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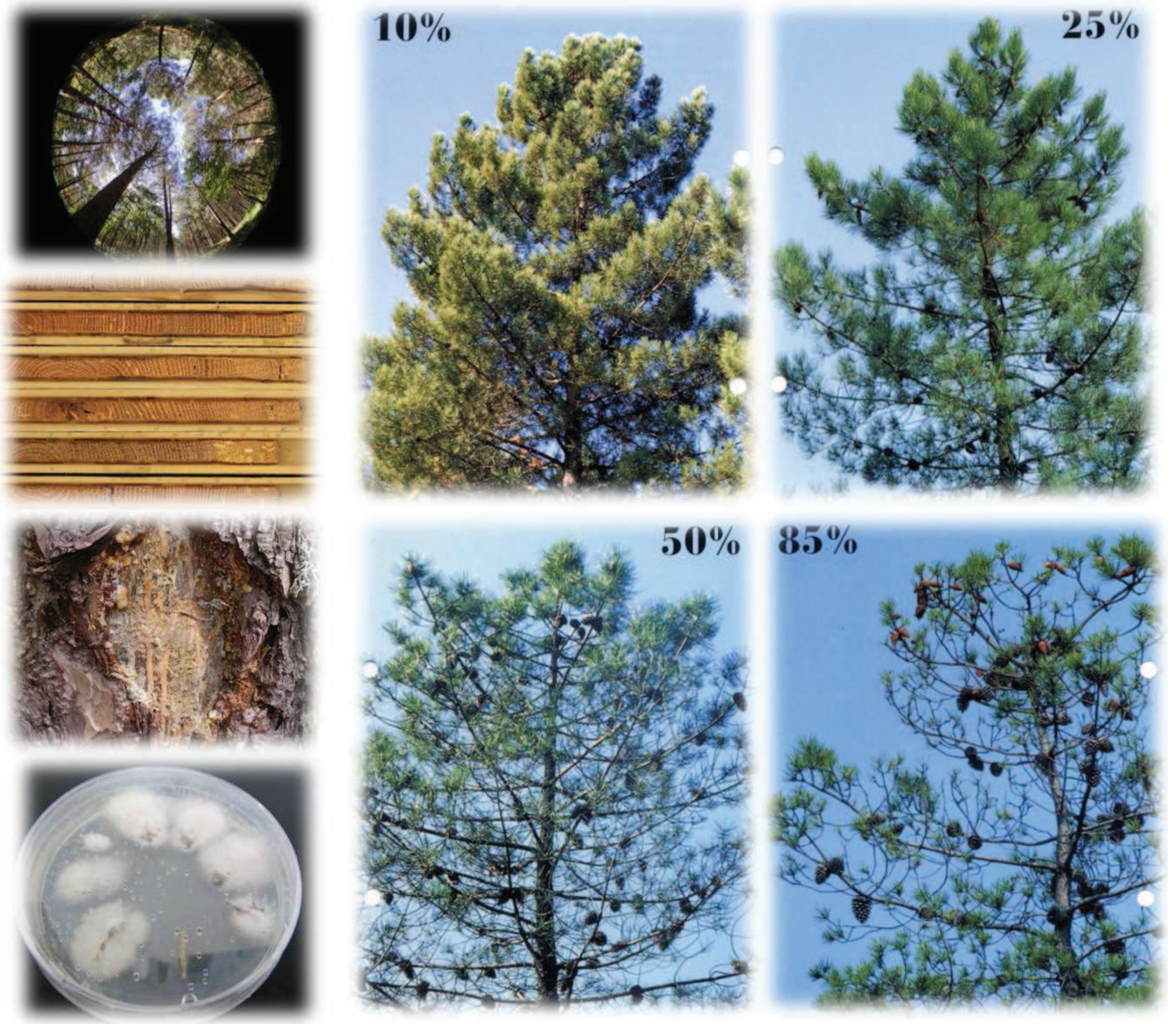
DOCTORAL THESIS

INFLUENCE OF BIOTIC AND ABIOTIC FACTORS ON HEALTH STATUS OF PINE FORESTS IN NORTHERN SPAIN



INFLUENCIA DE LOS FACTORES BIÓTICOS Y ABIÓTICOS EN EL ESTADO SANITARIO DE MASAS DE PINO DEL NORTE DE ESPAÑA

TESIS DOCTORAL

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ABSTRACT

Forest health can be considered "a condition where biotic and abiotic influences on forests (e.g., pests, pollution, silvicultural treatments, harvesting) do not threaten management objectives now or in the future". This thesis deals with the influence of abiotic and biotic factors on the health status of pine forests in northern Spain, focusing on factors under which pines are growing habitually, including climate, soil and stand characteristics, and as biotic component the previously established endophytic community, which inhabits plant tissues, and their relationship to the host, i.e pines. The objectives were: 1) To indentify biotic and abiotic factors which are influencing forest health status of pine forests in northern Spain. 2) To assess the more representative damages and their comparison among *Pinus nigra*, *P. sylvestris* and *P. pinaster*, and to find out if the use of this information can help to decide species suitability for future reforestations in the area. 3) To find relationships between canopy condition and some climatic parameters and to contribute to the understanding of how these climatic parameters will affect under future climate change scenarios. 4) To identify the stand characteristics affecting canopy condition in declining *Pinus halepensis* stands. 5) To characterize the endophytic community of *Pinus sylvestris* twigs, and to find out if the structure of this community is related to the growth rate of Scots pine. 6) To identify associations between the fungal endophytic communities of *Pinus sylvestris* twigs with various site factors, including climate, soil and stand characteristics. Our results indicate that the main observed damages were canopy defoliation and discoloration, and stem forking, sinuosity and cankers. However, only defoliation and discoloration were evaluable in all sites and species, so they appear as the most suitable indicators of canopy condition and health status of pine forests. On the other hand, long term precipitations and temperatures do influence the health status, which is a result of past and present climatic conditions. Under future climate change scenarios, an increment of mean temperature by 2ºC would cause a 30% increment in defoliation, representing a remarkable reduction of canopy and tree vigour.

Stand characteristics influencing health status of declining Aleppo pine stands were age, basal area, canopy depth and elevation, factors that if not well managed may predispose tree into decline. Additionally, decline is incited by frosts and the loss of canopy and favored by the presence of primary and secondary pathogens. Finally, 43 endophytic fungi were isolated from internal tissues, being three of them reported for the first time in the Pinaceae, and additionally six species were first reported in *Pinus sylvestris*. Some of these endophytes could be promoting tree growth, as is suggested for *Phoma herbarum*, while other species, such as *Hormonema dematioides*, might be protecting the tree against pathogens. The endophyte distribution is determined by climatic factors, such as precipitation and temperatures, and by soil conditions in a way that locations with favorable conditions for tree development showed a higher abundance of beneficial endophytes, while those under stressful conditions enhance the proliferation of other pathogens and opportunistic fungi.

RESUMEN

La sanidad forestal puede ser definida como un estado en el que la influencia biótica y abiótica (plagas, polución, tratamientos selvícolas, etc) no amenacen los objetivos de manejo presentes y futuros. Esta tesis trata de la influencia de los factores bióticos y abióticos en el estado sanitario de los pinares en el norte de España, prestando especial atención a los factores a los que las masas de pino están sometidos de forma habitual, incluyendo el clima, suelo y características del rodal, y como componente biótico los habitantes ya presentes en los tejidos internos de los árboles y su relación con sus hospedantes, los pinos. Los objetivos son: 1) Identificar los factores bióticos y abióticos que influyen en el estado sanitario de las masas de pino del norte de España. 2) Evaluar los daños más representativos y su comparación, tanto cualitativa como cuantitativamente, entre parcelas de *P. nigra, P. sylvestris* y *P. pinaster* en el norte de España, así como averiguar si el uso de esta información podría resultar útil como criterio que ayude a la decisión del uso de estas especies en futuras repoblaciones forestales en la zona. 3) Explorar las relaciones entre la condición de copa y los parámetros climáticos a largo plazo, y contribuir al conocimiento de cómo estos parámetros influirán bajo escenarios futuros de cambio climático. 4) Identificar los factores de sitio que afectan a la condición de copa en rodales de pino carrasco (*Pinus halepensis* Mill.) en decaimiento. 5) Caracterizar la comunidad fúngica endófita de ramillos de *Pinus sylvestris* L., y determinar si la estructura de esta comunidad está relacionada con su tasa de crecimiento. 6) Estudiar la influencia de los factores de sitio, incluyendo parámetros climáticos, edáficos y dasométricos (del rodal), en la distribución de organismos endófitos de *P. sylvetsris.*

Los principales daños encontrados fueron bifurcaciones, defoliaciones, malformaciones del tronco, decoloraciones y chancros, pero solamente defoliación y decoloración fueron encontradas en todas las parcelas y especies evaluadas, por lo que éstos son los más adecuados para su uso como indicadores de la condición de copa y el estado sanitario de las masas de pino. Las precipitaciones y temperaturas de los últimos 15-20 años influyen sobre el estado sanitario actual, el cual es una mezcla de condiciones presentes y pasadas. En escenarios de cambio climático, La tendencia de la defoliación es a aumentar considerablemente, siguiendo la de las temperaturas y precipitaciones previstas.

Por otro lado, las características de rodal que más influyen sobre el estado sanitario de masas de pino carrasco en decaimiento son la edad, el área basimétrica, la altura de copa viva y la elevación, factores que si no son bien gestionados pueden predisponer los árboles hacia un decaimiento, el cual también es incitado por las heladas y por la defoliación sufrida previamente, y que además se ve favorecido por la presencia de patógenos primarios y secundarios.

En los tejidos internos de pino silvestre fueron aislados 43 hongos endófitos distintos, siendo tres de ellos descritos por primera vez en la familia Pinaceae, y otros seis nuevos en *Pinus sylvestris*. Algunos de estos hongos podrían estar promoviendo el crecimiento, como se sugiere para *Phoma herbarum*, mientras que otros, como *Hormonema dematioides* podrían tener un papel protector ante patógenos. La distribución de hongos endófitos se encontró asociada al crecimiento del árbol, y está influenciada por factores climáticos, como precipitación y temperatura y por las condiciones edáficas, de modo que bajo condiciones favorables para el pino aparecen más abundantemente organismos beneficiosos, como los anteriormente citados, mientras que bajo condiciones estresantes se favorece la proliferación de organismos patógenos y oportunistas.

AGRADECIMIENTOS

Esta tesis jamás podría haber sido realizada sin el apoyo de mi tutor, Julio Diez Casero, quien un día decidió confiar en mí, y a quien agradezco su dedicación y su inmejorable trato. Además de su apoyo, en primer lugar he de agradecer a Encarna Rodríguez no solo su apoyo anímico y técnico, sino su compañía, comprensión y cariño, que durante todos estos años me han ido dando fuerza para continuar, siempre juntos, adelante. Por supuesto el apoyo de mis padres ha sido fundamental, en especial de mi madre, Manuela Ros Moreno, a quien se lo debo todo, y quien más ha sufrido esta tesis, por ser la causante de no tenerme a su lado. Agradezco mucho el apoyo de Michael Müller, del METLA en Finlandia, por una de las mejores experiencias de mi vida, que fue poder vivir allí unos meses, aprender y disfrutar todo lo que es posible. También a Fernando Valladares por su colaboración científica y su buen trato. No olvido a los compañeros del doctorado. Todos ellos, cada uno a su manera, ha ido aportando cosas buenas a mi desarrollo personal y profesional, de modo que han influido (significativamente, $P<0,05$) en la persona que ahora soy, y por lo tanto en el desarrollo de esta tesis. Así que agradezco a Jorge Martín García por su amistad en lo personal y su apoyo profesional, que siempre he apreciado enormemente. A Celia Herrero por su apoyo en campo y en gabiente, y por otros buenos momentos, a Antonio Urchaga, María Gómez, Iñaki Etxeveste, Leticia Botella, Luis Pacios (Moriarti, que ha sido como un compañero mas y un amigo), Pablo Martínez, Carmen Romeralo, Diana Bezos, Lucia Risio, Estela Husillos, Ana Isabel de Lucas, Cristóbal Ordóñez, Gonzalo Álvarez, Nico Cattaneo, Masum Haque, Norlan, Félix, María Fernanda (Mafe), y otra larga lista de personas que he conocido a raíz de esta tesis, que también han aportado su granito de arena a la montaña de la vida. Ha sido también muy importante el apoyo recibido por otras personas de la UVA, como Felipe Bravo y Celia Redondo, que siempre están ahí para ayudar en lo que haga falta, asi como a otros profesores que han sido una buena influencia, como Helena Hidalgo, Pablo Martínez Zurimendi, Fernando Alves, Pablo Pinto, o Carlos del Peso. Agradezco también el apoyo de los compañeros del Centro de Sanidad Forestal de Calabazazos, con quienes convivo diariamente y que me acompañan en este viaje a veces maravilloso, a veces no, que es la vida. A todos ellos, mil gracias, os quiero!.

NOTE TO READERS

This thesis is based on five original works (which are referred to in the text with Roman numerals) some of them published in international journals, as book chapters or conference proceedings. Each article constitutes one of the studies or chapters of the thesis (chapter I written in Spanish, and chapters II, III, IV and V written in English). Authors, co-authors, and the stage of the publication (submitted, under revision, accepted, published) are presented below.

Previously to the presentation of each study, the reader will find the outline of the thesis (written in English and Spanish), and a general introduction written in Spanish. After that there are listed the objectives of the thesis (written in English and Spanish). To conclude, after the presentation of the studies, the reader will find a general discussion written in Spanish, and the general conclusions written in English. The thesis may be easily followed by both English and Spanish speaking readers.

NOTA A LOS LECTORES

Esta tesis está basada en cinco trabajos originales (los cuales se citan en el texto con números romanos), algunos publicados en revistas de impacto internacional, otros como capítulo de libro o como anales de congreso. Cada artículo constituye uno de los estudios o capítulos de la tesis (el capítulo I escrito en español y los capítulos III, IV y V en inglés). Los autores, coautores y el estado de la publicación (enviado, en revisión, aceptado, publicado) se presentan a continuación.

Previamente a la presentación de cada capítulo, el lector encontrará unas líneas generales de la tesis (inglés y español), una introducción escrita en español, describiendo el esquema general de la tesis, y los objetivos generales y específicos de cada estudio (en español). Para concluir, después de presentar cada capítulo, el lector encontrará una discusión general escrita en español, y las conclusiones generales de la tesis en inglés. El manuscrito de la tesis está estructurado de forma que pueda ser entendida en su totalidad por anglo e hispanoparlantes.

LIST OF ORIGINAL STUDIES

I- Sanz-Ros, A.V., Pajares, J.A., Díez Casero, J. (2008a). Comparación de daños forestales entre especies de pino en repoblaciones del norte de España. *Cuad. Soc. Esp. Cienc. For. 26: 91-96.* Actas de la I Reunión sobre Sanidad Forestal. (Chapter **I**).

II- Sanz-Ros, A.V, Pajares, J. & Diez, J. (2008b): Influence of Climatic Variables on Crown Condition in Pine Forests of Northern Spain. In: Bravo, F., LeMay, V., Jandl, R. & Gadow, K. (Eds.), *Managing Forest Ecosystems: The Challenge of Climate Change*. Springer, Netherlands. pp. 103–115. (Chapter **II**).

III- Sanz-Ros, A.V, Valladares, F. & Diez, J. Stand factors influencing *Pinus halepensis* decline in north-western Spain. *Forest Pathology* (under revision after reviewers' comments). (Chapter **III**).

IV- Sanz-Ros, A.V., Müller, M., San Martín, R. & Diez, J. (2015): Fungal endophytic communities on twigs of fast and slow growing Scots pine (*Pinus sylvestris* L.) in northern Spain. *Fungal Biology*. http://doi.org/10.1016/j.funbio.2015.06.008. (Chapter **IV**).

V- Sanz-Ros, A.V., Müller, M., San Martín, R. & Diez, J. (XXXX). Influence of site factors on fungal endophytic community of *Pinus sylvestris* L. twigs in northern Spain. (This chapter is in the course of being submitted to the journal Fungal Ecology). (Chapter **V**).

OUTLINE OF THE THESIS

This thesis deals with the influence of abiotic and biotic factors on the health status of pine forests in northern Spain. Forest Health usually deals with specific biotic agents, extreme climatic conditions and ecosystem perturbations influencing forest health. Despite of that, there are a number of factors which could influence in pine health status. These factors include the environmental conditions under which pines are growing, such as climate, soil and stand features (density, basal area, etc), as well as other biotic component, the endophytes, as the main inhabitants of plant tissues, and their relations to their host, pine trees (Figure 1). This thesis is composed by five chapters, which are described below:

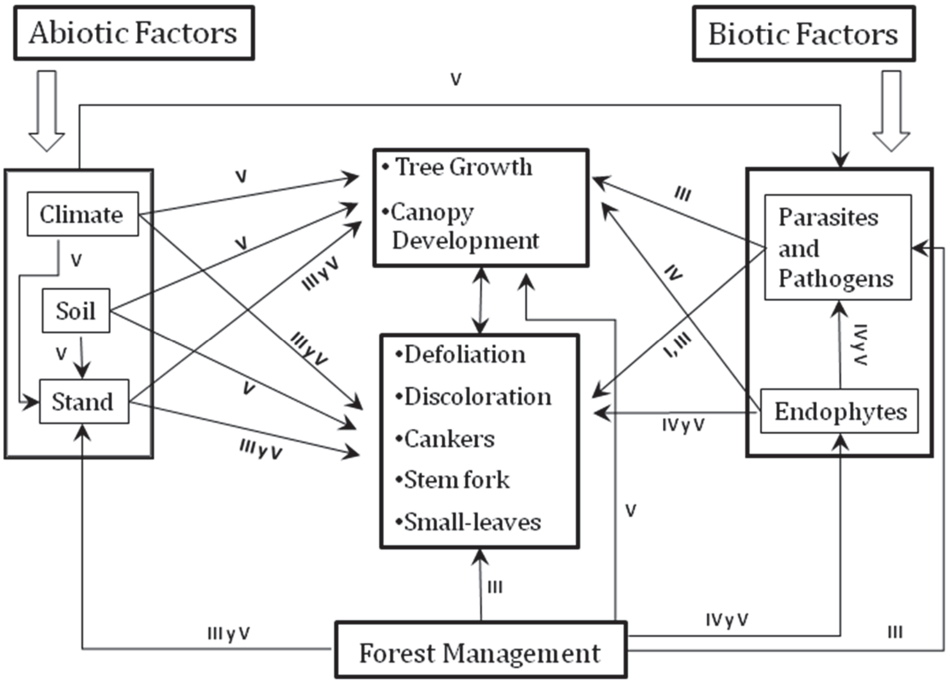


Figure 1: Conceptual map. Arrows represent influences of one factor in other, following the arrow direction. Roman number next to each arrow indicates the chapter of the thesis where this influence is discussed.

Chapter **I** focused on the types of damages that canopy use to show in pine stands. It is important to know this information in order to make a correct evaluation of forest health, which is assessed through the estimation of certain indicators of canopy condition, and this study try to help with the selection of the most suitable ones. The more frequently found damages were defoliation, discoloration, stem forking, sinuosity and cankers. Defoliation was the best indicator of canopy condition since it is quantifiable in every plot and every species and its assessment is reproducible following the criteria stated by ICP Forests.

Chapter **II** deals with the influence of climatic factors in canopy condition of pine stands. Evaluating canopy condition along a climatic gradient allows us to assess the long term climatic parameters influence. It has been demonstrated that long term climatic data is determining part of the present health status of pine stands, in a way that little increments in mean temperatures or little decreases in mean precipitations led to significant canopy reduction. Under future climate change scenarios the canopy condition might be significantly affected.

In Chapter **III** we studied the influence of stand characteristics in declining Aleppo pine forests. The etiology of its decline is not well understood, and in this chapter we attempt to unravel the influence of the stand features in this process. Besides of evaluating canopy condition through the estimation of defoliation, we used an optical method, the hemispherical photography, to compute the leaf area index, which is used as indicator of canopy development. A number of stand features were found to be influencing canopy condition, such as age, basal area, canopy depth and elevation. These parameters may be modulated by forest management. Factors involved in the observed decline were the presence of fungal primary and secondary pathogens, poor soil conditions, frosts days and forest management, determining stand features such as those previously cited. Some management strategies are proposed in order to minimize the impact of the decline.

Chapter **IV** is about internal inhabitants of healthy Scots pine twigs, the endophytes, and their relation with host growth rate. We characterize fungal endophytic community through the estimation of its diversity, evenness, species composition and abundance. Differences in fungal endophytic community were found between fast and slow growing pines, concretely in species composition, abundance and evenness, but not in diversity. Some species were found to be associated to trees showing high or low growth rates, so a biological role is expected for them, such their possible influence on tree growth or protecting against pathogens. Three fungal endophytic species were reported for the first time for the Pinaceae, and additional six species were first reported inhabiting *Pinus sylvestris*, which remind us that we don't know most of the fungal diversity inhabiting plant species.

In Chapter **V** we have studied the influence of site factors in the fungal endophytes' distribution in Scots pine twigs. Environmental factor determining growth and development of pine trees might influence the distribution of their inhabitants. These factors include climate, soil and stand characteristics, being the last determined by forest management. Our results indicate that the presence and abundance of some fungal endophytes were associated to specific combinations of environmental factors. Fungal endophytes playing a beneficial role to their host were associated to favorable environmental conditions, while those playing a pathogenic or damaging role were associated to more stressful environments, including drought and poor soil conditions.

LÍNEAS GENERALES DE LA TESIS

Esta tesis trata de la influencia de los factores bióticos y abióticos en el estado sanitario de los pinares en el norte de España. La sanidad forestal clásica suele atender a problemas concretos causados por patógenos, plagas, eventos climáticos extremos o perturbaciones severas del hábitat, sin embargo se ha prestado una menor atención a la influencia de los factores a los que las masas de pino están sometidas de forma habitual. Independientemente de si están siendo atacadas o no por agentes bióticos, como patógenos o plagas, existen una serie de factores que pueden influir en el estado de salud de los pinares. Estos factores incluyen, por un lado, las condiciones ambientales en las que crecen estos pinares, como son clima, suelo y características del rodal, y por otro los habitantes internos de los tejidos de los árboles y su relación con el hospedante (Figura 1). La tesis consta de cinco estudios o capítulos, cuyas líneas generales son descritas a continuación.

El capítulo **I** se centra en los tipos de daños más frecuentes que nos podemos encontrar en los pinares de la zona de estudio, incluyendo *Pinus nigra*, *P. pinaster* y *P. sylvestris*. Es importante conocer esta información para llevar a cabo una correcta evaluación sanitaria de las masas y al mismo tiempo ver cuáles son los parámetros más indicados para cuantificar el estado del dosel arbóreo. Los daños encontrados fueron principalmente defoliación, decoloración y bifurcaciones, siendo la defoliación el indicador que parece más adecuado para la evaluación de las masas, ya que es observable en todas las parcelas y reproducible en su evaluación, siempre que se sigan los criterios y métodos descritos. La tesis consta de cinco estudios o capítulos.

El capítulo **II** trata sobre la influencia de los factores climáticos en la condición de copa de los pinares. La condición de copa fue evaluada a lo largo de un gradiente climático, lo cual nos permite comparar dicho estado con el clima a largo plazo existente en cada punto. Se ha observado que existe una influencia de este clima a largo plazo en la condición de copa, de modo que pequeños aumentos de las temperaturas medias o reducciones de las precipitaciones medias provocan aumentos considerables de la defoliación, lo cual nos sugiere que la condición de copa podría empeorar bajo escenarios de cambio climático.

En el capítulo **III** se aborda la influencia de las características del rodal sobre el estado sanitario de pinares en decaimiento. En este caso, además de la cuantificación de la defoliación, fue usado un método óptico para la evaluación del índice de área foliar como medida del desarrollo de la copa. Se observo que hay ciertos parámetros que influyen en la condición de copa, como son la edad, la altura de copa, el área basimétrica y la elevación. La presencia de organismos patógenos primarios y secundarios en esta zona, junto con las pobres condiciones del suelo, la sequía y los parámetros del rodal, los cuales están directamente influenciados por el manejo que se hace de éste, son los factores que se discuten para intentar describir el proceso observado del decaimiento del pino carrasco, que viene produciéndose desde hace bastantes años en el norte de España e intentar sugerir líneas de gestión con el fin de minimizar el impacto del decaimiento.

El capítulo **IV** trata sobre los organismos endófitos que habitan en los tejidos vegetales y su relación con el hospedador, en este caso el pino silvestre (*P. sylvestris*). Se estudió la diversidad y equitatividad de la comunidad fúngica endófita, así como su composición de especies y abundancia en árboles con diferentes tasas de crecimiento, para dilucidar si existía alguna diferencia en la comunidad fúngica endófita. Se observaron diferencias entre árboles que crecen rápido y los que crecen lento en cuanto a la composición de especies fúngicas, su abundancia y la equitatividad de su distribución, sin embargo no fueron encontradas diferencias en su diversidad. Ciertos hongos endófitos fueron asociados a altas o a bajas tasas de crecimiento, lo cual hace interesante su estudio para evaluar cuales son los mecanismos por lo que estos hongos pueden influir sobre el crecimiento vegetal y sus posibles aplicaciones. Además se describen tres nuevas especies fúngicas endófitas para la familia Pinaceae, y seis nuevas para el pino silvestre, lo cual nos recuerda que desconocemos a día de hoy una gran parte de la diversidad fúngica endófita.

El capítulo **V** trata la influencia de los factores de sitio sobre la distribución de hongos endófitos en ramillos de pino silvestre. Los factores ambientales bajo los cuales crecen los pinos podrían influir en la distribución de hongos endófitos, ya que influyen sobre el desarrollo de su hospedador. Estos factores de sitio incluyen el suelo, el clima y las características del rodal, las cuales van a estar determinadas por la gestión forestal realizada. Los resultados indican que la presencia y abundancia de algunas especies endófitas está ligada a ciertas combinaciones de factores ambientales, con lo cual se confirma la influencia de dichos factores sobre la distribución de endófitos. Además se observa que especies endófitas que tienen un efecto positivo sobre el árbol, bien a través de la mejora de su crecimiento o bien como protector ante patógenos, aparecen más abundantemente cuando las condiciones climáticas y edafológicas son favorables, mientras las especies que ejercen una influencia negativa o patogénica están asociadas a suelos pobres y ambientes estresantes.

Introducción General

INTRODUCCIÓN GENERAL

LOS PINARES EN EL NORTE DE ESPAÑA

Los pinares son elementos importantes en ecosistemas naturales Mediterráneos (Barbero et al., 1998), y muchas veces dominantes en numerosos ecosistemas del hemisferio norte (Richardson, 1998). Existen descritas 111 especies de pino, y cada una de ellas posee unos requerimientos ecológicos diferentes, lo cual hace que estén tan extendidas. Por otro lado, existe una variación considerable en la plasticidad fenotípica entre especies, siendo capaces de colonizar un mayor rango de ambientes diferentes aquella con mayor plasticidad (Soto et al., 2010).

Los pinares ejercen una influencia sobre los ecosistemas de diversas maneras. Influyen en los ciclos biogeoquímicos, en los regímenes hidrológicos y en los de incendios (Fernandes, et al., 2008). Además, van a producir una serie de servicios ecosistémicos, entre ellos la producción de oxígeno, fijación de CO_2, fijación del suelo para evitar la erosión, filtrado de la escorrentía, el desarrollo de suelos muy pobres como especie pionera o el nicho que representan para muchas otras especies animales, vegetales y fúngicas, contribuyendo así a la biodiversidad. En definitiva, el género *Pinus* puede ser considerado como el género arbóreo más significativo a nivel ecológico y económico en el mundo (Richardson, 1998). También cabe citar la producción de recursos maderables y no maderables, que suponen una fuente renovable de recursos inestimable para el desarrollo humano, así como su función como lugares de esparcimiento para la especie humana (servicios culturales), e incluso en algunas culturas de tipo espiritual.

En España, según datos del Tercer Inventario Forestal Nacional (IFN 3) hay alrededor de 20 millones de hectáreas (mill. ha.) de superficie forestal arbolada. La comunidad de Castilla y León (CyL) tiene 5 mill. ha. de superficie forestal, de las cuales el 62% son arboladas, es decir 2.982 mill. ha. de bosque, que suponen el 15 % de la superficie forestal arbolada de toda España. Un 35 % de dicha superficie forestal arbolada en CyL son coníferas (alrededor de 1 mill. ha.), principalmente representadas por los géneros *Pinus*, *Juniperus* y *Cupressus*. Las especies de pino más representadas en Castilla y León, por orden de abundancia, son *Pinus sylvestris*, *P. pinaster*, *P. nigra*, *P. pinea*, *P. halepensis* y *P. radiata* según IFN3.

La gran mayoría de las masas de pino existentes en España son plantaciones, cuya gestión es determinante para asegurar su funcionalidad, sostenibilidad y permanencia en el tiempo. La vocación de cada rodal, es decir, el objetivo con el que el ser humano establece y gestiona cada masa, varía en función de diversos factores ambientales y antrópicos, pudiendo ser conservacionista, proteccionista o productivista.

En cuanto al aprovechamiento de cada especie en la comunidad de Castilla y León, la producción maderera es el tipo más importante económicamente. Las especies que más madera producen son el pino albar (*P. sylvestris*) con 41 millones de m^3 (mill. m^3), seguido por pino resinero (*P. pinaster*) (39 mill. m^3) (IFN3). Aunque existen otras especies muy importantes por otros motivos, como son el pino piñonero (*P. pinea*) por la producción de piñón, el pino salgareño (*P. nigra*), por su tolerancia a terrenos calizos, el pino resinero (*P. pinaster*), por la producción de madera y resina y el pino carrasco que, en el norte de España ha sido usado para la estabilización de taludes y la repoblación para el control de la erosión, en general en sitios con condiciones edafológicas e hidrológicas muy adversas, en las que otras especies no se desarrollan convenientemente (Serrada, Montero & Reque, 2008).

ESTADO SANITARIO DE LAS MASAS DE PINO

El estado sanitario de las masas de pino (así como en cualquier masa forestal compuesta por otras especies) es el resultado de la interacción dinámica entre el árbol y un amplio rango de patógenos bajo una serie de condiciones ambientales cambiantes (Pautasso et al., 2010). El estado sanitario es un factor clave para la persistencia de las masas, influyendo en procesos como lo fijación de CO_2 (Pinkard et al., 2014), la fotosíntesis, el crecimiento (Wiley et al., 2013) o la tolerancia hacia otros factores de estrés, ya que los arboles debilitados son más probablemente atacados por otras plagas o enfermedades (Houston, 1992). El estado de salud del bosque es un concepto general y complejo que puede verse influido por muchos y diversos factores, siendo su manifestación más clara la que se puede observar en el estado del dosel, al cual se hace referencia comúnmente como “condición de copa” o “condición del dosel”. El seguimiento del estado sanitario de las masas viene realizándose anualmente desde 1986 en la Red de Seguimiento de Daños a los Bosques (ICP Forests, 1992), desarrollada en el marco de la Convención de Ginebra por el Comité Permanente Forestal de la Unión Europea, quien elaboró la metodología común para toda Europa. Esta red se compone de una red de Nivel I y otra de Nivel II.

La Red de Nivel I nace como respuesta al deterioro observado en el estado fitosanitario de gran parte de los bosques en países industrializados desde la década de los 70, el cual se agravó durante los 80. Esta red se evalúa en toda Europa sobre una malla de 16x16 km tomados a partir de un punto “cero” tomado al azar en Europa (SPCAN, 2002), mientras que los países miembros deben realizar como mínimo la evaluación sobre esa malla, pudiendo aumentar la intensidad de muestreo. En España se evalúan unas 600 parcelas de Nivel I. En Castilla y León se realiza sobre una malla de 8x8 Km en masas certificadas mediante criterios PEFC (Red de Rango I), excepto en espacios naturales protegidos, donde se evalúa sobre una malla de 4x4 km (red de Rango II). Esta red trata de evaluar las condiciones ecológicas de la estación y la vitalidad del arbolado y su estado sanitario.

La red de Nivel II es una red de unas 53 parcelas permanentes de vigilancia intensiva, en la cual están representados los principales sistemas forestales. En ellas se evalúan un número mayor de parámetros con el fin de caracterizar la masa forestal y su historia, el arbolado y su follaje, la vegetación, el suelo, el clima, la composición química del agua de lluvia al descubierto, bajo el dosel de las copas y el de las aguas de drenaje (SPCAN, 2002).

Generalmente los pies que crecen bajo condiciones estresantes suelen mostrar una reducción en su copa, la cual es cuantificada en estas redes de evaluación de daños mediante el uso de algunos indicadores, siendo la defoliación y la decoloración los más comunes. La decoloración es un parámetro más difícilmente interpretable y plantea una serie de inconvenientes si no se evalúa en la época adecuada. Sin embargo la defoliación es un método mucho más reproducible. El uso de este indicador también ha suscitado cierto criticismo, sin embargo estudios recientes demuestran su validez y homogeneidad a lo largo del tiempo (Eickenscheidt & Wellbrock, 2014).

La defoliación se define como la pérdida de hoja provocada por la acción de factores bióticos y abióticos, pero también incluye la perdida prematura de hoja o una reducción en el periodo en que está unida a los ramillos (Ferretti, 1994). Además se considera que un pie defoliado tiene una menor probabilidad de supervivencia y es más sensible al ataque de patógenos o insectos (Houston, 1992). Por ello, la defoliación ha llegado a ser el indicador de sanidad forestal y condición de copa más ampliamente usado, habiéndose cuantificado en las redes de Nivel I en toda Europa desde 1986 (Fisher, Petrini & Sutton, 1993; Marco Ferretti, 1997; Eichhorn et al., 2010; Ferretti, 2010; Fischer et al., 2010).

MÉTODOS DE EVALUACIÓN DE LAS MASAS

La evaluación del estado sanitario de las masas se ha venido haciendo de forma tradicional por personal altamente especializado en este campo, ya que son muchos y complejos los factores que pueden producir daños en los árboles. Generalmente se suele realizar la evaluación sobre la copa viva, la denominada “copa evaluable”.

El parámetro más ampliamente usado en la evaluación de las masas es la defoliación (SPCAN, 2002), cuya estimación se lleva a cabo de forma visual mediante comparación con fotografías de referencia (Figura 2) para cada especie (Ferretti, 1994), las cuales muestran diferentes porcentajes de defoliación en intervalos del 5% y que se han usado como referencia común para todo el territorio circunmediterráneo (Innes, 1990; Innes, Landmann & Mettendorf, 1993). Sin embargo, la realización de la evaluación de forma visual siempre implica la posibilidad de un sesgo o cierta subjetividad dependiente del observador, por ello han sido desarrollados otros métodos cuya finalidad es automatizar esta evaluación, tema tratado en el Capítulo III de esta tesis. Algunos de estos métodos están basados en el estudio del ambiente luminoso y la radiación fotosintéticamente activa (RFA, o PAR según sus siglas en inglés), con el fin de obtener el índice de área foliar (IAF, o LAI por sus siglas en inglés), parámetro usado como indicador del desarrollo de la copa (Waring, 1985).

