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FORESTAL SOSTENIBLE

TESIS DOCTORAL:

Biotic, Abiotic and Management Factors Involved in *Pinus pinaster* Decline in the Iberian Peninsula

Factores Bióticos, Abióticos y de Gestión Involucrados en el Decaimiento de *Pinus pinaster* en la Península Ibérica

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Contents

Abstract

Forest decline is a complex disease caused by the interaction of both abiotic and biotic factors leading to gradual deterioration of the forest. These factors have been classified as predisposing, inciting or contributing factors. *Pinus pinaster* Ait. is one of the most important forest species in the Iberian Peninsula taking into account both the protection it provides and the timber it produces. It is also the main conifer species in Spain in terms of area covered. In recent years, several Maritime pine stands in the center of the Iberian Peninsula have experienced a general decline, manifested as transparency at the crown, smaller needles than normal, foliage discoloration and premature tree death. The main objective of this work was to develop a better understanding of the influence of both biotic (pathogens) and abiotic factors, including anthropogenic ones (climate, soil properties and forest management) on *Pinus pinaster* decline in the Iberian Peninsula, shedding light upon the role each factor plays in this process (i.e. if it acts as a predisposing, inciting or contributing factor). Various parameters were measured in 27 circular plots with radii of 15 m in natural stands of *Pinus pinaster* ssp. *mesogeensis*. Regarding abiotic and management factors; the results suggested that predisposing factors such as competition and water deficit have triggered the decline of *Pinus pinaster*. On the other hand, the silvicultural and environmental factors influencing the health status at the tree level were: size of the tree (diameter at breast height), stand structure (mean total height) and climate (seasonal summer precipitation). Correlations between tree growth and aridity index generated significant responses related to health status conditions and defoliation levels; Thus, drought events at the beginning of the 1990s, in 1994 and in 2005 could have acted as inciting factors to Maritime pine decline. Regarding biotic factors; *Heterobasidion annosum,* one of the most important causal agents of root and butt rot in the Northern Hemisphere, was found to be involved in Maritime pine decline, and was described on record for the first time in Spain as *Heterobasidion annosum* on *Pinus pinaster*. On the other hand, fifteen species were identified during the study and the main pathogenic species belonged to the group of Ophiostomatoid fungi*: Ophiostoma minus, Ophiostoma ips, Ophiostoma piliferum and Ophiostoma ranaculosum*. The root rot pathogen, *Heterobasidion annosum,* was also identified in the study area. We tested the pathogenicity of two Spanish isolates of *Heterobasidion annosum sensu strict*o in two-year-old *Pinus pinaster* seedlings. The mortality rates, lesion lengths and the percentage of fine root growth in the inoculated seedlings differed significantly from the control seedlings. These results demonstrated the susceptibility of Mediterranean Maritime pine to *Heterobasidion annosum* and indicated that this fungus could be the most important contributing factor to *Pinus pinaster* decline in the Iberian Peninsula.

Resumen Extendido

El decaimiento de un bosque se puede definir, como una enfermedad causada por la interacción de un número intercambiable de factores, tanto abióticos como bióticos que producen el deterioro general y gradual de una masa forestal, y cuya última consecuencia puede ser la muerte de los árboles. Estos factores pueden ser de predisposición, de incitación o contributivos. A largo plazo, el clima histórico, el sitio, la edad de la masa o la predisposición genética, puede que no causen problemas evidentes, pero sí que pueden predisponer a los árboles a sufrir decaimiento. Por otro lado, a corto plazo, los daños abióticos provocados por sucesos climáticos como heladas, vendavales, inundaciones o periodos de sequía, y los producidos por competencia y falta de recursos, se pueden considerar como factores que inducen a los árboles a entrar en un estado de decaimiento en un momento dado, del cual se pueden recuperar o por el contrario pueden dejarles unas secuelas que les incitarían a pasar a un estado de deterioro progresivo. Y por último, los factores contributivos como por ejemplo los hongos patógenos, las plantas parásitas y/o los insectos oportunistas, son los que finalmente desencadenarían un mayor debilitamiento de los árboles y la posterior muerte.

Pinus pinaster Ait., es una de las especies forestales más relevantes en España, tanto por su valor ecológico y protector como por su producción maderera. El Pino Negral es la segunda especie con la distribución más extendida en España tras la Encina, cubriendo una superficie de más 700.000 hectáreas en masas puras y 600.000 hectáreas en masas mixtas. Estas masas pueden ser tanto naturales como plantadas y pueden diferenciarse tres grupos dependiendo de la función que ejercen. Un primer grupo es el compuesto por los pinares de regeneración natural en la Meseta Castellana, dedicados históricamente a la extracción de resina. Por otro lado, están los pinares de serranía, tanto naturales como de repoblación, típicos del Sistema Central y del Sistema Ibérico, dedicados a la producción maderera. Por último, existen pinares en la zona costera mediterránea, en los que la función principal es la protección del suelo y la creación de masas mixtas con frondosas, combinada con niveles bajos de aprovechamiento maderero.

Durante las últimas décadas, el Pino Negral ha sufrido un proceso de decaimiento progresivo en la Península Ibérica con unos síntomas característicos: la inusual transparencia de copa, el enanismo en las acículas, la decoloración del follaje y la muerte prematura del árbol. Hasta el momento no se sabe qué factores están implicados en el decaimiento del pino Negral y qué papel juegan, además no existen hasta la fecha estudios que relacionen estos estados de decaimiento con los factores ambientales, la estructura de las masas y los regímenes selvícolas.

El objetivo general de esta tesis doctoral fue el de revelar los factores tanto bióticos (agentes patógenos) como abióticos incluyendo los factores antropogénicos (clima, propiedades del suelo y manejo forestal), involucrados en el decaimiento *Pinus pinaster* en la Península Ibérica. Así como, desentramar que papel está jugando cada uno de ellos, es decir, si actúa como factor de predisposición, de incitación o de contribución al decaimiento, para intentar dar una solución al problema, y proponer alternativas de control sanitario y gestión forestal sostenible, con el fin de garantizar el futuro de estos bosques de gran valor ecológico.

Este objetivo general se ha dividido en los siguientes estudios específicos; **a)** Para los factores abióticos y de gestión: **(1)** Desentramar las asociaciones que existen entre el clima, las propiedades del suelo y el manejo forestal en el decaimiento de *Pinus pinaster* en la Península Ibérica a nivel de masa forestal. **(2)** Determinar los factores que influyen en el decaimiento de *Pinus pinaster*, mediante la caracterización de las parcelas de estudio y la construcción de un modelo logístico multinomial que permita predecir el estado sanitario a nivel de árbol. **(3)** Comprender a través de herramientas dendrocronológicas las alteraciones en el crecimiento de *Pinus pinaster* considerando diferentes fases de decaimiento en relación con los cambios en la disponibilidad de agua durante las últimas décadas. Y **b)** para los factores bióticos: **(4)** Aislar e identificar por primera vez en España, el hongo patógeno *Heterobasidion annosum* involucrado en el decaimiento de *Pinus pinaster*. **(5)** Identificar morfológica y molecularmente los hongos patógenos asociados al decaimiento de *Pinus pinaster* y detectar las zonas más afectadas en relación con las variables climáticas y el estado sanitario. Y **(6)** Evaluar la patogenicidad de aislados españoles de *Heterobasidion annosum* sobre plántulas de *Pinus pinaster*.

Para ello, se han muestreado 27 parcelas circulares de 15 m de radio ubicadas en las principales áreas de distribución natural de la especie en Castilla y León. Dentro de cada parcela se han tomado las medidas dasométricas y dendrométricas de todos los árboles (1208 en total), se han evaluado sanitariamente 24 árboles (648 en total) y se han tomado muestras de 3 árboles sanos, 3 con síntomas de decaimiento y 3 muertos en píe. Estas muestras fueron de dos tipos: dos canutillos por cada árbol a la altura de pecho mediante una barrena Pressler, para obtener el crecimiento de los 20 últimos años en pies con distinto estado de decaimiento (486 en total) y un chaspe de madera tomado con un hacha en la base del tronco, para detectar a través de cámara húmeda, aislamiento en placa y posterior análisis molecular, la presencia de hongos patógenos (243 en total).

Para obtener los parámetros edáficos, se recogió una muestra de suelo a 30 cm de profundidad por cada una de las parcelas estudiadas y se envió al laboratorio del ITAGRA (Centro Tecnológico Agrario y Alimentario) para analizar los parámetros básicos (textura ISSS, pH, CE; P, K, Na, Mg, Ca, MO, CT, CaO), elementos gruesos, arena fina, Fe, N total, C/N y CCC. Los datos climáticos de cada una de las parcelas de estudio, se obtuvieron a través del atlas climático digital de la península ibérica considerado las precipitaciones, las temperaturas y la radiación solar, tanto mensuales y estacionales, como anuales. Y para cuantificar la relación entre decaimiento-clima-crecimiento, se solicitaron además a la Agencia Estatal de Meteorología (AEMET), los datos climáticos de los últimos 30 años de las estaciones más cercanas a cada una de las zonas de muestreo.

Por otro lado, se realizó un muestreo dirigido a dos zonas con claros síntomas de decaimiento para aislar e identificar tanto morfológica como molecularmente uno de los principales patógenos (*Heterobasidion annosum*) que se cree asociado al decaimiento de *Pinus pinaster*. Para ello se derribaron 11 árboles de diferentes estados sanitarios (sanos, sintomáticos, muertos) y se tomaron muestras de madera infectada para su posterior análisis en el laboratorio. Las muestras se colocaron en cámaras húmedas en condiciones óptimas de humedad y temperatura y depués de 20 días de incubación se observaron los cuerpos de fructificación. Con los aislados obtenidos se procedió a realizar la extracción de ADN para identificarlos molecularmente e introducirlos en la base de datos mundial Genbank.

Una vez confirmada mediante técnicas moleculares la presencia de *Heterobasidion annosum,* con los aislados obtenidos, se realizaron las pruebas de patogenicidad de este hongo en plántulas de *Pinus pinaster* de dos años de edad. Se inocularon un total de 120 plántulas con 2 aislados españoles de *Heterobasidion annosum* y se utilizaron dos tipos de inóculo (trozos de madera y serrín), por dos vías diferentes (en el tallo y en el suelo).

Las técnicas estadísticas utilizadas para la obtención de los resultados fueron las siguientes: Análisis de Componentes Principales y Método de Broken-Stick (Objetivos **1** y **2**). Escalamiento Multidimensional No Métrico (NMDS) y Procedimientos de Permutación de Respuestas Múltiples (MRPP) (Objetivo **1**). Agrupación Jerárquica y Modelo de Regresión Logística Multinomial (Objetivo **2**). Algoritmo Multi-Nivel: BIOdry (Objetivo **3**). Análisis de Correspondencias Canónicas y Test de Monte Carlo (Objetivo **5**). Análisis de Varianza, Test de Tukey y Modelos Lineales Mixtos (Objetivo **6**).

Los principales resultados de esta tesis según los diferentes objetivos han sido:

a) Factores abióticos y de gestión:

(1) Se confirmó que las masas muestreadas presentaban decaimiento, con casi el 40 % de los árboles muertos o dañados (10 y 30 %, respectivamente). No se encontraron diferencias significativas en cuanto a regiones procedencia de la especie. Sin embargo, si se observaron diferencias significativas según la zona de muestreo, siendo la zona de la Ribera del Duero en Burgos una de las más afectadas.

Se observó un mejor estado sanitario en masas puras que en masas mixtas. A su vez, las masas en las que se habían realizado claras, presentaban mejor estado sanitario que las masas donde no se habían aplicado claras. En particular, los altos valores de la altura total y de la precipitación de primavera parecen indicar un mejor estado sanitario, mientras que la alta densidad de la masa se asoció a estados fitosanitarios más pobres.

Estos resultados parecen sugerir que el déficit de agua y la competencia estarían actuando como factores de predisposición al decaimiento de *Pinus pinaster* en la Península Ibérica.

(2) Las parcelas de estudio se agruparon de acuerdo a las variables selvícolas, de suelo, de clima y las variables fitosanitarias, según las distintas zonas geográficas, divisiones provinciales y regiones de procedencia de la especie.

Las variables retenidas en el análisis de componentes principales para introducirlas en el modelo predictivo fueron las siguientes: el diámetro a la altura del pecho para la caracterización a nivel de árbol individual; la densidad, la altura media de la parcela y la altura media de la copa para la caracterización a nivel de masa; la capacidad de intercambio catiónico y el pH para la caracterización edáfica; y la temperatura media anual y las precipitaciones estacionales medias de primavera y de verano para la caracterización climática.

Finalmente el mejor modelo de regresión logística multinomial a nivel de árbol individual fue construido con las variables significativas: diámetro a la altura de pecho, altura media de la parcela y precipitación estacional de verano.

(3) Se detectaron diferencias en los patrones de crecimiento entre los árboles con diferentes condiciones fitosanitarias dentro de las diferentes zonas de muestreo. Las fluctuaciones en el crecimiento de los árboles en las zonas centrales de muestreo estaban distribuidas simétricamente a lo largo de los años de formación de los anillos. Por el contrario las fluctuaciones en el crecimiento de los árboles en las zonas de sierras montañosas fueron generalmente más estrechas y asimétricas.

Se observaron de forma frecuente y constante eventos de alta aridez principalmente durante dos períodos: a principios de la década de los 90 (1989-1991) y durante el año 2005. Durante el año 1994 también se observaron eventos de alta aridez, aunque no tan pronunciados. Los eventos de menor aridez se produjeron principalmente entre los años 1996 a 1998. En las zonas localizadas en sierras montañosas se observaron menos eventos de alta aridez que en las zonas centrales.

Los correlogramas de Mantel entre el crecimiento y la aridez generaron respuestas significativas en función del estado sanitario de los árboles. Las correlaciones significativas de los árboles muertos se localizaron a lo largo de un intervalo estrecho del eje del tiempo, mientras que los arboles sintomáticos y sanos se localizaron en intervalos medios y más amplios, respectivamente, en comparación con los intervalos de los árboles muertos.

Nuestros resultados tuvieron implicaciones en las relaciones de competencia-crecimiento-clima ya que se observaron valores significativos en los correlogramas de Mantel entre el crecimiento de los árboles y el índice de aridez según las diferentes clases densidad. Parcelas con altos índices de densidad mostraron una respuesta significativa más concentrada que las parcelas con índices de densidad medios y bajos, en las que las repuestas significativas se produjeron en intervalos de tiempo más amplios.

Estos resultados sugirieron que los periodos de sequía de las dos últimas décadas, podrían estar actuando como factores de incitación al decaimiento.

b) Factores bióticos:

(4) Se aisló e identificó mediante técnicas morfológicas y moleculares la presencia de *Heterobasidion annosum* en *Pinus pinaster* por primera vez en España.

Este hongo es uno de los patógenos más importantes que afectan a las coníferas en el hemisferio norte y fue detectado asociado al decaimiento de *Pinus pinaster* en el centro de España.

(5) Se identificaron quince especies durante el estudio, que se dividieron en dos grupos distintos en función de su ecología: especies saprófitas (seis especies) y especies patógenas con diferentes grados de virulencia (nueve especies).

Las especies patógenas identificadas pertenecieron principalmente al grupo de los hongos Ophiostomatales o del azulado de la madera destacando entre ellas *Ophiostoma minus*, *Ophiostoma ips*, *Ophiostoma piliferum* y *Ophiostoma ranaculosum*. También se identificó el hongo de pudrición de la raíz *Heterobasidion annosum* y fue encontrado junto con *Ophiostoma minus* y *Ophiostoma piliferum* en la misma zona geográfica. La zona de muestreo de la Ribera del Duero en Burgos albergó el mayor número de especies de hongos patógenos mientras que la zona de Soria fue la que presentó el menor número de especies de hongos patógenos.

Los resultados del análisis de correspondencias canónicas mostraron que las variables climáticas tales como la temperatura, la precipitación y la altitud no afectaban significativamente las comunidades de hongos patógenos. Sin embargo, mostraron una tendencia en cuanto a la precipitación, ya que se pudo observar que el mayor número de especies patógenas se agrupaba en zonas donde la precipitación era menor.

(6) Las tasas de mortalidad de las plántulas inoculadas en el tallo difirieron significativamente de los controles, mientras que las plántulas infectadas vía suelo no mostraron diferencias significativas con respecto a las plantas control.

Para ambos tipos de inoculación en el tallo (chips de madera y serrín), la longitud de la necrosis fue mayor que en los controles, y los síntomas de marchitamiento de las acículas también fueron significativamente más graves que en las plantas control.

La asignación de biomasa en plantas inoculadas en el tallo no difirió significativamente entre las plántulas infectadas y las de control. Sin embargo, en plantas infectadas vía suelo, el porcentaje de raíces finas fue significativamente menor en las plantas infectadas que en las plantas control.

Estos resultados han demostrado la susceptibilidad de *Pinus pinaster* al patógeno *Heterobasidion annosum,* y nos han sugerido que podría tratarse de uno de los principales agentes contributivos al decaimiento.

PART I: Background and Goals

Chapter 1: Introduction

Forest decline

Forest decline can be defined as a complex disease caused by the interaction of a number of interchangeable factors, both abiotic and biotic (Thomas et al., 2002), leading to gradual deterioration of the forest and, ultimately, the death of trees (Manion, 1991). Forest decline was first described in Central Europe and North America in the 1980s and, since then, has been widely reported around the world (Blank, 1985; Cook and Johnson, 1989; Hertel, 1988). These factors have been classified as predisposing, inciting or contributing factors (Manion, 1991). In the long term, historical climate, site management, stand age and genetic predisposition may not cause overtly evident problems, yet they may predispose the trees to decline (Hennon et al., 2009). On the other hand, inciting factors can be consider as severe short-term damage caused by abiotic climatic events (such as frost, windstorms, floods and periods of drought) and the detriment produced by competition for resources may cause trees to enter into a state of decline at any given time; After, the trees may recover or enter a more progressive state of decline (Hennon et al., 2012). Finally, contributing factors such as pathogenic fungi, parasitic plants and opportunistic insects may also contribute to the further weakening and subsequent death of the trees (Hennon and McWilliams, 1999).

Forest decline has been widely studied, but the first studies were conducted in North America on *Pinus palustris* associated with ophiostomatoid fungi (Otrosina et al., 1999) and in Canada, where they evaluated this phenomenon on sugar maples (Payette et al., 1996) and then assessed the tree basal area increments over time as a good indicator of the health and vigour of the tree (Duchesne et al., 2003). Besides, the decline of western white pine, often referred to as pole blight, had already been observed in the 1930s. In this case, drought acted as an inciting factor, whereas shallow soils and secondary fungi were predisposing and contributing factors, respectively (Kliejunas et al., 2009). More recently, extreme climatic conditions resulting from a warm drought has also acted as an inciting factor in the sudden aspen decline observed in North America (Rehfeldt et al., 2009; Worrall et al., 2010). On the other hand, in the Spanish forests this phenomenon has appeared along side environmental changes (Costa et al., 2010; Galiano et al., 2010; Oliva and Colinas, 2007). In recent years, *Pinus pinaster* decline, characterized by unusual transparency at the crown, small needles, foliage discoloration and premature tree death, has been observed in association with a high mortality rate in several forests in the center of the Iberian Peninsula (Álvarez et al., 2015; Prieto-Recio et al., 2015, 2012) (Figure 1.1).

Figure 1.1. Maritime pine forest decline at the Castilian plateau (Spain). Healthy, symptomatic and dead trees mixed in the same stand.

Forest health assessment of *Pinus pinaster* decline

The concept of "forest health" was developed in the mid-80s and 90s (Kolb et al., 1994) from several points-of-views (DellaSala et al., 1995; LaSalle, 1998; Staebler, 1994; Warren, 2007). Nevertheless, after much controversy, forest health is now being monitored practically all over the world through forest health monitoring programs. The International Cooperative Programme, ICP Forest, has been accepted by the United Nations Economic Commission for Europe (UNECE) and updated from the 1985 version (ICP Forest, 2015). The evaluation of forest health status in Spain traditionally has been done by specialized operators because of the complex factors involved in its assessment and the possible subjectivity of the observer, following the Levels I and II of the European network methodology (Área de Inventario y Estadísticas Forestales (AIEF), 2013; Servicio de Sanidad Forestal y Equilibrios Biológicos (SSF), 2000).

During the last three decades, Maritime pines have suffered progressive decline in the Iberian Peninsula with some characteristic symptoms: unusual transparency of the crown or defoliation, dwarfism in the needles or microphylls, discoloration of foliage and premature death of the tree (Figure 1.2). Defoliation is the most commonly measured symptom of decline applied to both conifers and broadleaves (Innes, 1993). This variable for the main Mediterranean forest species can be visually assessed in twenty classes of 5% interval width based on an absolute reference tree (Sánchez Peña et al., 1994). Discoloration is usually measured in four ranges of chlorosis percentage (0-10% no discoloration, 11-25% light, 26-60% moderate and 60-100% severe), according to Level I of the European network methodology (Eichhorn et al., 2010). Visual assessment of crown condition has widely been used as an indicator of forest health (Dobbertin and Brang, 2001); However, defoliation and discoloration conditions can change quickly as a result of insect outbreaks and, on its own, may be not a good indicator of cumulative effects. Furthermore, taking into account that factors involved in mortality may differ among sites, the use of different vitality indicators is highly recommended (Cailleret et al., 2014). Hence, other phytosanitary variables such as needle retention from year to year, the presence or absence of microphylls, terminal bud damage, dieback, cankers, cracking of the bark caused by wood compression and number of pinecones, have all been symptoms considered in the assessment of forest decline (Álvarez et al., 2015; Oliva and Colinas, 2007; Prieto-Recio et al., 2015).

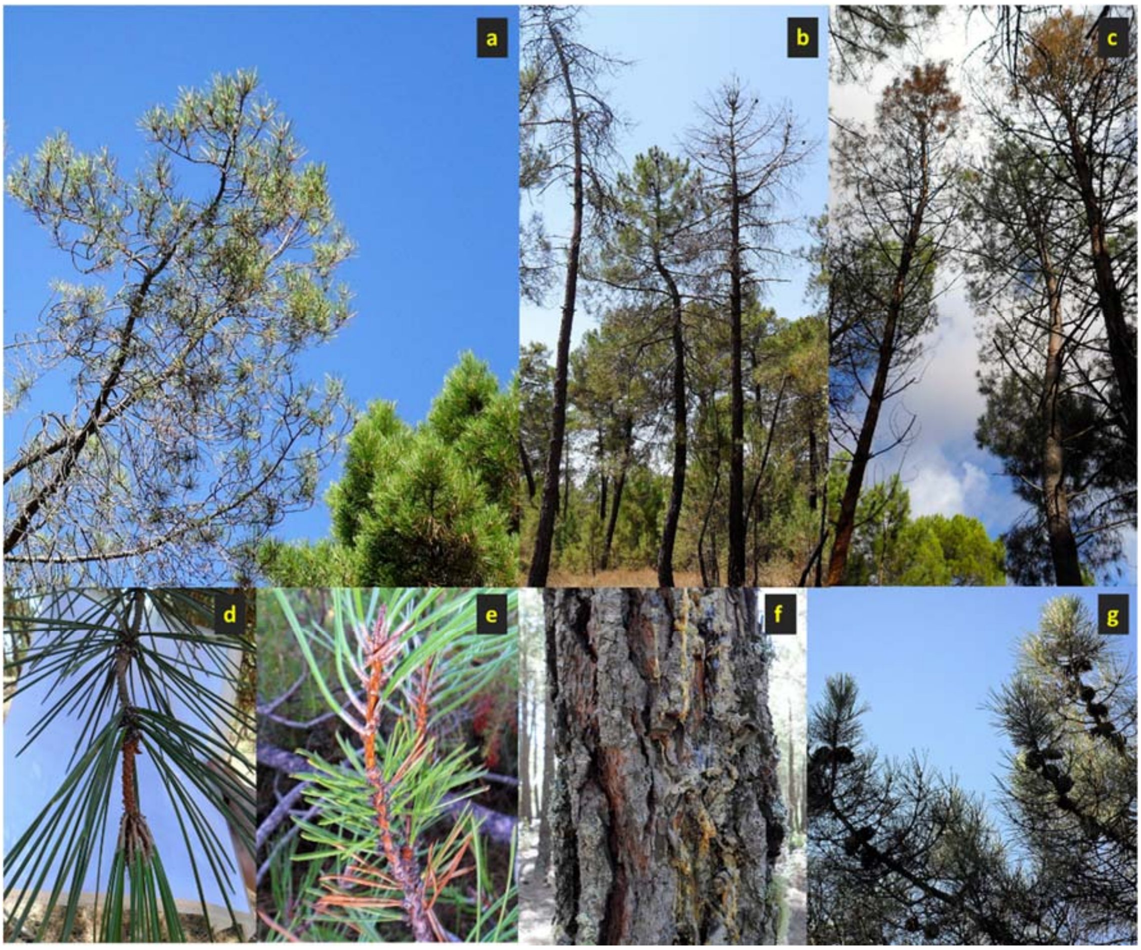


Figure 1.2. a) Defoliation, discoloration and microphylls. **b)** Defoliated trees. **c)** Discoloration at the crown. **d)** Needle retention (years). **e)** Small needles (microphylls). **f)** Cracks and cankers in the bark. **g)** Increase in the number of pinecones.

The host: *Pinus pinaster* Ait.

Maritime pine (*Pinus pinaster* Aiton), extends naturally throughout the western Mediterranean Basin, Southern Europe and Africa, and along the Atlantic coast in Portugal, Spain and France (Alía and Martín, 2003) (Figure 1.3). Its current spontaneous distribution covers territories that have been highly humanized ever since antiquity, and the species' genetic variation, characterized by different growth and shape, separates populations according to different regions of origins in Spain (Alia et al., 2001).

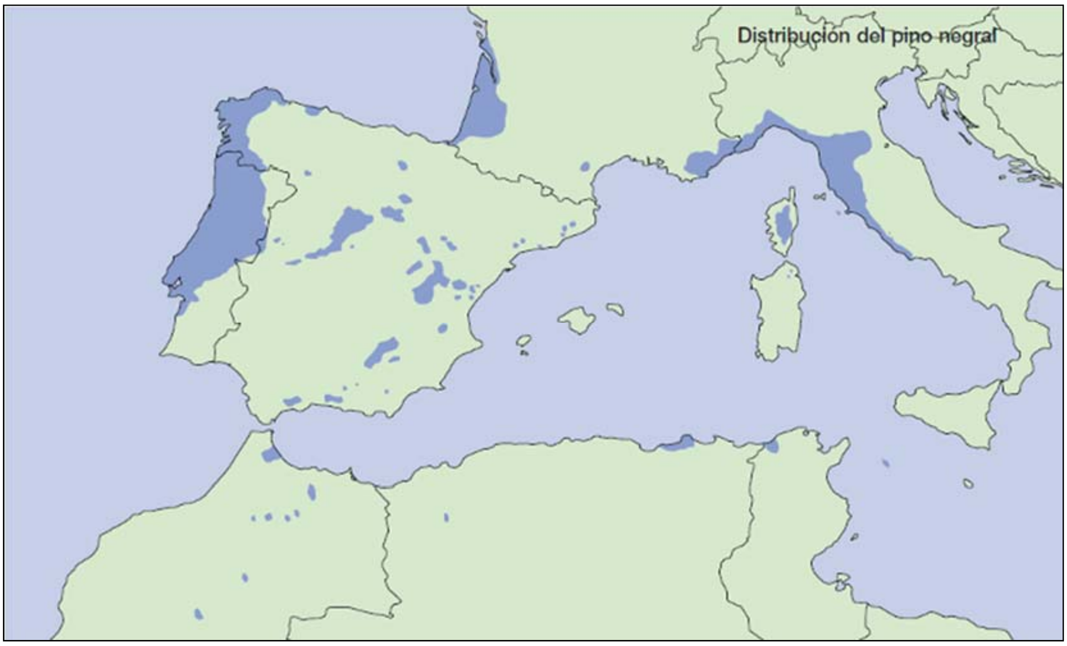


Figure 1.3. Natural distribution of *Pinus pinaster* Ait. (Alía and Martín, 2003).

Maritime pine is a species characteristic of the Mediterranean forests mainly located in the Iberian Peninsula (Blanco et al., 1997), and shows a wide ecological range (Nicolás and Gandullo, 1967). *Pinus pinaster* Ait. is divided into two subspecies with a different ecological and silvicultural behaviour: *Pinus pinaster* ssp. *atlantica* H. de Vill and *Pinus pinaster* ssp. *mesogeensis* Fieschi & Gaussen (Alía Miranda et al., 2009) (Figure 1.4). This species is highly plastic and can survive high or low temperatures, and regular or variable rainfall as well as severe droughts. It is adapted to the extremely cold winters of the continental climate in central Spain and to the mild, temperate winters of the Atlantic coast in the western Iberian Peninsula (Alía et al., 1996; Blanco et al., 1997).

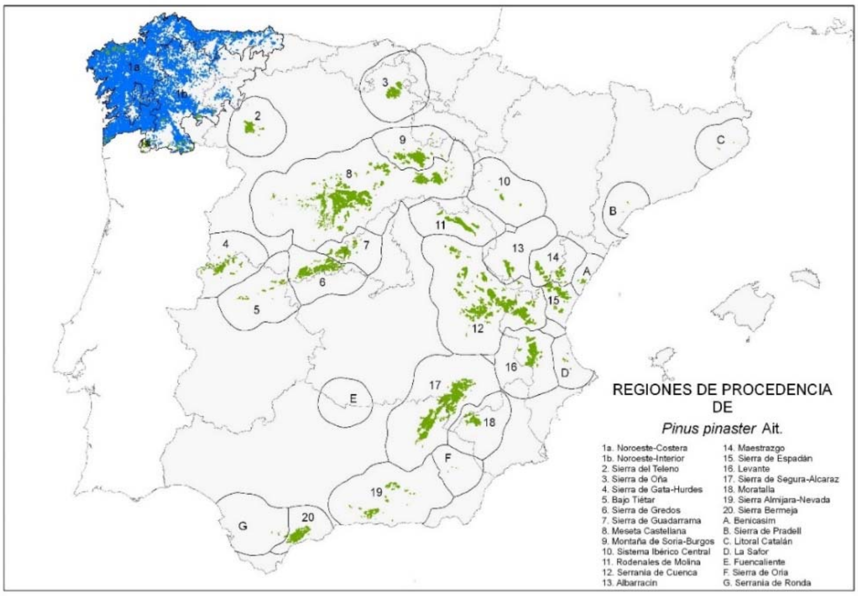


Figure 1.4. Distribution of *Pinus pinaster* ssp. *atlantica* (blue dots) and *Pinus pinaster* ssp. *mesogeensis* (green dots), and their provenance regions (Alía Miranda et al., 2009).

In Spain, Maritime pines are the most widespread conifer and are found in pure and mixed woodlands that are both natural (1 million ha) and planted (0.6 million ha) (DGCONA, 2002). Moreover, three different types of *Pinus pinaster* ssp. *mesogeensis* forests have been identified in Spain according to the function performed. The first group is composed of pines from natural regeneration in the Meseta Castellana, historically dedicated to the extraction of the resin. Moreover, there are the pines from the mountains, typical of the Central System and the Iberian System, used for timber production. Finally, there are pine forests in the Mediterranean coastal area, where the main function is the protection and creation of mixed stands with hardwoods, combined with low levels of logging (Rodríguez-Soalleiro et al., 2008). The natural distribution of this subspecies is centered mainly on the northern Spanish plateau at up to 1500 m altitude, in preferably sandy, stony and well-drained soils, where the weather is characterized by seasonal irregular rainfalls and high temperatures in the summer.

The ecological importance of this subspecies due to its role in conservation and maintaining biodiversity is widely accepted. Mediterranean pine forests, particularly those comprised of *Pinus pinaster* Ait., are classified as natural habitats of community interest and their conservation requires the designation of special conservation areas referred to as "Mediterranean pine forests with endemic Mesogean pines" (EU Council Directive 92/43/EEC on the conservation of natural habitats and wild fauna and flora).

Factors influencing forest decline

Abiotic factors: Environmental conditions

The reality of climate change is now recognised worldwide; However, regional consequences have not yet been studied in depth, especially in the case of forest ecosystems. Specific threats such as the increased frequency of abiotic risks (e.g. strong winds, droughts, floods and fires) are being observed (Canales et al., 2011; Jactel et al., 2009). In addition, new pests and pathogenic agents (Scherm, 2004) are causing general decline in numerous forest stands. Lower adaptability of tree species to the new climatic conditions is also being observed (Hansen et al., 2001). We know that the responses of forest ecosystems to climate change are complicated because of the interactions between different populations of organisms and their different biological characteristics (Roe and Baker, 2007). The potential impact climate change could have on forest diseases is affecting many aspects, such as the modification of populations and community structures, micro-evolutionary processes and plant dynamics (Chakraborty et al., 2000). Many Mediterranean and Sub-Mediterranean forest species from the Iberian Peninsula may suffer decline and drastic modification of their distribution area (Benito Garzón et al., 2009).

An increase in average global temperatures by up to 4.8°C above current levels and a decrease in mean precipitation in many mid-latitude arid and semi-arid regions is predicted to occur in this century (Stocker et al., 2013). Moreover, climatic extremes may occur even more frequently in hotspots such as the European Mediterranean areas (Giorgi and Lionello, 2008). Mediterranean regions, like transitional climate zones between arid and humid regions are especially important for the study of the relationships between climate, tree growth and vigour because in those areas water is a limiting resource (Boisvenue and Running, 2006; Rozas et al., 2011). Furthermore in this area, the climate change phenomenon is increasing the frequency and severity of extreme climatic events such as droughts (Manrique and Fernandez-Cancio, 2000). Such changes alter forest ecosystems' dynamics (Hampe and Petit, 2005) producing high mortality rates and forest decline (Allen et al., 2010). Therefore, taking into account that the forest decline in Mediterranean areas may be influenced mainly by changing climatic conditions, such as drought and outbreaks of pests and disease, it is expected that climate change will trigger the onset of forest decline in the coming years (Allen et al., 2010; Ayres and Lombardero, 2000; Desprez-Loustau et al., 2006; Garbelotto and Pautasso, 2012; Helfer, 2014; Sturrock et al., 2011).

Abiotic factors: Forest management

Decline in Mediterranean pine forests has recently been reported to be associated with the absence of silvicultural practices and high stand density, which has thus triggered the mortality and decline of these forest (Bravo-Oviedo et al., 2006; Camarero et al., 2011; Linares et al., 2009; Prieto-Recio et al., 2015). The response of tree growth and vigour to limiting factors can be influenced by forest management as the effect of different silvicultural scenarios and stand densities are key factors for forest preservation mainly in the driest areas (Gea-Izquierdo et al., 2014, 2009). However, the influence of stand density on tree growth in response to climate change and drought is not a simple issue, since different approaches, such as ecophysiological and dendroecological models, may give different points-of-view (Misson et al., 2003; Moreno and Cubera, 2008). Nonetheless, by maintaining sustainable forestry, the increased resistance and resilience of forests will increase economic and social benefits (Spiecker, 2003). The concept of sustainable forest management as an instrument that harmonizes ecological and socio-economic concerns is relatively new. The change in perspective occurred at the beginning of the 1990s, coinciding with the deterioration of the environment through alarming loss of forest resources in Europe (EU commission, 2011). Forest structure, past forest dynamics and forest management all affect the adaptive capacity of trees to adverse climatic conditions (Lindner et al., 2009). Hence, it would be suitable to have a sustainable forest management plan for these Mediterranean forests in order to promote their conservation and prevent their decline.

Biotic factors: Pathogenic fungi

Among the contributing factors to forest decline are many diseases produced by fungi, nematodes, bacteria and viruses, although fungi are recognised as the most destructive agents in the forest stands (Manion, 1991; Muñoz et al., 2007; Tainter and Baker, 1996). Fungi that affect trees can be classified according to feeding type: symbionts, parasites and saprophytes (Deacon, 2013). Fungal communities in forests are formed by endophytes together with saprotrophic and pathogenic species. Knowing the species composition and the factors influencing the presence of different fungal communities is important for understanding the role that fungi play on the regulation of other organisms (Arnold, 2007). Fungi that attack the wood (e.g. blue-stain or Ophiostomatoid fungi) and fungi that cause root rot (e.g. *Heterobasidion annosum*) are frequently involved in forest decline and cause high economic loss and ecological damage (Oliva and Colinas, 2007; Otrosina et al., 1999; Woodward et al., 1998) (Figure 1.5).

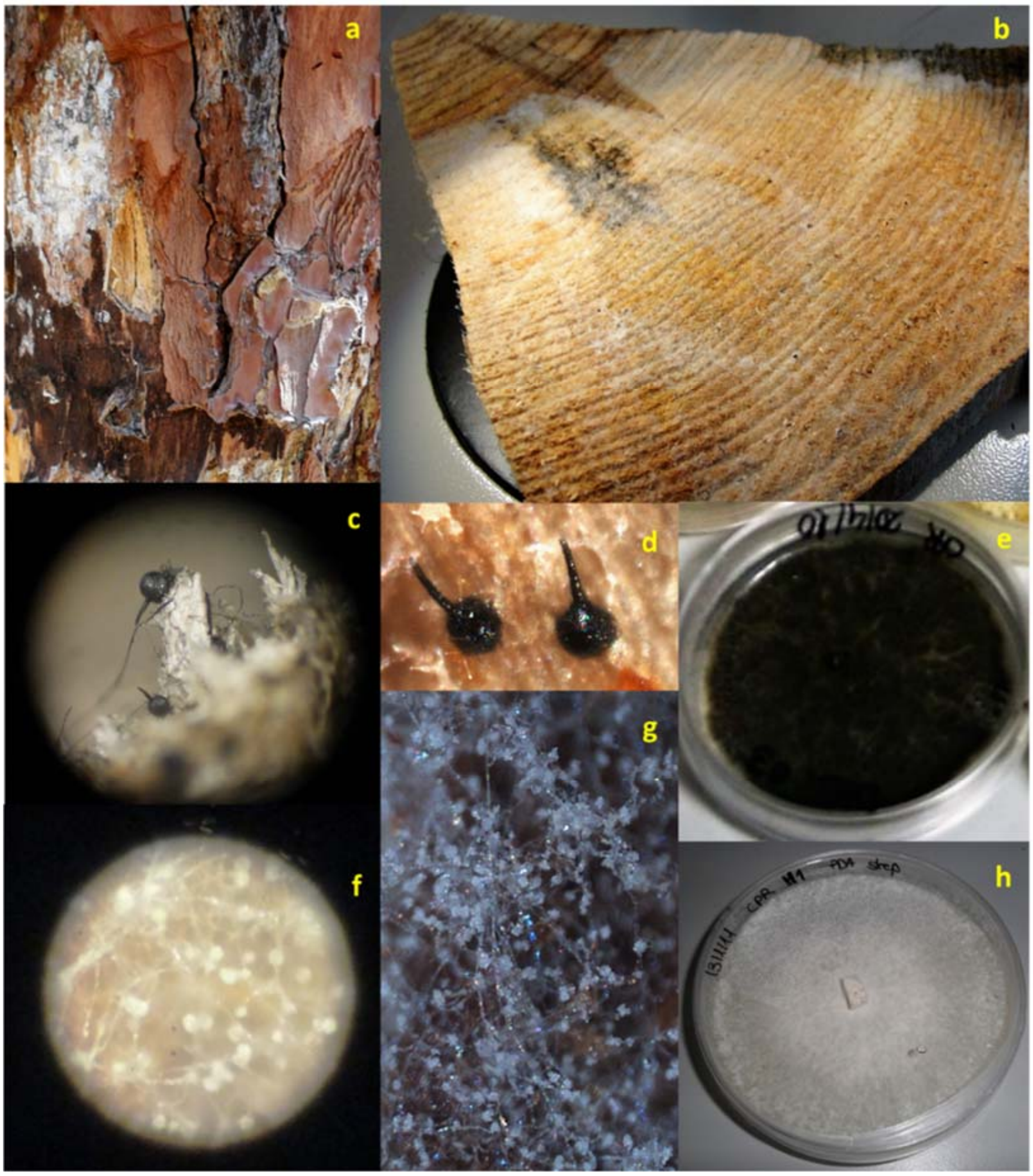


Figure 1.5. a) Debarked tree infected by blue-stain (dark area) and root rot fungi (white mycelium). **b)** Fungi after humid chamber: *Heterobasidion annosum* (white upholstered) and *Ophiostoma* sp. (dark zones). **c)** and **d)** Peritecia of *Ophiostoma* sp. (sexual stage). **e)** *Ophiostoma* sp. on culture media (MEA). **f)** and **g)** *Spiniger meineckellum* (anamorphic form of *Heterobasidion annosum*). **h)** *Heterobasidion annosum* on culture media (PDA).

The main species that cause blue-stain in stems or roots in conifers belong to the genera *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis*, the anamorphic or asexual states of which are respectively *Graphium* spp., *Leptographium* spp. and *Chalara* spp. (Grylls and Seifert, 1999; Solheim et al., 1993; Wingfield et al., 1993). Species like *Leptographium wingfieldii* have been commonly associated with *Pinus sylvestris* decline in Poland (Jankowiak et al., 2007), and *Leptographium wagenerii,* which causes black-stain in roots, has been associated with several situations of decline in conifers in North America (Otrosina et al., 1999). Furthermore, *Ophiostoma minus* has been reported as one of the most virulent ophiostomatoid fungi on pines (Masuya et al., 2003) and has been related to pine forest decline in France (Piou and Lieutier, 1989) and in Spain (Álvarez et al., 2015).

The root rot fungus *Heterobasidion annosum sensu lato* is a complex species formed by five species: *Heterobasidion irregulare* Otrosina & Garbelotto, *Heterobasidion occidentale* Otrosina & Garbelotto, *Heterobasidion annosum* (Fr.) Bref. *sensu stricto*, *Heterobasidion parviporum* Niemelä & Korhonen, and *Heterobasidion abietinum* Niemelä & Korhonen (Korhonen and Stenlid, 1998; Otrosina and Garbelotto, 2010). *Heterobasidion annosum s. l.* produces both sexual (basidiospores) and asexual spores (conidia). Perennial sporocarps or fruiting bodies are often found on the roots of windthrown trees, stumps, logs and dead or diseased trees. The anamorphic form (*Spiniger meineckellum* (A. Olson) Stalpers) is occasionally observed on broken roots, in insect galleries and on the stumps of recently felled trees under moist conditions (Woodward et al., 1998). This hymenomycete fungus, which causes root and butt rot in conifer temperate forests, is responsible for high economic loss in the forestry sector of Europe, Asia and North America. In Europe alone, *Heterobasidion* infections in forest stands are estimated to cause economic losses of up to 800 million euros annually (Asiegbu et al., 2005). *Heterobasidion annosum* has occasionally been recorded on *Pinus sylvestris* (Benito Martínez, 1943) and *Pinus nigra* in Spain (Oliva et al., 2008). The presence of this fungus on *Pinus pinaster* in association with forest decline is a very recent finding (Prieto-Recio et al., 2012). This pathogen is very aggressive on Pinaceae; however, some pine species such as *Pinus pinea* and *Pinus halepensis* have shown lesser susceptibility (Scirè et al., 2011).

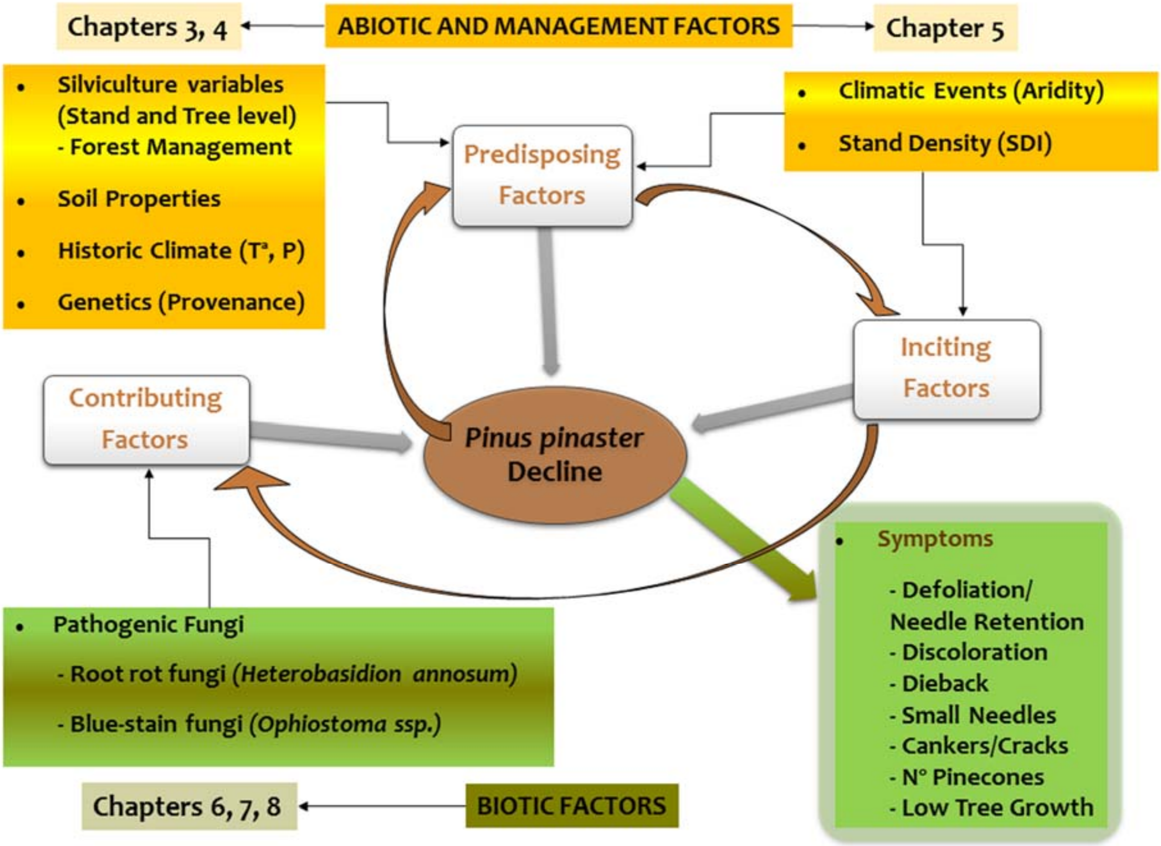
Forest pathology involves problems arising from the damage that disease inflicts on trees (Boa, 2003). Early detection of the problem, identification of the pathogens involved with morphological and molecular tools, and assessment of populations of harmful agents are therefore essential in order to protect forests and the associated products and services (Muñoz et al., 2007).

Management of forest decline

Forest decline is a complex problem, and in order to manage and control it, it is necessary to take into account the interaction of the all involved factors, both biotic and abiotic (Manion, 1991). The classical methods of management and control are based on the fight against biotic factors such as pathogenic fungi, parasitic plants and pests. The main concepts of plant disease control are focused on prevention, exclusion, eradication, protection, resistance and treatment (Tainter and Baker, 1996). The best way of addressing the problem of decline would be with an integrated management approach of both silvicultural treatment and biological control together, due to the advantages it has over the use of chemicals. Nevertheless, decision-making requires estimation and appraisal of the potential impacts (both economic and ecological) of forest decline, taking into account that management practice recommendations will differ among diseases, types of decline and between managed and natural forests.

Silvicultural control is focussed on the prevention of disease establishment and the reduction of the dispersion of diseases that reduce the amount of inoculum (Tainter and Baker, 1996). Through sustainable forest management it is possible to maintain the health and vigour of trees and conserve their genetic resources. Thinnings that remove dead or damaged trees are a common practice to improve the forest health status. But it should be carefully done because some root rot pathogens as *Heterobasidion* spp. colonize the stumps of freshly cut trees, allowing the pathogen to establish itself. In these cases, stump surfaces should be totally removed or treated with biological control methods (Oliva et al., 2010). Biological control can be define as the reduction of a disease through one or more organisms other than man (Cook and Baker, 1983). This is a friendly alternative method to combat forest pests and diseases, especially considering growing public concern over the potentially harmful effects that some chemical pesticides pose to human health and the environment (Omkar, 2016). Nevertheless, to perform an integrated management approach, the environmental factors that are influencing decline processes must be taken into account. Once these environmental factors and the biotic agents are identified, then we can focus silviculture management to prevent by increasing stand resilience and resistance, and biological control to treat and eradicate the problem.

Conceptual model of the thesis



References

Alía Miranda, R., García del Barrio, J.M., Iglesias Sauce, S., Mancha Núñez, J.A., de Miguel y del Ángel, J., Nicolás Peragón, J.L., Pérez Martín, F., Sánchez de Ron, D., 2009. Regiones de procedencia de especies forestales en España.

Alía, R., Martín, S., 2003. EUFORGEN: Technical guidelines for genetic conservation and use for Maritime pine (*Pinus pinaster*), in: International Plant Genetic Resources Institute. p. 6.

Alía, R., Martín, S., De Miguel, J., Galera, R., Agúndez, D., Gordo, J., Salvador, L., Catalán, G., Gil, L., 1996. Regiones de procedencia, *Pinus pinaster* Aiton., Ministerio de Medio Ambiente, Organismo Autónomo Parques Nacionales (DGCONA), Madrid.

Alia, R., Moro, J., Denis, J.B., 2001. Ensayos de procedencias de *Pinus pinaster* Ait. en el centro de España: resultados a la edad de 32 años. Investig. Agrar. Sist. y Recur. For. 10, 333–354.

Allen, C.D., Macalady, A.K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D.D., Hogg, E.H. (Ted), Gonzalez, P., Fensham, R., Zhang, Z., Castro, J., Demidova, N., Lim, J.-H.H., Allard, G., Running, S.W., Semerci, A., Cobb, N., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. For. Ecol. Manage. 259, 660–684. doi:10.1016/j.foreco.2009.09.001

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1–9.

Área de Inventario y Estadísticas Forestales (AIEF), 2013. Red de Seguimiento a gran Escala de Daños en los Bosques (Red de Nivel I). Manual de Campo. Ministerio de Agricultura, Alimentación y Medio Ambiente.

Arnold, A.E., 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol. Rev. 21, 51–66.

Asiegbu, F.O., Adomas, A., Stenlid, J., 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. s. l. Mol. Plant Pathol. 6, 395–409. doi:10.1111/j.1364-3703.2005.00295.x

Ayres, M.P., Lombardero, M.J., 2000. Assessing the consequences of global change for forest disturbance from herbivores and patogens. Sci. Total Environ. 262, 263–286.

Benito Garzón, M., Sánchez de Dios, R., Sainz Ollero, H., 2009. Effects of climate change on the distribution of Iberian tree species. Appl. Veg. Sci. 11, 169–178. doi:10.3170/2008-7-18348

Benito Martínez, J., 1943. El *Fomes annosus* Fr. (*Trametes radiciperda* Hart.) en España. An. del Jardín Botánico Madrid 3, 23–49.

Blanco, E., Casado, M., Costa, M., Escribano, R., García-Antón, M., Génova, M., Gómez-Manzaneque, F., Moreno, J., Morla, C., Regato, P., Sainz-Ollero, H., 1997. Los bosques ibéricos. Una interpretación geobotánica. Editorial Planeta, Barcelona.

Blank, L.W., 1985. A New Type of Forest Decline in Germany. Nature 314, 311–314.

Boa, E., 2003. Reconocimiento e interpretación de síntomas y daños. Servicio de Diagnóstico y Asesoría CABI Bioscensce. Egham, Surrey, Reino Unido. Organización de las Naciones

Unidas para la Agricoltura y la Alimentación- FAO; Organismo Internacional Regional de Sanidad Agropecuaria – OIRSA. Traducido por Dinora Alicia V.

Boisvenue, C., Running, S.W., 2006. Impacts of climate change on natural forest productivity - evidence since the middle of the 20th century. Glob. Chang. Biol. 12, 862–882. doi:10.1111/j.1365-2486.2006.01134.x

Bravo-Oviedo, A., Sterba, H., del Río, M., Bravo, F., 2006. Competition-induced mortality for Mediterranean *Pinus pinaster* Ait. and *P. sylvestris* L. For. Ecol. Manage. 222, 88–98. doi:10.1016/j.foreco.2005.10.016

Cailleret, M., Heurich, M., Bugmann, H., 2014. Reduction in browsing intensity may not compensate climate change effects on tree species composition in the Bavarian Forest National Park. For. Ecol. Manage. 328, 179–192.

Camarero, J.J., Bigler, C., Linares, J.C., Gil-Pelegrin, E., 2011. Synergistic effects of past historical logging and drought on the decline of Pyrenean silver fir forests. For. Ecol. Manage. 262, 759–769.

Canales, J., Avila, C., Canovas, F.M., 2011. A Maritime pine antimicrobial peptide involved in ammonium nutrition. Plant Cell Environ. 34, 1443–1453.

Chakraborty, S., Tiedemann, A. V, Teng, P.S., 2000. Climate change: potential impact on plant diseases. Environ. Pollut. 108, 317–326.

Cook, E.R., Johnson, A.H., 1989. Climate Change and Forest Decline - a Review of the Red Spruce Case. Water Air Soil Pollut. 48, 127–140.

Cook, R.J., Baker, K.F., 1983. The Nature and Practice of Biological Control of Plant Pathogens. APS Press.

Costa, A., Pereira, H., Madeira, M., 2010. Analysis of spatial patterns of oak decline in cork oak woodlands in Mediterranean conditions. Ann. For. Sci. 67, 204. doi:10.1051/forest/2009097

Deacon, J.W., 2013. Fungal biology. John Wiley & Sons.

DellaSala, D.A., Olson, D.M., Barth, S.E., Crane, S.L., Primm, S.A., 1995. Forest health: moving beyond rhetoric to restore healthy landscapes in the Inland Northwest. Wildl. Soc. Bull. 23, 346–356.

Desprez-Loustau, M.-L., Marcais, B., de la Rocque, S., 2006. Climatic factors and diseases in natural ecosystems and agroecosystems. Biofutur 37–40.

DGCONA, 2002. Mapa Forestal Español. Base de datos de la naturaleza, Ministerio de Medio Ambiente. Madrid.

Dobbertin, M., Brang, P., 2001. Crown defoliation improves tree mortality models. For. Ecol. Manage. 141, 271–284.

Duchesne, L., Ouimet, R., Morneau, C., 2003. Assessment of sugar maple health based on basal area growth pattern. Can. J. For. Res. Can. Rech. For. 33, 2074–2080.

Eichhorn, J., Roskams, P., Ferretti, M., Mues, V., Szepesi, A., Durrant, D., 2010. Manual Part IV: Visual Assessment of Crown Condition and Damaging Agents, in: Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg, p. 49.

EU commission, 2011. State of Europe's Forests 2011. doi:10.1007/s13398-014-0173-7.2

Galiano, L., Martínez-Vilalta, J., Lloret, F., Martinez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor Decline of Scots Pine in the Pyrenees and Potential Vegetation Change by the Expansion of Co-occurring Oak Species. Ecosystems 13, 978–991. doi:10.1007/s10021-010-9368-8

Garbelotto, M., Pautasso, M., 2012. Impacts of exotic forest pathogens on Mediterranean ecosystems: four case studies. Eur. J. Plant Pathol. 133, 101–116.

