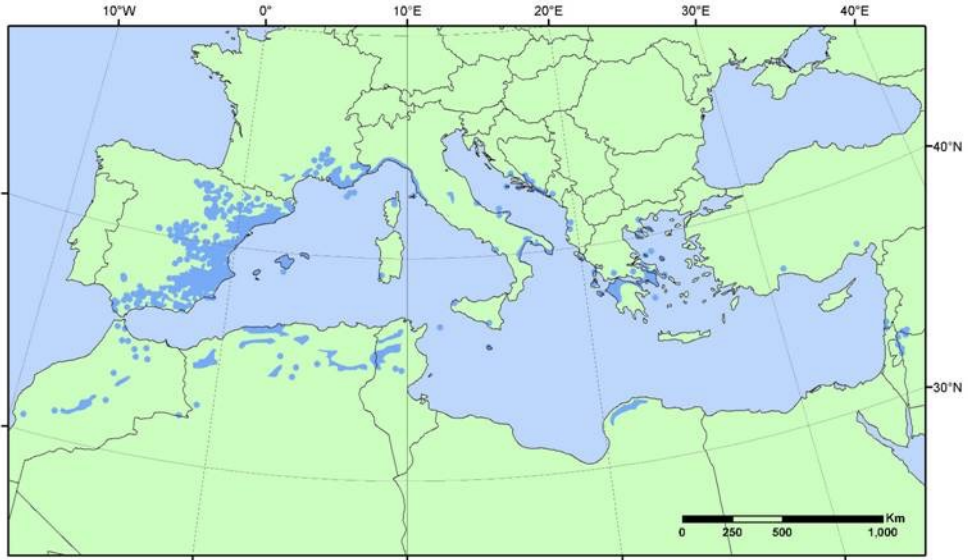


Figure 1.3: Worldwide distribution of *Pinus halepensis* (Source: euforgen.org).

Pine forests can grow from sea level up to 1.000 m although their optimum growth occurs around 800 m. Their maximum altitude has been found in Morocco at 2.600 m in the Atlas Mountains. The species normally can be regarded as thermophile, xerophile and calcicolous although it can tolerate harsh conditions, acidic substrates, and thermal variations. Nevertheless, its most distinctive feature is its tolerance to heat and drought (Gil et al., 1996). A total of 19 provenances were established in Spain based both on geography and on population phenotypes (Figure 1.4).

During the second part of the 20th century, as a result of its adaptability, it was used for afforestation beyond its natural habitat, especially in arid areas in order to prevent soil erosion, for socioeconomic reasons and in some cases for forest productivity (Ruiz de la Torre, 1979; Maestre and Cortina, 2004). Nevertheless, these plantations have been controversial due to the ecological impacts on the native flora and fauna, including fire regimes and

incidence of pests and diseases, associated with the extensive out planting of this species (Maestre and Cortina, 2004). Most of these negative impacts are not directly related to the species itself but with the way the afforestations were carried out by the use of single species, the intense site preparation that altered the previous vegetation and ecosystem functions and the election of unsuitable provenances (Maestre and Cortina, 2004).

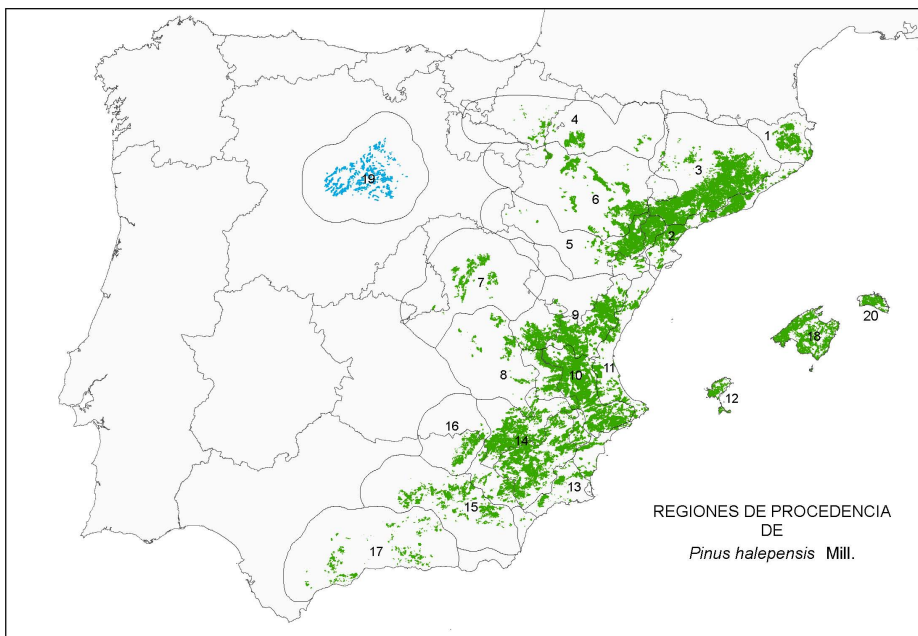


Figure 1.4: Provenance regions of Aleppo pine in Spain (Source: magrama.gob.es).

1.3. Defense mechanisms of plants

Plants employ several methods to defend themselves against pathogens. Defenses can be preformed or constitutive; they already exist in the plant before the attack, or induced, i.e. activated once the infection has happened. Both types of defenses imply physical and chemical responses. Constitutive defenses consist of the structural characteristics of the plant, such

as the cuticle and the epidermal cell walls, as well as some preexisting chemical defenses. Plants can respond to infection (induced defenses) blocking or slowing down the fungal growth by two ways: (i) by placing barriers; increasing cell wall thickness through lignification, i.e. the production of lignin, by the formation of cork, or by the creation of tyloses and (ii) by eliminating live cells already colonized or in the process of being colonized by a pathogen; e.g. producing abscission layers or killing the infected cells and their immediate neighbors accumulating fungitoxic compounds, a process known as hypersensitive response. The induced defense mechanism leads to the production of some hormones such as salicylic acid, jasmonic acid and ethylene to extend the communication within the plant and serve as prevention against future infections a process called called systemic acquired resistance (Agrios, 1997; Franceschi et al., 2005; Deacon, 2006).

Plant metabolites can be categorized as primary or secondary. Primary metabolites are substances produced by all plant cells that are directly involved in growth, development, or reproduction. Examples include sugars, proteins, amino acids, and nucleic acids. Secondary metabolites are not directly involved in growth or reproduction but they are often associated with plant defense. These compounds usually belong to one of three large chemical classes: terpenoids, phenolics, and alkaloids (Freeman and Beattie, 2007). Some secondary metabolites are preformed compounds, called phytoanticipins. They are present either as active compounds or as precursors that are rapidly converted to active compounds in response to infection. A second group of compounds are the phytoalexins. They are antimicrobial substances that are produced *de novo* by the plant after the infection and are extremely diverse. Some compounds are phytoalexins in one species and

phytoanticipins in another (Franceschi et al., 2005; Deacon, 2006; Gwinn et al., 2007, Witzell and Martín, 2008).

Induced defense mechanisms have been previously reported in several types of tissue from multiple pine species in response to infection by *G. abietina*. These include both physical alterations such as the sporadic formation of traumatic resin channels in the xylem and ligno-suberized barriers, and chemical reactions including the accumulation of large quantities of phenols in cell walls and changes in polyamines (Ylimartimo et al., 1997, Laflamme et al., 2006; Cvikrová et al., 2006, 2010).

1.4. Control and Management of Forest Diseases

Disease management in the forest requires an understanding of environment, host and pathogen interaction as well as pathogen biology and ecology (Manion, 1981; Rizzo et al., 2005). Methods of control vary considerably from one disease to another, depending on the kind of pathogen, the host, interactions between pathogens and hosts, environments, or presence of humans. Almost all control methods are aimed at protecting plants from becoming diseased rather than curing them after they have become diseased (Tainter and Baker, 1996). Classical concepts of plant disease control include avoidance, exclusion, eradication, protection, resistance and therapy (Tainter and Baker, 1996). The methods to achieve these objectives can be summarized in 4 categories: silvicultural, chemical, biological and host resistance (Table 1.2). Decision-making requires estimation of the potential impacts of the disease (i.e., setting priorities for management targets) and the ability to fit the disease into the context of other management goals within the broader landscape (Rizzo et al., 2005).

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Table 1.2: Disease management actions and strategies (based on Tainter and Baker, 1996; Agrios, 1997; Trigiano et al., 2007).

Actions	Definition	Practice
Avoidance	Avoid the contact between the pathogen and the host.	<ul style="list-style-type: none"> • Planting in areas where the disease is not present or the environment does not favor disease development. Avoid planting susceptible species. • Timing management activities to avoid having susceptible trees or tissues during disease infection periods. • Reduce the source of inoculum: pruning, host elimination.
Exclusion	Keep out a disease from an area.	<ul style="list-style-type: none"> • Quarantine, inspection and certification of commercial plants. • Use of pathogen-free material when restoring a new area.
Eradication	Total removal of the disease from a site.	<ul style="list-style-type: none"> • Cutting and removing all infected trees. • Only in nurseries with chemicals or biological products.
Protection	Application of a substance or technique to protect a plant. Prevent the infection.	<ul style="list-style-type: none"> • Applications of fungicides, nematicides, insecticides. • Mycorrhizae and fertilizers. • Thinning, pruning lower branches.
Resistance	A resistant phenotype reduces the growth, reproduction and/or activity of the pathogen.	<ul style="list-style-type: none"> • Found genetic resistance in a population. • Selective breeding: select resistant species/individuals/provenances. • Improve the resistance of the host: induced resistance.
Therapy	Cure the plants after they have become diseased.	<ul style="list-style-type: none"> • Treat with chemicals/biological products or heat. • Remove diseased branches/pruning.

1.4.1. *Silvicultural control*

Silvicultural methods in disease management are aimed at preventing diseases and at reducing the expansion of the disease once a stand is infected (Tainter and Baker, 1996). In order to prevent disease establishment, some of the management guidelines include avoiding planting susceptible species, to maintain the health and vigor of the trees as well as to protect the genetic resources (Tainter and Baker, 1996; Brown and Webber, 2007; Zeglen et al., 2010). On the other hand, it is important to reduce the amount of inoculum to limit disease spread within an infested site. This could be achieved by thinnings that remove dead or damaged trees or the alternate hosts (e.g. rusts). In other cases stumps may be the original infection court allowing for a pathogen to become established (e.g. in the case of *Heterobasidion* spp.) or to build up inoculum (e.g. *Armillaria* species), hence stump surfaces should be treated or removed to avoid the establishment of some root pathogens. In some cases it is recommended to prune infected branches, although this practice should be carried out with caution because it could wound the trees and promote a point of infection to fungi (Oliva et al., 2013). However, management practice recommendations will differ among pathogens and between managed and natural forests. It is important to know the pathogen life cycle in order to time management activities to avoid having susceptible trees or tissues during disease infection periods. Furthermore, managed forests are different from natural forests in many ways, like tree species diversity, age distribution, canopy structure, amount of dead wood or fungal community. Therefore, they are subjected to different silvicultural operations (Oliva et al., 2013).

Damage from *G. abietina* can be prevented or reduced using silvicultural methods but often efficacy requires they are combined with other

disease management approaches. These include maintaining a proper density of the stand and avoiding planting in cool and wet sites (Butin, 1995). Race of the pathogen may also make a difference: for instance the pruning of low branches performed in Quebec is effective, due to the specific etiology of the North American race (Laflamme, 1999). Furthermore, it is useful to know the time and level of spore dispersal of *G. abietina* to plan control operations (Petäistö and Heinonen, 2003).

1.4.2. Chemical control

The term fungicide is used in a broad sense for any compound that kills or inactivates fungi. Most of these compounds are chemically synthesized, but some are modified derivatives of naturally occurring compounds (Deacon, 2006). Fungicides are categorized in several ways based on different characteristics. The main classifications are based on the following (McGrath, 2004):

1) Antifungal target of the fungicide or mode of action: (i) the cell membrane; several fungicides affect lipid peroxidation, phospholipids biosynthesis or cell membrane permeability; (ii) the microtubules and microtubule-associated proteins, which are disrupted by inhibition of β -tubulin assembly during mitosis and cell division; (iii) mitochondrial respiration, targeted by many fungicides that block the steps in the mitochondrial electron transport chain or that inhibit ATP synthesis; (iv) fungal cell wall components, especially β 1-3 glucans; (v) general metabolism; several fungicides disrupt basic metabolic processes such as the production of sterols or chitin and lastly (vi) induction of the systemic acquired resistance of the plant, by mimicking the chemical signals (Deacon, 2006; McGrath, 2004; Hirooka and Ishii, 2013).

2) Mobility of the fungicide in the plant: contact or systemic. Contact fungicides act only near the site where they are applied. Fungi do not develop resistance to them easily because they interfere with basic metabolic processes and often have multiple sites of action in fungal cells. Systemic fungicides are absorbed by plants and are distributed internally, usually by upward movement in the xylem, where they can help to protect new growth (Deacon, 2006).

3) Role in protection: preventive or curative. Contact fungicides are preventive because they work by contact action on the surface of the plant to which they have been applied or to control an established infection. They need to be applied over the whole plant surface and must be re-applied to protect any new growth of the plant. Due to their ability to penetrate plants, systemic fungicides can eradicate existing infections and protect against subsequent infections (Deacon, 2006; McGrath, 2004).

4) Breadth of activity: single or multi-site. Single-sites fungicides are active against only one specific step in the metabolism of the fungi they control or against only one critical enzyme or protein needed for the fungus. They are highly specific in their toxicity and are mainly systemic fungicides. Because of this, fungi can develop resistance to them quite easily, often by a single gen mutation (Agrios, 1997; Deacon, 2006; McGrath, 2004). Typically, older fungicides have multi-site activity; they interfere with several metabolic processes, and thus usually affect many fungi (McGrath, 2004).

5) Type of chemical: organic or inorganic. Organic molecules are those containing carbon atoms in their structure whereas inorganic compounds do not. Many of the first fungicides to be used to control plant diseases were inorganic with sulfur, copper salts and mercury, although some of them have been banned because of their extreme toxicity (Deacon, 2006).

Their mode of action is based on the disruption of several basic metabolic processes, so fungi do not develop resistance to them easily. The organic compounds include both contact and systemic fungicides.

However, although the protection of plants against harmful organisms is absolutely necessary, the EU Parliament and Council Directive 2009/128/EC introduced new legislative provisions to achieve the sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and environment. The proposal suggests that member states should give priority to non-chemical methods of plant protection and pest management (Directive 2009/128/EC). Chemical control approaches may have deleterious impacts on the biodiversity, a negative effect on pathogen resistance to the fungicide and harmful consequences to non-target fungi (Agrios, 1997). For these reasons, the application of fungicides should be limited to nurseries, using field applications only in situations where very high disease incidence occurs (Santamaría et al., 2007).

For *G. abietina*, Kohn (1964) studied the use of Maneb, a multi-site contact fungicide, as the best chemical against the pathogen in nurseries. Other authors recommended the application of contact fungicides such as Maneb, Ziram, and Chlorothalonil to prevent the dispersion of the pathogen and reduce the incidence of the pathogen in seedlings (Skilling and Waddell, 1970; Dorworth, 1971; Hopkin and McKenney, 1995). Lastly, Santamaría et al (2007) studied the effect of several fungicides to prevent the growth of Spanish isolates of *G. abietina* in *in vitro* experiments and concluded that both contact fungicides Chlorothalonil and Daconil were suitable methods to reduce the growth of the pathogen.

1.4.3. *Biological control*

Biological control is the use of living organisms to fight against a disease. However, some authors broaden the definition and include not only the use of antagonistic micro-organisms; but also the application of naturally derived bioactive compounds; and the induction of natural resistance of the plants (Talibi et al., 2014). Biological control has become an alternative method to fight diseases because of the growing public concern over the potentially harmful effects that some chemical pesticides pose to human health and the environment (Ownley and Windham, 2007). Nevertheless, the viability of biological control as an effective way to manage tree diseases in a forest setting has been confirmed only in a handful of cases (for instance with *Heterobasidion* or *Cryphonectria parasitica*). Biological control agents are not usually pathogen specific, and use and combine different modes of action. These include: (i) parasitism, which is the feeding of one organism on another; (ii) competition, the result of two or more organisms trying to use the same source of food (carbon and nitrogen) or occupy the same niche or infection site; (iii) antibiosis, the inhibition or destruction of one organism by a metabolite produced by another organism; (iv) induction of plant defense system, the stimulation of the host plant defenses by nonpathogenic fungi; (v) the physical barrier effect, produced by the presence of the mycorrhizas (Schoeman et al., 1999; Alabouvette et al., 2006; Ownley and Windham, 2007; Heydari and Pessaraki, 2010; Diez and Alves-Santos, 2011).

Other mechanisms of biological control include hypovirulence that is the reduction of the virulence of a pathogen-strain by the presence of a virus. Fungal viruses, also called mycoviruses which obligately reside in host cells of fungi, have a genome of DNA, single stranded (ss) RNA or double stranded (ds) RNA (Ghabrial and Suzuki, 2009; Pearson et al., 2009). Their spread

occurs either through the cytoplasm when two hyphae fuse through a process called anastomosis or they can be found in spores when the fungus sporulate. Their presence can confer different properties to the pathogenic fungi such as the reduction (hypovirulence) or the enhancement of the virulence (hypervirulence) (Ghabrial and Suzuki, 2009; Zhang et al., 2010). The hypovirulence is achieved when a hypovirulent strain of a fungal pathogen fuses by anastomosis with a virulent strain and transmits the hypovirulent condition to the virulent strain. The classic example of hypovirulence is biocontrol of chestnut blight in Europe, by the fungal pathogen *Cryphonectria parasitica*. Hypovirulent strains of this fungus resulted in cankers that were only superficial. In addition, when hyphae from a virulent strain were allowed to fuse with hyphae of a hypovirulent strain, the virulent became hypovirulent because of the transmission of dsRNA by hyphal anastomosis (Agrios, 1997; Ownley and Windham, 2007). In essence, the infectious transmissive efficacy of dsRNA by hypovirulent strains needs to outweigh the decreased sporulation potential associated with viral infection, in order for this control approach to be self-sustained and viable. In order to be suitable for biological control they must fulfill two requirements: firstly, to have the ability to decrease the fitness of the pathogenic fungus and secondly, to transmit the dsRNA efficiently enough to be maintained in a large proportion of the pathogen population (McCabe et al., 1999).

Biological control agents (BCAs) include bacteria, fungi, nematodes, protozoa and viruses. Among BCAs, fungal endophytes have been used for this purpose and have been described by several authors as organisms that live inside the plant tissue and maintain a neutral, beneficial or detrimental relationship with the plant (Backman and Sikora, 2008). Other authors define them as fungi that live inside plant parts that produce no symptoms or signs of

infection (Arnold et al., 2003; Deacon, 2006; Sieber, 2007). Biological control by means of fungal endophytes has already demonstrated its suitability against *G. abietina* infections in some experiments. When tested on seedlings of red pine, the fungal endophyte *Phaeotheca dimorphospora* Desrochers and Ouellette has been described to inhibit the *in vitro* growth, the germination of spores, and ultimately the spread of *G. abietina* (*Pinus resinosa* Ait.) (Yang et al., 1995). Furthermore, it has also been reported that several endophytes including *Trichoderma*, *Aureobasidion*, *Cladosporium* and some unknown fungus called 20.1 can reduce or inhibit the growth of the Spanish isolates of *G. abietina* (Santamaría et al., 2007).

The release of BCAs is, in general terms, positive since they are considered less toxic to humans and to the environment than synthetic chemical pesticides. BCAs may also represent an acceptable and effective means of disease management since microbial organisms may control resistant pests and reduce the possibility of development of further resistance (Brimner and Boland, 2003). However, some problems are related to the use of BCAs and have to be taken into account. These include compatibility of the host plant and the BCA due to host plant genotype, mutation of the biocontrol organism resulting in a loss of effectiveness, resistance of the pathogen to biocontrol mechanisms, vulnerability of the biocontrol agent to defense mechanisms of the pathogen and effects of the environment on survival and effectiveness of the BCA (Ownley and Windham, 2007). Furthermore, the application of BCAs could have detrimental effects on non-target organisms, such as mycorrhizas and saprophytic fungi. So, it is important for researchers to attempt to identify as many possible non-target effects associated with the release of any BCA (Cook et al., 1996; Brimen and Boland, 2003).

1.4.4. Host resistance

Disease resistance is one of the most important factors contributing to the long-term survival of trees in plantations (Pataky and Carson, 2007). In most cases, genetic resistance means immunity, where a plant has inherited a trait that confers the ability of an individual to prevent infection. Resistance to a pathogen can involve several different processes: avoidance or inhibition, killing the threat, limiting spread, or host repair. Depending on the success of the employment of each mechanism, the host will be defined as resistant, or susceptible if it can not afford the development of any of them (Telford et al., 2014). Other traits permit the plant to become infected but the damaged is limited. For instance, the growth rate of the infected plant will be greater than the expansion rate of symptoms. Such plants are considered tolerant to the disease (Tainter and Baker, 1996).

The resistance of some plants could be improved by incorporating in the plant, through genetic engineering, genes obtained from plants, other pathogens, or other organisms that code for the production of enzymes, peptides, or toxins interfering with infection by the pathogen. Nevertheless, this approach is more frequent in agricultural crops rather than in forest trees (Agrios, 1997). Even so, by far the most common improvement of host resistance to almost any pathogen is brought by breeding and using resistance varieties or provenances (Bution, 1995; Agrios, 1997). However, resistance breeding does not stop once a resistance variety is released. It is important to monitor the performance of the new variety as it is used in the field (Manion, 1981).

Concerning *G. abietina* there have been studies to explore possible tolerant species or provenances and breeding programs. Resistance of plant hosts to *G. abietina* is likely to be correlated to: (a) the production of ligno-

suberized tissues that help the tree to compartmentalize the invaded tissues and (b) the secretion of molecules capable of degrading or altering the fibrillar matrix of the extracellular sheath of the pathogen that contains chitin, galactose, proteins, lipids and polygalacturonic acids (Simard et al., 2001; Laflamme et al., 2006; Bernhold et al., 2006; Simard et al., 2013). In some studies, both *P. contorta* and *P. banksiana* were found to be resistant to the pathogen (Simard et al., 2001; Laflamme et al., 2006; Bernhold et al., 2009). Some countries such as Canada, Germany, Sweden, Norway and United States have developed breeding programs to find and select species and individuals resistant to the disease (Dietrichson, 1968, Teich and Smerlis, 1969; Roll-Hansen, 1972; Dorworth, 1974; Laflamme and Blais, 2000). With regards to the susceptibility of Spanish pine species to the pathogen, *P. halepensis* has been reported to be the most susceptible followed by *P. pinea* while *P. pinaster* and *P. nigra* have been reported to be less susceptible (Santamaría et al., 2006).

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Chapter 2 Objectives

The need for management and control of *G. abietina* infections in a sustainable and balanced way together with the European recommendations of using non-chemical methods (Directive 2009/128/EC), make it necessary to provide alternative methods to fight the disease. Thus, the main objective of this thesis was to explore the use of alternative methods to control *G. abietina* infections. Some of these methods include the use of biological control agents such as fungal endophytes, mycoviruses and the selection of resistant provenances. In order to achieve the general objective several specific goals and questions were proposed:

Biological control agents

Endophytes

1) To evaluate the use of endophytes as biological control agents for the treatment of *G. abietina* infections on seedlings. Do fungal endophytes reduce the advance of the pathogen in the plant? What is the mode of action of these biological antagonisms? (Chapter 3).

Endophytes' filtrates

2) To study the effect of fungal filtrates on infected seedlings. Do the fungal filtrates reduce or prevent the infection? What compounds are responsible for this reduction in growth of the pathogen? (Chapter 4).

Mitoviruses

3) To study the effect of mitovirus-presence (i.e. mycoviruses from genus *Mitovirus*) on *G. abietina* isolates under different conditions and on the pathogenicity of the disease. Do mitovirus promote a change in phenotype of fungal isolates *in vitro* and *in vivo*? (Chapter 5).

Host resistance

4) To explore the susceptibility of different Aleppo pines provenances to the infection. How do less susceptible provenances tolerate infection? How do they defend themselves from the attack of this organism? (Chapter 6).

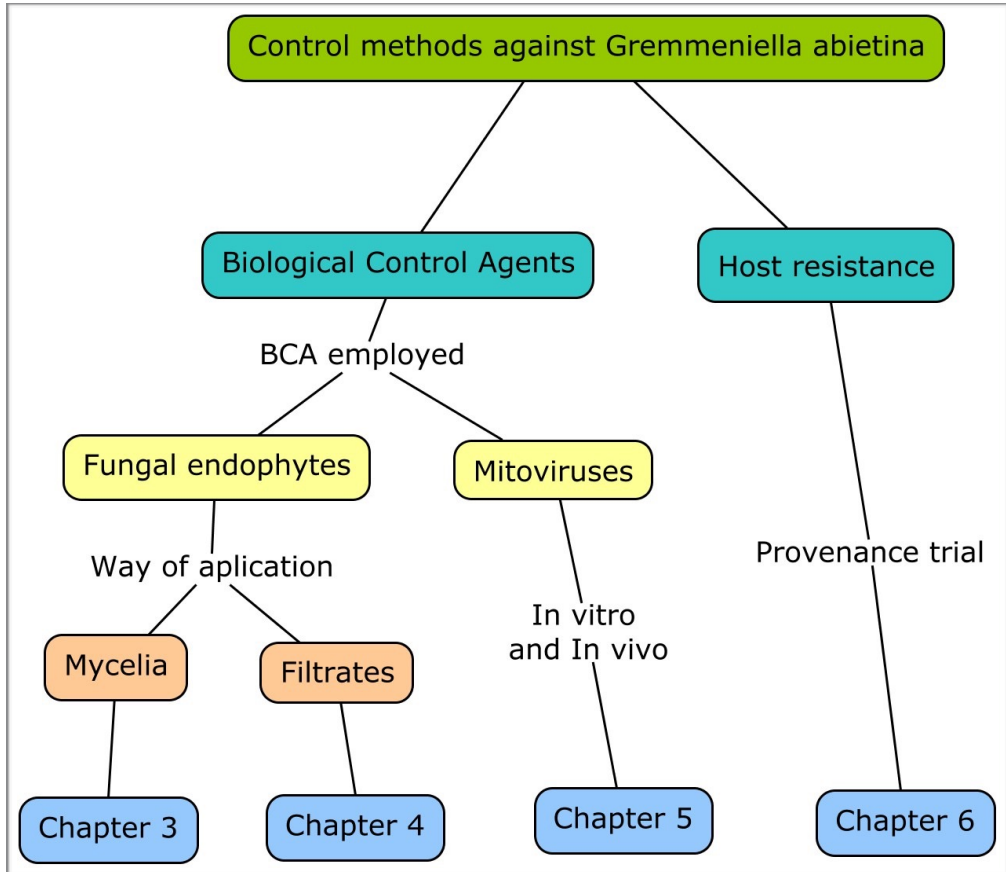


Figure 2.1: Conceptual model of the thesis. Control measures employed in the study: biological and host resistance. Biological control agent employed: micoviruses or fungal endophytes; way of application and the resulting chapters.