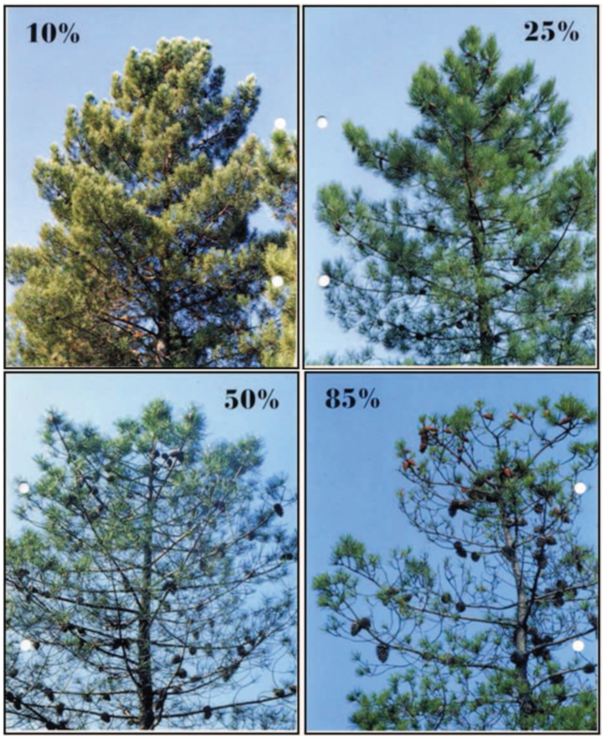
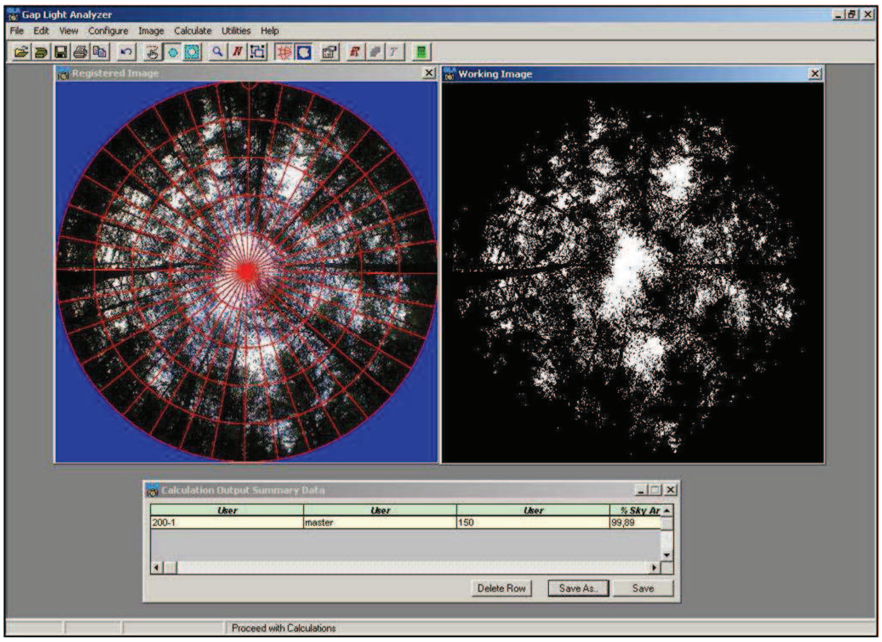


Figura 2: Fotografías de referencia para la evaluación de la copa de *Pinus pinaster*: los números indican el porcentaje de defoliación (Fuente: Ferretti, 1994).

Estos métodos pueden ser clasificados en tres grandes grupos en función de la posición y la tecnología empleados. Un primer grupo está formado por los métodos basados en las imágenes de satélite, entre ellos cabe citar lo sensores de alta resolución NOAA/AVHRR, SPOT/VGT, Envisat/MERIS y EOS/MODIS, los cuales tienen una resolución de entre 250 m y 1 Km, y ha sido investigado su uso con el fin de realizar detección rápida de daños por insectos cuando son producidos sobre grandes áreas (Eklundh, Johansson & Solberg, 2009). Sin embargo con esta metodología se ha conseguido detectar, pero no cuantificar, la defoliación producida por insectos. El segundo grupo estaría compuesto por métodos aéreos, dentro de los cuales se pueden incluir los basados en tecnología láser, denominado como Lidar (Riaño et al, 2004; Solberg et al., 2006). Por último están los métodos tomados desde el suelo, entre los cuales cabe destacar el LAI-2000 (López Serrano et al., 2000; Stenberg et al., 1994) y la fotografía hemisférica (Chen, Black, & Adams, 1991), ambos usan la intercepción de la luz que produce el dosel arbóreo para estimar la fracción de huecos (gap fraction) y de ahí computar el LAI efectivo. Existen diferentes programas informáticos para el análisis de las imágenes producidas, entre ellos Gap Light Analyzer (Frazer et al., 1999) (Figura 3).



User	User	User	% Sky Ar
200-1	master	150	99,89

Figura 3: Imagen del análisis de la fotografía hemisférica llevado a cabo con el programa Gap Light Analyzer

El LAI efectivo se computa asumiendo una distribución aleatoria de las acículas que no tiene en cuenta el índice de agrupamiento de las mismas (Weiss et al., 2004), el cual puede aplicarse posteriormente a los valores obtenidos (Chen & Black, 1992).

El desarrollo de estos métodos ha conseguido obtener de forma automática el índice de área foliar; incluso algunos autores han conseguido computar la defoliación, producida por un ataque severo de insectos, como cambios en el LAI a lo largo del tiempo (Solberg et al., 2006). Sin embargo la relación entre ambos parámetros en otras situaciones distintas no ha sido estudiada previamente. Este tema es abordado en el Capítulo III de esta tesis.

FACTORES DE ESTRÉS I: COMPONENTES ABIÓTICOS

Cuando los bosques, al igual que el resto de plantas, se ven afectados por factores abióticos adversos se considera que están bajo estrés. Ante dicho estrés se desencadenan en los árboles mecanismos de compensación, generalmente de tipo fisiológico o metabólico, con el fin de tolerar dicho estrés antes de que su situación llegue a ser crítica y peligre su persistencia. Los árboles, tanto entre especies distintas como dentro de una misma especie, poseen una mayor o menor plasticidad fenotípica según su tolerancia a cambios ambientales u otros factores de estrés abiótico. Dentro de cada especie se observa una mayor plasticidad fenotípica en poblaciones sometidas a considerables variaciones interanuales en la precipitación y las temperaturas extremas (Baythavong, 2011). En este sentido las coníferas poseen generalmente una elevada plasticidad fenotípica (Santos-Del-Blanco et al., 2013), lo cual les ha permitido colonizar amplias zonas a lo largo de la superficie terrestre. Sin embargo ciertas combinaciones de factores abióticos pueden generar un estrés suficiente como para superar estos mecanismos de plasticidad.

Los factores estresantes de tipo abiótico son muy diversos. De entre los más comunes, sequías y heladas suelen provocar desequilibrios fisiológicos que producen un descenso de vigor del árbol, e incluso su muerte. Otros factores como fuertes vientos, granizadas, nevadas o incendios pueden producir un daño físico directo que indudablemente repercute sobre el vigor del árbol. En ambos casos, el árbol necesita emplear toda su energía en compensar el daño y defenderse ante otros posibles invasores de tipo biótico, por lo que su crecimiento se ve severamente afectado (Andreu et al., 2007).

Los escenarios previstos de cambio climático pueden incrementar las condiciones estresantes, ya que la tendencia de la precipitación es decreciente, de forma general en el sur de Europa (Buffoni, Maugeri & Nanni, 1999; Piervitali, Colacino & Conte, 1997; Sarris, Christodoulakis, & Körner, 2007; Schönwiese & Rapp, 1997), y en la Península Ibérica (Esteban-Parra, Rodrigo, & Castro-Díez, 1998; Mossman, 2002; Parry, 2000). Además la tendencia de las temperaturas se sitúa claramente al alza (IPCC, 2013), habiéndose observado un aumento un incremento de 1,6 ºC en España a lo largo del último siglo (Hulme & Sheard, 1999; Parry, 2000). La combinación de ambos factores aumentará la evapotranspiración, por lo que el agua disponible para los sistemas forestales será menor. Aunque la eficiencia del uso del agua será mayor al aumentar las concentraciones de CO_2 atmosférico, todo parece indicar que la competencia inter e intraespecífica de las comunidades vegetales será más aguda, especialmente durante la regeneración de los bosques (Serrada, Montero & Reque, 2008).

La influencia de los factores climáticos a largo plazo sobre la condición de copa es estudiada y cuantificada en el Capítulo II de esta tesis, ya que se espera que la influencia de estos factores sea diferente en función de la zona, y la previsión de cambios en el clima presentará una elevada variabilidad espacial (Andreu et al., 2007).

FACTORES DE ESTRÉS II: COMPONENTES BIÓTICOS

Los factores estresantes de tipo biótico se corresponden con organismos que causan daños a los árboles. Estos organismos pueden pertenecer a diversos grupos taxonómicos, como plantas parásitas, insectos, nematodos, hongos, oomicetos, bacterias, fitoplasmas, mollicutes o virus. En los pinares, sin embargo, los agentes bióticos parásitos y patógenos están principalmente compuestos por insectos, hongos, nematodos y alguna planta parásita, como es el caso del muérdago (Muñoz et al., 2007). Todos ellos pueden causar enfermedades o plagas que estresan y debilitan los pies afectados. En la mayoría de ocasiones los árboles suelen sufrir una reducción de su copa como consecuencia de este ataque (Kaitera & Jalkanen, 1992; Solberg et al., 2006), produciendo un aumento de la defoliación. En algunos casos estos agentes bióticos son capaces de matar un porcentaje de elevado de pies, situación que se acrecienta en los casos de nuevas introducciones de plagas o patógenos, pudiendo causar la muerte de grandes extensiones de bosque, como ha sido el caso con *Phytophthora ramorum* o el nematodo de la madera del pino, *Bursaphelenchus xylophilus* (Brasier & Webber, 2010; Mota et al., 1999; Yokota, 1975). En este sentido, la globalización está produciendo un incremento en la introducción de nuevos patógenos (Anderson et al., 2004; Wingfield et al., 2015) a un ritmo que compromete la capacidad que tenemos de comprender su ecología para poder desarrollar estrategias de gestión adecuadas con el fin de combatir su expansión y minimizar los daños producidos, tanto a nivel ecológico como económico.

Estos organismos pueden causar daños en prácticamente todos los tejidos de un árbol, como raíces, tallos, hojas, yemas y flores (piñas en caso de los pinos). Los daños más comúnmente encontrados en diversas especies de pino en el norte de España son tratados en el Capítulo I de esta tesis (Sanz-Ros, Pajares & Diez, 2008).

No se hará referencia al elevado número de insectos y hongos patógenos que pueden atacar a pinares, los cuales pueden ser consultados en tratados de sanidad forestal (Smith, 1991; Muñoz et al., 2007), sino que esta tesis se centra más en los daños observados, en los métodos para su evaluación, en la identificación de la influencia de los factores abióticos de la estación y en el papel de los organismos que habitan los tejidos internos del árbol, y que pueden interaccionar con los organismos patógenos que ataquen al árbol. El hecho de que estos patógenos produzcan enfermedades se puede ver afectado por diversos factores, como son I) la variación de las condiciones ambientales, como el microclima y el suelo (Kaitera, 1997; Ranta & Neuvonen, 1994), II) la densidad, el patrón espacial, la etapa del desarrollo y la edad de la población del hospedante (Burdon & Chilvers, 1982; Burdon & Thrall, 1999), y III) las interacciones con otros organismos como vectores y antagonistas, muchos de los cuales son organismos endófitos (Ingvarsson & Ericson, 1998; Jalkanen & Laakso, 1986).

OTROS FACTORES BIÓTICOS: ORGANISMOS ENDÓFITOS

Los organismos endófitos son aquellos que habitan los tejidos vegetales, durante al menos parte de su ciclo vital, sin producir sintomatología ni daños al hospedante (Petrini, 1991; Wilson, 1995). Como su propio nombre indica endo- (dentro de) –phytos (planta) son habitantes habituales de los tejidos vegetales, y desde su descubrimiento por de Bary en 1866, ha sido descubierta una enorme diversidad de estos organismos en prácticamente todas las especies vegetales estudiadas. Principalmente son organismos fúngicos o bacterianos, siendo estos últimos menos frecuentes y principalmente localizados en tejidos de la raíz y el sistema vascular de plantas no leguminosas (Hallman, Quadt-Hallman, Mahaffee, & Kloepper, 1997; James & Olivares, 1997), aunque también han sido localizados en yemas de coníferas (Pirttilä, 2001).

Los endófitos fúngicos de especies herbáceas han sido ampliamente estudiados (Brosi et al., 2011; Clay, 2009; Faeth & Saari, 2012). En cuanto a su distribución en pinos, han sido encontrados en prácticamente todos los tejidos y órganos, entre ellos raíz, tallo, ramillos, hojas, yemas y piñas (Addy, Piercey, &

Currah, 2005; Ganley & Newcombe, 2006; Peršoh, Melcher, Flessa, & Rambold, 2010; Pirttilä, 2001; Zamora, Martínez-Ruiz, & Diez, 2008).

El estudio de los hongos endófitos ha sido abordado hasta ahora en muchos de los estudios, mediante cultivos "in vitro" en diferentes medios ricos en nutrientes, previa esterilización en superficie del material vegetal, con el fin de eliminar los epifitos y otros contaminantes presentes en el aire. Por este motivo se ha considerado como endófitos a algunos hongos que pudieran haber escapado de forma accidental a esta esterilización por encontrarse protegidos, por ejemplo, en la cavidad estomática (Schulz & Boyle, 2005). Otros métodos de estudio, como la PCR ambiental, mostraron una mayor diversidad sobre todo de hongos Basidiomicetes, aunque también se subestimó la presencia de Ascomicetos del orden Sordariomicetes (Arnold et al., 2007), por lo que no está claro cuál de ambos métodos puede representar mejor la comunidad endófita, aunque otros estudios muestran ambas técnicas como complementarias (Allen et al., 2003).

El papel desempeñado por los endófitos es, en la mayoría de casos, desconocido, de modo que hasta para las especies vegetales más comunes, aún permanece parcialmente desconocida su comunidad fúngica endofítica, y a día de hoy se siguen encontrando nuevas especies fúngicas, como se puede observar en el Capítulo IV de esta tesis (Sanz-Ros, Müller & Diez, 2015). Muchas especies consideradas como endófitas pueden ser simplemente parásitos tolerados por el hospedante, mientras que otras pueden ser patógenos latentes u oportunistas, o bien actuar como endófitos en un hospedante ser patógenos en otras especies vegetales (Carroll, 1988; Ganley, Brunsfeld, & Newcombe, 2004; Slippers & Wingfield, 2007). En este sentido, se considera que la planta controla los hongos que habitan en sus tejidos internos, habiéndose descrito esta relación como un delicado equilibrio, donde la planta no solamente tolera los metabolitos producidos por el endófito, sino que retarda su crecimiento sin llegar a erradicarlo (Schulz et al., 2002). Cuando este equilibrio se rompe, bien por un descenso en las defensas de la planta o por un incremento en la virulencia del hongo, se produce enfermedad.

Algunos estudios han demostrado que algunos de estos organismos pueden ser descomponedores primarios que simplemente están esperando la senescencia de los tejidos para comenzar su degradación (Müller, Valjakka, Suokko, & Hantula, 2001). Incluso algunos no ejercen ningún tipo de función en el hospedante, estando presentes simplemente como esporas no germinadas (Schulz & Boyle, 2005).

Sin embargo en otros casos sí que se atribuye a hongos endófitos diversas funciones biológicas que pueden ser determinantes en la historia vital de sus hospedantes. Algunos estudios muestran cómo ciertas especies endófitas pueden mejorar el estado de salud de su hospedante, por ejemplo haciendo que los herbívoros no los ramoneen mediante la producción de metabolitos tóxicos (Carroll, 1988; Miller, Sumarah, & Adams, 2008), o actuando como antagonistas ante organismos patógenos (Arnold et al., 2003; Webber, 1981) o bien produciendo una resistencia mediada por endófitos ante ciertos patógenos (Ganley, Sniezko, & Newcombe, 2008).

Por otro lado, una característica de los endófitos es que suelen ser capaces de producir compuestos activos biológicamente en pruebas realizadas para medir actividad fungicida, antibacteriana y herbicida (Krohn et al., 1992; Schulz et al., 2002). Esta característica los hace merecedores de ser objeto de estudio, ya que las posibles aplicaciones de estos compuestos son muy diversas, desde su uso como micofungicidas para el control biológico de otros patógenos e insectos, hasta su aplicación como biofertilizantes (Hallsworth & Magan, 1994; Santamarıía et al., 2007; Kaewchai, Soytong & Hyde, 2009; Prusky & Gullino, 2010).

En plantas no leñosas ha sido ampliamente descrita la capacidad de algunos hongos endófitos de incrementar el crecimiento de sus hospedantes o su tolerancia al estrés (Ernst, Mendgen, & Wirsel, 2003; Gasoni & De Gurfinkel, 1997; Mucciarelli, Scannerini, Bertea, & Maffei, 2003; Rademacher, 1994). En plantas leñosas, algunos ensayos de inoculación con endófitos han conseguido incrementar el crecimiento de su hospedante; la inoculación con cepas binucleadas de *Rhizoctonia* incrementó el crecimiento de plántulas de *Pinus sylvestris* y *Picea abies* (Gronberg, Kaparakis, & Sen, 2006), y la inoculación conjunta de *Trichodema harzianum* y *Laccaria laccata* produjo el mismo efecto en plántulas de *Pinus wallichiana*, además de un incremento en su biomasa. La relación de los hongos endófitos y el crecimiento de *Pinus sylvetsris* adultos se trata en el Capítulo IV de esta tesis (Sanz-Ros et al., 2015).

Algunas especies fúngicas y bacterianas asociadas a tejidos vegetales tienen la capacidad de producir reguladores del crecimiento vegetal (Rim et al., 2005; Hamayun et al., 2009; Ahmad et al., 2010; You, 2012; Kang et al., 2014). La producción de estos compuestos suele ser producida de forma conjunta por la planta y el organismo endófito. De *Populus trichocarpa* han sido aisladas algunas levaduras capaces de favorecer el crecimiento de plántulas mediante la producción de ácido indol acético (AIA), una hormona vegetal (auxina). Para producir esta auxina la levadura necesita L-triptófano, el cual es producido por el árbol, de modo que la levadura produce AIA mediante la ruta del indol-3-piruvato (Furukawa et al., 1996). Las especies con esta capacidad de síntesis resultan especialmente interesantes en cuanto a su potencial uso como biofertilizantes (Vessey, 2003; Kaewchai et al., 2009).

Por otro lado, los factores de sitio pueden influir sobre la distribución de organismos endófitos. La colonización de tejidos vegetales por hongos endófitos se puede ver influida por diversos factores, que podrían incluir casi cualquier componente del medio circundante. Los factores de sitio, incluyendo aquellos relacionados con el suelo, el clima, las características del rodal (dasometría) y el manejo forestal, podrían estar determinando la relación de los arboles con sus organismos endófitos. Además está descrito que la posición de un hongo endófito en un ecosistema y su respuesta ante los recursos y los enemigos (nicho) puede variar debido a que el organismo endófito continuamente afecta y es afectado por los factores bióticos y abióticos presentes (Saikkonen, 2007). Esta interacción entre los organismos endófitos y los factores del sitio donde crece el hospedador es tratada con más detalle en el Capítulo V de esta tesis.

INFLUENCIA DEL CAMBIO CLIMÁTICO EN LA CONDICIÓN DE COPA

Los registros históricos de temperaturas muestran un incremento en la temperatura media global de entre 0,4-0,8 ºC durante el siglo XX, el cual no puede ser atribuido a la variabilidad interna del sistema climático (Panel on Reconciling Temperature Observations, 2000; Parry, 2000). Sin embargo otros estudios muestran un calentamiento de entre 0,3-0,6 ºC desde el siglo XIX, el cual se atribuye a causas de tipo antropogénico (IPCC, 2001) o a causas d tipo astronómico

(Landscheidt, 2000; Soon et al., 2000). Otros indican a la década 1995-2006 como la más cálida nunca registrada (IPCC, 2007), aunque las tendencias de la temperatura global en superficie suelen ser calculadas usando periodos mucho más largos, los cuales muestran una variabilidad considerable, tanto entre diferentes años como entre décadas, habiéndose obtenido un incremento de 0,85 ºC en el periodo entre 1880 y 2012 (IPCC, 2013).

Este incremento de la temperatura global no estará homogéneamente distribuido en la superficie terrestre, sino que variará entre zonas, por lo que los modelos climáticos actualmente han predicho diferentes aumentos de la temperatura a diferentes escalas. Las proyecciones más recientemente elaboradas para el año 2100 en Europa muestran un incremento de entre 0,3-4,8 ºC (IPCC, 2013), mientras que modelos desarrollados anteriormente habían previsto un aumento de entre 1,5-3 ºC (Kattenberg et al., 1996), o entre 1-3,5 ºC para regiones de latitudes medias (Watson et al., 1997). Para la Península Ibérica, los modelos indican un aumento de la temperatura media de alrededor de 1,6 ºC durante los últimos 100 años, con aumentos algo mayores en verano (unos 2 ºC) y algo menores durante el invierno (Hulme & Sheard, 1999; Parry, 2000). Estos aumentos también se ven reflejados en los valores extremos, que dependerán de las condiciones locales, mostrando tendencias significativas en algunas regiones del globo pero no en otras, donde no han sido previstos cambios significativos (De Gaetano, 1996; Heino et al., 1999; Bonsal et al., 2001).

En cuanto a las precipitaciones, algunos modelos predicen un descenso generalizado en el sur de Europa hacia el Mediterráneo (Piervitali et al., 1997; Schönwiese & Rapp, 1997; Buffoni et al., 1999; Brunetti, Maugeri & Nanni, 2000, 2001; Sarris et al., 2007), al igual que lo previsto para la Península Ibérica. Dicha disminución será más intensa durante el verano, mientras que los inviernos tenderán a ser más húmedos (Karas, 1997; Esteban-Parra et al., 1998; Hulme & Sheard, 1999; Parry, 2000; IPCC, 2001; Mossman, 2002). En lo referente a las precipitaciones, al igual que ocurre con las temperaturas, el cambio climático no va a afectar del mismo modo a todos los lugares ni lo va a hacer tampoco a diferentes escalas. A nivel global se prevé un incremento de la variabilidad en la precipitación (Dore, 2005).

El cambio climático está produciendo diversos efectos en la vegetación natural, principalmente en los bosques, en los cuales está desencadenando fenómenos como el desplazamiento de los límites de las especies o el cambio en los patrones de biodiversidad actuales (Araújo & Rahbek, 2006). En este sentido, en Europa se está produciendo un desplazamiento hacia el norte de ciertos sistemas Mediterráneos, que actualmente están reconocidos como puntos calientes de biodiversidad (Myers et al., 2000), y que están yendo hacia la Región Eurosiberiana, que resulta comparativamente mucho más pobre en riqueza de especies (Thuiller et al., 2005).

El desplazamiento de los límites de una especie implica la colonización de zonas nuevas, lo cual conlleva superar una serie de factores limitantes: I) en la zona a colonizar ya hay una vegetación establecida, con la que tendrá que competir y II) la distancia hasta la nueva zona climáticamente adecuada puede ser más o menos elevada, y podría suponer el desplazamiento a través de áreas cuyas condiciones ambientales podrían no ser favorables para la espacie colonizadora. Todo ello aumenta el riesgo de fracaso en este proceso de colonización y por lo tanto, según algunos modelos predictivos, aumenta el riesgo de pérdida de especies. Entre las coníferas, *Abies alba* es la que mayor riesgo corre de desaparecer, ya que se predice una pérdida del 99% del área cuyas condiciones serían adecuadas para esta especie, mientras que otras especies se pueden ver favorecidas, como es el caso de *Pinus halepensis*, cuyo área potencial puede aumentar entre 300-450% (Ohlemüller et al., 2006).

Por otro lado, el cambio global afectará al estado sanitario de los bosques. Como se ha descrito anteriormente, todas las previsiones apuntan hacia un incremento de las temperaturas y hacia cambios en los regímenes de precipitaciones, los cuales podrían ejercer efectos diferentes sobre los distintos tipos de vegetación, por ejemplo sobre angiospermas y coníferas (gimnospermas). Algunos estudios prevén que las angiospermas podrían aumentar su crecimiento a causa del incremento de las temperaturas, mientras que el efecto sobre coníferas puede ser nulo o negativo (Gómez-Aparicio et al., 2011; Carnicer et al., 2013). El efecto de variables climáticas sobre el estado sanitario de masas de pino de diferentes especies es tratado en el Capítulo II de esta tesis (Sanz-Ros, Pajares, & Diez, 2008), donde también se discuten escenarios de cambio climático.

Además el calentamiento global y el cambio en los regímenes de precipitaciones también afectará a otros componentes del ecosistema, como son los herbívoros y los patógenos, de diversas formas: I) mediante el efecto directo sobre la supervivencia de herbívoros y patógenos, II) mediante cambios fisiológicos en las defensas del árbol y III) mediante efectos indirectos debidos a la abundancia de enemigos naturales, como parasitoides, mutualistas, vectores de patógenos y competidores (Ayres & Lombardero, 2000).

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OBJECTIVES OF THE THESIS

Below there are listed the objectives of the thesis, and after each objective there is one or more roman numbers between parentheses, which indicate the chapter of this thesis in which this objective is addressed.

1. To indentify which biotic and abiotic factors are influencing forest health status of pine forests in northern Spain (**I, II, III, IV, and V**).

2. To assess the more representative damages in pine stands and their qualitative and quantitative comparison among *Pinus nigra*, *P. sylvestris* and *P. pinaster* in northern Spain. In addition, to find out if the use of this information can help to decide species suitability for future reforestations in the area (**I**).

3. To find relationships between canopy condition and some climatic parameters to identify those having a main influence on canopy condition. In addition: To contribute to the understanding of how these climatic parameters will affect under future climate change scenarios (**II**).

4. To identify the site factors affecting canopy condition in declining *Pinus halepensis* stands located in north-western Spain (**III**).

5. To characterize the endophytic community of *Pinus sylvestris* twigs, and to find out if the structure of this community is related to the growth rate of Scots pine (**IV**).

6. To identify site factors influencing the fungal endophytic community of *Pinus sylvestris* twigs, including climate, soil and stand characteristics (**V**).

OBJETIVOS DE LA TESIS

A continuación se enumeran los objetivos de la tesis y se muestra, al final de cada objetivo un número romano que indica el capítulo en el que se aborda ese objetivo.

1. Identificar los factores bióticos y abióticos que influyen en el estado sanitario de las masas de pino del norte de España (**I, II, III, IV** y **V**).

2. Evaluar los daños más representativos y su comparación, tanto cualitativa como cuantitativamente, entre parcelas de *P. nigra, P. sylvestris* y *P. pinaster* en la zona de Páramos y valles, Palencia, España. Objetivo secundario: Averiguar si el uso de esta información podría resultar útil como criterio que ayude a la decisión del uso de estas especies en futuras repoblaciones forestales en la zona (**I**).

3. Explorar las relaciones entre la condición de copa y los parámetros climáticos a largo plazo para identificar cuáles de ellos tiene una mayor influencia. Objetivo secundario, contribuir al conocimiento de cómo estos parámetros influirán bajo escenarios futuros de cambio climático (**II**).

4. Identificar los factores de sitio que afectan a la condición de copa en rodales de pino carrasco (*Pinus halepensis* Mill.) en decaimiento (**III**).

5. Caracterizar la comunidad fúngica endófita de ramillos de pino albar (*Pinus sylvestris* L.), y, determinar si la estructura de esta comunidad está relacionada con la tasa de crecimiento de *P. sylvestris* (**IV**).

6. Estudiar la influencia de los factores de sitio, incluyendo parámetros climáticos, edáficos y dasométricos (del rodal), en la distribución de organismos endófitos de *P. sylvetsris* (**V**).

Capítulo I

(“No esperes hasta que las condiciones sean perfectas para comenzar,
el empezar hace las condiciones perfectas “)

(Alan Cohen)

COMPARACIÓN DE DAÑOS FORESTALES ENTRE ESPECIES DE PINO EN REPOBLACIONES DEL NORTE DE ESPAÑA[♣]

RESUMEN

La sostenibilidad de la gestión implica la preservación de los ecosistemas forestales y con ello el correcto mantenimiento de su estado sanitario. Las masas deben ser tan vigorosas como sea posible, siendo así más tolerantes ante ataques de insectos, hongos y otros agentes. Se estudia en este trabajo la influencia de la especie arbórea principal en la frecuencia y tipos de daños encontrados en plantaciones de coníferas del género *Pinus*. El área de estudio está situada en la comarca de Páramos y Valles, Palencia (España), tiene 186.642 ha, clima mediterráneo con influencia atlántica, precipitación media anual de 519 mm y temperatura anual media de 11,49 ºC. Para este estudio fueron muestreadas 37 parcelas de *P. sylvestris*, 22 de *P. nigra* y 9 de *P. pinaster*, obtenidas al azar de una malla sistemática de 2x2 km superpuesta a la del Inventario Forestal Nacional (IFN). Los daños encontrados difirieron entre las tres especies de pino estudiadas (*P. sylvestris*, *P. nigra* y *P. pinaster*), al menos en el porcentaje de cada tipo de daño observado. Los daños más importantes encontrados afectaron al dosel, como son defoliación y decoloración, o a zonas de crecimiento, como la muerte del meristemo apical. Debido a la vocación productivista de estas masas, las bifurcaciones y malformaciones del tronco fueron consideradas perjudiciales, sobre todo teniendo en cuenta el elevado porcentaje de árboles que los presentan. Al agrupar las parcelas en aquellas que tienen tratamientos selvícolas previos y aquellas que no, se observó que las parcelas con tratamientos selvícolas previos presentaban niveles menores de defoliación que las que no habían sido intervenidas.

[♣] Sanz-Ros, A.V., Pajares, J.A., Diez Casero, J.J. (2008). Comparación de daños forestales. *Cuad. Soc. Esp. Cienc. For.* ***26****: 91-96.* Actas de la I Reunión sobre Sanidad Forestal. Sociedad Española de Ciencias Forestales.

INTRODUCCIÓN

La sostenibilidad de la gestión implica la preservación de los ecosistemas forestales y con ello el correcto mantenimiento de su estado sanitario. Las masas deben ser tan vigorosas como sea posible, siendo así más tolerantes ante ataques de insectos, hongos y otros agentes. Se estudia en este trabajo la influencia de la especie arbórea principal en la frecuencia y tipos de daños encontrados en plantaciones de coníferas del género *Pinus*. El área de estudio está situada en la comarca de Páramos y Valles, Palencia (España), tiene 186.642 ha, clima mediterráneo con influencia atlántica, precipitación media anual de 519 mm y temperatura anual media de 11,49 ºC. Para este estudio fueron muestreadas 37 parcelas de *P. sylvestris*, 22 de *P. nigra* y 9 de *P. pinaster*, obtenidas al azar de una malla sistemática de 2x2 km superpuesta a la del Inventario Forestal Nacional (IFN). Los daños encontrados difirieron entre las tres especies de pino estudiadas (*P. sylvestris*, *P. nigra* y *P. pinaster*), al menos en el porcentaje de cada tipo de daño observado. Los daños más importantes encontrados afectaron al dosel, como son defoliación y decoloración, o a zonas de crecimiento, como la muerte del meristemo apical. Debido a la vocación productivista de estas masas, las bifurcaciones y malformaciones del tronco fueron consideradas perjudiciales, sobre todo teniendo en cuenta el elevado porcentaje de árboles que los presentan. Al agrupar las parcelas en aquellas que tienen tratamientos selvícolas previos y aquellas que no, se observó que las parcelas con tratamientos selvícolas previos presentaban niveles menores de defoliación que las que no habían sido intervenidas. La sostenibilidad de la gestión implica la preservación de los ecosistemas forestales y con ello el correcto mantenimiento de su estado sanitario. Las masas deben ser tan vigorosas como sea posible, resultando así más tolerantes ante ataques de insectos, hongos y otros agentes (SPCAN–DGCN, 2002)., evitando de este modo su debilitamiento y decaimiento. Desde 1986 se lleva a cabo el Programa Europeo para el Monitoreo Intensivo de Ecosistemas Forestales, Nivel I (EU-ICP Forests, 1998), en el cual se realiza una evaluación del estado sanitario a través de la evaluación visual del estado de la copa basada principalmente en la medida de la defoliación y decoloración (UN-ECE, 1994), así como otros síntomas que puedan aparecer, tales como chancros, microfilia, muerte del meristemo apical, bifurcaciones y deformaciones del tronco. En la década de los 70 se llevaron a cabo políticas de repoblación forestal en las cuales se usaron diversas especies de pino como pioneras, con el fin de favorecer la evolución del suelo, siendo la vocación de estas repoblaciones principalmente productivista, aunque las que caen dentro de zonas protegidas puedan tener otras vocaciones, como pueden ser la conservacionista o simplemente como regulación hidrológica o protección contra la erosión. Dependiendo de la composición de especies de pino usadas, que en la zona de estudio son básicamente *Pinus sylvestris*, *P. nigra* y *P. pinaster*, son encontrados en las evaluaciones diferentes tipos y porcentajes de daño, que pueden ser atribuidos a diversos y numerosos factores, de origen biótico, abiótico o antrópico, que determinan el estado sanitario actual de las masas. Este trabajo fue realizado con el objetivo de comparar los daños encontrados, tanto cualitativa como cuantitativamente, en parcelas del Inventario Forestal Nacional cubiertas por tres especies del género *Pinus* (*P. sylvestris*, *P. nigra*, *P. pinaster*).

Los objetivos de este estudio fueron:

1) Evaluar los daños forestales encontrados, su comparación, tanto cualitativa como cuantitativamente, entre parcelas de *P. nigra*, *P. sylvestris* y *P. pinaster* en la zona de Paramos y valles, Palencia, España. 2) Averiguar si el uso de esta información podría resultar útil como criterio que ayude a la decisión del uso de estas especies en futuras repoblaciones forestales en la zona.