Gea-Izquierdo, G., Martín-Benito, D., Cherubini, P., Cañellas, I., 2009. Climate-growth variability in *Quercus ilex* L. west Iberian open woodlands of different stand density. Ann. For. Sci. 66, 802–802. doi:10.1051/forest/2009080

Gea-Izquierdo, G., Viguera, B., Cabrera, M., Canellas, I., 2014. Drought induced decline could portend widespread pine mortality at the xeric ecotone in managed mediterranean pine-oak woodlands. For. Ecol. Manage. 320, 70–82.

Giorgi, F., Lionello, P., 2008. Climate Change Projections for the Mediterranean Region. Glob. Planet. Change 63, 90–104.

Grylls, B., Seifert, K.A., 1999. A synoptic key to species of *Ophiostoma, Ceratocystis* and *Ceratocystiopsis*, in: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity. pp 261–268.

Hampe, A., Petit, R.J., 2005. Conserving biodiversity under climate change: the rear edge matters. Ecol. Lett. 8, 461–7. doi:10.1111/j.1461-0248.2005.00739.x

Hansen, A.J., Neilson, R.R., Dale, V.H., Flather, C.H., Iverson, L.R., Currie, D.J., Shafer, S., Cook, R., Bartlein, P.J., 2001. Global change in forests: Responses of species, communities, and biomes. Bioscience 51, 765–779.

Helfer, S., 2014. Rust fungi and global change. New Phytol. 201, 770–780.

Hennon, P.E., D'Amore, D.V., Schaberg, P.G., Wittwer, D.T., Shanley, C.S., 2012. Shifting Climate, Altered Niche, and a Dynamic Conservation Strategy for Yellow-Cedar in the North Pacific Coastal Rainforest. Bioscience 62, 147–158.

Hennon, P.E., McWilliams, M.G., 1999. Decline symptoms do not develop with grafting from dying yellow-cedar. Can. J. For. Res. Can. Rech. For. 29, 1985–1988.

Hennon, P.E., Wittwer, D.T., D'Amore, D.V., Lamb, M., 2009. Yellow-cedar decline: Key landscape features and snow modeling of a climate-induced forest decline on a dormant volcano. Phytopathology 99, S52–S52.

Hertel, G., 1988. Proceedings of the US/FRG research symposium: effects of atmospheric pollutants on the spruce-fir forests of the Eastern United States and the Federal Republic of Germany. 1987 October 19-23; Burlington, VT., in: USDA For. Serv., Gen. Tech. Rep. NE-120.

ICP Forest, 2015. Forest Condition in Europe: Technical Report.

Innes, J.L., 1993. Forest health: its assessment and status. Cab International.

Jactel, H., Nicoll, B.C., Branco, M., Gonzalez-Olabarria, J.R., Grodzki, W., Langstrom, B., Moreira, F., Netherer, S., Orazio, C., Piou, D., Santos, H., Schelhaas, M.J., Tojic, K., Vodde, F., 2009.

The influences of forest stand management on biotic and abiotic risks of damage. Ann. For. Sci. 66.

Jankowiak, R., Rossa, R., Bilanski, P., 2007. Contribution to pathogenicity of three blue-stain fungi associated with the pine sawyer beetle (*Monochamus galloprovincialis*) (*Coleoptera: Cerambycidae*) to Scots pine in Poland. Phytopathol. Pol. 46, 6.

Kliejunas, J.T., Geils, B.W., Micales, J., Goheen, E.M., Hennon, P., Kim, M., Kope, H., Stone, J., Sturrock, R., Frankel, S.J., 2009. Review of literature on climate change and forest diseases of western North America 60.

Kolb, T.E., Wagner, M.R., Covington, W.W., 1994. Concepts of forest health: Utilitarian and ecosystem perspectives. Society of American Foresters.

Korhonen, K., Stenlid, J., 1998. Biology of *Heterobasidion annosum*, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*, Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK., pp. 43–70.

LaSalle, V.J., 1998. End the forest health debate. J. For. 96, 1–50.

Linares, J.C., Camarero, J.J., Carreira, J.A., 2009. Interacting effects of changes in climate and forest cover on mortality and growth of the southernmost European fir forests. Glob. Ecol. Biogeogr. 18, 485–497.

Lindner, M., Maroschek, M., Netherer, S., Kremer, A., Barbati, A., Garcia-Gonzalo, J., Seidl, R., Delzon, S., Corona, P., 2009. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For. Ecol. Manage. 259, 698–709. doi:10.1016/j.foreco.2009.09.023

Manion, P.D., 1991. Tree Disease Concepts, Prentice-Hall.

Manrique, E., Fernandez-Cancio, A., 2000. Extreme climatic events in dendroclimatic reconstructions from Spain. Clim. Change 44, 123–138.

Masuya, H., Kaneko, S., Yamaoka, Y., 2003. Comparative virulence of blue-stain fungi isolated from Japanese red pine. J. For. Res. 8, 83–88. doi:10.1007/s103100300009

Misson, L., Nicault, A., Guiot, J., 2003. Effects of different thinning intensities on drought response in Norway spruce (*Picea abies* (L.) Karst.). For. Ecol. Manage. 183, 47–60.

Moreno, G., Cubera, E., 2008. Impact of stand density on water status and leaf gas exchange in *Quercus ilex*. For. Ecol. Manage. 254, 74–84.

Muñoz, L.C., Perez, F. V., Cobos, S.P., Hernandez, A.R., Sanchez, G., 2007. Sanidad forestal. Guía en imágenes de plagas, enfermedades y otros agentes presentes en los bosques. Madrid – Ediciones Mundi-Prensa.

Nicolás, A., Gandullo, J.M., 1967. Ecología de los pinares españoles I: *Pinus pinaster* Ait. I.F.I.E., Madrid.

Oliva, J., Colinas, C., 2007. Decline of silver fir (*Abies alba* Mill.) stands in the Spanish Pyrenees: Role of management, historic dynamics and pathogens. For. Ecol. Manage. 252, 84–97.

Oliva, J., Ibarra, N., Colinas, C., Martín, E., 2008. Detección molecular de *Heterobasidion annosum s. s.* en claras de *Pinus nigra* en el Pirineo Aragonés. Boletín Sanid. Veg. Plagas. 34, 415–416.

Oliva, J., Thor, M., Stenlid, J., 2010. Long-term effects of mechanized stump treatment against *Heterobasidion annosum* root rot in *Picea abies*. Can. J. For. Res. Can. Rech. For. 40, 1020–1033.

Omkar, 2016. Ecofriendly Pest Management for Food Security. Elsevier Science.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with decline of longleaf pine in the southeastern United States. Plant Soil 217, 145–150.

Otrosina, W.J., Garbelotto, M., 2010. *Heterobasidion occidentale* sp nov and *Heterobasidion irregulare* nom. nov.: A disposition of North American Heterobasidion biological species. Fungal Biol. 114, 16–25.

Payette, S., Fortin, M.J., Morneau, C., 1996. The recent sugar maple decline in southern Quebec: Probable causes deduced from tree rings. Can. J. For. Res. Can. Rech. For. 26, 1069–1078.

Piou, D., Lieutier, F., 1989. Symptomatological observations and possible roles of *Ophiostoma minus* and *Tomicus piniperda* in the decline of Scots pine in the forest of Orleans. Ann. des Sci. For. 46, 39–53.

Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. doi:10.1016/j.foreco.2015.07.033

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770. doi:10.1094/PDIS-10-11-0890-PDN

Rehfeldt, G.E., Ferguson, D.E., Crookston, N.L., 2009. Aspen, climate, and sudden decline in western USA. For. Ecol. Manage. 258, 2353–2364. doi: 10.1016/j.foreco.2009.06.005

Rodríguez-Soalleiro, R., Lucas, J.A., Alejano, R., del Río, M., Torres, E., Cantero, A., Serrada, R.J., 2008. Selvicultura de *Pinus pinaster* subespecie *mesogeensis*, in: Montero, G., Serrada, R., Reque, J.A. (Eds.), Compendio de Selvicultura Aplicada En España. INIA y Fundacion del Conde del Valle de Salazar, pp. 399–430.

Roe, G.H., Baker, M.B., 2007. Why is climate sensitivity so unpredictable? Science (80-.). 318, 629–632.

Rozas, V., Zas, R., Garcia-Gonzalez, I., 2011. Contrasting effects of water availability on *Pinus pinaster* radial growth near the transition between the Atlantic and Mediterranean biogeographical regions in NW Spain. Eur. J. For. Res. 130, 959–970.

Sánchez Peña, G., Economou, A., Beccu, E., Canu, G., Cocco, S., Bussotti, F., Cenni, E., Cozzi, A., Ferretti, M., Conceicao, M., 1994. Especies Forestales Mediterráneas. Guía para la Evaluación de Copas., CEC-UN/ECE, Brussels, Geneva.

Scherm, H., 2004. Climate change: can we predict the impacts on plant pathology and pest management? Can. J. Plant Pathol. 26, 267–273.

Scirè, M., Motta, E., D’Amico, L., 2011. Behaviour of *Heterobasidion annosum* and *Heterobasidion irregulare* isolates from central Italy in inoculated *Pinus pinea* seedlings. Mycol. Prog. 10, 85–91. doi:10.1007/s11557-010-0678-1

Servicio de Sanidad Forestal y Equilibrios Biológicos (SSF), 2000. Manual Red de Nivel II. Red de Parcelas Permanentes para el Seguimiento Intensivo y Continuo de los Ecosistemas Forestales. Ministerio de Agricultura, Alimentación y Medio Ambiente.

Solheim, H., Langstrom, B., Hellqvist, C., 1993. Pathogenicity of the blue-stain fungi *Leptographium wingfieldii* and *Ophiostoma minus* to Scots pine - Effect of Tree Pruning and Inoculum Density. Can. J. For. Res. 23, 1438–1443.

Spiecker, H., 2003. Silvicultural management in maintaining biodiversity and resistance of forests in Europe - temperate zone. J. Environ. Manage. 67, 55–65.

Staebler, R.N., 1994. Forest health: everyone wants it, but what is it? J. For. 92, 5–5.

Stocker, T.F., Qin, D., Plattner, G.K., Alexander, L. V, Allen, S.K., Bindoff, N.L., Breon, F.M., Chuch, J.A., Cubasch, U., Emori, S., Forster, P., Friedlingstein, P., Gillet, N., Gregory, J.M., Hartmann, D.L., Jansen, E., Kirtman, B., Knutti, R., Krishna Kumar, K., Lemke, P., Marotzke, J., Masson-Delmotte, V., Meehl, G.A., Mokhov, I.I., Piao, S., Ramaswamy, V., Randall, D., Rhein, M., Rojas, M., Sabine, C., Shindell, D., Talley, L.D., Vaughan, D.G., Xie, S.-P., 2013. Technical summary. Climate Change 2013. Phys. Sci. Basis. Contrib. Work. Gr. I to Fifth Assess. Rep. Intergov. Panel Clim. Chang.

Sturrock, R.N., Frankel, S.J., Brown, A. V, Hennon, P.E., Kliejunas, J.T., Lewis, K.J., Worrall, J.J., Woods, A.J., 2011. Climate change and forest diseases. Plant Pathol. 60, 133–149.

Tainter, F.H., Baker, F.A., 1996. Principles of Forest Pathology. New York, NY: John Wiley and Sons, Inc.

Thomas, F.M., Blank, R., Hartmann, G., 2002. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. 32, 277–307.

Warren, W.A., 2007. What is a healthy forest?: Definitions, rationales and lifeworld. Soc. Nat. Resour. 20, 99–117.

Wingfield, M.J., Seifert, K.A., Webber, J.F., 1993. *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity. American Phytopathological Press, St. Paul, Minnesota, U.S.A.

Woodward, S., Stenlid, J., Karjalainen, R. Hütterman, A., 1998. *Heterobasidion annosum*, Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK.

Worrall, J.J., Marchetti, S.B., Egeland, L., Mask, R. a., Eager, T., Howell, B., 2010. Effects and etiology of sudden aspen decline in southwestern Colorado, USA. For. Ecol. Manage. 260, 638–648. doi:10.1016/j.foreco.2010.05.020

Chapter 2: Objectives

The main objective of this work is to develop a better understanding of the influence of both biotic (pathogens) and abiotic factors, including anthropogenic ones (climate, soil properties and forest management), on *Pinus pinaster* decline in the Iberian Peninsula, shedding light upon the role each factor plays in this process (i.e. if it acts as a predisposing, inciting or contributing factor).

Thus, the specific objectives of this work were:

For abiotic and management factors:

1- To disentangle the role of predisposing, inciting and contributing factors in Mediterranean Maritime pine decline in the Iberian Peninsula, especially the associations between climate, soil properties and forest management. (**Chapter 3**).

2- To determine the factors involved in *Pinus pinaster* decline by characterizing the study plots and constructing a Multinomial Logistic Regression model to predict the health status at the individual tree level. (**Chapter 4**).

3- To understand alterations in growth of *Pinus pinaster* by considering different phases of decline in relation to the changes in water availability during the last decades using dendrochronological methods. (**Chapter 5**).

For biotic factors:

4- To isolate and identify the root rot pathogen *Heterobasidion annosum* involved in Maritime pine decline (*Pinus pinaster)* in Spain. (**Chapter 6**).

5- To identify the pathogens associated with *Pinus pinaster* decline and detect the most strongly infected zones in relation to tree health status and climate conditions. (**Chapter 7**).

6- To explore the pathogenicity and virulence of Spanish isolates of *Heterobasidion annosum* in two-year-old *Pinus pinaster* seedlings. (**Chapter 8**).

PART II: Abiotic and Management Factors

Chapter 3: Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula

Abstract

Forest decline may be a complex disease caused by the interaction of a number of interchangeable factors (both abiotic and biotic), leading to gradual deterioration of the forest. These factors have been classified as predisposing, inciting or contributing factors. *Pinus pinaster* Ait. is one of the most important forest species in Spain because of its protective value and the high timber yields obtained. Maritime pine decline has been observed in several forests in the center of the Iberian Peninsula, manifested as transparency at the crown, small needles, foliage discoloration and premature tree death. The aim of this study was to unravel the role of predisposing, inciting and contributing factors in Mediterranean Maritime pine decline in Spain. Various parameters were measured in 27 circular plots of radius 15 m in natural stands of *Pinus pinaster* ssp. *mesogeensis*. The data obtained indicated better forest health status in pure stands, in which thinning has been carried out, than in unthinned mixed stands. High values of total height and spring precipitation showed better forest health, whereas high tree density was associated with the poorest forest health status. The results suggest that predisposing factors such as competition and water deficit have triggered the decline of *Pinus pinaster*.

Keywords: *Maritime pine, natural stands, predisposing, inciting and contributing factors, NMDS.*

Introduction

Forest decline was first described in Central Europe and North America in the 1980s and since then has been widely reported around the world (Blank, 1985; Cook and Johnson, 1989; Hertel, 1988). This decline can be defined as a complex disease caused by the interaction of a number of interchangeable factors, both abiotic and biotic (Thomas et al., 2002), leading to gradual deterioration of the forest and, ultimately, the death of trees (Manion, 1991). These factors have been classified as predisposing, inciting or contributing factors. In the long term, climate, site, management, stand age and genetic predisposition may not cause overt problems but may predispose the trees to suffering decline (Hennon et al., 2009). On the other hand, some inciting factors cause severe, short-term damage, for example abiotic climatic events such as frost, windstorms, floods and drought. The detriment produced by competition for resources also causes trees to enter a state of decline at a given time. The trees may then recover or enter a progressive state of decline (Hennon et al., 2012). Although damage caused by inciting factors may be reversed relatively quickly, if both inciting and predisposing factors act together, the recovery is much slower. Finally, contributing factors like root pathogenic fungi, parasitic plants and opportunistic insects may also contribute to the further weakening and subsequent death of trees (Hennon and McWilliams, 1999). In short, it is thought that a combination of both abiotic and biotic factors (including anthropogenic factors) may interact to cause the phenomenon of forest decline (Arnold, 2007; Manion, 1991; Thomas et al., 2002).

An increase in average global temperatures by up to 4.8°C above current levels and a decrease in mean precipitation in many mid-latitude arid and semi-arid regions is predicted to occur in the 21st century (Stocker et al., 2013). Moreover, climatic extremes may occur even more frequently in hotspots such as European Mediterranean areas (Giorgi and Lionello, 2008). Therefore, taking into account that some forest decline in Mediterranean areas has been recently associated with changing climatic conditions, such as drought and outbreaks of pests and disease, it is expected that climate change will trigger the occurrence of forest decline in the coming years (Allen et al., 2010; Ayres and Lombardero, 2000; Desprez-Loustau et al., 2006; Helfer, 2014; Jactel et al., 2012; Pautasso et al., 2012; Sturrock et al., 2011).

Maritime pine extends naturally through the western Mediterranean Basin, southern Europe, and Africa, as well as along the Atlantic coast in Portugal, Spain, and France (Alía and Martín, 2003). On the Iberian Peninsula, the spread point seems to be located in the “Serranía de Cuenca” (Gil et al., 1990), although the Maritime pine species has been widely used in reforestation throughout the Iberian Peninsula, particularly in its center area (Solis, 2003). Its current spontaneous distribution is through territories that have been highly influenced by human beings since antiquity. Maritime pine presents a high genetic variability, resulting in different growth patterns, forms of trees, wood quality, etc. according to the provenance (Alía et al., 2001).

Pinus pinaster Ait., which is the most widespread conifer in Spain, is subdivided into two subspecies, *Pinus pinaster* ssp. *atlantica* H. de Vill and *Pinus pinaster* ssp. *mesogeensis* Fieschi & Gaussen. Moreover, three different types of *Pinus pinaster* ssp. *mesogeensis* forests have been differentiated in Spain according to functional considerations. The first group comprises pines derived from natural regeneration in the Castilian plateau and historically used for resin extraction. The second group, typical of the Central System and the Iberian System, consists of pines growing in mountainous areas and destined for timber production. The third group includes pine forests in the Mediterranean Coastal Area, where the main function is the protection and creation of mixed stands with hardwoods, combined with low levels of logging (Rodríguez-Soalleiro et al., 2008). The species is mainly distributed on the northern Spanish plateau up to an elevation of 1500 m, preferably on sandy, stony and well drained soils in areas where the weather is characterized by seasonal irregular rainfall and high temperatures in summer.

The ecological importance of Mediterranean pine forests because of their role in conservation and maintaining biodiversity is well known. Mediterranean pine forests, particular those comprising *Pinus pinaster* Ait., are classified as natural habitats of community interest and their conservation requires the designation of special areas of conservation referred to as “Mediterranean pine forests with endemic Mesogean pines” (EU Council Directive 92/43/EEC on the conservation of natural habitats and wild fauna and flora). However, the decline of other Mediterranean pine forests, such as *Pinus sylvestris*, *Pinus nigra* and *Pinus halepensis*, has recently been reported to be associated with adverse climatic conditions (Girard et al., 2012; Linares and Tíscar, 2010; Navarro-Cerrillo et al., 2007; Sánchez-Salguero et al., 2012a, 2013). Likewise, over the last few years, severe *Pinus pinaster* decline has been observed in several forests in the center of the Iberian Peninsula (Álvarez et al., 2015; Madrigal-González and Zavala, 2014; Prieto-Recio et al., 2012; 2014). Diseased trees characteristically display defoliation, unusual transparency and discoloration at the crown, die-back, presence of microphylls, premature senescence and early tree death.

Models have been developed to predict mortality or survival of several conifer species (e.g. Mediterranean Maritime pine, Scots pine, Monterey pine and Silver fir) through forest structure (Bravo-Oviedo et al., 2006; Dieguez-Aranda et al., 2005; Gea-Izquierdo et al., 2014; González et al., 2004), climatic variables (Reich and Oleksyn, 2008; Ruiz-Benito et al., 2013) and soil properties (Cailleret et al., 2014; Galiano et al., 2010). However, little attention has been paid to the potential interactions between these factors, and further research is therefore needed to study the combined effects.

As already mentioned, numerous stands of *Pinus pinaster* have shown symptoms of decline in the center of the Iberian Peninsula in recent years, and this may compromise the future of these forests of great ecological and economic importance. We hypothesized that abiotic and anthropogenic factors such as forest management (i.e. stand structure), climate, and soil properties play a key role in *Pinus pinaster* decline. The aim of the work reported here was to research into the role of predisposing, inciting and contributing factors in Mediterranean Maritime pine decline in Spain. For this, a new three-step approach was used. This approach was based on principal component analysis to avoid multicollinearity problems, then proceeded to focusing on the best correlations, and, lastly, explored the relationships between forest health conditions and the explanatory factors using non-metric multidimensional scaling analysis.

Material and methods

Site description and sampling procedure

The present study was carried out in the center of the Iberian Peninsula (Castile and Leon, NW Spain). Twenty-seven circular plots of radius 15 m were selected and installed in twenty-seven natural stands of *Pinus pinaster* ssp. *mesogeensis* covering most of the distribution of the provenance regions in the center of the Iberian Peninsula (Table 3.1 and Figure 3.1). Ten of the stands belong to the Permanent Sample Plots Network of the Sustainable Forest Management Research Institute (University of Valladolid-INIA, Spain) and the other seventeen belong to the Spanish National Forest Inventory (NFI) network. As it is forbidden to mark and sample trees within NFI plots, new plots were established as close as possible to the original NFI plots (ca. 100 – 500 metres). Sampling was grouped into 7 zones according to the provenance and province. There was one zone per each provenance, except for Meseta Castellana. This provenance region is widespread throughout three provinces, each with different management practices and different climatic and soil conditions (see Figure 3.1).

Table 3.1. Location of the study sites and *Pinus pinaster* provenance codes (Alia et al.,1996).

Zone	Provenance	P. Code	Province	Location	X Coordinate	Y Coordinate
1	Meseta Castellana	P8	Burgos	Quintana del Pidio	3° 44’ 48”	41° 46’ 18”
1	Meseta Castellana	P8	Burgos	La Horra 2	3° 52’ 00”	41° 45’ 46”
1	Meseta Castellana	P8	Burgos	Villalba de Duero	3° 46’ 09”	41° 42’ 05”
1	Meseta Castellana	P8	Burgos	La Horra 1	3° 51’ 18”	41° 44’ 44”
1	Meseta Castellana	P8	Burgos	Gumiel de Izan	3° 40’ 42”	41° 43’ 40”
1	Meseta Castellana	P8	Burgos	Aranda de Duero	3° 43’ 26”	41° 42’ 01”
4	Meseta Castellana	P8	Segovia	Iscar	4° 29’ 06”	41° 22’ 29”
4	Meseta Castellana	P8	Segovia	Cuellar	4° 16’ 52”	41° 22’ 47”
4	Meseta Castellana	P8	Segovia	Arroyo de Cuellar	4° 20’ 23”	41° 21’ 13”
6	Meseta Castellana	P8	Soria	Quintana Redonda	2° 38’ 25”	41° 37’ 58”
6	Meseta Castellana	P8	Soria	Tardelcuende	2° 37’ 14”	41° 35’ 01”
6	Meseta Castellana	P8	Soria	Matamala de Almazán	2° 36’ 49”	41° 30’ 07”
6	Meseta Castellana	P8	Soria	Hortezuela	2° 50’ 58”	41° 29’ 56”
6	Meseta Castellana	P8	Soria	Tajueco	2° 49’ 42”	41° 32’ 54”
6	Meseta Castellana	P8	Soria	Bayubas de arriba	2° 55’ 22”	41° 33’ 40”
7	Montaña de Soria - Burgos	P9	Burgos	Salas de los Infantes	3° 18’ 37”	42° 2’ 36”
7	Montaña de Soria - Burgos	P9	Burgos	Gete	3° 17’ 28”	41° 56’ 48”
7	Montaña de Soria - Burgos	P9	Burgos	Huerta del Rey	3° 21’ 36”	41° 50’ 22”
3	Sierra de Gredos	P6	Ávila	El Hornillo 1	5° 7’ 22”	40° 14’ 43”
3	Sierra de Gredos	P6	Ávila	El Hornillo 3	5° 7’ 33”	40° 15’ 34”
3	Sierra de Gredos	P6	Ávila	El Arenal	5° 4’ 45”	40° 14’ 54”
2	Sierra de Oña	P3	Burgos	Pino de Bureba	3° 25’ 46”	42° 42’ 08”
2	Sierra de Oña	P3	Burgos	Aguas Candidas	3° 29’ 11”	43° 43’ 10”
2	Sierra de Oña	P3	Burgos	Trespaderne	3° 24’ 21”	42° 48’ 06”
5	Sierra del Teleno	P2	León	Pinilla de la Valdería	6° 5’ 36”	42° 13’ 10”
5	Sierra del Teleno	P2	León	Castrocontrigo	6° 12’ 59”	42° 12’ 13”
5	Sierra del Teleno	P2	León	Nogarejas	6° 9’ 55”	42° 12’ 13”

Note: Sampling was grouped into 7 zones according to the provenance and province.

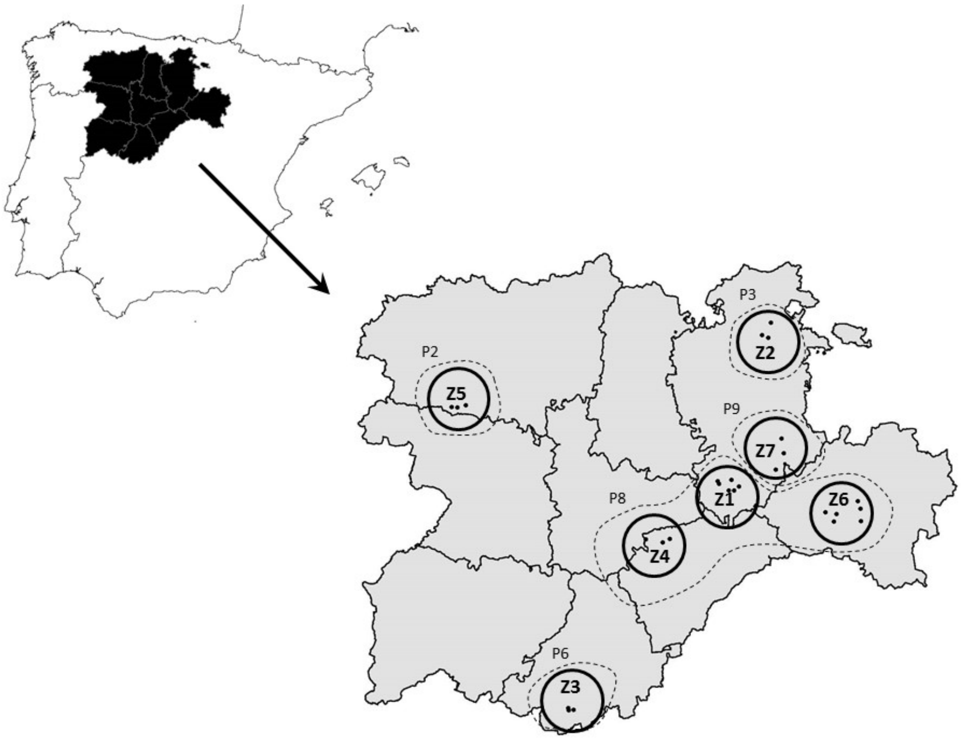


Figure 3.1. Geographical location of the study stands within the seven different zones (continuous circles) and provenance regions (discontinuous curves) See Table 3.1.

The diameter at breast height, total height, and crown height as well as crown area were measured for all trees within each 15 m radius plot (a total of 1208 trees). At the same time, forest health variables were assessed in 24 trees per plot (a total of 648 trees) (Table 3.2). In particular, six trees per quadrant closest to the center of the plot were chosen in accordance with Level I of the European network methodology (SPCAN-DGB, 2007).

Forest health was evaluated by visual assessment of crown conditions, such as crown defoliation and discoloration and live crown ratio. The variables were visually estimated, by only one operator, in twenty classes of 5 % interval width, based on an absolute reference tree (Sánchez et. al., 1994), according to Level I of the European network methodology (Eichhorn et al. 2010). Crown defoliation has been widely used as an indicator of forest health (Dobbertin and Brang, 2001); however, defoliation can vary widely as a result of insect outbreaks and may not by itself be a good indicator of cumulative effects. Furthermore, taking into account that factors involved in mortality may differ between sites, the use of different vitality indicators is highly recommended (Cailleret et al., 2014). Hence, other phytosanitary variables were also assessed, such as the presence of cankers, cracks in the bark (caused by wood compression), terminal bud damage, dieback, presence of microphylls, needle retention, number of pine cones and percentage of healthy, damaged and dead trees (Table 3.2).

Social classes (dominant, predominant, codominant and suppressed) were assessed in consonance with the Level I manual of the European network methodology (SPCAN-DGB, 2007). On the other hand, the number of stumps, regeneration of *Pinus pinaster* (calculated as the number of seedlings within a radius of 5 m from the center of the plot) and the percentage of other tree species were estimated at stand level. Density, basal area and several indexes of interest for the study, such as Reineke, Hart, and slenderness ratio, were also estimated at stand level (Table 3.2). Silvicultural information was also recorded, including origin of the stand, type of forest (pure or mixed stands), forest age class, thinning, pruning and resin extraction.

Mineral soil samples were collected from the upper 30-cm soil layer in each stand (four samples per plot). The samples were mixed and homogenized to produce one composite sample per plot. The pH was determined potentiometrically with a pH meter, in a soil solution (1:2.5, soil/water). Organic matter was determined by the $K_2Cr_2O_7$ method. Total N was determined by Kjeldahl digestion. Soil available P was extracted by the Olsen procedure and determined photometrically by the molybdenum-blue method. Soil exchangeable cations (K^+, Na^{+2}, Ca^{+2} and Mg^{+2}) were extracted with ammonium acetate and determined by atomic absorption/emission spectroscopy. The soil Fe was extracted by the Diethylenetriaminepentaacetic acid (DTPA) extraction method (1:2 ratio), and the concentration was estimated by atomic absorption spectroscopy. Particle-size distribution was determined by the Bouyoucos method (hydrometer method), and the ISSS (International Society of Soil Science) classification was applied. The Cationic Exchange Capacity (CEC) was determined by Bascomb's method (i.e. the exchange cations were displaced by Ba ions, which were then displaced by Mg ions and the remaining concentration of Mg was determined by titration against EDTA), (Martín-García et al., 2012). The FAO classification for each soil was determined through the maps elaborated by the Technological Institute of Agriculture in Castile and Leon (ITACYL, http://suelos.itacyl.es/) (Table 3.2).

The environmental variables studied were elevation, aspect, mean temperature, minimum temperature, maximum temperature and mean precipitation (annual, spring, summer, autumn and winter) (Table 3.2). The climatic data were obtained from the Digital Climatic Atlas of the Iberian Peninsula (Ninyerola, 2005).

Table 3.2. Summary of forest health, stand level characteristics, soil properties, and climatic variables of the 27 plots under study, in relation to the study zone. Mean (±sd).

	All stands	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**
Forest health								
Crown defoliation (%)	36 (1.7)	43.1 (2.9)	29.2 (5.8)	32.9 (2.5)	30.6 (4.9)	41.9 (5)	34.4 (3.9)	34.3 (4.3)
Crown discoloration (%)	29.1 (1.8)	39.6 (3.3)	24.1 (2.4)	23.7 (3.9)	22.7 (2)	34.1 (3.1)	25.3 (4.2)	27.5 (5.1)
Live crown ratio (%)	86.8 (1.8)	77.6 (3.6)	87.5 (5.1)	86.7 (3.2)	96.7 (3.3)	84.7 (4)	90.8 (3.5)	88.6 (6.3)
Dieback (%)	9.1 (1.59)	15.3 (3)	15.3 (7.3)	4.2 (2.40)	2.8 (1.39)	16.7 (2.4)	3.5 (1.99)	5.6 (2.78)
Microphylls (%)	17.9 (4.3)	41.2 (10.6)	5.7 (3.65)	0 (0)	13.3 (7.9)	37.7 (17.6)	8.6 (3.58)	4.9 (3.06)
Cankers (%)	6.9 (1.75)	15.3 (2.8)	0 (0)	13.9 (11.9)	5.6 (2.78)	2.8 (1.39)	1.4 (1.38)	6.9 (1.39)
Cracks (%)	39.5 (5.4)	47.9 (8.7)	58.3 (12.5)	33.3 (33.3)	61.1 (7.7)	26.4 (13.2)	31.9 (10.7)	16.7 (6.4)
Number of pine cones (no)	4.6 (0.46)	2.9 (0.54)	5.7 (0.78)	5.6 (0.49)	6 (1.18)	8.6 (1.32)	3.5 (0.7)	3.1 (0.86)
Needle retention (years)	3.7 (0.13)	3.1 (0.27)	4.1 (0.22)	3.46 (0.27)	4.3 (0.19)	3.9 (0.36)	3.5 (0.21)	4.5 (0.21)
Dead trees (%)	9.4 (1.75)	15.3 (4.6)	6.9 (3.67)	6.9 (5.01)	0 (0)	12.5 (4.2)	8.3 (3.57)	11.1 (6.1)
Damaged trees (%)	28.2 (1.9)	41 (1.3)	26.4 (3.7)	22.2 (5.6)	27.8 (5.6)	27.8 (6.1)	22.2 (3.7)	23.6 (2.8)
Healthy trees (%)	62 (2.9)	43.7 (4.4)	66.7 (6.4)	70.8 (4.2)	69.4 (7.3)	59.7 (10)	69.4 (6.1)	65.3 (3.7)
Number of trees assessed	*648*	*144*	*72*	*72*	*72*	*72*	*144*	*72*
Stand level characterization								
Age (years)	66.9 (3.3)	53.3 (6.1)	61.7 (11.7)	85 (0)	75 (5)	50 (11.5)	77.5 (3.4)	68.3 (10.1)
Stumps (no)	10.5 (3.4)	2.8 (0.95)	0.67 (0.67)	15 (0.6)	4.3 (1.20)	1.3 (0.67)	11.3 (3.3)	44.7 (23.2)
Regeneration (trees/ha)	12968.2 (4539.2)	2228,2 (994,8)	382 (194.5)	7215 (5879.2)	31406.6 (29946.2)	24531.1 (9405.4)	22005.8 (12781.4)	4711 (2323.4)
Suppressed (%)	11.7 (2.1)	21.5 (1.6)	19.5 (3.8)	0 (0)	10.3 (8.1)	22 (6.3)	0.98 (0.98)	8.7 (3.8)
Other species (%)	2.4 (1.13)	7.7 (4.43)	1.5 (1.5)	0 (0)	2.6 (2.6)	0 (0)	0 (0)	2.2 (1.32)
Crown height (m)	5.4 (0.42)	4.9 (0.19)	5 (0.57)	8.2 (0.41)	8.7 (0.92)	4.6 (0.31)	2.9 (0.51)	6.3 (1.1)
Crown area (m^2)	21.4 (1.9)	16.4 (2.0)	15.9 (4.4)	35.8 (2.0)	33.7 (7.2)	15.6 (3.4)	20.8 (2.9)	17.7 (4.3)
Basal area (m^2/ha)	26.5 (2.0)	19 (2.1)	18 (1.2)	47.8 (2.0)	26.3 (2.0)	18.6 (1.7)	31.5 (2.4)	26.3 (4.8)

	All stands	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**
Density (trees/ha)	591.6 (61.5)	594.2 (129.8)	528.2(108.8)	448 (54.4)	386.7 (75.5)	702.6 (110.9)	648.4 (188.8)	773.4 (297.8)
Dm (cm)	29 (1.6)	23.6 (1.7)	24.1 (3.5)	43.6 (2.0)	34.7 (2.3)	20.5 (2.7)	32.4 (2.9)	26.2 (3.1)
Hm (m)	13.2 (0.8)	10 (0.6)	9.3 (1.83)	22.4 (0.3)	16.1 (0.6)	9.1 (0.85)	14.5 (0.8)	12.8 (1.3)
HoCorreg (m)	15.7 (0.6)	15.8 (1.0)	12.9 (1.8)	21.5 (0.8)	16.2 (1.3)	15.1 (2.2)	14.8 (0.5)	14.2 (1.7)
HART	31.3 (1.7)	34.3 (3.1)	41 (7.1)	20.8 (2.2)	31.5 (4.2)	33.6 (0.4)	28 (3.0)	30.5 (7.7)
REINEKE	521.7 (32.9)	406.9 (48.5)	381.4 (28.1)	813 (46.8)	489 (46.7)	413.8 (20.7)	613.9 (53.2)	556.4 (119.9)
CE	47.6 (1.4)	45.2 (2.0)	40.2 (2.7)	52.9 (2.9)	49.3 (5.9)	49.6 (3.0)	47.8 (4.1)	50 (2.1)
ICV	0.55 (0.03)	0.46 (0.03)	0.45 (0.04)	0.61 (0.02)	0.44 (0.07)	0.48 (0.05)	0.78 (0.04)	0.47 (0.04)
Number of trees measured	*1208*	*274*	*118*	*97*	*88*	*151*	*302*	*178*
Soil properties								
Slope (%)	8.5 (2.24)	2.8 (0.75)	21.3 (7.5)	24.3 (9.0)	0 (0)	0.67 (0.67)	1.7 (0.84)	21 (3.1)
Coarse fragments (%)	25.5 (4.2)	24.1 (11.1)	32.2 (13.2)	41.6 (3.2)	4.4 (1.20)	38.9 (11.5)	12.1 (4.8)	39.6 (16.0)
Sand (%)	85.0 (2.4)	93.7 (2.7)	73.5 (7.5)	79.2 (3.1)	95.5 (2.5)	71.5 (7.7)	92.5 (1.9)	72.5 (8.3)
Silt (%)	7.3 (1.21)	3.0 (1.34)	8.2 (3.71)	12.2 (1.67)	1.3 (0.28)	15.2 (3.9)	5.0 (1.63)	12.5 (4.5)
Clay (%)	7.8 (1.49)	3.2 (1.37)	18.3 (4.5)	8.6 (1.45)	3.2 (2.19)	13.3 (3.8)	2.4 (0.48)	15.1 (7.2)
pH	6.6 (0.19)	6.5 (0.12)	8.2 (0.41)	6.1 (0.08)	7.0 (0.81)	5.4 (0.15)	6.3 (0.21)	6.9 (0.71)
OM (%)	1.2 (0.27)	0.42 (0.07)	1.8 (0.57)	3.9 (1.57)	0.69 (0.21)	1.2 (0.12)	0.47 (0.05)	1.5 (0.36)
N (%)	0.05 (0.01)	0.03 (0.004)	0.08 (0.02)	0.13 (0.04)	0.04 (0.02)	0.06 (0.01)	0.02 (0.003)	0.08 (0.03)
C/N	25.3 (1.6)	29.9 (2.7)	19.0 (3.3)	22.8 (1.5)	21.1 (7.3)	22.5 (2.4)	29.3 (2.7)	23.6 (8.9)
P (mg kg^{-1})	4.1 (0.62)	2.0 (0.87)	2.6 (1.3)	7.4 (0.50)	8.1 (4.08)	4.1 (0.17)	3.3 (0.65)	3.9 (0.0)
K (mg kg^{-1})	65.2 (12.8)	34.3 (6.5)	85 (23.4)	187.3 (41.2)	27 (6.2)	23 (4.2)	28.8 (3.8)	138 (48.2)
Ca (cmol$_c$ kg^{-1})	7.5 (2.48)	1.7 (0.27)	31.5 (7.0)	2.2 (0.61)	10.3 (7.8)	1.1 (0.44)	1.7 (0.18)	16.2 (13.4)
Mg (cmol$_c$ kg^{-1})	0.42 (0.05)	0.33 (0.06)	0.62 (0.16)	0.48 (0.13)	0.74 (0.32)	0.15 (0.04)	0.32 (0.06)	0.54 (0.15)
CEC (cmol$_c$ kg^{-1})	9.5 (0.96)	6.4 (0.42)	13.2 (3.2)	17.7 (2.2)	9.43 (2.71)	10.0 (0.9)	5.2 (0.42)	11.7 (3.4)
Fe (mg kg^{-1})	30.3 (2.9)	22.7 (3.0)	30.7 (18.3)	38.9 (11.3)	26.7 (11.1)	43.2 (5.3)	25.6 (3.3)	37.0 (7.4)

	All stands	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**
Climatic variables								
Elevation (m)	880.6 (27.9)	860.3 (7.4)	671.3 (64.5)	1013.3 (28.5)	673 (102.5)	934.3 (23.0)	935.7 (30.5)	1041.7 (11.5)
Winter precipitation (mm)	172.3 (20.4)	113.3 (1.2)	175 (4.7)	455.4 (16.7)	109 (13.3)	172.8 (14.3)	122.2 (2.2)	168.1 (6.6)
Spring precipitation (mm)	169.9 (7.0)	144.2 (0.9)	173.3 (6.0)	262 (5.6)	139 (1.9)	148.2 (2.3)	169.9 (0.5)	178.4 (4.7)
Summer precipitation (mm)	81 (2.2)	71.6 (0.6)	102.4 (1.9)	84.2 (2.4)	68 (0.1)	68.4 (1.2)	86.5 (1.4)	89.5 (3.9)
Autumn precipitation (mm)	216.8 (24.5)	145.6 (2.3)	210 (6.3)	555.4 (24.0)	147.8 (1.5)	237.5 (13.6)	152 (2.4)	205.8 (6.8)
Annual precipitation (mm)	636.1 (52.8)	479.7 (6.1)	661.7 (22.4)	1366 (47.0)	478.7 (5.0)	556.3 (72.7)	531 (5.7)	641 (24.0)
Minimum winter temperature (°C)	0.03 (0.14)	0.13 (0.10)	1.3 (0.31)	0.61 (0.28)	0.48 (0.03)	-0.57 (0.12)	-0.65 (0.12)	-0.56 (0.16)
Minimum spring temperature (°C)	6.3 (0.14)	6.5 (0.08)	6.9 (0.32)	7.2 (0.31)	7.0 (0.02)	5.0 (0.14)	6.0 (0.13)	5.7 (0.09)
Minimum summer temperature (°C)	11.2 (0.2)	11.2 (0.1)	11.3 (0.3)	12.8 (0.3)	12 (0.1)	9.5 (0.14)	11.1 (0.2)	10.8 (0.3)
Minimum autumn temperature (°C)	2.8 (0.15)	2.7 (0.06)	4.3 (0.26)	3.3 (0.24)	3.1 (0.03)	2.2 (0.11)	2.1 (0.13)	2.1 (0.13)
Minimum annual temperature (°C)	5.1 (0.21)	5 (0)	5.7 (0.33)	7.3 (0.88)	5.7 (0.33)	4 (0)	4.7 (0.21)	4.3 (0.33)
Maximum winter temperature (°C)	10.3 (0.1)	10.4 (0.1)	10.3 (0.3)	10.8 (0.3)	11.1 (0.02)	10.3 (0.2)	9.8 (0.13)	9.3 (0.10)
Maximum spring temperature (°C)	19.5 (0.2)	19.1 (0.8)	18.9 (0.3)	21 (0.3)	21.1 (0.2)	18.9 (0.2)	19.5 (0.1)	18.6 (0.3)
Maximum summer temperature (°C)	27.4 (0.3)	27.6 (0.1)	25.2 (0.2)	29.8 (0.2)	29 (0.2)	26.1 (0.2)	27.6 (0.1)	26.6 (0.3)
Maximum autumn temperature (°C)	12.6 (0.2)	12.8 (0.1)	12.6 (0.3)	13.5 (0.3)	13.4 (0.1)	12.9 (0.2)	12.3 (0.2)	10.9 (1.2)
Maximum annual temperature (°C)	17.6 (0.2)	17.8 (0.2)	16.7 (0.3)	19 (0)	18.7 (0.3)	17 (0)	17.3 (0.2)	16.7 (0.3)
Mean winter temperature (°C)	5.1 (0.12)	5.2 (0.07)	5.9 (0.33)	5.7 (0.28)	5.8 (0.03)	4.9 (0.13)	4.6 (0.13)	4.4 (0.08)
Mean spring temperature (°C)	13.1 (0.2)	13.6 (0.5)	13 (0.3)	14.1 (0.3)	14 (0.1)	12 (0.2)	12.7 (0.1)	12.1 (0.2)
Mean summer temperature (°C)	19.3 (0.2)	19.4 (0.1)	18.3 (0.3)	21.3 (0.2)	20.5 (0.1)	17.8 (0.1)	19.4 (0.1)	18.7 (0.3)
Mean autumn temperature (°C)	7.7 (0.11)	7.7 (0.06)	8.5 (0.31)	8.4 (0.27)	8.2 (0.06)	7.6 (0.17)	7.2 (0.13)	7 (0.10)
Mean annual temperature (°C)	11.4 (0.1)	11.3 (0.2)	11.7 (0.3)	12.3 (0.3)	12 (0)	10.7 (0.3)	11.2 (0.2)	10.7 (0.3)

Statistical analysis

A three-step approach was used to evaluate the relative influence of silvicultural and stand level characterization, soil properties, and climatic data on Maritime pine decline (estimated as a set of variables: crown conditions, dieback, presence of cankers, needle retention, etc. [see Table 3.2]). First, principal component analysis (PCAs) was applied to the stand level characterization, soil properties and climatic variables to avoid the use of highly correlated variables in subsequent steps. These PCAs was carried out in R (R Core Team, 2014), with the "Vegan" package (Oksanen et al. 2015). Second, the most closely correlated variable of each of the retained axes were selected. Significant axes were also selected in R by applying the broken-stick method with the "BiodiversityR" package (Kindt, 2014). The PCA axes with higher percentages of variance than broken-stick variances are significant. Third, non-metric multidimensional scaling (NMDS) was performed, again with the "Vegan" package (Oksanen et al. 2015), to explore the relationships between Maritime pine decline and the explanatory factors (using the variables selected in the previous step and the discrete variables that were not included in the PCAs). NMDS was conducted using Bray-Curtis as the distance metric and the multivariate ordination was created with the metaMDS results. Environmental variables (qualitative variables and those quantitative variables selected from PCAs carried out for stand level characterization, soil properties and climatic variables) that were correlated (at $p\text{-value} < 0.1$) with one or more axes were overlaid on the ordination plot as vectors. The qualitative variables retained are points showing the average values of factor levels, whereas the quantitative variables are displayed as vectors.

At the same time, MRPPs were performed to test the null hypotheses of no differences in forest health between *Pinus pinaster* stands according to the provenance and zone (for zoning see Table 3.1 and Figure 3.1) with the "Vegan" package (Oksanen et al. 2015). MRPP was also performed using Bray-Curtis dissimilarity with 1000 permutations.

Results

Forest health conditions are as summarised in Table 3.2. A clear decline was confirmed, with high percentages of defoliation, discoloration, microphylls (almost 40, 30 and 20 %, respectively), and almost 40 % of the trees dead or damaged (ca. 10 and 30 %, respectively). NMDS ordination and the MRPP test did not show significant differences in the decline of *Pinus pinaster* according to the provenance (A = 0.03, *P* = 0.195; Figure 3.2a). However, clear differences were observed depending on the zone (A = 0.15, *P* = 0.002; Figure 3.2b). The health status of the stands in zone 1 and, to a lesser extent, zone 5 were poorer than in other zones.

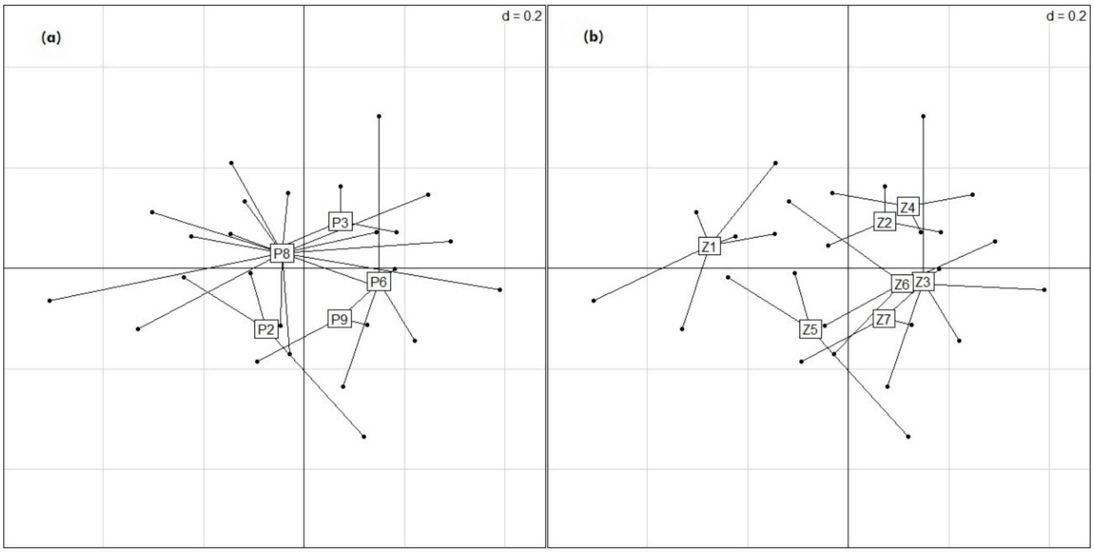


Figure 3.2. Non-metric multidimensional scaling (NMDS) ordination according to **(a)** provenance and **(b)** zone (for zoning see Table 3.1). Dissimilarity distance = Bray-Curtis.

Silvicultural and stand level characterization in the twenty-seven stands studied are shown in Table 3.2. The average age of the stands was almost 67 years-old, with the youngest stands included in zones 1 and 5 (ca. 50 year-old stands) and the oldest stands in zone 3 (85 year-old stands). One of the most important observations is the difference in most stand level variables (e.g. basal area, mean diameter, mean height, etc.) when comparing stands located in zone 3 (Sierra de Gredos provenance) with the other stands.

The soil textural classes of most of the stands were sandy and sandy loam, which implies low values of cationic exchange capacity (in most stands CEC < 10 $\text{cmol}_c\ \text{kg}^{-1}$). These low CEC values are related to the low contents of some cations, such as K (*r* = 0.75, *P* < 0.001), Ca (*r* = 0.49, *P* = 0.01) and Mg (*r* = 0.47, *P* = 0.01). The coarse fragment contents were relatively low, ranging between less than 5 % in zone 4 to more than 40 % in zone 3. Most of the soils were neutral or moderately acidic (6.3 - 7), with the exception of the alkaline and acidic soils found in zones 2 and 5, respectively. The soils were generally of poor quality, with low organic matter levels and N contents (again with the exception of zone 3), usually lower than 2 and 0.1 %, respectively. The C/N ratios were higher than 20, regardless of the pH. Data on the soil properties are also shown in Table 3.2.

The climatic data for the stands under study are summarised in Table 3.2. The elevation ranged from almost 700 m in zone 2 and 4 to more than 1000 m in zone 3. As noted above for soil properties, the climatic characteristics of the zone 3 are also quite different from those in the other zones. The average annual precipitation in zone 3 (1366 mm) is more than twice than in the other zones (between ca. 475 and 660 mm). Furthermore, mean annual temperature were slightly higher in zone 3 than the other zones.

Principal component analysis (PCAs) was performed to select the variables for use in the NMDS analysis. In particular, the broken-stick method retained three axes in the PCA for silvicultural and stand level characterization (cumulative percentage of variance, ca.75%) and climatic variables (cumulative percentage of variance, ca. 85 %) and two axes in the PCAs for soil properties (cumulative percentage of variance, ca. 62 %) (Table 3.3). The variables selected from the axes retained in the three PCAs were as follows: total height, density and crown height for silvicultural and stand level characterization (Table 3.4); CEC and pH for soil properties (Table 3.5); and annual mean temperature, average spring precipitation and average summer precipitation for climatic variables (Table 3.6).

Table 3.3. Selection of significant axes from principal component analysis by the broken-stick method.

	Axes							
	1	2	3	4	5	6	7	8
Silvicultural and stand level characterization								
Eigenvalue	6.32	3.77	1.89	1.19	0.95	0.67	0.57	0.19
Percentage of variance	**39.53**	**23.55**	**11.80**	7.44	5.94	4.19	3.55	1.18
Broken-stick percentage	**21.13**	**14.88**	**11.75**	9.67	8.11	6.86	5.82	4.92
Soil properties								
Eigenvalue	6.63	2.73	1.41	1.25	0.80	0.64	0.50	0.41
Percentage of variance	**44.23**	**18.19**	9.38	8.36	5.35	4.25	3.33	2.72
Broken-stick percentage	**22.12**	**15.45**	12.12	9.90	8.23	6.90	5.79	4.84
Climatic variables								
Eigenvalue	10.84	4.12	2.82	1.17	0.85	0.44	0.25	0.22
Percentage of variance	**51.64**	**19.62**	**13.45**	5.57	4.04	2.10	1.21	1.05
Broken-stick percentage	**17.36**	**12.60**	**10.22**	8.63	7.44	6.49	5.69	5.01

Note: PCA axes with larger percentages of variance than broken-stick variances are significant.

Table 3.4. Coefficients of correlation between silvicultural and stand level variables and the significant axes of the principal component analysis.

	PC1	**PC2**	**PC3**
Age (years)	-0.817	0.542	0.267
Stumps (no)	-0.312	-0.651	-0.015
Regeneration (trees/ha)	-0.082	0.090	-0.015
Suppressed (%)	0.865	-0.184	-0.479
Other species (%)	0.448	0.090	-0.412
Crown height (m)	-0.348	0.496	**-0.853**
Crown area (m^2)	-0.768	0.756	-0.145
Basal Area (m^2/ha)	-1.057	-0.277	-0.063
Density (trees/ha)	0.141	**-1.055**	0.060
Dm (cm)	-0.944	0.590	0.015
Hm (m)	**-1.073**	0.241	-0.175
Ho (70 years) (m)	-0.648	-0.367	-0.592
Hart	0.759	0.703	0.172
Reineke	-0.935	-0.580	-0.022
CE	-0.401	-0.767	-0.348
ICV	-0.646	-0.262	0.805

Note: The highest correlations are shown in bold type.

Table 3.5. Coefficients of correlation between soil properties and the significant axes of the principal component analysis.

	PC1	**PC2**
Slope (%)	0.877	-0.137
pH	0.485	**0.966**
Coarse fragments (%)	0.313	-0.411
Sand (%)	-0.955	0.130
Silt (%)	0.725	-0.427
Clay (%)	0.972	0.136
OM (%)	0.899	-0.454
P (mg kg^{-1})	0.106	-0.421
K (mg kg^{-1})	0.892	-0.182
Ca (cmolc kg^{-1})	0.705	0.739
Mg (cmolc kg^{-1})	0.549	0.548
N (%)	1.050	-0.177
C/N	-0.731	-0.503
CEC (cmolc kg^{-1})	**1.060**	-0.072
Fe (mg kg^{-1})	0.274	-0.838

Note: The highest correlations are shown in bold type.