Chapter 3

Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings

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ABSTRACT

Gremmeniella abietina (Lagerberg) Morelet is a pathogenic fungus that causes severe damage in coniferous forests, causing the death of the trees, in Central and Northern Europe, North America and Japan. Biological control (i.e. the use of biologically antagonistic organisms) is being considered as an alternative and an eco-friendly method to deal with plant diseases. Among such organisms several fungal endophytes have been successfully used to reduce or inhibit the growth of pathogens. Thus, the aim of this study was to evaluate the ability of several fungal endophytes to reduce the *G. abietina* spreading on pine seedlings, with the goal of exploring the mechanisms involved in that reduction. The experiment was carried out on two-year old *P. halepensis* seedlings under greenhouse conditions. Five fungal endophytes (*Trichoderma* spp., *Aureobasidium pullulans*, *Aureobasidium* spp., endophyte 20.1 and *Leotiomycece* spp.) obtained from healthy *P. halepensis* trees were used to evaluate their effect on six *G. abietina* isolates. The pathogen and the endophyte were both inoculated in every seedling. At the end of the

experiment seedlings were cut and brought to the laboratory where the necrosis length and total phenol content of the plant were measured. The pathogen presence was determined by a nested PCR with specific primers of *G. abietina*. The presence of all endophytes significantly reduced the necrosis length caused by *G. abietina* in most of the cases. However, the phenolic content of the plant, which is an indicator of the activation of the plant's defence mechanisms, had not increased with the endophyte inoculation. Therefore, the reduction in the necrosis observed could be attributed to a direct effect of the endophyte on the *Gremmeniella* spreading. These results suggest that the use of fungal endophytes could be an effective way to protect against *G. abietina* infections.

Keywords: Biological control, biological antagonisms, endophytes, forest pathogen.

3.1. Introduction

The ascomycete fungus *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnelt) is a pathogen whose infection produces cankers on stems and trunks, dieback and causes the death of the trees (Donaubauer, 1972). The fungus, which is native to Europe, has extended to most European countries, the east of North America and Japan (Yokota, 1975; Dorworth, 1978; Kaitera and Jalkanen, 1992). In some of the countries where it occurs severe damage has been registered in both natural forests and plantations, sometimes causing devastation of huge areas (Kaitera et al., 1998; Wulff et al., 2006). Trees from genera *Picea* and *Pinus* are their principal hosts although some damages have been also found in *Abies*, *Larix* and *Pseudotsuga* trees. In Spain, *G. abietina* was detected for first

time in 1929 causing damage to *Pinus pinaster* (Martinez, 1933), and to *P. halepensis* in 1999 on forest plantations (Santamaría et al., 2003). Although no epidemic outbreaks have been registered yet in Spain it causes serious damages to trees affected by the disease. The main symptoms of the infection in Spanish stands are crown defoliation, dieback and distortion of terminal twigs, which occasionally leads to the death of the tree (Santamaría et al., 2003). Control measures of the disease might include silvicultural practices, like pruning lower branches or removing dead trees, to avoid the expansion of the pathogen and to reduce the source of inoculum (Laflamme, 1999). The application of fungicides has been also used but mostly as an emergency measure on nurseries (Hopkin and Mckenney, 1995) although currently the application of chemical products is not recommended in the forests of the European Union (COM 659/2013, 20th of September).

Biological control is considered an alternative method in plant disease control. Since the use of chemical products has been reduced due to their harmful effect on the environment, the biological antagonisms are expected to become an important part of the control methods against plant pathogens (Cook, 1993). Fungi are being used more and more as commercial biological control agents, providing alternatives to chemical pesticides for combating insect pests, nematodes, and plant-pathogenic fungi (Deacon, 2006). Among them, fungal endophytes could be also used with this purpose. Fungal endophytes have been described by several authors as organisms that live inside the plant tissue and maintain a neutral, beneficial or detrimental relationship with the plant (Backman and Sikora, 2008). Other authors describe endophytes as fungi that live inside plant parts that produce no symptoms or signs of infection (Arnold et al., 2003; Deacon, 2006; Sieber, 2007). They have been previously used as biological control agents because

they can inhibit or reduce the pathogen growth by micoparasitism, antibiosis, metabolites production, competition for the nutrients and induced resistance of the plant (Heydari and Pessarakli, 2010). Biological control by means of fungal endophytes has already demonstrated its suitability against *G. abietina* infections. The fungal endophyte *Phaeotheca dimorphospora* Desrochers and Ouellette was previously described to have inhibited *in vitro* the growth of *G. abietina* colonies, the germination of the spores and the spread of the pathogen on seedlings of red pine (*Pinus resinosa* Ait.) (Yang et al., 1995). Furthermore, several endophytes produced a reduction or an inhibition of the mycelial growth of some Spanish isolates of *G. abietina* which belonged to the genus *Trichoderma*, *Aureobasidium* and some unknown genus (Santamaría et al., 2007).

The defense mechanisms of conifers against pathogens can be classified as constitutive which are already in the tree before the contact with the pathogen, and induced, that are activated as a consequence of the interaction with the pathogen. If the induced defences serve as prevention against future infections then is called acquired resistance. The activation of the systemic induced resistance (SIR) in the host can be due to the presence of fungal endophytes and this mechanism has been already found to be effective against other fungal pathogens like *Diplodia pinea* (Desmaz.) J. Kickx fil. (Muñoz et al. 2008; Regliński et al. 2012). This activation produces either new compounds or an increase of the concentration of others that already existed in the plant (Franceschi et al., 2005). As a result of the activation of the plant's defence system an increase of the phenolic compounds has been frequently observed in lesion margins and transition zones in living sapwood of many trees, (Pearce, 1996). Other frequent responses include: an increase of the peroxidase and chitinase activity, a

higher lignin content, a higher amount of glucanases that can degrade components of living organisms, the presence of toxic proteins and inhibitors of enzymes (Takahama and Oniki, 2000; Howell, 2003; Franceschi et al. 2005; Adomas and Asiegbu, 2006). In infections caused by *G. abietina* the defence mechanisms of conifers include an increase in the peroxidase activity and in lignin content with the accumulation of phenolic compounds in some cell walls (Cvikrová et al., 2006). Furthermore, the formation of a lignosuberized barrier has been shown to be a limiting component of the progression of *G. abietina* (Ylimartimo et al., 1997).

The reduction of *G. abietina* growth because of the endophyte antagonism has been previously studied in *in vitro* experiments (Santamaría et al., 2007) nevertheless no inoculation tests have been carried out yet. Thus, the main objectives of this study were (i) to evaluate the potential of several fungal endophytes to be used as biocontrol agents by testing the effect of their presence on the necrosis produced by *G. abietina* on the seedlings and (ii) to observe if the inoculation of the endophytes was able to activate the defence system of the plant by means of the measurement of the concentration of the total phenolic compounds.

3.2. Material and Methods

3.2.1. Plant and fungal material

To test the antagonistic effect of the endophytes and the pathogen *in vivo*, we performed artificial inoculations in healthy *Pinus halepensis* seedlings. The experiment was carried out in the greenhouse located at the University of Valladolid [Universal Transverse Mercator (UTM) coordinates: 4607558, 353022]. Containerized two-year old seedlings of *P. halepensis* (height: 16.77 ± 2.49 mm (mean \pm standard error); diameter: 3.16 ± 0.74

mm) provided by “El Serranillo” Nursery from the Ministry of Agriculture and Environment and the Central Nursery from the regional government of Castilla y León were used for the experiment. Seedlings had been grown according to nursery practice and six months before inoculation, any fungicides, pesticides or herbicides were not applied to them. The seedlings in the experimental greenhouse were watered to field capacity every 2 to 3 days.

The fungal material (Table 3.1) consisted of six Spanish isolates of *G. abietina* isolated from adult trees showing the typical symptoms of *Gremmeniella* disease and five fungal endophytes previously isolated from symptomless *P. halepensis* trees and identified by Santamaría et al., (2007), Botella and Diez, (2011) and Botella et al., (2010). The fungal endophytes corresponded to *Trichoderma* spp., 20.1 (not identified), *Aureobasidium* spp., *Aureobasidium pullulans* (de Bary) G. Arnaud and *Leotiomycece* spp. species.

Table 3.1: Characteristics of the isolates.

Behaviour	Isolate	Name	Species	Origin	Province	Year of isolation
Pathogen	G1	Z0-10-01	<i>G. abietina</i>	Valle de Cerrato	Palencia	2010
	G2	Z0-10-02	<i>G. abietina</i>	Valle de Cerrato	Palencia	2010
	G3	P1-8	<i>G. abietina</i>	Valle de Cerrato	Palencia	2007
	G4	P1-12	<i>G. abietina</i>	Valle de Cerrato	Palencia	2007
	G5	VAI-13	<i>G. abietina</i>	Villalba de los Alcores	Valladolid	2003
	G6	00P-7	<i>G. abietina</i>	Valle de Cerrato	Palencia	2001
Endophytes	E1	1778 AB	<i>Trichoderma</i> sp.	Tordehumos	Valladolid	2009
	E2	1077 4A	<i>Aureobasidium pullulans</i>	Valle de Cerrato	Palencia	2009
	E3	1812 RA 1-b	<i>Aureobasidium</i> sp.	Valle de Cerrato	Palencia	2009
	E4	20.1	Unknown Deuteromycete 1	Quintanilla de Onésimo	Valladolid	2004
	E5	638 AB 2-b	<i>Leotiomycece</i> sp.	Tordehumos	Valladolid	2009

Some of these species had previously been shown reduction of mycelial growth of Spanish *G. abietina* in *in vitro* experiments (Santamaría et al., 2007). Isolates were sub cultivated in culture media MOS-agar (modified orange serum-agar, Müller et al., 1994) and PDA (potato, dextrose, and agar, Scharlau) some weeks before the inoculations in order to have enough mycelium.

3.2.2. Experimental design and inoculations

The experiment was arranged in a completely randomized factorial design with eight repetitions per treatment and two factors: 'pathogen' (six isolates + control), and 'endophyte species' (five endophytes species + control). Every treatment consisted of the artificial inoculation of one of the 42 combination of "pathogen * endophyte species". Seedlings were placed 5 cm apart, without any direct contact between them, to avoid secondary infections. For inoculations, two wounds were made on the same side of the shoot axis; *Gremmeniella* isolate was placed at 10 cm below the shoot apex and the endophyte at 8 cm below the shoot apex. Therefore both fungal organisms were separated by 2 cm, which is considered an appropriate distance to test interaction between them. Each wound was made with a sterile scalpel. After, a small piece of culture medium from the margin of an actively growing colony was placed in the wound and finally the wounds were covered with Parafilm®. Control treatments were performed with sterile culture media. Inoculations were made in December in order to mimic the natural behaviour of the fungus (Ranta et al., 2000). Three weeks after the first inoculations, the experiment was repeated. Therefore, two times of inoculation were performed. Thus, a total of 672 seedlings were inoculated twice in the two rounds of this experiment.

3.2.3. Necrosis length and other plant-related measurements

Six months after the inoculations the plants were cut and brought to the laboratory. The total length of the seedlings, the diameter at root collar and the necrosis length were measured in all the seedlings. In order to get a more accurate observation of the necrosis length, shoots were halved lengthwise before taking the measurements. The necrosis produced by the fungus was considered a quality indicator of the disease's advance because *G. abietina* is an organ-specialized pathogen with a necrotrophic behavior that kills stem tissue during colonization (Adomas and Asiegbu, 2006). The relationship among necrosis and total length of the seedling was defined as relative necrosis length and was used as response variable as previously described (Santamaría et al., 2006).

3.2.4. Total phenols extraction and quantification

Changes in the concentration of total phenols were used to measure the activation of the defence mechanisms of the plants to the inoculation of *G. abietina* and the endophytes. Six seedlings of every combination (a total of 252 samples) were selected and were analyzed for phenol content. The preparation of the samples and the total phenols extraction was done following the protocol described by Peñuelas et al. (1996) and Robles et al. (2003) slightly modified. Firstly the samples were dried in the oven at 40°C for a week and then ground into powder. To extract the total phenols, 20ml 70% (v/v) methanol solution (acidified with some drops of 1M HCl) were added to every sample. The samples were left in the orbital shaker at continuous movement for 1.5 h and then filtered. To quantify the phenolic compounds we used the protocol described by Singleton and Rossi, (1965) with some modifications. The reagents Folin-Ciocalteu (Sigma-Aldrich) and Na₂CO₃ (20% w/v) were used to produce the colorimetric reaction in the

samples. After 1.5 h in darkness at room temperature the absorbance at 760 nm was measured 4 times for each sample with a spectrophotometer (Spectrum SP-2000UV, LAN Optics). To quantify the results, Gallic acid was used as the standard.

3.2.5. Re-isolation of *G. abietina*: DNA extraction and nested PCR

In order to confirm that *G. abietina* was the fungus responsible for the damage in the seedlings two methods were performed: (i) observation of the fruiting bodies and (ii) DNA extraction and amplification of the fungus. The observation of fruiting bodies was done in all the seedlings of the experiment. The fruiting bodies produced in the seedlings were taken and observed under the microscope to confirm morphologically that they belonged to *G. abietina*. The DNA extraction was made directly from the necrotic tissue from 168 seedlings. A 10 cm piece of the seedling was cut, freeze-dried for 24h and ground into a fine powder with tungsten beads. Then, DNA was extracted following the Hamelin et al. (2000) protocol. To heighten sensitivity of detection, a nested PCR was used to amplify the 18S region (840bp) of the rDNA. Amplifications were performed as described in Zeng et al. (2005) protocol but instead of using DNA products to perform the first round of PCR, we used dilutions of 1:100 and/or 1:1000 from DNA extracts. Nested PCR was carried out with specific primers for *G. abietina* (Zeng et al. 2005): NS. Grem 3 (5'-AACCTTGAACCTTGGTTGGTT-3'), NS. Grem 4 (5'-TGGTGGAGTGTTCCTACT-3') in the first round followed by a second round of PCR with the primers NS. Grem 5 (5'-CACTGATCCGACCGGGT-3') and NS. Grem 6 (5'-CCTTTCGGACAAGGAAGG-3'). PCR products (5 µl) were analyzed by electrophoresis on 1.2% agarose gels in 1× TAE buffer. The gels were stained with GelRed and visualized under UV light.

3.2.6. Statistical analysis

All statistical analyses were performed with SAS 9.2 Inc (2004) program. Due to the high variance heterogeneity of the data, the effect of time of inoculation, isolates, endophytes and their interactions on necrosis length and phenols content was evaluated by a linear mixed model with the MIXED procedure in SAS. The mathematical formulation of the model was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \epsilon_{ijkl}.$$

with $i=1, 2$ for the time of inoculation, $j=1, \dots, 6$ for the type of endophyte and $k=1, \dots, 7$ for the isolates, and:

Y_{ijkl} = the observed value of the dependent variable for the replication l of the time of inoculation i , the type of endophyte j and the isolates k ; μ =general mean effect; α_i =main effect of the time of inoculation i ; β_j =main effect of the type of endophyte j ; γ_k =main effect of the isolate k ; $\alpha\beta_{ij}$ = interaction effect of the time of inoculation i and the type of endophyte j ; $\alpha\gamma_{ik}$ =interaction effect of the time of inoculation i with the isolate k ; $\beta\gamma_{jk}$ = interaction effect of the type of endophyte j and the isolate k ; $\alpha\beta\gamma_{ijk}$ = triple interaction effect of the time of inoculation i , the type of endophyte j and the isolate k ; ϵ_{ijkl} =random error in the dependent variable for the replication l of the time of inoculation i , the type of endophyte j and the isolate k .

We suppose that the random errors ϵ_{ijkl} are independent, with normal distribution $\epsilon_{ijkl} \sim N(0, \sigma^2_{ij})$ for the relative necrosis length and $\epsilon_{ijkl} \sim N(0, \sigma^2_{ik})$ for the total phenolic content. Therefore the mixed linear models have 12 parameters of variance for the relative necrosis and 14 parameters of variance for the total phenolic content.

To choose the best model among the possibilities we used the lowest Bayesian (BIC) and the Akaike information criterion (AIC). Normality, linearity and homoscedasticity of the residuals were checked by the Kolmogorov-Smirnov test and graphical procedures. A 5% level of significance was used in the statistical analyses. A Tukey-Kramer HSD test was applied for the comparisons of means when significant differences were found in the ANOVA table. To explore a possible relationship among necrosis length and total phenols content a non-parametric Spearman's correlation test ($p=0.05$) was performed.

3.3. Results

3.3.1. Effectiveness of the *Gremmeniella* infections

The first symptoms caused by the *G. abietina* infection were observed a few weeks after the inoculations. Symptoms observed consisted of dried needles, discoloration, necrosis, cankers and the death of some plants. We found fruiting bodies of *G. abietina* in the 42% of the seedlings inoculated in time of inoculation 1 while only the 3% of seedlings had pycnidia in the time of inoculation 2. There were not fruiting bodies on control seedlings. A first attempt of re-isolating the fungus by means of traditional subculture on PDA was performed but nested PCR had to be used based on the lower percentage of re-isolation obtained. The results from nested PCR showed that 53.52 % of the samples with *G.abietina* inoculations showed the presence of the specific band of approx. 840 bp (Figure 3.1) in the gel electrophoresis. We found some differences based on the time of inoculations; in round one, the 58.33% of the samples were successfully amplified as compared to 47.22% of the samples from round two.

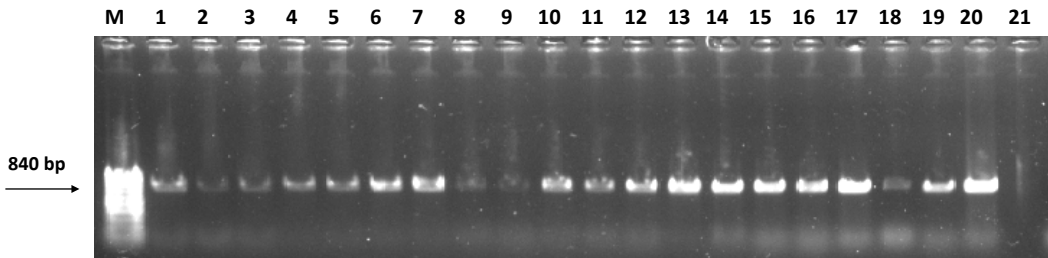


Figure 3.1: Detection of *G.abietina* in *P. halepensis* seedlings. PCR products of nested PCR. Lanes 1-19: Samples from infected seedlings. Lane 20: Positive control using *G. abietina* DNA. Lane 21: Negative control. M: marker 100 bp DNA Ladder.

3.3.2. Relative necrosis length

The best linear mixed model was selected for relative necrosis length (RLN) according to the lowest AIC and BIC values. A linear mixed model with 12 variance parameters (one variance for every time of inoculation-isolate) and no random effects was used to test the effect of the time of inoculation, the *Gremmeniella* isolate, the endophyte species and their interactions on the necrosis length. The ANOVA table showed that all the main effects: time of inoculation, endophyte and isolate as well as the interactions time of inoculation*isolate and endophyte*isolate were all statistically significant variables (Table 3.2).

Table 3.2: ANOVA table for relative necrosis length total phenols content.

Dependent variable	Effect	DF	F-value	Pr > F
Relative Necrosis Length	Time of inoculation (TI)	1	98.95	< 0.0001
	Endophyte	5	11.31	< 0.0001
	Isolate	6	64.16	< 0.0001
	TI*Endophyte	5	1.32	0.2547
	TI*Isolate	6	14.13	< 0.0001
	Endophyte*Isolate	30	1.72	0.0108
	TI*Endophyte*Isolate	30	1.04	0.4072
Total Phenols Content	Time of inoculation (TI)	1	44.78	< 0.0001
	Endophyte	5	2.1	0.0683
	Isolate	6	3.52	0.0049
	TI*Endophyte	5	11.91	< 0.0001
	TI*Isolate	6	9.01	< 0.0001
	Endophyte*Isolate	30	0.97	0.5218
	TI*Endophyte*Isolate	30	0.97	0.5116

The Tukey-Kramer test revealed that, on average, seedlings inoculated on time of inoculation 1 had significantly higher relative necrosis length measurement than those inoculated in time of inoculation 2 (Table 3.3). Regarding the *Gremmeniella* isolates, seedlings inoculated with any of them, regardless which endophyte was also inoculated, showed relative necrosis length higher than that observed in the controls (G-control) (Table 3.3). In both times of inoculation, G-control seedlings had less RLN than the seedlings inoculated with the *G.abietina* isolates (Table 3.3). Among the *G.abietina* isolates we also found some differences; G1 had lower RLN than G2, G3, G4, G5 and G6. Among them, G2 produced the significantly highest necrosis length in the seedlings, regardless the time of inoculation (Table 3.3). Differences in necrosis were also observed among the other isolates, although it was dependent on the time of inoculation (Table 3.3).

Chapter 3 Fungal endophytes reduce necrosis length

Table 3.3: Relative necrosis length. Mean values \pm standard error. ¹ Average necrosis when combining all the times of inoculation together. ² Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ³ Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁴ Average necrosis when combining all the *G.abietina* isolates together.

Isolate	Time of inoculation		Total ¹
	T1	T2	
G1	0.0544 \pm 0.015 b ² A ³	0.091 \pm 0.0087 b B	0.0727 \pm 0.0087 b
G2	0.2676 \pm 0.015 d B	0.1445 \pm 0.0087 d A	0.206 \pm 0.0087 d
G3	0.2383 \pm 0.015 cd B	0.1288 \pm 0.0087 cd A	0.1835 \pm 0.0087 cd
G4	0.236 \pm 0.015 d B	0.1184 \pm 0.0087 c A	0.1772 \pm 0.0087 c
G5	0.2239 \pm 0.015 c B	0.1313 \pm 0.0087 cd A	0.1776 \pm 0.0087 c
G6	0.1985 \pm 0.015 c B	0.1329 \pm 0.0087 cd A	0.1657 \pm 0.0087 c
G-Control	0.0108 \pm 0.015 a A	0.0255 \pm 0.0087 a B	0.0182 \pm 0.0087 a
TOTAL ⁴	0.1757 \pm 0.0057 B	0.1104 \pm 0.0033 A	

The presence of all endophytes reduced significantly the necrosis produced by *G. abietina* (Table 3.4) but no differences were found among the various endophyte species (Figure 3.2). When analyzed separately, the data showed that the effect of each endophyte was different for each *Gremmeniella* isolate. In the case of isolate G1, only the endophyte E5 (*Leotiomycece* spp.) was significantly effective reducing the RLN caused by the pathogen. In the case of G2, two of the endophytes (E3 (*Aureobasidium* spp.) and E4 (endophyte 20.1) reduced significantly the RLN caused by the pathogen. For G3, none of the endophytes was able to reduce significantly the necrosis length; whereas for G4, G5, and G6, the five endophytes were able to reduce the necrosis caused by the pathogen (Table 3.4) compared to the control seedlings. The susceptibility of the different *Gremmeniella* isolates to each endophyte can be also analyzed in Table 3.4. The isolate G1 was the most susceptible to the influence of all the endophytes. Even for three cases

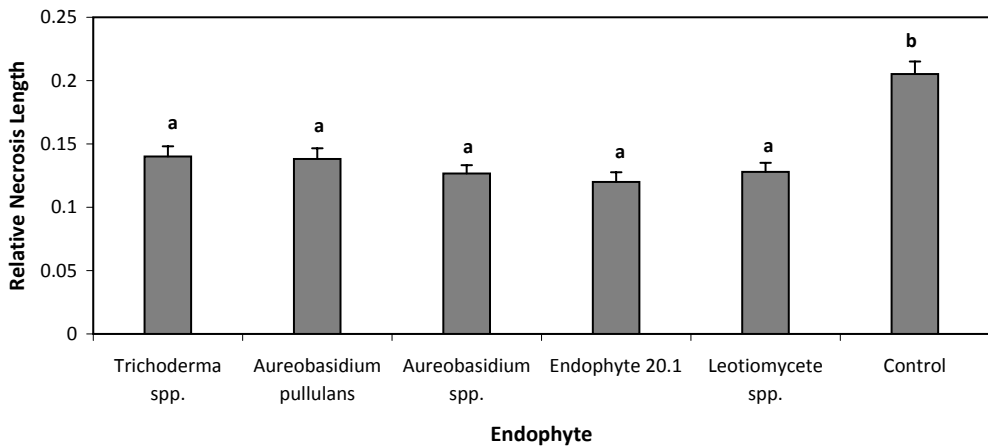


Figure 3.2: Relative necrosis length found in every seedling when inoculating both *G.abietina* isolates and different endophytes. Control seedlings had no endophyte but *G. abietina* isolate. Means with a different letter were significantly different from $p < 0.05$ (Tukey's HSD Test). Bars represent standard error.

(for E2 (*Aureobasidium pullulans*), E4 (endophyte 20.1), and E5 (*Leotiomycece* spp.) the endophyte reduced the necrosis length of this isolate to that observed in the controls (Table 3.4). In contrast, the isolate G2 seemed to be the most resistant to the influence of any endophyte antagonism, especially for E2 (*A. pullulans*) and E5 (*Leotiomycece* spp.). The rest of the *Gremmeniella* isolates showed a susceptibility to the endophyte influence in between those two extremes. None of the endophytes were pathogenic for *P. halepensis* seedlings as no significant differences were found in the RLN between the E-control and the G-control of either endophyte.

3.3.3. Total Phenols Content

A linear mixed model with 14 variance parameters (one variance for every combination isolate * endophyte) and no random effects was selected according to AIC and BIC criteria to test the effect of the time of inoculation, the *Gremmeniella* isolate, the endophytes species and their interactions on

the total phenols content. The ANOVA table (Table 3.2) for the linear mixed model showed that all the following variables to be significant: time of inoculation, isolate, time of inoculation*endophyte and time of inoculation*isolate. On the contrary, the endophyte species and the rest of interactions did not show significant influence on the total phenol concentration. On average, plants inoculated at the time of inoculation 2 had higher total phenolic content than those inoculated on time of inoculation 1 (Table 3.5). Among the isolates, G-control seedlings and the ones inoculated with isolate G1 presented the highest concentrations of total phenols (Table 3.5). At the time of inoculation 1 the G-control seedlings (no *Gremmeniella* isolate) and the ones inoculated with the isolate G1 had a significantly higher content of total phenols than the seedlings inoculated with the rest of *G. abietina* isolates. There were differences also among the other isolates. Seedlings inoculated with G6 had higher phenolic content than the seedlings inoculated with G2, G4 and G5. In time of inoculation 2 G-control seedlings had higher phenolic content than seedlings with G3, but there were no differences between G-control and the rest of the *Gremmeniella* isolates.

Table 4: Relative necrosis length. Mean value ± standard error (SE).

