Factors influencing the epidemiology of *Fusarium circinatum* in northern Spain

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DOCTORAL DISSERTATION/TESIS DOCTORAL

Factors influencing the epidemiology of *Fusarium circinatum* in northern Spain

Factores que afectan a la epidemiología de *Fusarium circinatum* en el norte de España

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Agradecimientos

La investigación exige esfuerzo, tiempo, dedicación y responsabilidad; implica compromiso, disponibilidad, ilusión y generosidad; proporciona alegrías, disgustos, satisfacciones y decepciones; requiere apoyo, respeto, confianza, complicidad y amistad. Me gustaría dedicar estas líneas a todas las personas que a lo largo de mi corta experiencia en este mundo han compartido todo esto conmigo.

En primer lugar a mis directores, Julio Diez y Mercedes Fernández, por darme la oportunidad de acercarme a la Patología y a la Entomología Forestal, por la oportunidad de asistir a congresos y a cursos y, sobre todo, por su tiempo y por sus consejos. También quisiera agradecer a mis supervisores de la estancia en el Instituto Finlandés de Investigación Forestal, Jarkko Hantula y Eeva Vainio, su disponibilidad y su generosidad.

Estos años han sido inolvidables gracias a mis compañeras y compañeros de laboratorio, de oficina, de pasillo, de máster, del club de té y de la estancia en el METLA. Muchas gracias por vuestro apoyo y por vuestra amistad, ha sido muy divertido. No tengo palabras para agradecer la generosidad de las personas que me han ayudado en el trabajo de campo, en el análisis de los datos, en las crisis del trabajo de laboratorio y con los nervios (y demás incidentes) previos a las charlas.

A mi gran familia (y a mi pequeña familia) quiero agradecerles que siempre respeten mis decisiones, que me ayuden en todo lo que pueden y más y, sobre todas las cosas, que siempre se ilusionen conmigo.

La investigación no debería entenderse sin financiación aunque la Ciencia se ejerza por inquietud personal, por lo que en último lugar me gustaría nombrar la Encomienda de Gestión entre Ministerio de Medioambiente, Medio Rural y Marino y el INIA "Etiología, Epidemiología y Control de *Fusarium circinatum*" y el proyecto del Ministerio de Economía y Competitividad "AGL2012-39912", gracias a los cuales pude llevar a cabo mi trabajo. También a las acciones Cost "FA1103", "FP1102" y "FP1406" que han facilitado mi formación en Patología Forestal. Además me gustaría recordar las instituciones que han hecho posible este trabajo y agradecer la disponibilidad y el trabajo de las personas que forman parte de ellas: el Servicio de Montes del Gobierno de Cantabria, la Universidad de Valladolid, el Centro de Sanidad Forestal de Calabazanos y el Instituto Universitario de Gestión Forestal Sostenible.

Abstract

Fusarium circinatum is an ascomycete fungus belonging to the *Gibberella fujikuroi* clade that causes pitch canker disease (PCD) in pines. The main symptom of PCD in adult trees is the presence of pitch soaked cankers which may girdle the trees. The disease can affect the crown when suitable wounds are available for infection, causing dieback that can lead to tree dead. *Fusarium circinatum* poses a threat to pine plantations and natural stands throughout the world, especially to *Pinus radiata* D. Don plantations due to the high susceptibility of this species. In regard to PCD epidemiology, there are two major aspects that have to be considered: abiotic factors, like forest management and environmental conditions, and biotic factors, like the presence of fungal communities and bark beetles. The main objective was to study the influence of several factors (abiotic and biotic) on the distribution of pitch canker disease caused by *F. circinatum* in northern Spain. Four specific aims were established: i) to determine the effect of pruning on *P. radiata* plantations infected with PCD, ii) to evaluate the fungal communities, with special attention to *Fusarium* species, present on bark beetles and their galleries in PCD affected stands, iii) to determine whether the pine shoot beetle *Tomicus piniperda* L. may vector the pitch canker pathogen and iv) to study the association between *Pityophthorus pubescens* Marsham and *F. circinatum* in PCD affected plantations.

For the first objective, 50 *P. radiata* plots (pruned and unpruned) affected by *F. circinatum* in the Cantabria region (northern Spain) were evaluated. Symptoms of PCD, such as dieback, cankers and red shoots were evaluated in 25 trees per plot and related to pruning. Regarding the second objective, funnel traps and logs were placed in a *P. radiata* plot affected by *F. circinatum*. Traps were baited with different attractive compounds: (E)-pityol and ethanol and alfa-pinene. In addition, fresh green shoots with *T. piniperda* feeding galleries were collected from the ground in 25 *P. radiata*-affected plots. The insects and gallery tissues were processed and cultured with the aim of isolating and identifying associated fungi. After this general sampling in which 12 insect species were studied in relation to *F. circinatum*, two of them were selected for more accurate evaluation: *T. piniperda*, due to its association with *F. circinatum* and healthy trees, and *P. pubescens*, due to the importance of this genus as *F. circinatum* vectors in California. To study the potential role of *T. piniperda*, fresh green shoots fallen to the ground with feeding gallery, breeding galleries from diseased trees and insects in their dispersion flight were collected and processed. Moreover, a laboratory experiment in which specimens of *T. piniperda* were inoculated with the pathogen prior to feeding on shoots was conducted. Regarding the role of *P. pubescens*, funnel traps baited with (E)-pityol were established and twigs from infested trees were sampled to collect insects and plant material. Both of them were plated on culture media with the aim of isolating *F. circinatum*. Moreover, an experiment was carried out under natural conditions in which *P. radiata* trees were baited with (E)-pityol in order to evaluate PCD symptoms 4 different times.

Taking into account the results of the first objective, a significant effect of pruning on the number of cankers per tree was observed. Moreover, the presence of resin outside the cankers was also higher in pruned plots. Regarding the biotic factors, a total of 24 different fungal species were obtained from bark beetle´s galleries while 18 were obtained from the insects' exoskeletons. Ten different species of *Fusarium* were isolated from tissues and insects. From them, *F. circinatum* was isolated from several bark beetles´ bodies and from their galleries. In regard to the third objective, *T. piniperda* appeared associated with both diseased and healthy *P. radiata* trees. *Fusarium circinatum* was present on *T. piniperda*'s exoskeleton in nature and the laboratory experiment evi-

denced the ability of *T. piniperda* to transfer the pathogen to healthy shoots. Results from the experiment carried out with *P. pubescens* indicated that the pathogen appeared on 1 % and 2 % of the collected insects in funnel traps during 2010 and 2012, respectively. Regarding the collected twigs, *F. circinatum* was found only in 3 galleries. Results from the baiting experiment showed that symptoms in the crown were more influenced by (E)-pityol than symptoms on the trunk.

In conclusion, both abiotic, like pruning, and biotic factors, like the presence of fungal communities and bark beetles, resulted in an important association with PCD epidemiology. *Tomicus piniperda* seems to have a more plausible role as a vector of *F. circinatum* than other bark beetles species in our study area, due to its maturation feeding behaviour.

Resumen

Fusarium circinatum es un hongo ascomicete perteneciente al complejo de especies de *Gibberella fujikuroi*, siendo el agente causante de la enfermedad del chancro resinoso del pino. En árboles adultos, el principal síntoma de esta enfermedad es la presencia de chancros con abundante resina en el tronco así como en ramas gruesas que pueden causar el anillamiento del árbol. Además, esta enfermedad puede causar daños en las copas al haber heridas susceptibles de ser infectadas por el patógeno, causando puntisecado que puede derivar en la muerte del árbol. *Fusarium circinatum* supone una amenaza para los bosques y plantaciones de todo el mundo, especialmente para las plantaciones de *Pinus radiata* D. Don debido a la gran susceptibilidad de esta especie. En relación con la epidemiología de esta enfermedad, hay que considerar dos aspectos principales: los factores abióticos, como la gestión forestal o las condiciones ambientales, y los factores bióticos, como la presencia de comunidades fúngicas y la presencia de escolítidos en las masas afectadas por la enfermedad. El principal objetivo de este estudio fue determinar la influencia de los diferentes factores, tanto abióticos como bióticos, en la distribución de dicha enfermedad en el norte de España. Para ello, se plantearon cuatro objetivos específicos: i) evaluar el efecto de la poda en plantaciones afectadas por la enfermedad, ii) estudiar las comunidades fúngicas, especialmente las especies de *Fusarium,* asociadas a los barrenillos (Coleoptera; Scolytinae) y sus galerías en plantaciones afectadas por el chancro resinoso, iii) determinar si *Tomicus piniperda* puede ser un agente vector de *F. circinatum* y iv) estudiar la asociación entre *Pityophthorus pubescens* Marsham y *F. circinatum* en plantaciones afectadas.