Table 3.6. Coefficients of correlation between climatic variables and the significant axes of the principal component analysis.

	PC1	**PC2**	**PC3**
Elevation (m)	0.363	0.856	-0.221
Winter precipitation (mm)	-0.611	0.778	0.206
Spring precipitation (mm)	-0.532	**0.859**	0.255
Summer precipitation (mm)	0.059	0.321	**0.840**
Autumn precipitation (mm)	-0.601	0.770	0.174
Annual precipitation (mm)	-0.629	0.787	0.234
Minimum winter temperature (°C)	-0.793	-0.386	0.540
Minimum spring temperature (°C)	-0.947	-0.214	0.143
Minimum summer temperature (°C)	-0.939	0.122	-0.051
Minimum autumn temperature (°C)	-0.743	-0.348	0.647
Minimum annual temperature (°C)	-0.891	0.226	0.140
Maximum winter temperature (°C)	-0.859	-0.440	-0.204
Maximum spring temperature (°C)	-0.595	0.103	-0.353
Maximum summer temperature (°C)	-0.718	0.285	-0.683
Maximum autumn temperature (°C)	-0.702	-0.277	-0.305
Maximum annual temperature (°C)	-0.843	0.041	-0.554
Mean winter temperature (°C)	-0.895	-0.440	0.243
Mean spring temperature (°C)	-0.826	-0.249	-0.219
Mean summer temperature (°C)	-0.856	0.248	-0.457
Mean autumn temperature (°C)	-0.914	-0.327	0.304
Mean annual temperature (°C)	**-0.951**	-0.158	-0.036

Note: The highest correlations are shown in bold type.

Two qualitative variables were retained in the NMDS analysis, in particular the type of forest (pure or mixed stands) ($P = 0.018$) and thinning ($P = 0.018$). The ordination biplot shows that forest health is mainly determined by the horizontal axis: negative values are associated with the poorest conditions and vice versa (Figure 3.3). Thus, the spatial ordination appears to indicate better forest health in pure stands, in which thinning has been carried out, than in unthinned mixed stands (Figure 3.3). Density, total height and average spring precipitation were also retained ($P = 0.018$, $P = 0.086$ and $P = 0.088$, respectively). In particular, high values of total height and spring precipitation appear to indicate better forest health, whereas high tree density is associated with the poorest forest health (Figure 3.3).

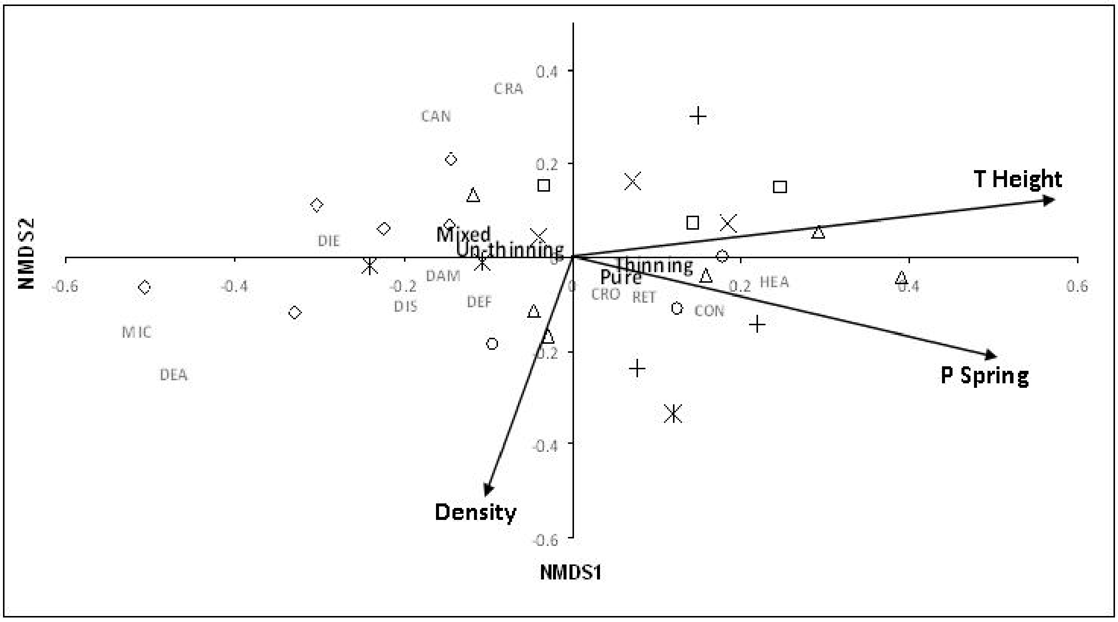


Figure 3.3. Ordination of phytosanitary variables, stands and the environmental variables retained in the NMDS analysis. Quantitative variables are represented by vectors (Total Medium Height, Density and Spring Precipitation) and qualitative variables as points indicating the average values of factor levels (Pure and Mixed stands and Thinned and Unthinned stands). Dissimilarity distance = Bray-Curtis. Stands are represented by zones (z1=rhombii, z2=crosses, z3=plusses, z4=squares, z5=asterisks, z6=triangles and z7=circles). The abbreviations for phytosanitary variables are as follows: DEF: Crown defoliation (%), DIS: Crown discoloration (%), CRO: Live crown ratio (%), DIE: Dieback (%), MIC: Microphylls (%), CAN: Cankers (%), CRA: Cracks (%), CON: Number of pine cones, RET: Needle retention (years), DEA: Dead trees (%), DAM: Damaged trees (%), HEA: Healthy trees (%).

Discussion

Water deficit has previously been associated with forest decline in the Iberian Peninsula (Camarero et al., 2011; Cándel-Pérez et al., 2012; Carnicer et al., 2011; Galiano et al., 2010; Linares and Tíscar, 2010; Navarro-Cerrillo et al., 2007; Sánchez-Salguero et al. 2012a, 2013). Water availability for trees not only depends on water input (i.e. rainfall or groundwater) but also the soil water-holding capacity. The study area, and Castile and Leon in general, is characterized by a continental climate with long cold winters and hot, dry summers, so that growth takes place during spring and, to a lesser extent, autumn. A positive trend in the annual mean temperature and a negative trend in the annual precipitation over the last 50 years have been demonstrated in Castile and Leon. Indeed, the greatest increase in temperatures and decrease in precipitation have occurred in spring (del Río et al., 2005, 2009, 2011). Furthermore, a low water-holding capacity is expected in the study stands, characterized by soils of sandy texture and containing little organic matter and a high percentage of course fragments (Saxton and Rawls, 2005). This supports our finding that rainfall, particularly spring precipitation, plays a key role in *Pinus pinaster* decline. It is well known that precipitation in the growing season is crucial for radial growth of trees and vigour in *Pinus pinaster* (Bogino and Bravo, 2008; Rozas et al., 2011; Sánchez-Salguero et al., 2010, 2012a). The study area, most of which is located on a superficial aquifer, is characterized by a variable groundwater table near the soil surface and highly permeable soils. The aquifers have been overused over the last few decades for irrigating crops, and changes in the levels of groundwater table have been suggested by other authors to threaten the stability and persistence of some trees (Gómez-Sanz and García-Viñas, 2011). This threat is even more important for the genus *Pinus*, and particularly *Pinus pinaster*, which is vulnerable to xylem embolism (Choat et al., 2012; Martínez-Vilalta et al., 2004). Other authors have also pointed out that the productivity of Maritime pine stands may depend on having access to a groundwater table during the growing season (Álvarez-Álvarez et al., 2009; Bravo-Oviedo et al., 2010, 2011). On the other hand, the fact that MRPP analysis has demonstrated that *Pinus pinaster* decline is determined by the location, regardless of the provenance, seems to suggest that genetic features are not as important as local site conditions. The present study has also pointed out that stand structure resulting from historical forest management might also be playing a fundamental role in *Pinus pinaster* decline. Indeed, several variables associated with competition were retained in the NMDS analysis. In particular, dense stands displayed the highest tree mortality rates and decline symptoms, as already reported in other cases of forest decline (Camarero et al., 2011; Galiano et al., 2010; Gómez-Aparicio et al., 2011; Linares et al., 2009; Madrigal-González and Zavala, 2014; Martínez-Vilalta et al., 2012; Sánchez-Salguero et al., 2012b, 2013). Competition can take place for aboveground (i.e. light) and belowground resources (i.e. soil nutrients and water). However in Mediterranean pines, and in particular *Pinus pinaster*, limitation is due to competition for belowground resources rather than to aboveground resources (Bravo-Oviedo et al., 2006; Cailleret et al., 2014; Sánchez-Salguero et al., 2012b). In the stands considered here, water availability seems to be more limiting than soil nutrients, as no soil variables were retained in the NMDS analysis. This may be due to the fact that *Pinus pinaster* has low mineral requirements and is able to grow in nutrient-poor soils (Álvarez-Álvarez et al., 2010).

Land-use modifications (mainly changes in the intensity of logging) have been associated with forest decline in other Mediterranean pines in the Iberian Peninsula, (Camarero et al., 2011; Galiano et al., 2010; Sánchez-Salguero et al., 2012b, 2013). The current density of *Pinus pinaster* forests is expected to be higher than it was 40-50 years ago because the exploitation of resin almost collapsed in the 1970s (Picardo and Pinillos, 2013) and a lower intensity of thinning is applied to forests focused mainly on the timber production (Rodríguez et al., 2008). In addition, the timber market in this area has decreased over the last few years.

Total height was also retained in the NMDS analysis, and the lower total height of the stands, the greater the decline. The close correlation between total height and age ($R^2_{adj} = 0.59$, $P < 0.001$) indicates an indirect effect. Other studies have demonstrated that smaller trees are more vulnerable to decline than larger ones (Bravo-Oviedo et al., 2006; Cailleret et al., 2014; Coomes and Allen, 2007; Galiano et al., 2010; Gómez-Aparicio et al., 2011; Madrigal-González and Zavala, 2014; Martínez-Vilalta and Piñol, 2002). This may be because *Pinus pinaster* preserves its taproot until maturity for supply water and nutrients from the groundwater table or deep soils (Gómez-Sanz and Garcia-Viñas, 2011), conferring mature trees a considerable competitive advantage over young trees in xeric sites.

In the present study, phytosanitary conditions were poorer in mixed stands than in pure stands. This does not concur with previous findings, which indicated that mixed stands are much less affected by changing climate conditions and pests and diseases than pure stands (Jactel et al., 2005; Neuner et al., 2015; Pautasso et al., 2005). This discrepancy may be due to the fact that one of the causes of the origin of mixed stands in the present study was probably the previous mortality of dominant species, which may lead to misinterpretation of the cause-and-effect relationship as a result of an indirect effect.

Manion's theory has been widely used in order to unravel forest decline processes. The decline of western white pine, often referred to as pole blight, was already observed in the 1930's. In this case, drought acted as an inciting factor, whereas shallow soils and secondary fungi were predisposing and contributing factors, respectively (Kliejunas et al., 2009). More recently, extreme climatic conditions resulting from a warm drought also acted as inciting factors in the sudden aspen decline observed in North America (Rehfeldt et al., 2009; Worrall et al., 2010).

Some recent studies have suggested alternative hypotheses to the traditional Manion model in order to explain forest declines in the Iberian Peninsula. In particular, Linares et al. (2010) suggested that competition and fungal infections caused by *Heterobasidion abietinum* act as predisposing factors in *Abies pinsapo* decline, with climatic variability acting as a contributing factor. Sánchez-Salguero et al. (2012b) suggested that stand structure and competition act as predisposing factors, with drought events occurring as inciting factors and intense drought episode as contributing factors.

In the present study, although competition clearly appeared to operate as a predisposing factor, the role water deficit played was not obvious. The possibility that water deficit was acting as an inciting factor (i.e. severe, short-term stress) is unlikely because *Pinus pinaster* is considered a drought-avoiding species, one able to cope with intense, short-term drought pulses but not with multiple droughts (Madrigal-González and Zavala, 2014). Likewise, the possibility that water deficit was acting as a contributing factor also seems unlikely because, if that were the case, *Pinus pinaster* would have been replaced by drier thermophyllous species. However, Maritime pine was mainly replaced by *Quercus ilex*, which has similar water requirements (Bravo-Oviedo and Montero, 2008; Ruiz-Labourdette et al., 2012, 2013). Therefore, we hypothesize that water deficit also acted as a predisposing factor.

Contributing factors have not been identified in this study; however, pathogenic fungi, and in particular root pathogens, have previously been widely identified as contributing factors (Desprez-Loustau et al., 2006). Recent studies have pointed out that a root fungal pathogen (*Heterobasidion annosum* [Fr.] Bref) may be involved in the decline of *Pinus pinaster* stands in Spain (Mesanza and Iturritxa, 2012; Prieto-Recio et al., 2012, 2014). *Heterobasidion* spp. is known to be favoured by drought-related stress and poor soils (Gori et al., 2013; Korhonen and Stenlid, 1998; Linares et al., 2010; Puddu et al., 2003), which are consistent with current conditions in our study area. Furthermore, Álvarez et al. (2015) suggested that *Ophiostoma minus* [Hedgcock] H. & P. may be involved in *Pinus pinaster* decline in Castile and Leon. Similarly, Soria et al. (2001) demonstrated that mortality of *Pinus pinaster* trees in the mountains of National Heritage of the Escorial (Madrid, Spain) increased in midsummer as a result of the presence of *Ophiostoma minus* and low rainfall and high temperatures. In this regard, it is important to note that *Ips sexdentatus* is one of the main vectors of ophiostomatoid fungi (Bueno et al., 2010). The presence of mistletoe has also frequently been related to forest decline in the Mediterranean forests (Durand-Gillmann et al., 2014; Galiano et al., 2010; Gea-Izquierdo et al., 2014; Oliva and Colinas, 2007; Sangüesa-Barreda et al., 2012). However, despite the presence of mistletoe on trees in stands suffering decline in the study area, no specific studies have been carried out to shed light on the role of this parasitic species.

Conclusions

We hypothesize that *Pinus pinaster* stands located in xeric sites may be close to their physiological tolerance threshold. Thus, decreased water availability in the last few decades as a result of climate change and/or overuse of aquifers, together with increased water requirements as a result of high stand densities brought about by land-use modifications, may have led to forest decline and long-term changes in tree species composition.

Forest managers are increasingly required to integrate both adaptation and mitigation strategies to cope with new scenarios and threats such as climate change and land-use modifications (Cavers and Cottrell, 2014; D'Amato et al., 2011; Keenan, 2015; Millar et al., 2007; Seidl and Lexer, 2013). This study provides critical new information that may be useful for establishing and implementing forest action plans and policies. In particular, intensive thinning and an extension of the rotation period could be used to enhance the resistance and resilience of Maritime pine forests.

The present study has pointed out that competition and water deficit might trigger the mortality of *Pinus pinaster*; however, further studies are required to determine the contributing factors involved in Maritime pine decline in the Iberian Peninsula.

References

Alía, R., Martín, S., 2003. EUFORGEN: Technical guidelines for genetic conservation and use for Maritime pine (*Pinus pinaster*). International Plant Genetic Resources Institute. Roma, p. 6.

Alía, R., Martín, S., De Miguel, J., Galera, R., Agúndez, D., Gordo, J., Salvador, L., Catalán, G., Gil, L., 1996. Regiones de procedencia, *Pinus pinaster* Aiton. Ministerio Medio Ambiente. Organismo Autónomo Parques Nacionales. Madrid, p. 75.

Alía, R., Moro, J., Denis, J.B., 2001. Ensayos de procedencias de *Pinus pinaster* Ait. en el centro de España: resultados a la edad de 32 años. Invest. Agr.: Sist. Recur. For. 10, 333-354.

Allen, C.D., Macalady, A.K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D.D., Hogg, E.H., González, P., Fensham, R., Zhang, Z., Castro, J., Demidova, N., Lim, J.-H., Allard, G., Running, S.W., Semerci, A., Cobb, N., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. For. Ecol. Manage. 259, 660-684.

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1-9.

Álvarez-Álvarez, P., Afif-Khouri, E., Camara-Obregon, A., Castedo-Dorado, F., Barrio-Anta, M., 2010. Effects of foliar nutrients and environmental factors on site productivity in *Pinus pinaster* Ait. stands in Asturias (NW Spain). Ann. For. Sci. 68, 497-509.

Arnold, A.E., 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol. Rev. 21, 51-66.

Ayres, M.P., Lombardero, M.J., 2000. Assessing the consequences of global change for forest disturbance from herbivores and pathogens. Sci. Total Environ. 262, 263-286.

Blank, L.W., 1985. A New Type of Forest Decline in Germany. Nature 314, 311-314.

Bogino, S.M., Bravo, F., 2008. Growth response of *Pinus pinaster* Ait. to climatic variables in central Spanish forests. Ann. For. Sci. 65, 506.

Bravo-Oviedo, A., Gallardo-Andres, C., del Río, M., Montero, G., 2010. Regional changes of *Pinus pinaster* site index in Spain using a climate-based dominant height model. . Can. J. For. Res. Can. Rech. For. 40, 2036-2048.

Bravo-Oviedo, A., Montero, G., 2008. Descripción de los Caracteres Culturales de las Principales Especies Forestales de España, in Serrada, R., Montero, G. and Reque, J.A. (Eds.), Compendio de Selvicultura Aplicada en España. INIA & Fundación del Conde del Valle de Salazar, pp. 1039-1114.

Bravo-Oviedo, A., Roig, S., Bravo, F., Montero, G., del Río, M., 2011. Environmental variability and its relationship to site index in Mediterranean Maritime pine. For. Syst. 20, 50-64.

Bravo-Oviedo, A., Sterba, H., del Río, M., Bravo, F., 2006. Competition-induced mortality for Mediterranean *Pinus pinaster* Ait. and *P. sylvestris* L. For. Ecol. Manage. 222, 88-98.

Bueno, A., Diez, J.J., Fernández, M.M., 2010. Ophiostomatoid fungi transported by *Ips sexdentatus* (Coleoptera; Scolytidae) in *Pinus pinaster* in NW Spain. Silva Fenn. 44, 387-397.

Cailleret, M., Heurich, M., Bugmann, H., 2014. Reduction in browsing intensity may not compensate climate change effects on tree species composition in the Bavarian Forest National Park. For. Ecol. Manage. 328, 179-192.

Camarero, J.J., Bigler, C., Linares, J.C., Gil-Pelegrin, E., 2011. Synergistic effects of past historical logging and drought on the decline of Pyrenean silver fir forests. For. Ecol. Manage. 262, 759-769.

Candel-Pérez, D., Linares, J.C., Vinegla, B., Lucas-Borja, M.E., 2012. Assessing climate-growth relationships under contrasting stands of co-occurring Iberian pines along an altitudinal gradient. For. Ecol. Manage. 274, 48-57.

Carnicer, J., Coll, M., Ninyerola, M., Pons, X., Sánchez, G., Peñuelas, J., 2011. Widespread crown condition decline, food web disruption, and amplified tree mortality with increased climate change-type drought, in: Proceedings of the National Academy of Sciences of the United States of America, pp. 1474–1478.

Cavers, S., Cottrell, J.E., 2014. The basis of resilience in forest tree species and its use in adaptive forest management in Britain. Forestry 88, 13-26.

Choat, B., Jansen, S., Brodribb, T.J., Cochard, H., Delzon, S., Bhaskar, R., Bucci, S.J., Feild, T.S., Gleason, S.M., Hacke, U.G., Jacobsen, A.L., Lens, F., Maherali, H., Martínez-Vilalta, J., Mayr, S., Mencuccini, M., Mitchell, P.J., Nardini, A., Pittermann, J., Pratt, R.B., Sperry, J.S., Westoby, M., Wright, I.J., Zanne, A.E., 2010. Global convergence in the vulnerability of forests to drought. Nature 491, 752-755.

Cook, E.R., Johnson, A.H., 1989. Climate Change and Forest Decline - a Review of the Red Spruce Case. Water Air Soil Pollut. 48, 127-140.

Coomes, D.A., Allen, R.B., 2007. Effects of size, competition and altitude on tree growth. J. Ecol. 95, 1084–1097.

D'Amato, A.W., Bradford, J.B., Fraver, S., Palik, B.J., 2011. Forest management for mitigation and adaptation to climate change: Insights from long-term silviculture experiments. For. Ecol. Manage. 262, 803-816.

del Río, S., Herrero, L., Penas, A., 2009. Recent climatic trends in Castile and Leon (Spain) and its possible influence on the potential natural vegetation. Acta Bot. Gall. 156, 625-636.

del Río, S., Herrero, L., Pinto-Comes, C., Penas, A., 2011. Spatial analysis of mean temperature trends in Spain over the period 1961-2006. Glob. Planet. Change 78, 65-75.

del Río, S., Penas, A., Fraile, R., 2005. Analysis of recent climatic variations in Castile and Leon (Spain). Atmos. Res. 73, 69-85.

Desprez-Loustau, M.L., Marcais, B., Nageleisen, L.M., Piou, D., Vannini, A., 2006. Interactive effects of drought and pathogens in forest trees. Ann. For. Sci. 63, 597-612.

Dieguez-Aranda, U., Castedo-Dorado, F., Álvarez-González, J.G., Rodríguez-Soalleiro, R., 2005. Modelling mortality of Scots pine (*Pinus sylvestris* L.) plantations in the northwest of Spain. Eur. J. For. Res. 124, 143-153.

Dobbertin, M., Brang, P., 2001. Crown defoliation improves tree mortality models. For. Ecol. Manage. 141, 271-284.

Durand-Gillmann, M., Cailleret, M., Boivin, T., Nageleisen, L.M., Davi, H., 2014. Individual vulnerability factors of Silver fir (*Abies alba* Mill.) to parasitism by two contrasting biotic agents: mistletoe (*Viscum album* L. ssp *abietis*) and bark beetles (*Coleoptera: Curculionidae: Scolytinae*) during a decline process. Ann. For. Sci. 71, 659-673.

Eichhorn, J., Roskams, P., Ferretti, M., Mues, V., Szepesi, A., Durrant, D., 2010. Manual Part IV: Visual Assessment of Crown Condition and Damaging Agents, in: Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg, p. 49.

Galiano, L., Martínez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor Decline of Scots Pine in the Pyrenees and Potential Vegetation Change by the Expansion of Co-occurring Oak Species. Ecosystems 13, 978-991.

Gea-Izquierdo, G., Viguera, B., Cabrera, M., Canellas, I., 2014. Drought induced decline could portend widespread pine mortality at the xeric ecotone in managed Mediterranean pine-oak woodlands. For. Ecol. Manage. 320, 70-82.

Gil, L., Gordo, J., Alía, R., Catalán, G., Pardos, J.A. 1990. *Pinus pinaster* Aiton en el paisaje vegetal de la Península Ibérica. Ecología 1, 469-495.

Giorgi, F., Lionello, P., 2008. Climate Change Projections for the Mediterranean Region. Global. Planet. Change 63, 90-104.

Girard, F., Vennetier, M., Guibal, F., Corona, C., Ouarmim, S., Herrero, A., 2012. *Pinus halepensis* Mill. crown development and fruiting declined with repeated drought in Mediterranean France. Eur. J. For. Res. 131, 919-931.

Gómez-Aparicio, L., García-Valdes, R., Ruiz-Benito, P., Zavala, M.A., 2011. Disentangling the relative importance of climate, size and competition on tree growth in Iberian forests: implications for forest management under global change. Glob. Chang. Biol. 17, 2400-2414.

Gómez-Sanz, V., García-Viñas, J.I., 2011. Soil moisture spatio-temporal behavior of *Pinus pinaster* stands on sandy flatlands of central Spain. For. Syst. 20, 293-302.

González, J., Dorado, F., González, A., Sánchez, C., von Gadow, K., 2004. A two-step mortality model for even-aged stands of *Pinus radiata* D. Don in Galicia (Northwestern Spain). Ann. For. Sci. 61, 439-448.

Gori, Y., Cherubini, P., Camin, F., La Porta, N., 2013. Fungal root pathogen (*Heterobasidion parviporum*) increases drought stress in Norway spruce stand at low elevation in the Alps. Eur. J. For. Res. 132, 607-619.

Helfer, S., 2014. Rust fungi and global change. New Phytol. 201, 770-780.

Hennon, P.E., D'Amore, D.V., Schaberg, P.G., Wittwer, D.T., Shanley, C.S., 2012. Shifting Climate, Altered Niche, and a Dynamic Conservation Strategy for Yellow-Cedar in the North Pacific Coastal Rainforest. Bioscience 62, 147-158.

Hennon, P.E., McWilliams, M.G., 1999. Decline symptoms do not develop with grafting from dying yellow-cedar. . Can. J. For. Res. Can. Rech. For. 29, 1985-1988.

Hennon, P.E., Wittwer, D.T., D'Amore, D.V., Lamb, M., 2009. Yellow-cedar decline: Key landscape features and snow modeling of a climate-induced forest decline on a dormant volcano. Phytopathology 99, S52.

Hertel, G., 1988. Proceedings of the US/FRG research symposium: effects of atmospheric pollutants on the spruce-fir forests of the Eastern United States and the Federal Republic of Germany. 1987 October 19-23; Burlington, VT. Gen. Tech. Rep. NE-120. Broomall, PA: U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station, p. 543.

Jactel, H., Brockerhoff, E., Duelli, P., 2005. A test of the biodiversity-stability theory: meta-analysis of tree species diversity effects on insect pest infestations, and reexamination of responsible factors, in: Scherer-Lorenzen M, Körner C, Schulze E-D (Eds) For. Divers. Funct. Temp. Boreal Syst. Springer, Berlin 176, pp. 235–262.

Jactel, H., Petit, J., Desprez-Loustau, M.L., Delzon, S., Piou, D., Battisti, A., Koricheva, J., 2012. Drought effects on damage by forest insects and pathogens: a meta-analysis. Glob. Chang. Biol. 18, 267-276.

Keenan, R.J., 2015. Climate change impacts and adaptation in forest management: a review. Ann. For. Sci. 72, 145-167.

Kindt, R., 2014. Package 'BiodiversityR'. Package for Community Ecology and Suitability Analysis. CRAN.

Kliejunas, J.T., Geils, B.W., Micales, J., Goheen, E.M., Hennon, P., Kim, M., Kope, H., Stone, J., Sturrock, R., Frankel, S.J., 2009. Review of literature on climate change and forest diseases of western North America. USDA., p. 60.

Korhonen, K., Stenlid, J., 1998. Biology of *Heterobasidion annosum*, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*, Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK., pp. 43–70.

Linares, J.C., Camarero, J.J., Bowker, M.A., Ochoa, V., Carreira, J.A., 2010. Stand-structural effects on *Heterobasidion abietinum*-related mortality following drought events in *Abies pinsapo*. Oecologia 164, 1107-1119.

Linares, J.C., Camarero, J.J., Carreira, J.A., 2009. Interacting effects of changes in climate and forest cover on mortality and growth of the southernmost European fir forests. Glob. Ecol. Biogeogr. 18, 485-497.

Linares, J.C., Tíscar, P.A., 2010. Climate change impacts and vulnerability of the southern populations of *Pinus nigra* subsp. *salzmannii*. Tree Physiol. 30, 795-806.

Madrigal-González, J., Zavala, M.A., 2014. Competition and tree age modulated last century pine growth responses to high frequency of dry years in a water limited forest ecosystem. Agric. For. Meteorol. 192, 18-26.

Manion, P., 1991. Tree Disease Concepts. Prentice-Hall 2nd edition, p. 402.

Martín-García, J., Merino, A., Javier Diez, J., 2012. Relating visual crown conditions to nutritional status and site quality in monoclonal poplar plantations (*Populus x euramericana*). Eur. J. For. Res. 131, 1185-1198.

Martínez-Vilalta, J., Lloret, F., Breshears, D.D., 2012. Drought-induced forest decline: causes, scope and implications. Biol. Lett. 8, 689-691.

Martínez-Vilalta, J., Piñol, J., 2002. Drought-induced mortality and hydraulic architecture in pine populations of the NE Iberian Peninsula. For. Ecol. Manage. 161, 247–256.

Martínez-Vilalta, J., Sala, A., Pinol, J., 2004. The hydraulic architecture of *Pinaceae* - a review. Plant Ecol. 171, 3-13.

Mesanza, N., Iturritxa, E., 2012. Root and butt rot caused by *Heterobasidion annosum* in Atlantic coniferous ecosystems of Spain. For. Pathol. 42, 514-520.

Millar, C.I., Stephenson, N.L., Stephens, S.L., 2007. Climate change and forests of the future: Managing in the face of uncertainty. Ecol. Appl. 17, 2145-2151.

Navarro-Cerrillo, R.M., Varo, M.A., Lanjeri, S., Hernandez-Clemente, R., 2007. Cartografía de defoliación en los pinares de pino silvestre (*Pinus sylvestris* L.) y pino salgareño (*Pinus nigra* Arnold.) en la Sierra de los Filabres. Ecosistemas 16, 163-171.

Neuner, S., Albrecht, A., Cullmann, D., Engels, F., Griess, V.C., Hahn, W.A., Hanewinkel, M., Haertl, F., Koelling, C., Staupendahl, K., Knoke, T., 2015. Survival of Norway spruce remains higher in mixed stands under a dryer and warmer climate. Glob. Chang. Biol. 21, 935-946.

Ninyerola, M., Pons, X., Roure, J.M., 2005. Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Univ. Autónoma de Barcelona, Bellaterra. ISBN 932860-8-7.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2015. Community Ecology Package. Package 'vegan'. Version 2.2-1. CRAN.

Oliva, J., Colinas, C., 2007. Decline of silver fir (*Abies alba* Mill.) stands in the Spanish Pyrenees: Role of management, historic dynamics and pathogens. For. Ecol. Manage. 252, 84-97.

Pautasso, M., Doering, T.F., Garbelotto, M., Pellis, L., Jeger, M.J., 2012. Impacts of climate change on plant diseases-opinions and trends. Eur. J. Plant Pathol. 133, 295-313.

Picardo, A., Pinillos, F. M., 2013. La resinación en España y en el mundo en 2013: Situación y perspectivas. II Simposio Internacional de Resinas Naturales, Segovia, pp. 30-53.

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770.

Prieto-Recio, C., Martín-García, J., Diez, J.J., 2014. Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings. For. Pathol. 44, 163-165.

Puddu, A., Luisi, N., Capretti, P., Santini, A., 2003. Environmental factors related to damage by *Heterobasidion abietinum* in *Abies alba* forests in Southern Italy. For. Ecol. Manage. 180, 37-44.

R Core Team, 2014. R: A language and environment for statistical computing: R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/.

Rehfeldt, G.E., Ferguson, D.E., Crookston, N.L., 2009. Aspen, climate, and sudden decline in western USA. For. Ecol. Manage. 258, 2353–2364.

Reich, P.B., Oleksyn, J., 2008. Climate warming will reduce growth and survival of Scots pine except in the far north. Ecol. Lett. 11, 588-597.

Rodríguez, R.J., Madrigal, A., 2008. Selvicultura de *Pinus pinaster* Ait. subsp. *atlantica* H. de Vill, in Serrada, R., Montero, G. and Reque, J.A. (Eds.), Compendio de Selvicultura Aplicada en España. INIA & Fundación del Conde del Valle de Salazar, pp. 376-398.

Rodríguez-Soalleiro, R.J.S., Lucas, J.A., Alejano, R., del Río, M., Torres, E., Cantero, A., 2008. Selvicultura de *Pinus pinaster* subespecie *mesogeensis*, in Serrada, R., Montero, G. and Reque, J.A. (Eds.), Compendio de Selvicultura Aplicada en España. INIA & Fundación del Conde del Valle de Salazar, pp. 399-430.

Rozas, V., Zas, R., García-González, I., 2011. Contrasting effects of water availability on *Pinus pinaster* radial growth near the transition between the Atlantic and Mediterranean biogeographical regions in NW Spain. Eur. J. For. Res. 130, 959-970.

Ruiz-Benito, P., Lines, E.R., Gómez-Aparicio, L., Zavala, M.A., Coomes, D.A., 2013. Patterns and Drivers of Tree Mortality in Iberian Forests: Climatic Effects Are Modified by Competition. Plos One 8.

Ruiz-Labourdette, D., Fe Schmitz, M., Pineda, F.D., 2013. Changes in tree species composition in Mediterranean mountains under climate change: Indicators for conservation planning. Ecol. Indic. 24, 310-323.

Ruiz-Labourdette, D., Nogues-Bravo, D., Sainz Ollero, H., Schmitz, M.F., Pineda, F.D., 2012. Forest composition in Mediterranean mountains is projected to shift along the entire elevational gradient under climate change. J. Biogeogr. 39, 162-176.

Sánchez, G., Economou, A., Beccu, E., Canu, G., Cocco, S., Bussotti, F., Cenni, E., Cozzi, A., Ferretti, M., Conceiçao, M., 1994. Especies forestales mediterráneas. Guía para la evaluación de las copas (Ed. Ferretti, M.). CEC – UN/ECE, Brussels, Geneva, p. 154.

Sánchez-Salguero, R., Camarero, J.J., Dobbertin, M., Fernandez-Cancio, A., Vila-Cabrera, A., Manzanedo, R.D., Zavala, M.A., Navarro-Cerrillo, R.M., 2013. Contrasting vulnerability and resilience to drought-induced decline of densely planted vs. natural rear-edge *Pinus nigra* forests. For. Ecol. Manage. 310, 956-967.

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Camarero, J.J., Fernandez-Concio, A., 2010. Drought-induced growth decline of Aleppo and Maritime pine forests in south-eastern Spain. For. Syst. 19, 458-469.

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Camarero, J.J., Fernandez-Cancio, A., 2012a. Selective drought-induced decline of pine species in southeastern Spain. Clim. Change 113, 767-785.

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Swetnam, T.W., Zavala, M.A., 2012b. Is drought the main decline factor at the rear edge of Europe? The case of southern Iberian pine plantations. For. Ecol. Manage. 271, 158-169.

Sangüesa-Barreda, G., Linares, J.C., Camarero, J.J., 2012. Mistletoe effects on Scots pine decline following drought events: insights from within-tree spatial patterns, growth and carbohydrates. Tree Physiol. 32, 585-598.

Saxton, K.E., Rawls, W.J., 2006. Soil water characteristic estimates by texture and organic matter for hydrologic solutions. Soil Sci. Soc. Am. J. 70, 1569-1578.

Seidl, R., Lexer, M.J., 2013. Forest management under climatic and social uncertainty: Trade-offs between reducing climate change impacts and fostering adaptive capacity. J. Environ. Manage. 114, 461-469.

Solis, A., 2003. Planteamientos sobre la regeneración en pinares de repoblación que alcanzan la edad de turno, in: Cuadernos de La Sociedad Española de Ciencias Forestales. Actas de

La III Reunión Sobre Regeneración Natural Y IV Reunión Sobre Ordenación de Montes. pp. 49-57.

Soria, S., Rodríguez Barreal, J.A., Saiz de Omeñaca, J.A., Rodríguez-Carmona, M.A., 2001. Estudio de las enfermedades que afectan a *Pinus pinaster* Ait. en montes de Patrimonio Nacional de el Escorial (Madrid) y determinación del agente o agente causales. Patrimonio Nacacional Servicio Jardines y Montes. Madrid.

SPCAN-DGB, 2007. Red de Seguimiento de Daños en los Montes (Red CE de Nivel I). Manual de Campo. Ministerio Medio Ambiente.

Stocker, T.F., Qin, D., Plattner, G.K., Alexander, L.V., Allen, S.K., Bindoff, N.L., Breon, F.M., Chuch, J.A., Cubasch, U., Emori, S., Forster, P., Friedlingstein, P., Gillet, N., Gregory, J.M., Hartmann, D.L., Jansen, E., Kirtman, B., Knutti, R., Krishna Kumar, K., Lemke, P., Marotzke, J., Masson-Delmotte, V., Meehl, G.A., Mokhov, I.I., Piao, S., Ramaswamy, V., Randall, D., Rhein, M., Rojas, M., Sabine, C., Shindell, D., Talley, L.D., Vaughan, D.G., Xie, S.P., 2013. Technical summary, in: Stocker, T.F., Quin, D., Plattner, G.K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), Climate Change 2013: Phys. Sci. Basis. Contrib. Work. Gr. I to Fifth Assess. Rep. Intergov. Panel Clim. Chang. Cambridge Univ. Press., Cambridge (UK) and New York (USA).

Sturrock, R.N., Frankel, S.J., Brown, A.V., Hennon, P.E., Kliejunas, J.T., Lewis, K.J., Worrall, J.J., Woods, A.J., 2011. Climate change and forest diseases. Plant Pathol. 60, 133-149.

Thomas, F.M., Blank, R., Hartmann, G., 2002. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. 32, 277-307.

Worrall, J.J., Marchetti, S.B., Egeland, L., Mask, R.A., Eager, T., Howell, B., 2010. Effects and etiology of sudden aspen decline in southwestern Colorado, USA. For. Ecol. Manage. 260, 638–648.

Chapter 4: Influence of environmental and silvicultural factors on *Pinus pinaster* health status at tree level in central Spain

Abstract

Pinus pinaster Ait. is one of the most important forest species in Spain and is particularly valued as a protective species and for timber production. However, Maritime pine decline has been observed in several forests in the center of the Iberian Peninsula. The characteristic decline symptoms, which include unusual transparency at the crown, small needles, foliage discoloration and early tree death, are associated with a high mortality rate in the species. The aim of this study was to determine the factors associated with *Pinus pinaster* decline by characterizing the study plots and constructing a model to predict individual tree health status. For this purpose, we assessed 27 circular plots of 15 m radius in the main provenance regions of the species in central Spain. Hierarchical cluster analysis grouped the plots according to the silvicultural, soil, climate and phytosanitary variables. The following most closely correlated variables were selected for testing in the model: diameter at breast height, for individual tree characterization; density, mean total height of the stand and mean height of the crown, for stand level characterization; cation exchange capacity and pH, as soil variables; and annual mean temperature, average spring precipitation and average summer, as climatic variables. Finally, the following variables were identified as significant by the best-performing multinomial logistic regression model: diameter at breast height, mean total height of the stand and seasonal summer precipitation. The significant variables and the estimated parameters were used to calculate the probability of occurrence of a particular health status (healthy, symptomatic or dead) at tree level.

Keywords: *Maritime pine decline, natural or naturalized stands, hierarchical clustering, Multinomial Logistic Regression (MLR).*

Introduction

The reality of climate change is now recognised worldwide; however, regional consequences have not yet been studied in detail, especially in the case of forest ecosystems. Specific threats such as the increased frequency of abiotic risks (e.g. strong winds, droughts, floods and fires) are being observed (Canales et al., 2011; Jactel et al., 2009). In addition, novel pests and pathogenic agents (Scherm, 2004) are causing general decline in numerous forest stands. Lower adaptability of tree species to the new climatic conditions is also being observed (Hansen et al., 2001). By contrast, other studies suggest that trees become more efficient in relation to water use efficiency when temperatures increase and rainfall decreases (Bogino and Bravo, 2014). We know that the responses of forest ecosystems to climate change are complicated because of the interactions between different populations of organisms and their different biological characteristics (Roe and Baker, 2007). The potential impacts of climate change on forest diseases are affecting many aspects such as the modification of populations and the community structures, micro-evolutionary processes and plant dynamics (Chakraborty et al., 2000). Many Mediterranean and Sub-Mediterranean forest species from the Iberian Peninsula may suffer decline and a drastic modification of the distribution area (Benito Garzón et al., 2009; del Río et al., 2009). However, other authors suggest that changes in forest productivity may vary positively or negatively depending on local environmental conditions (Bogino and Bravo, 2014; Bravo-Oviedo et al., 2010).

Forest decline was described for the first time in the 1970s and 1980s in Central Europe and North America. A series of pathological processes characterized by diverse symptoms (e.g. defoliation and leaf discoloration, changes in the architecture of the branches, death of roots, lower growth, premature senescence and high mortality of different species of trees) began to be observed in highly populated areas with intense industrial activity (Hertel, 1988). This phenomenon, apparently unusual and unprecedented, was designated forest decline. The term forest decline has since been defined as a complex disease caused by the interaction of an interchangeable number of biotic and abiotic factors (Thomas et al., 2002), that produce the general and gradual deterioration of forest stands, and whose final consequence may be death of the trees (Manion, 1991). The factors involved may be predispositional, inciting or contributory. In the long-term, the historical climate, site, stand age, lack of adequate management and genetic attributes may act as predisposing factors. These do not cause apparent damage, but can predispose the tree to gradual decline (Hennon et al., 2009). On the other hand, in the short term, the abiotic damage caused by climatic events (such as frost, gales, flooding and periods of drought), competition and lack of resources may induce the tree to undergo decline at a given time. The tree may recover or may be damaged and enter into a state of gradual deterioration (Hennon et al., 2012). Finally, contributing factors such as colonisation or attack by pathogenic fungi, parasitic plants, nematodes or opportunistic insects (e.g. bark beetles) ultimately weaken the tree and lead to its death (Hennon and McWilliams, 1999).

Forest decline has been widely investigated. For example, early studies investigated the possible causes of decline of sugar maple in Quebec (Canada) (Payette et al., 1996) and the association between ophiostomatoid fungi and decline of *Pinus palustris* in the southeastern United States (Otrosina et al., 1999). In Spanish forests, the occurrence of the phenomenon has appeared in parallel with the environmental changes (Oliva and Colinas, 2007; Prieto-Recio et al., 2015).

Pinus pinaster Ait. pine is the second most widely distributed tree species in Spain after *Quercus ilex*. It forms pure stands and also mixed forests with other conifers or broadleaved species. The stands can be natural (1 million of ha) or planted (0.6 million ha) (DGCONA, 2002). Three groups can be differentiated depending on forest use. One group comprises naturally regenerated pine woods in the Central plateau in Spain, historically dedicated to resin extraction. Another group of pine woods in Central and Iberian Systems (of natural origin or plantations) are dedicated to timber production. The third group of pine woods is located in the Mediterranean coastal area of Spain where the main function is protection and formation of mixed stands (Rodríguez-Soalleiro et al., 2008). These stands are mainly distributed in the central plateau, up to an elevation of 1500 m, preferably on sandy, stony and well drained soils. These areas are characterized by irregular seasonal rainfall and high temperatures in summer.

During the last few decades, Maritime pine has suffered a gradual process of decline in the Iberian Peninsula, with some characteristic symptoms: unusual transparency of the crown or defoliation, dwarf needles (microphylls), discoloration of foliage and premature death of the tree. *Pinus pinaster* decline has been associated with blue-stain fungi such as *Ophiostoma minus* (Hedgcock) H. & P. (Álvarez et al., 2015; Rodríguez Barreal et al., 2001). Another recently detected pathogenic root rot fungus (*Heterobasidion annosum* (Fr.) Bref) has also been associated with pine decline in central Spain (Prieto-Recio et al., 2015, 2012).

Some modelling studies have predicted the mortality or survival of Mediterranean conifer species such as *Pinus sylvestris* and *Pinus pinaster* through the interaction of different silvicultural variables (Bravo-Oviedo et al., 2006; Dieguez-Aranda et al., 2005). The growth-climate relationships in Mediterranean pines, including *Pinus pinaster*, have been also determined (Olivar et al., 2015). Multinomial Logistic Regression models can be used to determine the probability of transition of growth through attributes such as mortality (Boltz and Carter, 2006) and diameter growth (Escalante et al., 2011).

During the last three decades, Maritime pine decline (associated with high rates of mortality) has been observed in several stands in the center of the Iberian Peninsula, which could compromise the future of these valuable forests. The aim of this study was therefore to determine the factors influencing *Pinus pinaster* decline by characterizing the study plots and constructing a Multinomial Logistic Regression model to predict health status at the individual tree level.

Material and methods

Study sites

Twenty seven natural or naturalized stands of *Pinus pinaster* were selected throughout the region of Castile and Leon, covering the main provenance regions of the species in the center of the Iberian Peninsula (Figure 4.1).

Figure 4.1. Selected provenance regions of *Pinus pinaster* (Alía et al., 1996). P2=Sierra del Teleno, P3=Sierra de Oña, P6=Sierra de Gredos, P8=Meseta Castellana and P9=Montaña de Soria-Burgos.

The 27 circular plots of radius 15 m (10 belonging to the Permanent Sample Plots Network of the Sustainable Forest Management Research Institute, University of Valladolid-INIA, and 17 to the Spanish National Forest Inventory [NFI]) were measured and installed between July and October 2011. The sampling plots were selected at random in seven zones by the ArcGIS 9.3 program. Zoning was determined according to the provenance region of *Pinus pinaster* and province (Table 4.1 and Figure 4.2). One zone was established per provenance region, except for the Meseta Castellana. This provenance region is distributed across three provinces, each with different management practices and different climatic and soil conditions, so that three different zones were included within this region.

Table 4.1. Numeration, location, UTM coordinates (Huso 30T, European Datum 1950), elevation, orientation and slope of each plot.

Plot	Provenance region	Province	Zone	Location	UTM X	UTM Y	Elevation (m)	Orientation	Slope (%)
1	Meseta Castellana (P8)	Burgos	**z1**	Quintana del Pidio	438050	4624914	878	NE	5
2	Meseta Castellana (P8)	Burgos	**z1**	La Horra 2	428067	4623997	838	NE	1
3	Meseta Castellana (P8)	Burgos	**z1**	Villalba de Duero	436109	4617106	848	W	3
4	Meseta Castellana (P8)	Burgos	**z1**	La Horra 1	429010	4622082	846	S	1
5	Meseta Castellana (P8)	Burgos	**z1**	Gumiel de Izan	443698	4619970	877	NW	2
6	Meseta Castellana (P8)	Burgos	**z1**	Aranda de Duero	439870	4616948	875	W	5
7	Sierra de Oña (P3)	Burgos	**z2**	Pino de Bureba	464930	4728035	653	N	25
8	Sierra de Oña (P3)	Burgos	**z2**	Aguas Cándidas	460290	4729979	791	S	7
9	Sierra de Oña (P3)	Burgos	**z2**	Trespaderne	466925	4739067	570	NE	32
10	Sierra de Gredos (P6)	Ávila	**z3**	El Hornillo 1	319548	4457352	1070	NW	22
11	Sierra de Gredos (P6)	Ávila	**z3**	El Hornillo 3	319320	4458936	990	S	41
12	Sierra de Gredos (P6)	Ávila	**z3**	El Arenal	323267	4457600	980	SW	10
14	Sierra del Teleno (P2)	León	**z5**	Pinilla de la Valdería	244808	4678992	892	-	0
15	Sierra del Teleno (P2)	León	**z5**	Castrocontrigo	234580	4677595	971	-	0
16	Sierra del Teleno (P2)	León	**z5**	Nogarejas	238790	4677439	940	N	2
17	Meseta Castellana (P8)	Soria	**z6**	Quintana Redonda	530065	4609290	998	-	0
18	Meseta Castellana (P8)	Soria	**z6**	Tardelcuende	531744	4603836	964	S	3
19	Meseta Castellana (P8)	Soria	**z6**	Matamala-Almazán	532346	4594757	953	-	0
20	Meseta Castellana (P8)	Soria	**z6**	Hortezuela	512669	4594367	835	SW	2
21	Meseta Castellana (P8)	Soria	**z6**	Tajueco	514407	4599867	852	W	5
22	Meseta Castellana (P8)	Soria	**z6**	Bayubas de Arriba	506542	4601245	1012	-	0
23	Montaña de Soria - Burgos (P9)	Burgos	**z7**	Salas de los Infantes	474429	4654855	1059	W	23
24	Montaña de Soria - Burgos (P9)	Burgos	**z7**	Gete	475962	4644113	1020	W	15
25	Montaña de Soria - Burgos (P9)	Burgos	**z7**	Huerta del Rey	470226	4632229	1046	S	25
13	Meseta Castellana (P8)	Segovia	**z4**	Iscar	375925	4581622	757	-	0
26	Meseta Castellana (P8)	Segovia	**z4**	Cuellar	392975	4581898	793	-	0
27	Meseta Castellana (P8)	Segovia	**z4**	Arroyo de Cuellar	388039	4579097	469	-	0

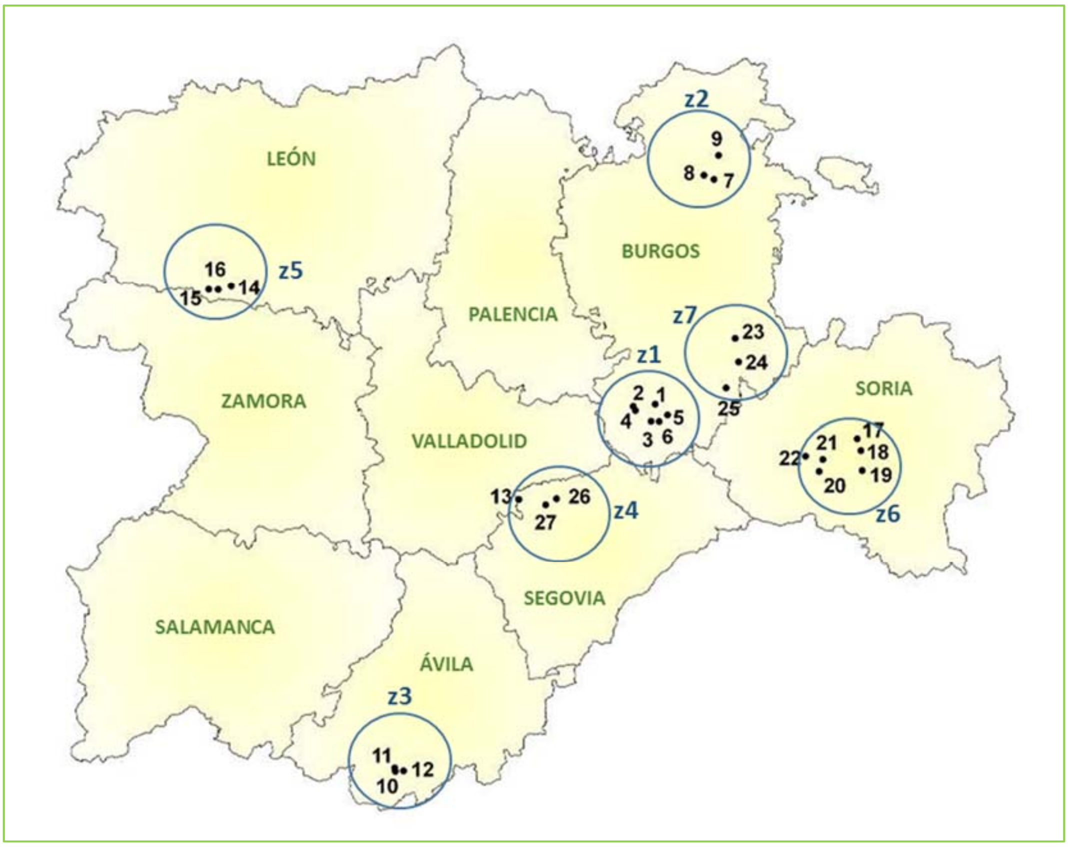


Figure 4.2. Location of the sampling plots (1 to 27), provinces (León, Palencia, Burgos, Zamora, Valladolid, Soria, Salamanca, Ávila and Segovia) and zones (z1=Ribera del Duero, z2=Oña, z3=Gredos, z4=Cuellar, z5=Teleno, z6=Soria, z7=Montaña Soriano-Burgalesa).

Field sampling

The center of each plot was marked and the UTM coordinates were recorded with a GPS receptor (E Trex H Personal Navigator. Garmin Corporation. Kansas, USA). To register the position of each tree, the angle (0 to 400 degrees) was measured using a compass fitted a fixed tripod. At the same time, the distance from the center to each tree was recorded using a VERTEX hypsometer. Each measured tree was marked with a numbered sheet at the base of the stem to enable identification during the present sampling and in future studies.

All trees within the 15 m radius were measured for silvicultural characterization (a total of 1208 trees) and 24 trees per plot were assessed by phytosanitary criteria (a total of 648 trees). Environmental variables (climate and soil properties) were also recorded at plot level.

Silvicultural characterization was conducted at the individual tree level and at stand level (Table 4.2). Dendrometric variables such as height (total, at the maximum crown width, and at the base of the crown), diameter at breast height and the four crown radii at the maximum crown width, were measured in all trees in each plot. The social classes of each tree (dominant, predominant, codominant and suppressed) were also assessed in accordance with Level I of the European network methodology (SPCAN-DGB, 2007). The density (trees/ha), basal area, dominant height, regeneration and several indexes such as Hart and Reineke, were also estimated at stand level. Silvicultural history (e.g. number of stumps, species composition and operations such as thinning, pruning and resin yield) was also recorded to characterize the forest management actions carried out in each plot.

Edaphic parameters were analysed by a soil sample extraction to 30 cm of depth (Martín-García et al., 2012) in each of the study plots. Soil samples in each plot were mixed, homogenized and stored in plastic bags, which were hermetically sealed and sent to the ITAGRA.CT laboratory for analysis of basic parameters (texture, pH, CE; P, K, Na, Mg, Ca, MO, CT, CaO), coarse fragments, Fe, N, C/N and CCC (Table 4.2). The pH was determined potentiometrically with a pH meter, in a soil solution (1:2.5, soil/water). Organic matter was determined by the $K_2Cr_2O_7$ method. Total N was determined by Kjeldahl digestion. Soil available P was extracted by the Olsen procedure and determined photometrically by the molybdenum-blue method. Soil exchangeable cations (K^+, Na^{+2}, Ca^{+2} and Mg^{+2}) were extracted with ammonium acetate and determined by atomic absorption/emission spectroscopy. The soil Fe was extracted by the Diethylenetriaminepentaacetic acid (DTPA) extraction method (1:2 ratio), and the concentration was estimated by atomic absorption spectroscopy. Particle-size distribution was determined by the Bouyoucos method (hydrometer method), and International Society of Soil Science (ISSS) classification was applied. The Cationic Exchange Capacity (CEC) was determined by Bascomb's method (i.e. the exchange cations were displaced by Ba ions, which were then displaced by Mg ions and the remaining concentration of Mg was determined by titration against EDTA).

The climatological data (i.e. monthly, seasonal and annual precipitation, temperatures and solar radiation) were obtained for each study plot (Table 4.2) from the Climatic Digital Atlas of the Iberian Peninsula (Ninyerola et al., 2005).

To perform forest health assessment, the 6 trees per quadrant closest to the center of the plot were chosen according to Level I of the European network methodology (SPCAN-DGB, 2007). In each of 24 trees per plot (6x4), diverse phytosanitary variables were assessed in the field to define the forest health status (Table 4.2). The most notable symptoms, such as defoliation, discoloration and microphylls, were detected using binoculars. Defoliation was visually estimated by a single operator throughout the whole study, in twenty classes of 5 % interval width, based on an absolute reference tree (Sánchez Peña et al., 1994), according to Level I of the European network methodology (Montoya et al., 1998). Discoloration was measured in four ranks of percentage (0-10% no discoloration, 11-25% light, 26-60% moderate and 60-100% severe). The presence of other variables such as microphylls, terminal bud damage, dieback, needle retention, wounds caused by resin extraction, number of pine cones and cracks in the bark (caused by wood compression) were assessed in the field. The presence of parasitic plants (mistletoe), bark beetle galleries and exit holes, and collar fungus (blue-stain and rot root fungus) were also noted for future reference.