Isolate	Endophyte						Total ¹
	E1	E2	E3	E4	E5	E-Control	
G1	0.082 ± 0.0213 b ² AB ³	0.0509 ± 0.0225 a AB	0.0807 ± 0.0173 b AB	0.0669 ± 0.0201 a AE	0.0408 ± 0.0191 a A	0.105 ± 0.0263 b B	0.0727 ± 0.0087 b
G2	0.1645 ± 0.0213 c A	0.2343 ± 0.0225 c BC	0.1476 ± 0.0173 c A	0.1798 ± 0.0201 b AE	0.2238 ± 0.0191 c BC	0.2863 ± 0.0263 cd C	0.206 ± 0.0087 d
G3	0.194 ± 0.0213 c A	0.1773 ± 0.0225 bc A	0.1863 ± 0.0173 c A	0.1565 ± 0.0201 b A	0.1171 ± 0.0191 bc A	0.216 ± 0.0263 c A	0.1638 ± 0.0087 c
G4	0.1742 ± 0.0213 c A	0.1594 ± 0.0225 b A	0.148 ± 0.0173 c A	0.1322 ± 0.0201 b A	0.1519 ± 0.0191 b A	0.2974 ± 0.0263 d B	0.1772 ± 0.0087 c
G5	0.2021 ± 0.0213 c B	0.1465 ± 0.0225 b AB	0.1715 ± 0.0173 c AB	0.1396 ± 0.0201 b A	0.1369 ± 0.0191 b A	0.2693 ± 0.0263 cd C	0.1776 ± 0.0087 c
G6	0.1446 ± 0.0213 c A	0.1574 ± 0.0225 b A	0.139 ± 0.0173 c A	0.1345 ± 0.0201 b A	0.1605 ± 0.0191 b A	0.2594 ± 0.0263 cd B	0.1657 ± 0.0087 c
G-Control	0.0193 ± 0.0213 a A	0.0307 ± 0.0225 a A	0.0137 ± 0.0173 a A	0.0307 ± 0.0201 a A	0.0103 ± 0.0191 a A	0.0044 ± 0.0263 a A	0.0182 ± 0.0087 a
TOTAL ⁴	0.1401 ± 0.008 A	0.1381 ± 0.0085 A	0.1287 ± 0.0085 A	0.12 ± 0.0076 A	0.1279 ± 0.0072 A	0.2052 ± 0.0099 B	

¹ Average growth when combining all the endophytes together.
² Means without a common small letter in the same column show values significantly different from p < 0.05 (ANOVA Tukey's HSD Test).
³ Means without a common capital letter in the same row values significantly different from p < 0.05 (ANOVA Tukey's HSD Test).
⁴ Average necrosis when combining all the *G. abietina* isolates together.

Table 3.5: Total phenols (equivalents Gallic acid (mg)/ dry weight of the sample (g)). Mean value \pm standard error (SE). ¹ Average necrosis when combining all the times of inoculation together. ² Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ³ Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁴ Average necrosis when combining all the *G.abietina* isolates together.

Isolate	Time of inoculation		Total ¹
	T1	T2	
G1	1282.44 \pm 98.78 a ² A ³	1043.03 \pm 63.93 ab B	1162.73 \pm 58.83 a
G2	349.81 \pm 45.99 c B	1038.47 \pm 89.56 ab A	694.14 \pm 50.34 bc
G3	515.87 \pm 115.74 bc B	888.19 \pm 78.43 b A	702.03 \pm 69.9 bc
G4	486.79 \pm 119.93 c B	1087.66 \pm 79.81 ab A	787.22 \pm 72.03 bc
G5	385.57 \pm 110.47 c B	983.55 \pm 74.11 ab A	684.56 \pm 66.51 c
G6	694.67 \pm 74.34 b B	1049.55 \pm 81.83 ab A	872.11 \pm 55.28 b
G-Control	1239.87 \pm 112.54 a A	1122.11 \pm 83.42 a A	1180.99 \pm 70.04 a
TOTAL ⁴	707.86 \pm 37.81 B	1030.37 \pm 29.89 A	

Among endophytes, at both times of inoculation, none of the endophytes were able to promote a higher phenolic production in the plants compared to E-controls (Figure 3.3, Table 3.6). Nevertheless, some differences among endophytes were found. At the time of inoculation 1, seedlings inoculated with E3 (*Aureobasidium* spp.) produced a higher total phenol content than seedlings inoculated with E1 (*Trichoderma* spp.), E2 (*A. pullulans*) and E5 (*Leotiomycece* spp.) (Table 3.6). In the second inoculation time, seedlings inoculated with E1 (*Trichoderma* spp.), E2 (*A. pullulans*), E5 (*Leotiomycece* spp.) and the E-control seedlings presented a significantly higher amount of total phenols than seedlings inoculated with E3 (*Aureobasidium* spp.) and E4 (endophyte 20.1) (Table 3.6). Relative necrosis

length showed a negative correlation ($r=-0.48002$; $p<0.0001$) with the total phenolic content (Figure 3.4).

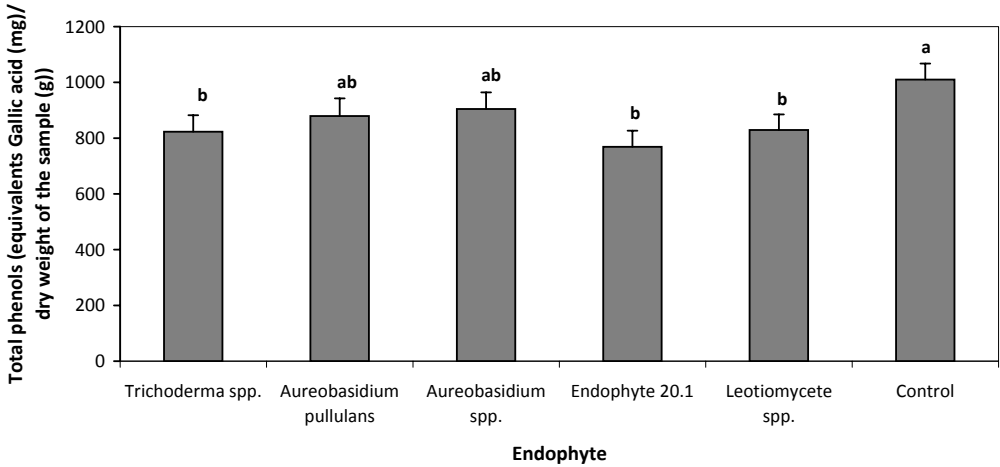


Figure 3.3.: Total phenols content, (equivalents Gallic acid (mg)/dry weight of the samples (g)) found in every seedling when inoculating both *G.abietina* isolates with the different endophytes. Control seedlings had no endophyte but *G. abietina* isolate. Means without a common letter were not significantly different from $p<0.05$ (Turkey’s HSD Test). Bars represent standard error.

Table 3.6: Total phenols (equivalents Gallic acid (mg)/ dry weight of the sample (g)). Mean value \pm standard error (SE). ¹ Average necrosis when combining all the times of inoculation together. ² Means without a common small letter in the same column show values significantly different from $p<0.05$ (ANOVA Tukey’s HSD Test). ³ Means without a common capital letter in the same row values significantly different from $p<0.05$ (ANOVA Tukey’s HSD Test). ⁴ Total phenols when combining all the endophytes together.

Endophyte	Time of inoculation		Total ¹
	T1	T2	
E1	585.01 \pm 93.28 b ² B ³	1060.75 \pm 72.76 a A	822.88 \pm 59.15 b
E2	621.42 \pm 104.29 b B	1137.07 \pm 72.76 a A	879.24 \pm 63.58 ab
E3	924.38 \pm 94.36 a A	884.68 \pm 72.76 b A	904.53 \pm 59.58 ab
E4	695.9 \pm 89.71 ab A	841.69 \pm 72.76 b A	768.8 \pm 57.76 b
E5	589.7 \pm 85.62 b B	1068.47 \pm 72.76 a A	829.08 \pm 56.18 b
E-Control	830.75 \pm 87.18 ab B	1189.54 \pm 75.42 a A	1010.14 \pm 57.64 a
TOTAL ⁴	707.86 \pm 37.81 B	1030.37 \pm 29.89 A	

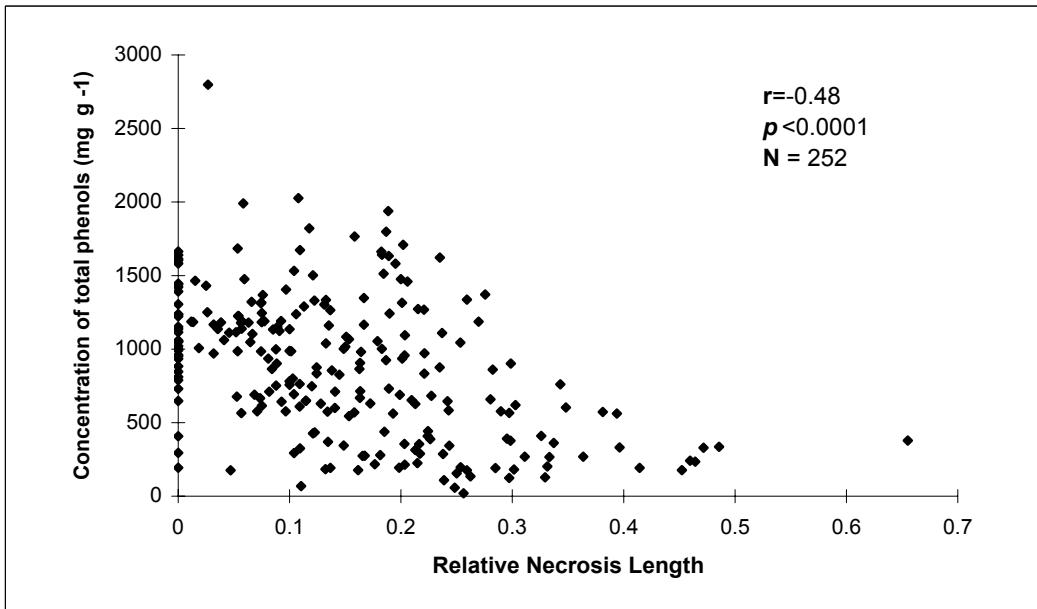


Figure 3.4: Correlation among concentration of total phenols (milligrams equivalents of Gallic acid per gram of dried sample) and Relative Necrosis Length.

3.4. Discussion

This study explores the effectiveness of biological control against *G. abietina* in Aleppo pine seedlings. Our results showed that the inoculation of *G. abietina* produced symptoms of chlorosis, dieback, necrosis, cankers and the death of some seedlings. These symptoms were similar to those previously reported in others studies (Uotila 1993; Santamaría et al., 2003; 2006). The results also showed that at the time of inoculation 1 there were fruiting bodies in 42% of the seedlings inoculated with *G.abietina* isolates while at the time of inoculation 2 this percentage was only 3%. Furthermore, the necrosis length was also statistically higher at the time of inoculation 1 than at the time of inoculation 2. Both facts confirm that in our study the time of infection was very important in terms of damage and control. The results of

DNA amplification were consistent with this statement: 58.33% of the samples from seedlings inoculated in the first round were successfully amplified whereas in round 2 the percentage was 47.22% of the samples. The longer the necrosis length, the greater the fruiting bodies production and the higher percentage of re-isolation by means of DNA amplification observed at the time of inoculation 1 could be explained by the fact that *G. abietina* is more active during the dormant season and its activity is influenced by the resource allocation process (Ranta et al., 2000). Therefore, when inoculations were carried out at the time of inoculation 1, the fungal pathogen was growing during a longer period in the optimal conditions for this fungus development.

The attempt of re-isolating the fungus by means of a traditional subculture on PDA media was a failure in our experiment as opposed to Santamaría et al. (2007) who obtained a 66% of re-isolation of *G. abietina* with this method. Because of that, the nested PCR was performed and 53% of the samples from the seedlings inoculated were successfully amplified with the specific *G. abietina* primers of the 18S region. Although the ITS region has been recently described as the international barcode for fungi and the marker of choice for studying the fungal diversity (Schoch et al. 2012; Kõljalg et al. 2013) the use of the 18S region to amplify the DNA is less likely to produce false negative detections (Zeng et al. 2005). Furthermore, the use of specific primers for *G. abietina* simplified the process as no-sequencing was needed, and the results could be visualized directly in the electrophoresis' gel.

Results from our experiment showed that generally speaking the presence of all the endophytes reduced the necrosis length produced by *G. abietina* although the efficacy of the control depended also on the time of inoculation. Several mechanisms could be responsible for that reduction. It

has been reported that biological control agents may produce substances that directly attacks the pathogens or that induce the systemic resistance which, in turn, reduce the pathogen incidence in the plant host (Paul and Sharma, 2002; Gao et al., 2010; Akila et al., 2011). For example, in seedlings of *Theobroma cacao* L., inoculation with endophytes significantly reduced leaf necrosis and mortality caused by a major foliar pathogen. On the pathogen-infected leaves that did survive, necrotic lesions were significantly larger on leaves without endophytes than on leaves with endophytes (Arnold et al 2003). Biocontrol agents employ an assortment of mechanisms to control plant diseases that vary with the host, the pathogen and also with the biocontrol agent involved in the interaction. In addition, these mechanisms are also influenced by the environmental conditions where the interaction is produced: leaf chemistry, soil type, temperature, pH, moisture of the plant and the possible occurrence of other endophytic species (Arnold et al. 2003; Howell, 2003; Miles et al. 2012).

In our study, the isolate from genera *Trichoderma* spp. was able to successfully control part of the spreading of *G. abietina*, as it caused a reduction of the necrosis produced by the pathogen in the seedlings compared to the control ones. Good results on *in vitro* experiments were previously shown in Santamaría et al. (2007) in the reduction of mycelial growth of *G. abietina* on dual culture tests. This genus has been reported as an effective tool against pathogens before. For instance, it was effective against *Fusarium circinatum* Nirenberg and O'Donnell on *in vitro* experiments (Martínez-Álvarez et al., 2012), against *Cytospora chrysosperma* (Pers.) Fr. both on *in vitro* and on *in vivo* tests (Yi and Chi, 2011) against *Botrytis cinerea* Pers. in Scots pine seedlings (Capiéau et al., 2004) against *Diplodia pinea* on *in vivo* experiments in *Pinus banksiana* Lamb. (Santamaría

et al., 2012) and against the causal agent of Dutch Elm disease in laboratory tests conducted under *in vitro* conditions (Diaz et al. 2013). It is especially effective against pathogens that colonize the rhizosphere, because it has been shown to increase the root growth of the plant as well as its systemic resistance (Inbar et al., 1994; Reglinski et al., 2012). The efficiency of *Trichoderma* as a biological control agent depends on the pathogen species they are confronted with. But in general terms, *Trichoderma* can be considered a good candidate to be used as biocontrol agent due to its high capacity and rate of reproduction; they are able to survive when the environmental conditions are not favorable, they can modify the rhizosphere, they present the ability to transport glucose rapidly which gives them an advantage in nutrient competition and they can promote plant growth and better defence mechanisms (Benitez et al. 2004). In some cases *Trichoderma* strains combine several of those mechanisms to successfully fight against the phytopathogenic fungi.

In addition to *Trichoderma*, our results showed that the inoculation with isolates of the *Aureobasidium* genus (both *A. pullulans* E2 and *Aureobasidium* spp. E3) also produced a reduction in the necrosis length caused by *G. abietina*. Previous studies have also pointed out the antagonistic activity of the species *A. pullulans* against several fungal pathogens such as *Penicillium expansum* Link (Mounir et al., 2007) and *Aspergillus carbonarius* (Bainier) Thom (Dimakopoulou et al., 2008). Miles et al. (2012) observed that *A. pullulans* was the most successful biocontrol agent based on the evaluation of its efficacy on *in vivo* test against *Rhizoctonia solani* J.G. Kühn, bacteria and oomycetes on tomato plants. This genus is especially effective on postharvest pathogens of several fruits and its mechanisms of success include the induction of plant defence responses and the competition of nutrients

(Banani et al. 2014). The endophyte named 20.1 also reduced the necrosis length produced by *G. abietina* in the seedlings compared to the control plants. Although no identification of the species has been made yet, a previous study performed *in vitro* by Santamaría et al. (2007), showed that in Petri Dishes, this endophyte completely inhibited *G. abietina*'s growth. Furthermore, this endophyte produced a brownish pigment around the colony on culture media so that *G. abietina* colonies became more compact and dense. These results suggested that the endophyte could produce some antifungal compounds which slow down the growth of *G. abietina*. Lastly, the isolate from *Leotiomycete* genus was also able to reduce the necrosis produced by *G. abietina* in the seedlings. Very scarce literature was found about *Leotiomycetes* fungus as a biocontrol agent. Nevertheless, Miles et al. (2012) tested several endophytes, including one member of *Leotiomycete* class, *Botrytis fabae* Sardiña which can acts as a plant pathogen as well, and concluded that it reduced the growth of other pathogens such as *Botrytis cinerea*, *Fusarium oxysporum* E.F. Sm. and Swingle, *Phytophthora infestans* (Mont.) de Bary, and *Rhizoctonia solani*.

Among the mechanisms which explain the reduction in the necrosis caused by the pathogen when an endophyte was also inoculated, several authors (Muñoz et al. 2008; Reglinski et al. 2012) have proposed that endophytes might activate a systemic induced resistance (SIR) mechanism in the host plant which might contribute to reduce the incidence of the disease. An increase in the concentration of phenolic compounds has been previously related to the activation of the induced defence mechanisms of the plant. For instance, *Trichoderma* spp. strains produce compounds that induce the synthesis and accumulation of phytoalexins, flavonoids, terpenoids, phenolic derivatives, aglycones and other antimicrobial compounds (Benitez et al.

2004). Nevertheless, the results of the present study showed that the presence of the endophytes, did not produce an increase of the total phenols. Therefore, although some phenolic production would have been instigated by the endophytes, it would not have been enough to retain the pathogen development. Consequently, in our case the reduction in the necrosis length caused by *G. abietina* observed when the endophyte was also inoculated could be likely caused, rather than by a systemic induced resistance mechanism, by the direct effect of the endophyte on the pathogen. In this case, competition and/or antibioses could be the mechanisms more likely involved in the observed antagonism.

On the other hand, in our study, the infection of the seedlings by *G. abietina* did not produce an increase in the total contents of phenolic acids compared to the control seedlings. This was inconsistent with the fact that an increase in phenolic compounds would be expected since they have been previously described as a part of conifer defence against *G. abietina* (Cvikrova et al. 2006). In addition, in most of woody plants phenols have been studied as markers for pathogen resistance (Witzell and Martin, 2008). It is known that wounding or an invasion of the bark has been shown to activate polyphenolic parenchyma cells, which includes cell expansion and accumulation of increased amounts of phenols. Thus, phenolic compounds can act as antifungal agents and can bind hydrolytic enzymes secreted by pathogens, thus inhibiting their spread into tissues (Franceschi et al. 2005). Nevertheless, relationships among such substances composition and resistance to pathogens are unclear and sometimes contradictory and some authors report finding no such correlations. For instance, there were no correlations between monoterpene composition and resistance to *Heterobasidion annosum* (Pearce, 1996). Varying relationships have also been

found between lesion length produced by other pathogens and secondary metabolites (Wallis et al. 2008; Witzell and Martin, 2008). The lack of response in the plant's phenols production by plants as a consequence of the pathogen inoculation could be explained by the fact that the seedlings used in the experiments were young (2-year old) and still developing. Therefore, the suberization of their cell walls may not have been complete. Furthermore, *G.abietina* has reported to have some phenoloxidase activity because it could grow through the bract cells surrounded by lignified and suberized cell walls and filled with phenols (Ylimartimo et al., 1997). In addition, Simard et al. (2013) observed that lignin, suberin and other phenolic compounds could be degraded by *G.abietina* in the transition zones of the infection. Thus, the low suberization of the cells linked to the ability to degrade phenolic compounds of *G. abietina* could explain the lack in response of the plants regarding phenolic production.

In the current study, the control seedlings without *G. abietina* inoculations showed the highest concentration of phenolic compounds. In our case, the higher phenolic content found in the not inoculated seedlings could be explained by the fact that the analyses were made six months after inoculation when plants were very affected by the pathogen. Under this condition it is supposed that the capacity of the plants to produce these defensive compounds could be reduced and if the analyses had been performed only some weeks after inoculation, the phenols contained in the plant tissues would have been higher. Therefore, it could be hypothesized that the induced resistance mechanisms of the plant are activated when the infection is produced. Nevertheless, further experiments including several samplings along the infection process should be carried out to confirm this hypothesis.

This study provides additional knowledge about the effects of the inoculation of *G. abietina* and fungal endophytes in Aleppo pine seedlings. It can be concluded that the use of fungal endophytes could be a suitable strategy to reduce the incidence of plant pathogens like *Gremmeniella abietina* in pine seedlings. This statement is made based on the results obtained in which the inoculation of an endophyte into a plant reduced the advance of the pathogen; although the efficiency of the control depended on the moment of inoculation. The inoculation with an endophyte did not promote a higher production of phenolic compounds, which are considered a good indicator of such induced resistance mechanism. However, it seemed that competition and/or antibiosis were the mechanisms responsible for that reduction, rather than a systemic induced resistance mechanism. Nevertheless, further studies of biological antagonisms are recommended, particularly about the mechanisms they employ to interact with the pathogens.

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Chapter 4

Antagonistic activity of fungal endophyte filtrates against *Gremmeniella abietina* infections on Aleppo pine seedlings

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ABSTRACT

Biological control agents (BCAs), and among them some species of fungal endophytes, are potential substitutes for chemical pesticides in the control of plant diseases due to their non toxicity to human beings and their surrounding environment. One mode of action of fungal BCAs is through their bioactive, extracellular products, which can inhibit the growth of pathogens. In this study, the effect of fungal filtrates from four endophyte isolates (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. and the unknown endophyte 20.1) on the advance of the pathogen *Gremmeniella abietina* on two-year *Pinus halepensis* seedlings was evaluated. Both preventive and therapeutic treatments of the filtrates were studied by applying the filtrates either before or after the pathogen inoculation, respectively. Since *G. abietina* is a necrotrophic fungus, the length of the necrosis produced by the pathogen was used as response variable in our experiment. In order to explore the chemical composition of the fungal filtrates, a simple HPLC screening of UV-absorbing components was conducted. The results of the

study showed that all fungal filtrates were able to reduce the advance of *G. abietina* when compared to the control seedlings, regardless of the time of inoculation and the treatment. Low-molecular weight phenolic compounds could be detected in some but not all filtrates, warranting further studies on the possible role of these compounds in fungal filtrates.

Keywords: Biological control agents, antibiosis, secondary metabolites, forest pathogens, *Trichoderma*, *Aureobasidium*.

4.1. Introduction

The Aleppo pine (*Pinus halepensis* Mill.) is one of the most common species in the Mediterranean, its forest area spanning more than 3 million ha and more than 800.000 ha in Spain (Gil et al. 1996). This species can withstand a wide variety of environmental conditions and soil features, and it presents a high resistance to drought. Because of its ecological plasticity, it has been used for reforestation in degraded areas and for plantations with commercial purposes in Spain (Gil et al. 1996). However, over the last few years, environmental conditions have been unfavourable for *P. halepensis*, especially in the north western part of the Iberian Peninsula where it grows outside its optimum natural habitat (Abelló 1998). In 1999, the fungal pathogen *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnelt) was detected and isolated from *P. halepensis* plantations in northern Spain causing defoliation, discoloration, terminal twig distortion and cankers (Santamaría et al. 2003). The fungus infects the trees during the spring, but the external symptoms appear after a latent period of the host (Ylimartimo et al. 1997). Ascomycetous fungi belonging to the genus *Gremmeniella* are all pathogens; they have been found all over the Northern Hemisphere spreading diseases on several conifer species. The most

important damages have been recorded on *Pinus*. Both seedlings and adult trees may be affected, and, on several occasions, epidemic outbreaks have led to the destruction of natural forests and restored stands (Yokota 1975; Dorworth 1979; Laflamme & Lachance 1987; Kaitera & Jalkanen, 1992; Kaitera et al. 1998; Wulff et al. 2006).

The control of *G. abietina* has varied from silvicultural to chemical practices. Some of the silvicultural techniques performed in the forests, like pruning and removing dead trees, may decrease the source of inoculum and thus slow the spread of the pathogen (Laflamme 1999). In some nurseries, the applications of synthetic fungicides such as chlorothalonil have been used to reduce *G. abietina* infections although mainly as an emergency measure (Skilling & Waddell 1970; Smerlis 1980). Nevertheless, there is currently an increasing interest in finding effective biological control methods, e.g. recent EU legislation (Council Directive 2009/128/EC) recommended sustainable forest management and protecting forests and their biodiversity giving priority to non-chemical methods of plant protection.

With the use of synthetic fungicides in forestry progressively more restricted by the strengthening of regulatory limitations and the risks of detrimental effects on the environment (Brimner & Boland 2003) more and more apparent, finding biological solutions is becoming an increasingly attractive control strategy against plant pathogens (Cook 1996; Pal & McSpadden 2006). Biological control is the use of living organisms to fight against a disease and is based on the antagonism of pathogens by the presence or the activities of other microorganisms. However, other authors broaden the definition and include not only the use of antagonistic microorganisms, but also the application of naturally derived bioactive compounds (Talibi et al. 2014). These microbial antagonists are known as biological control agents (BCAs). The interaction of a BCA and a pathogen

include: (i) mycoparasitism; the pathogen is directly attacked by a BCA that kills it or its propagules; (ii) antibiosis and metabolite production; i.e. the BCAs produce substances that are toxic to the pathogen; (iii) competition for nutrients; i.e. the BCAs occupy the same ecological niche of the pathogen and therefore deplete the nutrients necessary for its establishment; (iv) induction of the plant defence system; i.e. the stimulation of the host plant defences by the presence of the BCAs and (v) the barrier effect, caused by the presence of mycorrhizal fungi (Schoeman et al. 1999; Alabouvette et al. 2006; Ownley and Windham, 2007; Heydari & Pessaraki 2010; Diez & Alves-Santos 2011). Among the potential BCAs there are several fungal endophytes, i.e. fungi that live inside the plant tissue and maintain either a neutral, detrimental or beneficial relationship with the host plant (Sieber 2007; Backman & Sikora 2008). In other studies previously conducted, several species of fungal endophytes were able to reduce the growth of *G. abietina*. For example, *Phaeothea dimorphospora* Desrochers and Ouellette inhibited the mycelial growth of the colonies, the germination of the spores and the spread of the pathogen on seedlings of red pines (Yang et al. 1995). Santamaria et al. (2007) observed a reduction or even an inhibition of the growth of Spanish isolates of *G. abietina* on Petri dishes it was confronted with some endophytes such as *Trichoderma*, *Aureobasidion*, *Cladosporium* and some unknown fungus called 20.1. Lastly, Romeralo et al. (2015) observed that *Trichoderma viride*, *Aureobasidion pullulans*, the endophyte 20.1 and a *Leotiomyce* reduced the progression of *G. abietina* when inoculating both with mycelia on plants.

To protect themselves from the attack of the pathogens, plants have several defence mechanisms known as constitutive, if they already exist in the plant before the infection, or induced if they are produced as a consequence of it. The induced response leads to the production of some hormones to

extend the communication within the plant preparing it to prevent future infections which is called systemic acquired resistance (Agrios 1997; Franceschi et al. 2005). The presence of some BCAs has shown to activate this defence system effectively against other fungal pathogens (Muñoz et al. 2008; Regliński et al. 2012).