MATERIALES Y MÉTODOS

Para este estudio 68 parcelas fueron muestreadas, elegidas de una malla sistemática de 2x2 Km superpuesta a la malla del Inventario Forestal Nacional intentando reflejar la abundancia de especies en la zona, de modo que en 37 de ellas la especie dominante era *Pinus sylvestris*, en 22 *P. nigra* y en 9 *P. pinaster*). El área de estudio, denominada “Páramos y Valles”, está situada en la Provincia de Palencia (Castilla y León, España), y constituye una transición entre tierras agrícolas hacia el sur y la Montaña Palentina hacia el norte (Figura 1). Este área tiene una extensión 186.642 ha, clima mediterráneo, aunque con cierta influencia atlántica, cuya precipitación anual media es de 519 mm y su temperatura anual media de 11,5ºC. El dispositivo de muestreo utilizado consta de cuatro subparcelas circulares de 17,5 metros de radio, perpendiculares dos a dos y unidas entre si por dos transectos de 50 metros. De ellas, una es fija, la correspondiente a la parcela del Inventario

Forestal Nacional, y los otros 3 satélites pueden rotar con el fin de evitar caminos o cortafuegos donde la cubierta vegetal presenta discontinuidades (Figura 2). Dentro de cada subparcela fueron evaluados los 20 árboles mas cercanos al centro, de modo que el número total de árboles evaluados fue de 5.520. La metodología de evaluación de daños se basó en la utilizada en la Red Europea de Seguimiento de Daños a los Bosques (ICP Forests, 1998), y fue realizada entre el 15 de Julio y el 15 de septiembre del 2005.

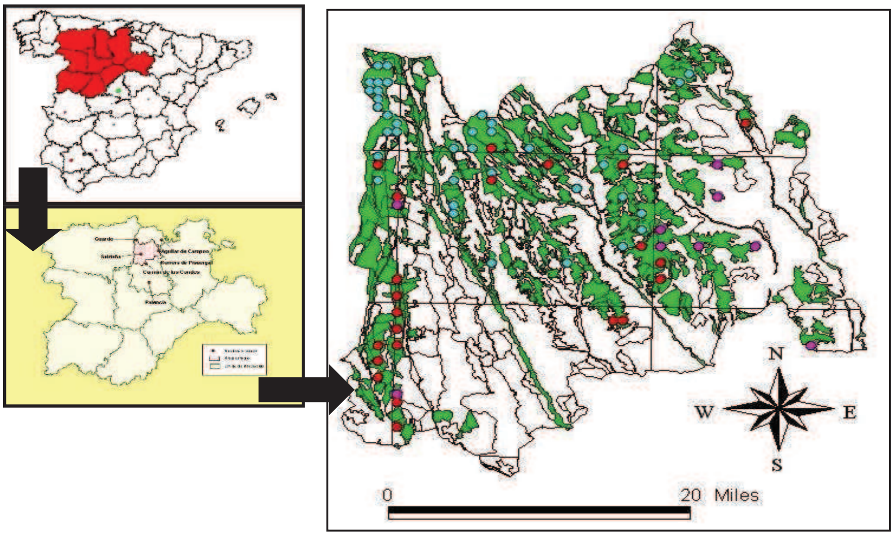

Figura 1. Localización de la Zona Piloto. La superficie verde se corresponde con la superficie con cubierta forestal arbolada. Los círculos representan parcelas de *Pinus. nigra*, los de *P. sylvestris* y los de *P. pinaster.*

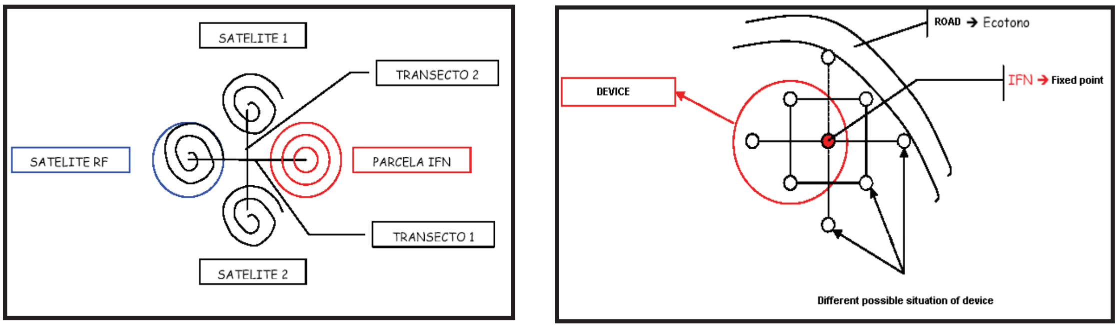

Figura 2. Dispositivo de muestreo utilizado en este trabajo, donde se muestra en rojo la parcela del IFN (Izquierda). Rotación del dispositivo con el fin de evitar caminos o cortafuegos (Derecha).

RESULTADOS

Los resultados obtenidos mostraron que los daños más importantes encontrados afectaban al dosel, como son defoliación y decoloración, o a zonas de crecimiento, como la muerte del meristemo apical. A pesar de ello, bifurcaciones y malformaciones del tallo resultaron ser los más numerosos (Figura 3).

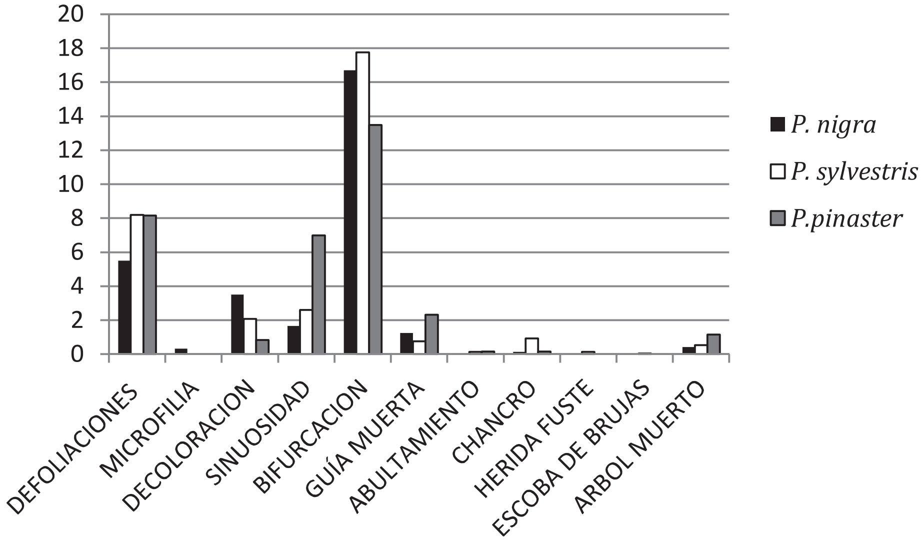

Figura 3. Comparación de frecuencia de aparición de los distintos síntomas para cada especie respecto del total de árboles.

Las estimaciones de la defoliación mostraron valores medios del 19,6% para pino silvestre (*P. sylvestris*), 22,9% para pino laricio (*P. nigra*) y 25,8% para el pino resinero (*P. pinaster*). Al agrupar las parcelas por especies quedó de manifiesto que en las masas de *P. nigra* estudiadas los daños más importantes son la defoliación y decoloración, además de ser la especie que mas porcentaje de bifurcaciones y decoloraciones presentó y en la única en la que se detectó microfilia. En las de *P. pinaster* los daños más significativos son la defoliación y la muerte del meristemo apical, siendo esta especie la que mas malformaciones del tronco, mayor número de guías muertas y mayores valores de defoliación mostró. En cuanto a las parcelas de *P. sylvestris*, los daños mas destacables fueron la defoliación y la decoloración, encontrándose en esta especie el mayor número de chancros en el tronco (Figura 4). Al agrupar las parcelas en aquellas que tienen tratamientos selvícolas previos y aquellas que no, se observó que las parcelas tratadas presentaban niveles menores de defoliación que las que no habían sido intervenidas, de modo que estos tratamientos selvícolas favorecieron la condición de copa. Se observa que el órgano más afectado en todas las especies, con valores similares que oscilan entre el 18,5–20,5% de los pies, fue el tronco (Figura 5, izquierda) y seguido del dosel (9–10,3% de los pies). En cuanto al porcentaje de pies que poseen dos o más síntomas, se observó que los valores obtenidos fue ron muy similares, variando entre un 4-5% de los pies totales (Figura 5, derecha), aunque se encontraron diferencias entre especies en cuanto al porcentaje que estos representaban cuando los valores se mostraban respecto del número de árboles sintomáticos.

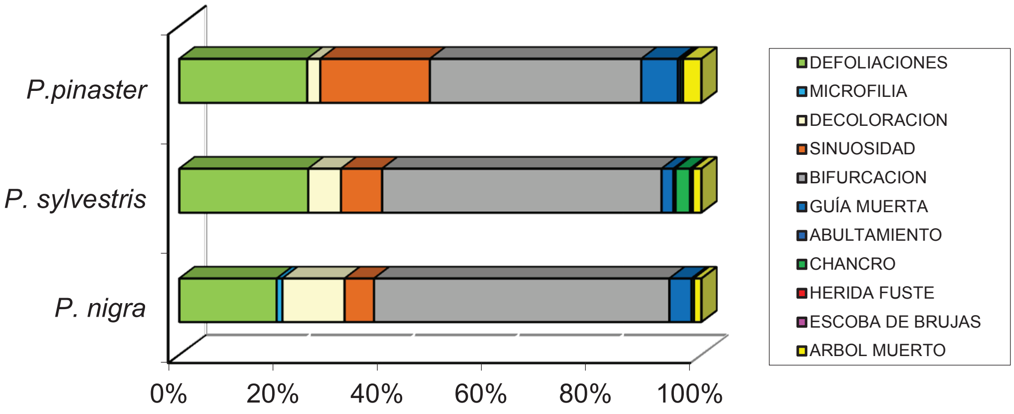

Figura 4: Porcentaje de tipos de daño observados para cada una de las tres especies principales respecto del número de árboles sintomáticos.

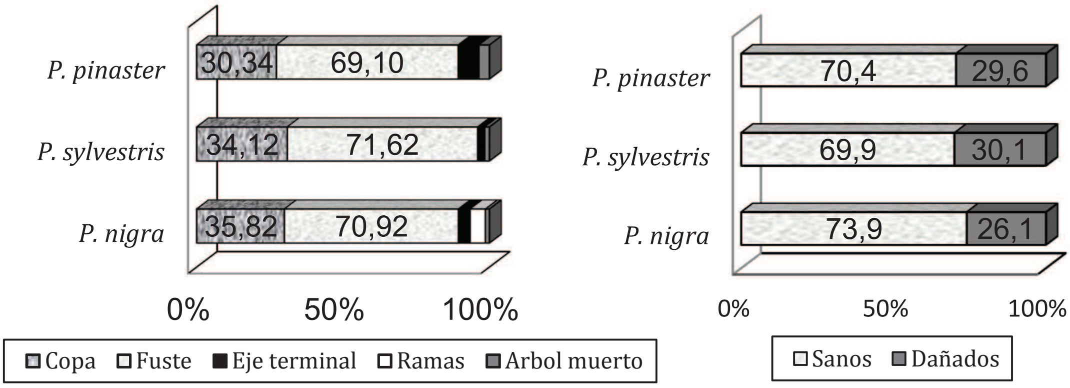

Figura 5: Porcentaje de órganos afectados respecto del número de árboles sintomáticos (Izquierda). Porcentaje de árboles dañados respecto del total de árboles evaluados (Derecha).

DISCUSIÓN

Los síntomas más representados en la presente evaluación fueron las bifurcaciones, defoliaciones, malformaciones del tronco y decoloraciones respectivamente. Al igual que en los resultados obtenidos por la Red Europea de Seguimiento de Daños a los Bosques (ICP Forests, 1998), se identifican principalmente defoliaciones y decoloraciones, como comunes a todas las especies evaluadas, por lo que éstos son usados como indicadores de la condición y el estado sanitario de las masas forestales. En cuanto a la intensidad de la defoliación, los valores medios de defoliación encontrados sitúan las masas de *P. sylvestris* y *P. nigra* en clases de defoliación leve, mientras que las masas de *P. pinaster* entrarían ya en la clase de defoliación moderada (25-60%). Además esta especie es la que más árboles muertos mostró, aunque esto puede ser debido a una selvicultura menos intensiva que la practicada para otras especies (Serrada, Montero & Reque, 2008). Respecto de la frecuencia de aparición de la defoliación, aunque su intensidad sea leve, el porcentaje de árboles con defoliación resultó bastante mas alto en pino silvestre y resinero que en el salgareño, probablemente debido a una peor adaptación de estas especies al terreno y la pluviosidad de la zona, que podría resultar mas favorable para *P. nigra* que para las otras dos. En cuanto al porcentaje de árboles con una decoloración identificable resultó bastante mayor en *P. nigra* que en *P. sylvestris*, coincidiendo este dato con otros estudios en los que se han evaluado ambas especies (Navarro et *al*., 2007), aunque en esta zona el porcentaje de decoloraciones es mucho menor debido a la mayor pluviosidad de esta zona de estudio. El hecho de que el órgano mas afectado en todas las especies sea el tronco es debido al elevado número de bifurcaciones y malformaciones del tronco, lo cual puede tener efectos económicos importantes en el futuro, aunque no hay evidencia de que ello represente algún problema de tipo fisiológico para el árbol o para su desarrollo. Sin embargo, debido a la vocación productivista de gran parte de estas masas, estas bifurcaciones y malformaciones del tronco podrían ser consideradas como síntomas perjudiciales, sobre todo teniendo en cuenta el elevado porcentaje de árboles que los presentan (13-18% pies, dependiendo de la especie), aunque no está claro hasta qué punto influyen éstos al funcionamiento fisiológico del árbol. El porcentaje de árboles con varios daños resultó similar entre las diferentes especies, aunque es ligeramente mayor en *P. nigra* debido al mayor número de bifurcaciones del tronco observadas. Existen estudios (Robredo, 1975, 1978) que revelan ataques graves por *Rhyacionia buoliana* en esta zona durante los años 70, posiblemente debido a la presencia de muchas masas jóvenes y coetáneas susceptibles de ser atacadas por este insecto. Este ataca al meristemo apical del árbol, de modo que al ser dañado suele ser sustituido por dos yemas situadas por debajo, produciendo bifurcaciones en el tronco.

Los resultados de este estudio mostraron que, de las tres especies que cubren la mayor parte de la superficie forestal arbolada del área de estudio, no se podría decidir cual de ellas es mas adecuada para la zona en base a la presencia de daños, ya que cada una presentó un tipo y porcentaje diferente de daños. Evidentemente hay que tener en cuenta otros factores para la elección de la especie a emplear, como el suelo y el clima, pero si atendemos solamente a las variables que se usan como indicadores de salud forestal (defoliación y decoloración) se observa que, el pino silvestre presentó una baja intensidad y frecuencia de defoliación, así como una muy baja aparición de decoloraciones. Los valores obtenidos en pino salgareño (*P. nigra* Arnold) son igualmente bajos en cuanto a intensidad de la defoliación, aunque el porcentaje de árboles defoliados resultó menor. Sin embargo, en cuanto a decoloraciones esta especie presentó un mayor porcentaje que el silvestre (aunque el número de árboles decolorados en general es muchísimo menor que el de los defoliados, por lo que este factor pesaría menos). En cuanto al pino resinero, presentó los valores mas altos de intensidad y frecuencia de defoliación, mostrando, sin embargo los más bajos en cuanto a decoloración.

Atendiendo al resto de daños se observó que cada especie mostró una serie de particularidades. El pino salgareño mostró un altísimo porcentaje de bifurcaciones, así como microfilia, daño que solo fue encontrado en ésta. El pino silvestre mostró una presencia mayor de chancros en el tronco. Por último el pino resinero mostró el mayor porcentaje de muertes del meristemo apical principal y malformaciones en el tronco, debidas al desarrollo en estadíos jóvenes y al ratio altura/densidad de pies (DEL RÍO et *al*., 2004).

CONCLUSIONES

1.- La especie usada en la repoblación influyó claramente en el tipo y porcentaje de daños estudiados, caracterizándose cada una por unos porcentajes de daño determinados, y resultando algunos tipos de daño encontrados solamente en ciertas especies.

2.- La defoliación es el síntoma mas importante encontrado en todas las especies, y sus valores medios difirieron entre las especies estudiadas, siendo mayores los encontrados en *Pinus pinaster* que en las otras dos. En esta especie, además fue detectado el mayor porcentaje de malformaciones en el tronco.

3.- En *P. sylvestris se detectó* una presencia muy superior de chancros en el tronco. Además, abultamientos en el mismo y escobas de bruja fueron detectadas, aunque en bajo número, solamente en parcelas de esta especie.

4.- *Pinus nigra* fue la especie que mayor número de bifurcaciones y decoloraciones presentó, observándose además microfilia únicamente como síntoma en esta especie.

5.- La comparación de daños no reveló datos suficientes para ayudar en la decisión de uso de especies a nivel de gestión forestal, al menos entre *P. sylvestris* y *P. nigra*, aunque sí mostró un estado sanitario mas deteriorado en parcelas de *P. pinaster* en esta zona.

AGRADECIMIENTOS

El presente trabajo ha sido cofinanciado por la Unión Europea y la Junta de Castilla y León, dentro del programa INTERREG IIIB Espacio Atlántico, proyecto FORSEE. Deseamos agradecer a todos los miembros del proyecto FORSEE su colaboración y su apoyo, y a Christophe en especial por su confianza y por la buena dirección e integración de dicho proyecto. A Celia Herrero por la ayuda con el trabajo de campo y a Jorge Martín por su asesoramiento.

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Chapter II

(“Procuremos más ser padres de nuestro porvenir que hijos de nuestro pasado)

(Miguel de Unamuno)

INFLUENCE OF CLIMATIC VARIABLES ON CANOPY CONDITION IN PINE FORESTS OF NORTHERN SPAIN [♣]

INTRODUCTION

Climate Change over the last century has created concern to the scientific community, as it could have a major impact on natural and social systems at local, regional and national scales. Greenhouse gasses emission has been modifying global climate, affecting ecosystems in very diverse ways (FAO, 2012). Current mitigation policies derived from Kyoto Protocol are following two main strategies: reduction of gas emissions, and implementation of a sustainable development assuring persistence of greenhouse carbon sinks, including forests lands.

Some models proposed that terrestrial ecosystems together with bioenergy systems, including capturing and storing carbon, may even neutralize unsustainable historical carbon emissions in the course of a century (Obersteiner et al, 2001), although the fifth assessment report of IPCC stated that the effects of climate change will remain for centuries, even if we stop emitting greenhouse gasses now (IPCC, 2013). Sustainable forest management is an essential tool to assure the permanence of our forests and to maintain properly their ecological functioning. They can offer a permanent carbon sink by the technological option of capturing carbon from biomass conversion facilities (Kraxner, F., Nilson, S., Obersteiner, M., 2003). The role of forest as a CO_2 sink could be influenced by the occurrence of forest pests and diseases causing tree defoliation and canopy reduction. In this sense climate change effects can increase the frequency of insect outbreaks due to drought and warming (Allen et al., 2010). Climatic factors could influence crown condition, question that is tried to answer with this work.

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Canopy condition is closely related to forest condition, and also, the contribution of each individual tree to CO2 sequestration depends on its crown development. Crown condition can be evaluated though the estimation of defoliation, and visual assessment of defoliation became accepted as the standard method for large scale intensive monitoring of forest condition in Europe, and it has been systematically assessed since 1986 throughout the whole Europe (EC and UN/ECE, 2000). There have been some criticism about the use of this indicator, but the consistency of this evaluation along time has been recently demonstrated (Eickenscheidt & Wellbrock, 2014).

The term defoliation is defined as the defoliative effects of biological agents, premature needle loss or reduction in the needle holding period (Ferretti, 1994). This parameter does not takes into consideration factors like unusual reduction of leaf size, presence of flowers and cones, branching deformation or shoot death (Ferretti, 1994). Estimation of defoliation, described by Innes (1990), and harmonized (Innes, 1993) using guidelines proposed by ICP Forests (1992), has been widely used as an indicator of the vitality of forest trees and of the degree of damage (Zierl, 2002). There are several causes of premature needle loss, sometimes they are well known (pests and diseases), but in many occasions they are far from clear, ranging from environmental stress (Zierl, 2004), such as low availability of water (drought) or extreme values of temperature, to other variables related to the management or disturbance events. Drought is a major factor in forest decline, making tree more vulnerable to fungi and pest attacks (Wellburn, 1994; Klap et al., 2000). In Mediterranean climate, growth of forest trees is subjected to many climatic constrains, particularly the availability of water (Gracia, et al., 1999).

It is known that some climatic factors can influence crown condition, but it is not known how this influence is, and which climatic parameters are the most determining for each region. It is expected that variation of the climatic factors would be different among the diverse regions in future climate change scenarios, as showed by Andreu et al., 2007, so it is needed to consider climate trends obtained by several surveys at different scales.

Some studies indicate that rainfall would have a general decrease in south Europe to the Mediterranean (Piervitali, Colacino, & Conte, 1997; Schönwiese and

Rapp, 1997; Buffoni, Maugeri & Nanni, 1999; Brunetti, Maugeri & Nanni, 2000; 2001; Sarris, Christodoulakis & Körner, 2007). It seems that, in Spain, annual rainfall shows a trend towards a decrease over the whole Iberian Peninsula, the greatest decreases occurring in summer, while the winters will become wetter (Karas, 1997; Esteban-Parra, Rodrigo & Castro-Díez, 1998; Hulme and Sheard, 1999; Parry, 2000; IPCC, 2001; Mossman, 2002). In any case, some review showed an increased variability of precipitation everywhere (Dore, 2005).

Temperature records show an increase in the global mean temperature between 0.4 and 0.8 ºC along the 20th century that cannot be attributed to the internal variability of the climate system (Panel on Reconciling Temperature Observations, 2000; Parry, 2000). Other studies showed a global warming rate of 0.3 - 0.6 ºC since the 19th century, due to either anthropogenic (IPCC, 2001) or to astronomic causes (Landscheidt, 2000; Soon et al., 2000). Some studies pointed that the decade 1995-2006 was the warmest record ever registered (IPCC, 2007), but global mean surface temperature trends are calculated using much longer periods, since it exhibits substantial decadal and interannual variability, showing an increase of 0.85 ºC over the period between 1880-2012 (IPCC, 2013).

This increase in the global temperature is not homogeneously distributed on the Earth surface, varying among the different regions and locations. According to this, climate models currently have predicted a temperature increase at different scales. In Europe, projections for year 2100 have shown an increase of 0.3 - 4.8 ºC (IPCC, 2013), whereas previous models have forecasted approximately an increase of 1.5 - 3 ºC (Kattenberg, 1996), or between 1 - 3.5 ºC for mid-latitude regions (Watson et al., 1997). For the Iberian Peninsula, results have indicated an increase in the annual mean temperature of about 1.6 ºC over the last hundred years, with highest increases in summer (approximately 2ºC) and the lowest in winter (Hulme and Sheard, 1999; Parry, 2000). This change is also reflected in the behavior of the extreme values, which depends on local conditions, showing significant trends in some regions of the globe but not in others, where no significant changes were detected (De Gaetano, 1996; Heino et al., 1999; Bonsal et al., 2001).

In some respects, these climate changes are likely to act as an important driving force on natural systems (Parmesan & Yohe, 2003). The increase of temperature along the next 100 years would be equivalent to a pole ward shift of the present geographic isotherms of approximately 150-155 km (Watson et al., 1997), causing changes in forest tree species distribution and limits. Risk of pests and diseases will be increased due to these limits displacement, so that many forested surface will be placed in a stressing environment. In this scenario, tree vigour of these species will decrease, leading to canopy decline manifested in symptoms as defoliation and discoloration.

The aim of this study was to find relationships between crown condition and some climatic parameters to identify which are those having a main influence on crown condition, and how this influence is shown in the tree (defoliation), and to contribute to the understanding of how these parameters will affect under future climate change scenarios.

MATERIALS AND METHODS

In this study, 68 National Forest Inventory (NFI) plots were sampled from July to mid September of 2005. All plots were placed in a pilot zone in Palencia province (northwest of Spain, Figure 1), and were covered by three Pinus species (37 by *P. sylvestris*, 22 by *P. nigra*, and 9 by *P. pinaster*). Most of the plots were pine plantations, in some cases mixed with different oak and pine species.

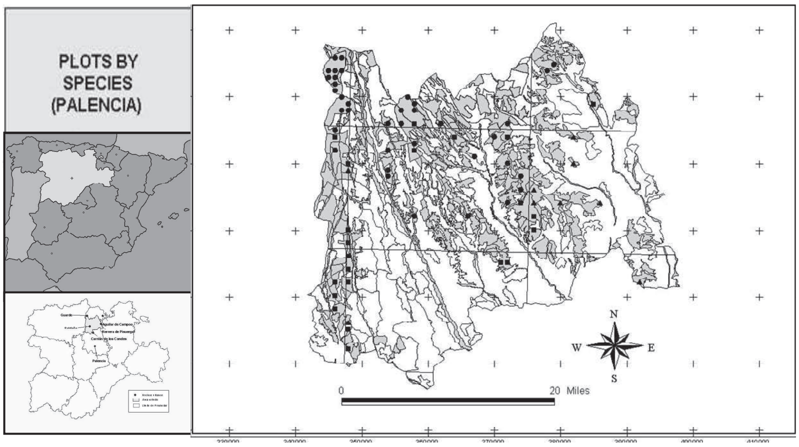


Figure 1: Distribution of plots in pilot zone in Palencia Province, Castilla y León, Spain. Plots were taken from a 2 km grid on tree covered area. *Pinus sylvestris* (●), *Pinus pinaster* (▲), and *P. nigra* (■).Gray surface is forest covered area.

This area is transitional between agricultural lands (southwards) and Cantabrian Mountains (northwards), and extends for 186.642 ha, 60.000 of them forested, showing enough climatic variations to study the influence of climatic factors in crown condition. This pilot zone is located between UTM coordinates 342.000, 4.685.000, and 398.000, 4.741.000, ranging in altitude from 800 to 1000 m.a.s.l. (Figure 1). The climate is Mediterranean with a slight Atlantic influence, with a mean temperature of 11.49 ºC and an annual rainfall of 519 mm.

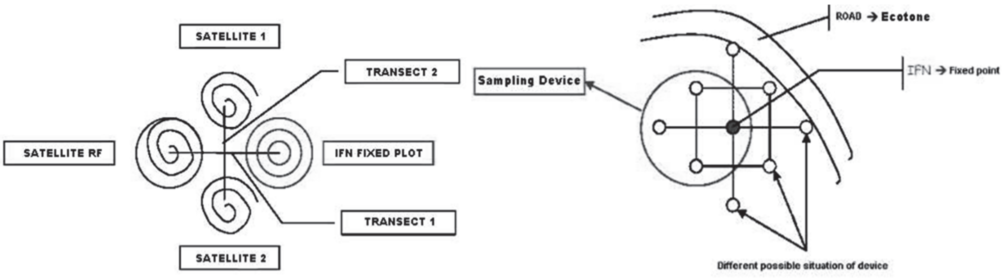


Figure 2: Sampling method with four subplots and two linear transects linking them (Left). IFN plot is a fixed plot, original transect orientation was N-S and E-W, but it was able to be rotated in order to avoid roads or firewalls (Right).

Sampling method involved four subplots (Figure 2). One fixed subplot of 25 m radius (National Forest Inventory plot) and three subplot of 17.5 m radius, linked by two orthogonal linear transects of 50 m. In each subplot, the 20 nearest trees in a spiral pattern were evaluated. Defoliation was estimated visually in the field according to the European Programme for the Intensive Monitoring of Forest Ecosystems, Level I (ICP forests, 1992). The visual estimation of the amount of light passing through the tree live crown was evaluated in 5% intervals and compared with a reference tree with complete foliage. Reference pictures of defoliation degrees for each species in Mediterranean areas were used in this comparison (Cadahia et al., 1991; Ferretti, 1994). The assessment was done by two different evaluators at a distance equal to tree height and avoiding to face the sun.

Data showed for plot defoliation for each plot were means of 20 evaluated trees. The establishment of one subplot in a road or firewall, where an edge effect is likely, was avoided by subplot rotation. However some other surveys have showed that there were no differences in defoliation between inside stand trees and edge trees (Durrant & Boswell, 2002).

Table 1: Likely predictor climatic variables used to find correlations with plot defoliation.

	Annual	December	January	February	June	July	August
Mean Temperature	x	x	x	x	x	x	x
Maximum Temperature	x	x	x	x	x	x	x
Minimum Temperature	x	x	x	x			
Rainfall	x	x	x	x	x	x	x
Solar Radiation	x						

Climatic long-term data for each plot were obtained from the Digital Climatic Atlas of Iberian Peninsula (Ninyerola, M., Pons, X. y Roure, J.M., 2005), a climatic model in which it is used data from all the meteorological stations from pilot zone, 15 of them within and other 31 in nearby areas. Rainfall values are refereed to means of the last twenty years, and temperatures to means the last fifteen previous years. Several climatic variables were chosen (Table 1) to study their possible relation to crown condition, including annual temperature means and monthly values of dry and cold seasons, its rainfall and solar radiation. All of these climatic variables were categorized in five homogeneous intervals (Table 2) with the aim of comparing plot defoliation among different levels of each climatic variable.

Plot defoliation values were transformed by decimal logarithm to obtain normal distribution and homocedasticity of data (Kolmogorov-Smirnov, Shapiro-Wilks and Bartlett tests). Analysis of Variance (ANOVA), with a signification level of 0.05, was carried out to know if there were statistically significant differences in plot defoliation among different levels of rainfall, temperatures and solar radiation. Finally, the Bonferroni test was used for multiple comparisons. To study the relationship between defoliation and climatic data, simple regression was used for each climatic variable, and multiple regression with backward selection was used with the aim of include several variables in the model to study cross effect among variables in defoliation.

Table 2: Homogeneous intervals of climatic variables obtained from plots values ranging. It is shown the range of temperature, rainfall and solar radiation among plots along the study area.

	Cat.	Annual	December	January	February	June	July	August
Mean Temperature (ºC)	1	9 – 9.4	2.4 – 2.76	1.6 – 1.94	2.7 – 3.1	14.2 – 14.74	17.5 – 18.1	17.2 – 17.82
	2	9.4 -9.8	2.76 – 3.12	1.94 – 2.28	3.1 – 3.5	14.74 – 15.28	18.1 – 18.7	17.82 – 18.44
	3	9.8 – 10.2	3.12 – 3.48	2.28 – 2.62	3.5 – 3.9	15.28 – 15.82	18.7 – 19.3	18.44 – 19.06
	4	10.2 – 10.6	3.48 – 3.84	2.62 – 2.96	3.9 – 4.3	15.82 – 16.36	19.3 – 19.9	19.06 – 19.68
	5	10.6 - 11	3.84 – 4.2	2.96 – 3.3	4.3 – 4.7	16.36 – 16.9	19.9 – 20.5	19.68 – 20.3
Maximum Temperature (ºC)	1	15.0 – 15.46	6.4 – 6.8	5.7 – 6.1	7.1 – 7.68	20.7 – 21.42	25.4 – 26.08	24.9 – 25.64
	2	15.46 – 15.92	6.8 – 7.2	6.1 – 6.5	7.68 – 8.26	21.42 – 22.14	26.08 – 26.76	25.64 – 26.38
	3	15.92 – 16.38	7.2 – 7.6	6.5 – 6.9	8.26 – 8.84	22.14 – 22.86	26.76 – 27.44	26.38 – 27.12
	4	16.38 – 16.84	7.6 – 8.0	6.9 – 7.3	8.84 – 9.42	22.86 – 23.58	27.44 – 28.12	27.12 – 27.86
	5	16.84 – 17.3	8.0 – 8.4	7.3 – 7.7	9.42 - 10	23.58 – 24.3	28.12 – 28.8	27.86 – 28.6
Minimum Temperature (ºC)	1	2.8 – 3.18	-1.7 – -1.36	-2.7 – -2.38	-2.1 – -1.76			
	2	3.18 – 3.56	-1.36 – -1.02	-2.38 – -2.06	-1.76 – -1.42			
	3	3.56 – 3.94	-1.02 – -0.68	-2.06 – -1.74	-1.42 – -1.08			
	4	3.94 – 4.32	-0.68 – -0.34	-1.74 – -1.42	-1.08 – -0.74			
	5	4.32 – 4.7	-0.34 – 0	-1.42 – -1.1	-0.74 – -0.4			
Rainfall (mm)	1	526.5 - 609.9	574 – 684.4	487 – 586.2	449 – 521	429 – 461.6	227 – 244.6	204 – 220
	2	609.9 – 693.5	684.4– 794.8	586.2 – 685.4	521 – 593	461.6 – 494.2	244.6 – 262.2	220 – 236
	3	693.5 – 776.9	794.8 – 905.2	685.4 – 784.6	593 – 665	494.2 – 526.8	262.2 – 279.8	236 – 252
	4	776.9 – 860.4	905.2 – 1015.6	784.6 – 883.8	665 – 737	526.8 – 559.4	279.8 – 297.4	252 – 268
	5	860.4 – 943.9	1015.6 - 1126	883.8 - 983	737 - 809	559.4 - 592	297.4 – 315	268 - 284
Solar Radiation Kj/m2*day*µm	1	2030 – 2046.2						
	2	2046.2 – 2062.4						
	3	2062.4 – 2078.6						
	4	2078.6 – 2094.8						
	5	2094.8 - 2111						

RESULTS

The Kolmogorov-Smirnov, Shapiro-Wilks and Bartlett tests proved normality and homocedasticity of the logarithm of mean plot defoliation (Def) data. The One Way ANOVA analysis showed that there were statistically significant differences between mean Def values of plots with different levels of July rainfall, Mean annual temperature and Mean august temperature, whereas there were no significant differences in plot Def in relation with the other variables analyzed, such as solar radiation, maximum and minimum annual, summer and winter temperatures or annual and winter rainfall (Table 3).

Table 3: Results of ANOVA between plot defoliation and 5 homogeneous levels of different climate variables.

	Annual	December	January	February	June	July	August
Mean Temp.	4.200	2.231	1.535	1.873	2.106	1.282	2.736
	0.004*	0.076	0.203	0.126	0.090	0.286	**0.036***
Max. Temp.	2.318	1.492	2.029	2.275	1.345	1.466	2.281
	0.067	0.215	0.101	0.071	0.263	0.223	0.070
Min. Temp.	1.952	1.878	1.979	1.538			
	0.113	0.125	0.108	0.202			
Rainfall	2.049	2.297	1.236	1.010	1.536	2.758	1.519
	0.098	0.069	0.305	0.409	0.202	**0.035***	0.207
Solar Rad.	2.246						
	0.091						

* Numbers show F value (up) and p-value (down). Numbers in bold with asterisk refer to p-values lower than 0.05. f.d. = 67 for all ANOVA. Abbreviations: Temp= temperature; Max= Maximum; Min= Minimum; Rad= Radiation.

In the analysis of rainfall, ANOVA showed significant differences in plot defoliation among different homogeneous levels of July rainfall (Figure 3, Top). Bonferroni test showed differences between levels 1 and 5 (Table 4), with a difference in precipitation of 60 mm. The erratic behavior of rainfall distribution (Dore, 2005) could preclude from making accurate predictions for the future.

Table 4: ANOVA between logarithm of defoliation and 5 July rainfall levels .There were significant (p<0.05) differences between defoliation in level 1 and 5, as it is showed by Bonferroni multiple comparison test.