Table 4.2. Summary of the main variables used to characterize stand level, environmental and health status in the 27 plots under study. Mean (sd) values are shown.

Stand level characterization															
Age (years)	Stumps (nº)	Regeneration (trees/ha)	Suppressed (%)	Other species (%)	Crown height (m)	Crown area (m^2)	Basal area (m^2/ha)	Density (trees/ha)	Dm (cm)	Hm (m)	HoCorreg (m)	HART	REINEKE	CE	ICV
66.9 (3.3)	10.5 (3.4)	12968.2 (4539.2)	11.7 (2.1)	2.4 (1.13)	5.4 (0.42)	21.4 (1.9)	26.5 (2.0)	591.6 (61.5)	29 (1.6)	13.2 (0.8)	15.7 (0.6)	31.3 (1.7)	521.7 (32.9)	47.6 (1.4)	0.55 (0.03)
Forest health assessment															
Crown defoliation (%)	Crown discoloration (%)	Live crown ratio (%)	Dieback (%)	Microphylls (%)	Cankers (%)	Cracks (%)	Number of pine cones (nº)	Needle retention (years)	Dead trees (%)	Damaged trees (%)	Healthy trees (%)				
36 (1.7)	29.1 (1.8)	86.8 (1.8)	9.1 (1.59)	17.9 (4.3)	6.9 (1.75)	39.5 (5.4)	4.6 (0.46)	3.7 (0.13)	9.8 (1.75)	28.2 (1.9)	62 (2.9)				
Environmental characterization															
Soil properties	Coarse fragments (%)	Sand (%)	Silt (%)	Clay (%)	pH	OM (%)	N (%)	C/N	P (mg kg^{-1})	K (mg kg^{-1})	Ca(cmol$_c$ kg^{-1})	Mg(cmol$_c$ kg^{-1})	CEC(cmol$_c$ kg^{-1})	Fe(mg kg^{-1})	
	25.5 (4.2)	85.0 (2.4)	7.3 (1.21)	7.8 (1.49)	6.6 (0.19)	1.2 (0.27)	0.05 (0.01)	25.3 (1.6)	4.1 (0.62)	65.2 (12.8)	7.5 (2.48)	0.42 (0.05)	9.5 (0.96)	30.3 (2.9)	
Climatic variables	Winter precipitation (mm)	Spring precipitation (mm)	Summer precipitation (mm)	Autumn precipitation (mm)	Annual precipitation (mm)	Minimum winter temperature (°C)	Minimum spring temperature (°C)	Minimum summer temperature (°C)	Minimum autumn temperature (°C)	Minimum annual temperature (°C)					
	172.3 (20.4)	169.9 (7.0)	81 (2.2)	216.8 (24.5)	636.1 (52.8)	0.03 (0.14)	6.3 (0.14)	11.2 (0.2)	2.8 (0.15)	5.1 (0.21)					
	Maximum winter temperature (°C)	Maximum spring temperature (°C)	Maximum summer temperature (°C)	Maximum autumn temperature (°C)	Maximum annual temperature (°C)	Mean winter temperature (°C)	Mean spring temperature (°C)	Mean summer temperature (°C)	Mean autumn temperature (°C)	Mean annual temperature (°C)					
	10.3 (0.1)	19.5 (0.2)	27.4 (0.3)	12.6 (0.2)	17.6 (0.2)	5.1 (0.12)	13.1 (0.2)	19.3 (0.2)	7.7 (0.11)	11.4 (0.1)					

Statistical analysis

Hierarchical cluster and principal component analysis (PCAs)

Principal component analysis (PCAs) was carried out with the aim of reducing the dimensionality of the dataset and to identify the main factors explaining the variation in the data for inclusion in the model explained below. The mean value of each variable per plot was calculated, and the percentage of trees with the corresponding symptomatology within each plot was estimated for the binary variables. Each plot was characterized in relation to the groups of variables defining them (stand level characterization, soil properties, climatic variables and health status assessment) to determine correlations within each group of variables. Associations between plots were assessed by cluster analysis based on the factorial axes defined by the variables retained in the previous PCAs. Hierarchical cluster analysis grouped the study plots using the Ward method by computing the Euclidean squared distances. The final grouping was obtained by the centroids of each cluster and k-means refining of groups. These analysis were carried out using R program Version 2.15.0; (R Development Core Team, 2012).

Multinomial Logistic Regression model (MLR)

A Multinomial Logistic Regression (MLR) model (Hosmer and Lemeshow, 1989) was constructed to predict the health status of *Pinus pinaster* at the individual tree level. The MLR technique was used to explore the influence of environmental attributes (soil properties and climatic variables) and the tree and stand level attributes, retained in the previous PCAs, on the categorical health status of the tree (healthy, symptomatic and dead). Significant axes were selected by applying the broken-stick method available in the “BiodiversityR” package (Kindt, 2014). The PCA axes with higher percentages of variance than broken-stick variances were considered significant. Variables with high absolute value (within each retained axe) were selected for inclusion in the MLR model.

The objective of using this technique was to calculate the probability of occurrence of a polytomous event, the presence or absence of various factors and the value of these factors. Hence, this method estimated the probability of occurrence of an event with more than two categories. In this case, analysis was carried out at the individual tree level, including all the phytosanitary trees assessed (648 in total).

The response categorical variable ($Y1, Y2, \dots, Yk$) was defined as Healthy (H), Symptomatic (S) or Dead (D), with healthy trees (H) considered as reference trees. The independent variables or covariables ($X = \{ X_1, X_2, \dots, X_n \}$) were selected in the previous PCAs.

We therefore had three categories, $k=3$, for calculating three probabilities:

$p1=p(Y=1)$

$p2=p(Y=2)$

$p3=1-(p1+p2)$

Where:

$p1 = \exp(Z1) / (1 + \exp(Z1) + \exp(Z2))$

$p2 = \exp(Z2) / (1 + \exp(Z1) + \exp(Z2))$

$p3 = 1 / (1 + \exp(Z1) + \exp(Z2))$

And with:

$Z1 = \text{logit}(p1) = \beta_{01} + \beta_{11} X_1 + \beta_{21} X_2 + \dots + \beta_{n1} X_n$

$Z2 = \text{logit}(p2) = \beta_{02} + \beta_{12} X_1 + \beta_{22} X_2 + \dots + \beta_{n2} X_n$

Where, $\beta_{01}, \beta_{11}, \beta_{21} \dots \beta_{n1}$ and $\beta_{02}, \beta_{12}, \beta_{22} \dots \beta_{n2}$ are the parameters that we wanted to estimate. Estimation by maximum likelihood was used to obtain the values of the dependent variable.

The independent variables were selected using the stepwise method. This method combines forward and backward processes for the selection of the variables and was based on conditional contrasts of likelihood. The models were compared using the Akaike Information Criterion (AIC), the Schwarz Criterion (SC) and the statistical likelihood -2 LOG L. The analysis were implemented using the PROC LOGISTIC procedure in SAS v.9.2 (SAS Institute INC. SAS/STAT® and USA., 2004).

Results

Characterization of the sampling plots

All variables were grouped as follows: silvicultural variables (for stand level characterization), soil properties and climatic variables (for environmental characterization) and phytosanitary variables (for forest health assessment). The cluster analysis separated the plots according to each of these four groups of study variables.

The dendrogram obtained by the hierarchical cluster analysis considering the silvicultural variables is shown in Figure 4.3. In this characterization, two groups of plots with similar stand level characteristics were observed. The first group (in black) was the set of plots geographically located in the south (provinces of Soria, Segovia and Avila), and the second group (in red) was associated with plots located geographically in the north (provinces of León and Burgos).

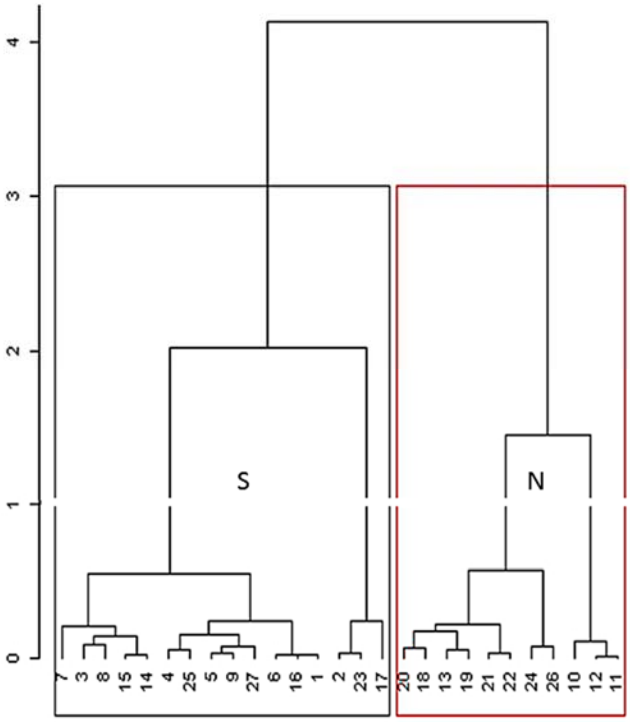


Figure 4.3. Hierarchical clustering of the study plots in relation to stand level characterization. S=plots located in the south. N=plots located in the north. The numbers below correspond to the 27 plots under study. For numbering, see Table 4.1 and Figure 4.2.

Hierarchical clustering in relation to the edaphic characterization was represented in a dendrogram (Figure 4.4). The sampling plots were grouped in two different clusters with similar soil properties. The first cluster (in black) corresponded to the plots located in the Castilian Plateau area (Meseta Castellana provenance). The second cluster (in red) corresponded to the plots in the Mountain Range areas (Sierra de Oña, Sierra del Teleno, Sierra de Gredos and Montaña de Soria-Burgos provenances).

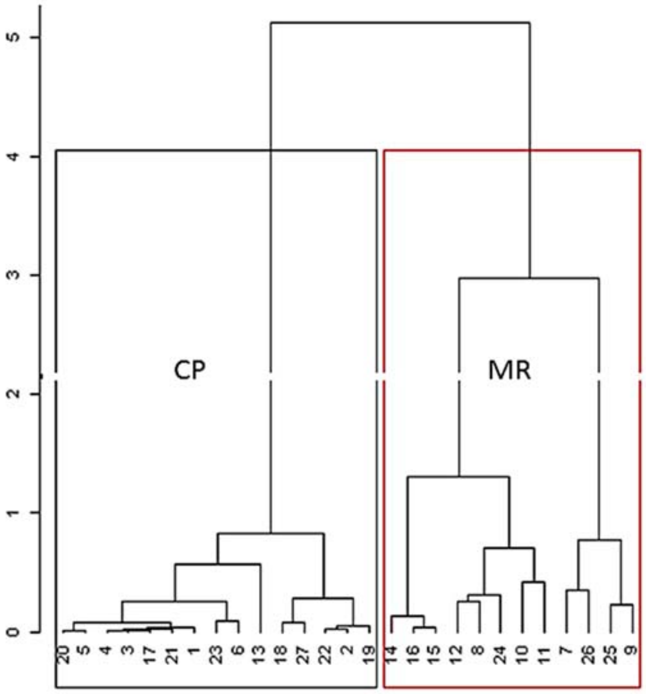


Figure 4.4. Hierarchical clustering of the study plots in relation to soil properties. CP=plots located in the Castilian Plateau area. MR=plots located in the Mountain Range areas. The numbers below correspond to the 27 plots under study. For numbering, see Table 4.1 and Figure 4.2.

Regarding climatic variables, the hierarchical clustering differentiated three groups (Figure 4.5). The first (in black) grouped plots located in the east of the region (zone z6 in Soria, and zone z1 in the east of Burgos). The second group (in red) matched plots from the west (z5 in León, z4 in Segovia and z2 and z7 in the west of Burgos). The third group (in green) represented the plots located in the south of the region, in the province of Avila (zone z3).

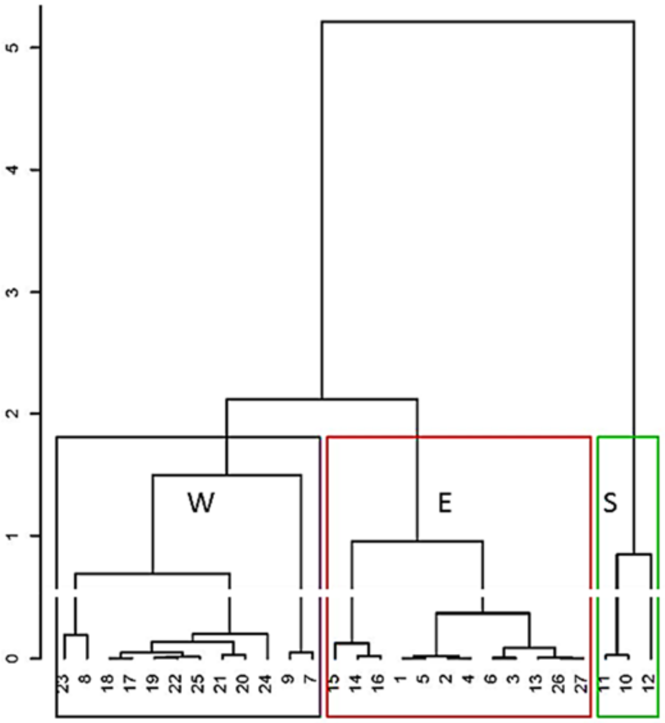


Figure 4.5. Hierarchical clustering of the study plots in relation to climatic variables. E=plots located in the east. W=plots located in the west. S=plots located in the south. The numbers below correspond to the 27 plots under study. For numbering, see Table 4.1 and Figure 4.2.

Finally, hierarchical clustering of health status variables classified the plots in three different groups with similar phytosanitary characteristics (dendrogram in Figure 4.6). The first group (in black) was represented by plots with good health status, mainly plots from zones z3, z4, z6 and z7. The second (in red) grouped plots with intermediate health conditions (mainly plots from z2 and z5). The third group (in green) represented plots with the poorest health conditions, including all the plots from zone z1 in Ribera del Duero (Burgos).

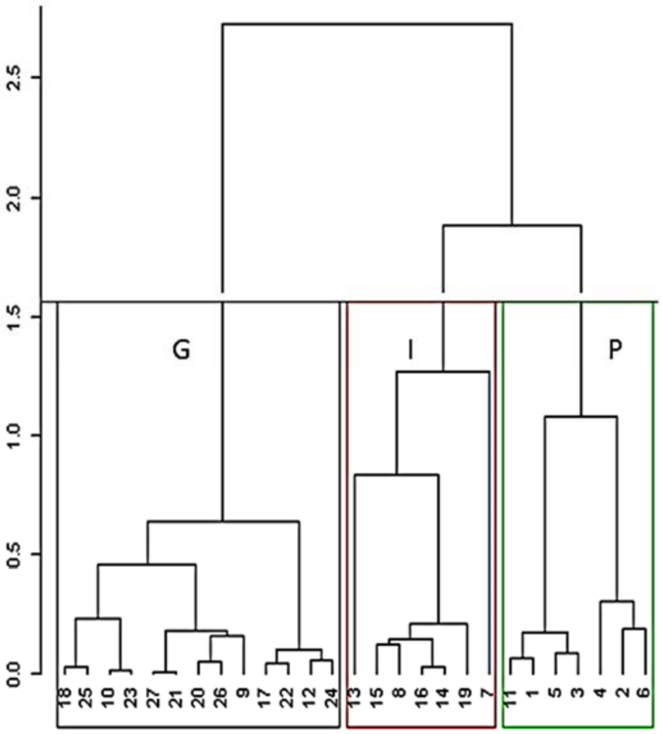


Figure 4.6. Hierarchical clustering of the study plots in relation to phytosanitary conditions. G=plots with good health status. I=plots with intermediate health status. P=plots with poor health status. The numbers below correspond to the 27 plots under study. For numbering, see Table 4.1 and Figure 4.2.

Selection of variables to construct the MLR model

Principal component analysis (PCAs) was applied to the silvicultural variables (stand and tree level characterization) and environmental variables (soil properties and climatic variables) to avoid the use of highly correlated variables in subsequent models.

The broken-stick method retained one axis in the PCA for individual tree level characterization (cumulative percentage of variance, ca.77%), and three axes in the PCA for stand level characterization (cumulative percentage of variance, ca.75%). For environmental variables, this method retained two axes in the PCA for soil properties (cumulative percentage of variance, ca.62 %), and three axes in the PCA for climatic variables (cumulative percentage of variance, ca.85 %) (Table 4.3).

For the next step, the following variables were selected from the axes retained in the PCAs: diameter at breast height (DBH), for individual tree level characterization; mean total height of the stand (Hm), stand density (trees/ha) and mean height of the crown, for stand level characterization (Table 4.4); cation exchange capacity (CEC) and pH, as soil variables; and annual mean temperature, average spring precipitation and average summer precipitation, as climatic variables (Table 4.5).

Table 4.3. Selection of significant axes from principal component analysis (PCAs) by the broken-stick method.

		Axes							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tree level characterization	Eigenvalue	3.08	0.66	0.15	0.11	0.00	0.00	0.00	0.00
	Percentage of variance	**76.96**	16.54	3.67	2.82	0.00	0.00	0.00	0.00
	Broken-stick percentage	**52.08**	27.08	14.58	6.25	0.00	0.00	0.00	0.00
Stand level characterization	Eigenvalue	6.32	3.77	1.89	1.19	0.95	0.67	0.57	0.19
	Percentage of variance	**39.53**	**23.55**	**11.80**	7.44	5.94	4.19	3.55	1.18
	Broken-stick percentage	**21.13**	**14.88**	**11.75**	9.67	8.11	6.86	5.82	4.92
Soil properties	Eigenvalue	6.63	2.73	1.41	1.25	0.80	0.64	0.50	0.41
	Percentage of variance	**44.23**	**18.19**	9.38	8.36	5.35	4.25	3.33	2.72
	Broken-stick percentage	**22.12**	**15.45**	12.12	9.90	8.23	6.90	5.79	4.84
Climatic variables	Eigenvalue	10.84	4.12	2.82	1.17	0.85	0.44	0.25	0.22
	Percentage of variance	**51.64**	**19.62**	**13.45**	5.57	4.04	2.10	1.21	1.05
	Broken-stick percentage	**17.36**	**12.60**	**10.22**	8.63	7.44	6.49	5.69	5.01

Note: PCA axes with higher percentages of variance than broken-stick variances are significant.

Table 4.4. Coefficients of correlation between silvicultural variables at tree level and at stand level, and the significant axes of the principal component analysis (PCAs).

			PC1		
Silvicultural Characterization	Tree level	**DBH** (cm)	**3.370**		
		DBH^{-1} (cm^{-1})	-3.346		
		Ratio H-D	2.761		
		Htotal (m)	2.866		
			PC1	PC2	PC3
	Stand level	Age (years)	-0.817	0.542	0.267
		Stumps (no)	-0.312	-0.651	-0.015
		Regeneration (trees/ha)	-0.082	0.090	-0.015
		Suppressed (%)	0.865	-0.184	-0.479
		Other species (%)	0.448	0.090	-0.412
		Crown height (m)	-0.348	0.496	**-0.853**
		Crown area (m^2)	-0.768	0.756	-0.145
		Basal Area (m^2/ha)	-1.057	-0.277	-0.063
		Density (trees/ha)	0.141	**-1.055**	0.060
		Dm (cm)	-0.944	0.590	0.015
		Hm (m)	**-1.073**	0.241	-0.175
		Ho (70 years) (m)	-0.648	-0.367	-0.592
		Hart	0.759	0.703	0.172
		Reineke	-0.935	-0.580	-0.022
		CE	-0.401	-0.767	-0.348
		ICV	-0.646	-0.262	0.805

Note: The highest correlations are shown in bold type. These variables were selected for constructing the MLR model.

Table 4.5. Coefficients of correlation between environmental variables (both edaphic and climatic) and the significant axes of the principal component analysis (PCAs).

			PC1	**PC2**	
Environmental Characterization	**Soil properties**	Slope (%)	0.877	-0.137	
		pH	0.485	**0.966**	
		Coarse fragments (%)	0.313	-0.411	
		Sand (%)	-0.955	0.130	
		Silt (%)	0.725	-0.427	
		Clay (%)	0.972	0.136	
		OM (%)	0.899	-0.454	
		P (mg kg^{-1})	0.106	-0.421	
		K (mg kg^{-1})	0.892	-0.182	
		Ca (cmolc kg^{-1})	0.705	0.739	
		Mg (cmolc kg^{-1})	0.549	0.548	
		N (%)	1.050	-0.177	
		C/N	-0.731	-0.503	
		CEC (cmolc kg^{-1})	**1.060**	-0.072	
		Fe (mg kg^{-1})	0.274	-0.838	
			PC1	**PC2**	**PC3**
	Climatic variables	Elevation (m)	0.363	0.856	-0.221
		Winter precipitation (mm)	-0.611	0.778	0.206
		Spring precipitation (mm)	-0.532	**0.859**	0.255
		Summer precipitation (mm)	0.059	0.321	**0.840**
		Autumn precipitation (mm)	-0.601	0.770	0.174
		Annual precipitation (mm)	-0.629	0.787	0.234
		Minimum winter temperature (°C)	-0.793	-0.386	0.540
		Minimum spring temperature (°C)	-0.947	-0.214	0.143
		Minimum summer temperature (°C)	-0.939	0.122	-0.051
		Minimum autumn temperature (°C)	-0.743	-0.348	0.647
		Minimum annual temperature (°C)	-0.891	0.226	0.140
		Maximum winter temperature (°C)	-0.859	-0.440	-0.204
		Maximum spring temperature (°C)	-0.595	0.103	-0.353
		Maximum summer temperature (°C)	-0.718	0.285	-0.683
		Maximum autumn temperature (°C)	-0.702	-0.277	-0.305
		Maximum annual temperature (°C)	-0.843	0.041	-0.554
		Mean winter temperature (°C)	-0.895	-0.440	0.243
		Mean spring temperature (°C)	-0.826	-0.249	-0.219
		Mean summer temperature (°C)	-0.856	0.248	-0.457
		Mean autumn temperature (°C)	-0.914	-0.327	0.304
		Mean annual temperature (°C)	**-0.951**	-0.158	-0.036

Note: The highest correlations are shown in bold type. These variables were selected for constructing the MLR model.

Development of the MLR model

The characteristics of analysed trees (648 in total) for constructing the model are described in Table 4.6. The approach using Multinomial Logistic Regression was able to model the health status condition (healthy, symptomatic or dead) of *Pinus pinaster* at tree level. The model response was multi-categorical and the results revealed that the size (DBH), mean total height of the stand (Hm) and the seasonal summer precipitation (Ps) influenced the health status of *Pinus pinaster*.

Table 4.6. Description of trees analysed (648 in total).

Health Status	Frequency	Percentage	Accumulated Percentage
Symptomatic (S)	183	28.24	28.24
Dead (D)	63	9.72	37.96
Healthy (H)	402	62.04	100

We used the stepwise method to select the independent variables, combining forward and backward processes to select the variables. The selection was based on conditional contrasts of likelihood and the models were compared using the Akaike Information Criterion (AIC), the Schwarz Criterion (SC) and the statistical likelihood -2 LOG L (Table 4.7). Finally, the AIC for model selection was chosen (Table 4.7). The best model, in which loss of information was minimal, was indicated by the lowest value of AIC.

Table 4.7. Model development and significant variables. Comparisons between models using the Akaike Information Criterion (AIC), the Schwarz Criterion (SC) and by the statistical likelihood -2 LOG L.

	Pr<ChiSq	**AIC**	**SC**	**-2 LOG L**
Independet terms	<.0001	1144.308	1153.256	1140.308
Tree Level variables				
DBH	<.0001	1098.124	1116.020	1090.124
Stand Level variables				
DBH + **Hm**	<.0001	1094.468	1121.311	1082.468
Environmental variables				
DBH + Hm + **Ps**	<.0001	1090.991	1126.782	1074.991

Note: DBH=diameter at breast height. Hm=mean total height of the stand. Ps= summer precipitation.

To construct the final model, parameters were compared using the Wald process and the coefficients, confidence intervals and p-values were calculated. The overall goodness of fit of the model was performed by Chi-square test based on the likelihood ratio (Table 4.8).

Table 4.8. Results of the selected model including the parameter estimators, analysis of the maximum likelihood estimator and significance.

Parameter	Health Status	Estimators	Standard Error	Wald ChiSq	Pr>ChiSq
Intercept	S	2.0864	0.7194	8.4120	0.0037
Intercept	D	0.8920	1.0485	0.7238	0.3949
DBH	S	-0.0731	0.2899	34.4344	<.0001
DBH	D	-0.0932	0.0186	25.1346	<.0001
Hm	S	0.0724	0.0300	5.8245	0.0158
Hm	D	0.1008	0.0454	4.9437	0.0262
Ps	S	-0.0213	0.0083	6.6227	0.0101
Ps	D	-0.0178	0.0122	2.1295	0.1445

Note: DBH=diameter at breast height. Hm=mean total height of the stand. Ps=seasonal summer precipitation. S=symptomatic. D=dead.

The final results were two probability equations used to predict the health status of *Pinus pinaster* at tree level, one for the symptomatic trees category in function of the reference category (healthy trees) (Equation (1)) and another for the category of dead trees according to the reference category (healthy trees) (Equation (2)). In these probability equations, a negative effect of both the diameter at breast height and the summer precipitation was observed. Hence, trees with lower values of DBH and Ps were more likely to be included as symptomatic or dead. The positive effect of the mean height of the stand indicated that the trees were more likely to be considered healthy as Hm values increased.

(1)

$$p\ (S/H)\ =\ \frac{\exp\ (2.0864 - 0.0731*(DBH) + 0.0724*(Hm) - 0.0213*(Ps))}{(1 + \exp\ (2.0864 - 0.0731*(DBH) + 0.0724*(Hm) - 0.0213*(Ps)) + \exp\ (-0.0932*(DBH) +0.1008*(Hm))}$$

(2)

$$p\ (D/H)\ =\ \frac{\exp\ (-0.0932*(DBH) +0.1008*(Hm))}{(1 + \exp\ (2.0864 - 0.0731*(DBH) + 0.0724*(Hm) - 0.0213*(Ps)) + \exp\ (-0.0932*(DBH) +0.1008*(Hm))}$$

Discussion

Principal component analysis was successfully used for hierarchical clustering of the plots and to reduce the dimensionality of the large dataset for constructing a predictive model of the health status of *Pinus pinaster* at tree level.

The hierarchical clustering by silvicultural characterization revealed that plots were grouped by geographic location. Most of the plots grouped in southern Castile and Leon (z6 in Soria, z4 in Segovia and z3 in Avila) correspond to areas belonging to the Permanent Sample Plots Network of the Sustainable Forest Management Research Institute (University of Valladolid-INIA). These areas have been managed over the years in the same way and are generally pure stands aimed at timber production and resin extraction. The phytosanitary status of this group of plots was better than that of the other cluster. The other cluster grouped plots from z1, z2 and z7 in the province of Burgos and z5 in the province of León. Intensity of management was lower in these zones, generally characterized by high tree densities, high levels of competition for the resources and high percentages of dead trees in the stands. The stand structure, resulting from historical forest management, plays a fundamental role in the health status of *Pinus pinaster*. Indeed, dense stands displayed the highest tree mortality rates and decline symptoms, as already reported in other studies (Camarero et al., 2011; Galiano et al., 2010; Linares et al., 2009; Prieto-Recio et al., 2015; Sánchez-Salguero et al., 2013, 2012a). Several studies have shown that tree mortality is directly related to the rate of tree growth (van Mantgem et al., 2003; Wyckoff and Clark, 2000). However, competition for resources in *Pinus pinaster* stands in the study area generally involves belowground resources such as water availability (Bravo-Oviedo et al., 2006; Sánchez-Salguero et al., 2012b).

The hierarchical ordination of environmental variables (both climatic and edaphic) also yielded interesting results. It is well known that environmental variables determine tree growth and vigour, thus influencing production in *Pinus pinaster* stands (Bogino and Bravo, 2008; Bravo-Oviedo et al., 2011; Lara et al., 2013).

Cluster analysis based on soil properties generated two groups of plots: those located in Mountain Range areas and those located in the Castilian Plateau area. The first cluster grouped zones z2, z3, z5 and z7 (Sierra de Oña, Sierra de Gredos, Sierra del Teleno y Montaña de Soria-Burgos provenances), generally characterized by clay soils. However, the second cluster grouped zones from the Meseta Castellana provenance (zones z1, z4 and z6), which have sandy soils with less organic matter and a lower cation exchange capacity. Soil characteristics can influence the health status of the trees, and nutrients play role regarding the growth and vigour of the trees (Acevedo-Sandoval et al., 2004; Martín-García et al., 2012). However, *Pinus pinaster* can grow in nutrient-poor soils with low mineral requirements (Alvarez-Alvarez et al., 2010).

According to climatic variables, the results revealed three groups defined by geographic zones and/or altitudinal gradient. One of these clusters grouped only plots from zone z3 in the Sierra de Gredos provenance (Avila), which are quite different from the other zones with twice the average annual precipitation of other zones and slightly higher temperatures than in the other sampling areas. Other cluster grouped zones z2, z6 and z7 located in the east of the region (Soria and eastern Burgos) where precipitation levels are high during summer and spring. The final cluster grouped plots located in western Castile and Leon (z1 in Burgos, z4 in Segovia and z5 in León) characterized by lower precipitation during summer and spring than in the other zones. Drought events occurring during tree growth period have often been considered to incite forest decline as they weaken the tree and facilitate the entry of pathogenic agents that contribute to the death of the tree (Desprez-Loustau et al., 2006). Other studies on Mediterranean conifers suggest that long periods of drought significantly reduce the growth diameter of the tree, with trees dominated or suppressed by competition being most affected (Linares et al., 2011; Martín-Benito et al., 2008). However, other authors have reported that water use efficiency increases under drought conditions (Bogino and Bravo, 2014). The variability in local climatic conditions must therefore take into account in such studies.

Hierarchical clustering according to phytosanitary variables grouped the plots within three different groups with similar forest health conditions. Variables quantifying Maritime pine decline or health status have been used in other studies of forest decline (Klepzig et al., 1991; Oliva and Colinas, 2007). Defoliation is one of the most representative variables for studying the tree decline process (Galiano et al., 2010; Sánchez-Salguero et al., 2013, 2012a). Clusters formed by plots from z1 in Ribera de Duero (Burgos) have the poorest phytosanitary conditions, with a rate of defoliation higher than 40 %. This zone was traditionally dedicated to resin production and to a lesser extent to timber production, and during the 1980s and 1990s, these stands were abandoned (Picardo and Pinillos, 2013). Resin extraction is again being carried out in some areas, but stands previously tapped are more weakened and display more symptoms of decline and a high percentage of dead trees. The presence of biotic agents such as mistletoe, blue-stain and root rot fungus were observed during field sampling in this zone. However, the cluster with good health status grouped plots from zones z3 in Avila, z4 in Segovia, z6 in Soria and z7 in Montaña de Soria-Burgos provenance, all with a tradition of timber production. The quality of the *Pinus pinaster* timber in these zones is good and the stands have been historically managed and maintained for timber production. Hence, defoliation and other symptoms of decline are less evident, partly because symptomatic or dead trees are usually eliminated. Finally, plots clustered in the intermediate health status class were mainly from z2 in Sierra de Oña and z5 in Sierra del Teleno. In these zones the wood quality is not as high, and management and maintenance are less intensive. Nevertheless, these stands are developing well due to the good climate and soil conditions. Most of the damage appears to be caused by abandonment of forest management practices. A lower intensity of thinning is thus causing high levels of competition, and symptomatic and dead trees are therefore more common in these stands (Prieto-Recio et al., 2015). Other studies investigating tree competition suggest similar behaviour (Bravo-Oviedo et al., 2006; Linares et al., 2010; Ruiz-Benito et al., 2013).

The model selected for predicting *Pinus pinaster* health status in the center of the Iberian Peninsula includes the following variables: diameter at breast height (DBH), mean total height of the stand (Hm) and historical seasonal summer precipitation of the plot (Ps). The silvicultural and environmental factors thus appear to explain the health status or the probability that a tree is healthy, has symptoms of decline or is dead. In the same way, other studies have yielded positive results by relating environmental and management factors to phytosanitary factors (Dieguez-Aranda et al., 2005; Fontes et al., 2010; Hennon et al., 2009; Scherm, 2004). The use of Multinomial Logistic Regression (MLR) models seems to indicate that this technique resolves problems related to the use of continuous and categorical variables and problems related to the use of individual and stand level variables together (Pando and San-Martín, 2004). Models simulating tree growth of

Mediterranean pine species have been developed with this technique, with interesting results (Escalante et al., 2011).

For tree and stand levels, the following silvicultural variables were retained in the principal component analysis (PCAs): diameter at breast height (DBH), mean total stand height (Hm), mean height of the crown and stand density (trees/ha). Thus, the size of the trees and the stand structure are the most important variables used to study many aspects of the forest, including forest health status. The present findings are consistent with those of Sánchez-Salguero et al., (2012a), who found that tree size variables such as DBH and crown height were good indicators for studying basal area increments comparing defoliated and non-defoliated trees in four Mediterranean pine species. The stand structure has also been assessed to predict the mortality of *Pinus sylvestris* and *Pinus pinaster* (Bravo-Oviedo et al., 2006). This study on Maritime pine demonstrated that the survival probability increased as the diameter of the tree increased and stand density decreased. However, in relation to intraspecific competition, stand density reduces the capacity of the trees to adapt to environmental stress (Linares et al., 2010). Our findings on silvicultural factors suggest similar behaviour, i.e. the larger trees and lower density for competing for resources will lead to a better health status of the trees and greater capacity to adapt to biotic and abiotic stress.

The following edaphic variables were retained in the PCA: cation exchange capacity (CEC) and pH. Both have been taken into account in other studies assessing the effect of environmental variability on site index of Mediterranean Maritime pine (Bravo-Oviedo et al., 2011) and to detect nutrition-related problems in the growth of poplars, based on visual crown conditions (Martín-García et al., 2012). As in our study, those variables are of interest for inclusion in the analysis, but did not prove significant in either case.

With respect to climatic variables, annual mean temperature, average spring precipitation and average summer precipitation were retained in the PCA with high correlation and maximum absolute values. Many studies at the global level have contemplated climate change with the variations in precipitation and temperatures and their influence on forest health status, determining whether they act as predisposing, inciting or contributing factors for forest decline (Auclair et al., 1996; Benito Garzón et al., 2009; Cook and Johnson, 1989; Lindner et al., 2009; Sánchez-Salguero et al., 2012b). Historical climate may act as a predisposing factor for Maritime pine decline, leading to a decrease in tree vigour and health status, while drought events caused by the climate during the past three decades may act as inciting or even contributing factors, as shown in previous studies with other species (Bigler et al., 2006; Sánchez-Salguero et al., 2012b). Average summer precipitation in the plot (Ps), obtained from historical climate data since 1950 (Ninyerola et al., 2005), was the most significant environmental variable explaining the health status of *Pinus pinaster*. This is consistent with other studies with similar results on the influence of summer precipitation on other types of forest decline (Helama et al., 2009; Rehfeldt et al., 2009).

The silvicultural and environmental factors influencing the health status of *Pinus pinaster* include the size of the tree (DBH), stand structure (Hm) and the climate (Ps), all of which play an important role in predicting Maritime pine decline. The negative effect of diameter at breast height indicates that tree with lower values of DBH are more likely to be symptomatic or dead. Nevertheless, mean stand height (Hm) has a positive effect indicating that as the Hm increases, the trees are less likely to be classified as symptomatic or dead status. Finally, seasonal summer precipitation also had a negative effect in the model, indicating that at lower values of Ps, the trees are more likely to be classified as symptomatic or dead.

Conclusions

The following environmental and silvicultural variables were related "*a priori*" to the *Pinus pinaster* decline: density (trees/ha), diameter at breast height, mean height of the crown and mean total height of the stand, for silvicultural characterization; cation exchange capacity and pH, for soil properties; and annual mean temperature, average spring precipitation and average summer precipitation, for the climatic variables.

We established different groups for characterizing the sampling plots by the sets of study variables (stand level characterization, soil properties, climatic variables and forest health assessment). Generally, hierarchical clustering separated the groups by different geographic areas or provinces and therefore related to the different provenance regions of *Pinus pinaster*. The results suggest that zones with a tradition of timber production, where the stands have been historically managed and maintained, have a better health status than zones with lower wood quality, where the lower intensity of thinning leads to symptomatic and dead trees being left in the stand, thus triggering high levels of competition for the resources.

A model was constructed to predict the probability of a particular health status for individual *Pinus pinaster* trees (healthy, symptomatic or dead). The model developed includes variables related to the size of the tree (diameter at breast height [DBH]), stand structure (mean total height of the stand [Hm]) and climate (seasonal summer precipitation of the plot [Ps]). DBH and Ps have a negative effect and Hm has a positive effect on the Multinomial Logistic Regression model for predicting Maritime pine decline.

A more detailed study of the variables considered, together with analysis of field core samples (essential for determining growth during the last 20 years) and wood samples (to identify the pathogenic fungi associated with the phenomenon) is necessary to shed further light on the problem of *Pinus pinaster* decline.

References

Acevedo-Sandoval, O., Ortiz-Hernández, E., Cruz-Sánchez, M., Cruz-Chávez, E., 2004. Role of Iron Oxides in Soils. Terra Latinoam. 485–497.

Alía, R., Martín, S., De Miguel, J., Galera, R., Agúndez, D., Gordo, J., Salvador, L., Catalán, G., Gil, L., 1996. Regiones de procedencia, *Pinus pinaster* Aiton., Ministerio de Medio Ambiente, Organismo Autónomo Parques Nacionales (DGCONA), Madrid.

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1–9.

Alvarez-Alvarez, P., Afif Khouri, E., Camara-Obregon, A., Castedo-Dorado, F., Barrio-Anta, M., 2010. Effects of foliar nutrients and environmental factors on site productivity in *Pinus pinaster* Ait. stands in Asturias (NW Spain). Ann. For. Sci. 68, 497–509.

Auclair, A., Lill, J., Revenga, C., 1996. The role of climate variability and global warming in the dieback of Northern Hardwoods. Water Air Soil Pollut. 91, 163–186.

Benito Garzón, M., Sánchez de Dios, R., Sainz Ollero, H., 2009. Effects of climate change on the distribution of Iberian tree species. Appl. Veg. Sci. 11, 169–178. doi:10.3170/2008-7-18348

Bigler, C., Bräker, O.U., Bugmann, H., Dobbertin, M., Rigling, A., 2006. Drought as an inciting mortality factor in scots pine stands of the Valais, Switzerland. Ecosystems 9, 330–343. doi:10.1007/s10021-005-0126-2

Bogino, S.M., Bravo, F., 2008. Growth response of *Pinus pinaster* Ait. to climatic variables in central Spanish forests. Ann. For. Sci. 65, 506.

Bogino, S.M., Bravo, F., 2014. Carbon stable isotope-climate association in tree rings of *Pinus pinaster* and *Pinus sylvestris* in Mediterranean environments. Bosque (Valdivia) 35, 175–184. doi:10.4067/S0717-92002014000200005

Boltz, F., Carter, D.R., 2006. Multinomial logit estimation of a matrix growth model for tropical dry forests of eastern Bolivia. Can. J. For. Res. Can. Rech. For. 36, 2623–2632.

Bravo-Oviedo, A., Gallardo-Andres, C., del Río, M., Montero, G., 2010. Regional changes of *Pinus pinaster* site index in Spain using a climate-based dominant height model. Can. J. For. Res. Can. Rech. For. 40, 2036–2048.

Bravo-Oviedo, A., Roig, S., Bravo, F., Montero, G., del Río, M., 2011. Environmental variability and its relationship to site index in Mediterranean Maritime pine. For. Syst. 20, 50–64.

Bravo-Oviedo, A., Sterba, H., del Río, M., Bravo, F., 2006. Competition-induced mortality for Mediterranean *Pinus pinaster* Ait. and *P. sylvestris* L. For. Ecol. Manage. 222, 88–98. doi:10.1016/j.foreco.2005.10.016

Camarero, J.J., Bigler, C., Linares, J.C., Gil-Pelegrin, E., 2011. Synergistic effects of past historical logging and drought on the decline of Pyrenean silver fir forests. For. Ecol. Manage. 262, 759–769.

Canales, J., Avila, C., Canovas, F.M., 2011. A Maritime pine antimicrobial peptide involved in ammonium nutrition. Plant Cell Environ. 34, 1443–1453.

Chakraborty, S., Tiedemann, A. V, Teng, P.S., 2000. Climate change: potential impact on plant diseases. Environ. Pollut. 108, 317–326.

Cook, E.R., Johnson, H., 1989. Climate Change and Forest Decline - a Review of the Red Spruce Case. Water Air Soil Pollut. 48, 127–140.

del Río, S., Herrero, L., Penas, A., 2009. Recent climatic trends in Castile and Leon (Spain) and its possible influence on the potential natural vegetation. Acta Bot. Gall. 156, 625–636.

Desprez-Loustau, M.-L., Marcais, B., de la Rocque, S., 2006. Climatic factors and diseases in natural ecosystems and agroecosystems. Biofutur 37–40.

DGCONA, 2002. Mapa Forestal Español. Base de datos de la naturaleza, Ministerio de Medio Ambiente. Madrid.

Dieguez-Aranda, U., Castedo-Dorado, F., Alvarez-Gonzalez, J.G., Rodriguez-Soalleiro, R., 2005. Modelling mortality of Scots pine (*Pinus sylvestris* L.) plantations in the northwest of Spain. Eur. J. For. Res. 124, 143–153.

Escalante, E., Pando, V., Ordonez, C., Bravo, F., 2011. Multinomial logit estimation of a diameter growth matrix model of two Mediterranean pine species in Spain. Ann. For. Sci. 68, 715–726.

Fontes, L., Bontemps, J., Bugmann, H., Oijen, M. Van, Gracia, C., Kramer, K., Lindner, M., Rötzer, T., Skovsgaard, J.P., 2010. Models for supporting forest management in a changing environment. For. Syst. 19, 8–29.

Galiano, L., Martínez-Vilalta, J., Lloret, F., Martinez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor decline of Scots pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems 13, 978–991. doi:10.1007/s10021-010-9368-8

Hansen, A.J., Neilson, R.R., Dale, V.H., Flather, C.H., Iverson, L.R., Currie, D.J., Shafer, S., Cook, R., Bartlein, P.J., 2001. Global change in forests: Responses of species, communities, and biomes. Bioscience 51, 765–779.

Helama, S., Laanelaid, A., Raisio, J., Tuomenvirta, H., 2009. Oak decline in Helsinki portrayed by tree-rings, climate and soil data. Plant Soil 319, 163–174.

Hennon, P.E., D'Amore, D.V., Schaberg, P.G., Wittwer, D.T., Shanley, C.S., 2012. Shifting climate, altered niche, and a dynamic conservation strategy for yellow-cedar in the North Pacific coastal Rainforest. Bioscience 62, 147–158.

Hennon, P.E., McWilliams, M.G., 1999. Decline symptoms do not develop with grafting from dying yellow-cedar. Can. J. For. Res. 29, 1985–1988.

Hennon, P.E., Wittwer, D.T., D'Amore, D.V., Lamb, M., 2009. Yellow-cedar decline: Key landscape features and snow modeling of a climate-induced forest decline on a dormant volcano. Phytopathology 99, S52–S52.

Hertel, G., 1988. Proceedings of the US/FRG research symposium: effects of atmospheric pollutants on the spruce-fir forests of the Eastern United States and the Federal Republic of Germany. 1987 October 19-23; Burlington, VT., in: USDA For. Serv., Gen. Tech. Rep. NE-120.

Hosmer, D.W., Lemeshow, S., 1989. Applied logistic regression. Willey Interscience. New York.

Jactel, H., Nicoll, B.C., Branco, M., Gonzalez-Olabarria, J.R., Grodzki, W., Langstrom, B., Moreira, F., Netherer, S., Orazio, C., Piou, D., Santos, H., Schelhaas, M.J., Tojic, K., Vodde, F., 2009. The influences of forest stand management on biotic and abiotic risks of damage. Ann. For. Sci. 66.

Kindt, R., 2014. Package "BiodiversityR". Package for Community Ecology and Suitability Analysis. CRAN.

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an Insect-Fungal Complex with Red Pine Decline in Wisconsin. For. Sci. 37, 1119–1139.

Lara, W., Bravo, F., Maguire, D.A., 2013. Modeling patterns between drought and tree biomass growth from dendrochronological data: A multilevel approach. Agric. For. Meteorol. 178-179, 140–151. doi:10.1016/j.agrformet.2013.04.017

Linares, J.C., Camarero, J.J., Carreira, J.A., 2009. Interacting effects of changes in climate and forest cover on mortality and growth of the southernmost European fir forests. Glob. Ecol. Biogeogr. 18, 485–497.

Linares, J.C., Camarero, J.J., Carreira, J.A., 2010. Competition modulates the adaptation capacity of forests to climatic stress: insights from recent growth decline and death in relict stands of the Mediterranean fir *Abies pinsapo*. J. Ecol. 98, 592–603.

Linares, J.C., Tiscar, P.A., Tíscar, P.A., 2011. Buffered climate change effects in a Mediterranean pine species: range limit implications from a tree-ring study. Oecologia 167, 847–859. doi:10.1007/s00442-011-2012-2

Lindner, M., Maroschek, M., Netherer, S., Kremer, A., Barbati, A., Garcia-Gonzalo, J., Seidl, R., Delzon, S., Corona, P., 2009. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For. Ecol. Manage. 259, 698–709. doi:10.1016/j.foreco.2009.09.023

Manion, P.D., 1991. Tree Disease Concepts, Prentice-Hall.

Martín-Benito, D., Cherubini, P., del Río, M., Canellas, I., 2008. Growth response to climate and drought in *Pinus nigra* Arn. trees of different crown classes. Trees-Structure Funct. 22, 363–373.

Martín-García, J., Merino, A.A., Diez, J.J., 2012. Relating visual crown conditions to nutritional status and site quality in monoclonal poplar plantations (*Populus x euramericana)*. Eur. J. For. Res. 131, 1185–1198. doi:10.1007/s10342-011-0590-5

Montoya, M., Sánchez, G., González, M.R., Jiménez, R., 1998. La Red Europea De Seguimiento De Daños En Los Bosques (Nivel 1) En España, 1987-1996. Ed. Org. Autónomo Parques Nac. (Ministerio Medio Ambient. Madrid, España. 557 pp.

Ninyerola, M., Pons, X., Roure, J.M., 2005. Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Univ. Autónoma Barcelona, Bellaterra. ISBN 932860-8-7.

Oliva, J., Colinas, C., 2007. Decline of silver fir (*Abies alba* Mill.) stands in the Spanish Pyrenees: Role of management, historic dynamics and pathogens. For. Ecol. Manage. 252, 84–97.

Olivar, J., Bogino, S., Spiecker, H., Bravo, F., 2015. Changes in climate-growth relationships and IADF formation over time of pine species (*Pinus halepensis, P. pinaster and P. sylvestris*) in Mediterranean environments. For. Syst. 24, 1–9.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with decline of longleaf pine in the southeastern United States. Plant Soil 217, 145–150.

Pando, V., San-Martín, R., 2004. Regresión Logística Multinomial. Cuad. la Soc. Española Ciencias For.

Payette, S., Fortin, M.J., Morneau, C., 1996. The recent sugar maple decline in southern Quebec: Probable causes deduced from tree rings. Can. J. For. Res. Can. Rech. For. 26, 1069–1078.

Picardo, A., Pinillos, F.M., 2013. La resinación en España y en el mundo en 2013: Situación y perspectivas, in: II Simposio Internacional de Resinas Naturales, Del 16 Al 18 de Abril de 2013, Coca - Segovia. pp. 30–53.

Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. doi:10.1016/j.foreco.2015.07.033

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770.

R Development Core Team, 2012. R: a language and environment for statistical computing. R Found. Stat. Comput. ISBN3-900051-07-0, URLhttp// www.r-project.org, Vienna.

Rehfeldt, G.E., Ferguson, D.E., Crookston, N.L., 2009. Aspen, climate, and sudden decline in western USA. For. Ecol. Manage. 258, 2353–2364. doi: 10.1016/j.foreco.2009.06.005

Rodríguez Barreal, J.A., Saiz de Omeñaca, J.A., Domínguez Núñez, J.A., Giaretta, M., Soria Carreras, S., Rodríguez-Carmona, Á., 2001. Estudio de las enfermedades que afectan a *Pinus pinaster* Ait. en montes de Patrimonio Nacional de el Escorial (Madrid) y determinación del agente o agente causales. Patrim. Nac. Serv. Jard. y montes. Madrid.

Rodríguez-Soalleiro, R., Lucas, J.A., Alejano, R., del Río, M., Torres, E., Cantero, A., Serrada, R.J., 2008. Selvicultura de *Pinus pinaster* subespecie *mesogeensis*, in: Montero, G., Serrada, R., Reque, J.A. (Eds.), Compendio de Selvicultura Aplicada En España. INIA y Fundacion del Conde del Valle de Salazar, pp. 399–430.

Roe, G.H., Baker, M.B., 2007. Why is climate sensitivity so unpredictable? Science (80-.). 318, 629–632.

Ruiz-Benito, P., Lines, E.R., Gomez-Aparicio, L., Zavala, M.A., Coomes, D.A., 2013. Patterns and drivers of tree mortality in Iberian forests: Climatic effects are modified by competition. PLoS One 8.

Sánchez Peña, G., Economou, A., Beccu, E., Canu, G., Cocco, S., Bussotti, F., Cenni, E., Cozzi, A., Ferretti, M., Conceicao, M., 1994. Especies Forestales Mediterráneas. Guía para la Evaluación de Copas., CEC-UN/ECE, Brussels, Geneva.

Sánchez-Salguero, R., Camarero, J.J., Dobbertin, M., Fernandez-Cancio, A., Vila-Cabrera, A., Manzanedo, R.D., Zavala, M.A., Navarro-Cerrillo, R.M., 2013. Contrasting vulnerability and resilience to drought-induced decline of densely planted vs. natural rear-edge *Pinus nigra* forests. For. Ecol. Manage. 310, 956–967.

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Camarero, J.J., Fernández-Cancio, Á., 2012a. Selective drought-induced decline of pine species in southeastern Spain. Clim. Change 113, 767–785. doi:10.1007/s10584-011-0372-6

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Swetnam, T.W., Zavala, M.A., 2012b. Is drought the main decline factor at the rear edge of Europe? The case of southern Iberian pine plantations. For. Ecol. Manage. 271, 158–169. doi:10.1016/j.foreco.2012.01.040

SAS Institute INC. SAS/STAT®, USA., I., 2004. User's Guide. Version 9.2. Cary, NC SAS Inst.

Scherm, H., 2004. Climate change: can we predict the impacts on plant pathology and pest management? Can. J. Plant Pathol. Can. Phytopathol. 26, 267–273.

SPCAN-DGB, 2007. Red de Seguimiento de Daños en los Montes (Red CE de Nivel I), Manual de Campo. Ministerio de Medio Ambiente.

Thomas, F.M., Blank, R., Hartmann, G., 2002. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. 32, 277–307.

van Mantgem, P.J., Stephenson, N.L., Mutch, L.S., Johnson, V.G., Esperanza, A.M., Parsons, D.J., 2003. Growth rate predicts mortality of *Abies concolor* in both burned and unburned stands. Can. J. For. Res. 33, 1029–1038.

Wyckoff, P.H., Clark, J.S., 2000. Predicting tree mortality from diameter growth: a comparison of maximum likelihood and Bayesian approaches. Can. J. For. Res. 30, 156–167.

Chapter 5: A multilevel approach for modelling tree growth and aridity in relation to stand competition and health status of *Pinus pinaster* in Spain

Abstract

Climatic stressors such as drought are considered a primary cause of forest decline worldwide. Forest decline is a complex process caused by the interaction of several interchangeable biotic and abiotic factors. For decades now, increased mortality and forest decline due to drought stress and competition have been affecting Maritime pine stands in the Iberian Peninsula. We applied the BIOdry multilevel algorithm to *Pinus pinaster* dendrochronological and climate data in order to: *i*) model and analyse growth patterns of trees in different phytosanitary conditions; *ii*) estimate historic water availability through annual aridity indexes covering the last three decades across the study sites; and *iii*) compute correlations between tree growth and aridity index at tree level, according to health status and density class. A total of 424 wood cores from healthy, symptomatic and dead trees were collected in 27 plots distributed throughout northern Spain. We detected variance in growth patterns among trees with different phytosanitary conditions within the sampling zones. Annual aridity index trends over time across the study sites revealed a clear chain of drought events in the early 1990s, in 1994 and in 2005. Mantel correlations between tree growth and aridity index showed significant responses at tree level in relation to health status and defoliation levels. We also observed significant values in Mantel correlograms between tree growth and aridity index according to Stand Density Index classes, pointing to the serious implications of competition-growth-climate relationships. These results may provide an essential tool for forest management.

Keywords: *Dendrochronology, BIOdry, CLIMATOL, Maritime pine decline, natural or naturalized stands, Stand Density Index.*

Introduction

Climate change during the 20th century has increased seasonal alterations in temperature and precipitation regimes and is affecting the productivity of forest ecosystems (Boisvenue and Running, 2006; Choat et al., 2010; Gonzalez-Munoz et al., 2014; Lenoir et al., 2008). Climatic stressors such as drought (water deficit) are considered a main cause of forest decline around the world (Adams et al., 2009; Allen et al., 2010; Ayres and Lombardero, 2000; Cailleret et al., 2014). Drought can be regarded as a transitory phenomenon related to meteorological variability, with and spatial and temporal repercussions of varying intensity and persistence. Aridity is the permanent or seasonal lack of rainfall and reflects local climate conditions (Cook et al., 2004).

Transitional climate zones between arid and humid regions, where water is a limiting resource, are especially interesting for studying the relationships between climate and tree growth and vigour (Bogino et al., 2009; Boisvenue and Running, 2006; Rozas et al., 2011b). Mediterranean regions can provide an appropriate laboratory for studying forest responses to water deficit, as climate change is increasing the frequency and severity of extreme climatic events such as droughts there (Manrique and Fernandez-Cancio, 2000). Continued change could alter Mediterranean forest ecosystem dynamics (Hampe and Petit, 2005), leading to high mortality episodes and forest decline processes (Allen et al., 2010).