Antibiotics, which are involved in the mechanisms employed by the BCAs, are microbial extracellular toxins that may eradicate other microbial cells. Most microbes produce and secrete one or more compounds with antibiotic activity. In some instances, antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens (Pal & McSpadden 2006). They include not only antibiotics *sensu stricto* but also bactericides, cell wall degrading enzymes, and volatile compounds with antifungal activity (Alabouvette et al. 2006). The role of antibiotics in biocontrol has been studied with genetic analyses by using mutants that do not produce antibiotics (Lo 1998). Apparently, antibiotic production is not specific to certain species. Different species may produce the same antibiotics or secondary metabolites, while products of different strains of the same species may turn out to be quite distinct (Lo 1998). Even different secondary metabolites produced by a single strain of a BCA might be responsible for the antagonistic activity towards different pathogens (Alabouvette et al. 2006). Examples of antifungal metabolites produced by either fungi or bacteria are: phenazine, produced by *Pseudomonas fluorescens* Migula; cladosporin produced by *Cladosporium cladosporioides* (Fresen.) G.A. de Vries; gliovirin and gliotoxin produced by *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx, and alkylpirones and peptaibol produced by *T. harzianum* Rifai (Lo 1998; Alabouvette et al. 2006; Wang et al. 2013).

Although biologically-based methods are desirable, there are only a few cases when they are applied in practice when managing forest diseases.

One example is the control of the root and butt rot pathogen *Heterobasidion annosum* (Fr.) Bref. with the fungus *Phlebiopsis gigantea* (Fr.) Jülich. In Scandinavia, *Phlebiopsis* stump treatment is commonly applied, as it may reduce *H. annosum* colonization on stump surfaces by 89-99% compared to untreated stumps (Thor & Stenlid 2005). Another example of biological control of forest disease in Europe is the control of the Chestnut blight fungus (*Cryphonectria parasitica* (Murr.) Barr.) using hypovirulent pathogen strains. The infection of the fungus produces cankers on stems and branches. Hypovirulent strains host viruses from the genus *Hypovirus* that reduce the virulence of these strains and are also transmissible by hyphal anastomosis (Anagnostakis & Day 1979; Polashock et al. 1997).

Since some endophytes had such good results in reducing the growth of the pathogen both *in vitro* (Santamaria et al. 2007) and *in vivo* (Romeralo et al. 2015) our hypothesis was that these endophyte's filtrates would be able to reduce or stop the progression of *G. abietina* once in the seedlings. Consequently, in the present study, the suitability of selected fungal endophytes filtrates in the control of the *G. abietina* is described. The specific goals of the present work were (i) to test if endophyte filtrates can provide preventive or therapeutic protection against *G. abietina* in *P. halepensis* seedlings and (ii) to screen the filtrates for UV-absorbing compounds to characterize the chemical composition of the fungal filtrates. The results are discussed with special emphasis on the potential use of the tested fungal filtrates as a novel, bio-based tool in the control of *G. abietina* in *P. halepensis* seedlings.

4.2. Materials and Methods

4.2.1. Plant material, fungal isolates and filtrates

The experiment was conducted in December 2011 (mean $T^{\circ}=4.4^{\circ}\text{C}$) and January 2012 (mean $T^{\circ}=3.4^{\circ}\text{C}$) in the shade cloth greenhouse of the College of Agricultural Engineering at the University of Valladolid, in Palencia, Spain. Two-year-old containerized Aleppo pine seedlings were used to perform this experiment obtained from the Central Nursery of the Castilla y León regional government. The seedlings ($n=840$) had a mean root collar diameter and height of $3.03 \text{ mm} \pm 0.73$ and $17.13 \text{ cm} \pm 2.64$ respectively (mean \pm standard deviation). Six months prior to the inoculations, all standard nursery treatments against pests and fungi were stopped. Once in the greenhouse, the seedlings were watered regularly.

All the *G. abietina* and the endophyte's isolates (Table 4.1) came from a collection at the University of Valladolid Forest Pathology Lab. The *G. abietina* isolates were selected randomly whereas the endophytes were the same used in previous experiments with success in reducing *G. abietina* mycelial growth *in vitro* (Santamaria et al. 2007) and *in vivo* (Romeralo et al. 2015). The endophytes *Trichoderma viride* Pers., *Aureobasidium* sp., *A. pullulans* (de Bary & Löwenthal) G. Arnaud and endophyte 20.1 (which did not match with any known fungus in the BLAST database) were grown on PDA (potato, dextrose, agar) at room temperature ($25 \pm 2^{\circ}\text{C}$) for two weeks while the *G. abietina* isolates were cultured on MOS-agar (modified orange, serum-agar) at 15°C (Müller et al. 1994). To obtain the fungal filtrates from the endophytes, several pieces of mycelial agar plugs were placed into Erlenmeyer flasks containing 250ml of PDB (potato, dextrose, broth) and incubated at room temperature in the orbital shaker with constant movement for 3 months. After this period, the broth culture was filtered twice with

Table 4.1: Characteristics of the *G. abietina* isolates and the endophytes, host, species, place of origin in Spain and year of isolation.

Behaviour	Isolate	Name	Species	Origin	Province	Year of isolation
Pathogen	G1	Z0-10-01	<i>G. abietina</i>	Valle de Cerrato	Palencia	2010
	G2	Z0-10-02	<i>G. abietina</i>	Valle de Cerrato	Palencia	2010
	G3	P1-8	<i>G. abietina</i>	Valle de Cerrato	Palencia	2007
	G4	P1-12	<i>G. abietina</i>	Valle de Cerrato	Palencia	2007
	G5	VAI-13	<i>G. abietina</i>	Villalba de los Alcores	Valladolid	2003
	G6	00P-7	<i>G. abietina</i>	Valle de Cerrato	Palencia	2001
Endophytes	E1	1778 AB	<i>Trichoderma viride</i>	Tordehumos	Valladolid	2009
	E2	1077 4A	<i>Aureobasidium pullulans</i>	Valle de Cerrato	Palencia	2009
	E3	1812 RA 1-b	<i>Aureobasidium</i> sp.	Valle de Cerrato	Palencia	2009
	E4	20.1	Unknown Deuteromycete	Quintanilla de Onésimo	Valladolid	2004

Whatman® qualitative filter paper, Grade 1 (Whatman International Ltd, Maidstone, UK), in order to separate the broth and mycelia. The filtrates were preserved in refrigerators at 4 °C until the time of inoculation.

4.2.2. Experimental design, *G. abietina* inoculations and application of fungal filtrates

In order to know if the presence of the endophytic filtrate was able to either prevent *G. abietina* infections or reduce its growth, two treatments were performed: (i) preventive; a primary treatment with the endophyte filtrates, followed by a challenge inoculation with the pathogen one week later, and (ii) therapeutic; primary inoculation of the pathogen, followed by treatment with the endophyte filtrates one week later. To perform the inoculation with the pathogen, we used mycelium to ensure that the infection

would take place; conidial suspension was found to be less effective in previous results obtained with Spanish isolates in our lab (unpublished data). Therefore, a small wound was made with a sterile scalpel at 10 cm from the shoot apex, and a small piece of 0.25 cm² of mycelial agar of *G. abietina* cultures was placed in the wound and covered with Parafilm® to avoid desiccation. Treatments with the fungal filtrates were done with a sterile syringe at 8 cm from the top after making a small wound with a sterile scalpel. Afterwards, four drops of the endophyte filtrates were placed into the wound that was covered with Parafilm. Control treatments were made with sterile agar and broth filtrates. The inoculations were performed in December and January in accordance with descriptions that the pathogen colonizes the living host tissues only during the dormant season (Ranta et al. 2000). Three weeks after all inoculations were finished, the whole experiment was repeated. The experiment had a completely randomized factorial design with six repetitions per combination and four factors: (i) pathogen (six *G. abietina* isolates + water-inoculated control), (ii) endophytes' filtrate (filtrate from four endophyte isolates + sterile broth filtrate as a control), (iii) time of inoculation (December or January) and (iv) treatment (preventive or therapeutic). Thus, every combination consisted of the artificial inoculation of one of the 70 possibilities of "pathogen / endophyte filtrate / treatment". In order to avoid uncontrolled infections among adjacent seedlings, the plants were placed 5 cm from each other.

4.2.3. Evaluation and measurement of the seedlings and re-isolation of the pathogen

Seedlings were kept under the shade cloth greenhouse at ambient temperature until symptoms of the disease started to appear. In June, the seedlings were cut and brought to the laboratory in order to quantify the

damages. Several parameters of the seedlings were measured and evaluated: (i) the total length of the plant (cm), (ii) the diameter at root collar (mm), (iii) the presence of cankers (presence/absence) and (iv) the length of the necrosis (cm). In order to measure the necrosis produced by the advance of the pathogen, the seedlings were cut lengthwise. Since *G. abietina* is a necrotroph, the necrosis length was considered to be an appropriate indicator of the progression of the disease (Adomas & Asiegbu 2007). The response variable of our experiment was the relative necrosis length and was defined as the relationship between the necrosis length vs. the total length of the plant (Santamaría et al. 2006).

To confirm Koch's postulates, (i.e., that the necroses were indeed produced by *G. abietina*) we proceeded to re-isolate the pathogen from four seedlings of every combination of pathogen / endophyte filtrate / treatment (280 seedlings in total). From every sample, a portion of 6 cm was cut and submerged into 100 ml of sterilized distilled water for 1 min; the surface was sterilized with 100 ml ethanol (96%) for 2 min then placed into MOS-agar plates, incubated at 15° C for 15 days and revised daily for the emergence of any *G. abietina* colonies.

4.2.4. Qualitative analysis of organic compounds of fungal filtrates by extraction

Given the expected low concentration of organic compounds in raw extracts (Pal & McSpadden 2006) the samples were subject to a concentration step prior to analysis. Concentration was determined in a total volume of 360 ml of *T. viride*, 90 ml of *Aureobasidium sp.*, 90 ml of *A. pullulans*, 360 ml of endophyte 20.1 and 90 ml of control broth. For the isolation of metabolites, multiple batches were needed. In each batch 45 ml of fungal filtrates were extracted with 25 ml of ethyl acetate (EtOAc). For that aim, a stirrer

Vibromatic 680-750 U/min (10 min x 6) was used. The interphases were also preserved and extracted with brine (40 ml). Later, the combined organic phases were filtered with a C18 solid phase extraction cartridge (Sigma-Aldrich) at vacuum pressure. Afterwards, 5ml of acetonitrile was used as elution buffer, and the samples were stored at 4 °C until needed for the chromatography analysis.

4.2.5. Screening of UV-absorbing phenolic compounds in the extracts

To elucidate the chemical characters of the EtOAc extracts, the samples were subjected to liquid chromatographic analysis, targeting the UV-absorbing phenolic compounds. The filtrates were first filtered through disposable filters (0.45 µ pore size) before their injection into HPLC. The HPLC system was a Merck Hitachi LaChrom device consisting of a D-7100 pump, D-7200 autosampler, D-7300 column oven at 40 °C, and a D-7455 DAD detector scanning the absorbance between 220 and 400 nm. Separation was achieved on a HyPurity C18 (Thermo Scientific, Waltham, MA, USA) column using the gradient of water (acidified with *o*-phosphoric acid to pH3; A) and methanol (B) as follows: 10% B (0–1 min); 10–70% B (1–20 min); 70% B (20–23 min); 70–100% B (23–30 min), followed by flushing and equilibration to initial conditions. The flow rate was 0.8 ml/min and the injection volume was 40 µl. UV-spectra, collected at 200 to 400 nm, were compared to the spectral data of a standard compound library.

4.2.6. Statistical analysis

To evaluate the effect of time of inoculation, treatment, *G. abietina* isolate, endophyte filtrate and their interactions on the relative necrosis length we performed a linear mixed model (MIXED procedure in SAS 9.2 Inc, 2004) because of the high heterogeneity of variances in some levels of our factors (Levene Test). In a linear model all levels of the factors should have the same

variance (homoscedasticity) thus; we used a linear mixed model that allows using different variances for any of the levels of the factors. By grouping our factors in pairs we obtain different combinations of variance parameters which produced different models. The best model was chosen according to the lowest values of the Bayesian Information Criterion (BIC) and in compliance to the normality, linearity and homoscedasticity of the residuals, checked by graphical procedures and the Kolmogorov-Smirnov test. Furthermore, in order to explore if the effect of the filtrates was different whether the pathogen was isolated or not, we divided the data into two subsets: samples with success in re-isolating *G. abietina* (Ga positive), and data without success (Ga negative). For every subset we performed a linear mixed model (because of the heteroscedasticity of the data) with the relative necrosis length as response variable and *G. abietina* isolate, endophyte filtrate and their interaction as explanatory ones.

The random errors of all models were supposed to be independent and with normal distribution for the relative necrosis length. In all the statistical analyses a 5% level of significance was used. When significant differences were found in the test type III table of the model, a Tukey-Kramer HSD test was applied to compare the means.

Lastly, a non parametric Kruskal-Wallis test was used to observe the effect of the extracts, time of inoculation, treatment and isolates on the visual severity (using the following scale: 0 symptomless; 1 chlorosis; 2 dieback; 3 dry needles; 4 dead plant) after it was found that the data did not follow a normal distribution in a Shapiro-Wilk test. Then, the same test was applied to compare the means of the factors that presented significant p-values. These analyses were performed with R software (version 3.1.2 R Development Core Team, Vienna, Austria, <http://www.r-project.org>).

4.3. Results

4.3.1. Symptoms of *G. abietina* infections and re-isolation of the pathogen

Four months after the artificial inoculations of *G. abietina*, a total of 740 (100 were symptomless) seedlings started to show symptoms of the disease such as chlorosis (61%) (Figure 4.1a), dieback (29%) (Figure 4.1b), dry needles (3%) and cankers (1%). No dead plants were found. Tissues around the inoculation site turned a brown colour (Figure 4.1c). The pathogen grew upwards in most of the seedlings; growth both upwards and downwards was found in 2 seedlings. The symptoms were attributed to *G. abietina* infections given that fruiting bodies were observed in 38% of the seedlings (Figure 1d) while no fruiting bodies were observed in the control inoculations. Fruiting bodies were found in 49% of the seedlings inoculated in December and in 28% inoculated in January. Moreover, *G. abietina* could be re-isolated in 20% of the samples; 22% of the seedlings that were inoculated in December and 18% of those from January and no *G.abietina* was isolated from the controls.

4.3.2. Effects of the factors on necrosis and visual severity

The effect of the four factors on necrosis length was explored by a linear mixed model, which was selected according to the lowest BIC value (Table 4.2). The best model had no random effects and 4-variance parameters, one variance for every time of inoculation-treatment combination. Three factors, time of inoculation, endophyte filtrate and *G. abietina* isolate, had a statistically significant effect on the relative necrosis length as well as the interaction time of inoculation*isolate (Table 4.3). The presence of the endophyte filtrates reduced the advance of the pathogen in the seedlings regardless of the endophyte isolate, time of inoculation, treatment and *G.*

abietina isolate (Table 4.3). The control seedlings (with no endophyte filtrate) presented a relative necrosis length greater than the seedlings which were inoculated with the filtrates of *T. viride*, *A. pullulans*, *Aureobasidium* sp. and the Endophyte 20.1 (Figure 4.2).

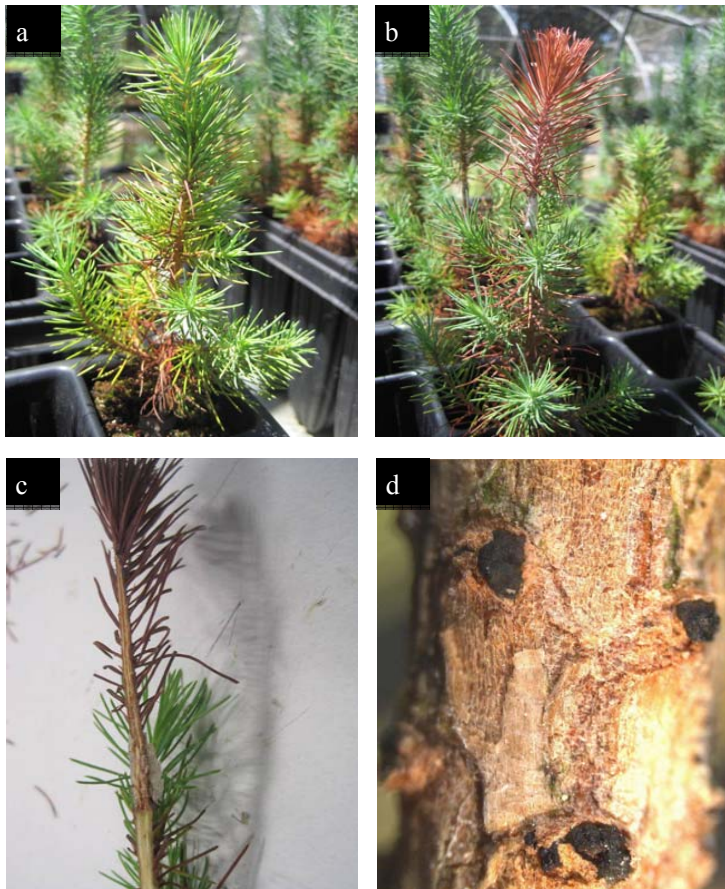
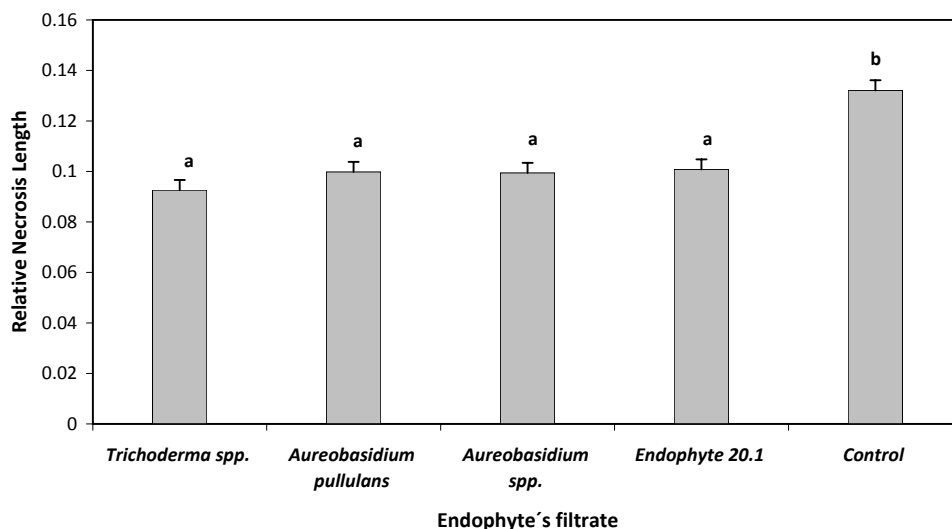


Figure 4.1: Symptoms of (a) chlorosis and (b) dieback; (c) brownish tissues in an endophyte-control plant and (d) *G. abietina* fruiting bodies (10x).

Table 4.2: Description and comparison of the models according to the Bayesian Information Criterion (BIC).

Factors	Group	Covariance parameters	Model selection criteria
			BIC
4	Time of inoculation_Isolate	14	-1780.8
4	Time of inoculation_Endophyte	10	-1785.9
4	Time of Inoculation_Treatment *	4	-1798.2
4	Treatment_Endophyte	10	-1795.3
4	Treatment_Isolate	14	-1778.6
4	Endophyte_Isolate	35	-1685.7

* Selected model

**Figure 4.2:** Average relative necrosis length found in *Pinus halepensis* seedlings when inoculating both *G.abietina* isolates with the different endophyte filtrates. Control seedlings had no endophyte but *G. abietina* isolate. Means with a different letter were significantly different from $p<0.05$ (Tukey's HSD Test). Bars represent standard error (n=70).

The inoculation with any isolate of *G. abietina* resulted in more extensive necrosis, as compared to the control seedlings (not *G. abietina*

Table 4.3: Test type 3 fixed effects for Relative Necrosis Length.

Dependent variable	Effect	DF	F-value	Pr > F
All seedlings (n=840)				
Relative Necrosis Length	Endophyte	4	13.4	< 0.0001
	Isolate	6	52.2	< 0.0001
	Time of inoculation (TI)	1	14.94	0.0001
	Treatment (T)	1	0.07	0.80
	Endophyte*Isolate	24	1.39	0.10
	Endophyte*TI	4	0.73	0.57
	Endophyte*T	4	2.23	0.63
	Isolate*TI	6	9.52	< 0.0001
	Isolate*T	6	1.62	0.14
	TI*T	1	0.24	0.62
	Endophyte*Isolate*TI	24	0.56	0.96
	Endophyte*Isolate*T	24	1.09	0.35
	Endophyte*TI*T	4	1.12	0.35
	Isolate*TI*T	6	2.01	0.06
	Endophyte*Isolate*TI*T	24	1.16	0.28

isolate inoculated) despite the time of inoculation, the treatment and the type of inoculated endophyte filtrate (Table 4.4). Nevertheless, some differences were found between the *G. abietina* isolates as indicated by the significant isolate effect (Table 4.3). Furthermore, the necrosis produced by isolates showed temporal variation, as indicated by the significant interaction between time of inoculation and isolate (Table 4.3). In December the *G. abietina* isolates G2, G3 and G5 resulted in more extensive necrosis than the rest of the isolates (G1, G4) whereas in January only G3 and G5 produced more necrosis than the rest; G2 was not as effective as in the first round.

The average relative necrosis length was significantly higher ($p < 0.001$) in seedlings inoculated in December (0.112 ± 0.003) (mean value \pm standard error) than in January (0.098 ± 0.003). Nevertheless, no difference in

necrosis length was found ($p=0.80$) between the preventive and therapeutic treatments, (0.106 ± 0.003 , and 0.104 ± 0.003 , respectively).

The results were very similar when analyzing the Ga-positive and the Ga-negative seedlings. There was a significant effect of the filtrates ($p=0.001$), the isolates ($p<0.001$) and their interaction ($p=0.01$) on the relative necrosis length of the Ga-positive seedlings. Furthermore, we also observed a significant effect of the filtrates ($p<0.001$), the *G. abietina* isolates ($p<0.001$) and their interaction ($p=0.001$) on the relative necrosis length on the seedlings without success in isolating the pathogen. The Tukey Kramer test revealed that in both models, the seedlings inoculated with any of the filtrates presented significantly lower necrosis than the controls although the efficacy depended on the isolate of *G.abietina* that was co-inoculated. In the Ga-positive seedlings the controls presented a higher necrosis length compared

Table 4.4: Relative Necrosis Length caused by six *G. abietina* isolates in two repeated experiments. Shown are the mean values \pm standard errors ($n=70$). ¹ Average necrosis when combining all the times of inoculation together. ² Means without a common small letter in the same column show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ³ Means without a common capital letter in the same row values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ⁴ Average necrosis when combining all the *G.abietina* isolates together.

Isolate	Time of inoculation		Total ¹
	T1	T2	
G1	0.097 \pm 0.008 b ² A ³	0.107 \pm 0.007 b B	0.102 \pm 0.005 b
G2	0.173 \pm 0.008 d B	0.099 \pm 0.007 b A	0.136 \pm 0.005 c
G3	0.145 \pm 0.008 cd B	0.109 \pm 0.007 bc A	0.127 \pm 0.005 c
G4	0.095 \pm 0.008 b A	0.099 \pm 0.007 b B	0.097 \pm 0.005 b
G5	0.134 \pm 0.008 c A	0.138 \pm 0.007 c B	0.136 \pm 0.005 c
G6	0.113 \pm 0.008 b B	0.096 \pm 0.007 b A	0.104 \pm 0.005 b
G-Control	0.028 \pm 0.008 a A	0.035 \pm 0.007 a B	0.032 \pm 0.005 a
TOTAL ⁴	0.112 \pm 0.003 B	0.098 \pm 0.003 A	

to one or more filtrate in seedlings inoculated with isolates G1, G2, G3, or G6. Furthermore, Ga-negative seedlings presented differences among control seedlings and the ones inoculated with any filtrate in isolates G1, G2, G4, G5, G6 and G7.

The Kruskal-Wallis test revealed that there were significant differences in the severity of the seedlings inoculated with different filtrates ($H=31.83$; $df.=4$; $p<0.001$) and *G. abietina* isolates ($H=96.36$; $df.=6$; $p<0.001$). Nevertheless, there were no significant differences between the two time of inoculations, December and January ($H=0.70$; $df.=1$; $p=0.40$) or the treatments, preventive or therapeutic ($H=0.003$; $df.=1$; $p=0.96$). The seedlings that were inoculated with the filtrate of *T. viride* had less mean visual severity than the ones inoculated with *Aureobasidium* sp. ($p=0.03$) or the Endophyte 20.1 ($p=0.02$). Furthermore, seedlings inoculated with the Endophyte 20.1 had less mean visual severity than the ones inoculated with the rest of the filtrates except the control ones. No differences were found between the controls and the seedlings inoculated with the rest of the filtrates. Regarding the *G. abietina* isolates, the control seedlings presented lower mean severity than the ones inoculated with the isolates G3 ($p=0.02$) and G5 ($p=0.05$).

4.3.3. UV-absorbing compounds of the filtrates

The identification of phenolic compounds was performed through the comparison of chromatographic retention times and UV spectra with those of commercial standards, when available. The HPLC analysis indicated that the EtOAc fractions of the fungal filtrates contained some phenolic compounds. In the filtrate from *T. viride* two peaks were found in the UV region (detection at 254 nm) (Figure 4.3), which were identified on basis of the UV-spectrum as hydroxybenzoic acids. In the filtrate from endophyte 20.1, three additional distinct peaks were found showing identical spectra but without a match in

library records. We did not detect any phenolic peaks in the filtrates of the two *Aureobasidium* endophytes or in the control broth.

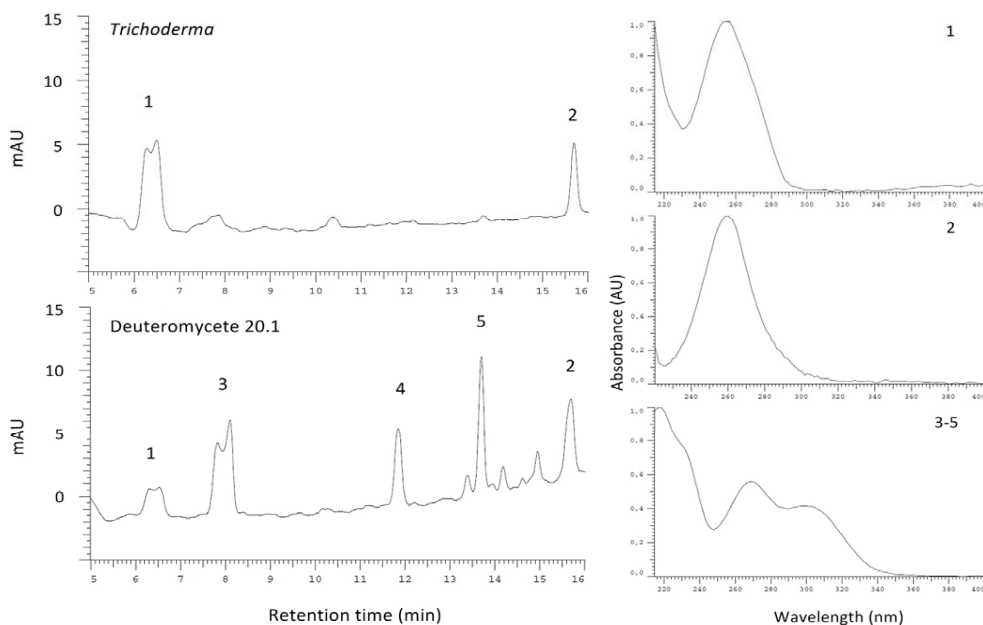


Figure 4.3: Chromatographic retention times and UV spectra of *T. viride* and Endophyte 20.1. Peaks 1 and 2 best matched with p-hydroxybenzoic acid in the library (over 98% of the spectrum form). Peaks 3-5 had a spectrum that did not match any of the library compounds.