July Rainfall	count	Mean	Homogeneous groups
1	13	1.27036	a
2	15	1.29274	ab
3	13	1.2988	ab
4	13	1.31593	ab
5	14	1.40098	b

On the other hand, there were significant differences in plot defoliation among Mean annual temperature levels (Figure 3, Medium). Duncan multiple comparison test demonstrated significant differences (p<0.05) between defoliation in level 1 and 5 (Table 5), being their difference in temperature of 1.6 ºC. If predictive models were accurate, this temperature increase, or even higher, could be reached in the next years.

Table 5: Bonferroni multiple comparison test results, which demonstrates significant differences in plot defoliation between levels 1 and 5 of mean annual temperatures.

Mean annual Temperature	count	Mean	Homogeneous groups
1	13	1.23130	a
2	17	1.28347	ab
3	18	1.34915	ab
4	16	1.35964	ab
5	4	1.41152	b

ANOVA results comparing defoliation among plots with different levels of Mean August temperature also showed significant (p<0.05) differences (Figure 3, Bottom). Bonferroni multiple comparison test revealed differences between levels 1 and 5 (Table 6), with a variation in August temperature of 2.48 ºC. The ANOVA analysis showed that there were no significant differences in defoliation values neither among levels of mean or minimum temperatures (annual, June, July, December, January and February) nor among different solar radiation or rainfall levels (annual, June, August, December, January and February).

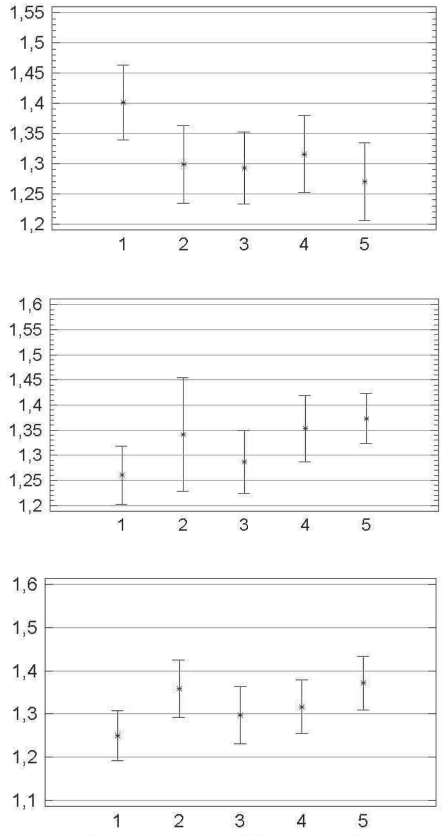


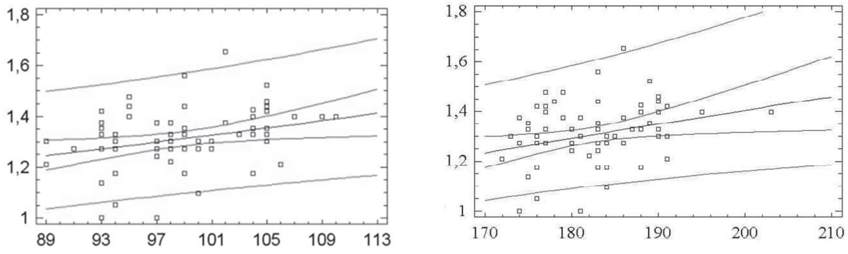
Figure 3: Confidence intervals of ANOVA analysis among the logarithm plot defoliation and July rainfall (Top), mean annual temperature (Medium), and mean August temperature levels (Bottom). Each variable was categorized in 5 homogeneous intervals. Letters indicate significant differences in defoliation among levels of climatic variables.

Table 6: Tukey multiple comparison test, showing significant differences in plot defoliation between levels 1 and 5 of mean August temperatures.

Mean August Temperature	count	Mean	Homogeneous groups
1	16	1.25	a
2	12	1.23	ab
3	14	1.32	ab
4	12	1.36	ab
5	14	1.37	b

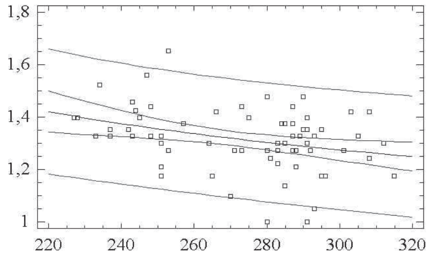
To assess the influence of these parameters in plot defoliation, a simple regression was done for each of the significant parameters. The regression models for defoliation versus July rainfall, mean annual and mean August temperatures were significant ($p<0.05$), with a negative slope for the precipitation model and positive for both temperature models. The R^2_{adj} for these models was only 10.66 % for July rainfall, 9.19 % for Maximum temperature and 8.01 % for August temperature (Figure 4), suggesting that defoliation was not only explained by climatic parameters, however there was an evident influence of these parameters on the observed defoliation.

Multiple regression analysis with backward selection showed that July Rainfall was the variable with higher effect on defoliation. However, although the model is significant, R^2 was quite low, with only 10.66%. A multiple regression model was built, but it is not improving R^2_{adj} of models with only one climatic variable.



Mean annual

Mean August



July rainfall

Figure 4: Regression models for defoliation and climatic parameters (mean annual and mean August temperatures and July rainfall). Reciprocal-Y regression model related to Mean temperature [log Def= 1 / (1.42482 – 0.000412949 * Mean temperature)](Top, Left). Double reciprocal model related to Mean August temperature [log Def = 1 / 0.155929 + 111.238/ Mean August temperature] (Top, Right). Reciprocal-X regression model related to July rainfall [log Def = 0.872209 + 120.684 / July rainfall] (Bottom, Center).

DISCUSSION

CURRENT STATE

According to ANOVA results, a negative deviation in July rainfall of 60 mm led to a significant increase of defoliation (about 10 %). Precipitation started to decrease in June, but temperatures remained at moderate levels. In July temperatures were higher but rainfall was much lower, thus the combination of drought and high temperatures creates a stressful environment, causing the decrease of tree vigour. Thus, July rainfall may act as a key factor to tree condition in Castile and Leon pine forests, mainly regarding tree vigour and tolerance to pest and disease. In this sense, July is not a suitable month for some management practices, as thinning and pruning in the pine forests of Castile and Leon, since the risk of insects attack will increase with the decreasing of tree vigour.

On the other hand, mean temperature also influenced defoliation. As it was showed by the ANOVA analysis, an increase of 0,4 ºC caused a significant increase in plot defoliation (6%), and an increase of 1,6 ºC (between levels 1 and 5) caused an increase of 9 %. This increment of temperatures corresponds exactly with the observed increase of temperatures in the Iberian Peninsula over the last hundred years (Hulme and Sheard, 1999). Plots with higher mean temperatures reached higher defoliation values, mainly when rainfall decrease was more acute. In addition, mean August temperature also was influencing canopy, as there was an increase in defoliation (about 6 %) when August temperature increased 2.48 ºC.

After the drought of July, August rainfall remained very low, and its high temperature could have enhanced the effect of dry conditions, leading to a decrease of tree vigour. Also, these variations in climatic factors may affect forest pathogens, mainly to their sporulation and colonization success, since these are influenced by changes in temperature, precipitation, soil moisture and relative humidity (Brasier, 1996; Lonsdale and Gibbs, 1996; Houston, 1998).

A pattern in temperature and precipitation was observed in relation to plot defoliation in the pilot zone. Thus, most of plots that were placed in warmer and drier conditions showed higher defoliation values, and those that were located in colder and wetter sites showed lower defoliation values. This agrees with results of the Programme for the Intensive Monitoring of Forest Ecosystems in Europe that showed that between 30-50% of the variation in defoliation could be explained by the variation in stand age, soil type, precipitation, N and S deposition and foliar chemistry, for pine, oak and beech. For Scots pine, only age, precipitation and foliar Nitrogen content showed a significant relationship with defoliation, and a model with these predictor variables fitted 21 % (R^2_{adj}) (De Vries et al., 2003).

The main achievement of our study is the identification of mean annual and mean August temperatures as important factors affecting crown condition of pine species. It becomes clear that there are many abiotic and biotic factors affecting canopy, and meteorological factors are just a small part of them, but these factors must be included in any predictive model for forest condition. Part of the low R2adj values from regression models could be derived from to the subjectivity of visual crown assessment, although it has been proved to be very low. Thus, more reliable methods are being developed for crown assessment. Most of those methods are based on indirect measures of light environment, and they have become more widely accepted, such as remote sensing based methods (De Santis and Chuvieco 2009; Somers et al. 2010), airborne laser methods (Solberg et al. 2006) and ground-based methods. Some ground-based methods use canopy light interception to estimate leaf area optically, e.g. by hemispherical photography (Chen et al. 1991; Kucharik, Norman & Gower, 1998; Valladares and Guzmán 2006; Montes et al. 2007).

Results obtained from this study suggest that defoliation is influenced by temperatures and precipitations of long term preceding years, not only by recent years conditions, as it is usually thought. Historical climatic conditions may influence the present crown condition, which is a result both of recent and past climatic conditions. Although the influence of these climatic variables on defoliation is demonstrated, other kind of variables must be included with the aim of predicting defoliation for a particular site, such as silvicultural, structural, nutritional and disturbance parameters. Even other factors, such as the presence of pest and diseases should be required.

CLIMATE CHANGE SCENARIOS

Climatic trends point to a likely temperature increase in south-eastern Europe of about 1-3 ºC (Kattenberg, 1996; Watson et al., 1997), and of 2 ºC in Spain, particularly during summer (Hulme and Sheard, 1999; Parry, 2000; Del Río, Penas & Fraile, 2004). Therefore, the results of this study suggest that defoliation will follow the temperature increasing trend, causing a reduction of tree vigour, and leading to an increase of pests and diseases attack risk. If there is a 2 ºC increase in mean temperature values, defoliation could increase about 30 %, which may represent an important reduction of canopy. A rise of maximum temperatures could also cause physiologic effects in trees, having a negative impact on primary processes as photosynthesis and causing the increase of respiration rates (Boonen et al., 2002).

Future projections predict that rainfall will be more erratic (Dore, 2005) with a decreasing trend in Spain (Schönwiese and Rapp, 1997), so it is likely that defoliation levels become higher due to this decrease. In Mediterranean ecosystems, summer is a marked dry season, and at the end of the summer there is already a notable reduction in the canopy of pine plantations (Bryant, Bhat and Jacobs, 2005), so an increase of drought may produce critical effects on forest health. In addition, it is advisable that crown assessment should be done during this period (August-September), so that canopy reduction as a result of summer drought and high temperatures will be recorded.

Forest managers must notice these trends and adapt forestry practices with the aim of minimize the impact of defoliation, which also may contribute to forest sustainability. Further surveys are required to predict defoliation values for a particular site, considering that many other kind of parameters are needed for building predictive models of crown condition, which could help to understand which are the main factors involved in this process. These surveys will be useful to help forest managers to minimize canopy reduction and to ensure the permanence of our forests in a good condition, thus helping to mitigate climate change effects over the atmosphere.

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Chapter III

(“El sabio no se sienta para lamentarse, sino que se pone
alegremente a su tarea de reparar el daño hecho“)

(William Shakespeare)

STAND FACTORS INFLUENCING *PINUS HALEPENSIS* MILL. DECLINE IN NORTH-WESTERN SPAIN♣

ABSTRACT

Although the decline of Aleppo pine has been observed long time ago and several climatic and biotic factors have been previously associated to this complex process, the site factors involved in this process remain poorly understood. The objective of this study is to identify site factors influencing canopy condition. Canopy condition was estimated both by a visual estimation of defoliation, and by an indirect estimation of leaf area index (LAI) and other stand and light-related parameters through the analysis of hemispherical photographs. A high percentage of damaged trees (81%) along with high levels of defoliation in plots (up to 53%) and trees (up to 85%) were recorded. Regression models showed that the site factors related to defoliation were basal area, age, crown depth and elevation, while those related to LAI were diameter at breast height, tree density and canopy openness. Hemispherical photography analysis was a good method for LAI estimation, but it was not for the estimation of defoliation because heterogeneous defoliation patterns caused by fungal pathogens were detected in the study area.

Soils and climatic conditions were common to all plots, so their influence could not be tested, but poor soil conditions and climatic restraints are described for this area, such as low productivity soil, summer drought and a high number of frosts days. Our results pointed several factors as involved in *Pinus halepensis* decline, including age, basal area, canopy openness, diameter, height and tree density. These factors can influence canopy condition, thus, they might be acting as predisposing factors for the decline. The modulation of these factors is possible if suitable forest

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♣Sanz-Ros, A.V., Pajares, J.A., Diez Casero, J. (2015). Stand factors influencing *Pinus halepensis* decline in north-western Spain. Forest Pathology, Under revision.

management strategies are selected, which could lead to a decrease of the decline incidence.

INTRODUCTION

Aleppo pine (*Pinus halepenesis*) is the most widespread pine species across the Mediterranean area, representing a distinctive and fundamental element of the Mediterranean forests (Serrada, Montero & Reque 2008). Aleppo pine has scarce soil requirements and can tolerate extreme dry and hot conditions (Cámara 1999). Although the species is susceptible to frost, mainly when it occurs in late spring or early autumn (Muñoz et al. 2007), it is able to grow well even under such climatic conditions. For this reason it has been used for afforestation and stabilization of slopes delimiting calcareous high plateau borders in north-western Spain, which represents the north-western limit of the species distribution in the Iberian Peninsula and the western limit in Europe (Gil et al. 1996).

A severe decline has been observed long time ago (Muñoz 1999; Santamaría, Pajares & Diez 2003). Its causes seem to be complex and not completely understood. The main symptoms observed in declining forest stands are dieback, branch death and high defoliation levels, producing a significant reduction in the tree canopy with the further reduction of tree growth and vigour. Several biotic agents have already been associated to the decline of this species, such as *Sirococcus conigenus* in central Spain (Muñoz 1997; 1999) and *Gremmeniella abietina* as primary pathogens in northern forest stands (Martínez 1933; Santamaría, Pajares & Diez 2003; Santamaría, Botella & Diez 2007; Botella, Santamaría & Diez 2010), as well as some secondary or weak pathogens, such as *Sclerophoma pithyophilla, Thyriopsis halepensis or Cenangium ferruginosum* (Sutton & Waterston 1970; Sinclair, Lyon & Johnson 1987;Phillips & Burdekin 1992;). However, the presence of these organisms does not fully explain the decline. Therefore, other site factors must be involved, for instance those related to soil, topography, climate or resources, i.e. light, water and nutrients, as well as disturbance factors, including those associated to silvicultural management (Barnes et al. 1998). Some combinations of these factors may represent a stressful environment, favouring the decline.

Trees under stressful conditions use to show a reduction of their canopy, which can be estimated through the use of a number of indicators, being defoliation one of the most common. The term defoliation is defined as the defoliative effects of biological agents, premature needle loss or reduction in the needle holding period (Ferretti, 1994). Furthermore, defoliated trees have a low chance of survival and are prone to be attacked by root pathogens and bark beetles (Houston, 1992). Thus, defoliation has become the most widely used indicator of forest health and canopy condition, for instance in the International Co-operative Program for the Intensive Monitoring of Forest Ecosystems (ICP Forests) since 1986 (Ferretti, 1997; Eichhorn et al., 2010; Fischer et al., 2010).

On the other hand, the amount of canopy, which is one of the most general indicators of stress (Waring 1985), has been most commonly estimated by leaf area index (LAI). This parameter is directly influenced by the water, carbon and energy exchange in a stand (Norman, 1992). Many stand ecological processes such as transpiration, competition, growth and self-thinning determine total stand leaf area (Waring, 1983; Long et al., 1984; Dean and Long, 1985).

Although LAI can be estimated by destructive sampling, this is extremely time-consuming and labour intensive in pine stands. Thus, indirect methods are required to increase the speed of LAI determination and avoid destructive tree harvesting. Different approaches have been used for LAI estimation: remote sensing (De Santis & Chuvieco, 2009; Somers et al., 2010), airborne laser scanning (LIDAR) (Riaño et al., 2004; Solberg et al., 2006) and ground-based methods. Some ground-based methods use photosynthetic active radiation (PAR) for LAI estimation (Chazdon and Field, 1987; Himmler, 1996; Chen & Cihlar, 2000), while hemispherical photography (Chen, Black & Adams, 1991) use canopy light interception to estimate leaf area optically. This optical technique has been widely used in studies of canopy structure and forest light transmission (Valladares & Guzmán, 2006; Montes et al., 2007), but its application to the assessment of canopy condition studies has not been previously evaluated.

The aim of this study was to identify the site factors affecting canopy condition in declining *Pinus halepensis* stands located in north-western Spain by the estimation of defoliation and LAI by visual and hemispherical photography methods respectively. We tested the hypothesis that site factors are influencing canopy condition.

MATERIALS AND METHODS

STUDY AREA

The study area is a located in the south-east of Palencia Province (Castilla y León, Spain) within latitudes within latitudes 41º 52’ 52”N- 41º 56’ 9”N and longitudes 4º 20’ 35”W-4º 28’ 51”W, where Aleppo pine trees suffer a severe decline. The study area is characterized by the presence of calcareous high plains with marlaceous slopes and some gypsum deposits. The altitude ranges between 775 and 965 m.a.s.l. The climate is Mediterranean with some continental features, with hot summers and cold winters, where frost days are very common (60 per year). Mean annual temperature is 12.3 ºC, and the mean annual precipitation is 400 mm. Soils are very stony, with a low percentage of organic matter and high content of calcium (Oria, Díez & Rodríguez, 1996), being classified as are classified as Inceptisol, Ochrept, Xerochrept following the Soil Taxonomy classification system (USDA, 1987).

SAMPLING METHOD AND DATA COLLECTION

During summer 2007 thirty circular plots of 15 m of radius were established in *P. halepensis* declining forests. In each plot, the 15 nearest trees to the plot centre were evaluated following a spiral track (Figure 1), therefore a total of 450 trees were examined. All plots were established over the same soil type (cited above). Location of plots and other plot features are shown in table 1.

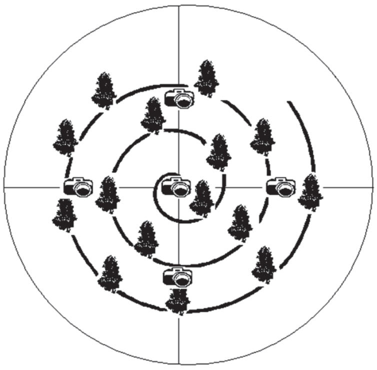

Figure 1. Distribution of the fifteen evaluated trees following a spiral path, and the five sampling points were hemispherical photographs were taken in each plot.

Table 1. Characteristics of studied *Pinus halepensis* plots.

Plot	coorX[a]	coorY[b]	Age (years)	Elevation (m.a.s.l)	Plot	coorX[a]	coorY[b]	Age (years)	Elevation (m.a.s.l)
1	387176	4640637	42.5	888	16	384350	4638884	38.0	887
2	387243	4640642	33.0	883	17	384193	4638842	38.0	894
3	387406	4640594	28.0	887	18	384328	4638802	36.5	884
4	387316	4640466	41.0	879	19	384424	4638817	25.0	874
5	387425	4640293	44.0	965	20	384425	4638899	35.0	883
6	387340	4640313	40.0	885	21	384510	4638887	29.0	883
7	387258	4640587	45.0	892	22	386415	4637153	27.0	797
8	387407	4640242	45.0	863	23	386244	4637069	31.0	806
9	387329	4640220	38.7	885	24	385591	4643447	36.3	832
10	387222	4640338	36.0	878	25	385630	4643401	45.5	828
11	387261	4640396	44.5	880	26	377651	4639996	36.7	782
12	384478	4639008	35.5	884	27	377782	4639795	39.5	799
13	384496	4638959	38.5	885	28	377615	4640137	30.7	783
14	384349	4638984	25.5	878	29	377734	4639484	27.0	775
15	384242	4638925	30.5	879	30	377694	4639545	31.0	780

In order to identify the site factors related to defoliation and LAI, each plot was characterized by a number of variables related to tree growth and canopy structure. Variables were calculated as the average value of the 15 evaluated trees (Table 2) and the they were: diameter at breast height (DBH), calculated as the mean value of two orthogonal stem diameters at 1.30 m above ground level; mean height (MH), as the arithmetic mean of tree height, using the Vertex III hypsometer (Haglöf), also used for canopy depth (CDe), the averaged height from the lowest live branch to the top of the crown; mean tree age (Age); tree density (D), expressed as the number of trees per hectare; basal area (BA) measured with a relascope and elevation (E), measured by a portable GPS.

Table 2. Description, abbreviations and units of plot parameters measured in field conditions and light-related variables obtained from the analysis of hemispherical photographs.

Variable	Description	Units
Age	Mean tree age	Years
BA	Basal area	m^2ha^{-1}
CDe	Mean canopy depth	m
D	Density	Trees/ha
E	Elevation	m.a.s.l
DBH	Mean tree diameter at breast height	cm
MH	Mean tree height	m
MDef	Mean defoliation	%
MCOp	Mean canopy openness	%
MLAI	Mean effective leaf area index	m^2m^{-2}
MDirT	Mean direct transmittance	%
MDifT	Mean diffuse transmittance	%
MTT	Mean total transmittance	%

EVALUATION OF CANOPY CONDITION

Canopy condition was evaluated through the estimation of plot defoliation and leaf area index (LAI). Defoliation was calculated as mean defoliation (MDef) of the 15 evaluated trees per plot. Tree defoliation was estimated visually at 5 % intervals, following the ICP Forest methodology (Ferretti, 1994; Eichhorn et al., 2006) from a distance at least equal to the height of the evaluated tree and avoiding to face the sun during the evaluation. The estimates were compared with reference tree photographs for *P. halepensis*. The reference tree is the best tree crown of this species in Mediterranean areas, with a maximum amount of foliage, which provides comparable data for *P. halepensis* through the Mediterranean area.

LAI was estimated through the analysis of hemispherical photographs. A total of 150 photographs (5 per plot, Figure 1) were taken as follows: one in the centre of the plot and one in each cardinal point (N, S, E, W) at a distance from the plot centre equal to half of the radius of the plot (7.5 m). Hemispherical photographs were taken with a Nikon camera (Coolpix 4500) equipped with a Nikon Fc-E8 fisheye adapter. The camera was mounted on a tripod with a levelling head, oriented to magnetic north and at a height of 1m above the ground (and above understory vegetation). Photographs were taken skywards at sunrise or sunset, or sometimes during the day, under overcast conditions and homogeneously distributed clouds, so that direct sunbeams were not captured in the photographs. An automatic exposure setting was used.

Hemispherical photographs were analyzed with Gap Light Analyzer (version 2.0) software (Frazer, Canham & Lertzman, 1999). The blue channel was used to maximize contrast between sky and canopy elements. A threshold level was selected for each photograph to distinguish between visible sky and vegetal elements, such as foliage, branches and stems. The uniform overcast distribution model (UOC) and a clear sky transmission value of 0.65 were used. The data obtained was combined to produce indirect estimates of growing-season light transmission and some parameters related to canopy structure. Effective leaf area index (MLAI), which was computed from 0–75° from the zenith as defined by Welles and Norman in 1991; mean canopy openness (MCOp), defined as the percentage of visible sky across the whole hemisphere; transmittance, defined as the percentage of transmitted direct, diffuse and total radiation (MDirT, MDifT and MTT) incident on a horizontal surface when light is blocked by the surrounding topography and overlying forest canopy (Frazer et al., 1999).

STATISTICAL ANALYSIS

A descriptive analysis was made at tree and plot levels and the Kolmogorov-Smirnov test was used to assess data normality. Variables which did not meet this assumption were logarithm, inverse or squared transformed. Secondly, a multivariate analysis, a Principal Components Analysis (PCA) was carried out in order to reduce the dimension of the observations, to identify gradients that explained the largest variation in the data, and to identify key factors that may influence on the response variables, defoliation and LAI. The PCA was based on the correlation matrix and the components were rotated according to the *Varimax* method. Finally, a multiple regression model was built for each of the response variables (defoliation and LAI), by using the forward stepwise selection method. For defoliation model were included all variables (Stand and hemispherical photography analysis) as explanatory, while for LAI model only was used the stand variables (excluding those obtained by hemispherical analysis). The Durbin-Watson test was used to assess residual autocorrelation, and variance inflation factors (VIFs) were examined to assess colinearity. The statistical package Statistica 6.0 (Statsoft, USA) was used for statistical analyses.

RESULTS

DEFOLIATION AND LEAF AREA INDEX

Canopy visual assessment in *P. halepensis* stands revealed a high level of defoliation in general, and a high percentage of damaged trees. The high defoliation levels found in the study area were mainly located in the upper part of the crown and in some cases it was observed a severe dieback downwards. The mean defoliation of plots ranged from 22 to 53% (Table 3), while defoliation ranged from 15 to 85% at tree level. The mean defoliation value was 37.74% in both tree and plot levels, although standard deviation of plots (plot SD) was 8%, while tree SD was 12%.

The assessment also showed that 19% of evaluated trees were defoliated between 10-25%, corresponding to Defoliation Class 1 (DC1), while 77.85 % of trees were defoliated between 25-60% (DC 2) and 3.13 % were defoliated over 60% (DC 3). LAI values of plots, determined by analysis of hemispherical photographs, ranged from 0.36 to 2.92 m^2/m^2 (Table 3), and the mean value was 1.71 (+0.48, standard deviation) m^2/m^2. Pearson correlation analysis revealed a very low correlation between defoliation and LAI (r=0.075; p>0.05) as shown in Figure 2.

Table 3. Stand parameters measured in the field and those obtained from the analysis of hemispherical photographs. Values given as averages are presented with standard deviation (±SD). Abbreviations are described in table 2.

	Stand parameters				Digital image analysis parameters				
Plot	MDef (%)	Density (trees/ha)	BA (m^2/ha)	CDepth (m)	MLAI	MCOp	MTT	MDirT	MDifT
1	37.86±5.45	198.1	12	3.06±0.87	0.36±0.06	58.20±2.68	22.93±1.30	73.39±4.94	70.08±4.56
2	42.33±7.53	339.5	23.5	4.26±1.88	1.11±0.12	31.40±2.24	11.98±1.34	37.82±10.28	37.12±3.15
3	36.67±6.17	863.0	20	3.45±1.24	1.80±0.09	17.93±0.94	8.61±1.35	27.76±7.67	26.09±1.60
4	48.00±9.78	1004.4	28	3.13±1.24	1.82±0.16	17.07±1.69	7.85±0.32	24.51±3.53	24.56±2.22
5	53.33±7.24	1867.4	36.5	2.33±0.95	2.12±0.26	14.77±1.78	7.20±0.36	22.67±1.49	22.37±1.56
6	53.00±7.26	1103.5	22	2.61±0.77	1.81±0.19	16.97±2.13	7.25±0.16	20.62±2.67	24.70±2.04
7	46.15±8.70	183.9	10.5	4.03±1.02	0.56±0.14	48.25±3.95	20.10±2.94	64.37±11.85	61.38±7.68
8	47.67±6.51	1457.1	36.5	2.19±0.50	1.75±0.15	17.89±1.20	8.28±0.53	26.1±3.64	25.65±1.22
9	43.00±7.75	1145.9	33	2.65±0.99	1.60±0.11	19.65±1.63	8.68±1.01	26.99±7.16	27.31±1.64
10	39.33±8.21	1414.7	26.5	2.78±1.45	1.87±0.07	15.87±0.72	6.04±0.56	22.12±4.94	24.53±1.05
11	47.67±11.93	1244.9	50	2.41±0.89	1.99±0.12	15.70±1.18	5.37±0.24	15.59±1.86	23.30±1.90
12	38.00±11.31	1174.2	45	2.87±1.22	2.22±0.17	14.84±1.05	5.71±0.43	17.49±2.90	22.03±0.49
13	44.67±16.95	1287.4	46	2.27±0.74	2.23±0.08	14.59±0.78	5.87±0.60	28.88±3.03	14.44±1.38
14	32.67±9.80	1216.6	33	3.34±1.27	1.69±0.15	21.03±3.31	7.32±0.72	21.74±2.03	28.98±6.38
15	38.33±6.73	1358.1	42	2.33±0.56	1.62±0.14	24.18±1.69	12.31±1.09	42.29±5.63	36.02±2.93
16	37.67±12.08	1782.5	43	2.62±1.13	2.92±0.26	13.23±0.72	11.22±0.47	31.61±2.47	21.45±0.59
17	31.00±7.37	1315.7	40	3.64±.082	1.81±0.18	19.25±1.99	13.06±1.74	34.94±6.77	26.81±2.50
18	34.67±7.67	933.7	44	2.25±0.75	2.02±0.11	16.43±0.65	6.86±0.70	22.71±5.23	24.82±1.12
19	30.00±6.55	778.1	24	3.72±0.78	1.08±0.08	31.79±2.21	13.11±1.81	45.77±10.95	44.99±3.47
20	41.67±8.16	1301.5	49.5	2.94±0.88	2.13±0.21	18.00±1.66	7.87±0.94	26.44±4.30	28.05±2.37
21	39.00±21.40	735.6	37.5	3.03±1.14	1.87±0.13	16.36±1.33	8.39±0.77	28.70±3.97	23.77±1.61
22	36.00±5.41	1061.0	26	3.97±1.06	1.47±0.15	24.57±1.83	13.04±1.95	39.26±7.88	36.30±4.36
23	22.33±5.30	721.5	25	5.14±1.43	1.79±0.46	20.54±7.50	8.14±2.75	22.53±4.61	25.88±13.22
24	25.33±6.67	749.8	39	5.87±1.10	1.66±0.05	18.93±1.05	9.67±1.28	31.01±8.37	26.50±1.52
25	30.00±8.86	1174.2	60	4.07±1.25	2.07±0.14	14.44±0.92	6.28±0.30	18.69±1.84	19.58±0.16
26	30.00±8.66	1216.6	30	3.36±1.4	1.58±0.19	20.81±2.38	8.90±0.70	29.12±2.72	27.92±3.02
27	37.33±9.61	820.5	36	3.04±0.93	1.63±0.07	19.59±1.20	6.58±0.36	20.36±3.42	26.23±1.68
28	33.00±5.61	1358.1	31	4.05±1.55	1.77±0.14	18.42±1.36	8.65±0.89	27.32±4.29	25.37±1.51
29	32.33±9.61	1103.5	35	3.31±1.26	1.71±0.16	18.28±1.31	9.26±0.92	30.37±5.07	25.69±0.79
30	24.33±6.23	749.8	33.5	3.85±1.08	1.52±0.18	21.45±2.58	9.99±1.02	30.45±6.24	29.13±2.06

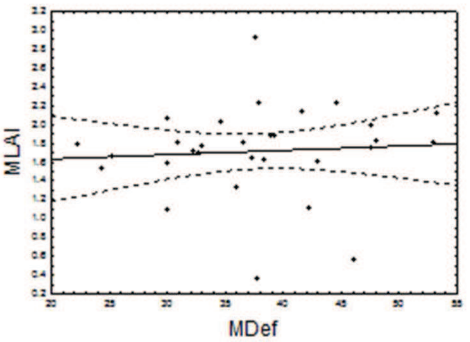

Figure 2. Scatter plot between defoliation and LAI. The continuous line represents the simple regression line for defoliation, using MLAI (obtained by analysis of hemispherical photographs) as the independent variable. Dashed lines represent the 95% confidence interval.

MULTIVARIATE ANALYSIS

The principal components analysis (PCA) identified three main axes which accounted for 80.96 % of the total variance (Table 4). The first principal component (40.12% of the total variance), was considered to be related to light environment under the canopy because of the high factor loadings for variables such as transmittance (MTT), canopy openness (MCOp), leaf area index (MLAI), density (D) and basal area (BA) (Table 4). The second component was interpreted as being related to canopy condition and associated factors, because high factor values were obtained for defoliation (MDef), elevation (E) and age (Age). The third component was related to stand growth and canopy development because the factor values were higher for mean height (MH), mean diameter (DBH) and canopy depth (CDe). Some abiotic factors, such as soil and rainfall, were homogeneous across all plots; therefore they were not included in the analysis.

Table 4. PCA components with eigenvalues and explained variance, and PCA factor loadings of variables. Bold numbers represent highest factor loadings of variables in each component. Abbreviated variables are described in Table2.

Variable	Factor 1	Factor 2	Factor 3
MCOp	**-0.898**	-0.313	0.139
MTT	**-0.861**	-0.291	0.186
CDe	-0.535	0.451	**-0.551**
DBH	-0.355	-0.458	**-0.693**
E	0.186	**-0.731**	0.243
Age	0.210	**-0.767**	-0.205
MDef	0.277	**-0.757**	0.428
MH	0.289	**-0.696**	-0.561
BA	**0.777**	0.051	-0.399
D	**0.869**	0.091	0.259
MLAI	**0.926**	0.147	-0.139
Eigenvalue	4.413	2.809	1.684
%TV[a]	40.116	25.539	15.309
%CTV[b]	40.116	65.656	80.966

a. %TV= percentage of total variance

b. %CTV= = percentage of cumulated total variance

Summarizing the results, PCA (Figure 3) suggested that the light environment, canopy condition and stand growth were the main components. Besides, this analysis showed a relationship between defoliation and some factors, such as elevation, age, tree height and crown depth. On the other hand, it showed a positive relationship of LAI with density and basal area, and a negative relationship with transmittance or canopy openness.

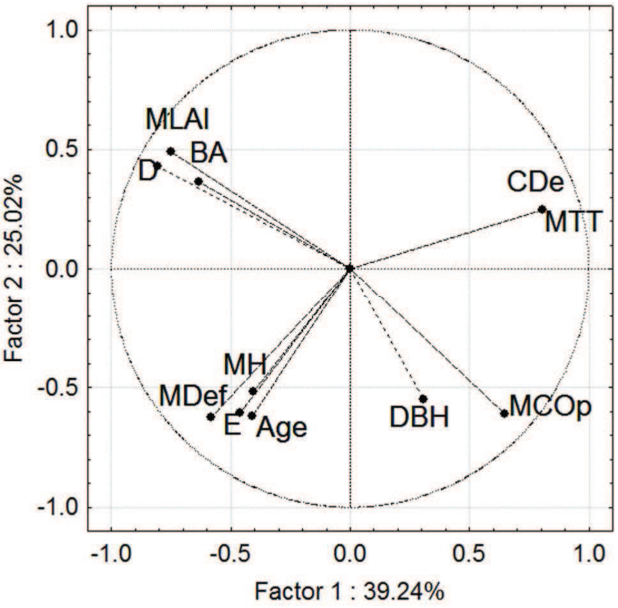

Figure 3. Biplot obtained from Principal Components analysis (Component 1 vs. 2). Variables are described in table 1.

DEFOLIATION AND LAI REGRESSION MODELS

With the aim of identifying the key factors influencing canopy condition, general regression models (GRM) were built for defoliation (Eq. 3) and LAI (Eq. 4). Regression model for defoliation included variables such as age (Age), Elevation (E), Basal Area (BA), and Canopy Depth (CDe). On the other hand, the LAI regression model includes Canopy Openness (MCOp), Mean Diameter (DBH) and Density (D).