Recent research has associated decline in Mediterranean pine forests with adverse climatic conditions (Girard et al., 2012; Linares et al., 2011; Navarro Cerrillo et al., 2007; Sánchez-Salguero et al., 2013, 2012a, 2012b). The absence of silvicultural practices and high stand density have also triggered mortality and decline processes (Bravo-Oviedo et al., 2006; Camarero et al., 2011; Linares et al., 2009; Prieto-Recio et al., 2015). Forest decline is a complex process caused by the interaction of several interchangeable biotic and abiotic factors (Manion, 1991). These factors can be classified into three categories: predisposing or long term factors such as historical climate or site, inciting factors such as severe short-term climatic events, and contributing factors such as pest and diseases that trigger tree death (Kliejunas et al., 2009).

The *Pinus pinaster* Ait (Maritime pine) forest ecosystem in Spain has suffered forest decline due to interactions between biotic/abiotic factors and water deficit (Álvarez et al., 2015; Madrigal-González and Zavala, 2014; Prieto-Recio et al., 2015, 2012). Affected trees of this species characteristically show defoliation, unusual transparency and discoloration at the crown, die-back symptoms, small needles (microphylls), premature senescence and early tree death. Maritime pine is the conifer that colonizes the largest area in the Iberian Peninsula. In Spain alone, the species covers over 700,000 hectares in pure stands and 600,000 hectares in mixed woods (del Río et al., 2004). *Pinus pinaster* and other Mediterranean pine forests constitute important ecosystems that are classified as natural habitats of community interest in EU Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora.

Dendrochronological methods have been used to evaluate the outcomes of drought stress on forest decline and tree defoliation (Dobbertin, 2005; Gea-Izquierdo et al., 2014; Sánchez-Salguero et al., 2012b; Sangüesa-Barreda et al., 2015b). Tree-ring width variability has been mainly related to climate oscillations (Fritts, 1976), but other studies suggest that climate-growth relationships can be altered by ecological changes from fires, competition, pests, or disease (Amoroso et al., 2015, 2011; Camarero et al., 2015b; Linares et al., 2010). Conventional dendrochronological analysis can be improved with new models and multivariate ordination techniques that solve statistical problems arising from the hierarchical factors observed during dendrochronological series comparisons (Lara et al., 2013).

The aim of this study is to understand alterations in *Pinus pinaster* growth by considering different phases of decline in relation to changes in water availability in recent decades. We simulated Maritime pine tree growth responses to drought using the BIOdry multilevel algorithm (Lara et al., 2013) in order to look at the effects of the sampling zones, provenances, stand density and health status on the relationships between tree growth and drought during last decades. This involved: *i*) modelling and analysis of tree growth and aridity patterns in trees with different phytosanitary conditions, *ii*) estimation of historic water availability based on annual aridity indexes for the last three decades across the study sites, and *iii*) analysis of tree-level relationships between tree growth and aridity based on health status and density classes.

Material and methods

Site description and sampling procedure

This study was carried out on natural or naturalized stands of *Pinus pinaster* ssp. *mesogeensis* Fieschi & Gaussen, distributed across most of the provenance regions in central Spain (Castile and Leon) (Figure 5.1). The sample design was validated in previous studies (Prieto-Recio et al., 2015). We sampled stands of Maritime pine that had previously been inventoried in the second and third stages of the Spanish National Forest Inventory (NFI), the most extensive inventory in the Iberian Peninsula, or stands that are monitored by the forestry research staff of the Sustainable Forest Management Research Institute (SFMRI). We sampled trees in 10 radial plots (15 m radius) previously established by the SFMRI, and established 17 new radial plots (15 m radius) in stands measured by the NFI.

From July to October 2011, variables for stand level characterization were measured for all trees in each radial plot, for a total sample of 1208 trees (Table 5.2). The forest health variables described below (Table 5.3) were assessed in 24 trees per plot, for a total of 648 trees. Two wood cores per tree were also taken from 9 trees per plot (3 healthy, 3 symptomatic or damaged and 3 dead, when available). A total of 424 wood cores were collected: 162 from healthy trees, 162 from symptomatic trees and 100 from standing, recently dead trees.

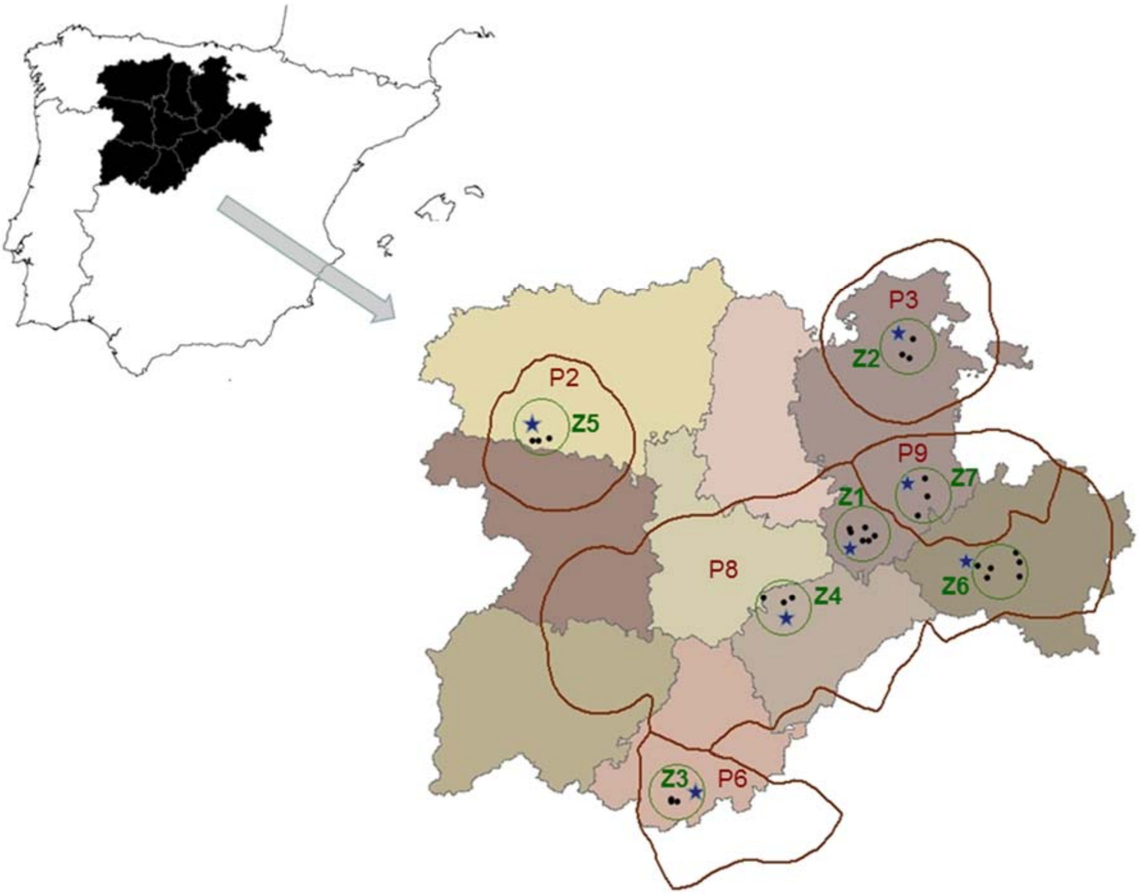


Figure 5.1. Geographic location of the study plots (black dots) and the meteorological stations (blue stars) within the seven different zones (green circles) and provenance regions (red lines). See Table 5.1 for zones and provenances and Table 5.4 for meteorological stations.

Zoning and silvicultural characterization

Sampling was grouped into seven zones according to the provenance region of *Pinus pinaster* and the provincial units of Spain (Table 5.1 and Figure 5.1). There was one zone per provenance region, except for the Meseta Castellana, the central plateau that extends across three provinces, each with different management practices, climate and soil conditions (Prieto-Recio et al., 2015). There, we maintained the provincial units in order to detect potential effects of actions related to forest management on tree growth-climate relationships.

Table 5.1. Location of the study plots: provenance region, province, zone and geographic coordinates.

Provenance	**Province**	**Zone**	**Location**	**Longitude (N)**	**Latitude (W)**	**Elevation (m)**
Meseta Castellana **(P8)**	Burgos	**z1**	Quintana del Pidio	3° 44’ 48”	41° 46’ 18”	878
			La Horra 2	3° 52’ 00”	41° 45’ 46”	838
			Villalba de Duero	3° 46’ 09”	41° 42’ 05”	848
			La Horra 1	3° 51’ 18”	41° 44’ 44”	846
			Gumiel de Izán	3° 40’ 42”	41° 43’ 40”	877
			Aranda de Duero	3° 43’ 26”	41° 42’ 01”	875
Meseta Castellana **(P8)**	Segovia	**z4**	Iscar	4° 29’ 06”	41° 22’ 29”	757
			Cuellar	4° 16’ 52”	41° 22’ 47”	793
			Arroyo de Cuellar	4° 20’ 23”	41° 21’ 13”	469
Meseta Castellana **(P8)**	Soria	**z6**	Quintana Redonda	2° 38’ 25”	41° 37’ 58”	998
			Tardelcuende	2° 37’ 14”	41° 35’ 01”	964
			Matamala de Almazán	2° 36’ 49”	41° 30’ 07”	953
			Hortezuela	2° 50’ 58”	41° 29’ 56”	835
			Tajueco	2° 49’ 42”	41° 32’ 54”	852
			Bayubas de Arriba	2° 55’ 22”	41° 33’ 40”	1012
Montaña de Soria-Burgos **(P9)**	Burgos	**z7**	Salas de los Infantes	3° 18’ 37”	42° 02’ 36”	1059
			Gete	3° 17’ 28”	41° 56’ 48”	1020
			Huerta del Rey	3° 21’ 36”	41° 50’ 22”	1046
Sierra de Gredos **(P6)**	Ávila	**z3**	El Hornillo 1	5° 07’ 22”	40° 14’ 43”	1070
			El Hornillo 3	5° 07’ 33”	40° 15’ 34”	990
			El Arenal	5° 04’ 45”	40° 14’ 54”	980
Sierra de Oña **(P3)**	Burgos	**z2**	Pino de Bureba	3° 25’ 46”	42° 42’ 08”	653
			Aguas Candidas	3° 29’ 11”	43° 43’ 10”	791
			Trespaderne	3° 24’ 21”	42° 48’ 06”	570
Sierra del Teleno **(P2)**	León	**z5**	Pinilla de la Valdería	6° 05’ 36”	42° 13’ 10”	892
			Castrocontrigo	6° 12’ 59”	42° 12’ 13”	971
			Nogarejas	6° 09’ 55”	42° 12’ 13”	940

For silvicultural characterization, diameter at breast height (DBH) and total height were measured for all trees. Social classes (dominant, predominant, codominant and suppressed) were assessed in consonance with the Level I manual of the European network methodology (SPCAN-DGB, 2007). The percentage of other tree species, density, basal area and the Hart and Reineke indexes, were also estimated at stand level (Table 5.2). Because density is a predisposing factor for triggering *Pinus pinaster* decline (Prieto-Recio et al., 2015), Stand Density Index classes were calculated and divided into H=high >700, M=medium 700-400, and L=low <400 (Valbuena et al., 2008). The upper limit of these classes was established at around 60% of the maximum Stand Density Index (SDI) to avoid overlap with density-related mortality (Thomas et al., 1993); the lower limit was set at 35% of maximum SDI when adequate site occupancy was maintained (Long, 1985).

Table 5.2. Silvicultural characterization in relation to the study zone, mean (sd).

	All stands	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Zone 6	Zone 7
Number of trees measured	*1208*	*274*	*118*	*97*	*88*	*151*	*302*	*178*
Age (years)	66.9 (3.3)	53.3 (6.1)	61.7 (11.7)	85 (0)	75 (5)	50 (11.5)	77.5 (3.4)	68.3 (10.1)
DBH (cm)	29 (1.6)	23.6 (1.7)	24.1 (3.5)	43.6 (2.0)	34.7 (2.3)	20.5 (2.7)	32.4 (2.9)	26.2 (3.1)
Height (m)	13.2 (0.8)	10 (0.6)	9.3 (1.83)	22.4 (0.3)	16.1 (0.6)	9.1 (0.85)	14.5 (0.8)	12.8 (1.3)
Density (trees/ha)	591.6 (61.5)	594.2 (129.8)	528.2 (108.8)	448 (54.4)	386.7 (75.5)	702.6 (110.9)	648.4 (188.8)	773.4 (297.8)
Basal area (m^2/ha)	26.5 (2.0)	19 (2.1)	18 (1.2)	47.8 (2.0)	26.3 (2.0)	18.6 (1.7)	31.5 (2.4)	26.3 (4.8)
Suppressed (%)	11.7 (2.1)	21.5 (1.6)	19.5 (3.8)	0 (0)	10.3 (8.1)	22 (6.3)	0.98 (0.98)	8.7 (3.8)
Other species (%)	2.4 (1.13)	7.7 (4.43)	1.5 (1.5)	0 (0)	2.6 (2.6)	0 (0)	0 (0)	2.2 (1.32)
HART	31.3 (1.7)	34.3 (3.1)	41 (7.1)	20.8 (2.2)	31.5 (4.2)	33.6 (0.4)	28 (3.0)	30.5 (7.7)
REINEKE (SDI)	521.7 (32.9)	406.9 (48.5)	381.4 (28.1)	813 (46.8)	489 (46.7)	413.8 (20.7)	613.9 (53.2)	556.4 (119.9)

Health status assessment

Crown defoliation was assessed visually by the first author in twenty classes of 5 % interval width, based on an absolute reference tree (Sánchez Peña et al., 1994), because defoliation estimates varied among observers. Other crown variables, such as discoloration, were measured according to Level I of the European network methodology (Eichhorn et al., 2010). Because the use of different vitality indicators is highly recommended (Cailleret et al., 2014), other phytosanitary variables or decline symptoms were also assessed, such as the presence of cankers, cracks in the bark (caused by wood compression), dieback, and small needles (microphylls) (Table 5.3). For this study, over 35% defoliation and over 25% discoloration were considered symptoms of decline. Trees were classified accordingly into three health status categories: healthy, symptomatic or damaged, and dead.

Table 5.3. Forest health assessment in relation to the study zone, mean (sd).

	All stands	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**
Number of trees phytosanitary assessed	*648*	*144*	*72*	*72*	*72*	*72*	*144*	*72*
Live crown ratio (%)	86.8 (1.8)	77.6 (3.6)	87.5 (5.1)	86.7 (3.2)	96.7 (3.3)	84.7 (4)	90.8 (3.5)	88.6 (6.3)
Crown defoliation (%)	36 (1.7)	43.1 (2.9)	29.2 (5.8)	32.9 (2.5)	30.6 (4.9)	41.9 (5)	34.4 (3.9)	34.3 (4.3)
Crown discoloration (%)	29.1 (1.8)	39.6 (3.3)	24.1 (2.4)	23.7 (3.9)	22.7 (2)	34.1 (3.1)	25.3 (4.2)	27.5 (5.1)
Dieback (%)	9.1 (1.59)	15.3 (3)	15.3 (7.3)	4.2 (2.40)	2.8 (1.39)	16.7 (2.4)	3.5 (1.99)	5.6 (2.78)
Microphylls (%)	17.9 (4.3)	41.2 (10.6)	5.7 (3.65)	0 (0)	13.3 (7.9)	37.7 (17.6)	8.6 (3.58)	4.9 (3.06)
Cankers (%)	6.9 (1.75)	15.3 (2.8)	0 (0)	13.9 (11.9)	5.6 (2.78)	2.8 (1.39)	1.4 (1.38)	6.9 (1.39)
Cracks (%)	39.5 (5.4)	47.9 (8.7)	58.3 (12.5)	33.3 (33.3)	61.1 (7.7)	26.4 (13.2)	31.9 (10.7)	16.7 (6.4)
Dead trees (%)	9.8 (1.75)	15.3 (4.6)	6.9 (3.67)	6.9 (5.01)	2.8 (3.9)	12.5 (4.2)	8.3 (3.57)	11.1 (6.1)
Symptomatic trees (%)	28.2 (1.9)	41 (1.3)	26.4 (3.7)	22.2 (5.6)	27.8 (5.6)	27.8 (6.1)	22.2 (3.7)	23.6 (2.8)
Healthy trees (%)	62 (2.9)	43.7 (4.4)	66.7 (6.4)	70.8 (4.2)	69.4 (7.3)	59.7 (10)	69.4 (6.1)	65.3 (3.7)

Dendrochronological methods

Two wood cores in each tree were extracted at 1.30 m above ground, forming a 120° angle between core samples (first core perpendicular to the centre of the plot, second core 120° to the right). Bark thickness for each core was measured in the field. The samples were then taken to the laboratory, air dried over absorbent paper for a month and mounted on wooden supports. A mechanical sander and sandpapers of varying grain were applied to each sample until its growth rings were clearly visible.

Tree-ring series were dated using a binocular microscope following standard dendrochronological procedures (Stokes and Smiley, 1968). The polished surfaces of the cores were subsequently scanned with an Epson Expression 1640 XL scanner (1100 dpi resolution). Annual growth rings from the 21 years prior so the study (1990 – 2010) were measured at 0.01 mm resolution using the WinDENDRO semiautomatic measurement programme (Regent Instrument Inc, 2002). For dead trees, the year of death was dated by synchronizing the narrow rings in these series with the series of healthy and symptomatic trees in the same plot. Broken and incomplete samples, mainly from dead trees, were discarded.

In order to detect dating and measurement errors, ring width series were cross-dated and analysed in the R environment for statistical computing (R Core Team, 2016) using the R-package dplR (Bunn, 2010). Summary statistics were calculated, including first-order autocorrelation and mean sensitivity. Each tree-ring series was cross-dated with the serial tree-ring width summary of all the other series belonging to the same plot. Serial data was assumed reliable at correlations above 0.5 for healthy trees and 0.3 for dead or symptomatic trees. Poorly correlated tree-ring series were checked and corrected with the WinDENDRO programme until the correlation was acceptable.

Climatic variables

Recorded meteorological data from 1980 to 2010 was provided by the National Meteorological Agency (AEMET, Spain). Meteorological stations were located within 40 km of the sampling sites and concordance between plot and station elevation was taken into account (Table 5.4).

Table 5.4. Location, geographic coordinates, and climatic data for the meteorological stations.

Zone	Location	Latitude (N)	Longitude (W)	Elevation (m)	Mean Precipitation (mm)	Mean Temperature (°C)
z1	Roa de Duero (Burgos)	41° 41' 23"	3° 55' 27"	780	418.1	11.7
z2	Oña (Burgos)	42° 43' 50"	3° 24' 47"	598	684.9	12.4
z3	Santa Cruz del Valle (Ávila)	40° 15' 12"	5° 00' 05"	725	1106.5	12.1
z4	Sanchonuño (Segovia)	41° 19' 25"	4° 18' 17"	803	464.0	12.3
z5	Tabuyo del Monte (León)	42° 17' 52"	6° 12' 47"	1020	635.7	10.5
z6	El Burgo de Osma (Soria)	41° 35' 10"	3° 04' 07"	895	502.0	10.7
z7	Santo Domingo de Silos (Burgos)	41° 57' 45"	3° 25' 12"	1003	648.4	11.0

Climatological data series were completed with data from the nearest stations and homogenised with CLIMATOL in the R package (Guijarro, 2011). Data series from the different zones were clustered into two groups in order to detect outliers and generate estimated data. Zones 1, 4, 6 and 7 formed a central plateau group, whereas zones 2, 3 and 5 composed a mountain range group. The *homogen* function was applied to automatically homogenize temperature and precipitation series for each group. This included filling in missing data along with detection and correction of outliers and shifts in the mean of the series.

Statistical analysis: BIOdry

We subtracted ring-width series trends and the computed annual aridity indexes (AAIs) using R-package BIOdry (Lara and Bravo, 2015; Lara et al., 2013). This package processes multilevel data frames (MDFs) containing serial records into initial columns, which is followed by recorded times (months, years, relative times, etc.), and ends with factor-column levels ordered from lower (usually a core-sample replicate, or an annual set of monthly meteorological records) to higher in the sampling hierarchy (plots, sites, or other spatial units). The package has several functions but we implemented only two of them to develop the trend subtraction and the multilevel correlograms (*modelFrame* and *muleMan*, respectively). The former function was implemented to normalize both the ring widths and the AAIs. The R-package (nlme) normalizes the series by fitting linear mixed-effects models with the *ringLme* function. Two kinds of model formulas, '*lmeForm*' and '*tdForm*', are available in the package to assist the detrending process; they implement functions with same names (Lara and Bravo, 2015). The *muleMan* function was used to compute the signatures between normalized series via multilevel correlograms.

Normalization of tree growth (Tree Radial Increments)

Multilevel normalization involved two principal steps: subtraction of long-term trends from ring-widths and serial assessment. The *modelFrame* function was applied for recursive multilevel modelling of tree radial increments (TRIs) (calculated from diameter at breast height without bark thickness) with health status, density and zone as factor-levels. The '*rtimes*','*scacum*' and '*amod*' arguments were applied: the first to calculate relative times from one to the number of observed years (up to 21 in this study); the second to scale the cumulative sums of radial increments as a constant value; the third to compute diameters based on simple allometric model parameters from TRIs. The formulas used to normalize the ring-widths in this step followed the '*tdForm*' (Time-decline formula) format.

Normalization of climatic variables (Annual Aridity Indexes)

Climatic variables normalized through aridity and Walter-Lieth climate diagrams, were used to compute annual aridity indexes (AAIs). The AAIs were calculated as the quotient between areas in dry and wet seasons, based on the graphs of average monthly temperature and total monthly precipitation (Walter and Lieth, 1967). The *modelFrame* function was used for recursive multilevel modelling and developed with the *'moveYr'* and *'wlai'* arguments, to order the months according to the hydrological year (October to September) and calculate AAI from Walter-Lieth diagrams, respectively. The formulas used to centre the AAIs in this step were formatted as: formula in form of covariate, *'lmeForm'*.

Correlation between tree growth and aridity

Mantel correlograms were computed from distance matrices of normalized series and permutation tests (Goslee and Urban, 2007). Thus, tree growth and aridity were compared on levels corresponding to the sample design of this study: by zone (zones 1 to 7), *Pinus pinaster* health status (healthy, symptomatic and dead) and Stand Density Index classes (high, medium and low). The *muleMan* function was applied to construct Mantel correlograms and compare patterns between TRI and AAI residuals.

Results

Tree growth

The differences in tree growth between sampling zones and health status are represented in Figure 5.2. Tree ring widths from 1990 to 2010 oscillated between 0.095 and 13.676 mm (sd = 1.23). Diameter at breast height (DBH) ranged from 7.7 to 57.3 cm (sd = 10.3) with bark and from 5.3 to 53.3 (sd = 9.55) without bark. Subtraction of the trends in the multiple levels of the TRI datasets (sampling zones and health status) produced common convex trends in the extracted fluctuations of tree growth (Figure 5.2). These were mostly centred around zero, generally distributed around +/- two standard deviations (sd), and slightly skewed towards the initial years of the series. The convex fluctuation patterns were characterized by negative extremes during the first recorded years (-4 sd), with maximal fluctuations observed from 1995 to 2000 (from +1.5 to +2 sd), and null values at year 2010 (0 sd).

The levels of agreement between extracted fluctuation patterns among individual trees depended on both sampling zones and health status (For zoning see Figure 5.1 and Table 5.1). From the perspective of sampling zones (Figure 5.2), the extracted fluctuations from trees belonging to z1, z4, z6 from the Meseta Castellana and z7 from the Montaña de Soria-Burgos exhibited greater uniformity than the corresponding extracted fluctuations from trees sampled in mountain range provenance zones 2, 3 and 5 (Sierra del Teleno, Sierra de Gredos and Sierra de Oña, respectively). Fluctuations from z1, z4, z6 and z7 were more widespread and symmetrically distributed along the recorded formation years of the measured tree rings than those observed in the remaining zones, suggesting greater tree-growth synchronization in these two neighbouring provenance regions (growth behaviour as even-aged stands) (see Figure 5.2). Conversely, fluctuations from the mountain range provenances were usually narrow and asymmetric (see Figure 5.2, z2, z3 and z5), suggesting the asynchronous tree growth behaviour of uneven-aged stands on these sites.

From the perspective of health status, the extracted fluctuation patterns from healthy or symptomatic living trees (Figure 5.2, Columns h and s) presented greater uniformity than the corresponding extracted fluctuations from dead trees (Figure 5.2, Column d). The higher number of sample replicates for living trees helped to depict trends in prevailing fluctuations more clearly, showing differences in the patterns of healthy and symptomatic trees according to the sampling zone. Uniformity of fluctuations among dead trees depended on both the sampling zones and the number of samples analysed per zone. In the zone with more viable samples of dead trees (Figure 5.2, Column d, z1), fluctuation patterns tended toward similarity with those of the living trees. The most obvious difference was the longer spans of the living trees. The remaining zones had fewer dead trees (Figure 5.2, see Column d, z2 to z7) and uniformity of fluctuations among trees was weakly depicted.

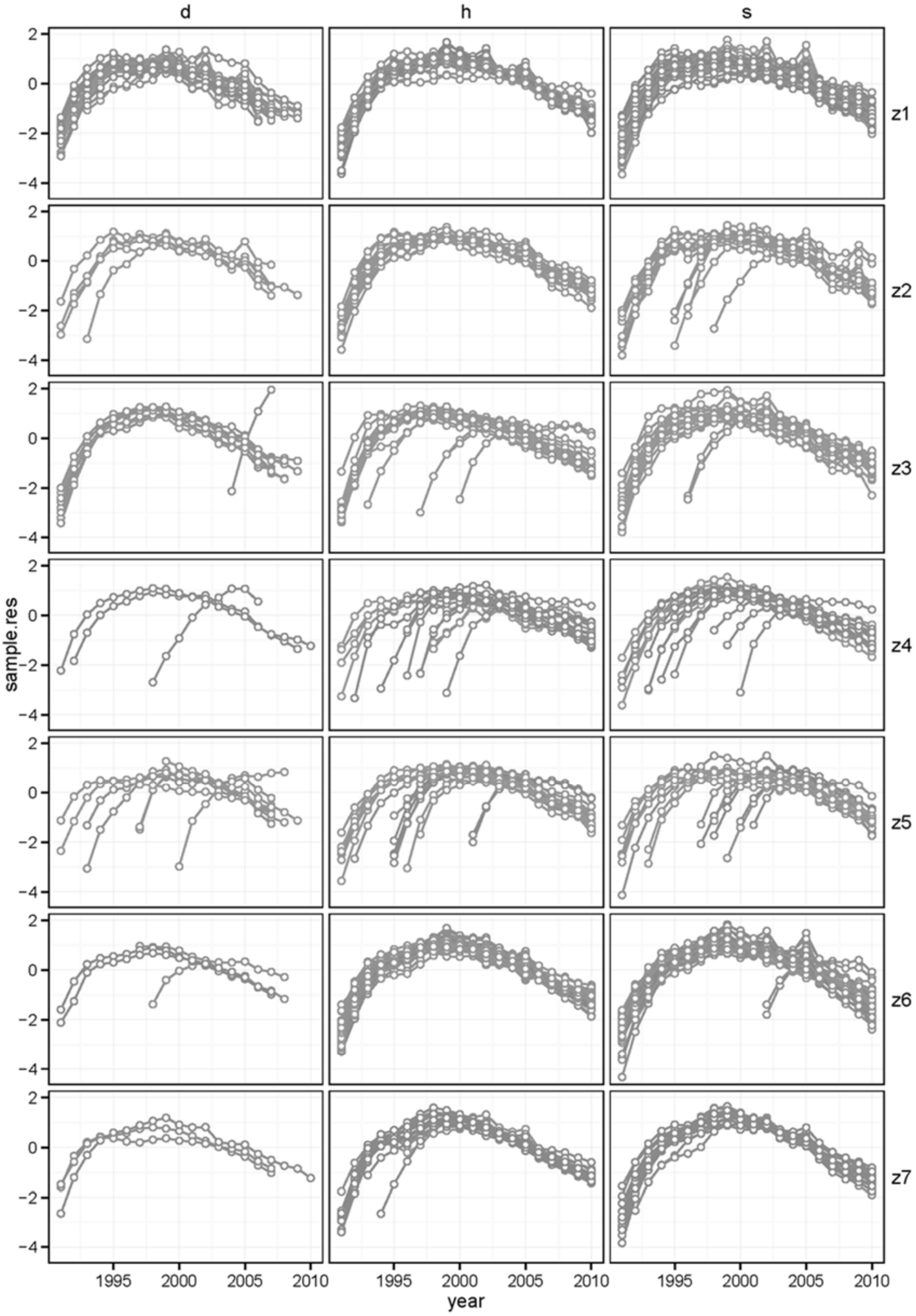


Figure 5.2. Studentized Residuals (sample.res) of the Tree Radial Increments (TRIs). Labels indicate sampling zones (z1 to z7) and health status (d=dead, h=healthy, s=symptomatic). Years are shown at the bottom (1990 to 2010). Standard deviations (sd) are indicated on the left side.

Aridity

The annual aridity indexes (AAIs) for 1980 to 2010 are represented in Figure 5.3, constructed from Walter-Lieth diagrams representing aridity based on seasonal rainfall and water deficits during the summer, when evapotranspiration exceeds precipitation. Higher AAI values correspond to more arid years when the water deficit was stronger. Trend subtraction across the multiple levels in the AAI datasets produced normalized trends in the extracted fluctuations for each of the zones studied (Figure 5.3). The normalized fluctuations in aridity were generally centred around zero and usually distributed between +/- two standard deviations, but also exhibited residual extremes outside this interval (extremes of aridity). Positive extremes indicated occurrences of high aridity while negative extremes suggested low aridity events.

High aridity events were frequently and consistently observed during two periods: around the beginning of the 1990s (1989-1991) and in 2005 (Figure 5.3, z1 to z7). The high aridity observed in 1994, was less pronounced than the other two periods cited. Lower aridity events primarily occurred between 1996 and 1998 (Figure 5.3, z1 to z7), along with other, less frequent low aridity events depending on the location of the sampling zones (Figure 5.3, see fluctuations around -2 sd). The mountain range zones (Figure 5.3, z2, z3 and z5) presented fewer high aridity events than the central areas (Figure 5.3, z1, z4, z6 and z7).

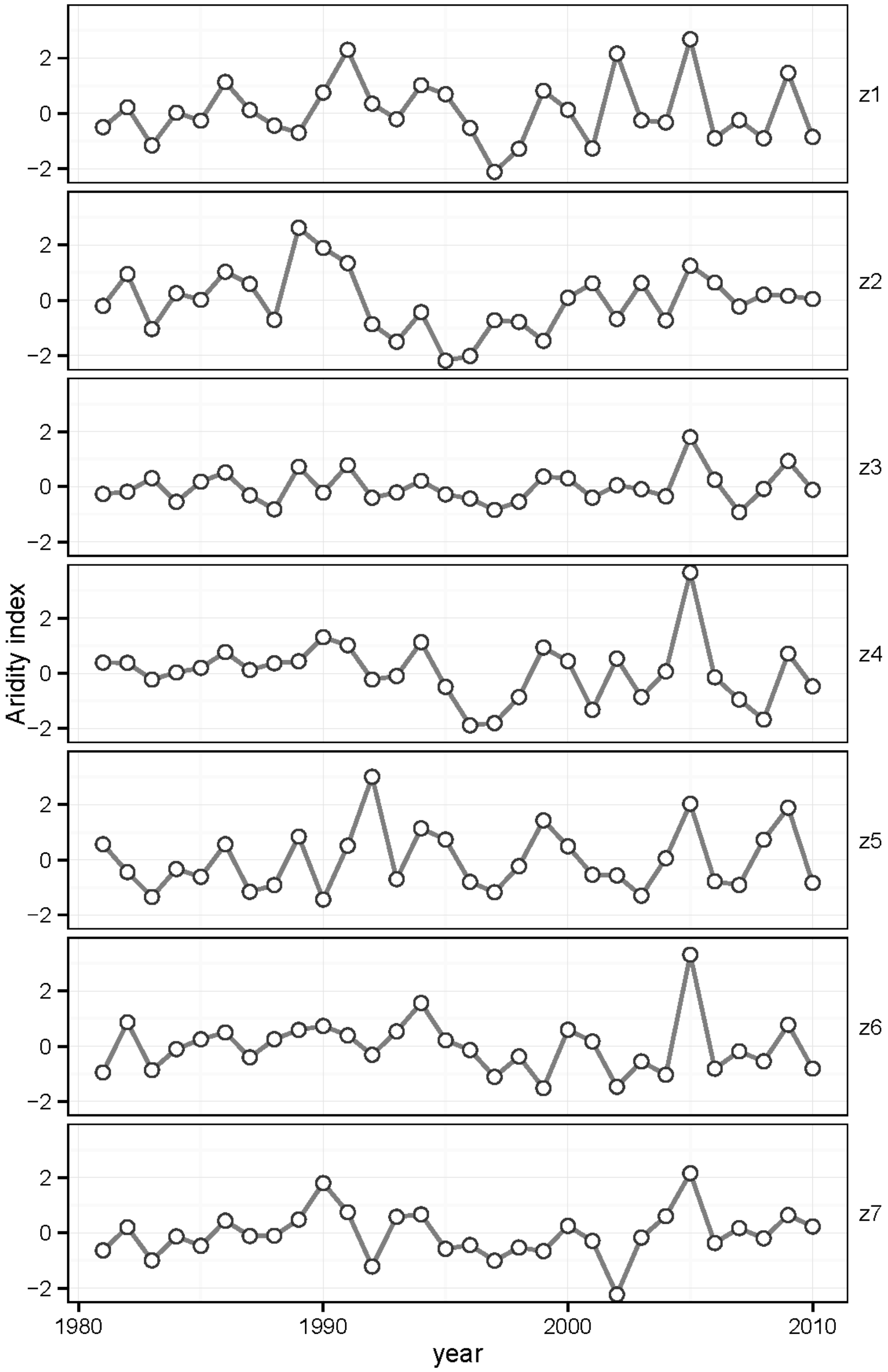


Figure 5.3. Studentized Residuals of Annual Aridity Index (AAI) for 1980 to 2010 in the different sampling zones (z1 to z7). Higher AAI values correspond to years where evapotranspiration exceeded precipitation.

Mantel correlations

Both health status and Stand Density Index (SDI) classes were used to construct Mantel correlograms in order to evaluate tree-growth responses to AAI variations. Comparison of tree growth and aridity fluctuations with the Mantel correlograms revealed cross-correlations between fluctuations across time-class lags that were defined by both patterns (or trends) of correlations and significances (Figures 5.4 to 5.7). The correlograms indicated that correlations between compared fluctuations generally oscillated around +/- twenty percent (Mantel correlation = 0.2). The occurrence of significant correlations depended on such trends and usually varied across the time-class lags. The stronger correlations depicted in the correlograms were predominantly negative, indicating that positive extremes (high aridity) usually correlated with negative extremes in the extracted tree growth fluctuations and vice versa: low aridity was mainly correlated to positive tree growth extremes. The relatively few significant positive correlations suggest that in some cases intermediate extremes of aridity were positively correlated with moderate extremes of tree growth.

To gain better understanding of the relationships between the correlogram patterns and the other levels studied (sampling zones, stand densities, and health status), we grouped the correlograms from individual sample replicates (usually two samples per tree) belonging to each level. The superimposed correlograms exhibited comparable patterns across the time-class lags, but the computed significances varied (see for example the isolated black circles in Figure 5.4). This suggests that the species dendroclimatic interaction responses were sensitive to the sample replicate position within the tree diameter.

When the Mantel correlograms were superimposed by zone and health status, proximate zones 1 and 7 in the Burgos province presented the most regular and significant trends (Figure 5.4). These appeared as oscillating pulses with frequencies that were significant for time-class lags greater than zero (usually 1 and 3). Correlograms of these two zones also suggested that healthy and symptomatic tree patterns were clearer, more rhythmic, and more significant than the corresponding patterns observed in the dead trees. Trends from correlograms grouped in the other zones where not as clear, and the number of significant correlations depended on interactions between health status and other specific zone conditions that were not accounted for by the plot effect (Figure 5.4, z2 to z6). Finally, one of the zones in the Soria province exhibited no significant correlations for any health status (Figure 5.4, z6).

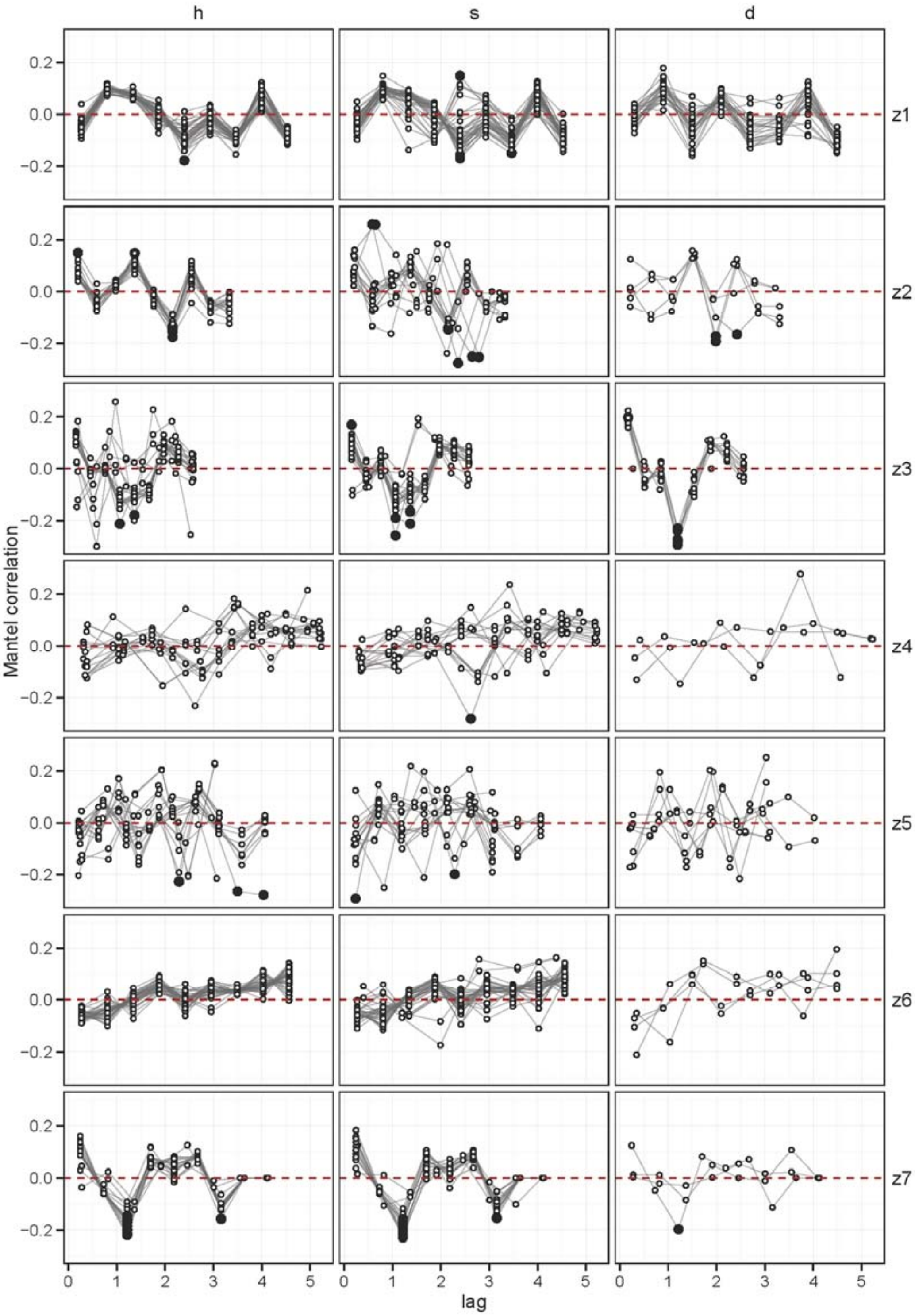


Figure 5.4. Mantel correlograms showing correlations (-0.2 to 0.2) and time class lag (0 to 5.5) for each sampling site (z1 to z7) and tree health status (h=healthy, s=symptomatic and d=dead) at tree level (grey lines). Trees with significant response to changes in AAIs are represented by large black dots, indicating significant Mantel statistics ($p \leq 0.05$).

Superimposing the Mantel correlograms by health status showed the predominance of significant negative correlations (Figure 5.5), suggesting that the significant correlation ranges along the time-class lags depended on tree health status. Significant correlations of dead trees were localized along a narrow interval on the time-class lag axis (0.2 to 2.4), while significant correlations of symptomatic trees occupied a wider interval (0.2 to 3.4) and significant correlations of healthy trees occupied the widest observed interval along the same axis (0.2 to 4).

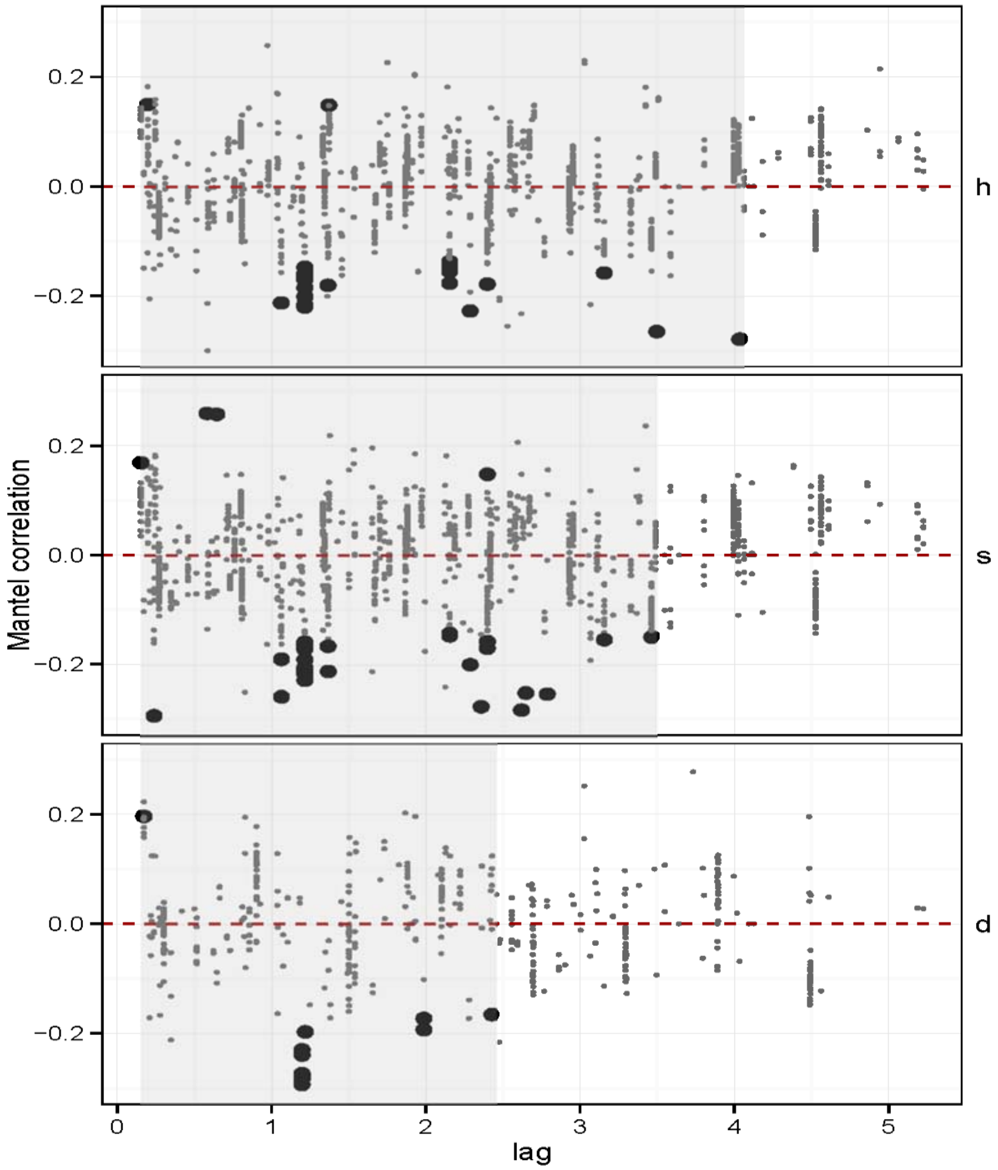


Figure 5.5. Mantel correlograms showing correlations (-0.2 to 0.2) and time class lag (0 to 5.5) for tree health status (h=healthy, s=symptomatic, d=dead) at tree level. Trees with significant response to changes in AAIs are represented by large black dots, indicating significant Mantel statistics (p≤0.05).

The differences observed between health status and sampling sites led us to examine the potential role of density, so Mantel correlograms according to SDI class (high, medium and low) and sampling site were also plotted (Figure 5.6). When they were grouped by zone and stand density, we found trends for zones and health status similar to those described in the previous paragraphs, but there were no individual trees in some of the stand density ranges (Figure 5.6). Once more, the proximate zones 1 and 7 in the Burgos province presented the most regular and significant trends, with the common trends showing pulses at time-class lags 1 and 3. Patterns in the remaining zones were unclear and numbers of significant correlations depended on interactions between stand density and other specific zone conditions which were not accounted for in the plots (Figure 5.6, z2 to z6). However, trees with significant response to AAI changes were found in all the SDI classes and sampling zones, except z6 in the Soria province and z4 in Segovia (virtually none).

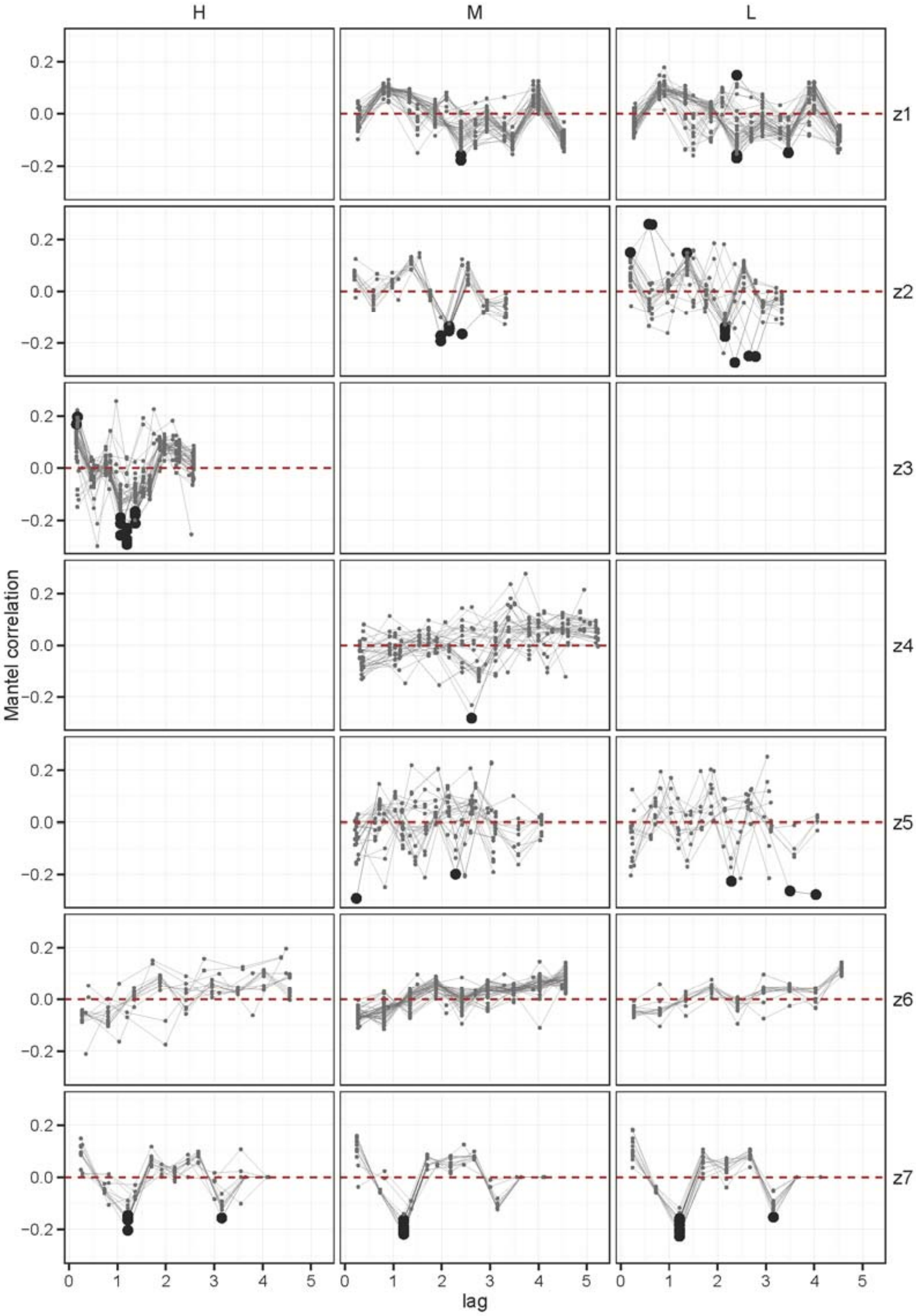


Figure 5.6. Mantel correlograms showing correlations (-0.2 to 0.2) and time class lag (0 to 5.5) for each sampling site (z1 to z7) and SDI class (H=high >700, M=medium 700-400, L=low <400) at tree level (grey lines). Trees with significant response to changes in AAIs are represented by large black dots, indicating significant Mantel statistics ($p \leq 0.05$).

A general trend also emerged when we analysed all trees together by SDI classes (Figure 5.7). Trees in high density stands presented significant, relatively fast responses, with time-lags mainly around 1.2, while tree response time in medium-density stands was intermediate, (most lags from 1.2 to 2.5) and trees in low density stands showed significant responses to aridity over a longer period of time (lags from 1.2 to 4). All of this was found to be dependent on tree health status; for instance, dead trees showed no significant response to low densities.

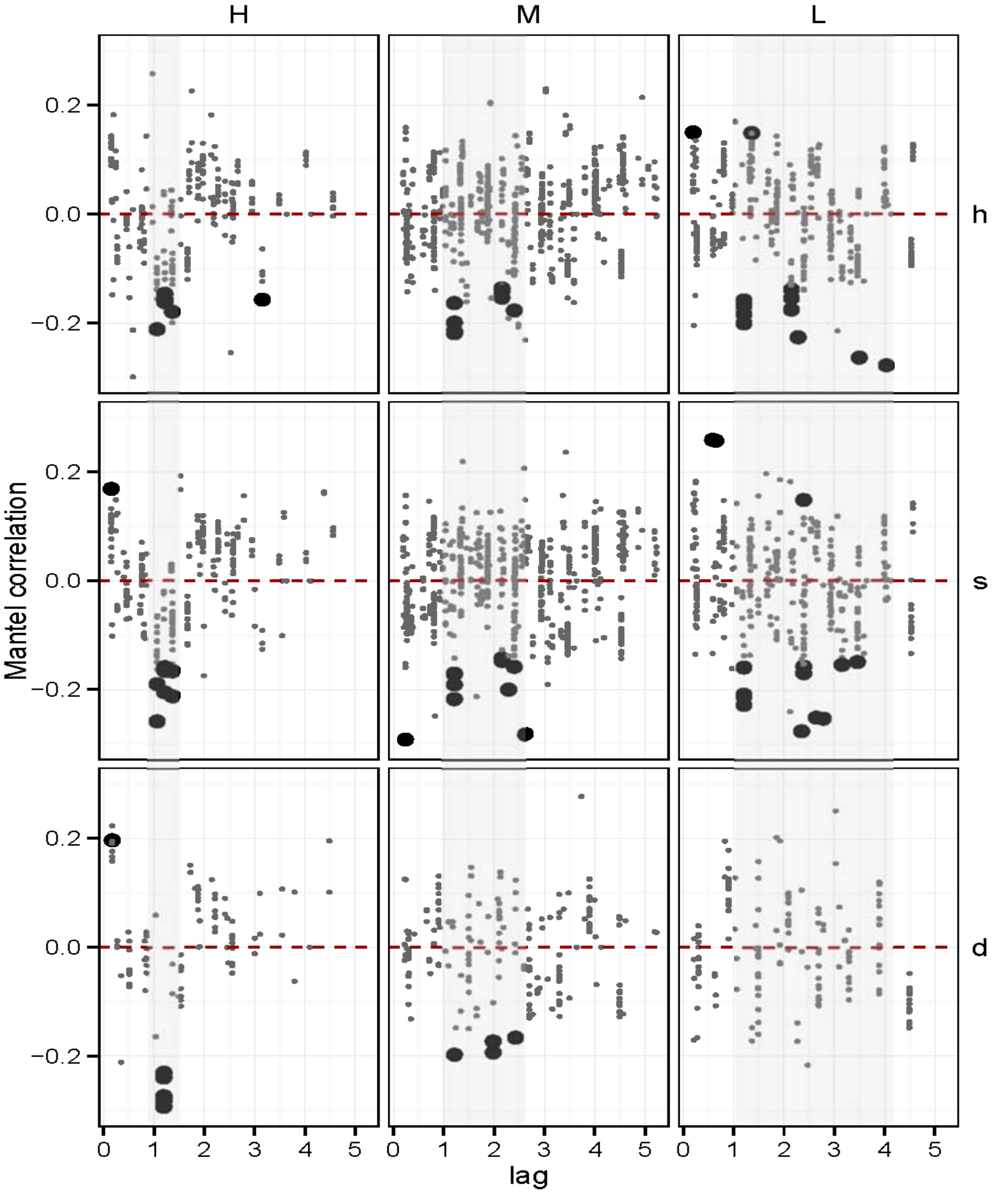


Figure 5.7. Mantel correlograms showing correlations (-0.2 to 0.2) and time class lag (0 to 5.5) for each SDI class (H=high >700, M=medium 700-400 and L=low <400) and tree health status (h=healthy, s= symptomatic and d= dead) at tree level. Trees with significant response to changes in AAIs are represented by large black dots, indicating significant Mantel statistics (p≤0.05).

Discussion

Our results for tree growth patterns indicate differences between geographic areas and health status of Maritime pine trees. We observed common behaviour in zones located in the central area (Meseta Castellana (z1, z4, z6) and Montaña de Soria-Burgos (z7)) that suggest conditions of tree-growth synchronicity in these two proximate provenance regions. The tree-ring measurements indicated symmetrical distribution along the recorded formation years. This can be considered even-aged stand behaviour and concurs with previous studies in Spain (Bogino and Bravo, 2008; Olivar et al., 2012). In contrast, the fluctuations of the mountain range provenances (Sierra de Oña (z2), Sierra del Teleno (z5) and to a lesser extent Sierra de Gredos (z3)), were usually narrow and asymmetric, suggesting asynchronous tree growth on these sites (Tessier et al., 1997) that can be considered typical growth-pattern behaviour for uneven-aged stands (Peng, 2000).