Para llevar a cabo el primer objetivo, se evaluaron 50 parcelas de *P. radiata* (podadas y no podadas) afectadas por *F. circinatum* en Cantabria (norte de España). En cada parcela se evaluó la presencia de los síntomas de la enfermedad en 25 árboles, la presencia de puntisecado, chancros y ramillos rojos, y se estudió su posible asociación con la poda. En relación con los factores bióticos y concretamente con el segundo objetivo, se colocaron trampas multiembudo y trozas cebo en una parcela afectada por la enfermedad. Las trampas se cebaron con diferentes compuestos atrayentes: cuatro con (E)-pityol y seis con etanol y alfa-pineno para capturar las distintas especies de barrenillos existentes en la zona de muestreo. Además, en 25 parcelas afectadas por *F. circinatum* se recogieron del suelo ramillos verdes y frescos horadados por *T. piniperda.* Los insectos y los tejidos vegetales de las galerías fueron cultivados con el objetivo de aislar e identificar los hongos asociados. Tras este muestreo general de escolítidos en el que se recogieron 12 especies diferentes, se seleccionaron dos de ellas para realizar un estudio más detallado. En primer lugar *T. piniperda*, debido a su posible asociación con *F. circinatum* y por la alimentación de maduración que realiza en las copas de árboles sanos, y en segundo lugar *P. pubescens*, debido a la importancia que tienen las especies de este género como vectores de la enfermedad en California. Para determinar el papel de *T. piniperda*, se recogieron del suelo ramillos verdes con galería de alimentación del insecto, galerías de cría realizadas en árboles enfermos y además, se capturaron insectos durante su vuelo de dispersión. También se llevó a cabo un experimento de laboratorio que consistió en inocular especímenes de *T. piniperda* con el patógeno previamente a su alimentación de maduración en ramillos. En relación con la participación de *P. pubescens*, en parcelas afectadas por la enfermedad se recogieron insectos utilizando tramas multiembudo cebadas con

(E)-pityol, además de ramillas atacadas por el insecto. Tanto los especímenes como el material vegetal se procesaron para aislar *F. circinatum*. Por último, se llevó a cabo un experimento en campo, en el que se cebaron con (E)-pityol árboles afectados, evaluándose los daños a lo largo de un año.

Los resultados del primer objetivo mostraron un efecto significativo de la poda sobre el número de chancros por árbol. Otros síntomas presente en el tronco, como el lagrimeo, también aparecieron en mayor número en las parcelas podadas. En relación con los factores bióticos, se obtuvieron un total de 24 especies fúngicas provenientes de las galerías horadadas por los escolítidos en las trozas y en los ramillos mientras que del exoesqueleto de los insectos, se obtuvieron 18 especies. Se identificaron diez especies distintas pertenecientes al género *Fusarium* procedentes de muestras tanto de material vegetal como de los insectos. Concretamente, *F. circinatum* fue aislado del exoesqueleto de varias especies de barrenillos, así como de sus galerías. En cuanto a los los resultados obtenidos del tercer objetivo planteado, *T. piniperda* apareció asociado tanto con árboles sanos como con árboles enfermos, a pesar de que *F. circinatum* fue detectado en pocos especímenes de *T. piniperda*. El experimento de laboratorio demostró la capacidad de *T. piniperda* para transferir el patógeno a ramillos sanos. Por lo que se refiere a *P. pubescens*, el patógeno fue aislado solamente del 1 % y 2 % de los insectos recogidos en embudos durante los años 2010 y 2012, respectivamente. En las ramillas recogidas que habían sido atacadas por *P. pubescens*, *F. circinatum* se aisló de tres galerías aunque no se obtuvo ningún aislado procedente directamente de los insectos recogidos en el interior de las mismas. Los resultados del experimento con árboles cebados mostraron una mayor influencia del atrayente sobre los síntomas de la copa que sobre los del tronco.

En conclusión, los factores abióticos como la poda y los factores bióticos, como la presencia de comunidades fúngicas y la presencia de escolítidos, influyeron de forma importante en la epidemiología de la enfermedad del chancro resinoso. El papel de *T. piniperda* como vector de *F. circinatum* resultó ser más plausible que el de otras especies de escolítidos presentes en el área de estudio debido a la alimentación de maduración que practica, pudiendo de esta forma, introducir la enfermedad en árboles sanos.

Chapter 1: Introduction

1.1-The pitch canker disease pathogen: *Fusarium circinatum*

Forest diseases affect native forests and plantations throughout the world; however specific conditions are required for the pathogen establishment. Thus, there are several events that occur for the pathogen to cause a disease under adequate conditions: pathogen inoculation, pre-penetration, penetration, establishment of the infection, growth and reproduction (colonization), dissemination of the pathogen and overwintering or oversummering (Agrios, 1997). Each one of these events is essential for the pathogen´s success and its significance must be deeply understood when a particular pathogen is under study, since disease management focuses on avoiding the pathogen success (Waring and O'Hara, 2005). In the case of the pitch canker disease (PCD) pathogen, *Fusarium circinatum* Nirenberg and O´Donnell (telomorph = *Gibberella circinata*), the successful establishment is associated to several factors influencing inoculation, penetration, colonization and dissemination, as for example, forest management, environmental conditions or presence of bark beetle vectors (Wingfield *et al.*, 2008; Gordon, 2011).

Fusarium circinatum is an ascomycete fungus belonging to the *Gibberella fujikuroi* clade that causes PCD on pines (Nirenberg and O'Donnell, 1998). *Fusarium circinatum* is a seedborne pathogen that can survive both superficially and internally in the seeds, causing seed high mortality rates (Gordon, 2011). Seedlings can also show die-back and die due to the girdling, but the main symptoms observed in seedlings are necrosis, chlorosis, wilting of needles, dieback and desiccation of the seedling tip (Viljoen *et al.*, 1994; Martín-Rodrigues *et al.*, 2013). The main symptom of PCD in adult trees is the presence of pitch soaked cankers in trunks and big branches which may girdle both trees and branches (Figure 1) (Wikler *et al.*, 2003). Trickles of resin can also be found on the trunks of diseased trees. The disease can affect the crown when suitable wounds are available for infection (Gordon *et al.*, 2001), causing dieback that can lead to tree dead. However, on small diameter branches a single infection may be sufficient to cause the death of the branch (Gordon, 2011). The increase in the resin production is due to the increment on the number of traumatic resin ducts (TRDs); this fact could benefit F.circinatum since epithelial cells surrounding the TRDs have starch that the fungus uses for feeding. The increment in the resin production restricts the water supply and leads to the desiccation of the infected tissue, causing the tree dead (Martín-Rodrigues *et al.*, 2013). *Fusarium circinatum* also causes growth reduction in adult trees in forest and plantations, leading to great economical and ecological losses.

Fusarium circinatum has a wide geographical distribution. This pathogen was first reported in North Carolina (Hepting and Roth, 1946) but has also been detected in Haiti (Hepting and Roth, 1953), California (McCain *et al.*, 1987), Japan (Muramoto and Dwinell, 1990), South Africa (Viljoen *et al.*, 1994), Mexico (Guerra-Santos, 1998), Chile (Wingfield *et al.*, 2002), Korea (Cho and Shin, 2004), France (EPPO, 2004), Spain (Landeras *et al.*, 2005), Italy (Carlucci *et al.*, 2007), Uruguay (Alonso and Bettucci, 2009), Portugal (Bragança *et al.*, 2009), Colombia (Steenkamp *et al.*, 2012) and Brasil (Pfenning *et al.*, 2014). Pitch canker disease poses a threat to pine plantations and natural stands throughout the world (Wingfield *et al.*, 2008), especially to *Pinus radiata* D. Don plantations due to the high susceptibility of this pine species (Viljoen *et al.*, 1995). However,

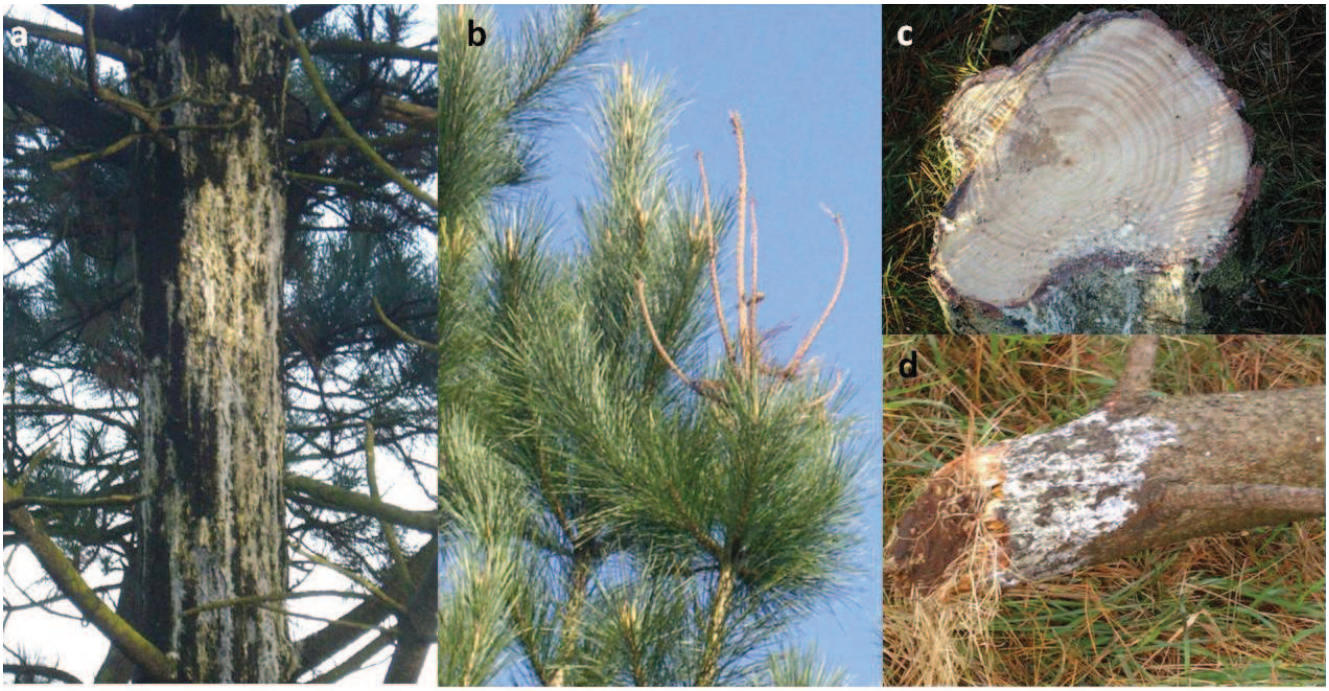

Figure 1. Pitch canker disease symptoms in *Pinus radiata* adult trees: a) pitch soaked canker on a trunk, b) crown dieback, c) trunk transversal section at the canker level, d) broken branch at the canker level.

other *Pinus* species like *Pinus pinaster* Ait. and *Pinus sylvestris* L. (Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007), as well as *Pseudotsuga menziesii* (Gordon *et al.*, 1996), are susceptible to the pathogen.