The multiple regression model for defoliation (Eq. 3) was statistically significant (F= 14.71; p<0.0001), with no colinearity (since variance inflation factors (VIFs) were lower than 3) and the residuals followed a normal distribution (Durbin-Watson, p-value>0.1). The regression provided a reasonable fit (R^2= 70.18 %), and the adjusted correlation coefficient (R^2_{adj}) was 65.42 % (Figure 4, left).

$$MDef = -2.671 - 4.39 \times CDe + 0.427 \times Age - 0.178 \times BA + 0.0531 \times E \qquad \text{[Eq.3]}$$

Where, MDef = Mean defoliation; CDe= Canopy depth; BA = Basal area; Age= Mean age; E= elevation. In this model basal area (BA) and canopy depth

(CDe) were negatively related to mean defoliation (MDef), while age (Age) and elevation (E) were positively related to this variable. Defoliation values were lower in plots with high BA, high tree density and thus composed by young trees. In addition, defoliation values were higher in older (ca. 40-45 years) and with a higher altitude.

The multiple regression model for LAI (Eq. 4) was statistically significant as well (F=35.78; $p<0.0001$), with no colinearity (VIFs< 2) and the residuals followed a normal distribution (Durbin-Watson, p-value>0.1). The regression model provided a good fit (R^2= 72.61 %), and the R^2_{adj} was 70.57 %.

$$MLAI = 0.396 + 0,00065 \times D + 0.0183 \times BA \qquad \text{[Eq.4]}$$

Where MLAI= mean effective leaf area index; D= stand density; BA=basal area. BA and D were positively related to LAI, indicating that plots that develop a higher amount of canopy were those with high density of big trees (Figure 4, right).

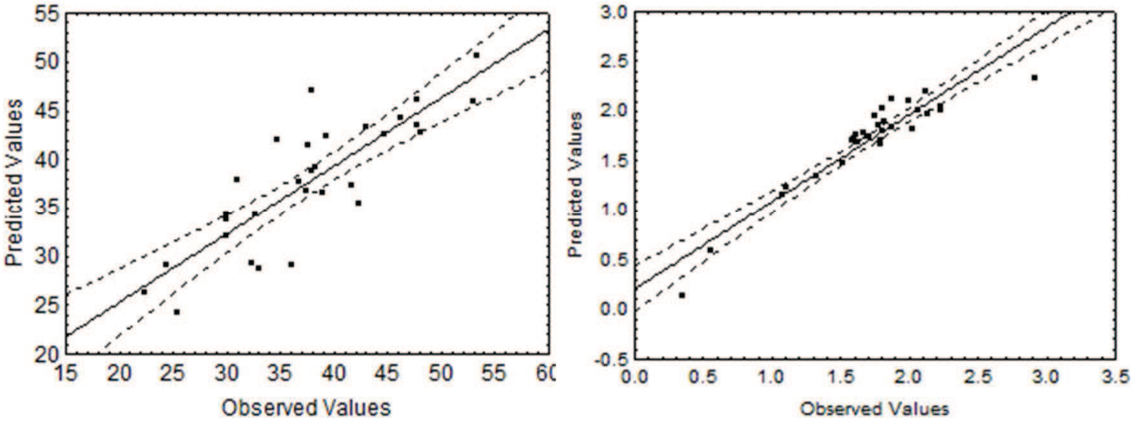

Figure 4: Predicted vs. observed values and regression line for defoliation model (Left). Predicted vs. observed values and regression line for LAI model (right).

DISCUSSION

The percentage of trees categorised as “damaged” by ICP Forests Commission, i.e. when belonged to defoliations classes DC2 and DC3 (Eichhorn et al., 2006), represented 81% of the evaluated trees. This percentage is much higher than the observed percentage (15.7%) for *Pinus* spp. in ICP Forest monitoring networks throughout Spain (Fischer et al., 2010).

The high defoliation levels observed in the upper part of the crown and in some cases with a dieback advancing downwards has been associated to the presence of some biotic agents. In this sense, *Gremmeniella abietina*, *Sclerophoma pithyophila, Thyriopsis halepensis and Cenangium ferruginosum*, have been previously isolated from affected tissues of declining trees in different studies performed previously in this area (Santamaría, Pajares & Diez, 2003; Santamaría, Botella & Diez, 2007; Santamaría et al., 2007; Botella, Diez & Santamaría, 2010). Type and location of symptoms were consistent with those caused by these biotic agents, including drying of needles and branches in the upper part of the crown, which leads to further defoliation and gradual dieback from the upper part of the crown (Santamaría et al., 2003; Santamaría et al., 2007). However, only the presence of those agents does not fully explain the decline, and the etiology of the decline is expected to be very complex.

The results of this study have identified a number of site factors influencing canopy condition that might act as decline predisposing factors. The models built in this study indicate that defoliation variation is explained by certain stand factors (Eq.3), while LAI variation is explained by other factors different than those explaining defoliation (Eq.4). These models supports our hypothesis, demonstrating that some site factors are affecting crown condition.

Identified factors affecting defoliation (Age, BA, CDe, and E) agree with the concept of decline, as explain by Manion in 1991, which states that only mature dominant trees will go into decline. Usually those mature trees are placed in stands with a low basal area, due to a low density of big trees. On the other hand, elevation may be related to a high exposure to frost of the more elevated plots compared to those located at lower positions. It is well known that *P. halepensis* usually displays twig tip wilting and general discoloration due to frost (Muñoz et al., 2007), and the studied area have shown a high number of frosts days (Santamaría, Botella & Diez, 2007). Moreover, some authors have recommended avoiding cold and wet locations for the establishment of pine plantations, with the aim of preventing damage caused by fungal pathogens (Karadžić & Milanović, 2008).

LAI values were high, as expected, in plots with a high density of big trees where the percentage of visible sky is very low. The mean LAI values of plots were 1.71±0.48 (SD), while other studies on healthy *P. halepensis* trees of similar diameter range have reported mean LAI values of 2.69 (López-Serrano et al., 2000). This reduction in LAI may be due to defoliation, but a cause-effect relation cannot be established, since many other factors, such as stand structural parameters (Long & Smith, 1984; Ford, 1985) or the method of estimating LAI (Fassnacht et al., 1994; Chen, 1996) may influence this relationship. Furthermore, the use of LAI as a health indicator requires reference values, since variations in LAI may be caused by factors other than defoliation, such as forest structure (Jack & Long, 1991; Dean & Baldwin, 1996; O'Hara, 1996), silvicultural treatments (Waring, 1985; Solberg et al., 2006) or forest fires (De Santis & Chuvieco, 2009).

Comparison between defoliation and LAI revealed that both variables were poorly related to each other, despite of the fact that the contrary was expected, and the models showed that the factors determining these two parameters were different. This low correlation may be due to the heterogeneous defoliation pattern that was observed, since most of the damage occurred in the upper part of the crown, thus supporting the idea of fungal pathogens as contributing factors to the decline. Given that LAI was estimated by photographs taken vertically from the ground towards the zenith, the overlapping of needles, twigs and branches may have impede the detection of dieback in the upper part of the crown. Is remarkable that Aleppo pine trees are keeping lower dead branches rather than natural pruning of them, thus increasing the superposition of branches and reducing the correlation between both variables.

In this sense, previous studies on defoliation after insect outbreaks in Scots pine explained defoliation in terms of changes in LAI, measured by airborne laser scanning, which could represent a useful tool for the early detection of canopy damage (Waring, 1985; Solberg et al., 2006; Eklundh, Johansson & Solberg, 2009), but this represents a different situation than the observed for Aleppo pine trees in northern Spain.

The hemispherical photography analysis is a suitable method for estimating LAI (Valladares & Guzmán, 2006; Montes et al., 2007), however it was not appropriate to estimate defoliation. Hemispherical photography, and probably some other ground based methods for canopy assessment, such as LAI 2000, might not be suitable for the detection of some defoliating agents, such as fungal pathogens causing dieback from the upper part of the crown downwards. On the other hand, it is likely that other methods such as remote sensing or airborne laser scanning were neither able to detect those heterogeneous defoliation patterns, mainly due to the same problem with background vegetation. However, more research is needed to test the ability of those methods for the detection of heterogeneous defoliation patterns. This remarks the complexity of forest health assessment and highlights the need for skilled evaluators in the field.

Overall, three kind of factors seems to be involved in the decline of *Pinus halepensis*: i) Predisposing factors, such as poor soil conditions. Soils are classified as xeric Inceptisols (Xerochrept), which are characterized by low productivity under a xeric climate (Soil Survey Staff USDA, 1987). The summer combination of high temperatures and low rainfall has been related to high defoliation levels in conifer species (Sanz-Ros, Pajares & Diez, 2008), and may produce a negative impact on tree physiology and growth (Borghetti et al., 1998). Our results point that some site factors, such as age, basal area, canopy openness, diameter, height, or tree density, can affect canopy condition, and might be considered also as predisposing factors. ii) Inciting factors suggested by our results include defoliation and frost damage. Although defoliation is caused by the combination of other biotic and abiotic factors, the reduction of the canopy led to a decrease in the photosynthetic activity, carbon sequestration and tree growth (Wiley et al., 2013; Pinkard et al., 2014), therefore reducing tree resources and vigour, and defoliated trees are prone to be attacked by other pest and diseases (Houston, 1992). On the other hand, the more elevated trees were more exposed to frosts and showed tip wilting, as previously described by Muñoz et al. in 2007. Both factors may incite trees to go into decline. iii) contributing factors, such as the presence of fungal primary and weak pathogens, previously isolated in this area, which may produce severe defoliation of trees (Santamaría, Pajares & Diez, 2003; Santamaría et al., 2007; Botella, Santamaría & Diez, 2010).

In Spain, future climate scenarios probably will increase the incidence of predisposing and inciting factors, since climate models predict an increase in temperature and a rainfall decrease (Kattenberg et al., 1996; Parry, 2000; Mossman, 2002; IPCC, 2013). In addition contributing factors also may be enhanced, as the combination of climatic factors may enhance the activity of weak pathogens.

In conclusion, the results of this study suggest that a number of site factors, including age, basal area, canopy openness, diameter, height and tree density can influence canopy condition. This influence might determine the final response of trees to stress and the severity of the observed decline. Even when contributing factors are present, only declining trees are not able to recover from its attack. Thus, suitable forest management strategies could be designed to modify certain site factors with the aim of decreasing the incidence of the decline, such as reducing tree density, pruning, create and heterogeneous stand structure (i.e. different tree sizes) in order to avoid proliferation of pathogens that attacks only young or mature trees (Hellgren & Högberg, 1995; Botella et al, 2010).

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Chapter IV

(“Nada está a salvo de la vida. Porque es vida lo que cava, quiebra y oscurece;
vida la humedad, los hongos que florecen en los altos ángulos pasivos…“)

(Santiago Kovadloff)

FUNGAL ENDOPHYTIC COMMUNITIES ON TWIGS OF FAST AND SLOW GROWING SCOTS PINE (*PINUS SYLVESTRIS* L.) IN NORTHERN SPAIN. ♣

ABSTRACT

For several decades it has been known that all plants have a diverse community of endophytic fungi both in their roots and shoots. Some of these endophytes may affect growth and health of their host. Still, even the most common endophyte species have not been characterized from the canopy of many ecologically and economically important tree species. This study describes the culturable fungal endophytic community of *Pinus sylvestris* L. twigs in northern Spain and its relationship with diametric growth of the host. Twig samples were collected from 20-34 years old Scots pines in fifteen stands. In all 360 twig samples were taken from 30 trees, surface sterilized and incubated on potato dextrose agar for isolation of endophytic fungi. Isolates were obtained from all twig samples investigated and they represent 43 fungal taxa as determined by morphogrouping and subsequent ITS rDNA sequencing.

All isolates were Ascomycetes of which Dothideomycetes was the most abundant and Sordariomycetes the most diverse class. Half of the species were host generalists while the others were conifer or pine specialists. We found three new endophytic species for the Pinaceae: *Biscogniauxia mediterranea*, *Phaemoniella effusa*, and *Plectania milleri* and additional six new species for *Pinus Sylvestris*: *Daldinia fissa*, *Hypocrea viridescens*, *Nygrospora oryzae*, *Ophiostoma nigrocarpum*, *Penicillium melinii* and *P. polonicum*. The endophytic community of fast and slow growing trees showed differences in species composition, abundance and evenness, but not in diversity. *Phoma herbarum* (the most common endophyte), occurred significantly more frequently in pines with high diametric growth than in trees with low growth rate.

♣Sanz-Ros, A.V., Müller, M., San Martín, R. & Diez, J. (2015): Fungal endophytic communities on twigs of fast and slow growing Scots pine (*Pinus sylvestris* L.) in northern Spain. *Fungal Biology*. http://doi.org/10.1016/j.funbio. 2015.06.008.

In contrast, *Hypocrea lixii* was found more frequently in trees with low diametric growth rate. Our results support the hypothesis that some endophytic species may affect growth of *P. sylvestris*.

INTRODUCTION

Research on endophytes of woody plants has so far concentrated mainly on describing species and infection rates of fungal inhabitants of various plant tissues, while in most cases their ecological role has remained poorly understood. Present knowledge on the microbial community structure of internal fungi of even the most common tree species is still patchy. Many species described as endophytes may simply be parasites tolerated by the host tree while other species can be regarded as opportunistic or latent pathogens, and some fungi, endophytic in one plant species, can be pathogenic to other plant species (Carroll, 1988; Ganley et al., 2004; Slippers & Wingfield, 2007). Some of them can also be primary decomposers waiting inactively for senescence of the tissue they have colonized (Müller et al., 2001). A number of fungal species described as endophytes on various plants may actually be insignificant to their host when occurring only as ungerminated spores which have survived surface sterilization procedures, for instance in a stomatal cavity (Schulz & Boyle, 2005).

A typical trait of endophytic fungi is their common ability to produce biologically active compounds in tests for fungicidal, antibacterial and herbicidal activities (Schulz et al., 2002). On the other hand, plants control their internal fungi and the relation has been described as a delicately balanced association where the host not only tolerates all the secondary metabolites excreted by the endophyte but retards growth of the invader without eradicating it (Schulz et al., 2002). When this balance is disturbed by either a decrease in plant defense or a rise in fungal virulence, disease develops.

The significance of the vast majority of fungal endophytes to their host trees is so far unknown. However, in some cases an endophytic fungus has been shown to promote host fitness, for instance by decreasing the palatability of the host tissue to herbivores through the production of toxic metabolites (Carroll, 1988; Miller et al., 2008), acting as antagonists against pathogens (Webber, 1981; Arnold et al., 2003) or inducing endophyte-mediated resistance against pathogens (Ganley et al., 2008).

In non-woody plants several endophytic fungi have shown the ability to increase plant growth as well as stress tolerance (Rademacher, 1994; Gasoni & de Gurfinkel, 1997; Ernst et al., 2003; Mucciarelli et al., 2003). In some studies the inoculation of woody plants with endophytic fungi has increased growth of the host. Binucleate *Rhizoctonia* strains enhanced considerably root growth of Scots pine and Norway spruce (*Picea abies*) seedlings (Gronberg et al., 2006). Also *Trichoderma harzianum* and *Laccaria laccata* when inoculated to blue pine seedling roots improved growth and biomass of the host (Ahangar et al., 2012). Moreover, dark septated root endophytes in Scots pine seedlings growing under elevated CO_2 concentrations produced an increase of nutrient use efficiency and a slight carbon gain (Alberton et al., 2009). Interestingly, also the diversity of microbes living in tree organs may be related to the growth rate of their host tree as shown by Korkama et al. (2006) who found higher species richness and diversity of ectomycorrhizal infections in roots of fast than in slow growing clones of Norway spruce.

Some fungal and bacterial species associated to plant tissues are able to produce plant growth regulators (Rim et al., 2005; Hamayun et al., 2009a; 2009b; Ahmad et al., 2010; You, 2012; Kang et al., 2014). Production of plant growth regulators can proceed jointly by both the host and the endophyte. Cottonwood (*Populus trichocarpa*) harbors yeasts which are able to enhance seedling growth by producing indol acetic acid, IAA, a plant growth regulator. For producing IAA, the yeast needs L-tryptophan, provided by the host (Furukawa et al., 1996).

The first step to investigate the interaction of endophytes with a certain host species is to find out what endophytic species thrive in various tissues and how abundant they are. Variation of the endophytic community between host individuals and different environments may give clues on the interaction between the host species and its various inhabitants. The aim of this study was 1) to characterize the endophytic community of *Pinus sylvestris* twigs, and 2) to find out if the structure of this community is related to the growth rate of Scots pine. P. sylvestris was chosen because of its high ecologic and economic significance in European forests. We tested the hypotheses that 1) Scots pine twigs inhabit a number of culturable fungal species not previously described in this host species and that 2) the endophytic community of fast and slow growing trees differs.

MATERIALS AND METHODS

STUDY AREA AND SAMPLING METHOD

The study was carried out in 2005 at Palencia province (northern Spain). The study area is located between UTM coordinates 4.685.000 to 4.741.000 (latitude) and 342.000 to 398.000 (longitude), where the altitude ranges from 800 to 1000 m.a.s.l., the climate is Mediterranean with a slight Atlantic influence, mean temperature is 11.5 ºC and annual rainfall 519 mm. This area represents a transition from agricultural lands (southwards) to Cantabric Mountains (northwards), where forest lands cover 32% of the surface, consisting of conifer, broadleaved and mixed forests including riparian sites, and shrublands (Sanz-Ros et al., 2008).

Fifteen circular Scots pine plots (Ø 15 m) were selected from the Spanish National Forest Inventory grid (NFI) (Figure 1). The selected Scots pine stands were uniform in age and height (20-32 y and 7.4-13.0 m, respectively) but varied in basal area (9.13-30.3 m^2/ha) and density (187-1368 stems/ha). Stand characteristics are presented in Table 1.

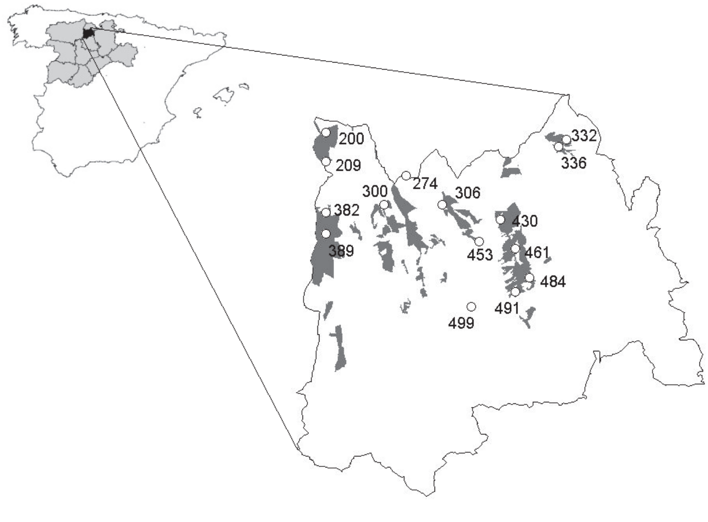

Figure 1: Location of the study area and sampling plots (O). In the left Spain and Balear Islands are represented, Castilla y León Region is showed in grey and the study area in black, located at Palencia Province. In the right grey areas represent Pinus sylvestris distribution, and numbers are Spanish NFI plot codes.

For twig sampling two healthy trees were randomly chosen from each plot, thus the intensity of the sampling was 1 tree/90 m2. Tree growth rate and age were measured from increment cores taken with a Pressler borer at 1,3 m height, mounted, sanded, and analyzed with the WinDendro® scanning software (Regent Instruments Canada Inc). Scanning results were used for calculation of tree diameter (without bark) (D), age of the tree, diametric growth rate (DGR) and relative diametric growth rate (RDGR), as shown in equations 1 to 3. Since the endophyte community is not likely to be influenced solely by the current year growth, both growth variables were calculated also for the last 5, 10 and 15 years before this study (Table 2). Equations for applied variables are as follows:

$$D_i = (\textstyle\sum_1^n Tree\ ring\ width)/N \qquad [Eq.1]$$

$$DGR_i = (D_n - D_i)/n - i \qquad [Eq.2]$$

$$RDGR_i = (D_n - D_i)/D_i \qquad [Eq.3]$$

Where n is the tree age in years, N is the number of trees, and i represents a reference age of the tree n-i years ago. Four healthy twigs with a diameter of 0.2-1.5 cm were cut from each sampling tree from the external part of the crown at 3-4 m above ground level during summer 2005. Each twig was divided with a sterilized knife into 1.5 cm segments, and three of them were randomly chosen for fungal isolation, resulting in 12 segments per tree. Twigs were stored at 4ºC and processed within the next 48 h. Summarizing, from each plot eight different twigs were selected from two different trees, of which 24 segments were randomly chosen for fungal isolation, making a total of 360 segments obtained from 30 different trees.

Table 2: Fungal endophytic community characteristics and Scots pine growth parameters.

Variable	Units	Description
Richness	Number of species	Number of endophytic species
DSim	-	Simpson's Diversity Index
ESim	-	Simpson's Evenness Index
DSha	-	Shannon's Diversity Index
ESha	-	Shannon's Equitability Index
Age	years	Averaged plot age
D5	mm	Tree diameter 5 years ago (without bark)
D10	mm	Tree diameter 10 years ago (without bark)
D15	mm	Tree diameter 15 years ago (without bark)
DGR5	mm/year	Diametric growth rate of the last 5 years period
DGR10	mm/year	Diametric growth rate of the last 10 years period
DGR15	mm/year	Diametric growth rate of the last 15 years period
RDGR5	mm/year	Relative diametric growth rate of the last 5 years
RDGR10	mm/year	Relative diametric growth rate of the last 10 years
RDGR15	mm/year	Relative diametric growth rate of the last 15 years
DGR5cat	-	Categorized DGR5
DGR10cat	-	Categorized DGR10
DGR15cat	-	Categorized DGR15
RDGR5cat	-	Categorized RDGR5
RDGR10cat	-	Categorized RDGR10
RDGR15cat	-	Categorized RDGR15
Density	Trees/ha	Plot density of trees per hectare
BA	m^2/ha	Basal area of plot
MH	meters	Mean height of plot
DH	meters	Dominant height of plot

ISOLATION AND MORPHOGROUPING OF THE ISOLATES

Twig segments were surface sterilized by shaking them for 1 minute in 96% ethanol, 5 minutes in 6% sodium hypochlorite, 1 minute in 70% ethanol, and finally two times for 2 minutes in sterile distilled water. Thereafter the segments were placed on Petri dishes of 9 cm diameter (4 segments per plate) containing a generalist medium for fungal culture, potato dextrose agar (PDA; Scharlau®), previously autoclaved for 20 minutes at 121 ºC. Cultures were incubated in darkness at 23 ºC.

After 4 days all outgrowing fungi were transferred by taking a ca. 9 mm^2 piece of agar from the edge of each colony to fresh medium, and during one month a weekly check was carried out in order to find new colonies. Fungal isolates were counted and stored at 4ºC. Finally, assemblages were grouped according to colony morphology on PDA, and they are called here as “colonial morphotypes” (CMs). Seventy eight CMs were distinguished based on colony colour, size, texture and presence of aerial hyphae (Wang et al. 2005).

The relative abundance (RA) of each fungal taxon (i.e. species identified by molecular means) is calculated as the number of fungal isolates expressed as percents of the total number of twig segments investigated from a plot.

IDENTIFICATION OF ISOLATES

The CMs were identified by molecular methods as follows. One isolate of each CM was transferred to a cellophane membrane (Surface Specialities, Wigton Cumbria, UK) on MOS-agar (Müller et al., 1994), and cultivated at 19 ºC in darkness for a week. Total DNA was isolated according to Vainio et al. (1998) including minor modifications. The protocol included I) cell disruption with quartz sand using a FastPrep® cell disrupter (Qbiogene, Inc., Cedex, France) for 2 x 20 s, II) two or three phenol:chloroform:isoamyl alcohol (25:24:1) and one chloroform:isoamyl alcohol (24:1) extraction (a third extraction with phenol:chloroform:isoamyl alcohol was done when dark pigments still existed in the extract after two extractions), III) precipitation with polyethylene glycol (PEG) and drying, and IV) resuspension of the DNA in 50 µl of TE-buffer (pH 8.0), containing 10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA).

The internal transcribed spacer (ITS) region of the rDNA was amplified with primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) according to White et al. (1990) and Vainio and Hantula (2000). Shortly, 1 µM of each primer, 0.4 mM of each deoxynucleotide (dNTP), 1 U of Dynazyme II Taq polymerase II (Finnzymes Ltd, Espoo, Finland), 1x Dynabuffer and 1 µl of template were mixed to 50 µl and subjected to a thermal program consisting of 10 min at 95 ºC, followed by 13 cycles at 95 ºC for 35 seconds (s), at 55 ºC for 55 s and at 72 ºC for 45 s, 13 cycles at 95 ºC for 35 s, at 55 ºC for 55 s, and at 72 ºC for 2 min, and finally 13 cycles at 95 ºC for 35 s, at 55 ºC for55 s and at 72 ºC for 3 min. PCR products were purified with the Jet Quick PCR Purification Spin Kit (Genomed, Löhne, Germany), according to the manufacturer's instructions.

DNA samples were sequenced with the automated sequencing apparatus (Li-Cor Global Edition IR2 System; Li-Cor Inc., Lincoln, NE, USA), using the Therm EXCEL™ II DNA Sequencing Kit-LC (Epicentre®; Madison, WI, USA) and following the manufacturer's instructions. Finally, the sequences were aligned with LI-COR software (ALIGN IR version 2.0). Consensus sequences were identified by blasting them against sequences deposited in GenBank (NCBI) database (Benson et al., 2002). The fungal assemblages were named according to the best GenBank match if at least 97.2 % matched with a sequence consisting of at least 393 bp. Sequences generated in this study have been submitted to GenBank (Table 3).

A maximum parsimony tree was constructed from the sequences in order to reveal the phylogenic relationships of the isolated endophytes. The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000) with search level 3, in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method (Nei & Kumar, 2000) and are in the units of the number of changes over the whole sequence. All alignment gaps were treated as missing data. There were a total of 642 positions in the final dataset, out of which 418 were parsimony informative.

For *Biscogniauxia mediterranea* sequences comparison, a Neighbor-Joining tree was built. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei & Kumar, 2000) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 641 positions in the final dataset. All Phylogenetic analyses were conducted in MEGA 4 (Tamura et al. 2007).

STATISTICAL ANALYSIS

Diversity of endophytes was obtained for each plot by calculation of the Shannon Diversity Index H (DSha, Eq. 4) and Simpson Diversity Index D (DSim, Eq. 5). Also species evenness was estimated calculating Shannon equitability index J (ESha, Eq.6) and Simpson equitability index E (ESim, Eq. 7) (Begon et al. 2006).

$$H=-\sum_{i=1}^{s} p_i * \ln p_i \qquad \text{Eq. [4]}$$

$$D= 1/\sum_{1}^{s} p_i^2 \qquad \text{Eq. [5]}$$

$$J= H/\ln S \qquad \text{Eq. [6]}$$

$$E= (1/\sum_{1}^{s} p_i^2)/S \qquad \text{Eq. [7]}$$

Where pi is the proportion of the i^{th} species and S is plot species richness. The species accumulation curves (rarefaction curves) and bootstrap estimates of total richness were inferred using the software EstimateS, version 9 (Colwell 2011).

Sample-based rarefaction curves were computed according to Gotelli & Colwell (2001), and the 95% confidence intervals according to Colwell et al. (2004). For inferred richness several non-parametric estimators were computed: Mao Tau Estimator (Colwell et al., 2004), Incidence-based Coverage Estimator of species richness, ICE (Chazdon et al. 1998; Chao et al., 2000), First and Second order Jackknife richness estimator, Jack 1 and Jack 2 (Burnham & Overton, 1978; Smith & van Belle, 1984), and Chao 1 and Chao 2 estimators (Chao, 1984; 1987).

For multivariate analysis we excluded those fungal taxa which were isolated less than 4 times. Principal Component analysis (rotated with varimax normalized) was used with the aim of reducing data dimensionality and to test which factors explained best the system variance. Variables included were summarized and described in Table 2.

Canonical correspondence analysis (CCA) was carried out to investigate if endophyte assemblages differed according to diametric growth (D, DGR and RDGR, see Table 2) and age. The statistical significance was tested by means of a Monte Carlo permutation test with 499 permutations of total inertia. Statistical analyses were carried out using the software Canoco 4.5 (Ter Braak & Šmilauer, 2002) and Statistica (release 6.0, Tulsa, KA, USA), both software were also used for building the figures included in the results section.

One-way analysis of variance (ANOVA) was used to determine the relationship between the relative abundance of a specific fungal species and the diametric growth rate (DGR) and relative diametric growth rate (RDGR). The variables were categorized into 3 levels (low, medium and high) so that medium level corresponded to mean ± SD, and the rest of values were assigned to low or high. Differences in growth among DGR categories were assessed by using ANOVA and Tukey HSD post-hoc multiple range comparison test.

Alignment of multiple DNA sequences and cluster analyses were done with Mega 4.0 software (Center for Evolutionary Functional Genomics, Biodesign Institute, Arizona State University). DNA alignments were submitted to TreeBase, submission number 16763.

RESULTS

Fungal isolates were obtained from all investigated Scots pine twigs. A total of 360 isolates were assigned into 77 different CMs and one representative of each CM was characterized by ITS sequencing. Ten CMs could not be identified due to their stunted growth and death or in a few cases due to bacterial contamination.

The number of these unidentified isolates represented 6% of all isolates. In average 2.3 colonial morphotypes represented one fungal taxon as 33 species were identified by blasting the ITS sequences in GenBank (Table 3). All identified species were Ascomycetes. Summarizing, 43 taxa were counted (including 33 ITS-sequences and 10 unknown CM groups). Sequences were included in a maximum parsimony tree to reveal their grouping into taxonomic units (Figure 2).

Table 3: Fungal endophytic taxa isolated from Pinus sylvestris twigs and identified according to BLAST of their ITS-sequences. The isolates are listed according to decreasing relative abundance (RA).

Fungal taxon[a]	Short Name	Class	Order	AN[b]	QC[c]	MI[d]	RA
Phoma herbarum Westend.	PhoHer	Dothideomycetes	Pleosporales	JX421706	100	99.1	36
Hormonema dematioides Lagerb. & Melin	HorDem	Dothideomycetes	Dothideales	JX421710	99.3	99.5	34
Alternaria infectoria E.G. Simmons	AltInf	Dothideomycetes	Pleosporales	JX421701	100	98.2	31
Biscogniauxia mediterranea (De Not.) Kuntze	BisMed	Sordariomycetes	Xylariales	JX421711	100	99.3	26
Hypocrea lixii Pat.	HypLix	Sordariomycetes	Hypocreales	JX421742	100	99.5	20
Leotiomycetes sp.	Leosp	Leotiomycetes	Unknown	JX421713	83.1	98	20
Alternaria alternata (Fr.) Keissl.	AltAlt	Dothideomycetes	Pleosporales	JX421734	100	100	20
Penicillium glabrum (Wehmer) Westling	PenGla	Eurotiomycetes	Eurotiales	JX421729	99.3	99.1	18
Trichoderma atroviride P. Karst	TriAtr	Sordariomycetes	Hypocreales	JX421707	99.6	99.7	17
Pezicula eucrita (P. Karst.)	PezEuc	Leotiomycetes	Helotiales	JX421708	99.8	100	16
Fusarium verticillioides (Sacc.) Nirenberg	FusVer	Sordariomycetes	Hypocreales	JX421719	99	99	12
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not	SorFim	Sordariomycetes	Sordariales	JX421743	100	99.7	11
Phaeomoniella effusa Damm & Crous	PhaEff	Chaetothyriomycetes	Chaetothyriales	JX421744	87.3	99.4	9
Aureobasidium pullulans (De Bary) G. Arnaud ex Cif., Ribaldi & Corte	AurPul	Dothideomycetes	Dothideales	JX421738	100	100	8
Beauveria bassiana (Bals.-Criv.) Vuill.	BeaBas	Sordariomycetes	Hypocreales	JX421709	99.8	99.5	6
Phoma macrostoma Mont.	PjoMac	Dothideomycetes	Pleosporales	JX421747	100	99.1	6
Geomyces sp.	GeoPan	Leotiomycetes	Not assigned	JX421704	90.6	100	6
Ophiostoma nigrocarpum (R.W. Davidson) de Hoog	OphNig	Sordariomycetes	Ophiostomatales	JX421721	100	100	5
Unidentified CM 64	UnkH64	-	-	-	-	-	5
Unidentified CM 66	UnkH66	-	-	-	-	-	5
Hypocrea viridescens Jaklitsch & Samuels	HypVir	Sordariomycetes	Hypocreales	JX421750	100	99.3	4
Fimetariella rabenhorstii (Niessl) N. Lundq.	FimRab	Sordariomycetes	Sordariales	JX421715	99.6	99.8	4
Sarocladium strictum (W. Gams) Summerbell	SarStr	Sordariomycetes	Hypocreales	JX421705	98.6	99.5	4
Lophodermium conigenum (Brunaud) Hilitzer	LopCon	Leotiomycetes	Rhytismatales	JX421746	100	97.2	4
*Sarea resinae (*Fr.) Kuntze	SarRes	Lecanoromycetes	Agyriales	JX421720	95.1	99.2	4
Unidentified CM 80	UnkH80	-	-	-	-	-	4
Trichoderma viride Pers.	TriVir	Sordariomycetes	Hypocreales	JX421740	100	99.1	3
Penicillium melinii Thom	PenMel	Eurotiomycetes	Eurotiales	JX421739	100	100	3
Daldinia fissa Lloyd	DalFis	Sordariomycetes	Xylariales	JX421716	98.9	98.9	2
Alternaria tenuissima (Nees) Wiltshire	AltTen	Dothideomycetes	Pleosporales	JX421745	100	99.8	2
Unidentified CM 77	UnkH77	-	-	-	-	-	2
Xylaria sp.	Xylsp1	Sordariomycetes	Xylariales	JX421748	100	98.4	1
Nigrospora oryzae (Berk. & Broome) Petch	NigOry	Sordariomycetes	Trichosphaeriales	JX421751	100	99.8	1
Plectania milleri Paden & Tylutki	PleMil	Pezizomycetes	Pezizales	JX421749	99.7	98	1
Penicillium polonicum K.M. Zalessky	PenPol	Eurotiomycetes	Eurotiales	JX421702	100	100	1
Penicillium minioluteum Dierckx	PenMin	Eurotiomycetes	Eurotiales	JX421741	100	99.8	1
Aspergillus fumigatus Fresen.	AspFum	Eurotiomycetes	Eurotiales	JX421732	100	99.5	1
Unidentified CM 33	UnkH33	-	-	-	-	-	1
Unidentified CM 82	UnkH82	-	-	-	-	-	1
Unidentified CM 84	UnkH84	-	-	-	-	-	1
Unidentified CM 87	UnkH87	-	-	-	-	-	1
Unidentified CM 88	UnkH88	-	-	-	-	-	1
Unidentified CM 89	UnkH89	-	-	-	-	-	1

[a] Closest related sequences in GenBank. Fungal species nomenclature follows National Center of Biotechnology information.