Looking at health status, we were able to assess the relative importance of the evaluated factors in *Pinus pinaster* decline. The extracted fluctuations of healthy or symptomatic trees showed more uniform tree growth patterns than those of dead trees. Also, patterns among trees of different health status varied according to local site conditions (Gea-Izquierdo et al., 2014). Living trees presented clearer trends in prevailing fluctuations and differences in the patterns of healthy and symptomatic trees depended on the sampling zone. Uniformity of fluctuations among dead trees depended on both the sampling zones and the number of samples that could be analysed per zone. Fewer samples were recorded for dead trees because there were not always three standing dead trees in each plot and several of the dead tree samples had to be discarded due to poor quality (Bigler, 2004). Zone 1 had more viable samples of dead trees and was the study zone with highest mortality (Table 5.3). There, fluctuations for dead trees presented patterns similar to those of living trees, but when we analysed the chronologies we could observe that dead trees began to decrease slightly before symptomatic and healthy trees. Symptomatic or damaged trees showed less growth during the final years of measurement, which is consistent with results from other studies (Camarero et al., 2015; Sangüesa-Barreda et al., 2015b).

Two of the most important environmental conditions for the growth of the trees; potential evapotranspiration and annual water availability, can be obtained by the relative magnitudes of the precipitation and the temperature. We built Walter-Lieth climatic diagrams for illustrating annual aridity indexes (AAIs) trends over the time, depicting intra-annual fluctuations of moisture and occurrences of seasonal extremes of drought. The influence of the precipitations and temperatures on the *Pinus pinaster* growth behaviour was already established in previous studies as (Bogino and Bravo, 2008; Olivar et al., 2012; Rozas et al., 2011a; Sánchez-Salguero et al., 2012a). Our results provide more information by comparing the tree growth with the aridity index. In this manner, we assess the measures of the interaction between the two climatic variables (precipitation and temperature) including the effect of water deficit periods. A high climatic variability year by year and an increasing in the aridity may reduce tree growth of pine species (Martín-Benito et al., 2008; Sánchez-Salguero et al., 2013), being growth decline caused by the more frequent occurrence of severe droughts. We have observed that events of high aridity in our study area have been frequently and consistently mainly during the beginning of the decade starting at 1990, in 1994 and during the year 2005. These severe drought events in the 90s were assessed in other studies (Camarero et al., 2013; Dobbertin, 2005; Sánchez-Salguero et al., 2012a; Sangüesa-Barreda et al., 2015b) showing a decrease in the radial growth and loss of vigour of the trees. On the other hand, the influence of the drought period on tree growth in 2005 has also been studied in other areas of Spain (Candel-Perez et al., 2012; Sánchez-Salguero et al., 2013). As well, the reduction in growth detected in trees from natural stands in response to the 2005 drought was significantly higher than in the rest of dry years (Sánchez-Salguero et al., 2013), which coincides with the observation of symptoms increase of *Pinus pinaster* decline in our study area. Therefore, reduction in tree growth and enhanced defoliation in pine forest in Spain caused by severe late-20th century droughts (1990s and 2000s) are inducing the pine stands to decline (Guada et al., 2016; Herguido et al., 2016; Sánchez-Salguero et al., 2012a, 2012b).

Multivariate cross-correlations between residual fluctuations of both tree radial increments (TRIs) and annual aridity indexes (AAIs) were graphed, revealing significant responses in Mantel correlograms depicting health status and Stand Density Index (SDI) classes. When we superimposed the Mantel correlograms by zone and health status, proximate zones 1 and 7 in the same province (Burgos) but from different provenance regions (Meseta Castellana and Montaña de Soria-Burgos, respectively) presented the most regular and significant trends. This significant and negative relation in a short time interval may indicate that high aridity events incite a downturn in tree vigour and growth, inducing the decline process one to three time-class lags after these events. Other studies have also demonstrated how drought periods induce tree decline (Cailleret et al., 2012; Dorman et al., 2013; Galiano et al., 2010; Gea-Izquierdo et al., 2014; Kharuk et al., 2013; Martínez-Vilalta et al., 2012) and how severe drought events play an inciting role in forest decline (Amoroso et al., 2015; Sánchez-Salguero et al., 2012b; Sangüesa-Barreda et al., 2015b). In contrast, trends from correlograms grouped in zones from mountain range provenances (z2 Sierra de Oña, z3 Sierra de Gredos, and z5 Sierra del Teleno) were less clearly defined and the number of significant correlations depended on interactions between health status and specific zone features. Local conditions such as topography and variability in precipitations and temperatures in the mountain range areas can influence tree response both positively and negatively (Hughes, 2002). Indeed, Linares and Tíscar (2010) found the negative effects of drought stress on tree growth to be greatly modulated by local factors such as topography. Finally, Meseta Castellana zones 4 and 6 presented almost no significant correlations for any health status. These areas are dedicated to forest production of timber and resin, and are more intensively managed; dead and weak trees are removed when necessary. The remaining trees do not have high levels of stress, so they may not show such clear responses to climate (Bigler, 2004). In this study, the sampling zones had greater influence than genetics or provenance regions, but were also influenced by forest management and local climate conditions (Prieto-Recio et al., 2015). We selected plots in natural or naturalized *Pinus pinaster* stands, but studies conducted in reforestation areas (which are mostly low quality sites) suggest that growth response to climatic stress can be highly conditioned by local site conditions (Bogino and Bravo, 2008; Tardif et al., 2003). In a similar study on *Pinus pinaster* in the Iberian Peninsula, analysis of the relationship between biomass increments and aridity in both Mediterranean and Atlantic climatic conditions showed significant relationships with clear Mantel statistics patterns (Lara et al., 2013) in the Mediterranean region. This coincides with our findings for the proximate zones 1 and 7 of our study. However, trees from the Atlantic region were less sensitive to annual droughts, which tended to be shorter in duration, and high variability in precipitation (Lara et al., 2013). In our study, this behaviour might resemble the areas located in mountain ranges.

In general, growth response to drought depended on the health status of the tree, or its state of decline. Defoliation is one of the most representative variables for studying tree decline processes (Camarero et al., 2015; Galiano et al., 2010; Sánchez-Salguero et al., 2012b, 2013; Sangüesa-Barreda et al., 2015a). As a general trend, our results showed that as tree health status declined, response time to aridity also decreased. Trees were considered symptomatic or damaged if defoliation exceeded 35% and they presented two or more symptoms of decline, so response to aridity might also be related to defoliation. Trees with high percentage of defoliation had faster, more acute, significant tree growth responses to aridity than healthy trees. Defoliated pines have premature cessation of cambial activity and wood formation and form narrow tree rings as the result of a shorter growing season (Eilmann et al., 2013). Defoliated trees also exhibited the highest sensitivity to water availability and the lowest post-drought resilience capacity (Guada et al., 2016). The *Pinus* genus in particular is the most vulnerable conifer to drought-induced xylem embolism (Martínez-Vilalta et al., 2004). Overall, our findings indicated that recently dead, completely defoliated trees and symptomatic trees with 35 to 99 % defoliation were more sensitive to high aridity caused by severe droughts in the 1990s and 2000s. This is consistent with behaviour observed in other studies on Mediterranean pines (Guada et al., 2016; Sánchez-Salguero et al., 2012a, 2012b), which have reported radial growth divergence among trees with different defoliation levels in the more xeric areas, where basal area increments and recent defoliation were negatively related.

Since differences between health status and sampling sites were found, Mantel correlograms were also plotted by SDI classes (high, medium and low) and sampling site in order to determine if density was playing a role. Stand structure resulting from historical forest management plays a fundamental role in *Pinus pinaster* decline (Prieto-Recio et al., 2015). Other studies have found that high-density stands displayed the highest tree mortality rates and symptoms of decline (Camarero et al., 2011; Galiano et al., 2010; Linares et al., 2009; Martínez-Vilalta et al., 2012; Sánchez-Salguero et al., 2012b, 2013), while competition also reduced tree growth (Gomez-Aparicio et al., 2011; Madrigal-González and Zavala, 2014). In our study, proximate zones 1 and 7 in the Burgos province showed the most regular and significant trends. Patterns in the remaining zones were less clearly defined and numbers of significant correlations depended on interactions between stand density and specific zone conditions that were not accounted for in the plots. Once again, our research confirms the great importance of local climatic conditions in relation to the effects of aridity on tree growth (Linares and Tíscar, 2010; Sánchez-Salguero et al., 2016). Forest structure and competition between trees within the stand have a significant effects on the individual tree growth-climate relationship. Hence, trees in conditions of lower competition would hold up better in the warmer conditions that are predicted as a result of climate change (Fernández-de-Uña et al., 2015). Past forest dynamics and management affect the adaptive capacity of trees to adverse climatic conditions because they altered stand structure and determined present competition levels (Lindner et al., 2009). In our study stands, we saw different types of management defined mainly by geographical areas or provinces. For example, zone 4 in the Segovia province and zone 6 in the Soria province are historically more intensively managed because the forests there are used for resin and timber production. In these zones, values for tree growth in response to climate were virtually non-significant, probably due to forest management aimed at forest production. In contrast, mountain range zones 2, 3, and 5 showed significant values with undefined patterns, probably due to the variability of local climatic and anthropogenic conditions (Sánchez-Salguero et al., 2016, 2015).

When the Mantel correlograms were superimposed to look at all zones together by SDI classes, we also observed a general trend. The significant correlations of trees in high-density stands were localized along a narrow interval on the time-class lag axis, whereas medium- and low-density stands responded in medium or long periods of time, respectively. For *Pinus pinaster*, competition takes place for belowground resources such as soil nutrients and water availability (Bravo-Oviedo et al., 2006; Cailleret et al., 2012; Sánchez-Salguero et al., 2012a). Trees in higher-density stands showed a faster, shorter response to negative AAIs, which could be inciting the decline process or making recovery difficult, eventually leading to death (Bravo-Oviedo et al., 2006). However, healthy and symptomatic trees that respond in medium and long time periods may be able to recover from stress events (Breda and Peiffer, 2014). Thus, drought events in combination with stand density may act as 'predisposing, inciting or contributing factors' *sensu* Manion (1991), depending on the SDI class. In very dense stands, competition might incite or even contribute to tree decline and death, while in low- and medium-density stands, competition could constitute a predisposing factor. Trees in very dense stands that have been affected by drought events respond quickly to aridity and are less able to recover, so growth decline persists and would not be influenced significantly by subsequent drought events. However, trees in medium- and low-density stands respond to drought, recover and return to normal growth rates, so subsequent drought events significantly affect them again. Thus, they would show greater sensitivity to aridity. Besides, some authors found that stands with lower competition showed greater growth responsiveness to climate, suggesting that tree-growth sensitivity to climate increases with decreasing competition intensity (Sánchez-Salguero et al., 2016). This is consistent with our findings, where significant growth responses to aridity were distributed across four time-class lags after drought events. The self-thinning line can be modified by recurrent drought events, so the stands arrive at the imminent mortality zone *sensu* Jack and Long (1996) at lower SDI values. This can lead to tree mortality at lower densities and serves to warn foresters regarding the need for more intense, early, and frequent thinning to reduce the impact of both predisposing and inciting factors.

Our results after implementation of BIOdry supported in all instances that water deficits caused by drought events significantly affect tree growth (Bogino and Bravo, 2008; Lara et al., 2013; Olivar et al., 2012; Rozas et al., 2011b; Sánchez-Salguero et al., 2012b). BIOdry applied to *Pinus pinaster* data detected and corrected the serial correlation observed in the residuals and ensured data normalization. The algorithm also efficiently addressed pseudoreplication in tree-ring data, as this standard dendrochronological sampling procedure introduced dependencies among model residuals that needed to be removed in order to compute valid variance estimates and extract stationary series (Lara and Bravo, 2015; Lara et al., 2013).

Conclusions

Differences in growth patterns between trees with different phytosanitary conditions in sampling zones were detected. Growth patterns in central zones were symmetrically distributed along the recorded formation years of the tree rings measured, exhibiting even-aged stand behaviour. In contrast, growth patterns in mountain range zones were usually narrow and asymmetric, suggesting the asynchronous tree growth conditions of uneven-aged stands.

We assessed the interactions between precipitation and temperature, including the effect of water deficit periods, using Walter-Lieth diagrams. Annual aridity index trends over time and across study sites revealed a clear chain of drought events in the early 1990s, then again in 1994 and in 2005.

Our findings of correlations between tree growth and aridity index indicate significant responses at tree level according to health status and in relation to defoliation levels. Completely defoliated, recently dead trees and symptomatic trees with 35-99% defoliation responded more quickly and acutely to high aridity. Site conditions also played a very important role in driving growth responses to climate. Central areas in Burgos showed a regular, significant trend within a short time period which might indicate that events of high aridity may incite reduced tree vigour and growth, inducing the trees into the decline process one to three time-class lags after these events. Trends in mountain range areas did not follow a clear pattern, probably due to local conditions such as topography and variability in precipitations and temperatures.

Our results also have implications for competition-growth-climate relationships. In Mantel correlograms we found significant values between tree growth and aridity index according to Stand Density Index classes. In very dense stands, competition might be acting as an inciting or contributing factor, while in the low- and medium-density stands competition may act as a 'predisposing factor' *sensu* Manion (1991). Hence, stands with lower competition levels show more growth responsiveness to aridity: significant responses were distributed over a longer period of time after the drought events. In contrast, very dense stands showed less growth responsiveness to aridity, suggesting a lack of recovery capacity. In that context, persistent growth decline would not be significantly influenced by subsequent drought events.

Forest managers are increasingly required to modify stand structure by reducing competition for water in the most declined Mediterranean pine forests. Regularly programmed and implemented thinning and shrub control could be used to enhance the resistance and resilience of *Pinus pinaster* stands. Further research is necessary to understand the key role of the biotic factors (pests and diseases) involved in *Pinus pinaster* decline, triggering tree death. Sustainable forest management plans linked to biological control should be implemented to ensure the future of these forests.

References

Adams, H.D., Guardiola-Claramonte, M., Barron-Gafford, G. a, Villegas, J.C., Breshears, D.D., Zou, C.B., Troch, P. a, Huxman, T.E., 2009. Temperature sensitivity of drought-induced tree mortality portends increased regional die-off under global-change-type drought. Proc. Natl. Acad. Sci. U. S. A. 106, 7063–7066. doi:10.1073/pnas.0901438106

Allen, C.D., Macalady, A.K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D.D., Hogg, E.H. (Ted), Gonzalez, P., Fensham, R., Zhang, Z., Castro, J., Demidova, N., Lim, J.-H.H., Allard, G., Running, S.W., Semerci, A., Cobb, N., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. For. Ecol. Manage. 259, 660–684. doi:10.1016/j.foreco.2009.09.001

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1–9.

Amoroso, M.M., Daniels, L.D., Bataineh, M., Andison, D.W., 2011. Evidence of mixed-severity fires in the foothills of the Rocky Mountains of west-central Alberta, Canada. For. Ecol. Manage. 262, 2240–2249. doi:10.1016/j.foreco.2011.08.016

Amoroso, M.M., Daniels, L.D., Villalba, R., Cherubini, P., 2015. Does drought incite tree decline and death in *Austrocedrus chilensis* forests? J. Veg. Sci. 26, 1171–1183. doi:10.1111/jvs.12320

Ayres, M.P., Lombardero, M.J., 2000. Assessing the consequences of global change for forest disturbance from herbivores and patogens. Sci. Total Environ. 262, 263–286.

Bigler, C., 2004. Predicting the time of tree death using dendrochronological data. Ecol. Appl. 14, 902–914.

Bogino, S.M., Bravo, F., 2008. Growth response of *Pinus pinaster* Ait. to climatic variables in central Spanish forests. Ann. For. Sci. 65, 506.

Bogino, S.M., Fernández-Nieto, M.J., Bravo, F., 2009. Climate effect on radial growth of *Pinus sylvestris* L. in the southern and western limits of its worldwide distribution. Silva Fenn. 43, 609–623.

Boisvenue, C., Running, S.W., 2006. Impacts of climate change on natural forest productivity - evidence since the middle of the 20th century. Glob. Chang. Biol. 12, 862–882. doi:10.1111/j.1365-2486.2006.01134.x

Bravo-Oviedo, A., Sterba, H., del Río, M., Bravo, F., 2006. Competition-induced mortality for Mediterranean *Pinus pinaster* Ait. and *P. sylvestris* L. For. Ecol. Manage. 222, 88–98. doi:10.1016/j.foreco.2005.10.016

Breda, N., Peiffer, M., 2014. Vulnerability to forest decline in a context of climate changes: new prospects about an old question in forest ecology. Ann. For. Sci. 71, 627–631.

Bunn, A.G., 2010. Statistical and visual crossdating in R using the dplR library. Dendrochronologia 28, 251–258. doi:10.1016/j.dendro.2009.12.001

Cailleret, M., Heurich, M., Bugmann, H., 2014. Reduction in browsing intensity may not compensate climate change effects on tree species composition in the Bavarian Forest National Park. For. Ecol. Manage. 328, 179–192.

Cailleret, M., Nourtier, M., Amm, A., Durand-Gillmann, M., Davi, H., 2012. Drought-induced decline and mortality of silver fir differ among three sites in Southern France. Ann. For. Sci. 71, 643–657.

Camarero, J.J., Bigler, C., Linares, J.C., Gil-Pelegrin, E., 2011. Synergistic effects of past historical logging and drought on the decline of Pyrenean silver fir forests. For. Ecol. Manage. 262, 759–769.

Camarero, J.J., García-Ruiz, J.M., Sangüesa-Barreda, G., Galván, J.D., Alla, A.Q., Sanjuán, Y., Beguería, S., Gutiérrez, E., 2015a. Recent and intense dynamics in a formerly static Pyrenean treeline. Arctic, Antarct. Alp. Res. 47, 773–783. doi:10.1657/AAAR0015-001

Camarero, J.J., Gazol, A., Sangüesa-Barreda, G., Oliva, J., Vicente-Serrano, S.M., 2015b. To die or not to die: Early warnings of tree dieback in response to a severe drought. J. Ecol. 103, 44–57. doi:10.1111/1365-2745.12295

Camarero, J.J., Manzanedo, R., Sánchez-Salguero, R., Navarro-Cerrillo, R.M., 2013. Growth response to climate and drought change along an aridity gradient in the southernmost *Pinus nigra* relict forests. Ann. For. Sci. 70, 769–780. doi:10.1007/s13595-013-0321-9

Candel-Perez, D., Linares, J.C., Vinegla, B., Lucas-Borja, M.E., Candel-Pérez, D., Linares, J.C., Viñegla, B., Lucas-Borja, M.E., 2012. Assessing climate-growth relationships under contrasting stands of co-occurring Iberian pines along an altitudinal gradient. For. Ecol. Manage. 274, 48–57. doi:10.1016/j.foreco.2012.02.010

Choat, B., Jansen, S., Brodribb, T.J., Cochard, H., Delzon, S., Bhaskar, R., Bucci, S.J., Feild, T.S., Gleason, S.M., Hacke, U.G., Jacobsen, A.L., Lens, F., Maherali, H., Martinez-Vilalta, J., Mayr, S., Mencuccini, M., Mitchell, P.J., Nardini, A., Pittermann, J., Pratt, R.B., Sperry, J.S., Westoby, M., Wright, I.J., Zanne, A.E., 2010. Global convergence in the vulnerability of forests to drought. Nature 491, 752–755.

Cook, E.R., Woodhouse, C.A., Eakin, C.M., Meko, D.M., Stahle, D.W., 2004. Long-term aridity changes in the Western United States. Science (80). 306, 1015–1018. doi:10.1126/science.1102586

del Río, M., Bravo, F., Pando, V., Sanz, G., Sierra de Grado, R., 2004. Influence of individual tree and stand attributes in stem straightness in *Pinus pinaster* Ait. stands. Ann. For. Sci. 61, 141–148.

Dobbertin, M., 2005. Tree growth as indicator of tree vitality and of tree reaction to environmental stress: a review. Eur. J. For. Res. 124, 319–333.

Dorman, M., Svoray, T., Perevolotsky, A., Sarris, D., 2013. Forest performance during two consecutive drought periods: Diverging long-term trends and short-term responses along a climatic gradient. For. Ecol. Manage. 310, 1–9. doi:10.1016/j.foreco.2013.08.009

Eichhorn, J., Roskams, P., Ferretti, M., Mues, V., Szepesi, A., Durrant, D., 2010. Manual Part IV: Visual Assessment of Crown Condition and Damaging Agents, in: Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg, p. 49.

Eilmann, B., Dobbertin, M., Rigling, A., 2013. Growth response of Scots pine with different crown transparency status to drought release. Ann. For. Sci. 70, 685–693.

Fernández-de-Uña, L., Cañellas, I., Gea-Izquierdo, G., 2015. Stand competition determines how different tree species will cope with a warming climate. PLoS One 10, 1–18. doi:10.1371/journal.pone.0122255

Fritts, H.C., 1976. Tree rings and climate. London, New York and San Francisco, Academic Press.

Galiano, L., Martínez-Vilalta, J., Lloret, F., Martinez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor decline of Scots pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems 13, 978–991. doi:10.1007/s10021-010-9368-8

Gea-Izquierdo, G., Viguera, B., Cabrera, M., Canellas, I., 2014. Drought induced decline could portend widespread pine mortality at the xeric ecotone in managed mediterranean pine-oak woodlands. For. Ecol. Manage. 320, 70–82.

Girard, F., Vennetier, M., Guibal, F., Corona, C., Ouarmim, S., Herrero, A., 2012. *Pinus halepensis* Mill. crown development and fruiting declined with repeated drought in Mediterranean France. Eur. J. For. Res. 131, 919–931.

Gomez-Aparicio, L., Garcia-Valdes, R., Ruiz-Benito, P., Zavala, M.A., 2011. Disentangling the relative importance of climate, size and competition on tree growth in Iberian forests: implications for forest management under global change. Glob. Chang. Biol. 17, 2400–2414.

Gonzalez-Munoz, N., Linares, J.C., Castro-Diez, P., Sass-Klaassen, U., 2014. Predicting climate change impacts on native and invasive tree species using radial growth and twenty-first century climate scenarios. Eur. J. For. Res. 133, 1073–1086.

Goslee, S.C., Urban, D.L., 2007. The ecodist package for dissimilarity-based analysis of ecological data. J. Stat. Softw. 22, 1–19. doi:10.18637/jss.v022.i07

Guada, G., Camarero, J.J., Sánchez-Salguero, R., Navarro-Cerrillo, R.M., 2016. Limited growth recovery after drought-induced forest dieback in very defoliated trees of two pine species. Front. Plant Sci. 7. doi:10.3389/fpls.2016.00418

Guijarro, J., 2011. User's guide to climatol.

Hampe, A., Petit, R.J., 2005. Conserving biodiversity under climate change: the rear edge matters. Ecol. Lett. 8, 461–7. doi:10.1111/j.1461-0248.2005.00739.x

Herguido, E., Granda, E., Benavides, R., García-Cervigón, A.I., Camarero, J.J., Valladares, F., 2016. Contrasting growth and mortality responses to climate warming of two pine species in a continental Mediterranean ecosystem. For. Ecol. Manage. 363, 149–158. doi:10.1016/j.foreco.2015.12.038

Hughes, M.K., 2002. Dendrochronology in climatology – the state of the art. Dendrochronologia 20, 95–116. doi:10.1078/1125-7865-00011

Jack, S.B., Long, J.N., 1996. Linkages between silviculture and ecology: an analysis of density management diagrams. For. Ecol. Manage. 86, 205–220.

Kharuk, V.I., Im, S.T., Oskorbin, P.A., Petrov, I.A., Ranson, K.J., 2013. Siberian pine decline and mortality in southern siberian mountains. For. Ecol. Manage. 310, 312–320. doi:10.1016/j.foreco.2013.08.042

Kliejunas, J.T., Geils, B.W., Micales, J., Goheen, E.M., Hennon, P., Kim, M., Kope, H., Stone, J., Sturrock, R., Frankel, S.J., 2009. Review of literature on climate change and forest diseases of western North America 60.

Lara, W., Bravo, F., 2015. R Package " BIOdry ". Multilevel Modelling of Dendroclimatical Fluctuations.

Lara, W., Bravo, F., Maguire, D.A., 2013. Modeling patterns between drought and tree biomass growth from dendrochronological data: A multilevel approach. Agric. For. Meteorol. 178-179, 140–151. doi:10.1016/j.agrformet.2013.04.017

Lenoir, J., Gégout, J.C., Marquet, P., de Ruffray, P., Brisse, H., 2008. A significant upward shift in plant species optimum elevation during the 20th century. Science (80-.). 320, 1768–71. doi:10.1126/science.1156831

Linares, J.C., Camarero, J.J., Carreira, J.A., 2009. Interacting effects of changes in climate and forest cover on mortality and growth of the southernmost European fir forests. Glob. Ecol. Biogeogr. 18, 485–497.

Linares, J.C., Camarero, J.J., Carreira, J.A., 2010. Competition modulates the adaptation capacity of forests to climatic stress: Insights from recent growth decline and death in relict stands of the Mediterranean fir *Abies pinsapo*. J. Ecol. 98, 592–603. doi:10.1111/j.1365-2745.2010.01645.x

Linares, J.C., Tíscar, P.A., 2010. Climate change impacts and vulnerability of the southern populations of *Pinus nigra* subsp. *salzmannii*. Tree Physiol. 30, 795–806.

Linares, J.C., Tiscar, P.A., 2011. Buffered climate change effects in a Mediterranean pine species: range limit implications from a tree-ring study. Oecologia 167, 847–859. doi:10.1007/s00442-011-2012-2

Lindner, M., Maroschek, M., Netherer, S., Kremer, A., Barbati, A., Garcia-Gonzalo, J., Seidl, R., Delzon, S., Corona, P., 2009. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For. Ecol. Manage. 259, 698–709. doi:10.1016/j.foreco.2009.09.023

Long, J.N., 1985. A Practical Approach to Density Management. For. Chron. 61, 23–27. doi:10.5558/tfc61023-1

Madrigal-González, J., Zavala, M.A., 2014. Competition and tree age modulated last century pine growth responses to high frequency of dry years in a water limited forest ecosystem. Agric. For. Meteorol. 192-193, 18–26. doi:10.1016/j.agrformet.2014.02.011

Manion, P.D., 1991. Tree Disease Concepts, Prentice-Hall.

Manrique, E., Fernandez-Cancio, A., 2000. Extreme climatic events in dendroclimatic reconstructions from Spain. Clim. Change 44, 123–138.

Martín-Benito, D., Cherubini, P., del Río, M., Canellas, I., 2008. Growth response to climate and drought in *Pinus nigra* Arn. trees of different crown classes. Trees-Structure Funct. 22, 363–373.

Martínez-Vilalta, J., Lloret, F., Breshears, D.D., 2012. Drought-induced forest decline: causes, scope and implications. Biol. Lett. 8, 689–691.

Martínez-Vilalta, J., Sala, A., Pinol, J., 2004. The hydraulic architecture of Pinaceae - a review. Plant Ecol. 171, 3–13.

Navarro Cerrillo, R.M., Varo, M.A., Lanjeri, S., Hernández Clemente, R., 2007. Cartografía de defoliación en los pinares de pino silvestre (*Pinus sylvestris* L.) y pino salgareño (*Pinus nigra* Arnold.) en la Sierra de los Filabres. Ecosistemas 16, 163–171.

Olivar, J., Bogino, S., Spiecker, H., Bravo, F., 2012. Climate impact on growth dynamic and intra-annual density fluctuations in Aleppo pine (*Pinus halepensis*) trees of different crown classes. Dendrochronologia 30, 35–47. doi:10.1016/j.dendro.2011.06.001

Peng, C., 2000. Growth and yield models for uneven-aged stands: Past, present and future. For. Ecol. Manage. 132, 259–279. doi:10.1016/S0378-1127(99)00229-7

Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. doi:10.1016/j.foreco.2015.07.033

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770.

R Core Team, 2014. R: A language and environment for statistical computing: R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/.

Regent Instrument Inc, 2002. Windendro TM v.2002a.

Rozas, V., García-González, I., Zas, R., 2011a. Climatic control of intra-annual wood density fluctuations of *Pinus pinaster* in NW Spain. Trees 25, 443–453. doi:10.1007/s00468-010-0519-5

Rozas, V., Zas, R., García-González, I., 2011b. Contrasting effects of water availability on *Pinus pinaster* radial growth near the transition between the Atlantic and Mediterranean biogeographical regions in NW Spain. Eur. J. For. Res. 130, 959–970.

Sánchez Peña, G., Economou, A., Beccu, E., Canu, G., Cocco, S., Bussotti, F., Cenni, E., Cozzi, A., Ferretti, M., Conceicao, M., 1994. Especies Forestales Mediterráneas. Guía para la Evaluación de Copas., CEC-UN/ECE, Brussels, Geneva.

Sánchez-Salguero, R., Camarero, J.J., Dobbertin, M., Fernandez-Cancio, A., Vila-Cabrera, A., Manzanedo, R.D., Zavala, M.A., Navarro-Cerrillo, R.M., 2013. Contrasting vulnerability and resilience to drought-induced decline of densely planted vs. natural rear-edge *Pinus nigra* forests. For. Ecol. Manage. 310, 956–967.

Sánchez-Salguero, R., Camarero, J.J., Hevia, A., Madrigal-González, J., Linares, J.C., Ballesteros-Canovas, J.A., Sánchez-Miranda, A., Alfaro-Sánchez, R., Sangüesa-Barreda, G., Galván, J.D., Gutiérrez, E., Génova, M., Rigling, A., 2015. What drives growth of Scots pine in continental Mediterranean climates: Drought, low temperatures or both? Agric. For. Meteorol. 206, 151–162. doi:10.1016/j.agrformet.2015.03.004

Sánchez-Salguero, R., Linares, J.C., Camarero, J.J., Madrigal-González, J., Hevia, A., Sánchez-Miranda, A., Ballesteros-Cánovas, J.A., Alfaro-Sánchez, R., García-Cervigón, A.I., Bigler, C., Rigling, A., 2016. Disentangling the effects of competition and climate on individual tree growth: A retrospective and dynamic approach in Scots pine. J. For. Sci. 358, 53–64. doi:10.17221/104/2015-JFS

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Camarero, J.J., Fernández-Cancio, Á., 2012a. Selective drought-induced decline of pine species in southeastern Spain. Clim. Change 113, 767–785. doi:10.1007/s10584-011-0372-6

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Swetnam, T.W., Zavala, M.A., 2012b. Is drought the main decline factor at the rear edge of Europe? The case of southern Iberian pine plantations. For. Ecol. Manage. 271, 158–169. doi:10.1016/j.foreco.2012.01.040

Sangüesa-Barreda, G., Camarero, J.J., Linares, J.C., Hernández, R., Oliva, J., Gazol, A., González de Andrés, E., Montes, F., García-Martín, A., de la Riva, J., 2015a. Role of biotic factors and droughts in the forest decline: contributions from dendroecology. Ecosistemas 24, 15–23. doi:10.7818/ECOS.2015.24-2.03

Sangüesa-Barreda, G., Camarero, J.J., Oliva, J., Montes, F., Gazol, A., 2015b. Past logging, drought and pathogens interact and contribute to forest dieback. Agric. For. Meteorol. 208, 85–94. doi:10.1016/j.agrformet.2015.04.011

SPCAN-DGB, 2007. Red de Seguimiento de Daños en los Montes (Red CE de Nivel I), Manual de Campo. Ministerio de Medio Ambiente.

Stokes, M.A., Smiley, T.L., 1968. An Introduction to Tree-Ring Dating. Tucson, UEA.

Tardif, J., Camarero, J.J., Ribas, M., Gutiérrez, E., 2003. Spatiotemporal variability in tree growth in the Central Pyrenees: Climatic and site influences. Ecol. Monogr. 73, 241–257. doi:10.1890/0012-9615(2003)073[0241:SVITGI]2.0.CO;2

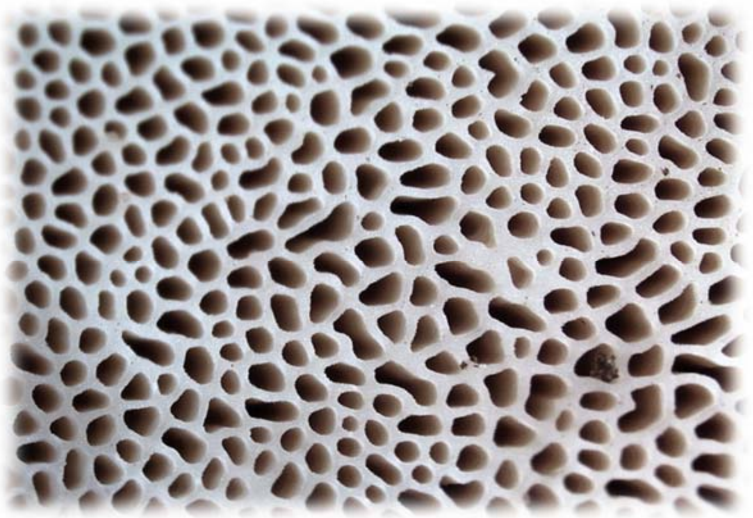
Tessier, L., Guibal, F., Schweingruber, F., 1997. Research strategies in dendroecology and dendroclimatology in mountain. Clim. Change 36, 499–517.

Thomas, D., Baldwin, J., Clark, J., 1993. Using a density- management diagram to develop thinning schedules for Loblolly pine plantations. Res. Pap. SO-275 12.

Valbuena, P., Peso, C., Bravo, F., 2008. Stand Density Management: Diagrams for two Mediterranean pine species in Eastern Spain. Investig. Agrar. Sist. y Recur. For. 17, 97–104. doi:10.5424/srf/2008172-01026

Walter, H., Lieth, H., 1967. Klimadiagramm Weltatlas. VEB Gustaf Fischer Verlag, Jena.

PART III: Biotic Factors



Chapter 6: First report of *Heterobasidion annosum* on *Pinus pinaster* in Spain

Disease note

The basidiomycete *Heterobasidion annosum* (Fr.) Bref. (= *Fomes annosus* (Fr.) Cooke) is one of the most important pathogens in coniferous forests in Europe, Asia and North America, causing root and butt rot. *Heterobasidion annosum* was first recorded on *Pinus pinaster* Ait. (commonly known as Maritime pine) in France and Great Britain (Spaulding, 1961) and in Portugal (Neves et al., 1986).

Pinus pinaster is the most widespread conifer in Spain, covering over 700.000 and 600.000 ha in pure and mixed stands respectively. Over the last few years, Maritime pine decline was observed in several stands in the center of the Iberian Peninsula. Unusual crown transparency, small needles, foliage discoloration and early tree death are characteristic decline symptoms associated with the high mortality rate on this species.

In June of 2010, eleven trees (40 – 60 years old) with a different degree of decline were felled in two zones (latitude 42°2′41″N, longitude 3°18′14″W, elevation 1096 m, and latitude 41°55′40″N, longitude 3°12′3″W, elevation 1128 m), and cut into sections (stump height, breast height and near the top).

Wood slices were removed from each section and carried to the laboratory. Afterwards, samples were placed in moist chambers under optimal conditions of humidity and temperature in order to enhance pathogen's growth. After 20 days of incubation in darkness at 25°C, *Heterobasidion annosum* anamorphic form (*Spiniger meineckellum* (A. Olson) Stalpers) occurred on most of these slices. Conidiophores with subglobose to pyriform conidia (5.8 x 4.2 µm) were observed under the compound microscope.

The fungus was isolated to extract DNA by disruption of the mycelium followed by washes with phenol: chloroform: isoamyl alcohol solution (25:24:1). Finally, DNA was precipitated with 20% polyethylene glycol solution. The polymerase chain reaction (PCR) was carried out according to the recommended conditions described by the manufacturer of Dynazyme II DNApolymerase (Finnzymes Ltd, Espoo, Finland) using the ITS primers, 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 4 (5′-TCCTCCGCTTATTGATATGC-3′). After DNA purification, samples were sequenced (SECUGEN, Madrid), and aligned and corrected with Geneious Pro™ 5.3 to obtain the consensus sequences. The resulting DNA sequences of two isolates were deposited in GenBank (Accession Nos. FR850494 and FR850495) and compared with a Blastn search at GenBank, showing 100% identity and 100% coverage with *Heterobasidion annosum sensu stricto*, former ISG-P (Intersterility Group of Pines).

In order to perform the pathogenicity tests, 10 seedlings (2 year-old) were inoculated with autoclaved *Pinus pinaster* wood chips colonized by *Heterobasidion annosum* and 10 control seedlings were inoculated with non-colonized wood chips. Inoculums were prepared by growing *Heterobasidion annosum* on 4 mm diameter wood chips placed on potato dextrose agar media during 3 weeks. Then, these wood chips were put inside an oblique incision made at 6 cm above the soil line and wrapped with Parafilm®. After 8 weeks in a growth chamber at 22.5°C with a 14-h photoperiod, the inoculated seedlings showed the typical symptoms of this disease, and 3 seedlings out of 10 were dead.

Heterobasidion annosum was previously recorded on *Pinus sylvestris* in Central Spain (Benito Martínez, 1943) causing needle drop, swelling at the stump height, and presence of dead trees by circular areas. This pathogen was also reported on *Pinus nigra* in North-Eastern Spain associated with defoliation and mortality (Oliva et al., 2008). This is the first record of *Heterobasidion annosum* on *Pinus pinaster* in Spain.

References

Benito Martín, J., 1943. El *Fomes annosus* Fr. (*Trametes radiciperda* Hart.) en España. An. del Jardín Botánico Madrid 3, 23–49.

Neves, N., Moniz, F., De Azevedo, N., Ferreira, M.C., Ferreira, G.W.S., 1986. Present phytosanitary situation of Portuguese forests. EPPO Bull. 16, 505–508.

Oliva, J., Ibarra, N., Colinas, C., Martín, E., 2008. Detección molecular de *Heterobasidion annosum s. s.* en claras de *Pinus nigra* en el Pirineo Aragonés. Boletín Sanid. Veg. Plagas. 34, 415–416.

Spaulding, P., 1961. Foreign diseases of forest trees of the world: an annotated list. Agric. handbook. U.S. Dept. Agric.

Chapter 7: Blue-stain and root rot fungi associated with *Pinus pinaster* decline in the Iberian Peninsula

Abstract

Pinus pinaster Ait. is one of the most important forest species in the Iberian Peninsula as regards both the protection it provides and the timber produced. It is also the main conifer species in Spain in terms of area covered. In recent years, several Maritime pine stands in the center of the Iberian Peninsula have undergone a general decline. The most important symptoms of the decline include unusually transparent foliage, smaller than usual needles, discoloration of foliage and premature death of the trees. A total of 212 samples of pine collar wood were collected from healthy, symptomatic and dead trees in 27 plots distributed throughout the region of Castile and Leon (northern Spain). Identification of the fungi was based on preliminary macroscopic examination of the colony cultures, morphological characterization by microscopic examination and molecular analysis. Fifteen species were identified during the study and were divided into two distinct groups according to their ecology: (1) ubiquitous saprophytic species (six species) and (2) pathogens displaying different degrees of virulence (nine species). The latter species mainly belong to the group of Ophiostomatoid fungi*: Ophiostoma minus, Ophiostoma ips, Ophiostoma piliferum and Ophiostoma ranaculosum*. The root pathogen *Heterobasidion annosum* was also identified. This pathogen is one of the main agents causing conifer death in Europe, but is a neophyte species in northern Spain, especially on *Pinus pinaster*. *Heterobasidion annosum* was found together with *Ophiostoma minus* and *Ophiostoma piliferum*, and all were isolated in the same geographical zone. The sampling zone of Ribera del Duero in Burgos harboured the greatest number of fungal species and the zone with the fewest fungal species was in the province of Soria. Further assays testing the pathogenicity of these fungi should be performed in order to establish their importance as causal agents of the symptoms of decline.

Keywords: *Maritime pine, Ophiostoma spp., Heterobasidion sp., forest pathology, tree mortality.*

Introduction

Pinus pinaster Ait. is widely distributed in the western Mediterranean basin: it extends along the Atlantic coast of Portugal, Spain and France and occurs on the western coast of Italy and in parts of North Africa. In the Mediterranean islands, the species is limited to Corsica and northern Sardinia (where it is represented by a very small population). There is a marginal population on the island of Pantelleria, near the Tunisian coast (Alía et al., 1997). Regarding genetic variation, large differences between populations have been observed at the level of molecular markers and in quantitative traits (Baradat and Marpeau, 1988). A high degree of genetic variation has been observed in survival, adaptation to different climatic conditions, growth, resistance to disease and tolerance to drought (Fernández et al., 1996). *Pinus pinaster* can be divided into three groups (Baradat and Marpeau, 1988): Atlantic (ssp. *atlantica* H. del Villar), Mediterranean and North African (both ssp. *mesogeensis* F. et Gaussen). Populations of all of these groups are found in central and southern Spain. Maritime pine is one of the most important forest species in Spain as regards both the protection provided and the timber and resin produced. It is also the main conifer in terms of area covered in Spain: over 700,000 hectares of pure stands and 600,000 hectares of mixed woods (del Río et al., 2004). Natural stands are highly fragmented and the species is characteristic of the Iberian forest landscape (Gil L., 1990). The species has also been widely used in reforestation throughout the Iberian Peninsula, particularly in the central part of the region (Solis, 2003).

Several Maritime pine stands in the center of the Iberian Peninsula have undergone a general decline in recent years. The most important symptoms of decline include unusually transparent foliage, smaller than usual needles, discoloration of foliage and premature death of the trees (Prieto-Recio et al., 2012). Forest decline, a phenomenon increasingly observed over the last forty years in Europe and North America, is defined as a complex process caused by the interaction of a number of abiotic and biotic factors (Manion, 1991). These factors lead to the gradual deterioration of forest ecosystems, including metabolic changes, reproductive problems and premature aging of trees, discoloration, impaired growth and loss of leaves, abnormal morphology of branches and foliage and, finally, death of trees (Innes, 1993). These factors can be classified as predisposing or long term factors (i.e. genetics, site, historical climate), factors inciting severe short-term damage (i.e. drought events, competition, frost) and contributing factors such as pathogenic fungi, parasitic plants and opportunistic insects (Kliejunas et al., 2009).

The main diseases are produced by fungi, nematodes, bacteria and viruses, although fungi are recognised as the most destructive agents in the forest stands (Manion, 1991; Muñoz et al., 2007; Tainter and Baker, 1996). Fungi that affect trees can be classified according to feeding type, as symbionts, parasites and saprophytes (Deacon, 2013). Fungi that attack the wood (e.g. blue-stain or Ophiostomatoid fungi) and fungi that cause root rot (e.g. *Heterobasidion annosum*) are frequently involved in forest decline and cause high economic and ecological losses (Oliva and Colinas, 2007; Otrosina et al., 1999). The main species that cause blue-stain in stems or roots in conifers belong to the genera *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis*, the anamorphic or asexual states of which are respectively *Graphium* spp., *Leptographium* spp. and *Chalara* spp. (Grylls and Seifert, 1999; Solheim et al., 1993; Wingfield et al., 1993). The root rot fungus *Heterobasidion annosum sensu lato* is a complex species formed by five species: *Heterobasidion irregulare* Otrosina & Garbelotto, *Heterobasidion occidentale* Otrosina & Garbelotto, *Heterobasidion annosum* (Fr.) Bref. *sensu stricto*, *Heterobasidion parviporum* Niemelä & Korhonen, and *Heterobasidion abietinum* Niemelä & Korhonen (Korhonen and Stenlid, 1998; Otrosina and Garbelotto, 2010). *Heterobasidion annosum s. l.* produces both sexual (basidiospores) and asexual spores (conidia). Perennial sporocarps or fruiting bodies are often found on the roots of windthrown trees, stumps, logs and dead or diseased trees. The anamorphic form (*Spiniger meineckellum* (A. Olson) Stalpers) is occasionally observed on broken roots, insect galleries and in the stumps of recently felled trees under moist conditions (Woodward et al., 1998).

Forest pathology involves problems arising from the damage that the disease inflicts on trees (Boa, 2003). Early detection of the problem, identification of the pathogens involved, by morphological and molecular tools, and assessment of populations of harmful agents are therefore essential in order to protect forests and the associated products and services (Muñoz et al., 2007). The objectives of the present study were as follows: (i) to identify the pathogens associated with *Pinus pinaster* decline, (ii) to associate the presence of the fungi identified with the health status of the tree, and (iii) to identify the areas most strongly affected by the pathogens in relation to climate conditions.

Material and methods

Description of the study site and sampling

This study was carried out in natural or naturalized stands of *Pinus pinaster* ssp. *mesogeensis* covering most of the distribution of the provenance regions in the center of the Iberian Peninsula (Castile and Leon, NW Spain) (Figure 7.1).

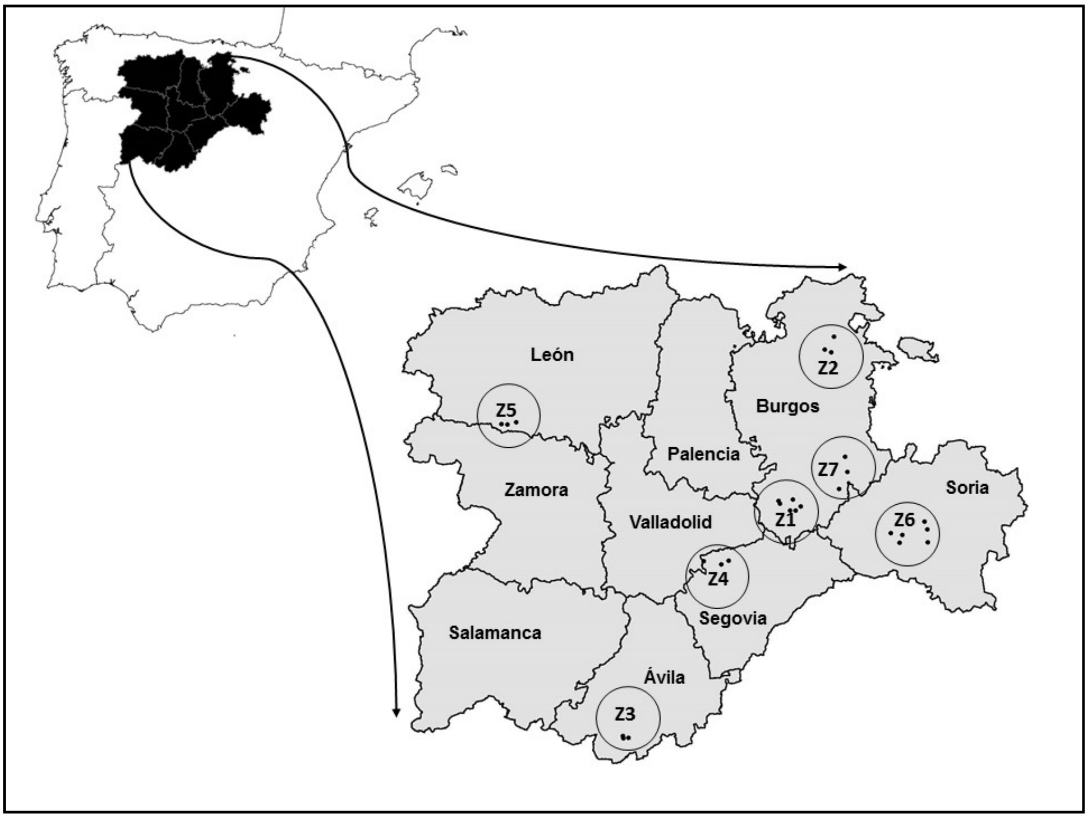


Figure 7.1. Geographical location of the plots in Castile and Leon within the sampling zones (Z1=Ribera del Duero, Z2=Oña, Z3=Gredos, Z4=Cuellar, Z5=Teleno, Z6=Soria, Z7=Montaña Soriano-Burgalesa), in the different provinces of the region (León, Burgos, Segovia, Soria y Ávila).

Sampling was carried out in seven zones according to the provenance region of *Pinus pinaster* and province (Table 7.1). Within these zones, 27 circular sampling plots of radius 15 m were randomly selected using ArcGIS 9.3.

Table 7.1. Location and coordinates of the 27 plots grouped by zones (Z1 - Z7).

Zone	Provenance	Province	Location	X Coordinate	Y Coordinate
Z1	Meseta Castellana	Burgos	Quintana del Pidio	3° 44’ 48”	41° 46’ 18”
Z1	Meseta Castellana	Burgos	La Horra 2	3° 52’ 00”	41° 45’ 46”
Z1	Meseta Castellana	Burgos	Villalba de Duero	3° 46’ 09”	41° 42’ 05”
Z1	Meseta Castellana	Burgos	La Horra 1	3° 51’ 18”	41° 44’ 44”
Z1	Meseta Castellana	Burgos	Gumiel de Izan	3° 40’ 42”	41° 43’ 40”
Z1	Meseta Castellana	Burgos	Aranda de Duero	3° 43’ 26”	41° 42’ 01”
Z2	Sierra de Oña	Burgos	Pino de Bureba	3° 25’ 46”	42° 42’ 08”
Z2	Sierra de Oña	Burgos	Aguas Candidas	3° 29’ 11”	43° 43’ 10”
Z2	Sierra de Oña	Burgos	Trespaderne	3° 24’ 21”	42° 48’ 06”
Z3	Sierra de Gredos	Ávila	El Hornillo 1	5° 7’ 22”	40° 14’ 43”
Z3	Sierra de Gredos	Ávila	El Hornillo 3	5° 7’ 33”	40° 15’ 34”
Z3	Sierra de Gredos	Ávila	El Arenal	5° 4’ 45”	40° 14’ 54”
Z4	Meseta Castellana	Segovia	Iscar	4° 29’ 06”	41° 22’ 29”
Z4	Meseta Castellana	Segovia	Cuellar	4° 16’ 52”	41° 22’ 47”
Z4	Meseta Castellana	Segovia	Arroyo de Cuellar	4° 20’ 23”	41° 21’ 13”
Z5	Sierra del Teleno	León	Pinilla de la Valdería	6° 5’ 36”	42° 13’ 10”
Z5	Sierra del Teleno	León	Castrocontrigo	6° 12’ 59”	42° 12’ 13”
Z5	Sierra del Teleno	León	Nogarejas	6° 9’ 55”	42° 12’ 13”
Z6	Meseta Castellana	Soria	Quintana Redonda	2° 38’ 25”	41° 37’ 58”
Z6	Meseta Castellana	Soria	Tardelcuende	2° 37’ 14”	41° 35’ 01”
Z6	Meseta Castellana	Soria	Matamala de Almazán	2° 36’ 49”	41° 30’ 07”
Z6	Meseta Castellana	Soria	Hortezuela	2° 50’ 58”	41° 29’ 56”
Z6	Meseta Castellana	Soria	Tajueco	2° 49’ 42”	41° 32’ 54”
Z6	Meseta Castellana	Soria	Bayubas de Arriba	2° 55’ 22”	41° 33’ 40”
Z7	Montaña de Soria - Burgos	Burgos	Salas de los Infantes	3° 18’ 37”	42° 2’ 36”
Z7	Montaña de Soria - Burgos	Burgos	Gete	3° 17’ 28”	41° 56’ 48”
Z7	Montaña de Soria - Burgos	Burgos	Huerta del Rey	3° 21’ 36”	41° 50’ 22”

Between July and October 2011, diameter at breast height and total height were measured in all trees within each sampling plot (a total of 1208 trees). Forest health variables were assessed in 24 trees per plot (a total of 648 trees) (Table 7.2). At the same time, wood samples were collected from 9 trees per plot (from 3 healthy, 3 symptomatic or damaged and 3 dead trees, if present). A total of 212 wood samples were collected (81 from healthy trees, 81 from symptomatic trees and 50 from recently dead, standing trees; note that not all stands included this type of tree).

For the phytosanitary assessment, crown defoliation was visually estimated, by a single operator, in twenty classes of 5 % interval width, relative to an absolute reference tree (Sánchez Peña, 1994). Other crown variables, such as discoloration, measured according to Level I of the European network methodology (Eichhorn et al., 2010). As the use of different vitality indicators is highly recommended (Cailleret et al., 2014), other phytosanitary variables were also assessed, including the presence of cankers, cracks in the bark (caused by wood compression), dieback, presence of microphylls, needle retention, and the proportion of healthy, symptomatic (or damaged) and dead trees (Table 7.2).

For environmental characterization, soil samples from each plot were analysed (Prieto-Recio et al., 2015). The climatic variables considered were elevation, mean temperature, and mean precipitation. The historical climatic data were obtained from the Digital Climatic Atlas of the Iberian Peninsula (Ninyerola et al., 2005). The values of the different environmental variables are summarized in Table 7.2.

Table 7.2. Summary of number of trees measured, forest health status and environmental characteristics of the 27 plots under study in each study zone (mean ± sd).

	All stands	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**
Number of trees measured	*1208*	*274*	*118*	*97*	*88*	*151*	*302*	*178*
Density (trees/ha)	591.6 (61.5)	594.2 (129.8)	528.2 (108.8)	448 (54.4)	386.7 (75.5)	702.6 (110.9)	648.4 (188.8)	773.4 (297.8)
Diameter (cm)	29 (1.6)	23.6 (1.7)	24.1 (3.5)	43.6 (2.0)	34.7 (2.3)	20.5 (2.7)	32.4 (2.9)	26.2 (3.1)
Height (m)	13.2 (0.8)	10 (0.6)	9.3 (1.83)	22.4 (0.3)	16.1 (0.6)	9.1 (0.85)	14.5 (0.8)	12.8 (1.3)
Number of trees assessed	*648*	*144*	*72*	*72*	*72*	*72*	*144*	*72*
Live crown ratio (%)	86.8 (1.8)	77.6 (3.6)	87.5 (5.1)	86.7 (3.2)	96.7 (3.3)	84.7 (4)	90.8 (3.5)	88.6 (6.3)
Crown defoliation (%)	36 (1.7)	43.1 (2.9)	29.2 (5.8)	32.9 (2.5)	30.6 (4.9)	41.9 (5)	34.4 (3.9)	34.3 (4.3)
Crown discoloration (%)	29.1 (1.8)	39.6 (3.3)	24.1 (2.4)	23.7 (3.9)	22.7 (2)	34.1 (3.1)	25.3 (4.2)	27.5 (5.1)
Dieback (%)	9.1 (1.59)	15.3 (3)	15.3 (7.3)	4.2 (2.40)	2.8 (1.39)	16.7 (2.4)	3.5 (1.99)	5.6 (2.78)
Microphylls (%)	17.9 (4.3)	41.2 (10.6)	5.7 (3.65)	0 (0)	13.3 (7.9)	37.7 (17.6)	8.6 (3.58)	4.9 (3.06)
Cankers (%)	6.9 (1.75)	15.3 (2.8)	0 (0)	13.9 (11.9)	5.6 (2.78)	2.8 (1.39)	1.4 (1.38)	6.9 (1.39)
Cracks (%)	39.5 (5.4)	47.9 (8.7)	58.3 (12.5)	33.3 (33.3)	61.1 (7.7)	26.4 (13.2)	31.9 (10.7)	16.7 (6.4)
Needle retention (years)	3.7 (0.13)	3.1 (0.27)	4.1 (0.22)	3.46 (0.27)	4.3 (0.19)	3.9 (0.36)	3.5 (0.21)	4.5 (0.21)
Dead trees (%)	9.8 (1.75)	15.3 (4.6)	6.9 (3.67)	6.9 (5.01)	2.8 (3.9)	12.5 (4.2)	8.3 (3.57)	11.1 (6.1)
Symptomatic trees (%)	28.2 (1.9)	41 (1.3)	26.4 (3.7)	22.2 (5.6)	27.8 (5.6)	27.8 (6.1)	22.2 (3.7)	23.6 (2.8)
Healthy trees (%)	62 (2.9)	43.7 (4.4)	66.7 (6.4)	70.8 (4.2)	69.4 (7.3)	59.7 (10)	69.4 (6.1)	65.3 (3.7)
Environmental characterization								
Elevation (m)	880.6 (27.9)	860.3 (7.4)	671.3 (64.5)	1013.3 (28.5)	673 (102.5)	934.3 (23.0)	935.7 (30.5)	1041.7 (11.5)
Annual precipitation (mm)	636.1 (52.8)	479.7 (6.1)	661.7 (22.4)	1366 (47.0)	478.7 (5.0)	556.3 (72.7)	531 (5.7)	641 (24.0)
Mean annual temperature (°C)	11.4 (0.1)	11.3 (0.2)	11.7 (0.3)	12.3 (0.3)	12 (0)	10.7 (0.3)	11.2 (0.2)	10.7 (0.3)

Samples of wood (15 x 15 cm) were removed from the base of each sampled tree with an axe. For each tree, piece of wood (including xylem and phloem) was wrapped in paper wetted with distilled water and placed in a plastic bag, which was then hermetically sealed. Sampling at the base of the trees was justified because most of the lethal infections of rot root and blue-stain pathogens arise at the level of the roots, the collar or the lower part of the stem (Grylls and Seifert, 1999; Woodward et al., 1998). Once in the laboratory, the wood samples were stored at 4°C within 24 h (Zamora et al., 2008).