4.4. Discussion

In this study, we explored the possibility of controlling the pathogenic fungus *G. abietina* with fungal filtrates from selected BCAs. According to the symptoms observed (i.e. chlorosis, dieback, cankers and death of the plants), the presence of fruiting bodies and absence of fungal signs in the controls, we concluded that it was likely that the infections were produced by *G. abietina*. The re-isolation of the fungus was lower than the percentage obtained in a

previous study by Santamaría et al (2007) who obtained 66%. Nevertheless, isolating *G. abietina* from vegetal material is especially challenging even when the sample material has fruiting bodies as previously observed in our lab (Romeralo et al. 2015). Besides the slow growth of the pathogen, another hypothesis explaining why it was so difficult to isolate it could be because the fungus was not alive until the end of the experiment. A successful activation of the plant defence mechanism after the dormancy period (around March) could be responsible for excluding the pathogen in some of the seedlings. This plant defence mechanism would include the formation of ligno-suberized boundaries followed by the restoration of cambial activity, tissue regeneration and the production of fungal degrading enzymes by the host previously described as being key factors in the resistance of Pine species to the pathogen (Simard et al. 2001, 2013). Isolates from this fungus grow very slowly in media, even if it is specific media like MOS-agar and the pathogen is growing in its optimal temperature of 15 °C. Even so, necrosis was apparently produced by the pathogen because the seedlings inoculated with *G. abietina* isolates had significantly greater necrosis length than the controls, which were not infected by the pathogen. The small necroses observed in the controls were probably the result of the wound made by the scalpel as also seen previously (Dogmus-Lehtijärvi et al. 2012). The different isolates of *G. abietina* also varied in their ability to cause necrosis in the tested plants. This concurs with previous reports (Terho & Uotila, 1999; Santamaría et al. 2006, 2007), which have shown that virulence can vary within isolates.

Other factors influenced the extent of necrosis in our study. Temporal variation was found in necrosis length: the seedlings inoculated in December exhibited longer necrosis and more fruiting bodies than the seedlings that were inoculated in January. This result coincides with those obtained by Dogmus-Lehtijärvi et al. (2012) who found that from several inoculations

made with Turkish isolates of *G. abietina* on several periods of the year (September, November, December and January), the ones made in December (mean $T^{\circ} = 4.1^{\circ}\text{C}$) presented the highest necrosis. In our experiment, the colder weather in January (mean $T^{\circ} = 3.1^{\circ}\text{C}$) than in December (mean temp. 4.4°C) seems unlikely to be a limitation for the development of the fungus since it has been reported to grow at temperatures as low as -6°C (Marosy et al. 1989). Therefore, the highest necrosis in December could be explained by the fact that the fungus had 3 weeks more to grow inside the plant until March when the temperatures started to increase and the defence system of the plant would be activated again.

In our experiment, there was not a significant effect of the treatment (preventive or therapeutic) on the necrosis length produced by the pathogen or the visual severity of the disease. Due to the short time between treatments (one week) and due to the fact that the plants were submerged in the dormancy period by the time of the inoculations, it is likely that there was no activation of the defence mechanism of the plants. Nevertheless, although pine dormancy is described as the absence of growth (and in the case of Aleppo pine the growth in height is known to stop at temperatures below 10°C) some activities have been reported to happen during dormancy in this species as opposed to other conifers. Puertolas et al (2005) found that Aleppo pine seedlings maintain their photosynthetic ability during cold hardening. Furthermore increases in shoot dry weight (which indicates some cambial activities) and in starch reserves have also been reported during this period (Tinus et al. 2000; Fernandez et al. 2003). Therefore, although some activity or activation of the defence system of the plants will remain during the dormancy period, it was not enough to lead to a different response among the treatments in our experiment.

The results of our study indicate that the filtrates of all the tested endophytes reduced the necrosis produced by *G. abietina* in the seedlings. The filtrates had a similar effect whether the pathogen was isolated from the seedlings or not, suggesting that the pathogen could be alive until the end of the experiment, but it was difficult to isolate because of the features of this fungus. Another explanation could be that the pathogen was not alive until the end of the experiment, and that the effects of the filtrates were produced during the first months after the inoculations. The biological control agents (BCAs) may antagonize the pathogens through several modes of action and revealing them is useful for easier registration procedures at the commercialization stage (Castoria et al. 2001). Our results show that the mechanisms of the studied BCAs were likely linked to production of extracellular metabolites, since the filtrates alone resulted in necrosis reduction whereas the competition for nutrients or the microbial antagonism would involve the presence of the BCAs themselves. Similar results were reported in other studies where the presence of fungal filtrates was able to decrease the mycelial growth of several pathogens like *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque (Campanile et al. 2007) or *Sclerotinia sclerotiorum* (Lib.) de Bary (Zhang et al. 2014). An induction of the resistance in plants has been reported as well as a consequence of the presence of fungal filtrates (Viecelli et al. 2009).

The visual severity was not a good indicator in our experiment, as most of the seedlings presented symptoms of chlorosis, and this was not enough to pinpoint a difference of effectivity of the filtrates or the damage produced by the different *G. abietina* isolates. A more accurate scale and the examination along a longer period of time (throughout the whole experiment) would be recommended to improve these results in future experiments.

The inoculation of the filtrates of *T. viride* in the seedlings was able to reduce the necrosis produced by *G. abietina* as compared to the controls. The success of *Trichoderma* filtrates was previously reported in reducing the spore germination or the mycelial growth of other plant pathogens such as *Claviceps africana* Freder., Mantle & De Milliano (Bhuiyan et al. 2003) or *Ophiostoma novo-ulmi* Brasier (Díaz et al. 2012). According to our results, the filtrates of *Trichoderma* spp. were found to have some phenolic compounds. Although these phenols might contribute to the observed antagonism, results from previous studies have pointed out the presence in the fungus' filtrates of other potential chemical agents. Indeed, a wide range of non-volatile and volatile antifungal substances produced by *Trichoderma* spp. have been identified (Reino et al. 2008; Howell 2003), such as gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Vinale et al. 2008) as well as hydrolytic enzymes such as chitinase and glucanase (Aziz et al. 1993; Schirmböck et al. 1994).

Our results showed that inoculation with *Aureobasidium* (both *A. pullulans* and *Aureobasidium* sp.) filtrates also resulted in a reduction of the necrosis length, as compared to the controls. In previous studies, an antagonistic behaviour of different isolates of this genus through different mechanisms has been reported, including the presence of volatile compounds (Mari et al. 2012), competition for nutrients (Bencheqroun et al. 2007; Zhang et al. 2010), and induction of phytoalexins (Rühmann et al. 2013). The results from the Castoria et al. (2001) study showed that *A. pullulans* was an effective BCA against postharvest fungal pathogens, most likely due to the production of enzymes such as β -1,3-glucanase(s) and nagase(s) that were acting against fungal walls. Nevertheless, the same authors reported that neither antibacterial nor antifungal compounds were present in ethylacetate filtrates obtained from the culture filtrate of the fungus;

which coincided with our results that we could not detect any UV-absorbing metabolites in the *Aureobasidium* filtrates.

The seedlings that were inoculated with the filtrate of the endophyte 20.1 exhibited reduced necrosis length compared to the controls. A previous study performed *in vitro* by Santamaria et al (2007) showed a complete inhibition of Spanish isolates of *G. abietina* on cultures when the filtrate of this fungus was present, suggesting that there was some antifungal compound in the filtrate. Furthermore, Romeralo et al. (2015) observed that the presence of the mycelia of this fungus resulted in a reduction of necrosis produced by *G. abietina* on *P. halepensis* seedlings. We found that the filtrates of this fungus did contain a few phenolic compounds. Therefore it is probable that antioxidant activity and toxicity of these compounds might have contributed to the apparent antagonistic activity of this fungus against the pathogen. Thus, their potential involvement in restriction of necrosis length should be studied further along with a more comprehensive chemical profiling of the filtrates.

In conclusion, both the preventive and therapeutic treatments of *P. halepensis* seedlings with filtrates of four endophyte isolates (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. and Endophyte 20.1) were effective against necrosis development caused by *G. abietina* infection. However, there was some temporal variability in responses, indicating the complexity of the system. Not all fungal filtrates contained phenolics in amounts that were detectable with our HPLC method, suggesting that such compounds were not a general factor behind the preventive or therapeutic effect or that they were in such low concentrations that we could not detect them. Further studies, including more inoculation intervals and shorter incubation periods, could provide more accurate results about the efficacy of the filtrates and timing of activation of the defence mechanisms. A more

comprehensive chemical profiling of the filtrates is recommended in the future.

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Chapter 5

Effect of putative mitoviruses on *in vitro* growth of *Gremmeniella abietina* isolates under different laboratory conditions and on its pathogenicity on *Pinus halepensis* seedlings

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ABSTRACT

Mitoviruses have been found in several forest pathogens (i.e. *Cryphonectria parasitica*, *Gremmeniella abietina*), and because they have been shown to reduce the virulence of host fungi there is a growing interest in studying their use as a biocontrol. This study was carried out to test the effect of temperature (5 °C, 15 °C, 25 °C and 35 °C), pH (4, 5, 7 and 9) and osmotic potential (-0.6, -1.2, -1.8 and -2.4 MPa) on the mycelial growth of seven *G. abietina* isolates under controlled laboratory conditions and to observe the effect of the presence of mitoviruses in the pathogenicity of *G. abietina* isolates inoculated to *Pinus halepensis* seedlings. Four of the isolates hosted mitoviruses and three of them did not. During the experiment, mycelial growth was recorded every week for a period of 8 weeks. In the greenhouse experiment, once the

seedlings started to show symptoms, disease severity was recorded during 5 weeks. At the end of the experiment, plants were carried to laboratory and necrosis length was measured in all of them. Results from *in vitro* experiment showed no differences in growth behavior between mitovirus infected and non-infected isolates when placed under different pH modifications. However, the mitovirus-infected isolates presented larger mycelial growth than the mitovirus-free ones when at the fungi's optimal growing temperature of 15 °C. When growing at certain osmotic potentials (-0.6 and -1.8 MPa) a reduction in growth of the mitovirus-infected isolates was observed. In the greenhouse experiment, no differences were found in visual disease severity among plants however larger necrosis lengths were observed in the plants inoculated with mitovirus infected isolates, suggesting a possible hypervirulence produced by the mitoviruses' presence. The results of this experiment provide further insight into the effects of mitovirus on *Gremmeniella abietina* isolates.

Key words: mitoviruses, Scleroderris canker, *in vitro*, biological control, *Gremmeniella abietina*, dsRNA.

5.1. Introduction

Gremmeniella abietina (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnel) is a pathogenic fungus which has caused destruction in plantations and natural conifer forests in Northern and Central Europe, North America, and Japan (Yokota, 1975; Dorworth, 1979; Kaitera and Jalkanen, 1992) producing symptoms such as stem cankers and shoot dieback (Donaubauer, 1972). This fungus has been divided into three races:

European, North American and Asian. Within the European race three biotypes have been determined based on the length of spores, number of septa, disease symptoms, and molecular markers. There is biotype A (LTT, large tree type), biotype B (STT, small tree type) and alpine biotype (Uotila, 1983; Hamelin et al., 1993; Hellgren and Hogberg, 1995; Kaitera and Jalkanen, 1996; Hantula and Muller, 1997). In Europe, the fungus mostly affects genera *Picea* spp. and *Pinus* spp. although it has also been found on genera *Abies* and *Larix*. In Spain, its presence on *Pinus pinaster* was first reported in 1929 (Martínez, 1933) and later on *Pinus halepensis* in 1999 (Santamaria et al., 2003). Notwithstanding, it has only been isolated from symptomatic *Pinus halepensis* trees. The symptoms observed generally consist of dry needles, branches with some distortion of terminal twigs and eventual dieback or death of the trees (Santamaria et al., 2003). Spanish *G. abietina* is currently recognized as part of the European race (Santamaria et al., 2005) and has recently been related to biotype A, although it has a unique genotype (Botella et al., 2010).

Mycoviruses, which are obligate parasites of fungi, are widespread in all major taxonomic groups of plant pathogenic fungi (Ghabrial and Suzuki, 2009; Pearson et al., 2009). They are transmitted through hyphal anastomosis and/or fungal sporulation (Zhang et al., 2010). Fungal viruses differ in their genomes, which can contain DNA, double-stranded (ds) RNA or single-stranded (ss) RNA genomes (Pearson et al., 2009). Eight families and one genus are currently described in the International Committee on Taxonomy of Viruses (ICTV) (2011): *Chrysoviridae*, *Endornaviridae*, *Hypoviridae*, *Narnaviridae*, *Barnaviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* and genus *Rhizidiovirus* (Hausner et al., 2000; Zhang et al., 2010). Mycoviruses usually produce latent infections in nature, affecting sometimes the host's phenotype and/or its growth (Aoki et al., 2009). Symptoms produced by the

presence of mycoviruses may vary from zero to severe effects on host physiology and may lead to attenuation (hypovirulence) or enhancement of fungal virulence (hypervirulence) (Ghabrial and Suzuki, 2009). Because some viruses are capable of reducing virulence of fungal pathogens they can potentially be used for control of fungal diseases (McCabe et al., 1999; Boland 2004; Zhang et al., 2010). However, they must fulfill two requirements in order to be suitable for biological control: firstly, have the ability to decrease the fitness of the pathogenic fungus and secondly, transmit the dsRNA efficiently enough to be maintained in a large proportion of the pathogen population (McCabe et al., 1999).

Members of genus *Mitovirus* are only found in fungi and belong to the family *Narnaviridae* (Ghabrial and Suzuki, 2009). They lack true virions, and have a (+) ssRNA genome of approximately 2.5 kb (Boland, 2004). Mitoviruses have been recorded in several phytopathogenic fungi such as *Cryphonectria parasitica* (Polashock and Hillman, 1994; Polashock et al., 1997), *Ophiostoma novo-ulmi* (Brasier, 1983; Rogers et al., 1987), *Sclerotinia homoeocarpa* (Deng et al., 2003; Deng and Boland, 2004), *Helicobasidium mompa* (Osaki et al., 2005), *Chalara elegans* (Park et al., 2006) and *Botrytis cinerea* (Castro et al., 2003; Wu et al., 2007). In most cases, the presence of mitoviruses is associated with reduction of fungal pathogenicity (Ghabrial and Suzuki, 2009; Wu et al., 2010). Members of the genus *Mitovirus* have also been isolated in *G. abietina* (Tuomivirta and Hantula, 2003) which, in the Spanish population, has recently been discovered to host (Botella et al., 2011).

Reduction of virulence could be related, among other reasons, to anomalous mycelial growth in the fungal pathogen caused by mitoviruses (Ghabrial and Suzuki, 2009; Pearson et al., 2009). However mycelial growth is also influenced by environmental and cellular conditions such as

temperature, pH and osmotic potential. Temperature limits mycelial growth and production of fruiting bodies in most fungi while the pH determines availability of elements such as nitrogen, calcium and magnesium among others (Carlile et al., 2001). Osmotic potential has also been identified as an important parameter in the ecology and growth of phytopathogenic fungi (Davis et al., 2000). For example, a decrease in the potential produces a reduction in fungal growth due to the subsequent energy increase needed to maintain the swelling of the hyphal cells (Lira-Méndez and Mayek-Pérez, 2006). In general, the effect of the mitovirus could be combined with the effects of these environmental parameters and therefore modify fungal behaviour.

Although some strains of *G. abietina* have been shown to host dsRNA mycoviruses, the effect these agents have on the virulence of this problematic phytopathogenic fungus has not yet been investigated. Accordingly, the main objectives of the present study were (i) to evaluate the effect of the occurrence of viral dsRNA molecules (the replicative form of *Mitovirus*) on the *in vitro* mycelial growth of *G. abietina* isolates under different temperature, pH and osmotic potential conditions and (ii) to observe the effect of the presence of mitoviruses in the pathogenicity of *G. abietina* isolates inoculated on *Pinus halepensis* seedlings.

5.2 Materials and Methods

5.2.1. Fungal material

To develop this study seven Spanish isolates of *G. abietina* were chosen: four isolates were naturally infected by putative mitoviral molecules (P3-12, 00P-07, Hon 3-3 and P1-12) and three were not (Hon 9-2; P1-8 and VAI-13) (Botella et al., 2010). All isolates were selected based on previous

studies developed in our laboratory in which RT-PCR and sequencing techniques confirmed the presence or absence of mitoviruses (Botella et al., 2011). The isolates were previously stored in 15% glycerol at -80 ° C and were reactivated on modified orange serum agar medium (MOS-agar; Müller et al., 1994) before performing the experiment. Thus, four weeks before the experiment fungi isolates were sub-cultured in MOS medium and kept in the dark at 15 °C in order to obtain sufficient amounts of mycelium.

5.2.2. Mycelial growth

At the bottom of every Petri dish containing 20 ml MOS medium two perpendicular lines were drawn, and a 1 mm squared piece of mycelium from each isolate was placed over the intersection of both lines. Mycelial growth was measured weekly for a period of 8 weeks. The Response variable was the growth area calculated by the following formula: $Area = \pi/4 (d_1 \times d_2)$ where d_1 and d_2 were the two diameters measured along the lines.

5.2.3. Culture conditions for monitoring mycelium growth

The effect of mitovirus infection on mycelial growth under different laboratory conditions was the main focus of this study. Three experiments were conducted, each taking into account a separate factor: changes in temperature, pH or osmotic potential. Within each experiment four variations were tested: four temperatures (5 °C, 15 °C, 25 °C and 35 °C), four pH values (4, 5, 7 and 9) and four osmotic potentials (-0.6, -1.2, -1.8 and -2.4MPa). The effect of temperature on mycelial growth was investigated by placing Petri dishes in several stoves at 5 °C, 15 °C, 25 °C and 35 °C. To examine the effect of pH, HCl or KOH 1N was added to MOS medium until the pH required was reached. All these Petri dishes were placed in the dark at 15 °C since it is the optimal temperature for fungal development (Santamaria et al., 2004). Finally, in order to evaluate the effect of different osmotic potential on

mycelial growth, different concentrations of KCl (250, 500, 750 and 1000mM) were added to MOS medium in order to reach the osmotic potential (Ψ_{π}) values of -0.6 MPa, -1.2 MPa, -1.8 MPa and -2.4 MPa (Lira-Méndez and Mayek-Pérez, 2006). Petri dishes were incubated at $15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in continuous darkness. Four repetitions of each combination “isolate x treatment” were completed.

5.2.4. DsRNA extractions

Fungal mycelium of mitovirus-infected isolates from significative treatments was incubated in MOS medium covered with cellophane for two weeks. Mycelia were first freeze-dried and then ground for 20 minutes into a fine powder. DsRNA was extracted following a modified version of the protocol described by Morris and Dodds (1979). The dsRNA presence in every isolate was verified by electrophoresis. Samples were loaded in a 1% agarose gel, which contained 1x TAE buffer and GelRed™ 10.000X. The test was run in a 1x TAE buffer during 60 min at 90V/30 cm, and immediately afterwards observed under UV light and photographed. The marker used to estimate the lengths of the dsRNA molecules was λ -DNA Hind III – Φ X174Hae III (DyNAzyme™).

5.2.5. In vivo pathogenicity tests

Pathogenicity tests were carried out in the greenhouse using 1-year old seedlings of *Pinus halepensis* and the same fungal isolates used in the laboratory experiment. Plant material was provided by the Serranillo nursery (Ministry of Agriculture, Food and Environment). Seedlings were placed in trays leaving spaces among them to avoid contact. In order to perform the inoculation, a wound was made with a sterile scalpel at 10 cm below the shoot apex. A 0.5 cm diameter piece of MOS with *Gremmeniella abietina* mycelium was placed on each wound and covered with Parafilm. Fifteen

repetitions of every combination were made and 15 plants were used as controls, thus a total of 120 plants were inoculated.

Two and a half months after inoculations, some of the plants started to show symptoms of decline. A visual evaluation was made over 5 weeks to measure the disease severity according to the following scale: 0, symptomless; 1, chlorosis; 2, advanced chlorosis; 3, dieback; 4, necrotic; 5, dead. Afterwards, plants were cut and carried to the laboratory in order to measure the necrosis produced by the pathogens and the plant length. Relationship among necrosis and total length was defined as relative necrosis length and was used, with the severity index, as response variable in the statistics analyses. In order to verify if *G. abietina* was the fungus producing the necrosis (Koch postulates), in half of the symptomatic seedlings samples of 1cm were cut, surface-sterilized and placed in Petri dishes with MOS media at 15 °C for several weeks for reisolation.

5.2.6. Statistical analysis

All statistical analyses were done with SAS program (SAS Institute Inc., 2004). The response variable in all models was growth area (mm²). A repeated-measures ANOVA for every treatment was calculated by means of Repeated Procedure by SAS to test the effect of the time on the mycelial growth of the isolates. In this case, the growth areas of every week were used as responses variables. Furthermore, for every experiment (temperature, pH and osmotic potential) a model was calculated to evaluate the effect of the putative presence of mitovirus (yes/no), the treatments (4) and their interactions by a two-way analysis of variance. A significance of 95% was taken in all of the analyses. A Tukey HSD test was used on means of factors when significant differences were found in the ANOVA model. Before the

analyses were performed, normality, linearity and homocedasticity for the residuals were probed with Shapiro-Wilk test and graphical procedures.

Results from pathogenicity tests were also analyzed with SAS program. Two models were made to evaluate the presence of mitoviruses (yes/no): first, severity index was used as response variable and second, relative necrosis length. In all the analysis a 95% of significance was considered. Normality, linearity and homocedasticity for the residuals were probed with Shapiro-Wilk test and graphical procedures. Since data did not fulfill these requirements, they were analyzed with a non-parametric test (the two-sample median test).

5.3. Results

5.3.1 Effect of temperature

A significant effect of time on the colony growth area ($p < 0.001$) was observed. Although the interaction between time and mitovirus presence was significant ($p = 0.017$) as well as the interaction between time and temperature ($p < 0.001$), only the effect of temperature and mitovirus presence on mycelial growth at the end of the experiment (eight weeks after plating) is shown in Table 5.1. The average growth from mitovirus-infected and mitovirus-free isolates is shown in Figure 5.1 at 5 °C (A), 15 °C (B), 25 °C (C) and 35 °C (D) throughout the eight weeks. Growth at 25 °C was minimal and there was no growth at 35 °C. Mean growth area was significantly different among mitovirus-infected and mitovirus-free isolates ($p = 0.0030$), temperatures ($p < 0.001$) and their interactions ($p < 0.001$). According to the Tukey test, the largest colony areas were found at 15 °C whereas the smallest were found at 35 °C. The overall mean colony size of mitoviruses-infected isolates was significantly bigger than that of the mitoviruses-free ones. When temperatures were considered separately, significant differences among mitoviruses-

infected and mitoviruses-free isolates were found only at 15°C ($p=0.0043$), the temperature that produced the most growth.

Table 5.1: Mycelial growth (mm²) after 8 weeks at different temperatures. Mean value \pm standard error (SE). Treatments tagged with * presented significant differences among isolates. ¹ If the isolate was naturally-infected with mitovirus. ² Average growth when combining all the temperatures together. ³ Different letters in the same column show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ⁴ Different letters in the same row show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ⁵ Average growth when combining all the isolates together.

Mitovirus ¹	Temperature				TOTAL ²
	5 ° C	15 ° C *	25 ° C	35 ° C	
Infected	2.41 \pm 0.69 a ³ B ⁴	11.7 \pm 5.62 a A	0.254 \pm 0.05 a B	0.196 \pm 0.00 a B	3.65 \pm 5.53 a
Mitovirus-free	2.51 \pm 0.57 a B	7.30 \pm 1.97 b A	0.223 \pm 0.03 a C	0.196 \pm 0.00 a C	2.56 \pm 3.09 b
TOTAL ⁵	2.45 \pm 0.63 B	9.83 \pm 4.92 A	0.249 \pm 0.05 C	0.196 \pm 0.00 C	

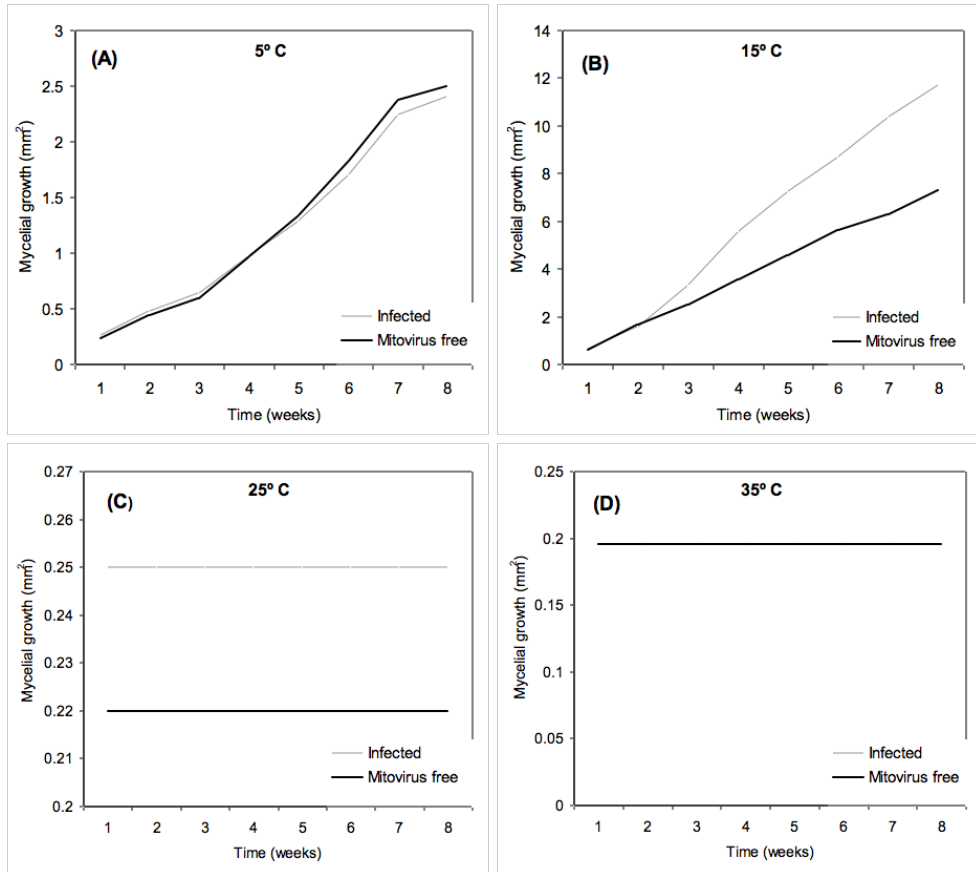


Figure 5.1: Average growth from mitovirus-infected and mitovirus-free isolates at 5 °C (A), 15 °C (B), 25 °C (C) and 35 °C (D) over the eight weeks.