Fusarium circinatum is microscopically characterised by the presence of sterile coiled hyphae, polyphialides in branched conidiophores, non-septate microconidia and multiseptate macroconidia (Figure 2). Sporodochia with macroconidia appear sparsely on carnation leaf agar (CLA) (Leslie and Summerell, 2006). In culture on potato dextrosa agar (PDA) *F. circinatum* produces aerial mycelium that is usually white to violet and can produce grey to dark pigmentation (Ganley, 2008). The sexual stage, *G. circinata*, has been produced in culture, but has not been observed in nature. This pathogen has a necrotrophic behavior, since fungi belonging to the genus *Fusarium* do not suffer differenciation of the hyphae for invading the host tissues, i.e. haustoria or appresoria (Mendgen *et al.*, 1996). However, they are characterized by the production of cell-degrading enzymes and mycotoxins. *Fusarium circinatum* produces poligalacturonasa for the degradation of the cell wall and subsequent penetration of the host (Leslie and Summerell, 2006). Mycotoxins are secondary metabolites that are released by the fungi after host penetration, e.g. beauvericin, which is the toxin most widely produced by *F. circinatum* and by other species from the genus, as well as fumonisin (Mirete *et al.*, 2003). Beauvericin induces cell death similar to apoptosis and causes cytolisis, having entomopathogenic and phytopathogenic properties and appears to be one of the most widely produced toxins by species of *Fusarium* (Logrieco *et al.*, 1998). Due to this mechanism, vegetal cells of the host are destroyed forming gaps where conidiophores grow, however the transformation of vegetative mycelia to conidiophores requires a change on the genetic expression pattern. Martín-Rodrigues *et al.* (2013) suggest that this transformation could occur when the pathogen feeds on the starch of the parenchyma cells in the pith of seedlings and reported the production of conidiophora orientated towards the hollow cavities of the pith at the moment when the first symptoms of disease appeared.

Monterey pine, *P. radiata*, is a widely planted conifer, with 215.000 ha in Spain, mainly in the northern coast where it provides a lot of benefits due to its rapid growth (Fernández and Sarmiento, 2004), since this pine species´ wood is well-known for its use as structural material in construction (Hermoso *et al.*, 2007). The use of monospecific *P. radiata* plantations is leading to the emergence of pests and diseases that threaten the crops (Dajoz, 2001; García-Serna, 2014). In Spain, the presence of *F. circinatum* in Monterey pine plantations and in nurseries has resulted in severe loss and in a reduction of revenues due to the ban on planting susceptible species in infected areas (Real Decreto 637/2006 and 65/2010), the high costs invested in monitoring and con-

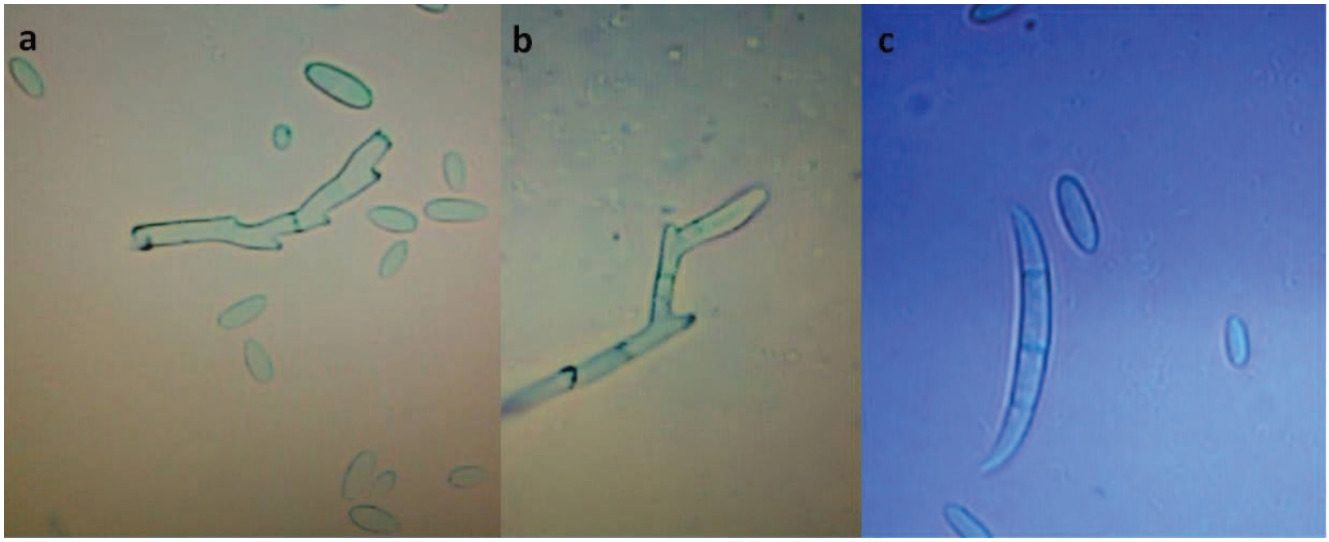

Figure 2. *Fusarium circinatum* microscopical structures on SNA. a) monophialides and microconidia, b) polyphialid, c) macroconidia and microconidia.

trol, and the restrictions on the export of timber. At present, the disease is causing damages in forests and nurseries in five regions within Spain; Galicia, Asturias, Cantabria, País Vasco and Castilla y León (Figure 3). The origin of the pathogen introduction in Spain has been deeply studied, showing two significantly differentiated populations regarding all the affected areas (Berbegal *et al.*, 2013) and a clonal population within País Vasco (Iturritxa *et al.*, 2011). Since it was first reported in Spain, several studies have been carried out to identify the factors influencing its distribution on the northern area (Romón *et al.*, 2007a) and in order to prevent the pathogen dispersal (Serrano *et al.*, 2014). But little is known regarding some of the factors influencing the epidemiology of the disease, as for instance, forest management or the specific role of bark beetles vectors.

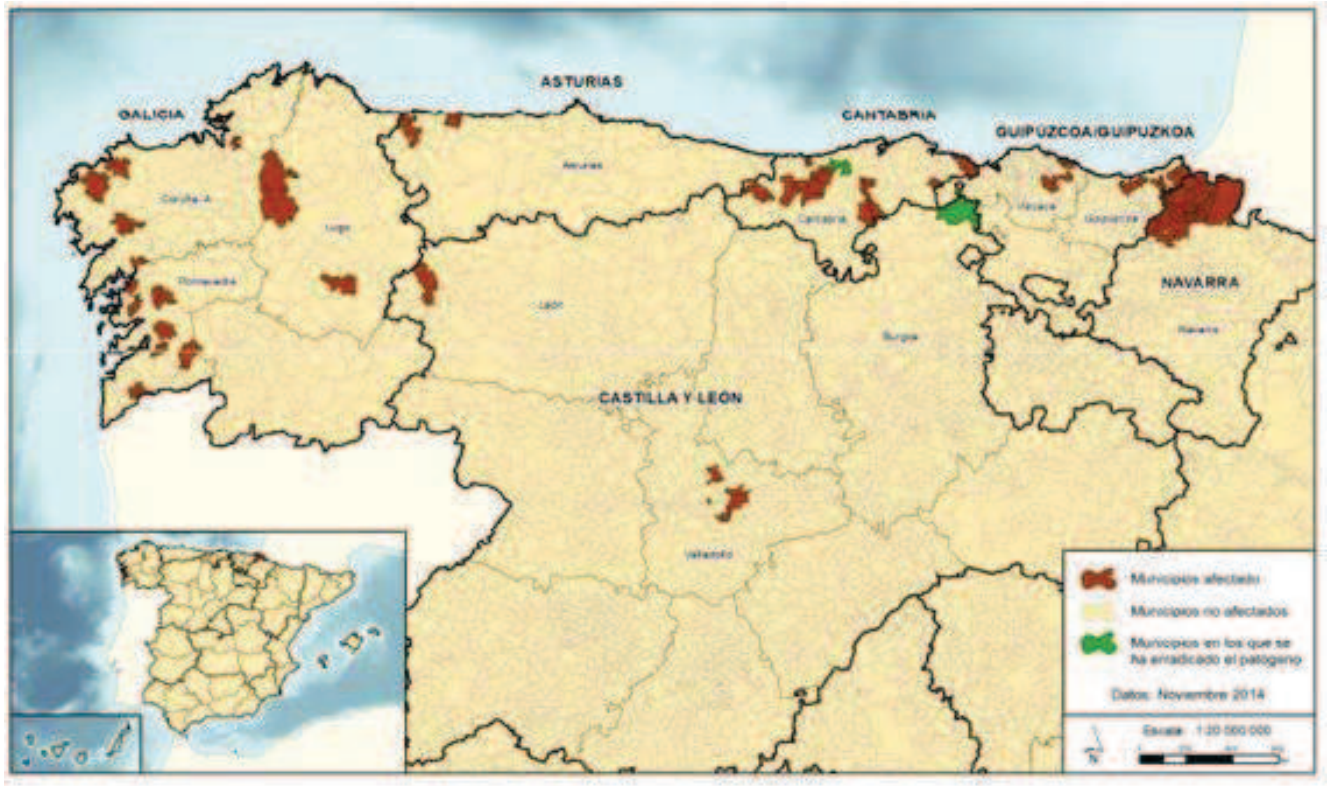

Figure 3. Distribution map of *Fusarium circinatum* in northern Spain. Municipalities are represented in different colours: affected (dark brown), non-affected (light brown) and pathogen eradicated (green). Source: Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA), Dirección General de Sanidad de la Producción Agraria, Subdirección General de Sanidad e Higiene Vegetal y Forestal, 2014.