Morphological identification and fungal isolation

The wood samples were processed and incubated in dark conditions in moist chambers (i.e. the plastic bags in which they were placed in the field) at room temperature (24 ± 2 °C) for two weeks to promote development of any fungal colonies present (Zhou et al., 2001). The samples were maintained under constant observation, and the paper surrounding the wood was wetted every 2-3 days with sterile distilled water. Completely hermetic conditions were avoided to prevent accumulation of excessive moisture, which could provide an advantage to generalist fungi. Once fruiting bodies were observed, the moist chambers were opened and the pieces of wood were placed under the binocular magnifying glass for examination. The fruiting bodies were removed with the aid of a scalpel and tweezers and placed on slides previously moistened with a drop of distilled water. The samples were then covered with coverslips and observed under an optical microscope. Morphological identification of the fungus was mainly based on the structure and composition of reproductive cells (conidia, ascospores, fruiting bodies). The microscope (Nikon Eclipse E-400 model) was connected to a camera and a video screen. Photographs of the different morphological structures were taken and the images were projected on to a calibrated monitor for direct measurement of the structures. Different taxonomic keys and books were used for fungal identification (Barnett and Hunter, 1998; Grylls and Seifert, 1999; Hanlin, 1998; Sinclair et al., 1987; Wingfield et al., 1993; Woodward et al., 1998).

Additionally, fungi were isolated in culture media for identification. Potato Dextrose Agar (PDA, 42 g per litre of distilled water) and Malt Extract Agar (MEA, 20 g/l Malt Extract, 15 g/l Agar per litre of distilled water) were prepared with streptomycin (0.6 g/l) to inhibit the growth of contaminants such as saprophytic bacteria. Fruiting bodies were removed from the wood samples and placed on culture media in Petri dishes with the aid of a scalpel or inoculating loop. The dishes were kept in the dark at room temperature (24 ± 2 °C) for one week and were then stored in the fridge at 4°C until examination. Identification of the fungi was based on macroscopic examination of the colony and its morphological characteristics such as the shape of the colony, colour of the surface and the texture and characteristics of the reproductive structures. The isolates were grouped by morphotypes and used for molecular characterization.

Genomic DNA extraction, PCR and sequencing

Single hyphae cultures were established by subculture of the isolates in Water Agar medium (15 g/l agar in a litre of distilled water) in darkness at room temperature for four days. The hyphae were thus dispersed, enabling transfer of the isolates on PDA Petri dishes with cellophane membrane (Surface Specialities, Wigton, Cumbria, UK) for extraction of DNA.

The DNA was extracted from the fungal culture by a modified version of the protocol proposed by Vainio et al. (1998). Briefly, a small piece of fungal material (2 x 1 mm) was cut from the colony and placed in a tube of 2 ml with 500 µl of sterile sand and 300 µl lysis buffer to disrupt the material. The tubes were centrifuged for 4 min at 30 Hz and then placed in a thermostatic bath (65 °C) for one hour. For extraction of nucleic acids, 350 µl and 250 µl aliquots of a mixture of phenol, chlorophorm and isoamyl alcohol (ratio 25:24:1) were added in successive steps. The mixture was then stirred in a Vortex mixer for 1 minute before being centrifuged for 2 min at 14000 rpm. The upper phase thus obtained was then transferred to a clean 1.5 µl tube, avoiding the white interphase. This procedure was repeated from 3 to 5 times. An aliquot (100 µl) of chlorophorm-isoamyl alcohol (24:1) was then added and the mixture was stirred for 2 min and centrifuged for 3 min. To precipitate the DNA, 100 µl of the upper phase. The tubes were preserved on ice for 20 min and centrifuged for 20 min at 30 Hz. The supernatant was removed and washed with 70 % ethanol. One ml of ethanol precipitate was then added to the tube, which was centrifuged for 5 min. Finally, the supernatant was again removed and dried at 50 °C for 10-15 minutes in a heater. The pellet was resuspended in 50 µl of TE solution and was then incubated at room temperature overnight.

The polymerase chain reaction (PCR) is used to amplify DNA sequences. In this case, ribosomal DNA Internal Transcribed Spacers (ITS) were amplified using the primers ITS-1F

(5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993; White et al., 1990). The polymerase chain reaction was carried out according to the recommendations of the manufacturer of Dynazyme II DNA-polymerase (Finnzymes Ltd, Espoo, Finland). The samples were denaturalized in a thermocycler for 10 min at 95 °C. Thirty-five (13+13+9=35) amplification cycles were then carried out, as follows: 13 cycles of 35 s at 95 °C, 55 s at 55 °C and 45s at 72 °C; 13 cycles of 35 s at 95 °C, 55 s at 55 °C and 2 min at 72 °C and finally 9 cycles of 35 s at 95 °C, 55 s at 55 °C and 3 min at 72 °C. In the final cycle the samples were held at 72 °C for 7 min (synthesis step) to ensure elongation.

PCR products (5 µl) were analysed by electrophoresis on 1.2% agarose gels in 1× TAE buffer. The molecular marker used to estimate the length of the products of amplification was λ-DNA Hind III – ΦX174Hae III (DyNAzyme™ DNA Polymerase Kit) (500 bp). The gels were stained with GelRed and visualized under UV light.

The final amplification products were purified with NucleoSpin® Extract II 10/2007 Rev. 06 (Macherey-Nagel Gmbh and Co.KG) according to the manufacturer's instructions and were then sent to SECUGEN S.A. (Madrid, Spain) for sequencing in both directions. Briefly, 1.5 µl of primer ITS-1F was added to 5 µM to a sample tube containing 15 µl of DNA to obtain the sequence 5'-3', and 1.5 µl of primer ITS- 4 was added to 5 µM in the other tube containing 15 µl of DNA of the same sample to obtain the complementary 3'-5' sequence.

The sequences were corrected, aligned and assembled using the Geneious Pro 5.4 program. Consensus sequences were identified by comparison with sequences deposited in GenBank® (NCBI) database by using the BLAST (Basic Local Alignment Search Tool) algorithm.

Data analysis

Saprophytic species were not included in the following analysis. The absolute and relative frequency of the fungal species, in relation to the health status of the sampled trees and to the sampling zone, were calculated and plotted using R software environment (R Core Team, 2014). Canonical Correspondence Analysis (CCA) was carried out to assess the influence of the main explanatory variables (zone, health status, climate conditions) on the fungal species encountered, and the Monte Carlo test was used to determine the significance of the results, with 499 permutations. The constrained ordinations were performed with CANOCO 4.5 software for Windows (Ter Braak and Smilauer, 2002).

Results and discussion

Description of species encountered

Analysis of the samples by molecular and morphological techniques led to the identification of fifteen distinct species. Nine of these were primary pathogens (indicated by * in the table) and the other six were saprophytes (Table 7.3). More detailed descriptions of the main species are included in an appendix at the end of the chapter.

Table 7.3. List of species identified: pathogenic species (marked with *) and saprophytic species, and their taxonomic classification. The sampling zones (Z1 - Z7) where the isolates originated and the proportion (%) of isolates of each species identified are also shown.

Species (S/P)	**Taxonomic classification**	**Zone(s) where isolated**	**Proportion of Isolates (%)**
Bionectria ochroleuca (Schwein.)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Bionectriaceae)*	Z1, Z3, Z7	3.774
* ***Ceratocystis brunneocrinita*** (E.F. Wright & Cai)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Microascales, Ceratocystidaceae)*	Z1	0.472
* ***Heterobasidion annosum*** (Fr.) Bref.	*(Basidiomycota, Agaricomycetes, Russulales, Bondarzewiaceae)*	Z4	0.472
* ***Ophiostoma fasciatum*** (Olchow. & J. Reid)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1	0.472
* ***Ophiostoma ips*** (Rumbold) Nannf.	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1	0.943
* ***Ophiostoma minus*** (Hedgcock)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1, Z3, Z4, Z5	3.302
* ***Ophiostoma pallidobrunneum*** (Olchow. & J. Reid)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1, Z2, Z6	1.887
* ***Ophiostoma piliferum*** (Fr. Syd. & P. Syd.)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1, Z3, Z4, Z5	2.830
****Ophiostoma ranaculosum*** (J.R. Bridges & T.J. Perry)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Sordariomycetidae, Ophiostomatales, Ophiostomataceae)*	Z1	2.358
Penicillum corylophilum (Dierckx.)	*(Ascomycota, Pezizomycotina, Eurotiomycetes, Eurotiomycetidae, Eurotiales, Trichocomaceae)*	Z1	0.472
* ***Pestalosphaeria maculiformans*** (Marincowitz, M.J. Wingf. & Crous.)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Xylariomycetidae, Xylariales, Amphisphaeriaceae)*	Z3, Z7	0.943
Trichoderma atroviride (P. Karst)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Hypocreaceae)*	Z1, Z4, Z5	1.415
Trichoderma harzianum (Rifai)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Hypocreaceae)*	Z1, Z3	1.415
Trichoderma viride (Pers.)	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Hypocreaceae)*	Z5	0.943
Trichoderma viridescens (A.S. Horne & H.S. Will.) Jaklitsch & Samuels	*(Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Hypocreaceae)*	Z1, Z2, Z3, Z4, Z5, Z6, Z7	10.849

Pathogens such as *Ophiostoma minus* and *Ophiostoma piliferum* appeared in several areas with a high relative proportion of isolates. Saprophytic fungi also often appeared as laboratory contaminants (i.e. *Trichoderma viridescens* was abundant in samples from all zones) probably because some samples were held in moist conditions for longer than optimal before microscopic examination. Therefore, for subsequent data analysis only pathogenic fungi were considered.

Relationship between species encountered, areas of isolation and tree health status

A total of 15 species were isolated and identified from 212 trees (81 healthy, 81 symptomatic and 50 recently dead). The nine pathogenic fungal species recovered from these pine trees, as well as their frequencies in the seven sampling zones, are plotted in Figure 7.2. The zones where pathogenic fungi were found only on dead trees are shown at the top of the chart. The sampling zones where pathogenic fungi were found on trees of all types (as regards health status) are shown at the bottom of the chart.

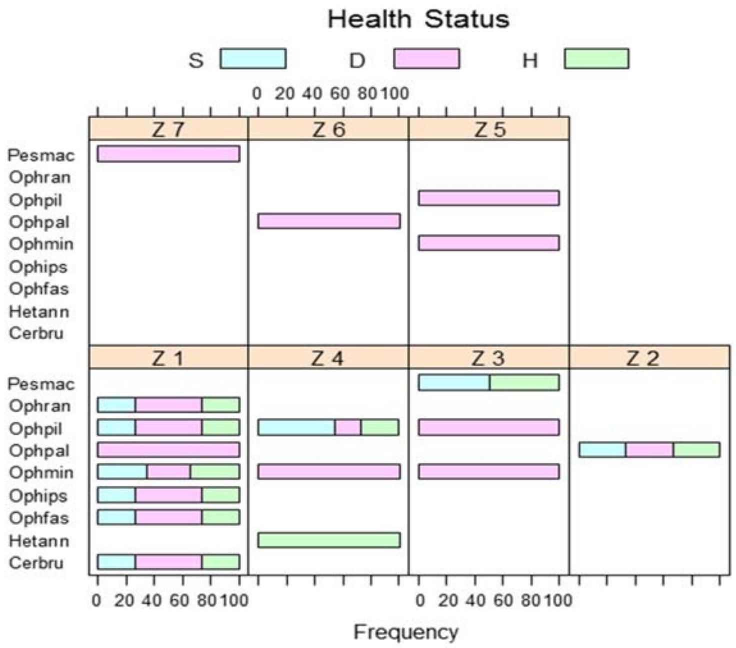


Figure 7.2. Bar chart of the relative frequency in percentage of the pathogenic species (Pesmac= *Pestalosphaeria maculiformans*, Ophran= *Ophiostoma ranaculosum*, Ophpil= *Ophiostoma piliferum*, Ophpal= *Ophiostoma pallidobrunneum*, Ophmin= *Ophiostoma minus*, Ophips= *Ophiostoma ips*, Ophfas= *Ophiostoma fasciatum*, Hetann= *Heterobasidion annosum* and Cerbru= *Ceratocystis brunneocrinita*), according to the health status of the trees (H= health, S= symptomatic and D= death) and the location of sampling sites (zones: Z1 to Z7). For zoning see Figure 7.1.

The sampling zone where the greatest number of pathogens was encountered was Z1 in Ribera del Duero (Burgos); the fungi isolated were obtained in equal numbers from healthy, symptomatic and dead *Pinus pinaster* trees. All of the *Ophiostoma* species identified in this study were present in this zone, and *Ophiostoma minus, Ophiostoma ips* and *Ophiostoma fasciatum* were strongly associated with *Pinus pinaster* decline as previously reported (Álvarez et al., 2015). In Z4 (Cuellar, Segovia) and Z3 (Gredos, Avila), *Ophiostoma minus* was mainly detected on dead trees, as previously observed (Álvarez et al., 2015). This pathogen was also found in Z5 (Teleno, León). Bark beetles such as *Ips sexdentatus,* which can carry fungal spores of this blue-staining fungi (Bueno et al., 2007), have previously been studied in the area (Etxebeste et al., 2012; Fernández et al., 2004). However, *Heterobasidion annosum,* which is known to be involved in *Pinus pinaster* decline throughout Castile and Leon (Prieto-Recio et al., 2012), was only found in Z4 (Cuellar). This species was isolated in samples from apparently healthy trees with no evident symptoms, indicating its strong virulence. The species was associated with *Ophiostoma minus* and *Ophiostoma piliferum* found in the same study site. The last area sampled was Z4 and the sampling was carried out early autumn, when fungal activity is greatest (Gonthier et al., 2005). The species probably did not appear in other zones because field sampling in these areas was conducted in summer and some of the samples may have been damaged due to high temperatures. In Z2 (Sierra de Oña, Burgos) and Z6 (Soria) only *Ophiostoma pallidobrunneum* was isolated and identified. In Z7 (Montaña Soriano-Burgalesa, Burgos), only *Pestalosphaeria maculiformans* was isolated and identified. These species are probably not primary pathogens responsible for the decline of the pine population, because the percentage of isolates in these zones was low and they were not associated with other pathogenic fungi.

Relationship between species encountered and the explanatory variables

The results of the Multivariate Canonical Correspondence Analysis showed that climate variables such as temperature, precipitation and elevation did not significantly affect the pathogenic fungal communities (Figure 7.3). The result of the Monte Carlo test, with 499 permutations, was not significant (P-value=0.378), and the cumulative percentage variance of species-environment relation showed that 98.4 % of the total variation was explained by the first two axes. The pathogenic species were mainly concentrated, although not significantly, in zone 1 (Ribera del Duero), where precipitation is relatively low, which is consistent with the results of a previous study (Prieto-Recio et al., 2015).

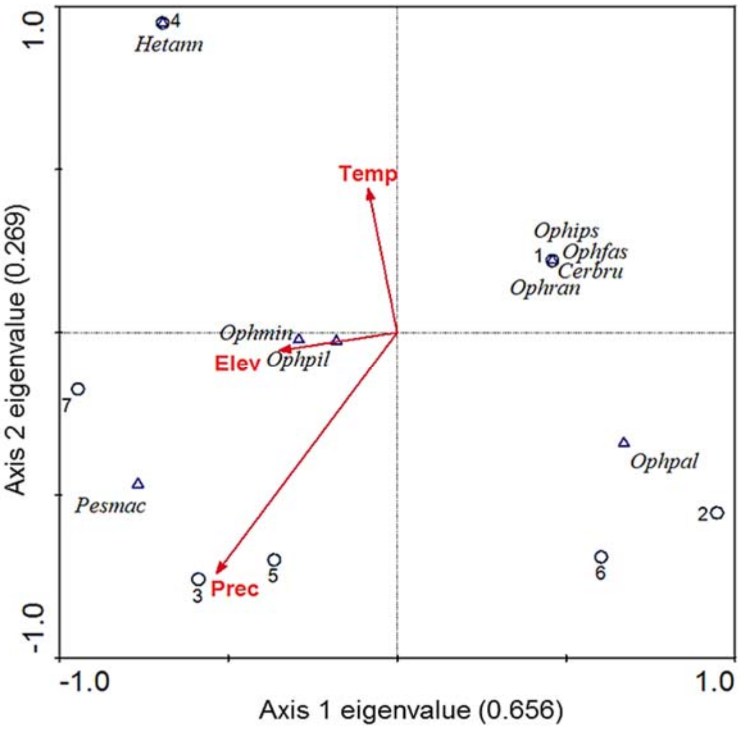


Figure 7.3. CANODRAW CCA triplot: full species, sampling sites and explanatory climate variables. Symbols: circles represent sampling zones (1 - 7); triangles represents species abundance. Species abbreviations, as in Figure 7.2 (first three letters of genus and first three letters of species names). Arrows: Prec=Mean annual precipitation, Temp=Mean annual temperature and Elev=Elevation.

The proportion of healthy, symptomatic and prematurely dead trees did not differ significantly between the pathogenic fungal communities sampled in each of the seven areas (P-value=0.458) according to the results of the Monte Carlo test (499 permutations). The relationship between cumulative percentage variance of species and health status showed that 93.9 % of the total variation was explained by the first two axes (Figure 7.4). Although the results were not significant, some trends were observed, as before. The Ophiostomatoid group detected in Z1 (Ribera del Duero) was mainly associated with high proportions of symptomatic and dead trees, which is consistent with the findings of other studies (e.g. Álvarez et al., 2015). Nevertheless, Ophiostomatoid fungi appeared in the center of the graph and were associated with trees representing all health conditions. *Heterobasidion annosum* was only found associated with healthy trees, although this primary pathogen is known to occur on healthy and damaged trees (Woodward et al., 1998). Furthermore, this fungus has been shown to be capable of killing *Pinus pinaster* seedlings from the same provenance region as the affected zone in this study (Prieto-Recio et al., 2014).

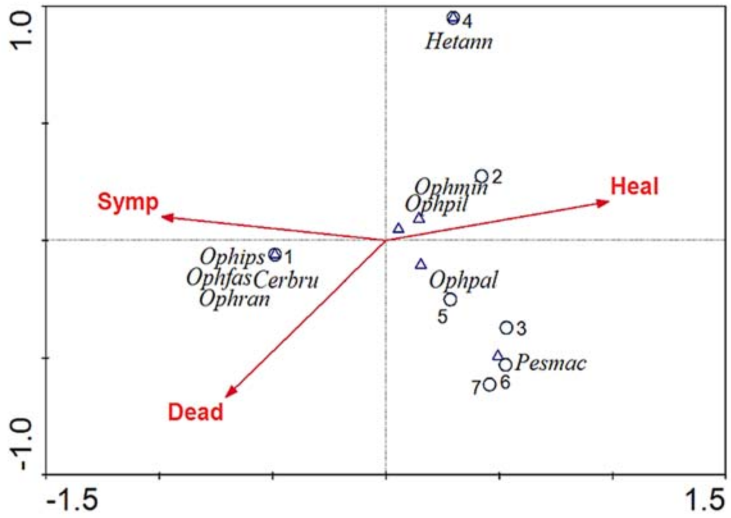


Figure 7.4. CANODRAW CCA triplot: species identified, sampling sites and explanatory heath status variables. Symbols: circles represent sampling zones (1 - 7); triangles represent species abundance. Species abbreviations, as in Figure 7.2 (first three letters of genus and first three letters of species names). Arrows: Heal= Percentage of healthy trees, Symp= Proportion of symptomatic trees (%) and Dead= Proportion of prematurely dead trees (%).

Conclusions and final remarks

The 15 species identified during the study were grouped according to their ecology: six species were saprophytes and the other nine were pathogens displaying different degree of virulence. The first group was not considered relevant to this study because as opportunists they are highly unlikely to be responsible for Maritime pine decline.

From a diagnostic point of view, the species of interest were those belonging to the group of Ophiostomatoid fungi, which attack wood and cause blue-stain symptoms (and are often considered as principal agents responsible for Maritime pine decline), and *Heterobasidion annosum*, which is thought to trigger the death of weakened trees in the decline process.

Four out of seven species belonging to the group of Ophiostomatoid fungi identified were particularly relevant to the objectives of this study: *Ophiostoma minus*, *Ophiostoma ranaculosum, Ophiostoma ips* and *Ophiostoma piliferum*. *Ophiostoma minus* was the most abundant species as a result of its strong competitive ability and its ability to kill invaded trees. The abundance of this species on symptomatic and dead trees indicates that it plays a crucial role in the decline of Maritime pine in the center of Iberian Peninsula. *Ophiostoma ranaculosum* was the second most abundant species and is described as a strong primary pathogen sometimes found in association with bark beetle communities. *Ophiostoma ips* and *Ophiostoma piliferum* were the least well represented species in this study but are also associated with Maritime pine decline in Spain.

Heterobasidion annosum was found in this study as primary pathogen in the same region as it was first recorded in association with *Pinus pinaster* decline in Spain (Prieto-Recio et al., 2012). Although it is unusual only to have found the species on a few trees despite the decline process being well established, the sampling period in the present study was probably not optimal. We can conclude that the presence of *Heterobasidion annosum* is fairly recent and specific sampling should be carried out in the near future.

We cannot exclude the possibility that Maritime pine decline is due to a combination of biotic factors (fungal, entomological and parasitic) and abiotic factors (edaphic, climatic and silvicultural) (Manion, 1991) as reported in several studies (e.g. Oliva and Colinas, 2007; Otrosina et al., 1999; Prieto-Recio et al., 2015). The present study should therefore be viewed as a first glimpse of the biotic factors involved in Maritime pine decline in the center of the Iberian Peninsula, thus providing the basis for more detailed studies.

Appendix: Description of the main species identified

Bionectria ochroleuca (Schwein.). (Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, *Bionectriaceae*). It colonizes live plants as an endophyte and decomposes organic matter in the soil as a saprophyte. It produces a large variety of antibiotic agents and can be used as an agent for the biological control of various pathogens of plants (Schroers, 2001). It is a filamentous fungus that produces widely white or yellowish colonies of fast growth and expansion. This microorganism has transparent and septate hyphae; erect conidiophores branched at the top. Conidia are unicellular, ovoid or cylindrical.

Ceratocystis brunneocrinita (E.F. Wright & Cai). (Ascomycota, Pezizomycotina, Sordariomycetes, *Hypocreomycetidae*, Microascales, *Ceratocystidaceae*). The genus *Ceratocystis sensu stricto*, includes a large number of pathogens of conifers and deciduous trees causing vascular withering, wood stains, cankers, root fruit and flower rots across the world. Many species produce volatile metabolites attracting a wide variety of insects to infected plant tissues. However, the association between insects and the different species of the genus *Ceratocystis* is highly variable, presenting several kinds of mutual associations. The high degree of morphological similarity between the species belonging to this group makes difficult a clear identification. Nevertheless, fortunately during the last decade, the use of phylogenetic studies based on the comparison of DNA contributed substantially to clarify the taxonomy of this group (Marín Montoya and Wingfield, 2006). Generally, they are susceptible to the antibiotic cycloheximide (Harrington, 1981) and include the anamorphs *Chalara / Thielaviopsis* with endoconidia (Zipfel et al., 2006). The base of the perithecium in the samples of *Ceratocystis brunneocrinita* varies from dark brown to black, from 70 to 150 µm of diameter. Neck has the same colour and length measures are around 100 to 450 µm. The base of the neck is 20 to 40 µm wide and from 11 to 25 µm at the apex. The ostiolares hyphae are long 25-55 µm and present a cylindrical shape. Ascospores are rectangular and with a size of 3.5 to 5 x 1.4 to 2.4 µm (Ohtaka et al., 2006).

Heterobasidion annosum (Fr.) Bref. (Basidiomycota, Agaricomycetes, Russulales, *Bondarzewiaceae*). *Heterobasidion annosum sensu lato* is a pathogenic fungi commonly known as “annosum root rot”. It mainly attacks conifers and is very widespread in the temperate regions of the northern hemisphere preferring species of the genera *Pinus*, *Picea*, *Abies*, *Cedrus* and *Pseudotsuga* (Asiegbu et al., 2005). *Heterobasidion annosum* was previously recorded on *Pinus sylvestris* in central Spain (Benito Martínez, 1943), causing needle drop, swelling at the stump height, and presence of dead trees by circular areas. This pathogen has been also reported in Spain on *Abies pinsapo* (Navarro et al., 2003) and *Abies alba* natural areas (Oliva and Colinas, 2007), on *Pinus nigra* northern stands (Oliva et al., 2008), on *Chamaecyparis lawsoniana, Pinus radiata, Pseudotsuga menziesii* and *Picea* *abies* (Mesanza and Iturritxa, 2012) and recently detected on *Pinus pinaster* (Prieto-Recio et al., 2012). The entrance way of the infection is frequently fresh stumps where basidiospores widespread by wind colonize the area. Instead, it is unusual the entry through wounds on the trunk. After that, it moves to the root system via mycelium, where can move long distances through the radical anastomosis of neighbour plants (Otrosina et al., 1999). *Spiniger meineckellus*, the name for of the asexual stage of this fungus, is produced on stumps when the conditions are moist, and the conidiophores that are produced are able to live in the soil for up to ten months. In addition, unlike the basidiospores, which are ejected actively and disperse with great ease in the air, the conidia are sessile and are independent from external agents (wind, rain, animals) (Korhonen and Stenlid, 1998). Colonization by the pathogen proceeds with different speed, depending on the size of the host and by its phytosanitary status: virulence and the diffusion will be as faster as the state of decay of the tree is advanced. On average passes from 6 to 10 years between infection and the appearance of the stump of fungal spots in the roots of the nearest tree, demanding an even more time for the appearance (rare) of the sporophores (Low, 1958). At the level of the collar it is possible to meet the fruiting bodies of the fungus, even if they appear generally hidden under the foliage. It is considered a pathogen of difficult detection, as the symptomatic side, covered by a typical white mycelium, is under the ground or in the first few centimetres of the collar (Hodges, 1999). The conidiophores observed in the samples contain conidia of subglobose to pyriform form measuring around 5.8 x 4.2 µm.

<u>Ophiostoma</u> (Syd. & P. Syd.). (Ascomycota, Pezizomycotina, Sordariomycetes, *Sordariomycetidae*, Ophiostomatales, *Ophiostomataceae*). The species of the group *Ophiostoma* have a quite complicated taxonomy and its species have been relocated frequently from one genre to another according to the character considered, according the scope of the observation and the technology available. More recent studies (Zipfel et al., 2006), based on the comparison of DNA sequences, defend the idea that there are three lineages more or less defined. A first line present the anamorph *Leptographium* in common with the teleomorph *Grosmannia* (typical species: *Grosmannia penicilliata*) that incorporate 27 species and 24 new combinations. A second line includes species sensitive to cycloheximide of the type of *Hyalorhinocladiella*, which have short perithecial necks and conic spores. The anamorph would be included in the genre *Ceratocystiopsis* (typical species: *Ceratocystiopsis minute*), counting 11 species and 3 new combinations. Equally sensitive to cycloheximide is *Ceratocystis*, with anamorphs *Chalara* and *Thielaviopsis*. A third line of species is represented by *Ophiostoma* and its anamorphes *Sporothrix* and *Pesotum*. The ascocarps are perithecia, varying slightly from dark brown to black, with bulbous base and erect necks of cylindrical shape from brown to black (Zipfel et al., 2006). The ascospores are hyaline, usually unicellular, although sometimes are bicellular (with a septum). They can assume different shapes including cylindrical, kidney shaped, half orange or pillow. In many species ascospores present various layers in the wall that look like a gelatinous sheath in the light of the microscope. These spores without pore germination, are produced in a mucilaginous matrix and oozing along the neck forming a viscous height droplet of perithecium (Upadhyay, 1999). Spores are mugillacinose and are spread through drillers insects. Although, some species use waterways transport systems or other animals as vectors (Upadhyay, 1999). *Ophiostoma* species colonize different ecological niches and are characterized by having two types of behavioural association: commensalism and parasitism. The latter behaviour provoke microphylls, yellowing and early fall of needles. Sometimes the plant exudes resin at the base of the trunk. The wood colonized by the agent of tracheomycosis looks tinted with clear dark veins determined by the intense pigmentation of the hyphae of the fungus (Nicolotti et al., 2008).

Ophiostoma fasciatum (Olchow. & J. Reid). The base of the perithecium and the neck varies from dark brown to black, respectively from 50 to 110 μm of diameter the first and 100 μm long and less than 10 μm wide at the apex. The ostiolares hyphae are parallel or converging of 50 μm of size. The ascospores are fusiform, apparently in shaped sheath, from 7 to 20 μm in length and from 1 to 2 μm in width (Grylls and Seifert, 1999).

Ophiostoma ips (Rumbold) Nannf. The base of perithecium is globose or subglobose, dark brown to black and 131 to 263 μm of diameter, usually ornamented by aseptate light brown hyphae measuring around 1.8 to 4.9 μm wide. The neck is generally black or dark brown; the length varies from 301 to 766 μm and 19 to 38 μm wide at the base. The hyphae observed in the samples are ostiolares and unicellular, hyaline, rectangular and slightly depressed at the center of 4.0 to 5.9 x 1.8 to 2 μm. The covering hyaline sheath forms small depressions at the corners giving a look of a pillow (Marmolejo and Butin, 1990). It is one of the fungi causing blue-stain of wood in conifers associated with some species of bark beetles of *Ips* and *Dendroctonus* species (Zhou et al., 2001). Its virulence has been recorded in various studies that registered a high mortality when overstep a standard threshold of inoculation (Fernández et al., 2004; Masuya et al., 2003) on seedlings, young and mature trees but also healthy plants. In some places like South Africa it is not especially aggressive; nevertheless in western Japan it is responsible for the death of the tree associated with *Ips* species (Fernández et al., 2004).

Ophiostoma minus (Hedgcock). The base of the perithecium and the neck veers from dark brown to black, respectively from 50 to 125 μm of diameter and 65 to 140 μm less and less 20 μm wide at the apex. Ostiolare hyphae are absent or when present are divergent and 50 μm long. Allantoidal or kidney-shaped ascospores measure 2 to 5.5 x 1.5 μm having no gelled sheath. This pathogen is considered one of the most virulent Ophiostomatoid fungi on pines (Masuya et al., 2003). Its virulence has been studied in Europe in combination with *Tomicus piniperda* (Romón et al., 2007) highlighting complex relations with host trees, insects and other fungi (Hausner et al., 2005). Evidences come from (Hofstetter et al., 2006) that tested these complicated relations underlying the mutual behaviour of *Ophiostoma minus* with its vector *Dendroctonus frontalis* (the Southern Pine Beetle) in North America in charge of transporter and neutralizer of host defences.

And recently has been related with important diseases like the pine wood nematode pathogen *Bursaphelenchus xylophilus* (Maehara et al., 2005) and Maritime pine decline (Álvarez et al., 2015).

Ophiostoma pallidobrunneum (Olchow. & J. Reid). The base of the perithecium varies from light brown to yellow, from 50 to 100 µm of diameter. The neck is light brown, 50 to100 µm long and 10 to 50 µm wide at the apex. The ostiolare hyphae are convergent and vary from brown to black or in some case are hyaline measuring 50 µm. Ascospores are fusiform gauging 10 to 20 x 2.1 µm (Grylls and Seifert, 1999).

Ophiostoma piliferum (Fr. Syd. & P. Syd.). The base of the perithecium changes from dark brown to black, from 50 up to 300 µm of diameter. The neck varies from dark brown to black from 300 to 500 µm in length and up to 20 µm of width at the top. The ostiolares hyphae are 100 µm long, hyaline, ajar or parallel. The ascospores are kidney shaped with the absence of the shaped sheath, from 3 to 7 m in length and 1 to 2 µm in width (Grylls and Seifert, 1999). It is a very aggressive primary pathogen that causes blooming wood of conifers, colonizing the parenchymal cells and resin channels (Fishcer et al., 1994). Studies conducted by (Blanchette et al., 1992) have shown that it reduces the amount of resin in the tree, devitalizing the immunological defences.

Ophiostoma ranaculosum (J.R. Bridges & T.J. Perry). The base of the perithecium varies from dark brown to black, from 50 to 110 µm of diameter. Likewise the neck varies from dark brown to black and measures 20 to 30 µm long and from 15 at 25 µm width at the apex. Ascospores measure 8 to 16 µm (Hsiau and Harrington, 1997). In nature is found in association with *Dendroctonus frontalis* and *Dendroctonus brevicomis*, bark beetle insects that attack pine forests. *Dendroctonus frontalis* digs tunnels under the bark of pine trees, laying its eggs to let the larvae feed protected under the bark. *Ophiostoma ranaculosum* usually grows in the galleries opened by bark beetle and serves as food for the larvae. In addition, there is a third fungus, *Ophiostoma minus*, which helps the insect to overcome the defences of the tree; the tree responds by secreting resin to seal wounds expelling it from the bark (Harrington, 2005).

Penicillium corylophilum (Dierckx.). (Ascomycota, Pezizomycotina, Eurotiomycetes, Eurotiomycetidae, Eurotiales, *Trichocomaceae*). It is a fungus from the rapid growth that generates initially white velvety colonies, covered with spores that progressively veer colour; while the end is characterized by a group of spores with a dusty look. The colony consists of white mycelium formed by thin and set hyphae. The classification of the whorl is a critical step, however it is possible to recognize four major groups: monoverticilate, asymmetric, biverticilate and poliverticilate (Arias and Piñeros, 2008). The conidiophores are biverticilates and asymmetric, with some monoverticilate structures. Conidia are smooth-walled, sub-globular of 2.5 to 3.2 x 2.5 to 3 µm of dimension. The species of the genus *Penicillium* produces various secondary metabolites, including cyclopiazonic acid, penicillin acid, cyclochlorotine, citroviridine, citrinin, griseofulvin, ochratoxin A and patulin (Pitt and Hocking, 2009). All these substances are generated from the organism to overwhelm the environment by inhibiting other organisms competing for the same substrate. Mycotoxins have a different chemical structure; their molecular weight is relatively low thus easily spread in the culture medium in laboratory (Samarajeewa, 1991).

Pestalosphaeria maculiformans (Marincowitz, M.J. Wingf. & Crous.). (Ascomycota, Pezizomycotina, Sordariomycetes, Xylariomycetidae, Xylariales, *Amphisphaeriaceae*). The genus *Pestalotiopsis* has received considerable attention in recent years, not only for his role of pathogen, but also for the one of endophyte, being a great producer of a wide range of chemical metabolites. To classify the species within the genus is necessary a morphological classification, with careful attention on its conidia (Maharachchikumbura et al., 2011). *Pestalosphaeria maculiformans* produces round light-brown spots of irregular shape varying from 0.25 to 2.5 cm of diameter (Steyaert, 1953). The acervuli are circular and produce black conidia with a fusiform shape with a size around 24 to 25 x 29 to 31 µm and 7 to 8 x 9.5 to 10 µm (Marincowitz et al., 2008). It is made up by four cells gauging 3 to 5.5 x 4 to 6 µm veering from brown to hyaline yellow. Sometimes the prolongations have widths from 13 to 24 µm at the top and a long peduncle of 16 to18 µm at the bottom (Marincowitz et al., 2008).

Trichoderma (Pers.). (Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, *Hypocreaceae*). The genus *Trichoderma* was introduced by Persoon 200 years ago and consists of anamorphic fungi isolated mainly from the soil and decaying organic matter (Grondona et al., 1997). In general, the colonies of *Trichoderma* initially present a hyaline aerial mycelium that can assume a flocculent appearance depending on the strain and the culture medium, and can take with time a green colouring more or less intense. The conidiophores are produced and widespread by the colony in clumps, pustules or more commonly in concentric rings in shades of green. Conidiophores are often formed by a central axis with regular lateral branching, where split into new several branches in a pyramidal structure. Phialides conidiogene are formed by hyaline cell appearing cylindrical, subglobose or ampoule-shaped. The conidia are unicellular, usually green or hyaline, rarely yellow. Their wall is usually smooth but sometimes wrinkled. The shape could be elliptic, elongated or cylindrical, rarely globose with a size of about 3 to 5 µm of diameter. Conidia appear generally dry, although some species are found together in gelatinous groups of heads. Chlamydospores (quiescent resistant structure of the fungus) are present in the hyphae, in the leap or at the end and can be unicellular or multicellular taking different forms and colours (green or pale yellow). Often chlamydospores are coated with a thickened wall. The vegetative hyphae are normally hyaline, with smooth wall and a width of 2 to10 µm. The optimum temperature of growing is around 25-30 °C until a limit of 35 °C where growth stops (Chaverri and Samuels, 2003). The species of this genus are natural inhabitants of soil and can behave as saprophytes, or parasites of other fungi. They are widely known for their character of antagonist used as biocontrol agents. Thanks to their ubiquity, their fast growth and the large number of substrates that colonizes they are easily isolated in laboratory (Harman et al., 2004). The identified species were ***Trichoderma atroviride, Trichoderma harzianum, Trichoderma viride, and Trichoderma viridescens.***

Trichoderma harzianum (Rifai) has been found in different organic materials and soils and it has been adapted to different environmental conditions facilitating its distribution. Some species prefer dry and temperate conditions, while other cold temperatures. This fungus is widely known as a producer of toxins and antibiotics (Seaby, 1989). In the initial stage, the colour of the mycelium is white and subsequently develops a dark green colour after the sporulation. It prefers an acidic pH of 4.5-5, however, it is spread also in areas with excessive moisture content and stagnation of carbon dioxide in the atmosphere. The size of the conidiophores ranges from 62.5 to 69 x 3 to 4.7 µm. Its colour is dark green and presents several perpendicular ramifications in groups of two or three apparently in a pyramidal shape. The phialides are large, elongated, asymmetrical that measure 6.3 to 15.6 x 2.7 to 3.4 µm with whorls ending with four lemon-shaped or subglobose conidia of 3.1 to 3.7 x 3.8 to 4 µm. The clamidiospore are formed from submerged mycelium and are subglobose, toothed and light green measuring 10 to 12.5 µm (Bissett, 1991).

References

Alía, R., Moro, J., Denis, J.B., 1997. Performance of *Pinus pinaster* provenances in Spain: interpretation of the genotype by environment interaction. Can. J. For. Res. 27, 1548–1559. doi:10.1139/cjfr-27-10-1548

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1–9.

Arias, E., Piñeros, P., 2008. Aislamiento e identificación de hongos filamentosos de muestras de suelo de los paramos de Guasca y Cruz Verde. Fac. Ciencias La Pontif. Univ. Javeriana 204.

Asiegbu, F.O., Adomas, A., Stenlid, J., 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. *s. l.* Mol. Plant Pathol. 6, 395–409. doi:10.1111/j.1364-3703.2005.00295.x

Baradat, P., Marpeau, A., 1988. Le Pin Maritime *Pinus pinaster* Ait. Biologie et génétique des terpénes pour la conaissance et l'amélioration de l'espéce. Université Bordeaux I, France.

Barnett, H.L., Hunter, B.B., 1998. Illustrated genera of imperfect fungi. The American Phytopathological Society. St. Paul, Minnesota. USA.

Benito Martínez, J., 1943. El *Fomes annosus* Fr. (*Trametes radiciperda* Hart.) en España. An. del Jardín Botánico Madrid 3, 23–49.

Bissett, J., 1991. A revision of the genus *Trichoderma*. II. Infrageneric classification. Can. J. Bot. 69, 2357–2372. doi:10.1139/b91-297

Blanchette, R.A., Farrell, R.L., Burnes, T.A., Wendler, P.A., Zimmerman, W., Brush, S., Snyder, A., 1992. Biological control in pulp and paper production by *Ophiostoma piliferum*. Tappi J. 75, 102–106.

Boa, E., 2003. Reconocimiento e interpretación de síntomas y daños. Servicio de Diagnóstico y Asesoría CABI Bioscensce. Egham, Surrey, Reino Unido. Organización de las Naciones Unidas para la Agricoltura y la Alimentación- FAO; Organismo Internacional Regional de Sanidad Agropecuaria – OIRSA. Traducido por Dinora Alicia V.

Bueno, A., Diez, J.J., Fernandez, M.M., 2007. Ophiostomatoid Fungi transported by *Ips sexdentatus* (*Coleoptera, Scolytidae*) in *Pinus pinaster* in NW Spain. Silva Fenn. 44, 387–397. doi:10.14214/sf.137

Cailleret, M., Heurich, M., Bugmann, H., 2014. Reduction in browsing intensity may not compensate climate change effects on tree species composition in the Bavarian Forest National Park. For. Ecol. Manage. 328, 179–192.

Chaverri, P., Samuels, G.J., 2003. *Hypocrea/Trichoderma* (*Ascomycota, Hypocreales, Hypocreaceae*): species with green ascospores. Stud. Mycol. 48, 116.

Deacon, J.W., 2013. Fungal biology. John Wiley & Sons.

del Río, M., Bravo, F., Pando, V., Sanz, G., Sierra de Grado, R., 2004. Influence of individual tree and stand attributes in stem straightness in *Pinus pinaster* Ait. stands. Ann. For. Sci. 61, 141–148.

Eichhorn, J., Roskams, P., Ferretti, M., Mues, V., Szepesi, A., Durrant, D., 2010. Manual Part IV:

Visual Assessment of Crown Condition and Damaging Agents, in: Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg, p. 49.

Etxebeste, I., Álvarez, G., Pérez, G., Pajares, J.A., 2012. Field response of the six-toothed pine bark beetle, *Ips sexdentatus* (*Col.: Curculionidae, Scolytinae*), to pheromonal blend candidates. J. Appl. Entomol. 136, 431–444. doi:10.1111/j.1439-0418.2011.01682.x

Fernández, M., Gil, L., Pardos, J.A., 1996. Supervivencia y crecimiento en vivero de plantas de cinco procedencias de *Pinus pinaster* Ait. bajo diferentes regímenes hídricos. Investig. Agrar. Sist. y Recur. For. 5, 19–44.

Fernández, M.M.F., García, A.E., Lieutier, F., 2004. Effects of various densities of *Ophiostoma ips* inoculations on *Pinus sylvestris* in north-western Spain. For. Pathol. 34, 213–223. doi:10.1111/j.1439-0329.2004.00360.x

Fishcer, K., Akhtar, M., Blanchette, R., Burnes, T., Messner, K., Kirk, T.K., 1994. Reduction of resin content in wood chips during experimental biological pulping processes. Holzforschung 48, 285–290.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118. doi:10.1111/j.1365-294X.1993.tb00005.x

Gil L., Gordo J., C.G. and P.J.A., 1990. *Pinus pinaster* Aiton en el paisaje vegetal de la Península Ibérica. Ecología 1, 469–495.

Gonthier, P., Garbelotto, M.M., Nicolotti, G., 2005. Seasonal patterns of spore deposition of *Heterobasidion* species in four forests of the Western Alps. Phytopathology 95, 759–767.

Grondona, I., Hermosa, R., Tejada, M., Gomis, M.D., Mateos, P.F., Bridge, P.D., Monte, E., Garcia-Acha, I., 1997. Physiological and biochemical characterization of *Trichoderma harzianum,* a biological control agent against soilborne fungal plant pathogens. Appl Env. Microbiol 63, 3189–3198.

Grylls, B., Seifert, K.A., 1999. A synoptic key to species of *Ophiostoma, Ceratocystis* and *Ceratocystiopsis*, in: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity. pp 261–268.

Hanlin, R.T., 1998. Combined keys to illustrated genera of ascomycetes: Volumes 1 & 2. American Phytopathological Society (APS Press).

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma* species--opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2, 43–56. doi:10.1038/nrmicro797

Harrington, T.C., 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73, 1123–1129.

Harrington, T.C., 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. Insect-fungal Assoc. Ecol. Evol. 257–291.

Hausner, G., Iranpour, M., Kim, J.J., Breuil, C., Davis, C.N., Gibb, E.A., Reid, J., Loewen, P.C., Hopkin, A.A., 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis, Leptographium truncatum,* and *Leptographium wingfieldii.* Can. J. Bot. 83, 1222–1237. doi:10.1139/b05-095

Hodges, C.S., 1999. *Heterobasidion annosum*. Biology, Ecology, Impact and Control. Plant Pathol. 48, 564–565.

Hofstetter, R.W., Cronin, J.T., Klepzig, K.D., Moser, J.C., Ayres, M.P., 2006. Antagonisms, mutualisms and commensalisms affect outbreak dynamics of the southern pine beetle. Oecologia 147, 679–691.

Hsiau, P.T.W., Harrington, T.C., 1997. *Ceratocystis brevicomi* sp. nov., a mycangia fungi from *Dendroctonus brevicomis* (*Coleoptera: Scolytidae*). Mycologia 89, 659–661.

Innes, J.L., 1993. Forest health: its assessment and status. Cab International.

Kliejunas, J.T., Geils, B.W., Micales, J., Goheen, E.M., Hennon, P., Kim, M., Kope, H., Stone, J., Sturrock, R., Frankel, S.J., 2009. Review of literature on climate change and forest diseases of western North America. 60 p.

Korhonen, K., Stenlid, J., 1998. Biology of *Heterobasidion annosum*, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*, Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK. pp. 43–70.

Low, J.D., 1958. *Fomes annosus*. Unasylva 12, 180–182.

Maehara, N., Hata, K., Futai, K., 2005. Effect of blue-stain fungi on the number of *Bursaphelenchus xylophilus* (*Nematoda: Aphelenchoididae*) carried by *Monochamus alternatus* (*Coleoptera: Cerambycidae*). Nematology 7, 161–167. doi: 10.1163/1568541054879557

Maharachchikumbura, S.S.N., Guo, L.D., Chukeatirote, E., Bahkali, A.H., Hyde, K.D., 2011. *Pestalotiopsis*: morphology, phylogeny, biochemistry and diversity. Fungal Divers. 50, 167–187. doi:10.1007/s13225-011-0125-x

Manion, P.D., 1991. Tree Disease Concepts, Prentice-Hall.

Marín Montoya, M., Wingfield, M.J., 2006. A review of *Ceratocystis sensu stricto* with special reference to the species complexes *C. coerulescens* and *C. fimbriata*. 3046-3075.tle. Rev. Fac. Nac. Agron. Medellin 59, 3046–3075.

Marincowitz, S., Crous, P.W., Groenewald, J.Z., Wingfield, M.J., 2008. Microfungi occurring on *Proteaceae* in the fynbos. CBS Fungal Biodiversity Centre Utrecht, the Netherlands.

Marmolejo, J.G., Butin, H., 1990. New conifer-inhabiting species of *Ophiostoma* and *Ceratocystiopsis (Ascomycetes, Microascales)* from Mexico. Sydowia 42, 193–199.

Masuya, H., Kaneko, S., Yamaoka, Y., 2003. Comparative virulence of blue-stain fungi isolated from Japanese red pine. J. For. Res. 8, 83–88. doi:10.1007/s103100300009

Mesanza, N., Iturritxa, E., 2012. Root and butt rot caused by *Heterobasidion annosum* in Atlantic coniferous ecosystems of Spain. For. Pathol. 42, 514–520.

Muñoz, L.C., Perez, F. V., Cobos, S.P., Hernandez, A.R., Sanchez, G., 2007. Sanidad forestal. Guía en imágenes de plagas, enfermedades y otros agentes presentes en los bosques. Madrid – Ediciones Mundi-Prensa.

Navarro, R., Calzado, C., Sánchez, M., López, J., Trapero, A., 2003. Censo de focos de *Heterobasidion annosum* (Fr.) Bref. en ecosistemas de pinsapo. Boletín Sanid. Veg. y Plagas 29, 581–592.

Nicolotti, G., Faccoli, M., Capretti, P., 2008. Specie invasive, rischi di introduzione e gestione delle emergenze, in: Atti Del Terzo Congresso Nazionale Di Selvicoltura. Taormina (ME). pp. 611–619.

Ninyerola, M., Pons, X., Roure, J.M., 2005. Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Univ. Autónoma Barcelona, Bellaterra. ISBN 932860-8-7.

Ohtaka, N., Masuya, H., Yamaoka, Y., Kaneko, S., 2006. Two new *Ophiostoma* species lacking conidial states isolated from bark beetles and bark beetle-infested *Abies* species in Japan. Can. J. Bot. 84, 282–293. doi:10.1139/b05-164

Oliva, J., Colinas, C., 2007. Decline of silver fir (*Abies alba* Mill.) stands in the Spanish Pyrenees: Role of management, historic dynamics and pathogens. For. Ecol. Manage. 252, 84–97.

Oliva, J., Ibarra, N., Colinas, C., Martín, E., 2008. Detección molecular de *Heterobasidion annosum s. s.* en claras de *Pinus nigra* en el Pirineo Aragonés. Boletín Sanid. Veg. Plagas. 34, 415–416.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with decline of longleaf pine in the southeastern United States. Plant Soil 217, 145–150.

Otrosina, W.J., Garbelotto, M., 2010. *Heterobasidion occidentale* sp nov. and *Heterobasidion irregulare* nom. nov.: A disposition of North American *Heterobasidion* biological species. Fungal Biol. 114, 16–25.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. Springer Science & Business Media.

Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. doi:10.1016/j.foreco.2015.07.033

Prieto-Recio, C., Martín-García, J., Diez, J.J., 2014. Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings. For. Pathol. 44, 163–165. doi:10.1111/efp.12091

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770.

Romón, P., Zhou, X., Iturrondobeitia, J.C., Wingfield, M.J., Goldarazena, A., 2007. *Ophiostoma* species (*Ascomycetes : Ophiostomatales*) associated with bark beetles (*Coleoptera : Scolytinae*) colonizing *Pinus radiata* in northern Spain. Can. J. Microbiol. 53, 756–767. doi:10.1139/W07-001

Samarajeewa, U., 1991. In situ degradation of mycotoxins by physical methods, in: Smith, I.E., Henderson, R.S. (Eds.), Mycotoxins and Animal Foods.

Schroers, H.J., 2001. A monograph of *Bionectria* (*Ascomycota, Hypocreales, Bionectriaceae*) and its Clonostachys anamorphs. Stud. Mycol. 1–214.

Seaby, D.A., 1989. Further observations on *Trichoderma*. Mushroom J. 197, 147–151.

Sinclair, W.A., Lyon, H.H., Johnson, W.T., 1987. Diseases of trees and shrubs. Comstock Pub. Associates.

Solheim, H., Langstrom, B., Hellqvist, C., 1993. Pathogenicity of the Blue-Stain Fungi *Leptographium* *wingfieldii* and *Ophiostoma minus* to Scots Pine - Effect of Tree Pruning and Inoculum Density. Can. J. For. Res. 23, 1438–1443.

Solis, A., 2003. Planteamientos sobre la regeneración en pinares de repoblación que alcanzan la edad de turno, in: Cuadernos de La Sociedad Española de Ciencias Forestales. Actas de La III Reunión Sobre Regeneración Natural Y IV Reunión Sobre Ordenación de Montes. pp. 49–57.

Steyaert, R.L., 1953. *Pestalotiopsis* from the Gold Coast and Togoland. Trans. Br. Mycol. Soc. 36, 235–242. doi:10.1016/S0007-1536(53)80008-2

Tainter, F.H., Baker, F.A., 1996. Principles of Forest Pathology. New York, NY: John Wiley and Sons, Inc.

Ter Braak, C.J.F., Smilauer, P., 2002. CANOCO Reference Manual and CanoDraw for Windows User's Guide: software for canonical community ordination (version 4.5).

Upadhyay, H.P., 1999. Classification of the Ophiostomatoid Fungi, in: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity.

Vainio, E.J., Korhonen, K., Hantula, J., 1998. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycol. Res. 102, 187–192.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols, a Guide to Methods and Applications. Academic, New York, pp. 315–322.

Wingfield, M.J., Seifert, K.A., Webber, J.F., 1993. *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity. American Phytopathological Press, St. Paul, Minnesota, U.S.A.

Woodward, S., Stenlid, J., Karjalainen, R. Hütterman, A., 1998. *Heterobasidion annosum*, Biology, Ecology, Impact and Control, CAB International, Wallingford, Oxon, UK.

Zamora, P., Martinez-Ruiz, C., Diez, J.J., 2008. Fungi in needles and twigs of pine plantations from northern Spain. Fungal Divers. 30, 171–184.

Zhou, X.D., de Beer, Z.W., Wingfield, B.D., Wingfield, M.J., 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. Sydowia 53, 290–300.

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud. Mycol. 55, 75–97. doi:10.3114/sim.55.1.75

Chapter 8: Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings

Abstract

The aim of this study was to test the pathogenicity of two Spanish isolates of *Heterobasidion annosum sensu stricto* in two-year-old *Pinus pinaster* seedlings. Two types of inoculum (woodchips and sawdust) were used to infect the seedlings by two different routes (stem inoculation and soil infestation). The mortality rates of the stem-inoculated seedlings differed significantly from controls, but those of the seedlings infected via soil infestation did not differ. For both types of inoculation, the lesions were longer and wilting symptoms were more severe in the seedlings inoculated with *Heterobasidion annosum* than in control seedlings. For stem inoculation, biomass allocation did not differ significantly between the infected and control seedlings. However, the percentage of fine roots was lower in seedlings infected via soil infestation than in the control seedlings. To our knowledge, this is the first pathogenicity test with *Heterobasidion annosum* isolates and *Pinus pinaster*.