[b] GenBank accession number for ITS rDNA sequences.

[c] Query coverage.

[d] Maximum identity.[e] Relative abundance

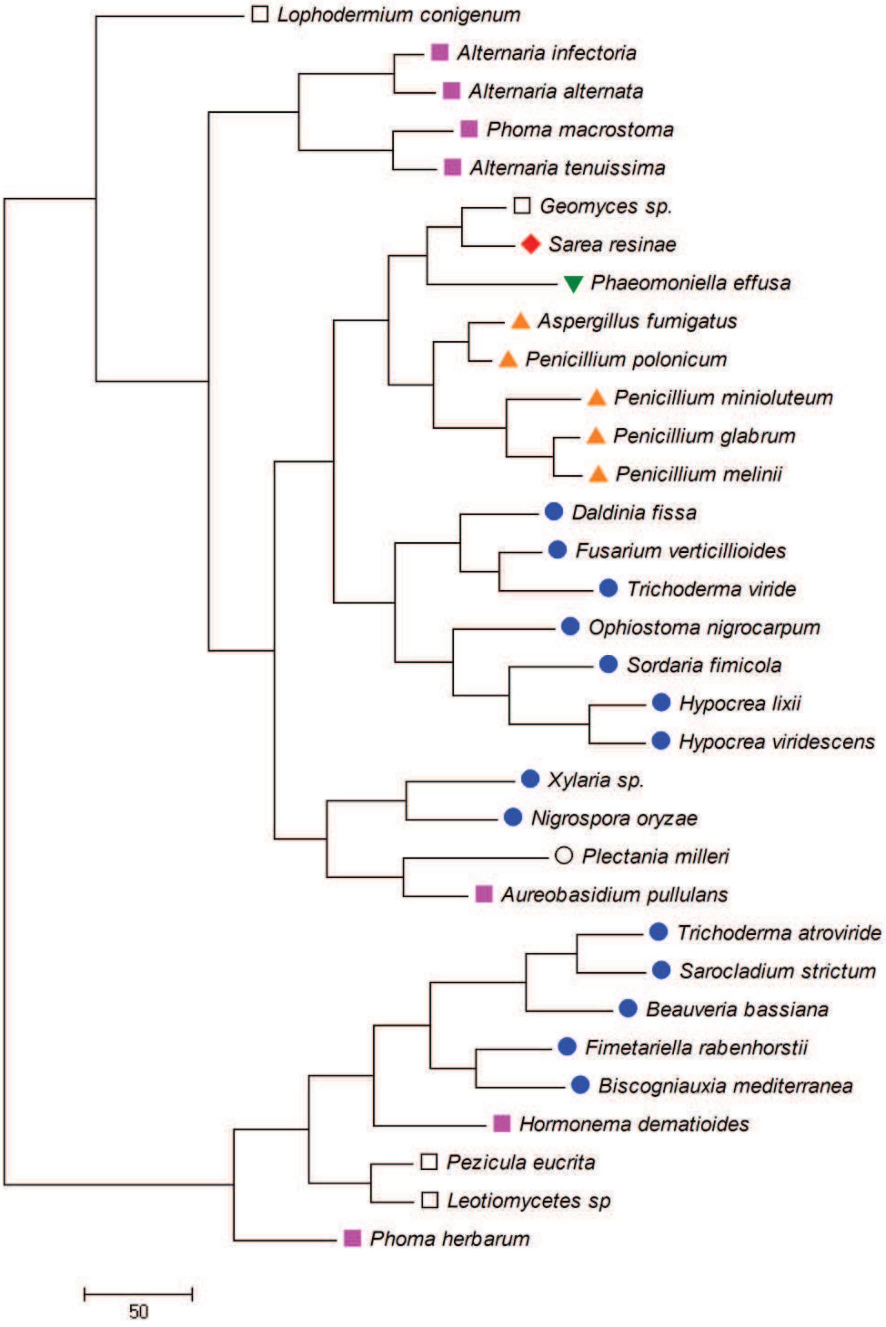

Figure 2: Maximum parsimony tree of the 33 different ITS -sequences found among all 360 endophytic isolates. Their best GenBank match is given and taxonomic class denoted by symbols (●=Sordariomycetes; ■=Dothideomycetes; ▲=Eurotiomycetes: ▼=Chaetothyriomycetes; ☐=Leotiomycetes: ◆=Lecanoromycetes; ○=Pezizomycetes). Branch lengths are given as numbers of changes over the whole sequence (scale given below the tree).

The most frequently isolated class was Dothideomycetes (43.1% of total number of isolates) followed by Sordariomycetes (32.1%), Eurotiomycetes (7.7%), Leotiomycetes (7.1%), Chaetothyriomycetes (2.5%), Lecanoromycetes (1.1%) and Pezizomycetes (0.3%) (Figure 3).

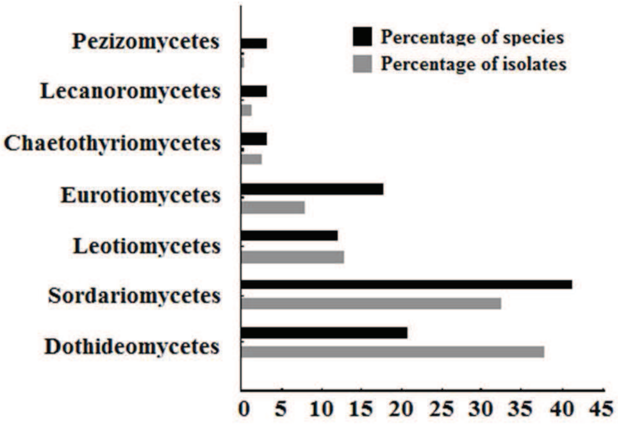

Figure 3: Characterization of Classes, showing relative abundance of total isolates collected orders and species of each class within Phylum Ascomycota, Subphylum Pezizomycotina.

The most frequently isolated species were *Phoma herbarum, followed by Hormonema dematioides, Alternaria infectoria, Biscogniauxia mediterranea, Hypocrea lixii, Leotiomycetes sp, Alternaria alternata* and *Penicillium glabrum*. These species were found in 27-60% of plots (i.e. in 4-9 of the 15 plots), and they represented 63% of the total number of isolates obtained. The number of fungal taxa obtained from individual trees ranged from 3 to 13, and plots from 4 to 16. From the 43 fungal taxa, 18 appeared solely in one plot and were named as “rare fungi”, representing 41.9% of all fungal taxa, but just 7.7 % of the 360 fungal isolates obtained. The 25 remaining taxa were referred to as “frequent fungi” and they represented 58.1% of all found taxa and 92.3 % of total fungal isolates, and were used for multivariate analysis. Rare taxa in each plot ranged from 0 to 5, and after removing them, the number of frequent taxa per plot still ranged from 3-13.

The class showing the highest percentage of fungal species was I) Sordariomycetes: including orders Hypocreales, Ophiostomatales, Sordariales, Trichosphaeriales and Xylariales. The second most numerous class was II) Dothideomycetes: including the orders Pleosporales and Dothideales, followed by class III) Eurotiomycetes: all belonging to the Order Eurotiales, and class IV) Leotiomycetes: including orders Helotiales, Rhytismatales and Leotiomycetes Incertae Sedis. Finally, the three less frequent classes were V) Chaetothyriomycetes, VI) Lecanoromycetes and VII) Pezizomycetes, including orders Chaetothyriales, Agyriales and Pezizales respectively.

The taxon accumulation curve (Figure 4) showed that our sampling intensity provided a reasonable estimate of fungal endophytes richness of the twigs *of Pinus sylvestris* in the study area. The actual species number was estimated to be 63, 67, 73, 76 and 90 using Jack1, ICE, Chao 1, Jack 2, and Chao 2 estimates respectively. This means that the 43 taxa found here represent ca. half of the species richness actually present. Fungal diversity and evenness varied largely between the sampling plots (Table 1).

Table 1: Mean stand characteristics of the Scots pine sampling plots and properties of the endophytic fungal community isolated from Scots pine twigs.

Scots pine stand characteristics						Endophytic fungi of Scots pine twigs				
Plot	Species[a] composition	Age (years)	Height (m)	Basal Area (m^2/ha)	Density (tres/ha)	Species Richness	Simpson Diversity	Simpson Evenness	Shannon Diversity	Shannon Evenness
P200	Pure Ps	27	12.2	34.5	987	12	9.00	0.75	4.67	1.88
P209	Ps, Pp, Pn	32	11	12.9	249	16	10.24	0.64	5.16	1.86
P274	Ps, Pn	20	8.9	12.3	516	7	5.83	0.83	3.69	1.90
P300	Ps, Pn	26	11.6	30.3	1022	11	6.19	0.56	4.21	1.75
P306	Ps, Qp	30	9.3	10.4	1085	13	7.52	0.58	4.56	1.78
P332	Ps, Pn, Qp	25	10	21.5	704	4	1.94	0.49	1.87	1.35
P336	Ps, Pn	31	13	24.6	690	13	6.03	0.46	4.29	1.67
P382	Ps, Qp, Pn	32	10.6	21.5	1093	7	6.00	0.86	3.73	1.92
P389	Pure Ps	32.5	7.4	20.1	1368	8	5.48	0.69	3.56	1.71
P430	Ps, Qp	29	11.2	18.6	810	9	7.72	0.86	4.21	1.91
P453	Ps, Pn	33.5	12.3	26.6	679	7	3.59	0.51	3.07	1.58
P461	Pure Ps	22	9.6	9.1	187	13	9.00	0.69	4.74	1.85
P484	Ps, Pn	28	12	27.6	704	8	5.19	0.65	3.21	1.54
P491	Ps, Pn	26.5	10.6	21	584	10	5.42	0.54	3.93	1.71
P499	Ps, Pn	25	8.4	23.4	1206	8	6.37	0.80	3.92	1.88

a. Tree Species are showed by its dominance in each plot. Ps= *Pinus sylvestris*; Pn=*Pinus nigra*; Pp= *Pinus pinaster*; Qp= *Quercus pyrenaica*.

The Shannon diversity index (DSha) ranged from 1.87 to 5.16 and Simpson diversity index (DSim) from 1.94-10.24. Evenness varied in the range 1.35-1.92

(Shannon's Equitability index, ESha) and 0.46-0.86 (Simpson's Equitability index, ESim).

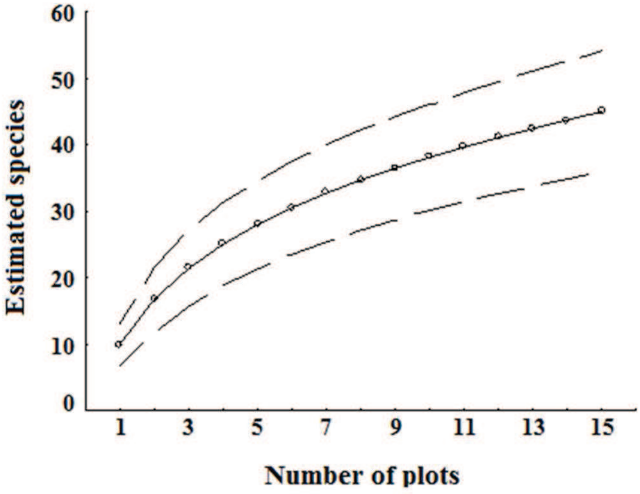

Figure 4: Taxon accumulation curve of fungal endophyte species as a function of the number of P. sylvestris plots sampled. Solid line: number of fungal taxa calculated by the Mao Tau estimator. Dashed lines: upper and lower 95 % confidence limits of the estimate of taxa number. The curve is based on 50 randomizations of sample order.

A principal component analysis (PCA) including endophyte isolation frequencies and tree characteristics in each plot revealed a relation between tree growth and the endophytic community structure. PCA yielded three factors (Figure 5) which accounted 69 % of total variance. The first principal component (explaining 30.4% of variance) separates accumulated growth (D5, D10 and D15) from tree relative growth rate variables (RDGR5, RDGR10 and RDGR15). The second principal component (explaining 22.9% of variance) separates plot diametric growth rate variables (DGR15, DGR10 and DGR5) from variables of fungal evenness (ESim).

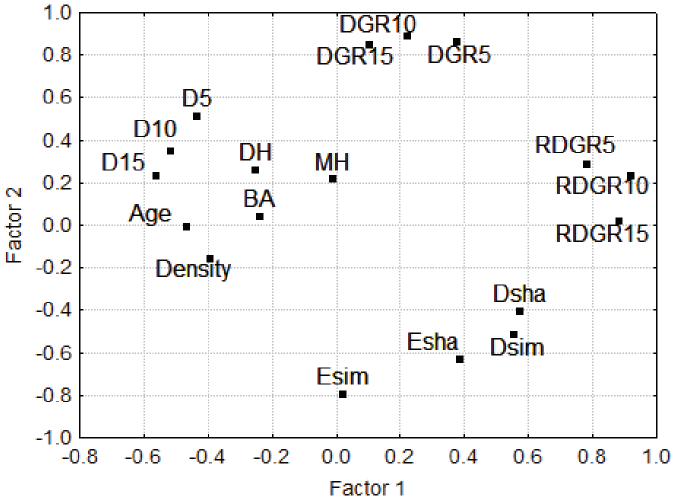

Figure 5: Relation between twig endophyte community characteristics and host tree growth features as revealed by principal component analysis. Host tree variables: BA= Basal area; MH= Mean height; DH= Dominat height; D5, D10 and D15= Plot average stem diameter 5,10 and 15 years ago, respectively; DGR5, DGR10 and DGR15= Plot average annual diametric growth during last 5, 10 and 15 years, respectively; RDGR5, RDGR10 and RDGR15= Plot relative diametric growth rate during last 5, 10 and 15 years, respectively. Endophyte community variables: Richness= number of fungal taxa/plot; DSha= Shannon's diversity index; DSim= Simpson's diversity index; ESha= Shannon's equitability index; ESim= Simpson's equitability index.

Factor 1 in Fig. 5 suggests a correlation between diversity (Richness, Dsha and Dsim) and relative diametric growth (RDGR) or negative correlation with tree size (D). In regression analyses, however, no significant relationships were found between fungal diversity and tree growth, size, basal area or tree density. Factor 2 suggests that trees with high diametric growth rate (DGR) harbour a higher proportion of dominating fungal species (low evenness, Esha and Esim) compared to trees with low DGR, in which the abundance of fungal species is more even. This results were supported by regression analysis between evenness and DGR ($p<0.05$). On the other hand, regression analyses did not showed significant relationships between fungal evenness and diameter, basal area or tree density.

The interrelationship between host diametric growth and the endophytic community structure of twigs is also revealed by a canonical correspondence analysis (CCA). Monte Carlo permutation test revealed significant relationships between diametric growth rate of the host tree (DGR) and the presence of certain endophyte species, some of which show a trend to be associated to high growth (Figure 6, lower circle cluster) and other to low growth (Figure 6, upper circle cluster).

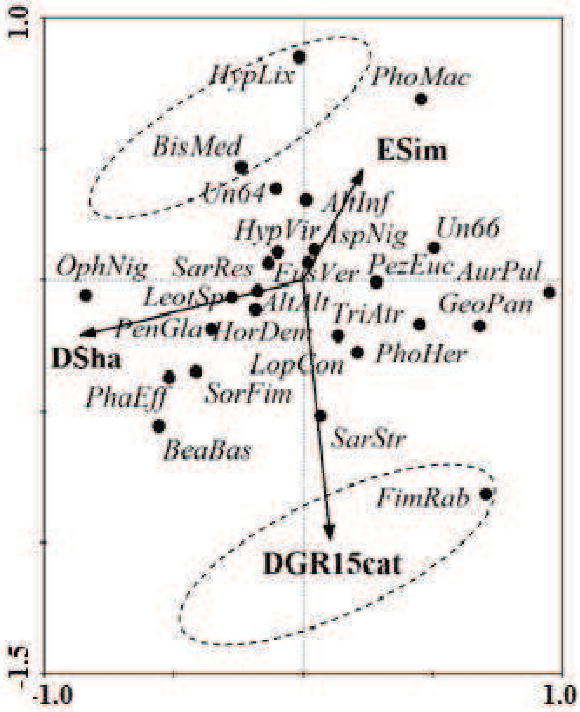

Figure 6: Biplot between fungal species and plot variables calculated by a canonical correspondence analysis (CCA) using all variables described in Table 2. Black circles and abbreviated names in italics represented fungal species (Table 3), and vectors with bold names represented the following plot variables: DSha= Shannon's diversity index; DGR15= Diametric growth rate of the trees (categorized); ESim= Simpson's equitability. Dash line ellipses indicate fungal endophytes associated to high diametric growth (lower ellipse) or low diametric growth (upper ellipse).

The presence of three endophytic species in twigs correlated significantly with DGR15 of the host tree. Both *Fimetariella rabenhorstii* and *Phoma herbarum* occurred more frequently in fast growing trees (p=0.008 and p=0.051, respectively, ANOVA). However, only four isolates of *F. rabenhorstii* (Table 2) were encountered and therefore this result should be verified with a larger data set. On the other hand, *Hypocrea lixii* was significantly associated (p=0.014) to trees with low diametric growth rate.

We examined the sequences identified as *B. mediterranea* more closely because this species was one of the most frequent ones in this study (Table 3) and because it is a well-known pathogen of oaks and has also been found in some other hosts, such as poplar, ash and apple trees (Figure 7). The twenty six isolates identified as *B. mediterranea* were grouped initially in four CMs, and four ITS-sequences were obtained, differing maximally by 14 base pairs. To visualize the relationship of our sequences with those of isolates occurring on other host species we ran a neighbor joining tree of our sequences together with other ITS-sequences deposited in GenBank (Figure 4).

Isolates obtained from Scots pine in this study are closely related to isolates from insects in Portugal, three of the CMs (corresponding to accession numbers JX421711, JX421730 and KP113664) representing 24 isolates, are closely related to those obtained from isolates of insects retrieved from a documents archive in Portugal and from *Quercus castaneifolia* forest in Iran, while the fourth CM (KP113665) is more related to isolates obtained from *Populus x euramericana* in Spain, but different to the other three CMs.

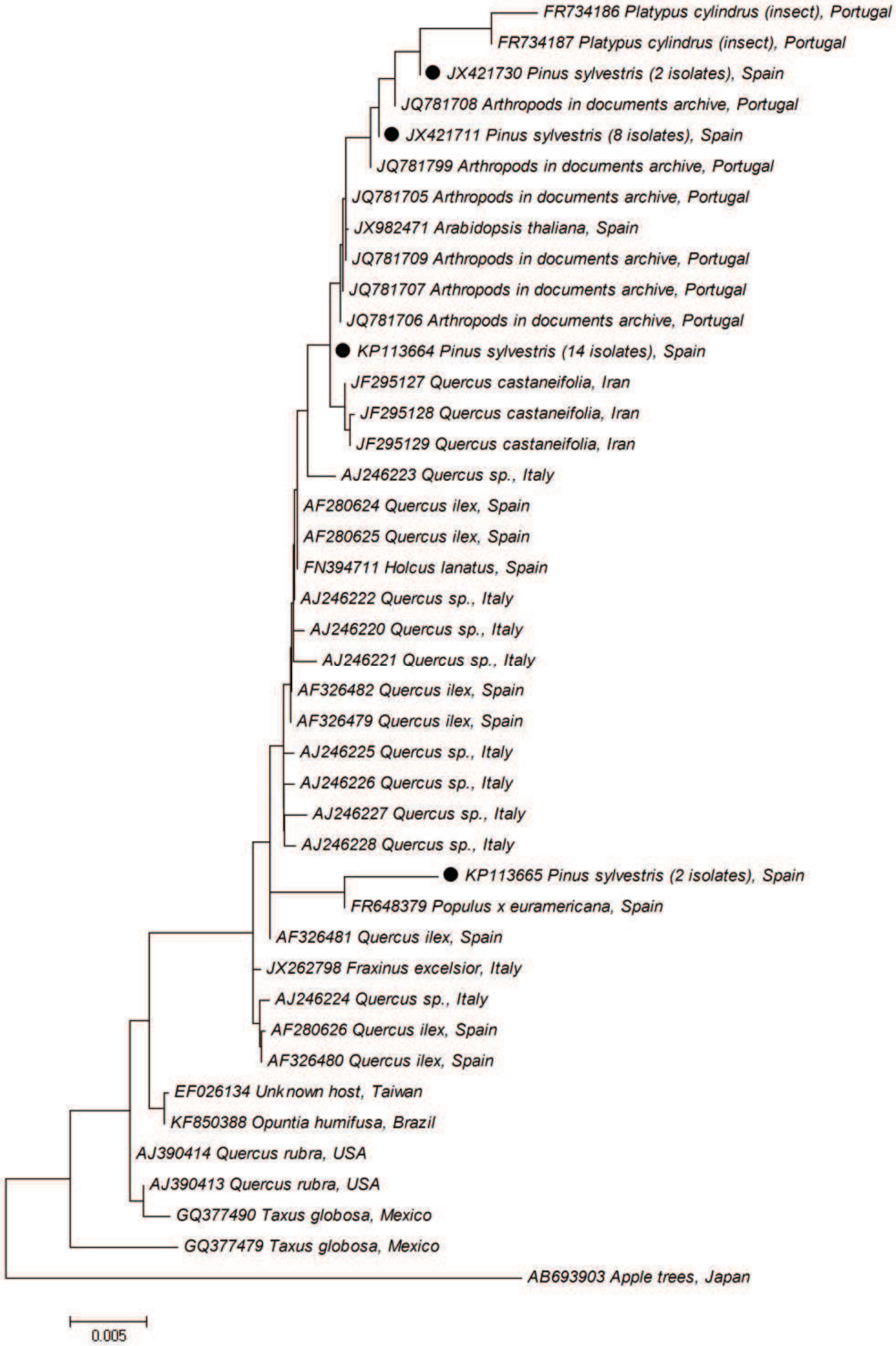

Figure 7: Neighbour joining tree (Bootstrap 1000 replicates) of *Biscogniauxia mediterranea* ITS sequences deposited in GenBank. Four of them were obtained in this study from pine and are indicated with a black dot (●). Each of them represents one colony morphotype and the number of total isolates is given in parenthesis. The branch lengths refer to the number of base substitutions per site (scale given below the tree).

DISCUSSION

A diverse fungal community including forty three different fungal endophytic taxa was found from twigs of healthy Scots pines in northern Spain. We found nine new fungal species not earlier described as endophytes of *P. sylvestris* supporting our first hypothesis. Species composition showed to differ between fast and slow growing pines and some of the species were found to be associated to tree diametric growth, Hence, both of our initial hypotheses were supported. Low fungal evenness and presence of *Phoma herbarum* are associated with high diametric growth, while high fungal evenness and presence of *Hypocrea lixii* are related to low diametric growth of their host trees.

The fungi identified in this study that have already been described as Scots pine endophytes in earlier reports were *Alternaria alternata, A. infectoria, A. tenuissima, Aspergillus fumigatus, Aureobasidium pullulans, Beauveria bassiana, Fimatariella rabenhorstii, Fusarium verticillioides, Geomyces sp., Hypocrea lixii, H. viridescens, Hormonema dematioides, Lophodermium conigenum, Phoma herbarum, P. macrostoma, Sarocladium strictum, Sordaria fimicola, Penicillium glabrum, P. minioluteum, Sarea resinae, Trichoderma atroviride, T. viride* and *Xylaria* sp (Cooke, 1973; Carroll et al., 1977; Russo & Bradley, 1979; Diwani & Millar, 1987; Land et al., 1993; Kubátová, 2000; Anderson et al., 2003; Pirttilä et al., 2003; Lygis et al., 2004; Kwasna, 2008; Zamora et al., 2008; Giordano et al., 2009; Peršoh et al., 2010; Menkis & Vasaitis, 2011; Terhonen et al., 2011), while the following species have been found in other pine species: *Daldinia fissa, Nygrospora oryzae, Ophiostoma nigrocarpum, Penicillium melinii, P. polonicum* and *Pezicula eucrita* (Smith, 1965; Carroll & Carroll, 1978; Kowalski & Zych, 2002; Guo et al., 2003; Kim et al., 2005; Wang et al., 2005; Zamora et al., 2008; Botella & Diez, 2010; Kowalski & Andruch, 2011).

Hence, 29 out of all 33 fungal taxa (excluding the 10 unidentified colonial morphotypes) obtained in this study have been earlier reported from pines, while 22 out of 33 were previously reported in Scots pine, so that nine species (27.3 % of identified taxa) were reported, to our knowledge, for the first time to inhabit Scots pine: *Biscogniauxia mediterranea, Daldinia fissa, Hypocrea viridescens, Nygrospora* *oryzae, Ophiostoma nigrocarpum, Phaeomoniella effusa, Penicillium melinii, P. polonicum* and *Plectania milleri*. Three of them *B. mediterranea, Phaeomoniella effusa and Plectania milleri* are new endophytic species for the Pinaceae.

Fifteen species found here can be regarded as host-generalists because they have been described also from broadleaved trees*: Alternaria alternata, A. tenuissima, Aureobasidium pullulans, Beauveria bassiana, Biscogniauxia mediterranea, Fusarium oxysporum, Hypocrea lixii, Hormonema dematioides, Nygrospora oryzae, Penicillium glabrum, Sarocladium strictum, Sordaria fimicola, Trichoderma harzianum, T. viride* and *Xylaria* sp. (Chapela, 1989; Fisher & Petrini, 1992; Bettucci & Alonso, 1997; Collado et al., 1999; Santamaría & Diez, 2005; Gonthier et al., 2006; Jaklitsch et al., 2006; Unterseher & Schnittler, 2010 Martín-García et al., 2011;). Therefore, half of the taxa inhabiting Scots pine twigs were host-generalists, and the other half were coniferous or pine specialists. In this sense, dominant endophytic species are usually presumed to be host-specialists because each host species usually harbours a characteristic assemblage of endophytic fungi (Saikkonen, 2007).

Regarding fungal classes distribution, our result is in accordance with earlier studies (Botella & Diez, 2010; Zamora et al., 2008) showing that the fungal community of the canopy of several pine species in northern Spain is dominated by Ascomycetes and more specifically, by the classes Dothideomycetes and Sordariomycetes, which represented 46% and 34% of total isolated fungi. Studies on pine species from other countries also showed them as the more frequently isolated classes (Ganley & Newcombe, 2006; Arnold et al., 2007).

The species richness and diversity found in this study for twig endophytes of *Pinus sylvestris* is similar to those obtained in previous studies on pine twigs (Table 4) (Zamora et al., 2008; Botella & Diez, 2010; Martínez-Álvarez et al., 2012;), and higher than in pine needles or cones (Ganley & Newcombe, 2006; Arnold et al., 2007; Peršoh et al., 2010), but lower than those obtained from roots or sapwood (Giordano et al., 2009; Menkis & Vasaitis, 2011).

Table 4: Summary of the number of fungal endophytes identified in previous studies in different forest tree species, obtained from different organs/tissues.

Tree species	Number of taxa	Tissue	Location	References
Quercus cerris	15	Bud, twigs and leaves	Northern Italy	Ragazzi et al., 2001
Fagus sylvatica	17	Branches	SW Britain and Southern France	Chapela & Boddy, 1988
Pinus monticola	21	Needles and cones	USA	Ganley and Newcombe, 2006
Pinus taeda	23	Needles	Eastern USA	Arnold et al., 2007
Pinus sylvestris and *Viscum album*	25	Needles and stem	Germany	Persoh et al, 2010
Quercus robur	25	Shoots, twigs and sapwood	Northern Italy	Gonthier et al., 2006
Quercus cerris, Q. pubescens and *Q. robur*	28	Leaves and twigs	Northern Italy	Ragazzi et al., 2003
Pinus nigra, P. pinaster, P. sylvestris and *P. uncinata*	45	Needles and twigs	Northern Spain	Zamora et al., 2008
Populus tremula	48	Leaves, twigs and bark	Northern Spain	Santamaría and Diez, 2005
Pinus nigra	49	Shoots	Southern Poland	Kowalsky & Zych, 2002
Eucalyptus grandis	52	Twigs	North Uruguay	Betucci & Alonso, 1997
Fagus sylvatica and *Pinus sylvestris*	53	Stem and xylem	United Kingdom	Petrini & Fisher, 1988
Quercus ilex and *Q. faginea*	54	Leaves, twigs and bark	Central Spain	Collado et al, 2000
Pinus sylvestris	56	Roots (nursery)	Western Lithuania	Menkis & Vasaitis, 2011
Pinus nigra, P. pinea, P. sylvestris, Quercus ilex and *Q. pyrenaica*	59	Leaves and stem (nursery)	Northern Spain	Martín-Pinto et al., 2004
Eucalyptus nitens	64	Leaves, branches and stem	SE Australia and SW Britain	Fisher et al., 1993
24 tree species of dry tropical forest	81	Leaves	Southern india	Suryanarayanan et al., 2003
Pinus sylvestris, *Betula pendula* and *Juniperus communis*	85	Roots	Eastern Lithuania	Lygis et al., 2004a
Abies alba	116	Needles	SE Poland	Kowalski & Andruch, 2011
Pinus sylvestris	143	Sapwood	Western Italy	Giordano et al, 2009
Quercus ilex	149	Leaves, twigs and bark	Central Spain	Collado et al, 2000
Heisteria concinna and *Ouratea lucens*	347	Leaves	Panama	Arnold et al., 2000

The cumulative curves for species richness (Figure 4) show that more species would have been found if more samples had been taken, but in this context the dominant and abundantly occurring species are of greatest interest because they are more likely to be host-specialists (Saikkonen, 2007). Even if half of the actual species remained undetected we can assume to have encountered most dominant species culturable on PDA because the majority of fungal species in healthy trees of natural environments generally occur at very low numbers and just a few are frequent (Müller & Hallaksela, 1998; Ragazzi et al., 2003; Saikkonen, 2007; Linaldeddu et al., 2010). The isolation method used in this study favors medium and fast growing fungi, and thus, some of the slow growing species may have remained undetected, as well as all species not culturable on PDA. The high proportion of successful isolations (i.e. isolates were obtained from every twig segment plated) and high number of species found (33 different sequences from 360 isolates) suggest that at least some of the twig segments may have inhabited more than one species. Therefore, the species richness and diversity indices found in this investigation must be regarded as minimum estimates. DNA methodology would have revealed a higher number of microbes in our samples but pure cultures of the organisms may be important for further studies on their significance as for instance inoculation studies.

Factor analysis showed that endophytic fungal diversity (Richness, DSim and DSha) is not significantly correlated to tree growth or tree size (Figure 5), while for mycorrhizal fungi a relationship between mycorrhizal diversity and spruce growth has been found (Korkama et al., 2006). Instead, fungal evenness (ESim) correlates negatively with diametric growth rate (DGR), ($p<0.05$), suggesting that there are some dominant species occurring more frequently in fast growing trees, while in slow growing trees the fungal species abundance is more even.

Our results suggest that particular species or clusters of them are related to the diametric growth rate of *P. sylvestris*. It is not possible based on the present results to speculate if there is any causality between pine growth and the twig endophyte community structure. One possibility is that the availability and/or quality of nutrients usable by fungi differ in slow and fast growing pines, or that tissue structure of fast/slow growing trees represent different niches for fungal growth. However, enhanced growth due to production of plant growth regulators by endophytes has been observed in roots and stems of woody plants (Hietala et al., 1994; Gronberg et al., 2006; Xin et al., 2009). *Phoma herbarum* and other *Phoma* species have been found to be able to produce gibberellins (Rim et al., 2007; Hamayun et al., 2009), and the presence of *Phoma* spp. has been previously detected in different pine tissues, such as roots (Menkis & Vasaitis, 2011), sapwood (Giordano et al., 2009), needles and twigs (Zamora et al., 2008). As *P. herbarum* was the most common endophyte in our samples of fast growing pines, further research on the mechanism of its influence on the host would be justified.

Hypocrea lixii (anamorph *Trichoderma harzianum*) is a cosmopolitan species and a well-known mycoparasite (Chet & Inbar, 1994) that has been found to promote growth of pine seedlings when inoculated together with the mycorrhizal *Laccaria laccata* (Ahangar et al., 2012). However, our results show an association of this species with low growth of pines, which is in accordance with previous studies describing decrease in growth of Scots pine trees infected with *H. lixii* (Giordano et al., 2009). Different genotypes of this species may have a different effect on host growth, or perhaps biotic interactions with other organisms are explaining its association to low growing trees.

The presence of some well-known pathogens, such as *Fusarium oxysporum*, *F. verticillioides* and *Biscogniauxia mediterranea* in healthy tissues of Scots pine is noteworthy. Fungi pathogenic to one host but endophytic in other host tree species are not uncommon (Chapela, 1989; Rodríguez et al., 2012). *B. mediterranea* is known as the causal agent of charcoal disease on *Quercus* spp. (Jiménez et al., 2005), mainly on *Q. suber* (Muñoz et al., 2007), and it has been reported as a pathogen also on *Pistacia vera*, *Juglans* sp. (FAO, 2007) and *Persea bombycina* as well (Dutta et al., 2010). Charcoal disease outbreak is often preceded by a long latent period, i.e. the fungus is able to exist as an endophyte in oak (Vannini et al., 2009). Still, its rather common presence (53% of investigated plots) also in Scots pine twigs as an endophyte appears surprising. *B. mediterranea* may be harmful to Scots pine, as it shows a trend (p=0.06, ANOVA) of occurring more frequently in trees with low diametric growth rates (Figure 6).

Recently, *B. mediterranea* has been reported as an endophyte in twigs of hybrid poplars in Spain (Martín-García et al., 2011) and also from grasses (Sánchez et al., 2010), so this fungus seems to be able to dwell in a broad range of different host species. According to their ITS sequences most of the isolates obtained from pine (24 out of 26) are more closely related to those obtained from insects (Portugal) and *Quercus castaneifolia* (Iran) than to those obtained from oak forests in Spain (Figure 4). But further investigations are needed to elucidate if they represent the same biological species, and if isolates from different tree species show any host specificity, i.e. can strains dwelling endophytic in pine or poplar be pathogenic to oak? Several *Quercus pyrenaica* stands are spread within the study area, but presence of *B. mediterranea* on oak was not investigated in our study. The significance of *B. mediterranea* to Scots pine deserves further investigations because our study shows that the presence of the fungus is associated with low growth of the pine host (Figure 6), and reduced ring growth is a well-known symptom of charcoal canker caused by this fungus on *Quercus* spp. (Vannini et al., 1996).

Our results revealed a number of new endophytic species living in *P. sylvestris* and suggest that some of the species are involved in mechanisms affecting host growth. Inoculation studies are needed to disclose possible causal effects by these fungi on host plant growth. Abundant presence of fungi in twigs of P. sylvestris as found with the applied culturing method does not exclude the possibility that additional species could be found using other methods.