1.2-Factors influencing the epidemiology of pitch canker disease

For a forest disease to occur, a combination of three factors must be present: susceptible plant, infective pathogen and favorable environment (Agrios, 1997). However, there are several other events that influence the incidence (number of infected plants), severity and dissemination of a disease. In regard to PCD epidemiology, there are two major aspects that have to be considered: biotic factors and abiotic factors.

1.2.1-Abiotic factors

1.2.1.1-Forest management

a- Movement of plant material

Infested seeds as well as latently-infected seedlings can serve as a vehicle for dissemination of *F. circinatum* over long distances (Gordon, 2011). However, the development of the disease coming from infected seedlings will depend on the environmental conditions. For instance, Chile, which is known to have *F. circinatum* in the nurseries but not in the plantation forests, was also predicted to have marginal to suitable climatic conditions for pitch canker establishment (Ganley *et al.*, 2009).

Fusarium circinatum has been dispersed around the world, probably, with pine infected seeds (Berbegal *et al.*, 2013). Genetic evidences have shown that Mexico is a plausible source of *F. circinatum* infection found in South Africa (Wikler and Gordon, 2000); however in Spain two independent introductions seemed to occur (Berbegal *et al.*, 2013).

b- Tree host selection

Up to 60 *Pinus* species have been reported to be susceptible to the PCD (Gordon, 2006), among them: *P. radiata*, *P. sylvestris* (Landeras *et al.*, 2005), *P. pinaster* (Vivas, 2012), *Pinus nigra* Arn. and *Pinus* uncinata Ram. (Martínez-Álvarez *et al.*, 2014) in Spain. *Pinus muricata* D. Don in California (Schmale and Gordon, 2003), *Pinus halepensis* Mill. and *P. pinea* in Italy (Carlucci *et al.*, 2007), *Pinus* rigida Mill. in Japan (Kim *et al.*, 2009) and *Pinus taeda* L. in Uruguay (Alonso and Bettucci, 2009) are also susceptible. Other coniferous trees, such as *Pseudotsuga menziesii* (Mirb.) Franco, can also suffer from this disease (Gordon *et al.*, 1996).

Although the pitch canker pathogen can cause disease in many coniferous species, not all the hosts are equally susceptible, being *P. radiata* a particularly susceptible species (Correll *et al.*, 1991; Martínez-Álvarez *et al.*, 2014). Thus, the risk of damage caused by this pathogen could be minimized using less susceptible species or selecting resistant genotypes (Gordon, 2011). Intraspecific variation has been observed regarding PCD susceptibility in a number of pine species. For instance, different *P. pinaster* families showed variation in resistance to *F. circinatum* in the study carried out by Vivas *et al.* (2012) and Bonello *et al.* (2001a) demonstrated that induced resistance could appear as a consequence of the previous presence of *F. circinatum*. Thus, the frequency of resistant individuals will influence the extensiveness of damage caused by the PCD (Gordon, 2006).

c- Pruning

Fusarium circinatum seems to require fresh wounds on trees as infection court (Dwinell *et al.*, 1985). These authors suggested that *F. circinatum* inoculum could infect wounds produced by pruning, mowing and harvesting, although little is known on this issue. Notwithstanding, the susceptibility of these wounds to infection could decrease significantly with wound age (Sakamoto and Gordon, 2006). Nonetheless, other studies carried out by Correll *et al.* (1991) suggest that branches with mechanical wounds are not susceptible to infection even if airborne inoculum is present, postulating that airborne spores are unable to infect wounds.

On the other hand, pruning could be considered for removing diseased branches, though this approach is not effective in eradicating the disease (Gordon *et al.*, 2001). Attempts to remove disease causing fungi have been made via tree pruning, though it was shown that this treatment does not completely eliminate the disease from the tree (Moorman and Lease, 1999). As such, forest management should be considered as an important factor for decreasing disease establishment and spread (Waring and O'Hara, 2005). The effect of pruning has not been deeply studied in Monterey pine plantations affected by PCD.

1.2.1.2: Environmental conditions

Environmental conditions taking place in both air and soil are determinant for the development of a disease after the contact of a pathogen with its host (Agrios, 1997). Regarding PCD development, temperature (20-25ºC for spore germination and fungal growth) and high humidity levels are chief factors for the pathogen to success (Wingfield *et al.*, 2008). Thus, it develops more rapidly in *P. radiata* plots closer to the coast than in plots located in land (Wikler *et al.*, 2003), being the fog a major factor influencing the disease distribution near the coast (Gordon, 2006).

Some other meteorological events can affect the incidence of the disease acting as wounding agents, for instance, hail or wind storms that increase the number of infection courts for the pathogen. Wounds caused by hurricanes or those resulting from wind-thrown needles are also thought to provide an infection court for the pathogen to infect the trees (Kelley and Williams, 1982). Environmental conditions also influence dissemination of *F. circinatum* spores, especially wind and rain (Gordon, 2011). Dispersal of airborne spores in *F. circinatum* and *Fusarium* spp. not only depends on the wind, but also on the rain, since macroconidia are adapted to the dispersion by wind, but before flight they require to be in touch with raindrops that carry the spores into the air (Deacon, 2006).

1.2.2-Biotic factors

1.2.2.1-Fungal communities

Fungal communities inhabiting *P. radiata* trees may be a determinant factor influencing PCD distribution in Spain (Figure 4). Fungal communities in forests are formed by endophytes together with saprotrophic and pathogenic species. Knowing the species composition and the factors influencing the presence of different fungal communities is important in terms of understanding the role that fungi play on the regulation of other organisms (Arnold, 2007). In general terms, interactions between two fungal species may occur in three different ways: i) by the exclusion of one species through the competition in exploting resources, ii) the exclusion by antagonism i.e. antibiotic production or parasitism and, finally, iii) by the ability of two species to coexist (commensalism) or to cause a profit to both (mutualism) (Deacon, 2006).

The study of fungal species present on PCD affected plantations could be crucial for the biological control of the disease. Endophytic species which do not cause any damage (Arnold, 2007), such as *Trichoderma viride* Bissett that could be used for biological control of *Fusarium* spp. (Martínez-Álvarez *et al.*, 2012), have been reported related to pitch canker diseased in *P. radiata* trees. *Trichoderma* spp. have antagonistic properties by means of antibiotic production, chitinase secretion or parasitism, the latter occurs when the hyphae of *Trichoderma* coil round the hyphae of another fungi and eventually penetrates it from these coils (Deacon, 2006). *Penicillium* spp. usually appear as saprotrophes in pines, and rarely occur as endophytes in healthy tissue (Zamora *et al.*, 2008). However, *Penicillium chrysogenum* Link. in association with *F. circinatum* in *P. radiata* resulted in antagonism and induced resistance against the pitch canker pathogen in the work carried out by Romón *et al.* (2008).

Other fungal species, e.g. *Diplodia pinea* (Desm.) Kickx, which may remain as a latent pathogen in pine trees, have been reported to be associated with *F. circinatum* in *P. radiata* plantations (García-Serna, 2014). *Diplodia pinea* is a saprotrophic fungus that can act as a parasite in stressed trees, causing shoot dieback. In *P. radiata*, the symptoms of this disease are the presence of resin drops and necrotic stem lesions (Chou, 1976; García-Serna, 2014).

Fusarium species in *P. radiata* stands affected by PCD may play a chief role as endophytes or as plant pathogens, depending on the species. The genus *Fusarium* includes important plant pathogens affecting both forest and agricultural species (Alves-Santos and Diez, 2012) because of the production of different types of wall-degrading enzymes (e.g. cellulases, glucanases or glucosidases) and mycotoxins like beauvericin or fumonisins (Mendgen *et al.*, 1996; Logrieco *et al.*, 1998). Regarding the association of *Fusarium* species with *F. circinatum*, it was found that *Fusarium lateritium* Nees, which is not pathogenic, inhibited the pathogen growth when it was introduced as a pioneer (Romón *et al.*, 2008).

1.2.2.2-Bark beetles

Bark beetles (Curculionidae; Scolytinae) have a worldwide distribution affecting forest dynamics, contributing to nutrient cycling, canopy thinning, gap dynamics, disturbance regimens and successional pathways (Raffa *et al.*, 2015). Several bark beetle species has been reported to be present in Spanish forests (Gil and Pajares, 1986), having determinant implications for forest management (López *et al.*, 2007).