Keywords: *lesion length, wilting symptoms, mortality, root biomass, root and butt rot.*

Introduction

Heterobasidion annosum sensu lato (Fr.) Bref. is a species complex that severely affects coniferous forests in Europe, Asia and North America. This hymenomycete fungus, which causes root and butt rot, is responsible for high economic losses in the forestry sector. In Europe alone, *Heterobasidion* infection of forest stands is estimated to cause economic losses of up to 800 million euros annually. *Pinus pinaster* Ait. is the most widespread conifer in Spain, covering over 700 000 ha in pure stands and 600 000 ha in mixed stands. Mediterranean Maritime pine is planted for soil conservation purposes and for timber and resin production. In recent years, *Pinus pinaster* decline, characterized by unusual transparency at the crown, small needles, foliage discoloration and early tree death, has been observed in association with a high mortality rate in several forests in the center of the Iberian Peninsula. Although *Heterobasidion annosum* has occasionally been recorded on *Pinus sylvestris* and *Pinus nigra* in Spain, the presence of the fungus on *Pinus pinaster* in association with forest decline is a very recent finding (Prieto-Recio et al., 2012). This pathogen is very aggressive on Pinaceae; however, some pine species such as *Pinus pinea* and *Pinus halepensis* have shown a lesser susceptibility (Scirè et al., 2011). To our knowledge, pathogenicity tests have not previously been carried out with *Heterobasidion annosum* isolates and *Pinus pinaster*. Therefore, the aim of this study was to test the virulence of Spanish isolates of *Heterobasidion annosum* in *Pinus pinaster* seedlings.

Material and methods

A total of 120 two-year-old *Pinus pinaster* seedlings (Meseta Castellana provenance, Spain) were obtained from a Government tree nursery in Castile and Leon. The Spanish isolates of *Heterobasidion* used in the study (H1 and H4) were previously isolated and identified as *Heterobasidion annosum s. s.* (Prieto-Recio et al., 2012). The sequences were deposited in the EMBL/GenBank database (GenBank Accession No. FR850494 and FR850495, respectively).

Two types of inoculum, woodchips and sawdust, were used to infect the seedlings via each of two routes, stem inoculation and soil infestation. Thus, 40 seedlings were inoculated with each *Heterobasidion annosum* isolate (10 for each type of inoculum and route of inoculation). Control seedlings (n=40) were prepared in the same way, but using sterile woodchips and sawdust. The inocula were prepared by growing the isolates of

Heterobasidion annosum on autoclaved woodchips (4 mm diameter, 4 mm depth) and sawdust, which were maintained on potato dextrose agar (PDA) for 3 weeks. For stem inoculations, the inocula were placed inside an oblique incision made in the stem at 6 cm above the soil line, and the inoculation site was then wrapped with Parafilm®. For soil infestation, four infected woodchips and four pieces of PDA with infected sawdust were placed in the soil near the stem at a depth of 1.5 cm. The seedlings were incubated in a growth chamber at 22.5°C with a 14-h photoperiod and watered twice a week. In months 1, 2, 4, 6, 10 and 18, the survival and the visual severity of symptoms (percentage of wilting or chlorosis) were assessed in each plant according to the following scale (where 0=healthy and 4 = dead): 0=0-10%, 1=11-33%, 2=34-66%, 3=67-99%, 4=100%. Disease progress curves for each plant were constructed by plotting the scores over time. The area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids.

Eighteen months after inoculation, seedlings were cut into two pieces at the root collar. The height, diameter and length from the inoculation point to the last withered needle on the leader were measured. The stems were also split lengthways, with a scalpel, to enable measurement of the length of the lesions. The aerial part were subdivided into stem and needles, was dried in an oven at 80°C for 38 hours for investigation of biomass allocation. Roots were washed to remove adhering soil, the main root length measured and the total root mass dried to estimate the biomass of primary, secondary and tertiary roots.

Log-linear analysis of frequency tables was used to test the effects of the isolates and the type of inoculum on the mortality rates. Analysis of variance (ANOVAs) and post hoc Tukey's HSD tests were used to test the effects of the isolates and the type of inoculum on the biomass allocations, wilting symptoms and lesion lengths. However, because heteroscedasticity was observed in the AUDPC, linear mixed models (PRO MIXED) with six variance parameters and no random effects were used to test for the effects of the isolates and the type of inoculum on this variable.

Results and discussion

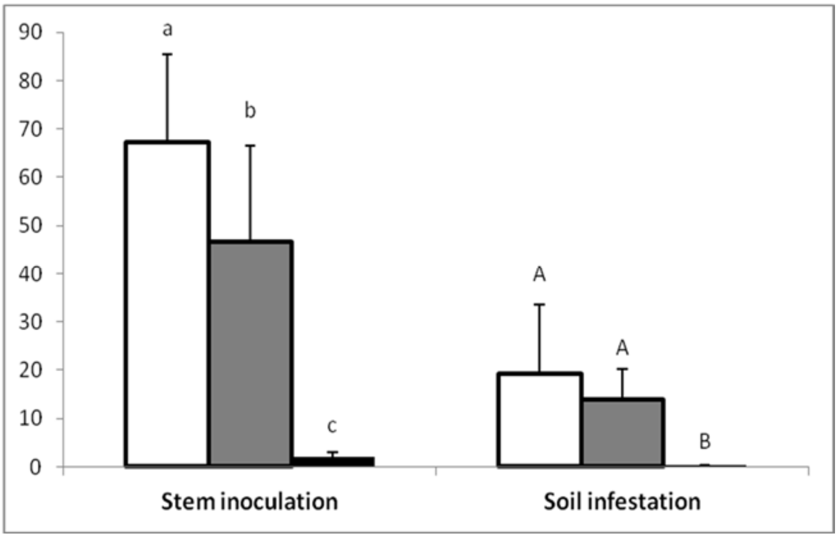
The mortality rate of seedlings infected with *Heterobasidion annosum* via stem inoculation differed significantly from that of the respective control seedlings ($\chi^2 = 3.96$, $p = 0.04$). However, there was no difference in the mortality rate of the infected and control seedlings treated by soil infestation. Moreover, seedling mortality did not occur after three months from the inoculations, which is consistent with previous findings of maximal mortality rates in young seedlings at between 5 and 14 weeks after inoculation, which was attributed to occlusion of the pathogen from the plant material after this length of time (Swedjemark et al., 2001).

The mortality rates (ca. 35 % and 10 % for stem inoculation and soil infestation, respectively) were lower than reported for some North American pine species, such as *Pinus elliottii*, *Pinus echinata*, *Pinus palustris*, *Pinus strobus* and *Pinus taeda* (Delatour et al., 1998) and, to a lesser extent, for *Pinus sylvestris* (Swedjemark and Stenlid, 1995), although inoculation methods were different from those used in this study. Nevertheless, rates were similar to or even higher than observed by other authors (Lehtijarvi et al., 2011; Scire et al., 2011), who reported low mortality rates in other pine species, such as *Pinus pinea*, *Pinus halepensis*, *Pinus nigra, Pinus sylvestris* and *Pinus brutia* seedlings. The fact that resin production was higher in the seedlings infected via stem inoculation than in the respective control seedlings ($\chi^2 = 5.64$, $P = 0.01$) may indicate that the relatively low mortality rate was due to physical and chemical host defences. Indeed, *Pinus pinaster* (also called resin pine) produces a large amount of oleoresin (62.8 % of resin acids), which acts as mechanical barrier, and monoterpenes (28.7 %), which may be fungitoxic (Asiegbu et al., 1998; Scirè et al., 2011).

In addition to the differences in mortality rates, the lesions in the infected seedlings were also longer than in the control seedlings for both types of inoculation (Figure 8.1a). The type of inoculum also affected lesion length: stem inoculation with woodchips caused more damage than stem inoculation sawdust ($N = 60$, $F = 10.14$, $p < 0.01$). The lesion lengths in stem inoculations were consistent with other findings in *Picea abies* (Swedjemark et al., 2001), where an average lesion length of approximately 50 mm 182 days was found after inoculation. However, the lesion lengths were substantially higher than those observed in other pine species in Turkey, such as *Pinus brutia* (9.4 mm), *Pinus nigra* (15.7 mm) and *Pinus sylvestris* (15 mm) (Lehtijarvi et al., 2011). These differences may be due to (*i*) host defences and/or (*ii*) the maximum incubation temperature which in previous study reached approximately 31°C, and may have halted the development of the disease. Furthermore, in the present study, the lesion lengths were measured 18 months after inoculation, whereas Lehtijarvi et al., (2011) harvested the seedlings 12 weeks after inoculation. Therefore, although the daily increase in lesion length was lower when plants were incubated for a long period of time (Swedjemark et al., 2001), an increase in the overall lesion length would be expected for longer incubation periods, depending on host species and defence mechanisms.

A similar finding was observed for AUDPC, which indicated that disease progress was greatest in the stem-inoculated seedlings (Figure 8.1b). Furthermore, the curve stabilized after four to six months, which seems to confirm that occlusion of the pathogen from the plant material occurs after that time. Likewise, wilting symptoms were more severe in inoculated seedlings than in control seedlings, for both stem inoculation (N = 60, F = 4.97, p = 0.01) and soil infestation (N = 60, F = 8.84, $p < 0.01$) treatments.

(a)



(b)

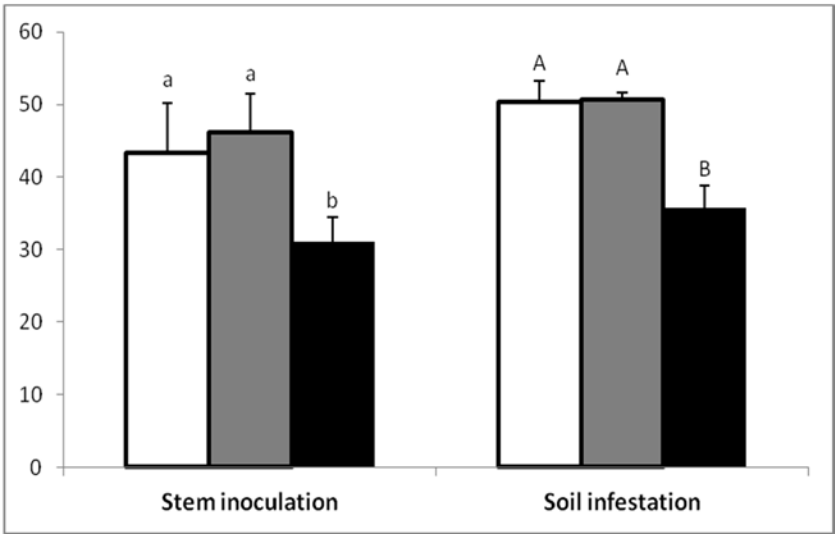


Figure 8.1. (a) Length of lesions (mm) and **(b)** Area Under the Disease Progress Curve (AUDPC) (±95 % confidence intervals) for seedlings artificially infected with *Heterobasidion annosum* isolate H1 (white bars) and *Heterobasidion annosum* isolate H4 (grey bars) and for the respective control seedlings (black bars). For each route of infection, different letters above the bars indicate significantly different means (two-tailed t-test, $\alpha = 0.05$).

For stem inoculation, biomass allocation did not differ significantly between the infected and control seedlings. However, the percentage of fine roots (tertiary) was lower in the seedlings infected via soil infestation than in the respective control seedlings (Figure 8.2). This difference may be the result of the ability of *Heterobasidion annosum* to parasitize fine roots in pine (Asiegbu et al., 1998) and also the inability of weakened seedlings (as a result of soil inoculation) to develop the same quantity of fine roots as control seedlings.

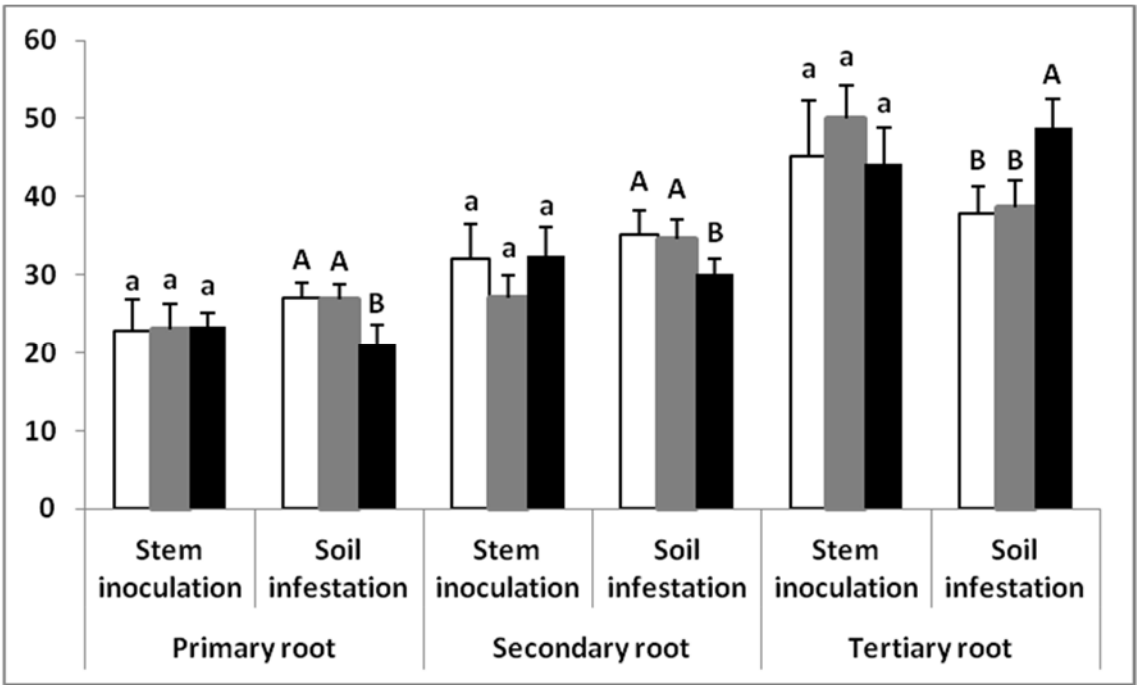


Figure 8.2. Rate of biomass of primary, secondary and tertiary roots (%) (± 95 % confidence intervals) in seedlings infected with *Heterobasidion annosum s. s.* isolate H1 (white bars) and *Heterobasidion annosum s. s.* isolate H4 (grey bars) and in the respective control seedlings (black bars). For each type of inoculation, different letters above the bars indicate significantly different means (post hoc Tukey's test, α = 0.05).

This study reports the first pathogenicity test with *Heterobasidion annosum* isolates and *Pinus pinaster* and the results demonstrate the susceptibility of Mediterranean Maritime pine to *Heterobasidion annosum*. Further studies and surveys are essential to determine the role that *Heterobasidion annosum* is playing in Maritime pine decline in Spain.

References

AsiegAsiegbu, F.O., Johansson, M., Woodward, S., Hüttermann, A., 1998. Biochemistry of the host-parasite interaction, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK, pp. 167–193.

Delatour, C., von Weissenberg, K., Dimitri, L., 1998. Host resistance and response to infection, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK, pp. 143–166.

Lehtijarvi, A., Aday, A.G., Dogmus-Lehtijarvi, H.T., 2011. *Cedrus libani*: the most susceptible Turkish conifer species to local *Heterobasidion* isolates in spring inoculations. For. Pathol. 41, 1–6. doi:10.1111/j.1439-0329.2009.00625.x

Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Dis. 96, 770. doi:10.1094/PDIS-10-11-0890-PDN

Scirè, M., Motta, E., D'Amico, L., 2011. Behaviour of *Heterobasidion annosum* and *Heterobasidion irregulare* isolates from central Italy in inoculated *Pinus pinea* seedlings. Mycol. Prog. 10, 85–91. doi:10.1007/s11557-010-0678-1

Swedjemark, G., Stenlid, J., 1995. Susceptibility of Conifer and Broadleaf Seedlings to Swedish S-Strain and P-Strain of *Heterobasidion annosum*. Plant Pathol. 44, 73–79.

Swedjemark, G., Stenlid, J., Karlsson, B., 2001. Variation in growth of *Heterobasidion annosum* among clones of *Picea abies* incubated for different periods of time. For. Pathol. 31, 163–175.

PART IV: Concluding Remarks

Chapter 9: Synthesis

In the present work, six studies were carried out with the aim of discovering the biotic, abiotic and management factors affecting *Pinus pinaster* decline in Spain. Thus, different studies, including the 'predisposing, inciting and contributing factors' *sensu* Manion (1991), were developed.

Abiotic and management factors, predisposing and/or inciting *Pinus pinaster* to decline

Associations between climate, soil properties and forest management at stand level

High values of total height and spring precipitation showed better forest health, whereas high tree density was associated with the poorest forest health status. The results suggest that predisposing factors such as high level of competition and water deficit have triggered the decline of *Pinus pinaster*.

Phytosanitary conditions were poorer in mixed stands than in pure stands. This does not concur with previous findings, which indicated that mixed stands are much less affected by changing climate conditions as well as pests and diseases than pure stands (Jactel et al., 2005; Neuner et al., 2015; Pautasso et al., 2005). This discrepancy may be due to the fact that one of the causes of the origin of mixed stands in the present study was probably the previous mortality of the dominant species, which may lead to a misinterpretation of the cause-and-effect relationship, as a result of an indirect effect.

Several variables associated with competition were retained in the non-metric multidimensional scaling (NMDS) analysis. In particular, dense stands displayed the highest tree mortality rates and symptoms of decline, as previously reported in other cases of forest decline (Camarero et al., 2011; Galiano et al., 2010; Gómez-Aparicio et al., 2011; Linares et al., 2009; Madrigal-González and Zavala, 2014; Martínez-Vilalta et al., 2012; Sánchez-Salguero et al., 2012b, 2013). Competition can take place for aboveground resources (i.e. light) and belowground resources (i.e. soil nutrients and water). However, in Mediterranean pines and in particular *Pinus pinaster*, limitation is normally due to competition for belowground resources (Bravo-Oviedo et al., 2006; Cailleret et al., 2014; Sánchez-Salguero et al., 2012b). In the stands considered in the current study, water availability seemed to be more limiting than soil nutrients, as no soil variables were retained in the NMDS analysis. This may be due to the fact that *Pinus pinaster* has low mineral requirements and is able to grow in nutrient-poor soils (Álvarez-Álvarez et al., 2010).

While competition appeared to operate as a predisposing factor, the role water deficit played was not obvious. The possibility that water deficit was an inciting factor (i.e. severe, short-term stress) was unlikely because *Pinus pinaster* is considered a drought-avoiding species, one able to cope with intense, short-term drought pulses but not with multiple droughts (Madrigal-González and Zavala, 2014). Likewise, the possibility that water deficit acted as a contributing factor also seemed unlikely because, if that were the case, *Pinus pinaster* would have been replaced by drier thermophyllous species. On the contrary, Maritime pine was mainly replaced by *Quercus ilex*, which has similar water requirements (Bravo-Oviedo and Montero, 2008; Ruiz-Labourdette et al., 2012, 2013). Therefore, water deficit could have also acted as a predisposing factor.

Influence of environmental and silvicultural factors at tree level

Results confirmed that the significant variables for the best Multinomial Logistic Regression (MLR) model to predict the health status at tree level were: diameter at breast height (DBH), mean total height of the stand (Hm) and seasonal summer precipitation (Ps).

Generally, hierarchical clustering was used to separate the groups by different geographic areas or provinces and therefore represented the different provenance regions of *Pinus pinaster*. Hierarchical clustering according to phytosanitary variables was used to divide the plots into three different groups with similar forest health conditions. Variables quantifying Maritime pine decline or health status have been used in other studies of forest decline (Klepzig et al., 1991; Oliva and Colinas, 2007). Defoliation is one of the most representative of these variables (Galiano et al., 2010; Sánchez-Salguero et al., 2013, 2012a). The poorest phytosanitary conditions can be found in clusters formed by plots in Ribera de Duero (Burgos), with a defoliation rate of higher than 40 %. This zone was traditionally dedicated to resin production and, to a lesser extent, timber production; During the 1980s and 1990s, these stands were abandoned (Picardo and Pinillos, 2013), yet resin extraction is again being carried out in some areas. Previously tapped stands have been found to be weaker, display more symptoms of decline and have a higher percentage of dead trees. However, the cluster with good health status was the grouped plots from the provinces of Avila, Segovia Soria and Montaña de Soria-Burgos provenance (Burgos), all with a history of timber production. The quality of the *Pinus pinaster* timber in these zones is good, and, historically, the stands have been managed and maintained for timber production. Hence, defoliation and other symptoms of decline are less evident, partly because symptomatic or dead trees are usually eliminated. Finally, plots clustered in the intermediate health status category were mainly from Sierra de Oña and Sierra del Teleno. In these zones the wood is lower quality and management and maintenance less intensive. Nevertheless, these stands are developing well due to the good climate and soil conditions. Most of the damage appears to be caused by abandonment of forest management practices. A lower intensity of thinning is thus causing high levels of competition, and symptomatic and dead trees are therefore more common in these stands.

On the other hand, the silvicultural and environmental factors influencing the health status of *Pinus pinaster* include the size of the tree (DBH), stand structure (Hm) and the climate (Ps), all of which play an important role in predicting Maritime pine decline and can be considered as predisposing factors to this pathology (Prieto-Recio et al., 2015). The negative effect on the model of diameter at breast height (DBH) indicated that tree with lower values of DBH are more likely to be symptomatic or die. Nevertheless, stand average height (Hm) was a positive indicator, meaning that as the Hm increased the trees were less likely to be classified as symptomatic or dead status. Finally, seasonal summer precipitation (Ps) also had a negative effect on the model, indicating that at Ps lower values, the trees were more likely to be classified as symptomatic or dead.

Modelling tree growth and aridity in relation to stand competition and health status

Dendrochronological methods and climate data from the last three decades (when the *Pinus pinaster* decline started to be observed in Spain) have been used to develop a more in-depth understanding of the influence of abiotic and management factors. In this study variance in growth patterns among trees with different phytosanitary conditions within the sampling zones was detected. Annual aridity index trends over time across the study sites revealed a clear chain of drought events in the early 1990s, in 1994, and in 2005 as well. Mantel correlations between tree growth and aridity index showed significant responses at tree level in relation to health status and defoliation levels. Significant values were also observed in Mantel correlograms between tree growth and aridity index according to Stand Density Index classes, highlighting the serious implications of competition-growth-climate relationships.

Tree growth patterns suggest differences between geographic areas and health status conditions of the trees. We have seen common behaviour in zones located in the central area (Meseta Castellana and Montaña de Soria-Burgos provenances), for example symmetrical distribution along the recorded formation years of the measured tree rings, suggesting conditions of tree-growth synchronicity across these two nearby provenance regions. Our results have provided more information by comparing the tree growth with the aridity index. In this way, we assess the amount of interaction between the two climatic variables (precipitation and temperature), including the effect of water deficit periods. High climatic variability year after year and increasing aridity may reduce tree growth of pine species (Martín-Benito et al., 2008; Sánchez-Salguero et al., 2013), for example growth decline caused by the more frequent occurrence of severe droughts. We have observed that the occurrence of high aridity in our study area have been frequent and consistent, mainly during the beginning of the 1990s, in 1994 and during the year 2005.

In general, the response of growth to drought depends on the health status of the trees. Defoliation is one of the most representative variables in the study of tree decline (Camarero et al., 2015; Galiano et al., 2010; Sánchez-Salguero et al., 2012b, 2013; Sangüesa-Barreda et al., 2015a). Our results have shown a general trend: the worse the health status, the faster the tree responded to aridity. Trees with a high percentage of defoliation have a faster and more acutely, significant reaction in tree growth to aridity than healthy trees. Defoliated pines have a premature cessation of cambial activity and wood formation and form narrow tree rings as the result of a shorter growing season (Eilmann et al., 2013). Also, most defoliated trees show a higher sensitivity to water availability and have a lower post-drought resilience capacity (Guada et al., 2016). Overall, our findings suggest that recently dead, totally defoliated trees and symptomatic trees with levels of defoliation between 35 to 99 %, are more sensitive to high aridity due to severe droughts in the 1990s and 2000s.

Stand structure resulting from previous forest management, plays a fundamental role in *Pinus pinaster* decline (Prieto-Recio et al., 2015). Other studies suggest that stands with high densities displayed the highest tree mortality rates and symptoms of decline (Camarero et al., 2011; Galiano et al., 2010; Gómez-Aparicio et al., 2011; Linares et al., 2009; Madrigal-Gonzalez & Zavala, 2014; Martínez-Vilalta et al., 2012; Sánchez-Salguero et al., 2012b, 2013). In our study, the significant correlations of trees within high density stands where localized along a narrow interval of time-class lag axis, whereas medium and low density stands can be responsive over a medium or long period of time, respectively. Thus, in highly dense stands, competition might be acting as an inciting factor in the decline and death of trees, while in the stands with low and medium densities, the competition could act as a predisposing factor.

Biotic factors contributing the *Pinus pinaster* decline

First report of *Heterobasidion annosum* on *Pinus pinaster* in Spain

In this study the *Heterobasidion annosum* anamorphic form (*Spiniger meineckellum* (A. Olson) Stalpers) was identified using morphological and molecular tools. This basidiomycete is one of the most important pathogens in coniferous forests in Europe, Asia and North America, causing root and butt rot. Over the last few years, *Pinus pinaster* decline was observed in several stands in the center of the Iberian Peninsula. Unusual crown transparency, small needles, foliage discoloration and premature tree death are characteristic decline symptoms associated with high mortality rate in this species.

Two zones in Castile and Leon with clear symptoms of decline were sampled. Infected wood samples were taken to the laboratory, and after incubation in humid chambers, conidiophores with subglobose to pyriform conidia could be observed (5.8 x 4.2 µm). The fungus was isolated in order to extract DNA, and the resulting sequences of two isolates were deposited in GenBank (Accession Nos. FR850494 and FR850495) and then compared with a Blastn search at GenBank, showing 100% identity and 100% coverage with *Heterobasidion annosum sensu stricto*, former ISG-P (Intersterility Group of Pines).

Heterobasidion annosum has previously been recorded on *Pinus sylvestris* in central Spain (Benito Martínez, 1943) causing needle drop, swelling at the stump height, and the presence of dead trees in circular areas. This pathogen has also reported on *Pinus nigra* in North-Eastern Spain in association with defoliation and mortality (Oliva et al., 2008). However, this is the first record of *Heterobasidion annosum* on *Pinus pinaster* in Spain associated with Maritime pine decline. In addition, it was hypothesized that this fungus may play a key role in the Maritime pine decline by acting as 'contributing factor' *sensu* Manion, (1991).

Blue-stain and root rot fungi associated with *Pinus pinaster* decline

Analysis of the samples using molecular and morphological techniques led to the identification of fifteen distinct species. Nine of these were primary pathogens and the other six were saprophytes. Pathogenic species mainly belong to the group of Ophiostomatoid fungi*: Ophiostoma minus, Ophiostoma ips, Ophiostoma piliferum and Ophiostoma ranaculosum*. The root pathogen *Heterobasidion annosum* was also identified. This pathogen is one of the main agents causing conifer death in Europe, but it is a neophyte species in northern Spain, especially on *Pinus pinaster*. *Heterobasidion annosum* was found together with *Ophiostoma minus* and *Ophiostoma piliferum*, and all were concentrated in the same geographical zone. The sampling zone where the greatest number of pathogens was found, was in Ribera del Duero (Burgos); the fungi isolated were obtained in equal numbers from healthy, symptomatic and dead *Pinus pinaster* trees. All of the *Ophiostoma* species identified in this study were present in this zone; *Ophiostoma minus, Ophiostoma ips* and *Ophiostoma fasciatum* were strongly associated with *Pinus pinaster* decline as previously reported (Álvarez et al., 2015). In the provinces of Segovia and Avila, *Ophiostoma minus* was mainly detected on dead trees, as previously observed (Álvarez et al., 2015). However, *Heterobasidion annosum,* which is known to be involved in *Pinus pinaster* decline throughout Castile and Leon (Prieto-Recio et al., 2012), was only found in Segovia province. This species was isolated in samples from apparently healthy trees with no evident symptoms, indicating its strong virulence. The species was associated with *Ophiostoma minus* and *Ophiostoma piliferum* found in the same study site. This zone was the last area sampled and the sampling was carried out in early autumn, when fungal activity is greatest (Gonthier et al., 2005). The species probably did not appear in other zones because field sampling in these areas was conducted in summer, and some of the samples may have been damaged due to high temperatures.

The results of the Multivariate Canonical Correspondence Analysis showed that climate variables such as temperature, precipitation and elevation did not significantly affect the pathogenic fungal communities. The pathogenic species were mainly concentrated, although not significantly, in the Ribera del Duero zone, where precipitation is relatively low, which is consistent with the results of previous studies (Prieto-Recio et al., 2015). The proportion of healthy, symptomatic and prematurely dead trees did not differ significantly between the pathogenic fungal communities sampled in each of the seven areas. Although the results were not significant, some trends were observed, as before. The Ophiostomatoid group detected in the zone of Ribera del Duero was mainly associated with high proportions of symptomatic and dead trees, which is consistent with the findings of other studies (e.g. Álvarez et al., 2015). *Heterobasidion annosum* was found to be only associated with healthy trees, although this primary pathogen is known to occur on healthy and damaged trees (Woodward et al., 1998). Furthermore, this fungus has been shown to be capable of killing *Pinus pinaster* seedlings from the same provenance region as the affected zone in this study (Prieto-Recio et al., 2014).

Pathogenicity of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings

One last study was conducted in order to test the *Heterobasidion annosum* pathogen's ability to kill Maritime pine seedlings. The mortality rate of seedlings infected with *Heterobasidion annosum* via stem inoculation differed significantly from that of the respective control seedlings. However, there was no difference in the mortality rate of the infected and control seedlings treated by soil infestation. Moreover, seedling mortality did not occur three months after the inoculations, which is consistent with previous findings of maximal mortality rates in young seedlings between 5 and 14 weeks after inoculation, which was attributed to the occlusion of the pathogen from the plant material after this length of time (Swedjemark et al., 2001). The mortality rates (ca. 35 % and 10 % for stem inoculation and soil infestation, respectively) were lower than reported in some North American pine species, such as *Pinus elliottii*, *Pinus echinata*, *Pinus palustris*, *Pinus strobus* and *Pinus taeda* (Delatour et al., 1998) and, to a lesser extent, in *Pinus sylvestris* (Swedjemark and Stenlid, 1995), although inoculation methods were different from those used in this study. Nevertheless, rates were similar to or even higher than those observed by other authors (Lehtijarvi et al., 2011; Scire et al., 2011), who reported low mortality rates in other pine species, such as *Pinus pinea*, *Pinus halepensis*, *Pinus nigra, Pinus sylvestris* and *Pinus brutia* seedlings.

In addition to the differences in mortality rates, the lesions in the infected seedlings were also longer than in the control seedlings for both types of inoculation. The type of inoculum also affected lesion length: stem inoculation with woodchips caused more damage than stem inoculation sawdust. The lesion lengths in stem inoculations were consistent with other findings in *Picea abies* (Swedjemark et al., 2001), where an average lesion length of approximately 50 mm 182 days was found after inoculation. However, the lesion lengths were substantially higher than those observed in other pine species in Turkey, such as *Pinus brutia* (9.4 mm), *Pinus nigra* (15.7 mm) and *Pinus sylvestris* (15 mm) (Lehtijarvi *et al.*, 2011).

Similar findings were observed for the area under the disease progress curve (AUDPC), which indicated that disease progress was greatest in the stem-inoculated seedlings. Furthermore, the curve stabilized after four to six months, which seems to confirm that occlusion of the pathogen from the plant material occurs after that time.

For stem inoculation, biomass allocation did not differ significantly between the infected and control seedlings. However, the percentage of fine roots (tertiary) was lower in the seedlings infected via soil infestation than in the respective control seedlings. This difference may be the result of the ability of *Heterobasidion annosum* to parasitize fine roots in pine (Asiegbu et al., 1998) and also the inability of weakened seedlings (as a result of soil inoculation) to develop the same quantity of fine roots as control seedlings.

This study reports the first pathogenicity test with *Heterobasidion annosum* isolates and *Pinus pinaster,* and the results demonstrate the susceptibility of Mediterranean Maritime pine to *Heterobasidion annosum*.

In short, our findings have brought the influence several biotic, abiotic and management factors have on *Pinus pinaster* decline to light. Water deficit and competition were shown to have a determinant role on the Maritime pine decline acting as predisposing and/or inciting factors. Moreover, biotic factors, like the presence of blue-stain and root rot fungi (*Heterobasidion annosum*) were shown to be contributing factors to forest decline. However, further studies taking into account these factors are necessary in order to achieve more suitable disease management.

References

Álvarez, G., Fernández, M., Diez, J.J., 2015. Ophiostomatoid fungi associated with declined *Pinus pinaster* stands in Spain. For. Syst. 24, 1–9.

Asiegbu, F.O., Johansson, M., Woodward, S., Hüttermann, A., 1998. Biochemistry of the host-parasite interaction, in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK, pp. 167–193.

Benito Martínez, J., 1943. El *Fomes annosus* Fr. (*Trametes radiciperda* Hart.) en España. An. del Jardín Botánico Madrid 3, 23–49.

Bogino, S.M., Bravo, F., 2008. Growth response of *Pinus pinaster* Ait. to climatic variables in central Spanish forests. Ann. For. Sci. 65, 506.

Camarero, J.J., García-Ruiz, J.M., Sangüesa-Barreda, G., Galván, J.D., Alla, A.Q., Sanjuán, Y., Beguería, S., Gutiérrez, E., 2015. Recent and Intense Dynamics in a Formerly Static Pyrenean Treeline. Arctic, Antarct. Alp. Res. 47, 773–783. doi:10.1657/AAAR0015-001

Delatour, C., von Weissenberg, K., Dimitri, L., 1998. Host resistance and response to infection, in: in: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.), *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, pp. 143–166.

Eilmann, B., Dobbertin, M., Rigling, A., 2013. Growth response of Scots pine with different crown transparency status to drought release. Ann. For. Sci. 70, 685–693.

Galiano, L., Martínez-Vilalta, J., Lloret, F., Martinez-Vilalta, J., Lloret, F., 2010. Drought-Induced Multifactor decline of Scots pine in the Pyrenees and potential vegetation change by the expansion of co-occurring oak species. Ecosystems 13, 978–991. doi:10.1007/s10021-010-9368-8

Gonthier, P., Garbelotto, M.M., Nicolotti, G., 2005. Seasonal patterns of spore deposition of *Heterobasidion* species in four forests of the Western Alps. Phytopathology 95, 759–767.

Guada, G., Camarero, J.J., Sánchez-Salguero, R., Navarro-Cerrillo, R.M., 2016. Limited growth recovery after drought-induced forest dieback in very defoliated trees of two pine species. Front. Plant Sci. 7. doi:10.3389/fpls.2016.00418

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an Insect-Fungal Complex with Red Pine Decline in Wisconsin. For. Sci. 37, 1119–1139.

Lehtijarvi, A., Aday, A.G., Dogmus-Lehtijarvi, H.T., 2011. *Cedrus libani:* the most susceptible Turkish conifer species to local *Heterobasidion* isolates in spring inoculations. For. Pathol. 41, 1–6. doi:10.1111/j.1439-0329.2009.00625.x

Manion, P.D., 1991. Tree Disease Concepts, Prentice-Hall.

Martín-Benito, D., Cherubini, P., del Río, M., Canellas, I., 2008. Growth response to climate and drought in *Pinus nigra* Arn. trees of different crown classes. Trees-Structure Funct. 22, 363–373.

Oliva, J., Colinas, C., 2007. Decline of silver fir (*Abies alba* Mill.) stands in the Spanish Pyrenees: Role of management, historic dynamics and pathogens. For. Ecol. Manage. 252, 84–97.

Oliva, J., Ibarra, N., Colinas, C., Martín, E., 2008. Detección molecular de *Heterobasidion annosum s. s.* en claras de *Pinus nigra* en el Pirineo Aragonés. Boletín Sanid. Veg. Plagas. 34, 415–416.

Olivar, J., Bogino, S., Spiecker, H., Bravo, F., 2012. Climate impact on growth dynamic and intra-annual density fluctuations in Aleppo pine (*Pinus halepensis*) trees of different crown classes. Dendrochronologia 30, 35–47. doi:10.1016/j.dendro.2011.06.001

Picardo, A., Pinillos, F.M., 2013. La resinación en España y en el mundo en 2013: Situación y perspectivas, in: II Simposio Internacional de Resinas Naturales, Del 16 Al 18 de Abril de 2013, Coca - Segovia. pp. 30–53.

Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. doi:10.1016/j.foreco.2015.07.033

Prieto-Recio, C., Martín-García, J., Diez, J.J., 2014. Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings. For. Pathol. 44, 163–165. doi:10.1111/efp.12091

Rozas, V., Zas, R., Garcia-Gonzalez, I., 2011. Contrasting effects of water availability on *Pinus pinaster* radial growth near the transition between the Atlantic and Mediterranean biogeographical regions in NW Spain. Eur. J. For. Res. 130, 959–970.

Sánchez-Salguero, R., Camarero, J.J., Dobbertin, M., Fernandez-Cancio, A., Vila-Cabrera, A., Manzanedo, R.D., Zavala, M.A., Navarro-Cerrillo, R.M., 2013. Contrasting vulnerability and resilience to drought-induced decline of densely planted vs. natural rear-edge *Pinus nigra* forests. For. Ecol. Manage. 310, 956–967.

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Camarero, J.J., Fernández-Cancio, Á., 2012a. Selective drought-induced decline of pine species in southeastern Spain. Clim. Change 113, 767–785. doi:10.1007/s10584-011-0372-6

Sánchez-Salguero, R., Navarro-Cerrillo, R.M., Swetnam, T.W., Zavala, M.A., 2012b. Is drought the main decline factor at the rear edge of Europe? The case of southern Iberian pine plantations. For. Ecol. Manage. 271, 158–169. doi:10.1016/j.foreco.2012.01.040

Sangüesa-Barreda, G., Camarero, J.J., Linares, J.C., Hernández, R., Oliva, J., Gazol, A., González de Andrés, E., Montes, F., García-Martín, A., de la Riva, J., 2015. Role of biotic factors and droughts in the forest decline: contributions from dendroecology. Ecosistemas 24, 15–23. doi:10.7818/ECOS.2015.24-2.03

Scirè, M., Motta, E., D'Amico, L., 2011. Behaviour of *Heterobasidion annosum* and *Heterobasidion irregulare* isolates from central Italy in inoculated *Pinus pinea* seedlings. Mycol. Prog. 10, 85–91. doi:10.1007/s11557-010-0678-1

Swedjemark, G., Stenlid, J., 1995. Susceptibility of conifer and broadleaf seedlings to Swedish S-Strain and P-Strain of *Heterobasidion annosum*. Plant Pathol. 44, 73–79.

Swedjemark, G., Stenlid, J., Karlsson, B., 2001. Variation in growth of *Heterobasidion annosum* among clones of *Picea abies* incubated for different periods of time. For. Pathol. 31, 163–175.

Woodward, S., Stenlid, J., Karjalainen, R. Hütterman, A., 1998. *Heterobasidion annosum*, Biology, Ecology, Impact and Control. CAB International, Wallingford, Oxon, UK.

Chapter 10: Conclusions

Abiotic and management factors, predisposing and/or inciting *Pinus pinaster* to decline

Associations between climate, soil properties and forest management at stand level

1. *Pinus pinaster* stands located in xeric sites may be close to their physiological tolerance threshold. Thus, decreased water availability in the last few decades as a result of climate change and/or overuse of aquifers, together with increased water requirements as a result of high stand densities brought about by land-use modifications, may have led to forest decline and long-term changes in tree species composition. The present study has pointed out that competition and water deficit might trigger the mortality of *Pinus pinaster* by acting as predisposing factors.

Influence of environmental and silvicultural factors at tree level

2. Different groups were established by characterizing the sampling plots according to the sets of study variables (stand level characterization, soil properties, climatic variables and forest health assessment). Generally, hierarchical clustering separated the groups according to different geographic areas or provinces, which corresponded to the different provenance regions of *Pinus pinaster*. Results suggested that zones with a tradition of timber production, where the stands have been historically managed, have a better health status than zones with lower wood quality, where lower intensity of thinning triggered high levels of competition for the resources.

3. A model was constructed to predict the probability of a particular health status for individual *Pinus pinaster* trees (healthy, symptomatic or dead). The model developed includes variables related to the size of the tree (diameter at breast height), stand structure (average height of the stand) and climate (seasonal summer precipitation).

Modelling tree growth and aridity in relation to stand competition and health status

4. Annual aridity index trends over time and across the study site, revealed a clear chain of drought events at the beginning of the 1990s, in 1994 and in 2005. The correlations found in this study between tree growth and aridity index indicated significant responses at tree level related to health status and defoliation levels. Completely defoliated trees that had recently died and symptomatic trees with 35-99% defoliation, responded more quickly and acutely to high aridity, suggesting that drought events may act as inciting factors in *Pinus pinaster* decline.

5. Our results also had implications for competition-growth-climate relationships. In very dense stands, competition might be acting as an inciting factor, whereas in the low- and medium-density stands competition may act as a predisposing factor. Hence, stands with lower competition levels showed more growth responsiveness to aridity: significant responses were distributed over a longer period of time after the drought events. In contrast, very dense stands showed less growth responsiveness to aridity, suggesting a lack of recovery capacity. In that context, persistent growth decline would not be significantly influenced by subsequent drought events.

Biotic factors contributing the *Pinus pinaster* decline

First report of *Heterobasidion annosum* on *Pinus pinaster* in Spain

6. *Heterobasidion annosum,* one of the most important causal agents of root and butt rot in the Northern Hemisphere, is involved in the mortality and decline process of Maritime pine (*Pinus pinaster*). This pathogen was previously recorded on *Pinus sylvestris* in central Spain and on *Pinus nigra* in northeast Spain, but this is the first record of *Heterobasidion annosum* on *Pinus pinaster* in Spain.

Blue-stain and root rot fungi associated with *Pinus pinaster* decline

7. The fifteen species identified during the study were grouped according to their ecology: six species were saprophytes and the other nine were pathogens displaying different degrees of virulence. From a diagnostic point-of-view, the species of interest were those belonging to the group of Ophiostomatoid fungi, which attack wood and cause blue-stain symptoms (principal agents responsible for Maritime pine decline in other studies), and *Heterobasidion annosum*, which causes root rot and is thought to trigger the death of weakened trees in the decline process.

8. Four out of seven species belonging to the group of Ophiostomatoid fungi identified were particularly relevant: *Ophiostoma minus*, *Ophiostoma ranaculosum, Ophiostoma ips* and *Ophiostoma piliferum*. *Ophiostoma minus* was the most abundant species on symptomatic and dead trees, indicating that it plays a crucial role in the decline of Maritime pine in the Iberian Peninsula. On the other hand, *Heterobasidion annosum* was the most pathogenic species detected in this study. This fungus could be the most important contributing factor of *Pinus pinaster* decline in the Iberian Peninsula.

Pathogenicity of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings

9. Pathogenicity tests performed on two-year-old seedlings of *Pinus pinaster* has confirmed the virulence of the isolates of *Heterobasidion annosum*. The mortality rate of seedlings infected with *Heterobasidion annosum* via stem inoculation differed significantly from that of the respective control seedlings. Lesion lengths in the infected seedlings were also longer than in the control seedlings for both types of inoculation (woodchips and sawdust). And the percentage of fine roots (tertiary) was lower in the seedlings infected via soil than in the respective control seedlings.

10. This study reports the first pathogenicity test with *Heterobasidion annosum* isolates and *Pinus pinaster,* and the results demonstrated the susceptibility of Mediterranean Maritime pine to *Heterobasidion annosum* in Spain. Therefore, this pathogen seems to be the most likely contributor to the decline of *Pinus pinaster* in Spain.

Recommendations

Forest managers are increasingly required to integrate both adaptation and mitigation strategies to cope with new scenarios and threats such as climate change, new diseases and land-use modifications.

This work provides critical new information that may be useful for establishing and implementing forest action plans and policies. In particular, it is recommended the modification of stand structure by reducing competition for water in the most declined Mediterranean pine forests. Regularly programmed and implemented thinning and shrub control could be used to enhance the resistance and resilience of *Pinus pinaster* stands.

Further research is necessary to deepen the key role of the biotic factors involved in *Pinus pinaster* decline. Sustainable forest management plans linked to biological control should be implemented to ensure the future of these valuable forests.

Conclusiones

Factores abióticos y de gestión que predisponen y/o incitan al decaimiento de *Pinus pinaster*

Asociaciones entre el clima, las propiedades del suelo y la gestión forestal a nivel de masa

1- Las masas de *Pinus pinaster* ubicadas en sitios séricos pueden estar cerca de su umbral de tolerancia fisiológica. Por lo tanto, la disminución de la disponibilidad de agua en las últimas décadas como consecuencia del cambio climático y/o el uso excesivo de los acuíferos, junto con el aumento de las necesidades de agua debido a las altas densidades del arbolado y provocada por las modificaciones del uso del suelo, puede haber conducido al decaimiento de estos bosques y a los cambios a largo plazo en la composición de especies. El presente estudio ha puesto de manifiesto que la competencia y el déficit de agua podrían estar desencadenando la mortalidad de *Pinus pinaster* actuando como factores de predisposición al decaimiento.

Influencia de los factores ambientales y selvícolas a nivel de árbol

2- Se establecieron grupos diferenciados para la caracterización de las parcelas de muestreo según los distintos conjuntos de variables estudiadas (caracterización selvícola, propiedades del suelo, variables climáticas y estado fitosanitario). En general, los agrupamientos jerárquicos separaron las parcelas de muestreo según las diferentes zonas geográficas, las provincias, y por lo tanto también según las diferentes regiones de procedencia de *Pinus pinaster*. Nuestros resultados sugirieron que las zonas con tradición maderera que han sido gestionadas históricamente tienen un mejor estado sanitario que las zonas en las que la calidad de la madera es menor, en las cuales, la menor intensidad en la gestión selvícola provoca altos niveles de competencia por los recursos.

3- Se ha construido un modelo para predecir la probabilidad de que un árbol se encuentre en un estado de sanitario determinado (sano, sintomático o muerto), a nivel de árbol individual. El modelo desarrollado incluye variables relacionadas con el tamaño del árbol (diámetro a la altura del pecho), con la estructura de la masa (altura media de la masa) y con el clima (precipitación estacional de verano).

Modelado del crecimiento y la aridez en relación con la densidad y el estado sanitario

4- Las tendencias de los índices de aridez anuales a lo largo del tiempo y a través de los diferentes sitios de estudio, revelaron una clara cadena de eventos de sequía desde comienzos de 1990, en 1994 y en 2005. Nuestros hallazgos indicaron que las correlaciones entre el crecimiento y el índice de aridez generan respuestas significativas a nivel de árbol de acuerdo a las condiciones fitosanitarias y en relación con los niveles de defoliación. Así, los árboles recién muertos totalmente defoliados y los arboles sintomáticos con niveles de defoliación entre el 35 y el 99%, tienen una respuesta más rápida y aguda a la alta aridez, sugiriendo que los eventos de sequía pueden actuar como factores de incitación al decaimiento de *Pinus pinaster*.

5- Nuestros resultados también tuvieron implicaciones en las relaciones entre competencia-crecimiento-clima. En las masas con alto índice de densidad, la competencia podría estar actuando como factor de incitación, mientras que en las masas con densidades medias y bajas, podría actuar como factor de predisposición al decaimiento. Las masas menos densas mostraron una mejor capacidad de respuesta del crecimiento a la aridez, ya que las respuestas significativas se distribuyeron en un período de tiempo más largo después de los eventos de sequía. Por el contrario, las masas más densas mostraron menos capacidad de respuesta del crecimiento a la aridez, lo que sugiere a su vez, una falta de capacidad de recuperación. En ese contexto, la disminución del crecimiento no se vería influenciada significativamente por los posteriores eventos de sequía.

Factores bióticos que contribuyen al decaimiento de *Pinus pinaster*

Primera cita de *Heterobasidion annosum* en *Pinus pinaster* en España

6- *Heterobasidion annosum*, uno de los agentes causantes de la pudrición del tocón y de la raíz más importantes en el hemisferio norte, también está involucrado en el proceso de mortalidad y decaimiento del pino Negral (*Pinus pinaster*) en España. Este patógeno se registró previamente en *Pinus sylvestris* en el centro de España y en *Pinus nigra* en el noreste de España, pero esta es la primera cita de *Heterobasidion annosum* en *Pinus pinaster* en España.

Hongos del azulado de la madera y hongos de pudrición de la raíz asociados al decaimiento de *Pinus pinaster*

7- Las quince especies identificadas durante este estudio fueron agrupadas según su ecología: seis especies fueron saprófitas y las otras nueve fueron patógenos con diferentes grados de virulencia. Desde el punto de vista del diagnóstico, las especies de interés fueron los hongos pertenecientes al grupo de los Ophiostomatales, que atacan la madera y causan síntomas de azulado (considerados en otros estudios como principales agentes responsables del decaimiento), y el hongo de pudrición de la raíz *Heterobasidion annosum*, que puede estar desencadenando la muerte de los árboles debilitados en el proceso de decaimiento.

8- Cuatro de las siete especies que pertenecen al grupo de los hongos Ophiostomatales identificados fueron particularmente relevantes: *Ophiostoma minus*, *Ophiostoma ranaculosum*, *Ophiostoma ips* y *Ophiostoma piliferum*. *Ophiostoma minus* fue la especie más abundante en árboles sintomáticos y muertos, y parece desempeñar un papel crucial en el decaimiento del pino Negral. Por otro lado, *Heterobasidion annosum* fue la especie más patógena detectada en este estudio. Este hongo también podría ser uno de los factores contributivos más importantes al decaimiento de *Pinus pinaster* en la Península Ibérica.

Patogenicidad de *Heterobasidion annosum* en plántulas de *Pinus pinaster*

9- Las pruebas de patogenicidad realizadas en plántulas de *Pinus pinaster* de dos años de edad han confirmado la virulencia de las cepas Españolas de *Heterobasidion annosum*. La tasa de mortalidad en las plántulas infectadas, fue significativamente diferente de la de las plantas control. Las longitudes de las necrosis provocadas en plántulas infectadas en el tallo, también fueron significativamente diferentes de las de las plantas control. Además, el porcentaje de raíces finas (terciarias), fue significativamente menor que en las respectivas plantas control.

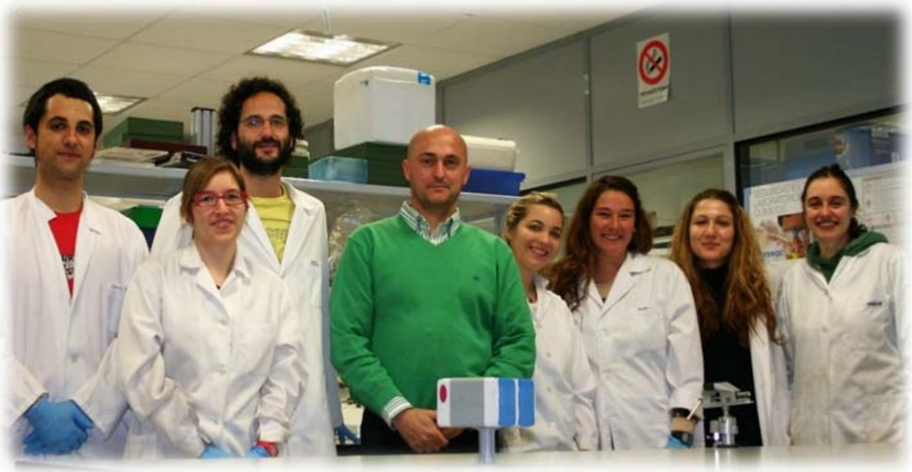
10- Este estudio muestra la primera prueba de patogenicidad con aislados españoles de *Heterobasidion annosum* en *Pinus pinaster,* demostrando la susceptibilidad del pino Negral a *Heterobasidion annosum* en España. Por lo tanto, este patógeno parece ser uno de los mejores candidatos para contribuir al decaimiento de *Pinus pinaster* en nuestro país.

Recomendaciones

Los gestores forestales están llamados cada vez más, a integrar en sus planes de gestión, estrategias de adaptación y mitigación para hacer frente a los nuevos escenarios y amenazas como; el cambio climático, las nuevas enfermedades y las modificaciones de uso del suelo.

Este trabajo proporciona nueva información relevante que puede ser útil para el establecimiento e implementación de planes de acción y políticas forestales. En particular, se recomienda la modificación de la estructura de las masas, reduciendo la competencia por el agua en los bosques de pinos mediterráneos con decaimiento. La aplicación de claras regularmente programadas y el control implementado de la vegetación arbustiva, podrían utilizarse para mejorar la resistencia y la capacidad de recuperación de la masas de *Pinus pinaster*.

Se necesita más investigación para profundizar en el papel clave de los factores bióticos implicados en el decaimiento de *Pinus pinaster*. Y planes de gestión forestal sostenible vinculados al control biológico, deberían ser implementados para asegurar el futuro de estos bosques de gran valor.



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Listado de publicaciones obtenidas a partir de esta tesis

- Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. Forest Ecology and Management. 356, 74–83. doi:10.1016/j.foreco.2015.07.033. Factor de impacto (JCR) 2.66.

- Prieto-Recio, C., Martín-García, J., Diez, J.J., 2014. Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings. Forest Pathology 44, 163–165. doi:10.1111/efp.12091. Factor de impacto (JCR) 1.37.

- Prieto-Recio, C., Romeralo, C., Bezos, D., Martín-García, J., Martínez-Álvarez, P., Botella, L., Diez, J.J., 2012. First Report of *Heterobasidion annosum* on *Pinus pinaster* in Spain. Plant Disease 96, 770. doi:10.1094/PDIS-10-11-0890-PDN. Factor de impacto (JCR) 3.02.

Próximas publicaciones:

- Prieto-Recio, C., Lara, W., Riofrío, J., Diez, J.J., Bravo, F., 2016. A multilevel approach for modelling tree growth and aridity in relation to stand competition and health status of *Pinus pinaster* in Spain. Próximo envío a la revista: Agricultural and Forest Meteorology. Factor de impacto (JCR) 3.76.

- Prieto-Recio, C., Roder, G., Diez, J.J., 2016. Blue-stain and root rot fungi associated with the *Pinus pinaster* decline in the Iberian Peninsula. Próximo envío a la revista: Silva Fennica. Factor de impacto (JCR) 1.18.

- Prieto-Recio, C., Bravo, F., Diez, J.J., Martín-García, J., 2016. Influence of environmental and silvicultural factors on *Pinus pinaster* health status at individual tree level in central Spain. Próximo envío a la revista: Annals of Forest Science. Factor de impacto (JCR) 1.98.