5.3.2. Effect of pH

A significant effect of time was observed on the growth area in the pH experiment ($p < 0.001$) and in its interaction with the pH treatments ($p < 0.001$) but not in the mitovirus presence ($p = 0.7265$). Average growth from mitovirus-infected and mitovirus-free isolates is shown in Figure 5.2 at pH 4 (A), pH 5 (B), pH 7 (C) and pH 9 (D) throughout the eight weeks. Only the data from the effect of pH values on mycelial growth at the end of the experiment (week 8) is shown in Table 5.2. The growth area was affected by the pH value

($p < 0.001$), but it was neither affected by the mitovirus presence ($p = 0.9459$) nor their interaction ($p = 0.2753$). The largest mycelial growth for all samples was observed at pH 4 while the smallest was shown at pH 9. No differences were shown between mitovirus-infected and mitovirus-free isolates in any pH treatment.

Table 5.2: Mycelial growth (mm²) after 8 weeks at different pHs. Mean value \pm standard error (SE). ¹ If the isolate was naturally-infected with mitovirus. ² Average growth when combining all the pH values together. ³ Different letters in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁴ Different letters in the same row show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁵ Average growth when combining all the isolates together.

Mitovirus ¹	pH				TOTAL ²
	pH 4	pH 5	pH 7	pH 9	
Infected	16.8 \pm 8.05 a ³ A ⁴	9.95 \pm 4.15 a B	9.65 \pm 4.64 a B	6.62 \pm 1.39 a B	10.76 \pm 6.26 a
Mitovirus-free	20.3 \pm 7.66 a A	8.95 \pm 3.52 a B	8.23 \pm 1.60 a B	6.45 \pm 2.12 a B	10.96 \pm 6.96 a
TOTAL ⁵	18.26 \pm 7.93 A	9.51 \pm 3.86 B	9.04 \pm 3.68 B	6.55 \pm 1.71 B	

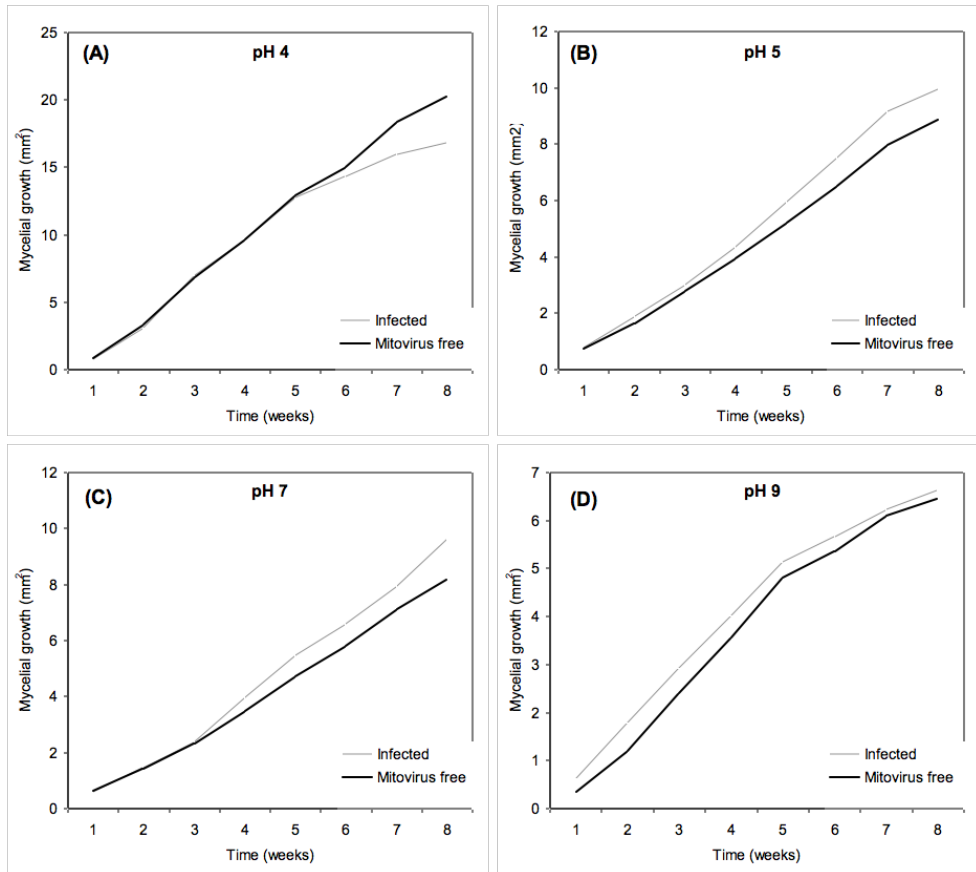


Figure 5.2: Average growth from mitovirus-infected and mitovirus-free isolates at pH 4 (A), pH 5 (B), pH 7 (C) and pH 9 (D) over the eight weeks.

5.3.3. Effect of osmotic potential ($\Psi\pi$)

In this experiment, time also affected the growth ($p < 0.001$) and interacted as well with the osmotic potential ($p < 0.001$) and the mitovirus presence ($p < 0.0447$). Data taken in the eight week showed the greatest differences (Table 5.3). The average growth of mitovirus-infected and mitovirus-free isolates is shown in Figure 5.3 at -0.6MPa (A), -1.2MPa (B), -1.8MPa (C) and -2.4MPa (D) throughout the eight weeks. The model was

significant ($p=0.027$) although it was not the mitovirus presence ($p=0.1378$) nor osmotic potential ($p=0.0805$), but the interaction was significant ($p=0.0034$), that is, the effect of mitovirus presence was different among the different osmotic potentials. When osmotic potential was considered separately at Ψ_{π} of -0.6 MPa ($p=0.0167$) and at -1.8 MPa ($p=0.0387$), mitovirus-free isolates presented a higher mycelial growth than the mitovirus-infected ones which did not happen at the osmotic potentials of -1.2MPa ($p=0.7515$) and -2.4MPa ($p=0.1004$).

Table 5.3: Mycelial growth (mm²) after 8 weeks at different osmotic potentials. Mean value \pm standard error (SE). Treatments tagged with * presented significant differences among isolates. ¹ If the isolate was naturally-infected with mitovirus. ² Average growth when combining all the pH values together. ³ Different letters in the same column show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ⁴ Different letters in the same row show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test). ⁵ Average growth when combining all the isolates together.

Mitovirus ¹	Osmotic potential (Ψ_{π})				TOTAL ²
	0.6 MPa *	1.2 MPa	1.8 MPa *	2.4 MPa	
Infected	7.66 \pm 0.79 b ³ AB ⁴	9.24 \pm 0.49 a AB	6.47 \pm 0.71 b B	9.96 \pm 0.77 a A	8.33 \pm 0.35 a
Mitovirus-free	10.7 \pm 0.80 a A	9.00 \pm 0.56 a AB	8.82 \pm 0.82 a AB	7.96 \pm 0.89 a B	9.13 \pm 0.40 a
TOTAL ⁵	8.98 \pm 0.53 A	9.14 \pm 0.53 A	7.48 \pm 0.53 A	9.10 \pm 0.53 A	

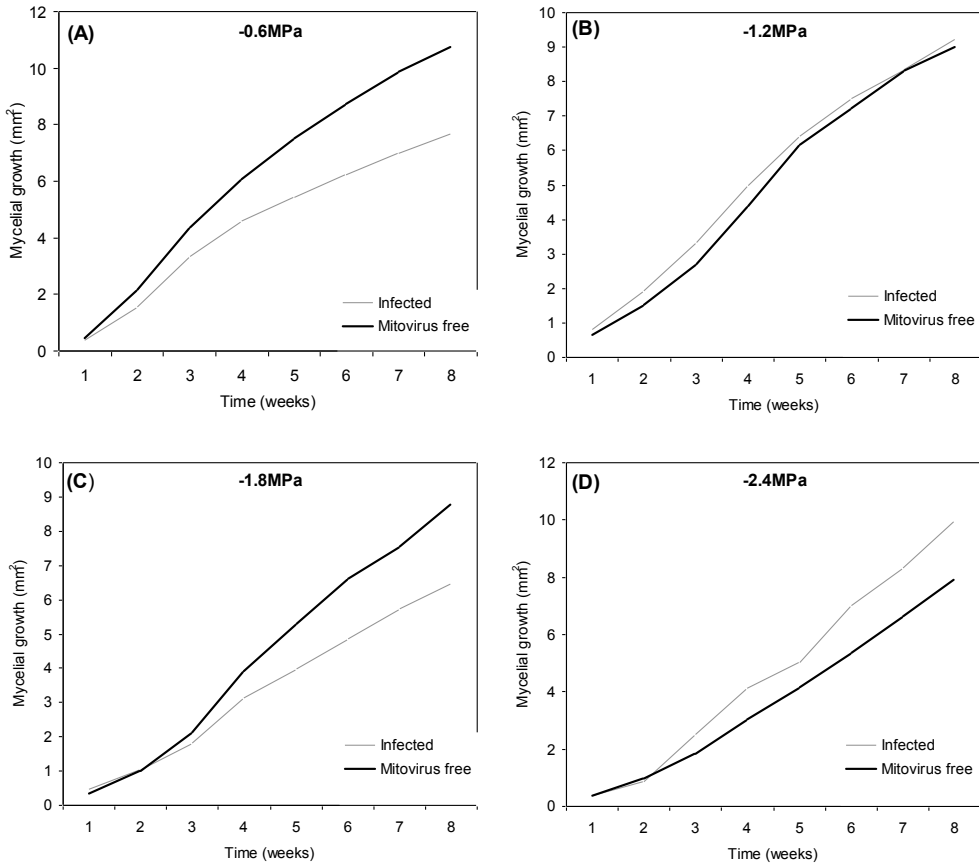


Figure 5.3: Average growth from mitovirus-infected and mitovirus-free isolates at -0.6MPa (A), -1.2MPa (B), -1.8MPa (C) and -2.4MPa (D) over the eight weeks.

5.3.4. DsRNA banding patterns

The presence of the different putative mitoviruses was confirmed by dsRNA extraction and gel electrophoresis after significant treatments were carried out (Figure 5.4). Isolate P3-12 was found to maintain a 2.5 kb band despite receiving the treatments of Ψ_{π} -0.6MPa (Lane 1) and -2.4 MPa (Lane 10). Conversely, the 2.5 kb band was not sustained in isolate Hon3-3 after treatments of Ψ_{π} -0.6MPa (Lane 2) and -1.8MPa (Lane 8). These results

suggested that putative mitovirus occurrence is not affected equally by similar osmotic potential and therefore KCL concentrations. In addition, dsRNA bands that appeared in P3-12 suggested the occurrence of other putative mycoviruses, which did not seem to be affected by the different treatments either. According to the size of the bands and the previous work developed in the laboratory (Botella et al., 2010) they possibly belonged to genera *Totivirus* (ca 6kb) and *Partitivirus* (three bands of ca 1-2 kb).

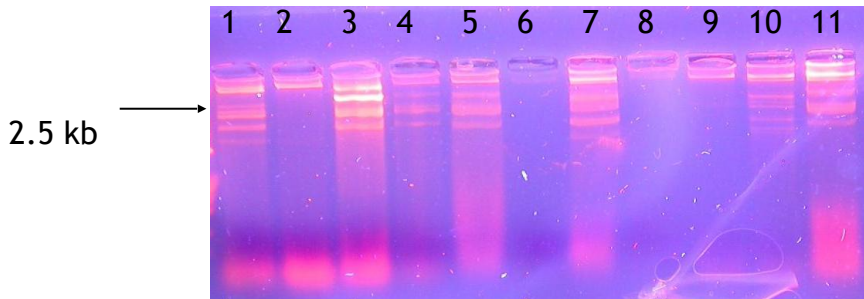


Figure 5.4. A GelRed-stained 1 % agarose gel showing the dsRNA banding patterns. Lane 1, P3-12 (-0.6MPa); lane 2, Hon 3-3 (-0.6MPa); lane 3, 00P-07 (-0.6MPa); lane 4, P1-12 (-0.6MPa); lane 5, P1-12 (15 °C); lane 6, Hon 3-3 (15 °C); lane 7, P3-12 (15 °C); lane 8, Hon 3-3 (-1.8MPa); lane 9, P1-12 (-1.8MPa); lane 10, P3-12 (-2.4MPa); lane 11, 00P-07 (-2.4MPa).

5.3.5. *In vivo* pathogenicity tests

The average relative necrosis length from seedlings inoculated with mitovirus infected and from non-infected isolates is shown in Figure 5.5. The necrosis length of seedlings inoculated with mitovirus-infected isolates was significantly larger ($p=0.0291$) than the necrosis from seedlings which were inoculated with non-infected isolates. In the second model, severity index was used as response variable, but it was not significant with a 95% level of significance. Seedlings inoculated with mitovirus infected isolates had a higher disease severity than the non-infected isolates but it was not statistically

significant (Fig 5.6). *G. abietina* was re-isolated from 35% of the symptomatic seedlings that were inoculated.

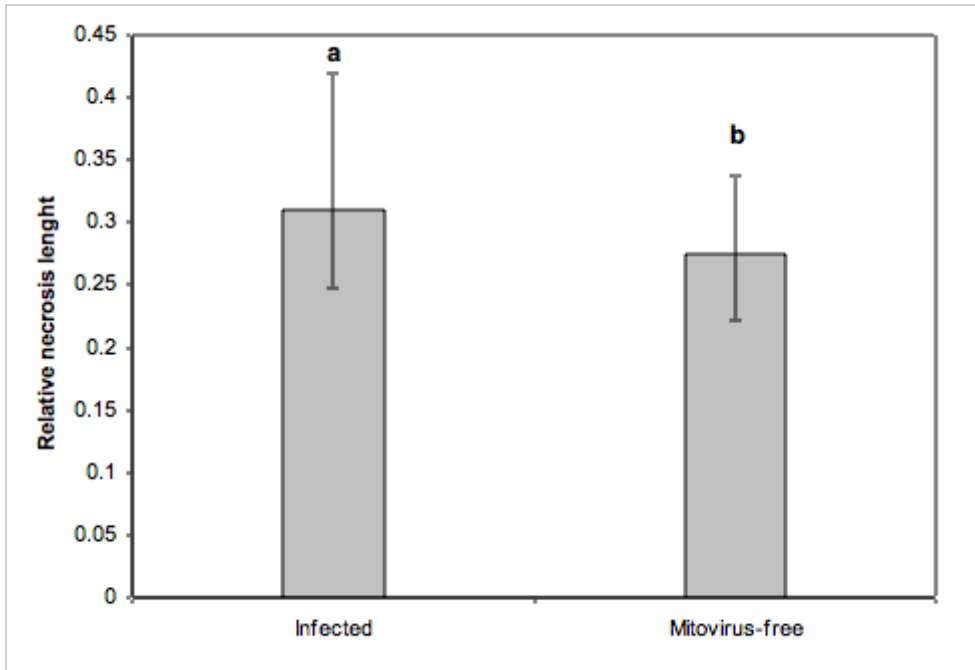


Figure 5.5. Mean relative necrosis length among seedlings inoculated with mitovirus infected and mitovirus-free isolates. Means with different letter were significantly different at $p < 0.05$ (non-parametric median test).

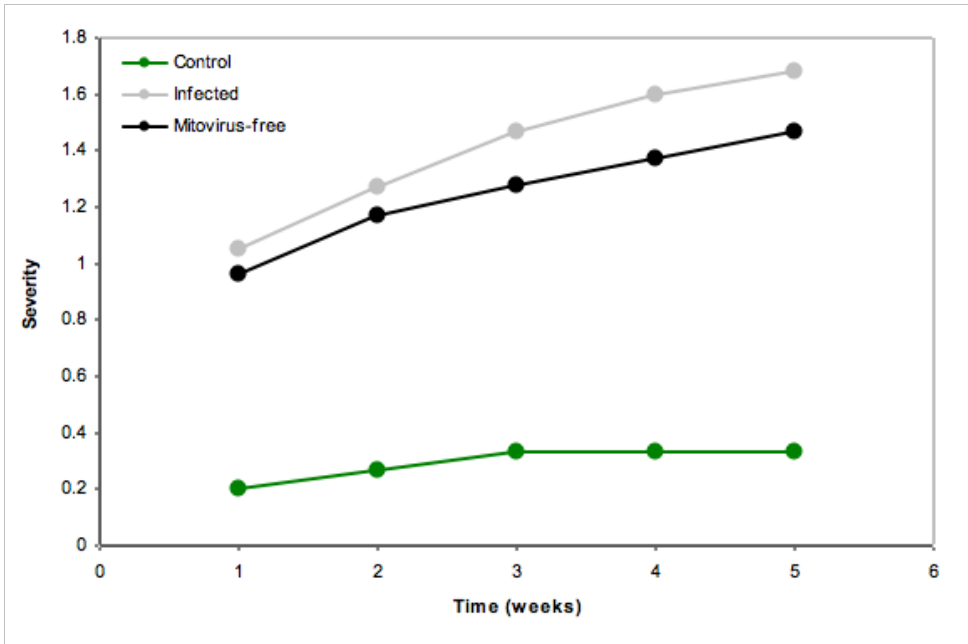


Figure 5.6. Mean severity index of each group (control, infected and mitovirus-free) over the 5 weeks.

5.4. Discussion

Mycelial growth depends on the temperature of the environment. In our study, all the isolates showed an optimal growth at 15 °C, which was in accordance with Santamaría et al. (2004) who demonstrated that Spanish isolates of *G. abietina* had the best growth at this particular temperature. Furthermore, the presence of mitovirus seemed to have a significant effect on *G. abietina* isolates at its optimal growing temperature of 15 °C because the isolates with mitovirus present had higher mycelial growth than isolates without mitoviruses. This increase in the mycelial growth of our isolates could be related to a higher virulence of the pathogen since, in general terms, a suppression of mycelial growth has been reported to be closely associated

with hypovirulence of fungi (Ghabrial et al., 2009; Pearson et al., 2009) although it could also be related to other factors (e.g., poor sporulation).

Heat tolerance was previously observed in several fungi among virus-infected and virus-free isolates (Marquez et al., 2007; Herrero et al., 2011) but in our study neither mitovirus-infected nor mitovirus-free isolates were able to endure the heat (few isolates hardly grew at 25 °C and no growth was observed at 35 °C). Marquez et al., (2007) observed that plants inoculated with the virus-infected wild type isolate of *Curvularia protuberata* R.R. Nelson and Hodges, with presence of the virus named CThTV, tolerated soils temperatures as high as 65°C for two weeks whereas plants inoculated with the virus-free isolate of the fungus dried-up and became chlorotic. Light evidence of heat tolerance was also observed in *Tolyposcladium cylindrosporium* W. Gams due to the different behaviors displayed between virus-infected and virus-free isolates at 30 °C (Herrero et al., 2011).

The pH value determines the availability of elements such as nitrogen, calcium and magnesium, among others, taken up by the fungus. In other fungi the effects of viruses have been shown to undergo variations when the composition of substrates, and therefore the availability of elements, differ. Van Diepeningen (2006) observed that abundance of available nutrients in rich medium could mask viral effects on *Aspergillus* isolates. In our study no statistical differences were shown between mitovirus-infected and mitovirus-free strain growth under any treatment variation. Fungal cellular activity measured by means of growth and metabolism rates tend to decrease if the fungi are grown at different pH values from their optimal (Perez et al., 2000). According to our results, highest mycelial growths of all the isolates were observed at the initial pH 4, which is consistent with the general statement that most fungi will grow properly over a broad pH range on the acidic side of neutrality, i.e., pH from 4 to 7 (Carlile et al., 2001). Nevertheless, it is

known that several species of isolates are able to modify the initial pH of the media in order to stabilize the acidity or alkalinity of the substrate (Carlile et al., 2001; Vazquez Garcia et al., 2002).

In our study, there wasn't any clear evidence that a decrease in osmotic potential produced a reduction of mycelial growth as previously observed in other fungal species (Imolehin et al., 1980; Lira-Mendez and Mayek-Perez, 2006; Palmero et al., 2008; Armengol et al., 2011). A reduction of the growth of the mitovirus-infected isolates was observed at -0.6 MPa and -1.8 MPa which can be linked to a decrease in the virulence of the isolates. Changes in behavior were also observed in isolates with and without viral infection when growing at certain osmotic potentials for *Monosporascus cannonballus* (Armengol et al., 2011).

Concerning in vivo pathogenicity test, mitovirus' infection resulted in any statistically significant difference in visual disease severity among seedlings. Necrosis length of seedlings inoculated with mitovirus-infected isolates was significantly larger than the necrosis from seedlings which were inoculated with non-infected isolates, that is, mitovirus infected isolates did not reduce the pathogen aggressiveness, which is the most desirable feature in control of plant pathogenic fungi (Xu et al., 2005). This behavior could suggest an advantage to the infected isolates and could lead to a hypervirulence of the pathogen. However additional studies are required to understand the role of this mitovirus in *G. abietina* features.

Plant pathologists have been interested for a long time in mycoviruses (and among them, the mitoviruses) because of their potential use as biological control agents (Pearson et al. 2009). Although many viruses produce no obvious phenotypic changes, it is reasonable to assume that many virus infections will have some effect on growth (McCabe et al. 1999). The results from this experiment suggest that the presence of mitoviruses

affects mycelial growth under different culture conditions as previously observed (Vainio et al. 2010). Nevertheless, the differences in growth among isolates may be also having been due to a genetic influence (Zharare et al., 2010) a possibility not tested due to not working with genetically similar strains. Previous studies have shown that viruses found in many fungi, e.g., *Cryphonectria parasitica*, *Fusarium graminearum* or *Botrytis cinerea*, produce several phenotypic changes such as reduction in growth and sporulation of the fungal strains they infect (Chu et al., 2002; Boland, 2004; Van Diepeningen et al., 2006; Robin et al., 2010; Wu et al., 2010; Zhang et al., 2010). In our study, the isolates growing at osmotic potential medium of -0.6 MPa and -1.8 MPa also showed a reduction of the mycelial growth. However, in the virus-infected *Fusarium oxysporum* strains when growing on PDA only slight morphological alterations were evident (Lee et al., 2011). Furthermore, it has been observed that several *Cryphonectria parasitica* virus-infected strains grow as well as virus free isolates on most artificial media although they are incapable of producing grilling cankers on chestnut trees and sporulate poorly (McCabe et al. 1999). In other cases, the presence of dsRNAs did not cause any fungal specific symptoms, such as reduced mycelial growth (Aoki et al., 2009). In some *Alternaria* spp. species there was no correlation between the radial growth of isolates and the presence of the dsRNAs (Zabalgozcoa, 1998). In contrast to hypovirulent interactions, there is evidence that some mycoviruses are beneficial to their hosts. Tan et al., (2007) observed statistically significant differences in *in vitro* growth rates of virus-infected versus uninfected isolates, with the infected cultures growing more rapidly. In our results, an increase of the mycelial growth was observed at treatment 15 °C, the optimal growing conditions of *G. abietina*.

This study provides additional knowledge on the effects of mitovirus infection on *G. abietina* isolates. However, further research including other

virulence-associated parameters such as sporulation rates and *in vivo* virulence are recommended to establish an association between mycovirus infection and fungal virulence in Spanish *G. abietina* isolates. The development of a biocontrol protocol may create opportunities for biological control of this disease.

In our study mycelial growth depended on the treatment and the presence of mitoviruses. The presence of mitoviruses did not reduce mycelial growth of *Gremmeniella abietina* at its optimal growing temperature of 15 °C. No effects of the occurrence of mitoviruses were shown among the mitovirus-infected and the mitovirus-free ones at any pH value. Variations in the behavior of the isolates with and without viral infection were observed when growing at certain osmotic potentials. A reduction in the growth of the mitovirus-infected isolates compared to the mitovirus-free ones was observed at the higher osmotic potentials (-0.6 and -1.8 MPa) while an increase was observed at the lower one (-2.4 MPa), when the conditions for the fungus were less favorable. No differences were found in visual disease severity among plants however larger necrosis lengths were observed in the plants inoculated with mitovirus infected isolates, suggesting a possible hypervirulence produced by the mitoviruses' presence. Further research including other virulence-associated parameters is recommended.

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Chapter 6

Aleppo pine provenances vary in resistance and chemical defense response to the infection of *Gremmeniella abietina*

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ABSTRACT

Aleppo pine is a thermophile species that has become a popular tree species in plantings even beyond its natural habitat. In 1999 the pathogenic fungus, *Gremmeniella abietina*, was isolated for first time in Aleppo pine plantations in Spain. The main objective of this study was to analyze the variation in susceptibility to *G. abietina* (European Race, biotype A) infections among five Aleppo pine regions of provenance. Artificial inoculations were performed at two different inoculation times in January of 2012. The susceptibility of the provenances was evaluated by recording the severity and internal stem necrosis produced by the pathogen. In addition, we quantified the concentration of two flavanone compounds as putative resistance indicator of the plants. The provenances and the different *G. abietina* isolates exhibited different levels of necrosis although the results were dependent on the time of inoculation. There was a relationship between the geographic origin of the provenances and their resistance and a pattern was found that the higher the

altitude of the provenance, the lower the resistance to the disease. The amount of naringenin flavanone was significantly different between provenances, suggesting that it is a possible indicator value for the resistance of the provenances. Conversely, no differences were found in the concentration of eriodictyol between provenances. Our results show that the provenance-dependent variation in the susceptibility of Aleppo pine to *G. abietina* should be considered that may determine the successful use of Aleppo pine in restoration.

Keywords: Provenance trials, severity, forest health, phytopathogens, flavonoids.

6.1. Introduction

Sustainable forest management should integrate planning, management and evaluation of natural resources and artificial plantations. To ensure the success of forest restorations and guarantee an adequate level of survival, adaptation and growth of future stands, the best available plant materials should be used, chosen on basis of ecological, economic and genetic criteria (Alía et al. 2009). One species that was used in forest restorations of arid areas in Spain is the Aleppo pine (*Pinus halepensis* Mill.) This pine exhibits high heat and drought tolerance and the ability to withstand dry soils. In Spain, the natural Aleppo pine stands are present throughout the coast and in the central parts of the country, but it has also been used for reforestation in other parts beyond the boundaries of its natural habitat. In some of these regions, such as the northern plateau, the pine stands face frost and other hostile weather conditions. In addition, several episodes of decline (i.e., defoliation, discoloration, distortion of terminal

twigs, presence of cankers) have been detected and attributed to Scleroderris canker disease (Santamaría et al. 2003). The infection, which is caused by the fungus *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnelt) can lead to the weakening and death of these trees. So far, no large-scale epidemic outbreaks caused by this pathogen have been recorded in Spain, but the devastation of huge areas of *Pinus*, *Picea* or *Abies* stands has been reported in other parts of the world (Yokota 1975; Laflamme & Lachance 1987; Kaitera et al. 1998). This fungus was divided into three races; European, North American and Asian (Dorworth and Krywienczyk 1975). Within the European race three biotypes have been determined: biotype A (LTT, large tree type), biotype B (STT, small tree type) and alpine biotype (Uotila 1983; Hellgren & Hogberg 1995; Hamelin et al. 1996). Spanish *G. abietina* belongs to the European race and has been related to biotype A, although it has a unique genotype (Botella et al. 2010).