Bark beetles are associated to several fungal species in native forests and plantations world-wide, in particular, with endophytic or pathogenic fungi, including *F. circinatum* (Jacobs *et al.*, 2004; Kirisits, 2004; Lieutier *et al.*, 1989; Romón *et al.*, 2007a). The interaction between fungal pathogens and insects is a complex relationship that has been widely studied, being, in many cases, a mutual relationship between the vector and the fungus that has ecological advantages for both organisms (Paine *et al.*, 1997). These pathogens have been traditionally considered allies of the insects, as they may serve to overcome tree resistance, facilitating the beetle's attack, since the successful colonization of the host by

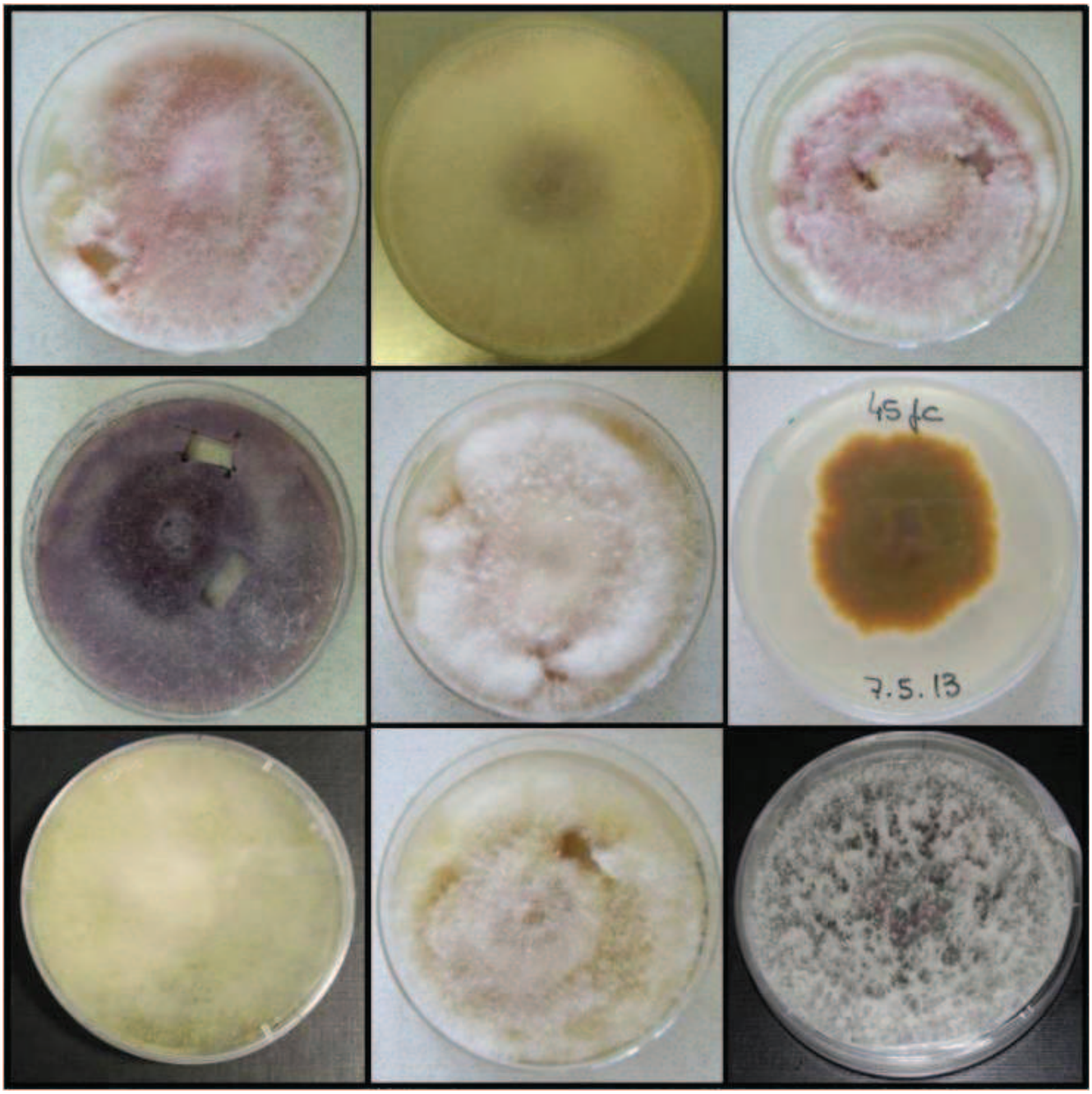

Figure 4. Fungal isolates obtained from *Pinus radiata* trees in Cantabria (Spain).

the insect depends on its ability to overcome tree resistance mechanisms (Långström and Hellqvist, 1993; Christiansen *et al.*, 1987; Franceschi *et al.*, 2005). As it has been proved that bark beetles can kill trees without any pathogenic fungi, other authors propose that this association could only benefit the fungus, allowing it to get to trees that it would not reach without an insect vector (Six and Wingfield, 2011). Lieutier *et al.* (2009) explained the role of pathogenic fungi in beetle establishment in terms of tree defence stimulation instead of in terms of defence overcoming.

To report the role of an insect species as vector of a pathogen, rules of proof for insect transmission described by Leach (1940) must be properly checked. Thus, i) a close, although not a constant, association of the insect with diseased plants must be demonstrated, ii) it must be demonstrated that the insect also regularly visits healthy plants under conditions suitable for the transmission of the disease, iii) the presence of the pathogen or virus in/on the insect in nature or following visitation to a diseased plant must be demonstrated and iv) the disease must be produced experimentally by in-

sect visitation under controlled conditions with adequate checks. Moreover, bark beetles not only act as vector or phoretic agents, but also they can act as wounding agents when bore their breeding or feeding galleries. Thus, the presence of these insect species in PCD affected stands could increase the incidence of the disease even if bark beetles are not carrying the pathogen.

Several species of *Fusarium* are associated with insects behaving in a mutualistic way, colonizing dead insects like saprophytes or acting as entomopathogens (Teetor-Barsch and Roberts, 1983). The importance of *Fusarium* spp. regarding its presence in PCD affected trees is highlighted by its entomopathogenic activity, due to the questionless role of bark beetles in *F. circinatum* spreading. These entomopathogenic fungi infecting bark beetles are usually ascomycetes, i.e. *Fusarium oxysporum*, whose infective unite are conidia that germinate on the insects' cuticle and penetrate the hemocele causing the insects death (Vega and Hofstetter, 2015). *Fusarium circinatum* has also been reported to be phoretically associated to several bark beetles species in *P. radiata* plantations in northern Spain, e.g. *Pityophthorus pubescens* (Marsham), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Boerner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* Erichson and *Orthotomicus erosus* (Wollaston) (Romón *et al.*, 2007a). We hypothesize that different bark beetle species living in these plantations could play a different role in the spreading of *F. circinatum*. The differences in their bioecology, e.g. *Hylastes* species feed on roots or trunks of declining trees whereas *Tomicus piniperda* L. feeds on shoots of healthy crowns (López *et al.*, 2007), as well as the population levels that may increase until epidemic levels (Raffa and Berryman, 1983), e.g. *I. sexdentatus*, will determine the spreading of the infections. The most relevant bark beetle species present in *P. radiata* stands in Cantabria, regarding *F. circinatum* distribution, are described below.

a- *Tomicus piniperda* L.

Tomicus piniperda is a serious pest affecting pines in Europe, Northern Africa and Asia (Långström, 1980; Bouhot *et al.*, 1988; Kirkendall *et al.*, 2008) and in the United States ever since it was introduced in 1992 (McCullough and Smitley, 1995). Its main host is *Pinus sylvestris* L. but other pine species are also suitable hosts, as, for example, *P. radiata*. *Tomicus piniperda* is a univoltine species that may present several sister broods. It is considered as a secondary pest on trunks and thick branches of weakened trees where it breeds (Figure 5), colonizing stressed or dying trees that have previously been attacked by other primary pest or pathogenic fungi (Paine *et al.*, 1997).

But, in terms of maturation feeding on the pith of the shoots *T. piniperda* acts as a primary species (Figure 6) (Långström, 1982; Lieutier *et al.*, 2015). This bark beetle becomes a primary pest capable of causing sharp reductions in growth and in carbon content, nitrogen loss, malformations and, in cases of high population densities, the death of the host (López *et al.*, 2007). The fact that *T. piniperda* causes weakness in the hosts after feeding on shoots also increases the number of reproductive niches susceptible to colonization, although shoot damage rarely exceed 50% (Långström, 1980). In regard to its life cycle in northern Spain, *T. piniperda* dispersion flight occurs in February/March, colonizing weakened trees for breeding. Subsequently, emerging young F1 beetles target the tops of nearby healthy trees to practice gonadal-maturation feeding and fat accumulation (Långström, 1982). This maturation feeding continues with the hibernation period (6-9 months within the shoots in our study area). In addition, each insect penetrates more than one shoot during the feeding phase, especially in the thicker and fresh current-year shoots (Tiberi *et al.*, 2009).