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Chapter V

(“Todo está conectado, todos los caminos se encuentran
y todos los ríos desembocan en el mismo mar.. …“)

(Paulo Coelho)

INFLUENCE OF SITE FACTORS ON FUNGAL ENDOPHYTIC COMMUNITY OF *PINUS SYLVESTRIS* L. TWIGS IN NORTHERN SPAIN

INTRODUCTION

Colonization of plant tissues by fungal endophytes has been demonstrated to be influenced by multiple factors, which may include numerous components of the surrounding environment. Forests are complex systems in which diverse types of factors may affect endophyte distribution. Site factors, including those related to soil, climate (Carroll & Carroll, 1978; Bills, 1996) forest health and stand characteristics (Müller & Hallaksela, 1998) among others, can determine the susceptibility of the host tree to fungal endophytes. On the other hand, the position of an endophytic fungus in an ecosystem, and how it responds to resources and enemies, (niche) may change because a fungus continuously affect, and is affected by both abiotic and biotic environment (Saikkonen, 2007).

The main climatic factors involved in tree development are well described, including temperature and humidity (determined by precipitation), but other factors are also involved, such as soil and silvicultural practices, all together determining the environment in which tree is growing. In Scots pine previous studies have shown that climatic conditions and latitude affect fungal colonization of needles (Kowalski, 1993; Terhonen, 2011). However there is little knowledge about twigs endophytes (Zamora et al., 2008) and factors determining their distribution in Scots pine. In other hosts the relationship between the distribution of foliar endophytes and precipitation has been shown (Suryanarayanan, Murali & Venkatesan, 2002), but there is little information about the relationship of twig endophytes and site factors.

Forest management can also influence the fungal endophytic colonization. The foliage of some coniferous trees seemed to be infected to a greater extent by fungal endophytes in hosts growing in areas with a higher density of vegetation. This can be attributed to microclimatic effects affected by the vegetation density (Müller &

Hallaksela, 1998) but perhaps also to the protective effect of the canopy, hindering sporal colonization by of other fungi especially when the vegetation is diverse (Petrini & Carroll, 1981; Müller & Hallaksela, 1998).

Fungal endophytes keep a tight relationship with their host since they live inside host tissues, so environmental factors that affect plants are likely to affect the community living inside them. The infection frequencies of endophytes in Douglas-fir collected over a wide geographical range are positively correlated with annual precipitation and negatively correlated with elevation, although this effect is attributed to effects of precipitation on fungal spread and not due to differences in tree susceptibility to fungal infection due to precipitation (Carroll & Carroll 1978).

Previously we reported (Sanz-Ros et al. 2015) differences between the fungal endophytic community of twigs of fast and slow growing Scots pines. Here we investigate the variation of the occurrence of twig endophytes in relation to climate, soil type and stand characteristics using the same fungal data set. The objective of this study is to identify associations between the fungal endophytic community of *Pinus sylvestris* twigs with various site factors, including climate, soil and stand characteristics.

MATERIALS AND METHODS

STUDY AREA, SAMPLING METHODS

The study site and experimental design is described previously (Sanz-Ros et al. 2015). Shortly, 15 circular Scots pine plots of 15 m radius were selected from the Spanish National Forest Inventory grid. Since the distribution of Scots pine in the study area is patchy, the plots were selected with the aim of representing the different forest patches covered by this species along the study area (Figure 1).

Soils of the study are included in three orders, Alfisol, Inceptisol and Entisol, based on soil Taxonomy classification system (USDA 1987). Soil classification at the different classifications levels are shown in table 1.

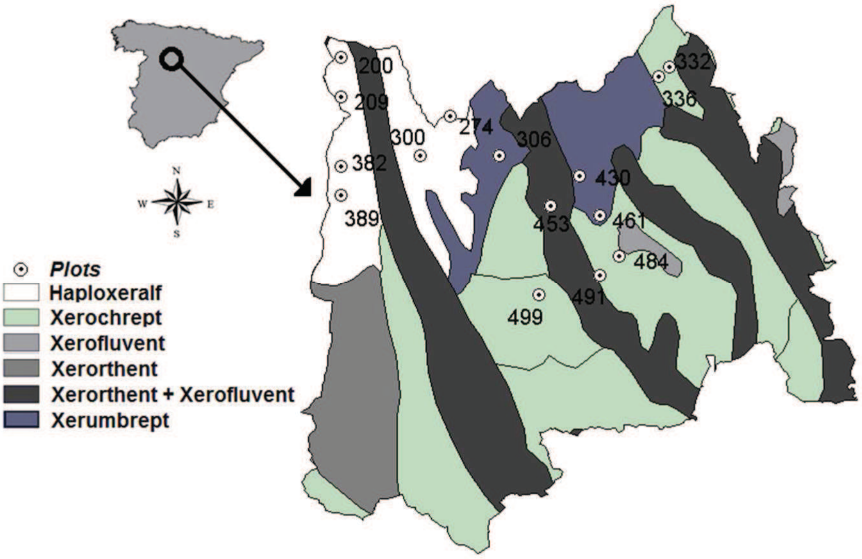

Figure 1: Location of the Scots pine plots in Palencia, Spain. Each plot is denoted with a code number used in the Spanish National Forest Inventory (NFI). Soil suborders are indicated by different colors as showed in the color legend.

Table 1: Soil classification and international textural class of each plot.

	Soil classification level				
Plot	Order	Suborder	Group	Association	Texture
200	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	sandy loam
209	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	sand
274	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	loamy sand
300	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	sandy loam
306	Inceptisol	Umbrept	Xerumbrept	Xerorthent + Xerochrept	loamy sand
332	Inceptisol	Ochrept	Xerochrept	Haploxeralf	loamy sand
336	Inceptisol	Ochrept	Xerochrept	Haploxeralf	loamy sand
382	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	sandy loam
389	Alfisol	Xeralf	Haploxeralf	Palexeralf + Ochraqualf	sandy loam
430	Inceptisol	Umbrept	Xerumbrept	Xerorthent + Xerochrept	loamy sand
453	Entisol	Orthent	Xerorthent + Xerofluvent	Xerochrept	loamy sand
461	Inceptisol	Umbrept	Xerumbrept	Xerorthent + Xerochrept	loamy sand
484	Inceptisol	Ochrept	Xerochrept	Xerorthent	sandy loam
491	Inceptisol	Ochrept	Xerochrept	Xerorthent	loamy sand
499	Inceptisol	Ochrept	Xerochrept	N/A	sandy loam

For the characterization of each plot several variables were measured, such as age (Age), diametric growth rate of the last 5, 10 and 15 years (DGR5, DGR 10 and DGR15), defoliation (MDef) and the presence and number of stumps (StNum) (Table 2). Other site characteristics were obtained from the 3rd Spanish National Forest Inventory including basal area (BA), tree density (Dens), Mean height (Mhe), dominant height (DH), Mean Diameter (DBH), and Reineke's Stand Density Index (Reineke).

Table 2: Climatic, dasometric, health and soil variables characterizing plots, abbreviations used, description and category.

Variable[1]	Description & units	Cat.[2]	Variable[1]	Description & units	Cat.[2]
Age	Age (years)	D	AnRain	Annual rainfall (mm)	CA
Dens	tree density of plots (tres.ha^{-1})	D	MTemp	Mean Temperature of the last 15 years	CA
Mhe	Mean height (m)	D	TMMax	Maximum of Mean Temperatures of the last 15 years	CA
Rad	Solar Radiation(10 kJ*m^{-2}*day^{-1} *μm^{-1})	D	TMMIN	Minimum of Mean Temperatures of the last 15 years	CA
StNum	Stump number	D	DecRain	December rainfall	CW
DBH	Mean Dameter (cm)	D	DecTM	Mean temperature of December	CW
DH	Dominant Height (m)	D	DecTMax	Maximum temperature of December	CW
BA	Basal area (m2*ha^{-1})	D	DecTMin	Minimum temperature of December	CW
DGR5	Diametric growth rate (average of the last 5 years)(mm*y^{-1})	D	JanRain	January rainfall	CW
DGR10	Diametric growth rate (average of the last 10 years) (mm*y^{-1})	D	JanTM	Mean temperature of January	CW
DGR15	Diametric growth rate (average of the last 15 years) (mm*y^{-1})	D	JanTMax	Maximum temperature of January	CW
Reineke	Reineke's stand density index	D	JanTMin	Minimum temperature of January	CW
Mdef	Plot defoliation (%)	H	FebRain	February rainfall	CW
Coarse	Coarse elements (%)	S	FebTM	Mean temperature of February	CW
Sand	Sand (%)	S	FebTMax	Maximum temperature of February	CW
Silt	Silt (%)	S	FebTMin	Minimum temperature of February	CW
Clay	Clay (%)	S	JuneRain	June rainfall	CS
pH	pH	S	JuneTM	Mean temperature of June	CS
P	Phosphorous (ppm)	S	JuneTMax	Maximum temperature of June	CS
K	Potasium (ppm)	S	JulyRain	July rainfall	CS
Ca	Clacium (meq/100 g)	S	JulyTM	Mean temperature of July	CS
Mg	Magnesium (meq/100 g)	S	JulyTMax	Maximum temperature of July	CS
Na	Sodium (meq/100 g)	S	AugRain	August rainfall	CS
CEC	Cationic Exchange Capacity (meq/100 g)	S	AugTM	Mean temperature of August	CS
N	Nitrogen (%)	S	AugTMax	Maximum temperature of August	CS
TotalC	Total Carbon (%)	S			

CANOPY EVALUATION AND CLIMATIC DATA

Defoliation was estimated following the ICP Forest Methodology in 5% intervals comparing with reference photographs of defoliation levels for Mediterranean ecosystems (Ferretti 1994; SPCAN-DGCN, 2002). Canopy evaluation was carried out by two different evaluators at a distance equal to tree height, avoiding facing the sun. Defoliation values of each plot were computed as averaged values of the fifteen nearest trees to the plot center

Climatic data were obtained from the Digital Climatic Atlas from the Iberian Peninsula (Ninyerola, Pons & Roure 2005), a climatic model which computes values from the last 15 years for temperatures and 20 years for precipitation. Variables obtained for climatic characterization of plots were: mean, minimum and maximum, monthly and annual temperatures, monthly (for summer and winter period) and annual rainfall, as well as solar radiation.

SOIL SAMPLING AND ANALYSIS METHODOLOGY

From each plot two soil samples were obtained at different depths, from 0-30 cm and from 0-60 cm. Each sample (i.e 0-30 cm) was taken at 1 m from the plot center towards the four cardinal directions, and properly mixed as a unique sample per plot. Various soil properties were determined by the Technological and Agricultural Laboratory (ITAGRA, Palencia). The determined characteristics were Cationic exchange capacity (CEC), pH, particle-size distribution, textural class, inorganic nutrients (Ca, K, Mg, Na, N, P) and total carbon and C/N ratio. CEC was determined by the Bascomb method (Bascomb 1964). Coarse elements were obtained using a 2 mm sieve, whereas sand, silt, clay and textural classes were determined following the International Soil Science Society (ISSS) methodology. Phosphorous content was determined by the Olsen method (Olsen & Sommers 1982), while Ca, Mg, K and Na were determined by the NH4-acetate test (MAPA 1994). Nitrogen content was determined by the Kjeldahl method and total Carbon was estimated by the easily oxidizable Carbon method, and finally C/N ratio was calculated.

Fungal isolation, grouping and identification are described earlier (Sanz-Ros et al. 2015). Shortly, twig segments were surface sterilized and outgrowing fungi were isolated on potato dextrose agar (PDA). The isolates were grouped according to colony morphology into colonial morphotypes (CMs) and isolate representatives of these morphotypes were identified based on comparison of their ribosomal DNA (rDNA) ITS sequences of with those in Genbank.

STATISTICAL ANALYSIS

A principal component analysis was carried out with the aim of reducing the dimensionality of the dataset and to identify main factors explaining variation of the data. A simple correspondence analysis (SCA) was carried out in order to observe the fungal distribution in plots. After that, a cluster analysis based on the factorial axes defined by the SCA analysis was carried out in order to group plots by their fungal community. This cluster analysis carried out using the Ward method and the Euclidean squared distances, finally, k-means method was used for group consolidation.

Further characterization of groups by environmental variables was used to identify relationships between fungi and environmental variables. For this characterization the mean value of each variable was contrasted with the mean of this variable in each plot cluster. Statistical analyses were carried out using SPAD 5.6.

RESULTS

Site characteristics describing climate, soil, tree health and stand properties are given in Table 3. PCA identified three main factors which explained 65 % of the total variation. The first factor accounted for 44. % of the variation, and separates the plots according to their temperature and precipitation (Figure 2). Factor 1 suggests for an association between some soil properties (high levels of Ca, Mg, P, K, pH and coarse elements) with high temperatures and low precipitation levels. On the other hand, low pH and high percentage of clay are associated to high precipitation and low temperature.

Table 3: Climatic, dasometric, health and soil variables characterizing plots. Mean, standard deviation, minimum and maximum values are shown.

Variable	Mean	SD	Minimum	Maximum	Variable	Mean	SD	Minimum	Maximum
Age	27.97	3.98	20	33.50	AnRain	716.63	109.92	586.60	943.90
BA	20.95	7.38	9.13	34.51	AugRain	248.73	27.89	204	283
DBH	19.49	3.66	13.45	25.43	AugTM	180.80	4.33	173	188
Dens	792.25	336.92	187.45	1368	AugTMax	259.60	3.94	254	266
DGR10	3.90	0.95	2.24	5.51	DecRain	807.27	151.55	611	1126
DGR15	3.59	0.83	2.11	4.99	DecTM	3.40	0.29	2.90	4.00
DGR5	4.14	1.13	2.44	6.05	DecTMax	7.63	0.27	7.10	8.20
DH	11.77	1.88	8.34	14.56	DecTMin	-0.81	0.32	-1.50	-0.20
Mhe	10.54	1.61	7.42	13.03	FebRain	655.20	86.75	517	779
Reineke	474.94	158.48	186.30	751.68	FebTM	3.63	0.36	2.90	4.40
StNum	80	62.5	0	210	FebTMax	8.50	0.37	7.80	9.30
Ca	2.72	1.84	0.57	7.58	FebTMin	-1.27	0.35	-2	-0.60
CEC	12.92	3.18	6.88	19.38	JanRain	756.13	112.34	612.	942.
Clay	8.68	3.01	4.35	16.65	JanTM	2.51	0.29	2	3.10
Coarse	48.79	21.62	5.59	81.73	JanTMax	6.91	0.28	6.40	7.50
K	67.44	34.72	32.40	173.82	JanTMin	-1.94	0.37	-2.70	-1.30
Mdef	21.25	5.26	15.00	36.25	JulyRain	281.27	23.03	237	315.
Mg	0.51	0.36	0.14	1.55	JulyTM	18.32	0.42	17.50	19
N	0.10	0.06	0.03	0.23	JulyTMax	26.27	0.39	25.60	26.90
Na	0.03	0.03	0.01	0.10	JuneTM	15.05	0.41	14.20	15.70
P	2.94	2.15	0	4.50	JuneTMax	21.97	0.41	21.20	22.60
pH	5.39	0.51	4.62	6.32	JunRain	513.47	42.46	429	592
Sand	76.53	6.68	62.85	85.10	MTemp	9.73	0.35	9	10.40
Silt	14.79	5.59	7.05	26.80	Rad	2049.40	16.05	2030	2076
TotalC	2.35	0.97	1.45	4.78	TMMax	15.79	0.35	15.10	16.40
					TMMIN	3.63	0.38	2.80	4.30

The second factor clusters the plots with slowly growing and defoliated dense stands on soils with high silt percentage, separating them from those plots with healthy and fast growing stands at low density on soils with high percentage of sand. The third factor groups together plots with big trees (high DBH and Mhe) having high basal area and growing slow on soils with low total carbon, and separates these from plots with high carbon content, low basal area and small trees with high diametric grow rates.

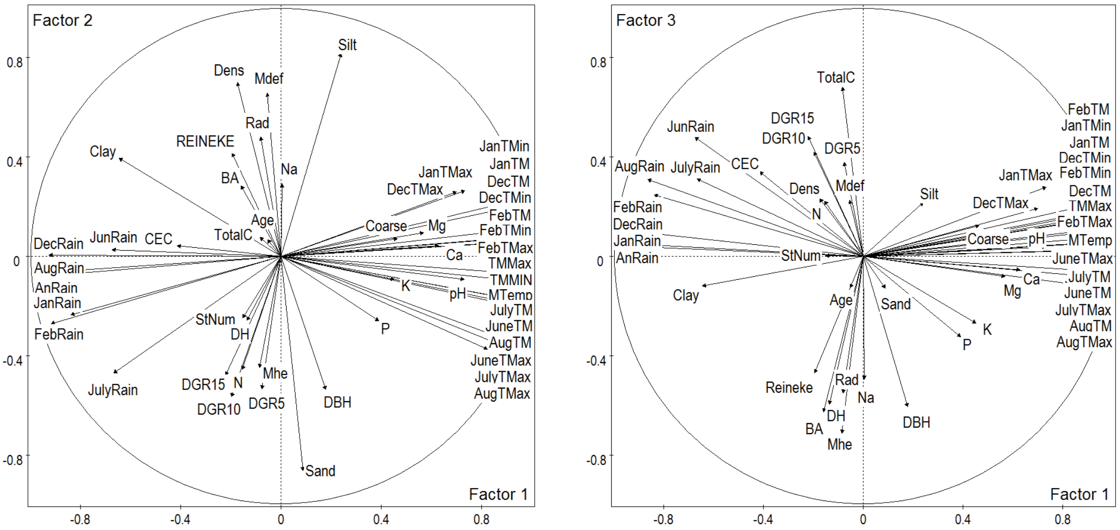

Figure 2: PCA-plot of the sampling sites showing associations of various stand characteristics (growth, health, density etc.) with soil and climatic site characteristics.

Cluster analysis grouped plots by their fungal community and later these groups were characterized by climatic, dasometric, health and soil variables (Figure 3). Both analyses combined show associations between fungal endophytes and plot characteristics.

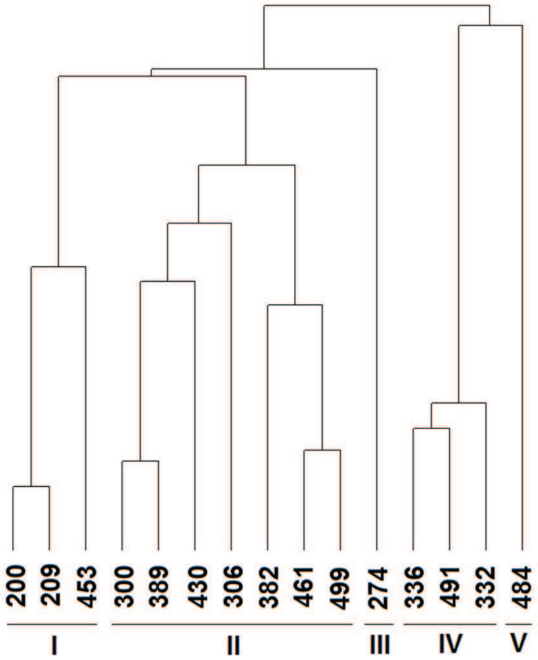

Figure 3: Cluster analysis in which the five classes of plots can be observed (roman numerals).

Cluster analysis (Figure 3) combined with correspondence analysis yielded 5 groups of forest plots (Figure 4) . The class I (plots 200, 209 and 453) showed a higher abundance of *Biscogniauxia mediterranea*, *Hypocrea lixii* and *Ophiostoma nigrocarpum*, and it was characterized by big trees (DBH and MHe) showing a low growth rate, placed in slightly acid soils with low content of carbon (C), magnesium (Mg) and nitrogen (N) , under high annual and winter rainfall and low temperatures. Trees in class II (including forest plots 300, 306, 382, 389, 430, 461 and 499) showed high abundance of *Alternaria alternata*, *A. infectoria*, *Trichodema atroviridae* and *Massarina corticola*, and are characterized by dense plots of small trees (MHe and DBH) growing slow and slightly more defoliated than the overall mean, placed in soils with high silt percentage and carbon content, but low levels of K, N and P, where summer temperatures are moderated and precipitations are scarce both annually and in winter.

Class III is a cluster with a single plot (274), and the more abundant endophytes were *Phoma macrostoma* and an unknown species (66), and is characterized by the presence of young and small trees at low tree density because the intensity of forest use (high StNum), growing at a medium rate in clay soils with high CEC, as well as N and C content, where precipitation is considerably high during summer, winter and annually, and temperatures are low in summer and winter.

Class IV (plots 332, 336 and 491) showed a high abundance of *Phoma herbarum* and *Hormonema dematioides*, but total absence of *Hypocrea lixii* and *Biscogniauxia mediterranea*. This class is characterized by plots with big trees (DBH and MHe) growing fast (high DGR) in sandy soils containing high levels of N, P and K and a low tree density. Temperatures are high during summer and winter, and rainfall is rather abundant during summer. Finally, Class V (plot 484) showed a high abundance of *Aspergillus niger* and *Penicillium glabrum*, and is characterized by big trees (high DBH and MHe), no recent logging (StNum = 0), slow growth (low DGR) in clay soils with high nutrient content (N,P, K, Ca, Mg) and high CEC, but where the temperature during summer and winter is high but the precipitation is scarce during both seasons.

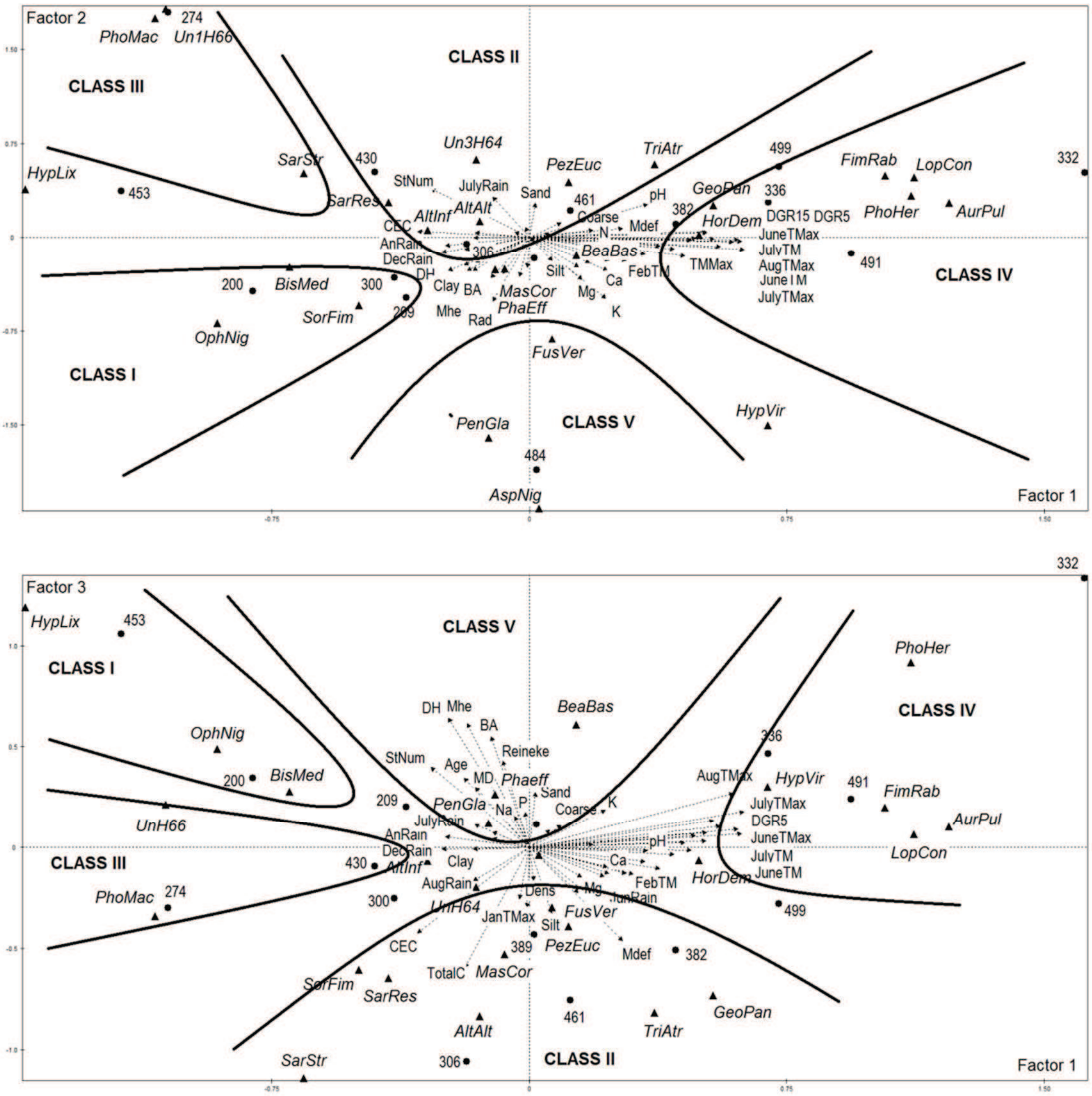

Figure 4: Triplot obtained from the correspondence analysis combined with cluster analysis. Plots (●) are separated by fungal endophytes (Δ), while continuous variables and plots classes are (→). Factor 1 vs 2 (top). Factor 1 vs 3 (bottom).

DISCUSSION

The most abundant species isolated from these forest plots are most likely playing an ecological role to their hosts and probably they are more resilient and long lasting within the host tree. Our results suggest that fungal endophytic distribution in *Pinus sylvestris* twigs is associated to particular environmental and site conditions. We have identified climatic, soil, health and stand characteristics influencing fungal distribution, as well as combinations of them which provide a particular niche for certain fungal endophytic species or species groups.

The main characteristics distinguishing the studied stands were temperature and precipitation and soil physical & chemical features as texture, pH, CEC and nutrient content. Among nutrients, carbon (C), nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) were the most influencing parameters on separation of forest plot clusters, thus on fungal distribution. A higher uptake of these nutrients have been demonstrated in grasses when particular fungal endophytic species have been inoculated (Rahman & Saiga, 2005). However studies on interactions between endophytic microbes and nutrient uptake by their host plants are scarce in coniferous and broadleaved tree species. Some association between fungal community and soil nutrients has been found previously (Martín-García, 2011).

In addition to this environmental framework, different forest structure can be found depending on the management carried out in each stand, which will determine factors such as tree density, basal area or Reineke index. Thinning usually allows a higher growth of a lower number of tree (Assmann, 1970), but our results suggest that depending on the combination of all previous parameters (abiotic and biotic), there are different tree growth rates and canopy development. In this sense it was observed that stands without thinning showed a low growth rate, despite of a high nutrient content and soil CEC, as usually occurs after silvicultural treatments usually applied to Pinus stands (Assmann, 1970; Serrada, Montero & Recke, 2008).

Some interactions between site factors were found and they deserve to be commented. Locations with high temperatures and low precipitations were associated to soils with higher pH values and high content of Ca, Mg, K and P, while high N content and high CEC were linked to locations with the opposite climatic features, i.e. low temperatures and high precipitations. Other soil elements were not associated to climatic parameters, such as C and sodium (Na). In addition hot and dry locations were linked to soils having a high percentage of coarse fractions, while wet and cold locations to those with high clay percentage. However, silt and sand percentages were not associated to temperatures or precipitations.

In general, we observed that final growth rate of Scots pine depends mainly on soil texture, carbon and nitrogen content, tree age and density, result which is largely in agreement with common knowledge on growth of Scots pine (Assmann, 1970). We can identify two main situations: the first is characterized by big trees (high DBH and Mhe) showing high growth rates in sandy soils with high nitrogen content and growing at a low density in managed stands (with silvicultural treatments), showing a good health status (low defoliation). On the other hand we have small trees (low DBH and Mhe) showing high growth rates in soils with high carbon and moderate nitrogen content. The effect of climate parameters, temperature and precipitation, is also determining tree growth. There are stand clusters growing slow both with high or low rainfall, as well as with medium or low temperatures, while fast growing stands were always in sites with high temperatures and precipitations.

On the other hand, mature trees growing on soils with a high percentage of fine mineral fractions (clay and silt) and low nutrient content (N, P, K) were associated to low growth rates. It is likely that a high content of fine materials can difficult root respiration or root development in this tree species. In other *Pinus* species a high content of organic matter were associated to high respiration rates (Lee & Jose 2003), but soils in the present study show, in general, low organic matter content. On the contrary, young trees are more influenced by soil C and N, and their growth is poorly affected by soil texture.

Our results suggest that the fungal community structure in Scots pine twigs is ruled by several site factors, characterized by different combinations of climatic, soil, health and stand variables. It has been previously demonstrated that climatic variables influence needle endophyte distribution (Carroll & Carroll, 1978; Bills 1996), however its influence on twig fungal endophytes remain poorly understood.

Phoma herbarum and *Hormonema dematioides* were associated to plots with big trees growing fast in sandy soils containing high levels of N, P and K. and low tree density. Temperatures on these sites were high during summer and winter, and rainfall was rather abundant during summer. *P. herbarum* and other *Phoma* species have been shown to produce plant phytohormones (Hamayun et al. 2009; Rim et al. 2007). However, as well as in the previous case, we can neither know if *P. herbarum* is helping to tree growth or it can be attributed only to the environmental conditions. More research is deserved in this issue in order to evaluate possible effects of endophytes on tree growth. It is remarkable that stands showing a high abundance of *Phoma herbarum* and *Hormonema dematioides* showed a total absence of *Biscogniauxia mediterranea*, suggesting a possible protective effect of *P. herbarum* and/or *H. dematioides* against fungal pathogens, role which has been previously proposed for *H. dematioides* in Scots pine buds (Pirttilä et al., 2003).

On the other hand, *Biscogniauxia mediterranea, Hypocrea lixii* and *Ophiostoma nigrocarpum*, were found to be associated to big trees growing slowly, placed in slightly acid soils with low content of carbon, magnesium and nitrogen, high annual and winter precipitations and low temperatures. Some pathogenic species are included in the genus *Ophiostoma*, and *B. mediterranea* is considered an important pathogen of *Quercus*. Thus, the presence of the last fungus may be influencing tree growth, since these species were associated to low growth. However, more research is needed to understand their influence, if any, in their host growth, since our sampling trees did not show disease symptoms and it is possible that these fungal species have just an endophytic life mode in Scots pine.

In conclusion, fungal endophytes distribution in Scots pine twigs is affected by numerous factors. First, climate parameters, including summer and winter temperatures and precipitation, followed by soil parameters, such as texture, pH or nutrient content, and stand structure parameters determined by the management of the stands, mainly density and basal area. Locations with high temperatures and high precipitation, high nutrient content and low tre density favours the abundance of *P. herbarum* and *H. dematioides*, which appears associated to high tree growth rates. Our results suggests that these fungi seem to have a positive interaction with the host, since might be excluding *B. mediterranea,* protecting tree from its presence. Such protective role has been proposed previously for *H. dematioides* in Scots pine buds (Pirttilä et al., 2003). In addition, *P. herbarum* has the ability of producing phytohormones and it was associated to fast growing trees, so it might be promoting host growth.

On the other hand, pathogenic fungi seems to be more abundant in locations with low precipitation and soil nutrient content, as well as to high temperatures, factors that combined may produce an stressful environment for the host and might represent an opportunity for the colonization by some pathogenic or opportunistic fungi. The study of this influence put some light on endophytes ecology and interactions host-endophyte and endophyte-other microorganisms. Thus, starting form this point, possible applications of particular species for diverse purposes can be evaluated.

So or results suggest that favourable conditions for tree growth and development also favor a higher abundance of beneficial fungi for the host. Similar results has been obtained in weeds infected by an endohyte that protect from herbivore feeding, but the protection effect is higher when soil conditions are favourable (Lehtonen, 2005). On the contrary, stress environmental conditions favor the proliferation of pathogenic organisms, which could be related with a decrease of tree vigor.

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Discusión General

DISCUSIÓN GENERAL

PRINCIPALES DAÑOS ENCONTRADOS EN PINARES DEL NORTE DE ESPAÑA, Y SU USO COMO INDICADORES DE CONDICIÓN DE COPA

Los principales daños encontrados fueron bifurcaciones, defoliaciones, malformaciones del tronco, decoloraciones y chancros, por orden de frecuencia. Sin embargo, al igual que en los resultados obtenidos por la Red Europea de Seguimiento de Daños a los Bosques (Fischer et al., 2010), solamente defoliación y decoloración fueron encontradas en todas las especies evaluadas, por lo que éstos van a ser los más adecuados para su uso como indicadores de la condición de copa y el estado sanitario de las masas forestales, siendo estos resultados acordes con las conclusiones obtenidas previamente en evaluaciones a lo largo de Europa (Fisher, Petrini & Sutton, 1993; Marco Ferretti, 1997; Eichhorn et al., 2010; Ferretti, 2010; Fischer et al., 2010). *P. sylvestris* y *P. nigra* se sitúan en clases de defoliación leve (0-25%), mientras que *P. pinaster* se sitúa en una clase moderada (25-60%), siendo además la especie que más árboles muertos mostró, aunque esto puede ser debido a una selvicultura menos intensiva que la practicada para otras especies.

El porcentaje de árboles con una decoloración identificable resultó bastante mayor en *P. nigra* que en *P. sylvestris*, coincidiendo este dato con otros estudios en los que se han evaluado ambas especies (Navarro et al., 2007), aunque en esta zona el porcentaje de decoloraciones es mucho menor, lo cual podría ser debido a la mayor pluviosidad de esta zona de estudio. Cabe destacar el elevado número de bifurcaciones y malformaciones del tronco, lo cual puede tener efectos económicos importantes en el futuro. Debido a la vocación productivista de gran parte de estas masas, estas bifurcaciones y malformaciones del tronco podrían ser consideradas como síntomas perjudiciales, sobre todo teniendo en cuenta el elevado porcentaje de árboles que los presentan (13-18% pies, dependiendo de la especie), aunque no está claro hasta qué punto influyen pueden influir negativamente o no estás bifurcaciones al funcionamiento fisiológico del árbol. Existen estudios (Robredo, 1975, 1978) que revelan ataques graves por *Rhyacionia buoliana* en esta zona durante los años 70, posiblemente debido a la presencia de muchas masas jóvenes y coetáneas susceptibles de ser atacadas por este insecto. La muerte del meristemo apical causada por este insecto y su sustitución por otros meristemos situados por debajo, que pasan a ser apicales, podría ser la causa del numero de bifurcaciones presentes actualmente.