The resistance of a tree to a pathogen infection is known to be genetically related and varies among species and attributes like the geographic source region of the seeds, denominated as provenance. A provenance region refers to the area or areas with uniform ecological conditions, where the tree seed crops present similar phenotypic and genetic characteristics (Alía et al. 2009). A total of 19 provenances were established in Spain based on both geography and population phenotypes. Most of the provenances are situated along the coast while some occupy sites at a high elevation (e.g., the provenance, Bética, is located within an elevation of 700 to 1900 m a.s.l.). Differences found between Spanish provenances include climatic features (e.g., annual precipitation, mean temperature), altitude, and resistance to drought (Alía et al. 2009). It is reported that there is a higher risk of infection when the tree species or provenances are growing outside its

optimal habitat (Witzell & Karlman 2000). In the case of the pathogen *G. abietina*, differential susceptibility of provenances has been reported for *Pinus* and other coniferous species in several countries (Hansson 1998; Santamaría et al. 2006; Laflamme et al. 2006; Bernhold et al. 2009). Furthermore, studies conducted with other pathogens have confirmed that the outcome of the tree's interactions with pests and pathogens vary within species and provenances (Wallis et al. 2010). However, little is known about the correlations of the provenance variation with physiological and biochemical indicators for *G. abietina* infections on Aleppo pine.

To defend themselves from infections, plants employ several methods to prevent and impede the advance and spread of pathogens, although the mechanisms vary between species and circumstances. One way to conceptualize these methods is according to their timing in relation to the infection. The preexisting or constitutive defenses, which include physical barriers and preexistent chemical defenses, are present in the plant before the attack of the pathogen. Once the infection has occurred, the induced defense system is activated after the plant's recognition of specific pathogen-produced signal molecules, known as elicitors. It implies not only physical changes like the suberization of some cells but also a chain of biochemical reactions with the production *de novo* of some antimicrobial substances called phytoalexins (Franceschi et al. 2005). After the plant has been infected, some hormones, such as salicylic acid, jasmonic acid and ethylene, are produced to extend the communication within the plant and prevent future infections. This is called systemic acquired resistance. The most common compounds involved in the chemical defense (both constitutive and induced) include phenolics, terpenoid resins, proteins and enzymes synthesized by three secondary metabolic pathways: the acetate-malonate route, the acetate-mevalonate route and the shikimic acid route (Franceschi et al. 2005; Witzell & Martin

2008). The functions of flavonoids in plants are diverse and some of them are known to have antifungal benefits because of their antioxidative properties, their inhibition of the pathogen's enzymes, spore development and mycelium hyphae elongation, and because they provide the host with quantitative resistance (Bollina & Kushalappa 2011; Mierziak et al. 2014 and references therein).

Regarding *G. abietina* infections, both physical and chemical reactions have been reported to happen in previous studies. According to these previous studies, the host's resistance to the *G. abietina* infection is likely to be related to: (i) the production of ligno-suberized tissues that help the tree compartmentalize the invaded tissues and (ii) the secretion of molecules such as phenolic compounds capable of degrading or altering the extracellular sheath of the pathogen which contains chitin, galactose, proteins and lipids (Simard et al. 2001; Laflamme et al. 2006; Bernhold et al. 2009; Simard et al. 2013). Nevertheless, to our knowledge, there are no studies comparing the content of phenol compounds in trees showing differential susceptibility to *G. abietina* infections.

In order to gain more insights into the extent of variation in susceptibility to *Gremmeniella* among provenances and to further explore the physiological basis of such variation, we explored the susceptibility of five provenances of Aleppo pine seedlings to the *G. abietina* infection. Seedlings of Aleppo pine were artificially inoculated with the *G. abietina* pathogen. The susceptibility of the provenances was quantified by measuring the necrosis length produced by the progression of *G. abietina*, and the concentration of some UV-absorbing compounds (secondary metabolites) in the seedlings produced in response to the infection was quantified.

6.2. Materials and Methods

6.2.1. Pine provenances and fungal material

The susceptibility of five Spanish Aleppo pine provenances (Figure 6.1) was evaluated. The provenances were selected (Table 6.1) to represent two distinct climate regimes in Spain: (i) the continental climate, with frequent frosts and droughts (provenances P1-Ibérico, P2-Alcarria and P3-Sudeste) and (ii) the extreme continental climate which has strong within-year variations in temperature and humidity (P4-Bética Sur and P5-Cazorla). The seeds were provided to us by the government nursery, “El Serranillo,” and were all grown under the same conditions in a shade-cloth greenhouse at the College of Forestry and Agricultural Sciences, in Palencia, Spain (41°59'19.1"N, 4°30'56.5"W). Prior to seed sowing, the soil, which was

Table 6.1: Characteristics of the different provenance regions: name of provenances, latitude, longitude, altitude, climatic conditions of the region, diameter of the seedlings (in mm) (mean value \pm standard error), length of the seedlings (in cm) (mean value \pm standard error). * Means without a common letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).

N	Prov.	Latitude	Longitude	Altitude	Climate Characteristics	Diameter seedlings (mm) *	Length seedlings (cm) *
P1	Ibérico	40°44'N-41°30'N	1°55' W-0°35' E	200-600 m	Continental climate, Frost and draught	1.23 \pm 0.02 ab	10.4 \pm 0.148 b
P2	Alcarria	40°05'N-40°48'N	3°15'W-2°13'W	600-1000 m	Continental climate, Frost and draught	1.23 \pm 0.02 ab	10.5 \pm 0.148 b
P3	Sudeste	37°15'N-38°35'N	2°18' W-0°06' W	0-400 m	Continental climate, Frost and draught	1.29 \pm 0.02 a	12.7 \pm 0.148 a
P4	Bética Sur	37°35'N-38°15'N	2°27' W-1°17' W	600-1200 m	Very continental climate, Strong variations	1.11 \pm 0.02 c	7.92 \pm 0.148 d
P5	Cazorla	37°58'N-38°28'N	3°02' W-2°30' W	600-800 m	Very continental climate, Strong variations	1.19 \pm 0.02 bc	9.61 \pm 0.148 c

composed of peat (50%) and vermiculite (50%), was sterilized in the autoclave for 60 min. at 120°C. The seeds were surface sterilized 30 min in H₂O₂ and washed several times with sterile distilled water. To ensure the germination and growth of the seedlings, three seeds were placed in each container, based on the 45% viability of the seeds and their 82% germination index (data provided by El Serranillo nursery). The seeds were sowed in July

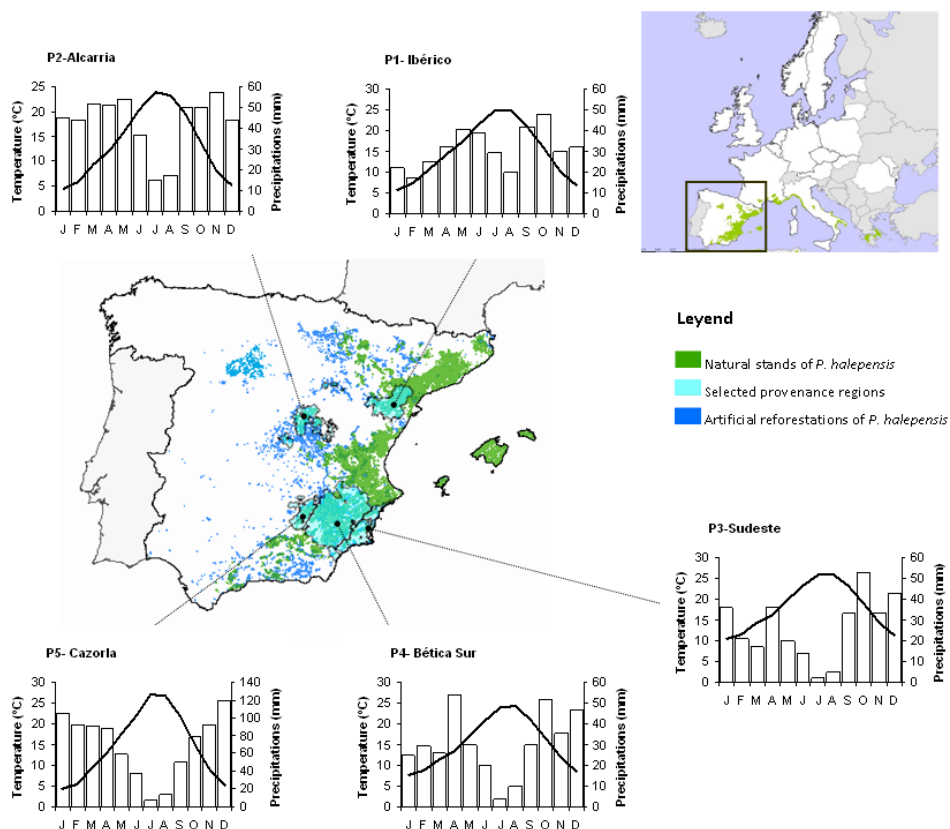


Figure 6.1: Maps showing the distribution of Aleppo pine in Europe and Spain as well as the location and climographs of the five provenances used in this experiment (P1-Ibérico, P2-Alcarria, P3-Sudeste, P4-Bética Sur and P5-Cazorla). The maps were generated with the platform SIG-Forest from the INIA from the Spanish Ministry of Science and Innovation.

of 2010 and visually examined through an 18-month period. Only visibly healthy seedlings were used in the experiment. To perform the pathogenicity tests, eight Spanish isolates of *G. abietina* (European race, biotype A) were randomly selected from the Forest Pathology Lab collection. All isolates were obtained from Aleppo pine trees from the Palencia and Valladolid provinces and isolated between 2001 and 2010. Isolates were grown for three weeks on MOS-agar (modified orange serum-agar) at 15 °C in the laboratory before the inoculation.

6.2.2. Experimental design and inoculation assays

The experiments were carried out in a shade-cloth greenhouse in Palencia, Spain at ambient temperature (mean daily=3.4°C). The artificial inoculations of the disease were performed with mycelium from colonies of *G. abietina*, due to the fact that we could not previously obtain homogenous infections using conidial suspensions from our isolates (Romeralo et al. unpublished data). In every seedling, a 4mm² piece of MOS-agar with mycelium of the pathogen was placed at 10 cm from the shoot apex after removing the thin bark with a sterile scalpel. Then the wound was covered with Parafilm® (Bemis Company, Inc, Neenah, Wisconsin, USA). The inoculation process was done in January because *G. abietina* is likely to infect the plants during the dormant season (Ranta et al. 2000). Three weeks after the first inoculations, the experiment was repeated in order to verify the results. The experiment had a randomized factorial design with seven repetitions per combination and three factors: (i) pathogen (eight *G. abietina* isolates + agar control), (ii) provenance (five provenances: P1-Ibérico, P2-Alcarria, P3-Sudeste, P4-Bética Sur, P5-Cazorla), (iii) time of inoculation (two rounds). Therefore, every combination consisted of an artificial inoculation of one of the 90 possibilities of “pathogen / provenance / time of inoculation.” A

total of 630 Aleppo pine seedlings were included in these measurements.

6.2.3. Severity, necrosis measurements and re-isolation of the pathogen

Five months after the inoculations, when the symptoms started to appear, the severity of the disease was visually evaluated using the following scale: 0 symptomless; 1 chlorosis; 2 advanced chlorosis; 3 dieback; 4 necrosis; 5 dead plant. Afterwards, the seedlings were cut and carried to the laboratory for detailed measurement of the following parameters: (i) the total length (cm); (ii) the diameter at root collar (mm); (iii) the presence of fruiting bodies and, (iv) the length of the necrosis produced by the pathogen's progression (cm). Since not all seedlings had the same height, the relationship among the length of the necrosis and the total length of the plant was calculated, which we called relative necrosis length, and used as response variable in our studies. Furthermore, to ensure that the *G. abietina* pathogen was the fungus responsible for the necrosis, a 2 cm piece of the stem containing the zone between necrotic and healthy tissue was cut from one third of the plants (210 seedlings). Then, the samples were surface sterilized (1 min in sterilized distilled water followed by 2 min in 2% NaClO followed by 1 min in 96% ethanol) and put into a MOS-agar plate at 15° C in darkness. During the following weeks, the development of *G. abietina* colonies was monitored every three days.

6.2.4. HPLC analysis

To study the chemical defenses produced by the plants as response to the attack of the pathogen, a 10 cm piece of the stem containing both healthy and necrotic tissue was cut and process from 180 seedlings of the experiment. The plant material was freeze-dried for 24h and then milled into a homogenous powder using a Retsch ball mill (MM301, Retsch GmbH, Haan, Germany). A subsample of 10 mg was weighed into an Eppendorf vial.

Three glass pearls and 1 ml of cold methanol was added, and the samples were shaken in the mill for two minutes at speed 25 and centrifuged for two minutes at 18928 g, after which the clear supernatant was transferred into another vial. The procedure was repeated with a 500 μ l solvent and the supernatants were combined. The solvent was evaporated to dryness in a vacuum concentrator and stored at 4° C until analyzed (within 1-3 days from extraction). Before the analysis, the extracts were dissolved in 400 μ l of methanol:water (1:1, v:v) and passed through disposable filters (0.45 μ pore size) to remove any particles. The HPLC system consisted of a Merck Hitachi LaChrom device with a D-7100 pump, D-7200 autosampler, D-7300 column oven at 40 °C, and a D-7455 DAD detector scanning the absorbance between 220 and 400 nm. Separation was achieved on a HyPurity C18 (Thermo Scientific, Waltham, MA, USA) column using a gradient made of water (acidified with o-phosphoric acid to pH3; A) and methanol (B) as follows: 10% B (0–1 min); 10–70% B (1–20 min); 70% B (20–23 min); 70–100% B (23–30 min). This was followed by flushing and equilibration to initial conditions. The flow rate was 0.8 ml/min, and the injection volume was 20 μ l. Two peaks with 8.06 min and 11.16 min retention times dominated the HPLC profile detected at 280 nm. The closest matching standards in the in-house standard library were the flavanones eriodictyol and naringenin, correspondingly. These peaks were thus quantified on the basis of peak area and identified as eriodictyol and naringenin equivalents.

6.2.5. Statistical analysis

The program SAS 9.2 Inc (2004) was used to study the effect of *G. abietina* isolates, provenances and time of inoculation on the relative necrosis length. The α was set at 0.05 for this analysis. To examine the effect of the three factors (time of inoculation, isolates and provenances) and their

interaction on the relative necrosis length; a linear mixed model using the MIXED procedure in SAS was used due to the high heterogeneity of the variance of the data. The mathematical formulation of the model was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \varepsilon_{ijkl}.$$

with $i=1, 2$ for the time of inoculation, $j=1, \dots, 9$ for the *G. abietina* isolate and $k=1, \dots, 5$ for the provenance; Y_{ijkl} = The observed value of the relative necrosis length for the replication l of the time of inoculation i , the *G. abietina* isolate j , and the provenance k ; μ = general mean effect; α_i = main effect of the time of inoculation i ; β_j = main effect of the *G. abietina* isolate j ; γ_k = main effect of the provenance k ; $\alpha\beta_{ij}$ = interaction effect of the time of inoculation i and the *G. abietina* isolate j ; $\alpha\gamma_{ik}$ = interaction effect of the time of inoculation i and the provenance k ; $\beta\gamma_{jk}$ = interaction effect of *G. abietina* isolate j and the provenance k and $\alpha\beta\gamma_{ijk}$ = triple interaction effect of the time of inoculation i , the *G. abietina* isolate j and the provenance k ; ε_{ijkl} = Random error in the relative necrosis length for the replication m of the time of inoculation i , the *G. abietina* isolate j , and the provenance k .

The random errors ε_{ijkl} were supposed to be independent and with normal distribution $\varepsilon_{ijkl} \sim N(0, \sigma^2_{ij})$ for the relative necrosis length. The best model was chosen according to the value of the Akaike Information Criterion (AIC) and in compliance with the normality, linearity and homoscedasticity of the residuals. Normality was assessed by examining residual plots and histograms as well as using Kolmogorov-Smirnov's test of heterogeneity. The factors provenance, *G. abietina* isolate and time of inoculation were compared with a multiple range Tukey-Kramer HSD test.

To study the effect of the provenances, time of inoculation and isolates on the phenols (erioctydiol and naringenin) a non parametric Kruskal-Wallis test was used after it was found that the data did not follow a normal

distribution in a Shapiro-Wilk test). Then, a post-hoc Mann-Whitney test with Bonferroni correction was applied to compare the means of the factors that presented significant p-value in the test. These analyses were performed with R software (version 3.1.2 R Development Core Team, Vienna, Austria, <http://www.r-project.org>).

In addition, to explore possible relationships among the variables, a correlation matrix using the nonparametric Spearman's rank correlation coefficient was applied to the following variables from the provenances: UTM coordinates (XUTM and YUTM), altitude, mean temperature, total precipitation and precipitation in the summer months (June, July and August) as well as the following seedling variables: severity, diameter, length, necrosis, relative necrosis length and amount of eriodictyol and naringenin equivalents. These analyses were also performed with the R software.

6.3. Results

6.3.1. Re-isolation of the pathogen, seedling parameters and visual severity

Pycnidia of *G. abietina* were found in 15.71% of the seedlings. In addition, there was a 30.2% occurrence rate of *G. abietina* colonies in the Petri dishes. The seedlings from the provenance regions differed significantly in diameter at root collar ($p < 0.001$) and height ($p < 0.001$) (Table 6.1). Seedlings from the provenance region P3-Sudeste were the tallest and had the highest diameter value, whereas the ones from the provenance P4-Bética Sur were the smallest and had the lowest diameter value at root collar (Table 6.1). Based on visual examination, 3.8% of the seedlings were symptomless (0), 7.77% had chlorosis (1), 32.7% had advanced chlorosis (2), 44.76% had dieback (3), 10.32% were necrotic (4) and 0.63% were found completely dead (5). Thus, dieback was the most frequent symptom, being registered in

40.5 % of the seedlings from the P1-Ibérico, 60.3% from P2-Alcarria, 47.6 % from P3-Sudeste and in 36.5% of the seedlings from P5-Cazorla. The most frequently observed symptom in the seedlings from the P4-Bética Sur provenance was advanced chlorosis (40.5% of the seedlings). The most frequent symptoms (i.e. 3-dieback and 2-advanced chlorosis) were equally distributed and therefore, not useful in making a distinction between provenance susceptibility. The P1-Ibérico, P2-Alcarria and P4-Bética Sur provenances all presented symptoms different enough to make variation among them in terms of visual severity apparent.

6.3.2. Relative necrosis length

The best linear mixed model was selected according to the lowest AIC value and the requirements of normality, linearity and homoscedasticity. The model that met these conditions had 18 covariance parameters (one variance for each time of inoculation*isolate) and no random effects. The test type 3 fixed effects table (Table 6.2) showed that the factors *G. abietina* time of inoculation, isolate, provenance, interaction time of inoculation*isolate and time of inoculation* provenance were all statistically significant variables and had an effect on the relative necrosis length. The isolates of *Gremmeniella*

Table 6.2: Test type 3 fixed effects for relative necrosis length for time of inoculation, *G. abietina* isolate and provenances.

Dependent variable	Effect	DF	F-value	Pr > F
Relative Necrosis Length	Time of Inoculation	1	3.86	0.05
	Isolate	8	28.28	0.0001
	Provenance	4	9.86	0.0001
	Time of Inoculation*Isolate	8	4.13	0.0001
	Time of Inoculation*Provenance	4	3.19	0.01
	Isolate*Provenance	32	0.98	0.51
	Time of Inoculation*Isolate*Prov.	32	1.25	0.16

generally showed a different relative necrosis length value although it was dependent on the time of inoculation. All *G. abietina* isolates were capable of causing a necrotic response in Aleppo pine seedlings, but the extent of the necrosis was not identical in the two repeated experiments (Table 6.3). In the first experiment (inoculation 1) a significant difference was found between the control seedlings and the ones inoculated with *G. abietina* regardless of the isolate used. In the second experiment (inoculation 2) isolates G1 and G3 caused the largest necrosis (Table 6.3).

The relative necrosis length varied among provenances although it depended on the time of inoculation (Table 6.4). In the first inoculation, P1-Ibérico and P3-Sudeste were the provenances with the lowest necrosis.

Table 6.3: Relative necrosis length for each *G. abietina* isolate for every time of inoculation. Mean value \pm standard error (SE). ¹ Mean value \pm standard error (SE). ² Average necrosis when combining all the times of inoculation together. ³ Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁴ Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁵ Average necrosis when combining all the *G.abietina* isolates together.

Isolate	Time of inoculation ¹		Total ²
	T1	T2	
G1	0.117 \pm 0.012 b ³ A ⁴	0.175 \pm 0.013 d B	0.146 \pm 0.009 e
G2	0.145 \pm 0.012 b A	0.127 \pm 0.012 c A	0.136 \pm 0.008 de
G3	0.134 \pm 0.011 b A	0.159 \pm 0.012 d A	0.147 \pm 0.008 e
G4	0.133 \pm 0.016 b A	0.104 \pm 0.011 bc A	0.118 \pm 0.010 bcd
G5	0.14 \pm 0.012 b B	0.086 \pm 0.009 b A	0.113 \pm 0.007 bc
G6	0.136 \pm 0.013 b A	0.128 \pm 0.001 c A	0.132 \pm 0.008 cde
G7	0.127 \pm 0.012 b A	0.112 \pm 0.011 bc A	0.12 \pm 0.008 bcd
G8	0.13 \pm 0.012 b B	0.091 \pm 0.008 b A	0.111 \pm 0.007 b
G-Control	0.054 \pm 0.007 a A	0.04 \pm 0.006 a A	0.047 \pm 0.005 a
TOTAL ⁵	0.124 \pm 0.004 A	0.114 \pm 0.004 A	

Table 6.4: Relative necrosis length for each provenance region for every time of inoculation. Mean value \pm standard error (SE). ¹ Mean value \pm standard error (SE). ² Average necrosis when combining all the times of inoculation together. ³ Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁴ Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). ⁵ Average necrosis when combining all the provenances together.

Provenance	Time of inoculation ¹				Total ²	
	T1		T2			
P1- Ibérico	0.09	\pm 0.009 a ³ A ⁴	0.105	\pm 0.008 a A	0.098	\pm 0.006 a
P2- Alcarria	0.135	\pm 0.009 b B	0.112	\pm 0.008 a A	0.123	\pm 0.006 b
P3- Sudeste	0.099	\pm 0.009 a A	0.103	\pm 0.008 a A	0.101	\pm 0.006 a
P4- Bética	0.144	\pm 0.009 b A	0.132	\pm 0.008 b A	0.138	\pm 0.006 b
P5- Cazorla	0.152	\pm 0.009 b B	0.115	\pm 0.008 a A	0.134	\pm 0.006 b
TOTAL ⁵	0.124	\pm 0.004 A	0.114	\pm 0.004 A		

However, in inoculation 2, only the provenance P4-Bética Sur had more necrosis length compared to the other provenances (Table 6.4). Nevertheless, analysis of the data from both experiments showed that the P1-Ibérico and P3-Sudeste provenances had lower mean relative necrosis length than the P2-Alcarria, P4-Bética Sur and P5-Cazorla provenances (Table 6.4).

6.3.3. Chemical analysis

The Kruskal-Wallis test revealed that there were no significant differences in concentration of neither the eriodictyol ($H=0.12$; $df.=1$; $p=0.73$) nor in the naringenin ($H=0.25$; $df.=1$; $p=0.61$) between the two experimental rounds. No effect of the *G. abietina* isolate was found either on the concentration of eriodictyol ($H=5.88$; $df.=8$; $p=0.66$) or the naringenin ($H=9.61$; $df.=8$; $p=0.29$). Furthermore, the concentration of the eriodictyol compound did not differ among the provenances ($H=1.02$, $df.=4$; $p=0.91$)

Table 6.5: Concentration of each compound (mg/dry weight of the samples in grams) for each provenance region. ¹ Mean value \pm standard deviation (SD). ² Means without a common small letter in the same column show values significantly different from $p < 0.05$ (Mann-Whitney test).

Provenance	Compound (mg/g) ¹	
	Eriodictyol	Naringenin
P1- Ibérico	1.604 \pm 0.406 a ²	3.090 \pm 0.716 a
P2- Alcarria	1.571 \pm 0.756 a	2.466 \pm 0.660 ab
P3- Sudeste	1.597 \pm 0.866 a	2.421 \pm 0.884 b
P4- Bética	1.477 \pm 0.633 a	2.819 \pm 1.059 ab
P5- Cazorla	1.538 \pm 0.652 a	2.646 \pm 0.922 ab

However, a significant provenance effect was found on the concentration of the naringenin compound ($H=10.00$; $df.=4$; $p=0.04$). The post-hoc Mann-Whitney comparison test revealed that this difference was significantly higher in seedlings of the P1-Ibérico provenance as compared to the P3-Sudeste provenance (Table 6.5).

6.3.4. Correlation matrix

Relative necrosis length, the main response variable in our experiment, showed a significant positive correlation with the altitude of origin of the provenance ($r=0.20$, $p < 0.01$) and their precipitation ($r=0.12$, $p=0.05$). On the other hand, it showed a significant negative correlation with the XUTM ($r=-0.18$, $p < 0.01$) and the YUTM ($r=-0.20$, $p < 0.01$) coordinates and a very low but significant negative correlation with the diameter at root collar ($r=-0.085$, $p=0.04$). The visually-estimated disease severity had a negative correlation with the total precipitation ($r=-0.11$, $p=0.014$). The phenol compounds, eriodictyol and naringenin, had a positive correlation

($r=0.30$, $p<0.01$), and the naringenin was also positively correlated with the XUTM coordinate of the provenance ($r=0.15$, $p=0.05$). Furthermore, the diameter at root collar presented a significant negative correlation with the altitude ($r=-0.19$, $p<0.01$) and the YUTM ($r=-0.053$, $p<0.01$), but was positively correlated with the total length of the plant ($r=0.30$, $p<0.01$). Additionally, the total length of the seedling showed a negative correlation with the altitude ($r=-0.58$, $p<0.01$) and the precipitation ($r=-0.17$, $p<0.01$) but a positive correlation with the mean temperature ($r=0.37$, $p<0.01$), XUTM ($r=0.23$, $p<0.01$) and YUTM ($r=0.11$, $p<0.01$) coordinates.