In northern Spain, *T. piniperda* is a major *candida*te for being an effective vector of *F. circinatum* due to the maturation feeding it practices in the crowns of healthy pines and subsequent overwintering. Several authors have previously mentioned the association of *T. piniperda* with virulent ophiostomatoid fungi like, *Leptographium wingfieldii* Morelet (Lieutier *et al.*, 1989), *Ophiostoma minus* (Hedgc.) Syd. & P. Syd. (Solheim *et al.*, 2001) and L. guttulatum M.J. Wingf. & K. Jacobs (Romón *et al.*, 2014). This association occurs in the absence of mycangia, although some body structures present in the base of setae could be acting as fungi transport frames (Figure 7).

b- *Pityophthorus pubescens* (Marsham)

Twig beetles, *Pityophthorus* spp., are phloeophagus and myelophagus species (Vega and Hofstetter, 2015). These bark beetle species are widely distributed in Europe living in several *Pinus* species as *Pinus pinea* L, *P. pinaster* and *P. radiata* (Gil and Pajares, 1986). Most species of this genus are secondary species with a low economic impact (Vega and Hofstetter, 2015). *Pityophthorus pubescens* is present in the Iberian Peninsula being a secondary pest that attacks weakly trees and presents a remarkable sexual dimorphism (López *et al.*,

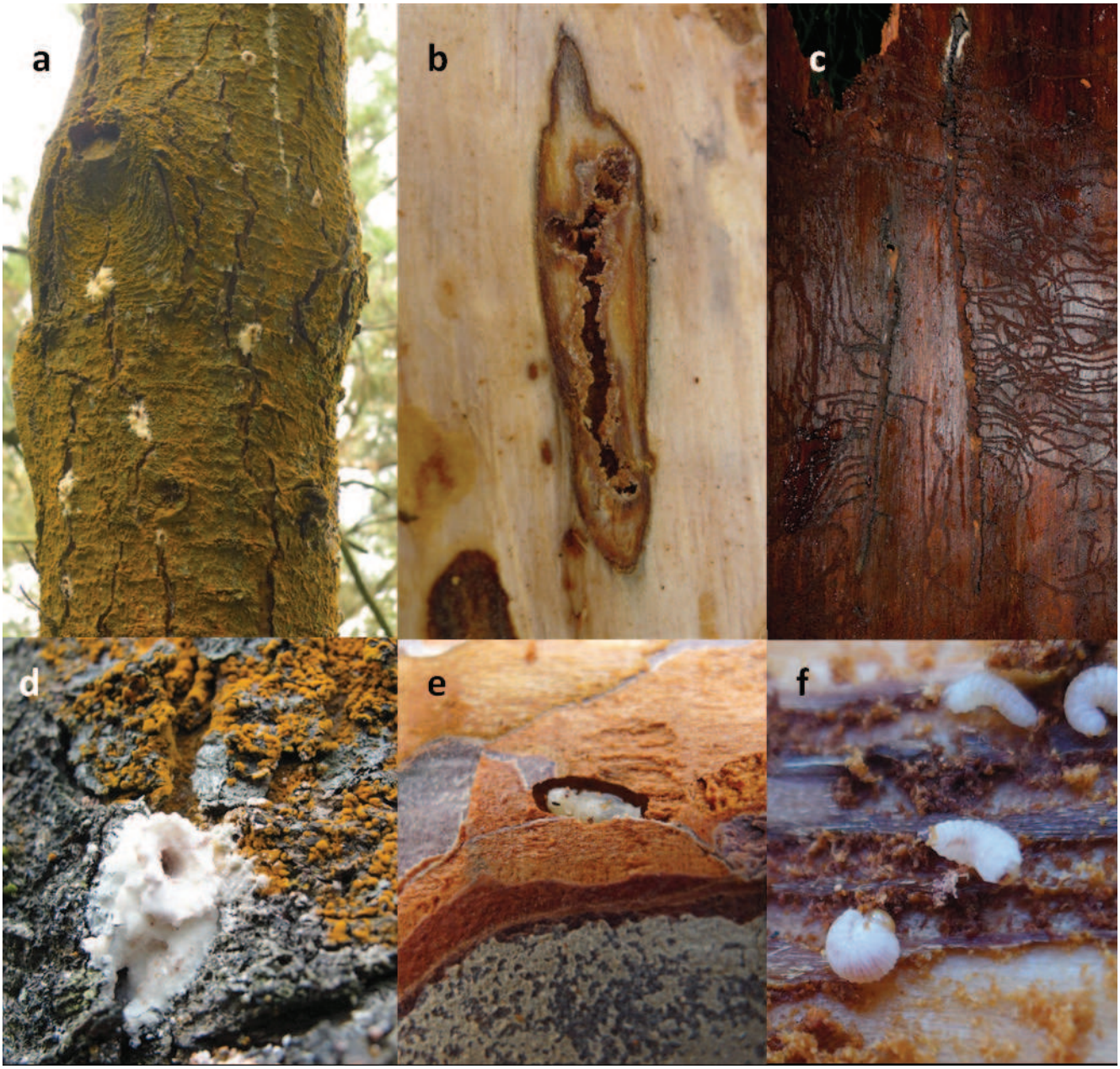

Figure 5. Trace of *Tomicus piniperda* presence during breeding on pine trunks: a) *Pinus radiata* trunk with *T. piniperda* entrance holes, b),c) *T. piniperda* breeding galleries: b) surrounded by necrotic tissue in *Pinus nigra*, c) parental and larval galleries in *P. radiata* d) detail of a *T. piniperda* entrance hole in *P. radiata* e) pupal chamber belonging to a breeding gallery in *P. nigra*, f) *T. piniperda* larvae in *P. nigra*.

2007). The presence of this insect species on the attacked crowns can be inferred by the presence of reddish twigs (Figure 8a). Twig beetles breed in shade-suppressed and broken branches as well as in branches of recently dead trees, but rarely cause tree mortality or even the death of individual branches (Storer *et al.*, 2004). They construct their galleries in the phloem or in the pith of small branches in the host tree (Figure 8b) (Sakamoto *et al.*, 2007). Overwintering in *P. pubescens* has been observed to occur in shoots, within *T. piniperda* feeding galleries (Balachowsky, 1962).

Fusarium circinatum has been reported to be phoretically associated with *P. pubescens* in Spain (Romón *et al.*, 2007a). The association of *Pityophthorus* spp. with PCD has also been observed in other affected areas, as for example in California where the importance of *Pityophthorus setosus* Blackman and *Pityophthorus carmeli* Swaine as *F. circinatum* vectors has been already demonstrated (Sakamoto *et al.*, 2007). Bonello *et al.* (2001b) reported the ability of *Pityophthorus* spp. in discriminating between healthy and pitch canker diseased branches, preferring symptomatic branches due to the

Figure 6. *Tomicus piniperda* within shoot feeding galleries: a) *Pinus nigra* and b) *Pinus radiata.*

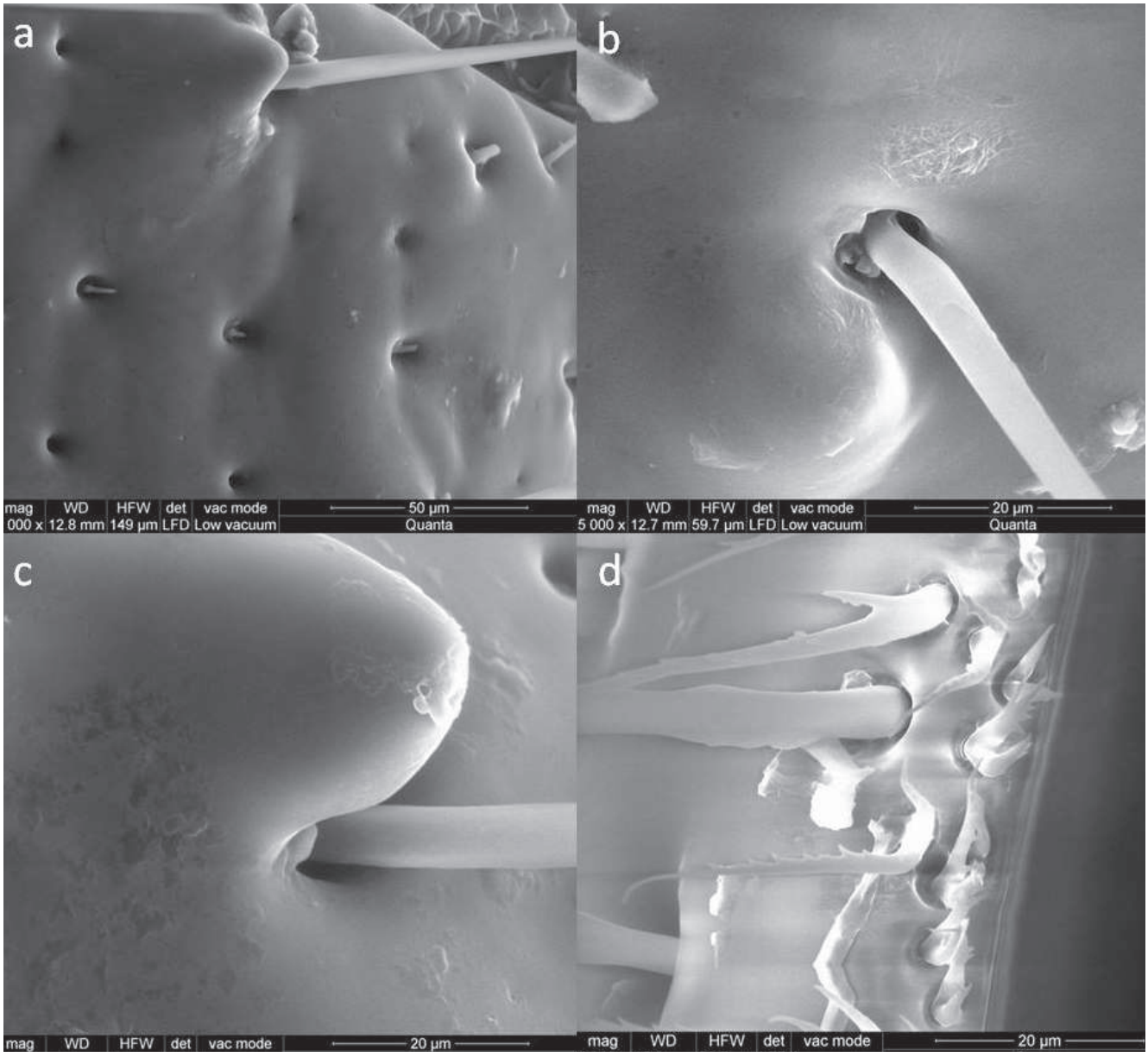

Figure 7. Scanning Electron Microscope pictures of *Tomicus piniperda*'s body structures: a), b) and c) on the elytra, d) at the base of the pronotum.