No se podría decidir cuál de estas tres especies de pino resulta más adecuada para la zona en base solamente a la presencia de daños, ya que cada una presentó un tipo y porcentaje diferente de daños. El pino silvestre presentó una baja intensidad y frecuencia de defoliación, así como una muy baja aparición de decoloraciones. El pino salgareño también presentó una baja intensidad de defoliación, y su frecuencia fue muy baja, sin embargo presentó un mayor porcentaje de decoloraciones que el silvestre (aunque el número de árboles decolorados en general es muchísimo menor que el de los defoliados, por lo que este factor pesaría menos). Finalmente, el pino resinero presentó los valores más altos de intensidad y frecuencia de defoliación, mostrando, sin embargo los más bajos en cuanto a decoloración. El resto de daños encontrados, aunque no tan frecuentes, mostró una serie de particularidades. El pino salgareño mostró un altísimo porcentaje de bifurcaciones, así como microfilia, daño que solo fue encontrado en esta especie. El pino silvestre mostró una presencia mayor de chancros en el tronco que las otras dos especies y el pino resinero mostró el mayor porcentaje de muertes del meristemo apical principal y malformaciones en el tronco, que son atribuidas al desarrollo en estadíos jóvenes y al ratio altura/densidad de pies (Del Río et al., 2004).

INFLUENCIA DE LOS FACTORES CLIMÁTICOS EN LA CONDICIÓN DE COPA

Los resultados mostraron que una pequeña desviación en la precipitación media de Julio, procedente de modelos a largo plazo (Ninyerola, Pons, & Roure, 2005), puede producir un incremento significativo de la defoliación. La combinación de altas temperaturas y bajas precipitaciones del mes de julio crean un ambiente estresante, lo cual provoca un descenso del vigor de los pies de pino, por lo que este factor podría ser clave en la condición de copa de los pinares de Castilla y León. Por ello durante este mes no se recomiendan tratamientos selvícolas como las cortas y las podas, ya que, al descender el vigor, la probabilidad de un ataque de insectos aumenta considerablemente (Houston, 1992). Por otro lado esta combinación afectaría de forma negativa a los patógenos fúngicos, sobre todo a su esporulación y al éxito de colonización, que son sensibles a cambios en la temperatura, precipitación, humedad del suelo y humedad relativa del aire (Brasier, 1996; Frankland, Magan & Gadd, 1996; Houston, 1998).

Por otro lado, la temperatura media también influyó en la defoliación encontrada. Los resultados indican que un incremento de 0,4 ºC en la temperatura media produciría incrementos del 6% en la defoliación, mientras que y un incremento de 1,6 ºC (el encontrado entre las categorías 1 y 5) produciría incrementos en la defoliación del 9%. Este incremento en la temperatura media se corresponde exactamente con el predicho por algunos modelos para la Península Ibérica a lo largo del último siglo (Hulme & Sheard, 1999).

Confirmando ambos efectos, se observó que los rodales situados en zonas más cálidas y secas mostraban valores de defoliación más elevados, mientras aquellos situados en zonas más frías y húmedas estaban menos defoliados. Esto concuerda con los resultados de la Red de seguimiento de daños a los Montes, en la cual ser concluye que entre el 30 y 50 % de la variación en la defoliación puede ser explicada por la variación en la edad del rodal, el suelo, la precipitación y la deposición de nitrógeno y azufre (De Vries et al., 2003), aunque nuestros resultados apuntan también hacia la temperatura como un factor que influye sobre la condición de copa. En este sentido la temperatura media anual y la media de agosto se muestran como factores que influyen sobre la condición de copa. Al ser numerosos los factores implicados en el estado sanitario de las masas, tanto de tipo abiótico como biótico, los parámetros climáticos a largo plazo solamente constituyen una pequeña parte de ellos, lo cual explica el bajo porcentaje de variación en la defoliación explicado por ellos.

Los resultados de este estudio apuntan hacia un efecto significativo de los parámetros climáticos a largo plazo, y no solamente de aquellos obtenidos de años recientes. Ello sugiere que la condición de copa es el resultado de factores climáticos pasados y presentes, entre otros factores que queda claro que ejercen su influencia, como son los bióticos (Solberg et al., 2006) o las perturbaciones del medio como los incendios o la contaminación atmosférica (Calvo et al., 2008; Zierl, 2002).

INFLUENCIA DEL CAMBIO CLIMÁTICO EN LA CONDICIÓN DE COPA

La tendencia climática apunta hacia un incremento de la temperatura en el sur de Europa de 1-3 ºC y de 2ºC en España (Kattenberg et al., 1996; Watson et al., 1997; Hulme & Sheard, 1999; Parry, 2000; Del Río, Penas & Fraile, 2004). Nuestros resultados indican que la defoliación seguirá dicha tendencia al alza, produciendo un descenso del vigor de las masas expuestas a estos cambios ambientales. Si el incremento de la temperatura media llega a ser de 2 ºC la defoliación tendería a aumentar un 30 %, lo cual puede suponer una importante reducción de la copa.

Además se espera un descenso de las precipitaciones en el sur de Europa hacia el mediterráneo (Schönwiese & Rapp, 1997; Sarris, Christodoulakis & Körner, 2007), y además éstas tienden a ser también mas erráticas en general (Dore, 2005). Según nuestros resultados esto también producirá un aumento de la defoliación a una tasa del 10% por cada descenso de 60 mm. En los ecosistemas mediterráneos el verano es una estación marcadamente seca, y ya de forma habitual al final de esta estación suele encontrarse una notable reducción en la copa de plantaciones de pino (Fischer et al., 2010), por lo que una variación como la esperada puede incrementar el daño considerablemente.

Estas tendencias deben ser tenidas en cuenta por los gestores de sistemas forestales con el fin de adoptar estrategias de gestión encaminadas a minimizar la defoliación, contribuyendo de a la sostenibilidad de los bosques y a la mitigación del cambio climático, y así poder evitar procesos de decaimiento en pinares y en otras masas.

INFLUENCIA DE LAS CARACTERÍSTICAS DEL RODAL EN EL ESTADO SANITARIO DE MASAS DE *PINUS HALEPENSIS* EN DECAIMIENTO

Las masas de pino carrasco sufren desde hace tiempo un proceso de decaimiento (Santamaría, Pajares & Diez, 2003). La evaluación de una muestra de estas masas en el norte de España mostró la presencia de un porcentaje muy elevado (81%) de los pies dentro de la categoría de "dañados" según los criterios de la Red Europea de Nivel I, es decir, entre un 25-60% de defoliación (Eichhorn et al., 2006), lo cual supera con creces los resultados de dicha red, que han recogido un porcentaje medio el 15,7% en *Pinus* spp. (Fischer et al., 2010). Por ello claramente no todas las especies de pino están dañadas en igual magnitud ni el daño es el mismo bajo condiciones ambientales diferentes.

En este caso, la defoliación encontrada se observó en la parte alta de la copa, donde se podía observar en muchos casos una muerte progresiva desde el meristemo apical hacia abajo (dieback, en inglés, y así nos referiremos a este síntoma de aquí en adelante). En esta zona han sido aislados repetidamente *Gremmeniella abietina*, un patógeno primario, y *Sclerophoma pithyophila*, *Thyriopsis halepensis* y *Cenangium ferruginosum*, considerados como patógenos de debilidad u oportunistas (Santamaría et al., 2003; Santamaría et al., 2007; Botella, Santamaría & Diez, 2010). Los tipos de daños son consistentes por los descritos para la combinación de estos patógenos, sin embargo su presencia no explica totalmente el proceso de decaimiento, ya que no todos los pies están decaídos aun estando presentes dichos patógenos, y además la etiología del decaimiento se espera que sea mucho más compleja. Nuestros resultados identifican una serie de factores de sitio que podrían actuar como factores que predisponen las masas a entrar en decaimiento. El modelo de regresión obtenido para la defoliación (Capítulo III) así lo explica, incluyendo variables como la edad, la altura de copa, el área basimétrica y la elevación. Esto confirma nuestra hipótesis de que hay algunos factores de sito que están influyendo en la condición de copa. Estos factores pueden ser modulados a través de la gestión forestal, de modo que se pueden adoptar ciertas estrategias con el fin de reducir el impacto del decaimiento, como pueden ser la reducción de la densidad de pies, evitar que las copas se toquen unas con otras, llevar a cabo podas para aumentar la aireación inferior o crear una estructura heterogénea que contenga pies de tamaños diferentes, lo cual va a dificultar la expansión de ciertos patógenos que solamente atacan a pies jóvenes o maduros (Hellgren & Högberg, 1995; Botella et al., 2010).

Nuestros resultados mostraron una mayor defoliación en pies maduros, lo cual concuerda con el concepto de decaimiento definido por Manion en 1991 en el que se describió que solamente los pies maduros entraban en decaimiento. Estos pies maduros están situados en parcelas con un área bisimétrica reducida, debida a una baja densidad de pies grandes. La elevación podría estar relacionada con la exposición a las heladas, ya que los pies situados en lo alto de las laderas que delimitan los cerros están sometidos a temperaturas más extremas que los situados en posiciones más bajas. Es bien sabida la susceptibilidad de esta especie de pino a las heladas, que suelen producir marchitamiento de los extremos de las ramas y una decoloración general, sobre todo cuando se producen en primavera tardía o en otoño temprano (Muñoz et al., 2007). Además en la zona de estudio han sido datados un elevado número de días con helada (Santamaría, Botella & Diez, 2007). Estas heladas también favorecen el ataque por *G. abietina*. En este sentido, algunos autores han recomendado evitar las localizaciones húmedas y frías para el establecimiento de plantaciones de pino, con el fin d prevenir daños por este patógenos fúngicos (Karadžić & Milanović, 2008).

El modelo de regresión para el índice de área foliar (IAF), sin embargo, incluyó como explicativas solamente la densidad y el área basimétrica. Los valores de IAF encontrados en la zona son bastante menores (1,71±0,48 m^2/m^2) que los documentados en otros estudios en pinares sanos de pino carrasco, 2,69 m^2/m^2 (López-serrano et al., 2000). Sin embargo la relación encontrada entre defoliación y el IAF es muy baja, al contrario de lo esperado. Esta baja correlación entre ambas variables podría ser explicada por el patrón heterogéneo de defoliación encontrado en las copas, localizándose en parte alta de la copa y avanzando hacia abajo. Dado que el IAF fue estimado mediante el análisis de fotografía hemisféricas, las cuales fueron tomadas a una altura de 1 m desde el suelo hacia arriba, la superposición de ramas secas, ramillos secos y con acículas y fustes hizo que no se pudieran detectar esos daños en la parte alta de la copa. Además el pino carrasco tiene una poda natural muy pobre, es decir, mantiene mucho tiempo las ramas muertas inferiores. Todo ello explicaría la baja correlación entre ambas variables.

En estudios previos realizados tras ataques de insectos en pino silvestre y medidos mediante escaneo por laser, si consiguieron explicar la defoliación como variaciones en el IAF a lo largo del tiempo, lo cual puede ser usado como herramienta de detección precoz ante ataques de insectos (Waring 1985; Solberg et al. 2006; Eklundh, Johansson & Solberg 2009), sin embargo esta situación es muy diferente de la encontrada en estos pinares de carrasco, por lo que es muy probable que otros métodos de estimación del IAF, como el láser, el LAI-2000, o el análisis de imágenes de satélite tampoco sean capaces de detectar estos patrones heterogéneos de defoliación causados por patógenos fúngicos que causan un “dieback”, aunque sería necesario investigar más para determinar si son o no capaces de realizar esa detección. Por ello concluimos que la fotografía hemisférica, a pesar de ser un buen método para estimar el IAF (Valladares & Guzman, 2006; Montes et al., 2007), no lo es para la estimación de la defoliación, al menos en este caso en concreto. Esto pone de manifiesto la complejidad de la evaluación de la sanidad en los montes y la necesidad de evaluadores experimentados en el campo para llevar acabo correctamente esta tarea.

En resumen, parece que son tres tipos de factores los implicados en el decaimiento del pino carrasco en el norte de España. El primer tipo pueden ser considerados como factores que predisponen a los pies a entrar en decaimiento. Entre ellos está el suelo, que en esta zona es un inceptisol (xerochrept), el cual esta caracterizado por una productividad muy baja y por un ambiente xérico (Soil Survey Staff USDA, 1987). También se incluye aquí la combinación de altas temperaturas con un descenso de las precipitaciones, que, como hemos visto en el capítulo II, influye claramente sobre el estado sanitario de las masas (Sanz-Ros, Pajares & Diez 2008), y puede producir un impacto negativo en la fisiología y el crecimiento de los pinares (Borghetti et al. 1998). Nuestros resultados indican que ciertas características del rodal, como la edad, el área basimétrica, la altura de copa y la elevación pueden afectar a la condición de copa y por ello podrían ser considerados como factores de predisposición. El segundo tipo de factores son lo que incitan al decaimiento, entre los que podrían incluirse las heladas y la defoliación. Las heladas está bien descrito que producen daños fiscos al pino carrasco, sobre todo cuando ocurren en primavera tardía o en otoño temprano (Muñoz et al., 2007). La defoliación, está determinada por una serie de factores abióticos y bióticos, sin embargo el hecho de que un árbol esté defoliado produce un descenso de la actividad fotosintética, la fijación de carbono y el crecimiento vegetal (Wiley et al. 2013; Pinkard et al. 2014), reduciendo de esta manera los recursos de los que el árbol dispone, y con ello su vigor. Además los pies defoliados tienen una mayor sensibilidad al ataque por otras plagas o patógenos (Houston, 1992), por lo que aumenta la probabilidad de que entre en decaimiento y muera. El tercer y último tipo de factores son los que contribuyen al decaimiento, en los que cabría citar la presencia de patógenos primarios y otros de debilidad en la zona, capaces de reducir la copa cuando atacan al pino carrasco (Santamaría, Pajares & Diez 2003; Santamaría et al. 2007; Botella; Santamaría & Diez 2010), contribuyendo a que se produzca el decaimiento.

En España, los escenarios futuros de cambio climático probablemente van a producir un aumento de la incidencia de los factores de predisposición y de los que incitan al decaimiento, ya que los modelos predicen un aumento de la temperatura y un descenso de la precipitación (Kattenberg et al. 1996; Parry 2000; Mossman 2002; IPCC 2013). En cuanto a los factores contributivos (presencia de hongos patógenos), por un lado se pueden ver afectados negativamente por un aumento de la xericidad (Brasier, 1996; Frankland, Magan & Gadd, 1996; Houston, 1998), aunque por otro hay estudios que sugieren un aumento de la actividad de patógenos oportunistas debida a un descenso en el vigor de los pinos (Santamaría et al., 2007).

CARACTERIZACIÓN DE LA COMUNIDAD FÚNGICA ENDÓFITA Y SU INFLUENCIA EN EL ESTADO SANITARIO Y CRECIMIENTO DE *PINUS SYLVESTRIS*

El estudio de la comunidad fúngica endófita de ramillos de pino silvestre mostró la presencia de 43 taxones fúngicos (33 identificados y otros 10 no identificados). Hemos encontrado tres especies que no habían sido encontradas nunca en la familia Pinaceae y nueve que no habían sido encontradas en pino silvestre anteriormente, lo cual confirma nuestra hipótesis de que debe haber hongos viviendo en los tejidos que no hayan sido encontrados e identificados hasta el momento para esta especie. La composición y abundancia de especies está relacionada con el crecimiento del pino silvestre, ya que difiere entre pinos con una alta tasa de crecimiento y los que la tienen baja, lo cual confirma nuestra hipótesis de que la comunidad fúngica endófita está relacionada con el crecimiento vegetal. Hay algunas especies que se mostraron claramente asociadas a altas o bajas tasas de crecimiento, por ejemplo, *Phoma herbarum* y *Hormonema dematioides* fueron encontrados asociados a crecimientos rápidos, mientras que *Hypocrea lixii* y *Ophiostoma nigrocarpum* se asociaban a crecimientos lentos.

Los hongos previamente encontrados en pino silvestre fueron *Alternaria alternata*, *A. infectoria*, *A. tenuissima*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Beauveria bassiana*, *Fimatariella rabenhorstii*, *Fusarium verticillioides*, *Geomyces* sp., *Hypocrea lixii*, *H. viridescens*, *Hormonema dematioides*, *Leotiomyetes sp.*, *Lophodermium conigenum*, *Phoma herbarum*, *P. macrostoma*, *Sarocladium strictum*, *Sordaria fimicola*, *Penicillium glabrum*, *P. minioluteum*, *Sarea resinae*, *Trichoderma atroviride*, *T. viride* y *Xylaria* sp. (Cooke, 1973; Carroll et al., 1977; Russo & Bradley, 1979; Diwani & Millar, 1987; Land et al., 1993; Kubátová, 2000; Anderson et al., 2003; Pirttilä et al., 2003; Lygis et al., 2004; Kwasna, 2008; Zamora et al., 2008; Giordano et al., 2009; Peršoh et al., 2010; Menkis & Vasaitis, 2011; Terhonen et al., 2011). Por otro lado, las siguientes especies ya habían sido descritas en otras especies de pino, pero no en pino silvestre: *Daldinia fissa, Nygrospora oryzae, Ophiostoma nigrocarpum, Penicillium melinii, P. polonicum, Pezicula eucrit*a (Smith, 1965; Carroll & Carroll, 1978; Kowalski & Zych, 2002; Guo et al., 2003; Kim et al., 2005; Wang et al., 2005; Zamora et al., 2008; Botella & Diez, 2010; Kowalski & Andruch, 2011).

Por lo tanto 30 de 33 especies (excluyendo las 10 no identificadas) habían sido previamente encontradas e identificadas en pinos, mientras que 24 de las 33 habían sido encontradas previamente en pino silvestre, por lo que nueve especies han sido encontradas en el pino silvestre por primera vez, desde nuestro conocimiento: *Biscogniauxia mediterranea, Daldinia fissa, Hypocrea viridescens, Nygrospora oryzae, Ophiostoma nigrocarpum, Phaeomoniella effusa, Penicillium melinii, P. polonicum* y *Plectania milleri*. Además, tres de ellas es la primera vez que han sido encontradas en la familia Pinaceae: *B. mediterranea, P. effusa* y *P. milleri*.

Por otro lado la mitad de estas especies pueden ser consideradas como generalistas en cuanto a su hospedador, ya que han sido encontradas también en planifolios, que son: *Alternaria alternata, A. tenuissima, Aureobasidium pullulans, Beauveria bassiana, Biscogniauxia mediterranea, Fusarium oxysporum, Hypocrea lixii, Hormonema dematioides, Nygrospora oryzae, Penicillium glabrum, Sarocladium strictum, Sordaria fimicola, Trichoderma harzianum, T. viride* y *Xylaria* sp. (Chapela, 1989; Fisher & Petrini, 1992; Bettucci & Alonso, 1997; Collado et al., 1999; Santamaría & Diez, 2005; Gonthier et al., 2006; Jaklitsch et al., 2006; Unterseher &

Schnittler, 2010; Martín-García et al., 2011). Por lo tanto la otra mitad de las especies encontradas son consideradas como especialistas de pinos o de pino silvestre. En este sentido, las especies dominantes suelen ser especialistas en cuanto al hospedador, ya que cada taxón vegetal suele portar una comunidad característica de hongos endófitos (Saikkonen, 2007).

En cuanto a la distribución de los hongos en Clases (a nivel taxonómico), nuestros resultados mostraron que las Clases Dothideomycetes y Sordariomycetes fueron las más abundantes, lo cual concuerda con otros resultados obtenidos previamente en especies de pinos de la Península Ibérica (Zamora et al., 2008; Botella & Diez, 2010), al igual que en otros países, donde también han sido encontradas estas clases como las más abundantes en pinos (Ganley & Newcombe, 2006; Arnold et al., 2007).

En lo referente a la diversidad de hongos, la encontrada en este estudio (Capítulo IV) resulta similar a la de otros estudios realizados en ramillos de pino (Zamora et al., 2008; Botella & Diez, 2010; Martínez-Álvarez et al., 2012), y mayor que otros estudios realizados en acículas o en piñas (Ganley & Newcombe, 2006; Arnold et al., 2007; Peršoh et al., 2010), aunque bastante inferior a los realizados en raíces o en la albura de pinos (Giordano et al., 2009; Menkis & Vasaitis, 2011).

La curva de rarefacción nos indica que si hubiera sido tomado un mayor número de muestras se habría obtenido un mayor número de especies fúngicas endófitas. En este contexto cabe tener en cuenta que las especies dominantes y más abundantes son de mayor interés ya que suelen se especialistas en cuanto al hospedador (Saikkonen, 2007). Aun asumiendo que hubiéramos obtenido solamente la mitad de las especies realmente presentes, asumimos el hecho de que la mayoría de especies dominantes han sido encontradas, ya que está descrito que en ambientes naturales la mayoría de endófitos de árboles sanos generalmente ocurren en un número muy bajo y solamente unas pocas especies son frecuentes (Müller & Hallaksela, 1998; Ragazzi et al., 2003; Saikkonen, 2007; Linaldeddu et al., 2010). Las que no han sido detectadas puede que no crezcan en medio de cultivo *in vitro*, o que crezcan muy lentas y hayan sido ocultadas por otras de crecimiento más rápido. Si se hubiera usado otra metodología para identificar las especies, como la PCR ambiental, seguramente habríamos obtenido un mayor número de especies, sin embargo el hecho de tener estos organismos en cultivos puros podría resultar interesante en caso de querer realizar otro tipo de ensayos con estos organismos, por ejemplo ensayos de patogenicidad o ensayos para evaluar su capacidad de modificar el crecimiento vegetal. En cualquier caso, la estimación de riqueza de especies y diversidad deben ser tenidos en cuenta como estimaciones mínimas.

El análisis factorial nos reveló que la diversidad fúngica no estaba relacionada con el tamaño del árbol ni con su tasa de crecimiento, relación que sí ha sido encontrada en estudios con especies fúngicas micorrícicas (Korkama et al., 2006). Sin embargo la equitatividad de la distribución de hongos endófitos sí que se relacionó negativamente con la tasa de crecimiento, sugiriendo que en árboles de crecimiento rápido hay unas pocas especies fúngicas dominantes, mientras que en árboles de crecimiento lento la comunidad fúngica está más uniformemente distribuida en cuanto a la abundancia de las especies que la conforman.

Nuestros resultados indican que ciertas especies o grupos de especies están asociados a altas o bajas tasas de crecimiento. No podemos saber en base a nuestros resultados si existe alguna relación causa-efecto entre la tasa de crecimiento y la comunidad fúngica endofítica. Una posibilidad es que la disponibilidad o la calidad de los nutrientes utilizables por los hongos difiera entre pinos que crecen rápido y lento, o que la estructura interna de los tejidos pueda representar un nicho diferente para los hongos según la tasa de crecimiento del pino. Sin embargo tampoco podemos excluir la posibilidad de que sea el hongo endófito el que esté contribuyendo a un mayor crecimiento del árbol. En este sentido ha sido descritas previamente mejoras en el crecimiento vegetal de plantas leñosas provocadas por la producción de reguladores del crecimiento vegetal sintetizados por hongos endófitos de raíces y tallos (Gronberg, Kaparakis & Sen, 2006; Hietala, Sen & Lilja, 1994; Xin, Glawe & Doty, 2009). Además *Phoma herbarum* y otras especies del género *Phoma* tienen la capacidad de producir giberelinas (Hamayun et al., 2009; Rim et al., 2007), y la presencia de *Phoma* spp. ha sido detectada previamente en diferentes tejidos en pinos, como son las raíces (Menkis & Vasaitis, 2011), la albura (Giordano et al., 2009), acículas y ramillos (Zamora et al., 2008). Teniendo en cuenta que *P. herbarum* es uno de los hongos más abundantes en nuestro estudio, y además se ha encontrado asociado a altas tasas de crecimiento, estaría justificado el estudio de los mecanismos que llevan a influir en el crecimiento vegetal y su posible aplicación como biofertilizante, en caso de demostrarse que finalmente ejerce dicho efecto sobre el crecimiento en pinos.

En el otro extremo se encuentra *Hypocrea lixii* (anamorfo *Trichoderma harzianum*), el cual ha sido asociado a pinos con bajas tasas de crecimiento, junto con otras dos especies, cuya asociación es un poco más débil, que son *Ophiostoma nigrocarpum* y *Biscogniauxia mediterranea*. Estudios previos han descrito que *H. lixii* es capaz de promover el crecimiento vegetal de plántulas de pino al ser inoculada en conjunto con *Laccaria laccata* (Ahangar et al., 2012). Además *H. lixii* es un conocido micoparásito (Chet & Inbar, 1994), por lo que su presencia en árboles con bajas tasas de crecimiento podría ser explicado por la presencia de otros patógenos, por ejemplo los dos anteriormente citados o *Fusarium verticillioides*, los cuales pudieran estar siendo combatidos por *H. lixii*. Otros estudios también han encontrado esta especie asociada a pinos con un crecimiento reducido (Giordano et al., 2009). Tampoco podemos excluir que diferentes genotipos de esta especie puedan tener diferentes efectos en el crecimiento de pinos, así como que su interacción con diferentes organismos pudiera resultar en diferentes efectos sobre dicho parámetro.

Resulta interesante la presencia de organismos patógenos, como *F. verticillioides* o *B. mediterranea* en tejidos sanos de *Pinus sylvestris*, aunque la presencia de organismos patógenos para una especie como endófitos en otras no es tan rara y ha sido descrita en numerosas ocasiones (Chapela, 1989; Rodríguez et al., 2012). Además, *B. mediterranea* es conocido como el agente causal del chancro carbonoso de *Quercus* spp. (Jiménez et al., 2005), atacando principalmente a *Q. suber* (Muñoz et al., 2007), aunque también ha sido identificado como patógeno en *Pistacia vera*, *Juglans* sp. (FAO, 2007) y *Persea bombycina* (Dutta et al., 2010). Normalmente un brote de chancro carbonoso suele ir precedido de un periodo de latencia relativamente largo, de modo que este organismo es capaz de vivir como endófito durante cierto tiempo en los tejidos de *Quercus* (Vannini et al., 2009), lo cual hace más rara su presencia en pino silvestre, donde además ha sido encontrado muy frecuentemente (53% de las parcelas), siendo una de las especies más abundantes del presente estudio. Teniendo en cuenta que *B. mediterranea* muestra una tendencia a ocurrir más frecuentemente en pinos con bajas tasas de crecimiento (p=0,06, ANOVA), se plantea la posibilidad de que pudiera estar afectando negativamente al crecimiento de pino silvestre, y por ello estaría justificada mas investigación en este sentido.

EFECTO DE LOS FACTORES DE SITIO EN LA DISTRIBUCIÓN DE HONGOS ENDÓFITOS DE *PINUS SYLVESTRIS* EN EL NORTE DE ESPAÑA

Los factores de sitio, incluyendo clima, suelo y características dasométricas del rodal influyen claramente sobre el crecimiento vegetal y en este estudio se pretende evaluar su influencia en la distribución de hongos endófitos de ramillos de pino silvestre. Nuestros resultados indican que la comunidad de hongos endófitos está claramente asociada a ciertas condiciones ambientales y factores de sitio. Han sido identificados diferentes factores influyendo la distribución de endófitos, los cuales incluyen variables de clima, suelo, condición de copa y características de rodal. Sin embargo estos diferentes factores parece que no ejercen su acción de forma independiente, sino que existen ciertas asociaciones entre ellos, formando combinaciones de factores que producen nichos determinados para ciertas especies o grupos de especies fúngicas endófitas.

Las principales características que diferencian unas parcelas de otras fueron la temperatura, precipitación y ciertos parámetros edafológicos, como la textura, el pH, la capacidad de intercambio catiónico (CIC en adelante) y el contenido de nutrientes. Entre éstos últimos el carbono (C), nitrógeno (N), fósforo (P), potasio (K), calcio (Ca) y magnesio (Mg) fueron los más influyentes en la separación de las parcelas y en la distribución de endófitos. En especies herbáceas ha sido encontrada una mayor tasa de asimilación de nutrientes cuando se inoculaban ciertos endófitos fúngicos (Rahman & Saiga, 2005). Sin embargo en especies leñosas son escasos los trabajos en los que esta interacción haya sido estudiada. En planifolios, en concreto en chopos, ha sido encontrada una relación similar a la descrita en herbáceas (Martín-García, 2011), aunque no han sido encontrados trabajos previos en coníferas.

Por otro lado, la estructura de los diferentes rodales es un reflejo del manejo selvícola llevado a cabo, el cual va a determinar factores como la densidad, el área basimétrica o el índice de Reineke en cada parcela. Normalmente las cortas suelen producir un mayor crecimiento de un número menor de pies (Assmann, 1970), y de hecho en el presente estudio ha sido observado que las parcelas en las que se habían llevado a cabo tratamientos selvícolas la tasa de crecimiento fue mayor, incluso aun teniendo un menor contenido de nutrientes en el suelo o una menor CIC, aunque esto es ampliamente sabido en la selvicultura de pinares (Assmann, 1970; Serrada, Montero & Recke, 2008).

Algunas interacciones entre factores merecen ser comentadas. Las parcelas con altas temperaturas medias y bajas precipitaciones estaban asociadas a suelos con un pH mayor, un mayor porcentaje de elementos gruesos y un alto contenido en Ca, Mg, K and P, mientras que parcelas con temperaturas más bajas y precipitaciones mayores estaban asociadas a un mayor porcentaje de arcilla, un mayor contenido de N y una alta CIC. Sin embargo otros elementos del suelo como el porcentaje de arena y limo, el C y el Na no se encontraron asociados a parámetros climáticos. En general observamos que el crecimiento del pino silvestre depende principalmente de la textura del suelo, el contenido de C y N y de la edad y densidad de la masa, lo cual coincide con lo previamente descrito para esta especie (Assmann, 1970; Serrada et al., 2008).

Según los resultados obtenidos la comunidad fúngica endófita está influenciada claramente por diversos factores ambientales. Aunque la influencia de los parámetros climáticos había sido previamente descrita en la distribución de hongos en acículas (Bills 1996; Carroll & Carroll, 1978), su influencia en endófitos de ramillos ha permanecido poco estudiada, lo cual justifica el presente estudio.

Endófitos como *Phoma herbarum* y *Hormonema dematioides* fueron encontrados asociados a árboles grandes creciendo rápido sobre suelos arenosos que poseían altos niveles de N, P y K y con altas temperaturas y precipitaciones. Como se ha comentado en el apartado anterior, *P. herbarum* y otras especies del género *Phoma* son capaces de producir reguladores del crecimiento vegetal (Hamayun et al. 2009; Rim et al., 2007). Sin embargo no podemos saber si esta especie está influyendo sobre el crecimiento o éste está solamente determinado por las condiciones ambientales. Por ello es necesario determinar cuál es el papel de este organismo en el crecimiento de pinos, ya que podría tener aplicaciones útiles como biofertilizante, en caso de confirmarse dicha capacidad. Además la presencia de *H. dematioides* está inversamente relacionada con la de *B. meditarreanea*, sugiriendo que el primero esta excluyendo al segundo, lo cual sugiere un papel protector de *H. dematioides* contra otros patógenos. Ese mismo papel ha sido sugerido anteriormente para esta especie fúngica en yemas de pino silvestre (Pirttilä et al., 2003).

Por otro lado, *Biscogniauxia mediterranea*, *Hypocrea lixii* y *Ophiostoma nigrocarpum* se encuentran asociados a árboles grandes que crecen lentamente, situados en suelos con un pH más ácido y un bajo contenido de C, Mg y N, así como a bajas temperaturas y altas precipitaciones. Algunas especies de *Ophiostoma* son considerados patógenos en pinos, y *B. mediterránea* es un patógeno de *Quercus*, sin embargo, al estar asociada a bajas tasas de crecimiento no se puede descartar que pueda resultar patógeno también sobre pinos.

En resumen, nuestros resultados sugieren que las parcelas en las que las condiciones climáticas, edafológicas y del rodal son más favorables para el crecimiento vegetal, como en este caso es la combinación de altas temperaturas y precipitaciones, un elevado contenido de nutrientes en el suelo y una densidad adecuada, se favorece una mayor abundancia de hongos con un efecto positivo sobre el hospedante, como *H. dematioides* o *P. herbarum*. Un efecto similar ha sido descrito en herbáceas, donde un endófito protege la planta ante el ramoneo levado a cabo por un herbívoro, pero lo hace de manera más intensa cuando las condiciones edáficas son más favorables, al existir un mayor contenido de nutrientes (Lehtonen, Helander & Saikkonen, 2005).

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Conclusions

CONCLUSIONS

1. There has been identified several abiotic and biotic factors influencing forest health, including climatic (long term temperatures and precipitations), soil (texture, pH and nutrient content) and stand characteristics (age, basal area, canopy depth and elevation), as well as fungal endophytes, which can modulate host development and its relation with other biotic harmful agents.

2. Defoliation and discoloration were the most suitable indicators of canopy condition, since they can be evaluated in all plots, while stem fork, sinuosity, cankers or the presence of reduced needles were found only in some plots. Damage type is not enough information for helping on the selection of a species for planting, since several and different damages were found in each species, and damage amount presented a low variation among pine species. *Pinus sylvestris* was the less defoliated species (i.e. the lowest defoliation intensity) and showed some stem cankers. *Pinus nigra* was the less frequently defoliated species (i.e. the lowest frequency of defoliated trees) but showed some discoloration and the highest percentage of stem forking. Finally, *P. pinaster* was the more defoliated species and showed the highest number of dead trees.

3. Canopy condition is influenced by temperatures and precipitations of long term preceding years, and not only by recent years' conditions, as it is usually thought. Historical climatic conditions may influence the present crown condition, which is a result both of recent and past climatic conditions. Therefore, defoliation will follow the temperature increasing trend, causing a reduction of tree vigor, and leading to an increase of pests and diseases attack risk. An increase of 2 ºC in mean temperature could produce a 30 % increase in defoliation, which may represent an important reduction of canopy.

4. Canopy condition is influenced by a number of stand characteristics, including age, basal area, canopy depth and elevation. This influence might determine the final response of trees to stress and the severity of the observed decline in *Pinus halepensis* stands. These factors may be considered as predisposing factors for the decline, while frosts and defoliation could be considered as inciting factors. Even when also contributing factors (primary and secondary fungal pathogens) are present, only declining trees are not able to recover from its attack. Thus, suitable forest management strategies could be designed to modify certain stand characteristics with the aim of decreasing the incidence of the decline, such as reducing tree density, pruning, create and heterogeneous stand structure (i.e. different tree sizes).

5. A moderate diversity of fungal endophytes was obtained from Scots pine twigs. Forty three different taxa were found, but it is likely that some more species remain unidentified. Nine new endophytic species living in *P. sylvestris* twigs were reported for the first time, from which three species were first reported in live trees of the Pinaceae. Fungal endophytic community was found to be related to tree growth, and some species might be involved in mechanisms affecting host growth and its relation with other harmful organisms. However, inoculation studies are needed to disclose possible causal effects by these fungi on their host.

6. Fungal endophytes distribution in Scots pine twigs is affected by numerous site factors. We can separate them in climate parameters (including summer and winter temperatures and precipitation), followed by soil parameters (such as texture, pH or nutrient content), and stand structure parameters (density and basal area). Locations with favorable conditions have shown a higher abundance of beneficial fungal endophytes, which may be promoting growth or protecting against pathogens. On the other hand, pathogenic fungi were more abundant in locations under stressful conditions that produce a decrease of tree vigour, which might represent an opportunity for the proliferation of some pathogenic or opportunistic fungi.