6.4. Discussion

The results of this study showed that the Aleppo pine provenances varied in their susceptibility to the disease produced by *G. abietina* in Spain. Mycelial inoculations were used in our experiment due to the fact that conidial infections with Spanish *G. abietina* isolates have not been successful in earlier experiments in our laboratory (unpublished data). However, the artificial inoculations produced symptoms typically related to *G. abietina* infection such as chlorosis, dieback, necrosis and death of the plants, as previously reported for Aleppo pine (Santamaría et al. 2006). Although the young plants in this study (18 months-old) may differ from adult trees in character a direct relationship has been established with disease incidence in seedlings and older plants under field conditions (Gordon et al. 1998). Thus, we consider it highly plausible that similar provenance-specific variations in susceptibility to *G. abietina* are also exhibited in older field populations of *P. halepensis*.

In our study, the provenances of Aleppo pine showed a different response to the *G. abietina* infection in terms of relative necrosis length. In general, the P1-Ibérico and P3- Sudeste provenances exhibited a low level of necrosis and P4-Bética Sur, the highest. On the other hand, the P2-Alcarria and P5-Cazorla provenances exhibited different behavior depending on the time of inoculation. Furthermore, differing degrees of visual severity caused by the pathogen were found among the provenances also. The susceptibility of each provenance to a certain pathogen could be related to the physical or chemical properties of the trees. In the case of Aleppo pine provenances, this different behavior could be related to morphological or anatomical features. It was previously confirmed that Spanish populations of *P. halepensis* growing in regions with different environmental conditions have developed specific morphological and anatomical adaptations, such as wall thickness, intertracheid wall strength and many other characteristics, which explain the differences between the species in its regions of provenance (García et al. 2010). In addition, the necroses found in the seedlings were correlated with UTM coordinates of the provenances. The lower the values of the coordinates Y and X (which corresponded to the south and west respectively), the more necrosis was found. This pattern of increasing damage caused by *G. abietina* as the latitude of origin decreases was also recorded in previous experiments with the *P. sylvestris* (Hansson 1998). Another similar example of a susceptibility pattern based on geographical distribution was also observed with Canadian provenances of lodgepole pine with *Lophodermella* needle blight severity (Wallis et al. 2010).

In general, the first inoculations caused more damage than the second ones, although the effect was dependent on the *G. abietina* isolate and the provenance. This could be a result of the host dormancy status and more suitable temperatures for fungal growth during the first inoculations. These

factors have previously been identified as significantly affecting symptom expression of *G. abietina* isolates from Turkey (Dogmus-Lehtijärvi et al. 2012). The virulence of the different *G. abietina* isolates was found to vary during the experiment, confirming that the degree of damage was dependent on the individual. Other studies have pointed out that different isolates of the same pathogen vary in pathogenicity, and, in some studies, this variation has been found to be related to the geographic area or the host from which the isolates originate (Capretti & Dorworth 1989; Terho & Uotila 1999). Nevertheless, although the degree of damage caused to the plants varied among our isolates, all of them caused higher necrosis than that which occurred in the control treatment. This, together with the observation of fruiting bodies and the re-isolation of the pathogen, verified that *G. abietina* was the cause of the observed necrosis. The relatively low success of re-isolation may have been related to common problems in isolation and the *in vitro* cultivation of the pathogen, which has occurred previously when the pathogen was artificially inoculated into the seedlings (Santamaría et al. 2006).

Some statistical tests were applied to the data and some associations were obtained between provenances and other parameters. For example, statistically significant differences were found in the dimensions of the seedlings from different provenances such as in diameter and length. Although they were in the same environment (all of them were grown in the shade-cloth greenhouse at the College, in Palencia), the seedlings did not grow the same. It was previously reported that provenances of Aleppo pine presented differences in terms of height and diameter growth (Weinstein 1989; García et al. 2010) in response to drought, different photosynthesis, transpiration rate, efficiency of water-use and survival (Atzmon et al. 2004; Schiller & Atzmon 2009). Such differences were due to the genetic variation within individual plants and provenances which, in turn, determine their

susceptibility to threats, including diseases and drought. Furthermore, the relative necrosis length showed a significant positive correlation with the altitude of origin of the provenance and precipitation. The provenances P1-Ibérico, P2-Alcarria and P3-Sudeste all have continental climate with frequent frost and drought, but, nevertheless, they have differences in altitude and precipitation of origin. This difference in resistance may be explained by the fact that these high- and low- altitude provenances presented significant differentiation in adaptive traits mediated by maternal effects (Correia et al. 2014). A similar pattern (the higher the altitude, the lower the resistance to disease) has been observed before in the provenances of *P. patula* and *P. tecunumanii* in a study on their resistance to pitch canker disease carried out by Hodge and Dvorak (2007); they found that the provenances from drier locations and lower altitudes were the most resistant to the disease. This pattern is interesting particularly in the context of climate change scenario and increasing temperatures.

Two flavanone (flavonoids) compounds, identified as eriodictyol and naringenin, were distinguished in Aleppo pine tissue samples using liquid chromatography. The presence of some flavonoids in plants is known to have antifungal benefits (Mierziak et al. 2014). In our study, naringenin was significantly different between two provenances, P1-Ibérico and P3-Sudeste. The P1-Ibérico provenance, which had the lowest values of necrosis, had the highest concentrations of this compound. The composition and concentration of flavonoids are also known to vary within plant populations, provenances and geographic areas and sometimes are related to the resistance that plants present to certain pathogens (Lattanzio et al. 2006; Wallis et al. 2010). On the other hand, no differences were found in the concentration of eriodictyol among provenances, suggesting that is not likely that this compound was responsible for the difference in susceptibility to the

disease observed in our study. Compound-specificity of phenolics in plant interactions with different pathogens or pests has also been previously reported (Witzell & Martín 2008 and references therein) and indicates that it is possible to find chemical markers for resistance. A more comprehensive study of the chemical responses in Aleppo pine should also include other compounds in their chemical pool, such as lignin and terpenoids.

In conclusion, our study revealed that Aleppo pine provenances vary and differ in susceptibility to the *G. abietina* infection. We also found that the concentration of the naringenin compound was different between provenances, suggesting that it may be possible to find simple chemical markers for resistance. In our case, the provenances' resistance to *G. abietina* infections was also related to the origin of the seed and followed a pattern previously observed in other pathogens and provenances. Our results suggest that the sustainability of future Aleppo pine plantations may be improved if their provenance-dependent disease-resistance is tested. It may also be possible to find easily detectable chemical indicators for high *G. abietina* resistance among the secondary chemical pool of the Aleppo pine. In our case, the number of provenances and chemical compounds tested are too small to draw conclusions about a trend; nevertheless, this study should be considered a first step in a longer process. Further studies with analysis of a broader array of defensive and stress-related chemicals are needed if we want to fully comprehend the defensive potential of the Aleppo pine.

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Chapter 7 Synthesis

7.1. Control measures against *G. abietina*

The need for management and control of *G. abietina* infections especially in nurseries, and the European recommendations of using non-chemical methods (Directive 2009/128/EC), makes necessary the promotion of alternative methods to fight the disease. Some of these alternative methods can be achieved by the use of biological control agents such as mycoviruses and fungal endophytes and by the selection of resistance provenances.

7.1.1. Biological control agents

a. Endophytes and fungal extracts

Results from our experiment showed that in general terms the presence of all the endophytes (Chapter 3) and its extracts (Chapter 4) reduced the necrosis length produced by *G. abietina* in Aleppo pine seedlings although this effectiveness depended on the pathogen isolate and the time of inoculation, suggesting that it is not a highly effective antagonism. Several mechanisms could be responsible for that reduction such as parasitism, antibiosis and metabolite production, competition for nutrients, induction of resistance in the host or barrier effect. With the aim of investigating whether the endophytes were inducing the defense system of the plant as previously observed (Benítez et al., 2004; Muñoz et al. 2008; Regliński et al., 2012), the total concentration of phenols was measured (Chapter 3). The results of our study showed that the presence of the endophytes did not produce an increase of the total phenols as apposed to what was expected. Therefore, although some phenolic production would have been instigated by the endophytes, it would not have been enough to retain the pathogen

development. Consequently, in this case the reduction in the necrosis could be likely caused, rather than by an induced resistance mechanism, by the direct effect of the endophyte on the pathogen like parasitism, competition and/or antibiosis. Furthermore, in Chapter 4, we concluded that the mechanisms used by the fungal extracts were probably linked to antibiosis and production of extracellular metabolites. This result coincides with earlier studies that show similar examples of fungal filtrates reducing the growth of pathogens (Campanile et al., 2007; Zhang et al., 2014). Therefore, it is likely that the endophytes used in our study were using several mechanisms to fight against *G. abietina*.

Regarding the genera, isolate from *T. viride* (Chapter 3) as well as its extracts (Chapter 4) were able to control part of the spreading of *G. abietina*. Furthermore, we found phenolic compounds that corresponded to hydroxybenzoic acids in the filtrate of *T. viride* (Chapter 4). Good results on *in vitro* experiments were previously shown in Santamaría et al., (2007) in the reduction of mycelial growth of Spanish isolates of *G. abietina* on dual culture tests. In general terms, *Trichoderma* can be considered a good candidate to be used as biocontrol agent because it combines several modes of action to fight against fungal pathogens (Benitez et al., 2004). For example, the genus has been reported to act by the inhibition of growth and spore germination of fungal pathogens (Bhuiyan et al., 2003; Howell, 2003; Capieau et al., 2004; Bailey et al., 2008; Yi and Chi, 2011; Díaz et al., 2012; Martínez-Álvarez et al., 2012; Regliński et al. 2012; Santamaría et al., 2012; El-Hassan et al., 2013). Furthermore, the isolates produce a wide range of non-volatile and volatile antifungal substances (Reino et al., 2008; Howell, 2003), such as gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Vinale et al., 2008) as well as hydrolytic enzymes such as chitinase and glucanase (Aziz et al., 1993; Schirmböck et al., 1994).

Our results showed that the inoculation with isolates of the *Aureobasidium* genus (Chapter 3) and its filtrates (Chapter 4) also produced a reduction in the necrosis length caused by *G. abietina*. However, we found no evidence that this effect would have been due to UV-absorbing metabolites (Chapter 4) as happened to Castoria et al., (2001). Previous studies have also pointed out the antagonistic activity of the species *A. pullulans* against several fungal pathogens (Mounir et al., 2007) Dimakopoulou et al., 2008; Miles et al., 2012). Its mechanisms of success include the production of enzymes that act against fungal walls, the production of volatile compounds, the induction of plant defence responses and the competition of nutrients (Castoria et al., 2001; Bencheqroun et al., 2007; Zhang et al., 2010; Mari et al., 2012; Rühmann et al., 2013; Banani et al., 2014).

The endophyte named 20.1 (Chapter 3) as well as its filtrate (Chapter 4) also reduced the necrosis length produced by *G. abietina* in the seedlings compared to the control plants. Although no identification of the species has been made yet, a previous study performed *in vitro* by Santamaría et al. (2007), showed that in Petri Dishes, this endophyte and its extract completely inhibited *G. abietina*'s growth. These results suggested that the endophyte could produce some antifungal compounds which slow down the growth of *G. abietina*. We found that the filtrates of this fungus did contain a few phenolic compounds (Chapter 4). Therefore it is likely that antioxidant activity and toxicity of these compounds might have contributed to the apparent antagonistic activity of this fungus against the pathogen. Thus, their potential involvement in restriction of necrosis length should be studied further along with a more comprehensive chemical profiling of the filtrates.

Lastly, the isolate from *Leotimycete* genus was also able to reduce the necrosis produced by *G. abietina* in the seedlings (Chapter 3). Very scarce

literature was found about *Leotiomyces* fungus as a biocontrol agent. Nevertheless, Miles et al., 2012 tested several endophytes, including one member of *Leotiomyces* class and concluded that it reduced the growth of other pathogens.

A broader study including more endophyte species, and a randomized application of endophytes and pathogens will provide more information about the success of the antagonistic relationships. In addition, although efficacy has been shown in an experimental setting, a higher efficacy compared to the control level would be desirable. Other response variables such as survival or vitality of the seedlings would lead to a broader evaluation of the efficacy of the BCAs and their products.

b. Mycoviruses

Plant pathologists have been interested for a long time in mycoviruses because of their potential use as biological control agents (Pearson et al., 2009). The presence of mycoviruses is recorded to produce some changes in the fungi they infect, such as reduction in growth and sporulation which can confer sometimes hyper or hypovirulence to the strains (McCabe et al., 1999; Chu et al., 2002; Boland, 2004; Van Diepeningen et al., 2006; Robin et al., 2010; Wu et al., 2010; Zhang et al., 2010). However, in other cases, the presence of viruses did not cause any fungal specific symptoms on *in vitro* cultures (Zabalgogezcoa, 1998; Aoki et al., 2009; Vainio et al., 2012). So, the behavior of the virus-infected isolates *in vitro* does not necessary mean that the virulence of the pathogen would be diminished although in some cases is related (McCabe et al., 1999).

In Chapter 5, our results suggested that the presence of mitoviruses (i.e., mycoviruses from the genus *Mitovirus*) in *G. abietina* isolates affects mycelial

growth under different culture conditions as previously observed (Vainio et al., 2010). The isolates with mitovirus presence had higher mycelial growth than isolates without mitoviruses at its optimal growing temperature of 15 °C which was also observed for other fungi in Tan et al., (2007). This increase in the mycelial growth of our isolates could be related to a higher virulence of the pathogen and in our case it was related to the higher necrosis length found in the seedlings inoculated with the mitovirus-infected isolates. This behavior could suggest an advantage to the infected isolates and could lead to a hypervirulence of the pathogen. Nevertheless, the differences in growth among isolates may be also having been due to a genetic influence (Zharare et al., 2010) a possibility not tested in our experiment due to not working with genetically similar strains. We tried to remove the mitoviruses from the *G. abetina* isolates to work with the same strains, without success.

The presence of mitoviruses was previously observed to confer a heat tolerance in several fungi (Marquez et al., 2007; Herrero et al., 2011) but in our study neither mitovirus-infected nor mitovirus-free isolates were able to endure more than 25 °C. Furthermore under a pH variation, which will determine the availability of elements taken up by the fungus and the cellular activity, no statistical differences were shown between mitovirus-infected and mitovirus-free strains mycelial growth under any pH variation as apposed to it was expected (Pérez et al., 2000). Lastly, in our study, there wasn't any clear evidence that a decrease in osmotic potential produced a reduction of mycelial growth as previously observed in other fungal species (Imolehin and Grogan, 1980; Lira-Mendez and Mayek-Perez, 2006; Palmero Llamas et al., 2008; Armengol et al., 2011). Nevertheless, at certain osmotic potentials (-0.6 and 1.8 MPa) mitovirus-free isolates presented statistically higher mycelial growth than mitovirus-infected isolates.

7.1.2. Host resistance

In our study, the provenances of Aleppo pine showed a different response to the infection of *G. abietina* in terms of relative necrosis length and visual severity (Chapter 6). The provenances followed a susceptibility pattern based on geographical distribution already observed in other species (Hansson, 1998) and pathogens (Wallis et al., 2010). In our case, the lower the latitude of origin the higher the necrosis observed. The susceptibility of each provenance to a certain pathogen could be related to chemical properties (in our case, to some flavonoids composition, as previously discussed) and morphological or anatomical features of the plants. The Spanish provenances of Aleppo pine have been reported to have specific morphological and anatomical adaptations, like the wall thickness, inter-tracheid wall strength and many others, as a consequence of the adaptation to different environmental conditions (Esteban et al., 2009). In our study, although we did not randomize the seedling growth, we tried to move the trays every 2 weeks, to avoid any effect of the location inside the greenhouse. Even all of the seedlings were grown in the same environment, they presented differences in diameter and height among provenances. This fact was previously reported (Weinstein, 1989; Esteban et al., 2009) as well as differences in response to drought based on the different photosynthesis, transpiration rate, water use efficiency and survival (Atzmon et al., 2004; Schiller and Atzmon, 2009). All these differences are due to the genetic variation within individuals and provenances which will determine their susceptibility to threats, including diseases and drought.

7.2. *G. abietina* pathogenicity

In order to test the different possibilities to control the pathogen, *G. abietina* was artificially inoculated in 2-year old Aleppo pine seedlings (Chapters 3, 4, 5 and 6). Inoculations were made with mycelia since the use of spore solution of Spanish *G. abietina* isolates has not been successful in earlier experiments in our laboratory (unpublished data). The artificial inoculations produced symptoms typically related to *G. abietina* infections such as chlorosis, dieback, necrosis, presence of cankers and the death of some seedlings. These symptoms were similar to those previously reported in others studies (Uotila, 1993; Santamaría et al., 2006). The fact that controls showed no symptoms, the observation of fruiting bodies and the re-isolation of the pathogen suggested that *G. abietina* was the cause of the observed symptoms. Nevertheless, the percentages of the seedlings that presented fruiting bodies was very low (less than 30%) and the re-isolation of the fungus was in general terms difficult to achieve by means of traditional subculture on PDA or MOS-agar media and lower than the percentage obtained in a previous study by Santamaría et al. (2007). Isolates from this fungus grow very slow in media, even if it was pathogen-specific media like MOS-agar, and a faster growth of other fungal species could invade the petri-dish faster than *G. abietina*. To avoid this problem, an alternative method to re-isolate the pathogen was set out in Chapter 4; the direct extraction of fungal DNA from the edges of the lesion parts of vegetal material and amplification with a nested PCR. In our case, the use of a nested PCR with specific primers of *G. abietina* increased the success of detection although it was not very high indeed. The primers used for that purpose belonged to the 18S region, which is less likely to produce false negative detections (Zeng et al., 2005) than the ITS region, recently described as the international barcode for fungi (Schoch

et al., 2012; Kõljalg et al., 2013). Furthermore, the use of specific primers for *G. abietina* simplified the process as no-sequencing was needed, and the results could be visualized directly in the electrophoresis' gel.

The results of our experiments (Chapters 3, 4, 5 and 6) confirmed that the different isolates of *G. abietina* varied in their ability to cause necrosis in the seedlings. This statement agrees with previous reports (Terho and Uotila, 1999; Santamaría et al., 2006, 2007) which have suggested that virulence can be genetically related and depends on the geographic area and host from where the isolates were obtained (Capretti and Dorworth, 1989; Terho and Uotila 1999). In our case, the Meseta region is the only place where the pathogen has been found and isolate in Spain; therefore we could not hypothesize about the pattern of virulence of the fungus regarding its origin.

7.3. Interaction plant-pathogen

In order to defend themselves from the attack of the pathogens, plants have a complex immune system which comprises preformed and induced defense systems with both chemical and physical responses. Some of these plant responses include cell expansion and accumulation of increased amounts of phenols, which can act as antifungal agents (Franceschi et al., 2005). In Chapter 3 the total phenol content was measured in the seedlings six months after the infection of *G. abietina*. The results showed that the infection did not produce an increase in the total contents of phenols as previously observed (Cvikrová et al., 2006). Nevertheless, relationships among phenol compounds and resistance to pathogens are unclear and sometimes contradictory (Pearce, 1996; Wallis et al., 2008; Witzell and Martín, 2008). In our study, the lack of response in the plant's phenols production could be explained by the fact that the measurements of phenol content were made 6 months after the inoculations and the capacity of the

plants to produce these defensive compounds could be reduced after this time. Furthermore, another explanation of the low responses of the plant could be that the seedlings used in the experiments were young (2-year old) and the suberization of their cell walls may not have been complete. Lastly, the phenols of the plant in response to the infection could have been degraded by the pathogen, since it has previously reported that *G.abietina* has ability to degrade some lignin, suberin and other phenolic compounds (Ylimartimo et al., 1997; Simard et al., 2013).

In Chapter 6, chemical defenses produced by different Aleppo pine provenances as a result of the inoculation of *G. abietina* were measured and compared. Two flavonoids identified as eriodictyol and naringenin were present in all the samples and were used to explore different responses among the provenances. In our study the naringenin was significantly different among provenances but no differences were found in eriodictyol concentration suggesting that is not likely that this compound was responsible for the different susceptibility to the disease. These results were in line with previous studies that observed that composition and concentrations of flavonoids are known to vary within plant populations, provenances and geographic areas (Hare, 2002; Latanzio et al., 2006; Wallis et al., 2010, 2011). The functions of flavonoids in the plant are extremely diverse and some of them are known to provide quantitative resistance to the host and to have antifungal activity (Treutter, 2006; Bollina, 2010; Bollina et al., 2011; Mierziak et al., 2014).

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Chapter 8 Conclusions

8.1. Biological control agents

8.1.1. Endophytes

1. The use of fungal endophytes could be a suitable strategy to reduce the incidence of plant pathogens like *G. abietina* in pine seedlings. This statement is made based on the results obtained in which the inoculation of an endophyte into a plant reduced the advance of the pathogen; although the efficiency of the control depended on the moment of inoculation. The inoculation with an endophyte did not promote a higher production of phenolic compounds, which are considered a good indicator of such induced resistance mechanism. However, it seemed that competition and/or antibiosis were the mechanisms responsible for that reduction, rather than a systemic induced resistance mechanism.

8.1.2. Endophytes' filtrates

2. Both the preventive and therapeutic treatments of *P. halepensis* seedlings with filtrates of four endophyte isolates (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. and Endophyte 20.1) were effective against necrosis development caused by *G. abietina* infection. However, there was some temporal variability in responses, indicating the complexity of the system. Not all fungal filtrates contained phenolics in amounts that were detectable with our HPLC method, suggesting that such compounds were not a general factor behind the preventive or therapeutic effect or that they were in such low concentrations that we could not detect them. Further studies, including more inoculation intervals and shorter incubation periods, could

provide more accurate results about the efficacy of the filtrates and timing of activation of the defence mechanisms. A more comprehensive chemical profiling of the filtrates is recommended in the future.

8.1.3. Mitoviruses

3. Mycelial growth of *G. abietina* cultures depended on the treatment and the presence of mitoviruses. The presence of mitoviruses increased the mycelial growth of the pathogen at its optimal growing temperature of 15 °C. No effects of the occurrence of mitoviruses were shown among the mitovirus-infected and the mitovirus-free ones at any pH value. Variations in the behavior of the isolates with and without viral infection were observed when growing at certain osmotic potentials. A reduction in the growth of the mitovirus-infected isolates compared to the mitovirus-free ones was observed at the higher osmotic potentials (-0.6 and -1.8 MPa) while an increase was observed at the lower one (-2.4 MPa), when the conditions for the fungus were less favorable. No differences were found in the severity produced by the infection however larger necrosis lengths were observed in the plants inoculated with mitovirus infected isolates, suggesting a possible hypervirulence produced by the mitoviruses' presence.

8.2. Host resistance

4. Aleppo pine provenances vary and differ in susceptibility to the *G. abietina* infection. We also found that the concentration of the naringenin compound was different between provenances, suggesting that it may be possible to find simple chemical markers for resistance. In our case, the provenances' resistance to *G. abietina* infections was also related to the origin of the seed and followed a pattern previously observed in other pathogens and provenances. Our results suggest that the sustainability of future Aleppo pine

plantations may be improved if their provenance-dependent disease-resistance is tested. It may also be possible to find easily detectable chemical indicators for high *G. abietina* resistance among the secondary chemical pool of the Aleppo pine. In our case, the number of provenances and chemical compounds tested are too small to draw conclusions about a trend; nevertheless, this study should be considered a first step in a longer process. Further studies with analysis of a broader array of defensive and stress-related chemicals are needed if we want to fully comprehend the defensive potential of the Aleppo pine.

Chapter 9 Conclusiones

9.1. Agentes de control biológico

9.1.1. Endófitos

1. El uso de hongos endófitos puede ser apropiado para reducir la incidencia de fitopatógenos como *G. abietina* en plántulas de pino. Esta afirmación está hecha en base a los resultados obtenidos en los que la inoculación de un hongo endófito en la planta infectada redujo la progresión del patógeno, aunque la eficacia dependió del momento en que se realizó la inoculación. Sin embargo, la presencia de los hongos endófitos, no produjo un incremento en la producción de compuestos fenólicos, los cuales son considerados un buen indicador de la activación del mecanismo de defensa inducida de la planta. Por lo tanto, parece que los mecanismos empleados por los endófitos en nuestro experimento para luchar contra *G. abietina* fueron la competencia o la antibiosis, más que la activación del sistema de defensa inducida de la planta.

9.1.2. Filtrados de los endófitos

2. Tanto los tratamientos preventivos como terapéuticos en plantas de pino carrasco con filtrados de los cuatro hongos endófitos (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. y el Endófito 20.1) fueron efectivos contra el desarrollo de la necrosis producida como consecuencia de la infección por *G. abietina*. Sin embargo, se observaron algunas variaciones temporales en las respuestas, indicando la complejidad del sistema. No todos los filtrados de los endófitos contuvieron fenoles en cantidades perceptibles por el HPLC, sugiriendo que estos compuestos no fueron un factor clave en la eficacia de los tratamientos o que estaban en

cantidades tan bajas, que no se pudieron detectar. Se recomienda realizar más estudios en el futuro que contemplen mayores intervalos entre las inoculaciones para observar la activación del sistema defensivo, así como una análisis más profundo de los compuestos químicos generados como consecuencia de la infección.

9.1.3. Mitovirus

3. El crecimiento micelial de *G. abietina* en placas de cultivo dependió de las condiciones de laboratorio y de la presencia de mitovirus. En el caso de la temperatura óptima de crecimiento del hongo, a 15°C, la presencia de mitovirus supuso un incremento del crecimiento del patógeno. No se observaron efectos de la presencia de mitovirus entre los aislados infectados y los no infectados en ningún valor del pH. Sin embargo, sí se observó una diferencia en el crecimiento de los aislados con y sin virus a distintos potenciales osmóticos. Se observó una reducción en el crecimiento de los aislados con mitovirus en comparación con los sin mitovirus en los potenciales osmóticos más altos (-0,6 y -1,2 MPa) mientras que un aumento en el potencial inferior (-2,4 MPa), en las condiciones menos favorables para el hongo. En cuanto al ensayo *in vivo* no se encontraron diferencias significativas en la severidad visual, no obstante, las plantas inoculadas con aislados con mitovirus presentaron mayores necrosis, sugiriendo una posible hipervirulencia del patógeno producida por la presencia de mitovirus.

9.2. Resistencia del hospedante

4. Las procedencias de pino carrasco usadas en nuestro experimento presentaron una variación en la susceptibilidad a la infección de *G. abietina* en base a la necrosis producida por el patógeno y a la severidad visual. La

concentración del compuesto naringenina fue diferente entre las procedencias, indicando que podría ser posible encontrar marcadores químicos para resistencia. En nuestro caso, la resistencia de las procedencias a las infecciones también estuvo relacionada con el origen de la semilla, siguiendo un patrón observado anteriormente con otras procedencias y otros patógenos. Nuestros resultados sugieren que para mejorar la sostenibilidad de las plantaciones de pino carrasco en el futuro se recomienda evaluar con anterioridad la resistencia a la enfermedad. Además, se podrían encontrar marcadores de la resistencia del árbol al patógeno entre el conjunto de compuestos químicos que se generan como consecuencia de la infección.

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