Figure 8. *Pinus radiata* attacked by Pityopthorus pubescens a) Reddish twigs, b) detail of the gallery burrowed by *P. pubescens.*

increasing of ethylene emission. The relevance of the role of *P. pubescens* in regard to *F. circinatum* spreading has to be assessed taking into account its feeding and breeding habits as well as its population level.

c- Other bark beetles in northern Spain

Ips sexdentatus (Figure 9) is a polygamous species with one to five generations per year, depending on the weather conditions (Vega and Hofstetter, 2015), completing three generations in the Mediterranean area (López *et al.*, 2007). Most of the life cycle of this insect occurs under the tree bark (Vega and Hofstetter, 2015). *Ips sexdentatus* is a secondary pest infesting *P. radiata* in northern Spain, however when population levels increase they become a primary pest that can kill healthy trees (Etxebeste and Pajares, 2011). Outbreaks usually occur after forest fires or adverse climatic conditions (Gil and Pajares, 1986; Fernández and Salgado, 1999; Etxebeste *et al.*, 2012).

The association of *Ips* species with fungi has been widely studied. Whitehill *et al.* (2007) reorted the role of *Ips pini* as a vector of *D. pinea* and several ophiostomatoid species were isolated from *I. sexdentatus*' exoskeleton in the work carried out by Romón *et al.* (2007b) and by Bueno *et al.* (2010). *Ips sexdentatus* was also found to be phoretically associated with *F. circinatum* in Spain in the sampling performed by Romón *et al.* (2007a), in which 8.5% of the collected insects carried the pathogen. Likewise, the importance of other species like *Ips mexicanus* (Hopkins) and *Ips paraconfusus* Lanier in association with *F. circinatum* has also been observed in PCD affected areas in California, where these species were reported as vectors of the pitch canker fungus (Fox *et al.*, 1991). The importance of the association of *I. sexdentatus* with fungi is highlighted by the presence of mycangia on the insects' exoskeleton.

Among the bark beetle species present on pitch canker disease affected stands there are four species from the genus *Hylastes* in the Iberian Peninsula (Figure 10) which are monogamous, phloeophagus and are secondary insects infesting diseased or felled trees (López *et al.*, 2007). *Hylastes ater* (Payk.), *Hylastes angustatus* Herbest and *Hylastes linearis* Erichson appear at the base of the trunk or roots, whereas *H. attenuatus* also attacks branches. *Hylastes ater* is the most dangerous species since adult beetles carry out their maturation feeding on the stems of seedlings prior to ovoposition. Sopow *et al.* (2014) demonstrated *H. ater* capability of attacking unstressed seedlings more frequently than stressed ones; however the attack to stressed seedlings caused substantial girdling-induced mortality. Moreover, *H. ater* has been reported to be associated to several pathogenic fungi e.g *Ophiostoma* spp. *Leptographium* spp. (Eckhardt *et al.*, 2004) as well as

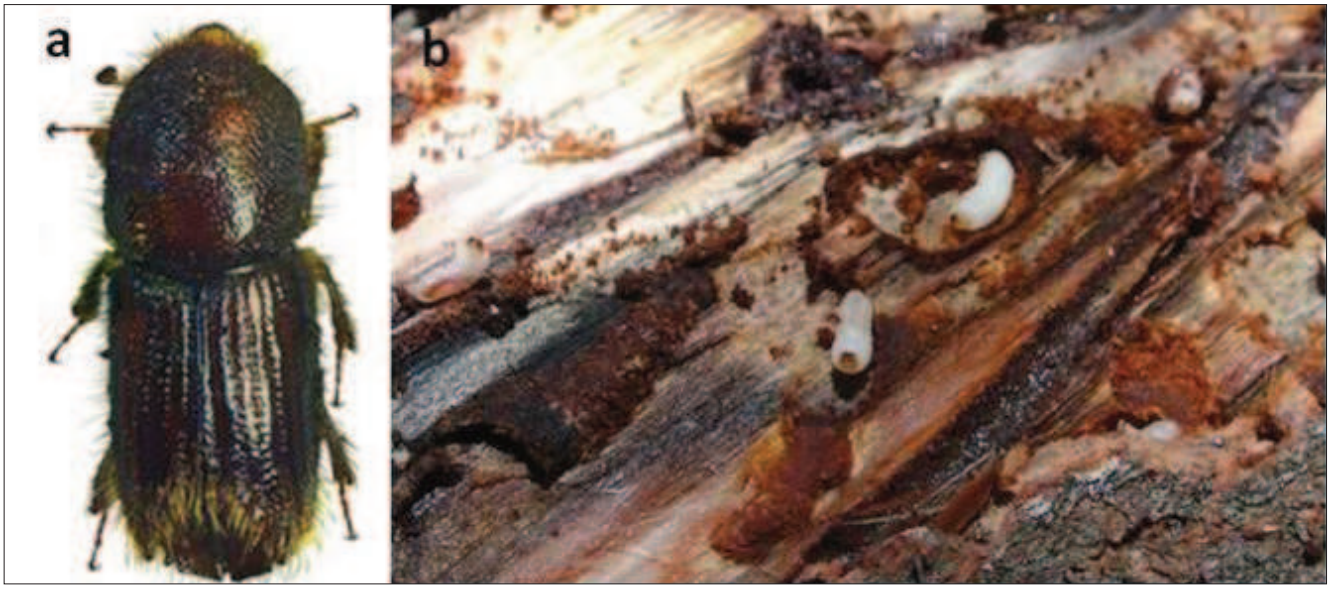

Figure 9. a) Adult specimen of *Ips sexdentatus* (Source: http://claude.schott.free.fr/Scolytidae Scolytidae_PL13.jpg), b) *Ips sexdentatus* larvae in a breeding gallery from *Pinus radiata*.

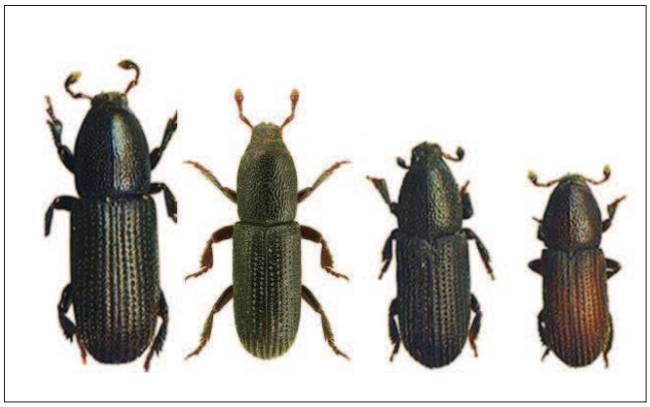

Figure 10. From left to right: *Hylastes ater*, *Hylastes linearis*, *Hylastes angustatus* and *Hylastes attenuatus*. Sources:http://claude.schott.free.fr/Scolytidae/Scolytidae_PL4.jpg http://coleoptera.ksib.pl/kfp/search.php?img=8512

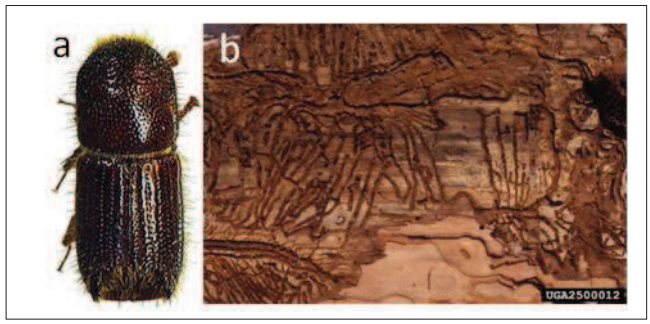

Figure 11. a) Adult specimen of *Orthotomicus erosus*. Source:http://claude.schott.free.fr/Scolytidae/Scolytidae_PL12.jpg, b) *O. erosus* breeding galleries. Source: http://www.invasive.org/browse/subinfo.cfm?sub=4071.

Fusarium spp (Romón *et al.*, 2007a; Romón *et al.*, 2014).

Orthotomicus erosus (Figure 11) is a polygamous species that generally acts as a secondary pest, infesting fallen or felled trees, but also attack living trees that suffer from stress due to fire, droughts or diseases (López *et al.*, 2007). Population densities may increase until epidemic levels, what would lead them to overcome the food store of weak trees and attack the healthy ones (Gil and Pajares, 1986). Maturation feeding of young adults occurs under the bark, in the phloem of the same tree where they were born or in another one from the same or different species (López *et al.*, 2007). This insect species has been reported to be associated to Ophiostomatoid fungi, i.e. *Ophiostoma* spp and *Leptographium* spp. (Kirisits, 2004; Romón *et al.*, 2014) as well as to several *Fusarium* spp., including *F. circinatum* (Romón *et al.*, 2007a).

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Chapter 2: Objectives

The main objective of this work was to study the influence of several factors (abiotic and biotic) on the epidemiology of the pitch canker disease caused by *Fusarium circinatum* in northern Spain. Thus, the specific objectives of this work were:

1) To determine the effect of pruning on the incidence and severity of PCD in *Pinus radiata* plantations.

2) To evaluate the fungal communities present on bark beetles and their galleries in *P. radiata* plantations affected by pitch canker disease, with special attention to *Fusarium* species.

3) To determine whether the pine shoot beetle *Tomicus piniperda* could vector the pitch canker pathogen *F. circinatum.*

4) To study the association between *Pityophthorus pubescens* and *F. circinatum* in PCD affected plantations in northern Spain.

Chapter 3: Effects of pruning in Monterey pine plantations affected by *Fusarium circinatum*

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Forest Systems 2012 21(3), 484-488

Abstract

Fusarium circinatum Nirenberg and O´Donnell (1998) is the causal agent of pitch canker disease (PCD) in *Pinus* species, producing damage to the main trunk and lateral branches as well as causing branch dieback. The disease has been detected recently in northern Spain in *Pinus* spp. seedlings at nurseries and in *Pinus radiata* D. Don adult trees in plantations. *Fusarium circinatum* seems to require a wound to enter the tree, not only that as caused by insects but also that resulting from damage by humans, i.e. mechanical wounds. However, the effects of pruning on the infection process have yet to be studied. The aim of the present study was to know how the presence of mechanical damage caused by pruning affects PCD occurrence and severity in *P. radiata* plantations. Fifty *P. radiata* plots (pruned and unpruned) distributed throughout 16 sites affected by *F. circinatum* in the Cantabria region (northern Spain) were studied. Symptoms of PCD presence, such as dieback, oozing cankers and trunk deformation were evaluated in 25 trees per plot and related to pruning effect. A significant relationship between pruning and the number of cankers per tree was observed, concluding that wounds caused by pruning increase the chance of pathogen infection. Other trunk symptoms, such as the presence of resin outside the cankers, were also higher in pruned plots. These results should be taken into account for future management of Monterey pine plantations.

Resumen

Fusarium circinatum Nirenberg and O´Donnell (1998) es el agente causante de la enfermedad del chancro resinoso del pino, que afecta a especies del género *Pinus* y provoca la aparición de chancros resinosos en el tronco y en ramas gruesas, además de puntisecado en la guía terminal. Esta enfermedad fue detectada recientemente en el norte de España asociada a plántulas de coníferas en vivero y a plantaciones de *Pinus radiata* D. Don. *Fusarium circinatum* suele requerir una herida en el árbol para poder infectarlo. Estas heridas pueden estar causadas por insectos o ser de origen antrópico, como las heridas mecánicas. Con la finalidad de conocer cómo las heridas producidas durante la poda afectan a la severidad de la enfermedad del chancro resinoso del pino, se estudiaron 50 parcelas de *P. radiata* (podadas y no podadas) distribuidas a lo largo de la provincia de Cantabria. En cada una de las parcelas fueron evaluados 25 árboles, en los que se estudiaron los síntomas más característicos de la enfermedad, como son puntisecado, presencia de chancros resinosos y deformación del tronco, relacionándolos con la presencia de poda. Se observó una relación significativa entre la poda y el número de chancros presentes en el árbol,

lo que indica que la herida producida en este tratamiento selvícola es susceptible de infección por parte del patógeno. Otros síntomas presentes en el tronco, como exudado de resina fuera del chancro, aparecieron también en mayor número en las parcelas podadas. Estos resultados son de gran trascendencia para el futuro manejo de las plantaciones de *P. radiata* afectadas por el chancro resinoso del pino.

3.1- Introduction

Pitch canker disease of pines is produced by the fungus *Fusarium circinatum* (teleomorph=*Gibberella circinata*) (Nirenberg and O´Donnell, 1998). This fungus poses a threat to pine plantations and forests throughout the world (Wingfield *et al.*, 2008). It was first reported in North Carolina (Hepting and Roth, 1946) but has also been observed in California (Mc Cain *et al.*, 1987), Chile (Wingfield *et al.*, 2002), South Africa (Viljoen and Wingfield, 1994), Japan (Muramoto and Dwinell, 1990), Mexico (Guerra-Santos, 1999), Portugal (Bragança *et al.*, 2009), France (EPPO, 2004) and northern Spain (Landeras *et al.*, 2005). Pitch canker commonly occurs in coastal rather than in inland areas (Wikler *et al.*, 2003), suggesting that the causal agent of this disease has some association with humidity and fog.

Pinus species like *P. pinaster*, *P. radiata* and *P. sylvestris* (Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007) as well as *Pseudotsuga menziesii* (Gordon *et al.*, 1996) show disease susceptibility. *Pinus radiata*, planted widely worldwide, is a species extremely sensitive to pitch canker disease (Viljoen *et al.*, 1995) and symptoms observed on these trees are very severe (Gordon *et al.*, 2001). Several *Pinus* species like Japanese black pine (P. thunbergiana) and Italian stone pine (*P. pinea*) are known for suffering little or no damage from the disease (Gordon *et al.*, 2001).

Fusarium circinatum symptoms include bleeding, resinous cankers with tree trunk deformation. The wood beneath the sunken bark of cankers is usually pitch-soaked. As these cankers grow they may girdle the larger shoots producing dieback (Blakeslee *et al.*, 1980). Defoliation and trickles of resin can also be found on diseased trees. These symptoms spoil the trees, and together with the premature trees' death, result in economic loss to the affected regions. The pathogen also causes damping-off and mortality in seedlings. Consequently, this fungus may be considered a threat to pine plantations and wood industry productivity throughout the world.

Fusarium circinatum seems to require fresh wounds on trees as infection court (Dwinell *et al.*, 1985), such as those caused by insects from the subfamily Scolytinae that have been found to be not only wounding agents but also vectors in California (Storer et al, 2004). Dwinell et al (1985) suggested that *F. circinatum* inoculum could infect wounds produced by pruning, mowing and harvesting, although no study was carried out on this issue. Wounds caused by hurricanes or those resulting from wind-thrown needles are also thought to provide an infection court for the pathogen to infect the trees (Kelley and Williams, 1982). Notwithstanding, the susceptibility of these wounds to infection could decrease significantly with wound age (Sakamoto and Gordon ,2006). Nonetheless, other studies as Correl *et al.* (1991) suggest that branches with mechanical wounds are not susceptible to infection even if airborne inoculum is present, postulating that airborne spores are unable to infect wounds. On the other hand, pruning could be considered for removing diseased branches, though this approach is not effective in eradicating the disease (Gordon *et al.*, 2001). Attempts to remove disease causing fungi have been made via tree pruning, though it was shown that this treatment does not completely eliminate the disease from the tree (Moorman and Lease, 1999). As such, forest management should be considered as an important factor for decreasing disease establishment and spread (Waring and O´Hara, 2005). The effect of pruning has never been studied in Monterey pine plantations where the disease is destroying the trees. Some reports regarding the effect of the presence of wounding agents have been made in the United States, where most Monterey pine appears in native stands (Gordon *et al.*, 2001).

The objective of this study was to check out the effect of pruning on *P. radiata* plantations infected with PCD to determine whether pruning wounds provide an infection court for *F. circinatum*, increasing the disease severity.

3.2- Materials and Methods

3.2.1- Plots selection

Data were collected from *P. radiata* plantations distributed throughout 16 sites affected by *F. circinatum* in the region of Cantabria (northern Spain) with high occurrence of severe PCD affected stands. From June to October, 2010, several factors related to the disease

were measured.

Fifty plots (pruned and unpruned) were selected among 16 sites (Figure 1) affected by *F. circinatum*, maintaining a distance of at least 500 meters between them. The location where the plot was set in each stand was randomly selected (Table 1).

3.2.2- Field work

Twenty five trees per plot were evaluated, considering dendrometric and forest health variables. A total of 1250 trees were measured against the below variables. Within the dendrometric variables, tree diameter, total height, first living branch height and pruning height were measured. The plant health variables included number and location (internodes or whorl) of cankers, flow of resin on the cankers (from 1 to 3 where 1=light, 2=medium, 3=abundant), percentage of trunk perimeter affected by the canker (<33 %, 33-66 % or >66 %), five degrees of defoliation (1=1-20 %, 2=21-40 %, 3=41-60 %, 4=61-80 %, 5=81-100 %), presence of trickles of resin outside the cankers (from 0 to 3, where 0= absence, 1=light, 2= medium, 3= abundant), presence of red shoots in the crown (from 0 to 3, where 0=absence, 1=on 1/3 of the crown, 2=on 2/3 of the crown, 3=on all the crown), dieback (from 0 to 3, where 0=absence, 1=on 1/3 of the crown, 2=on 2/3 of the crown, 3=on all the crown) and mortality.

Distance of each plot to the coast was calculated with Arc View 3.0 using UTM coordinates collected in the plots centre.

3.2.3- Statistical analysis

The effect of pruning on the disease occurrence was analyzed using both univariate and multivariate analyses. Data were subjected to analysis of variance (ANOVA) to assess the significance of differences in symptomatology between pruned and unpruned plots. Correlation analysis was used to establish the linear relationship between selected variables. The non-parametric Kruscal-Wallis test (data could not be transformed to fit a normal distribution) was used to determine whether significant differences existed between pruned and unpruned plots.

Non-parametric multivariate analysis, recommended for non-normal data, was performed to assess in detail the influence of pruning on plant health variables. A non-parametric multidimensional scaling (NMSD) analysis was executed including different forest health variables (number of cankers, trickles of resin, red shoots, defoliation and dieback). NMDS was carried out using Bray-Curtis distance.

Multiple response permutation procedure (MRPP) was used to tests whether there were differences between pruned and unpruned plots. Ordination diagrams, Ordihull combined with Ordispider were used to represent the items on a class and to combine the items to their class centroid (Oksanen, 2005).

All statistical analyses were carried out at the 0.05 level of significance. Data analyses were run on VEGAN package 2.0-2 of R version 2.14.1.

3.3- Results

The average number of cankers was significantly higher (n=50, F=5.232, p=0.026) in pruned than in unpruned plots (Table 2). It was also observed that the number of cankers present on whorls was significantly higher in pruned plots (n=50, F=4.256, p=0.044) than in unpruned ones while the number of cankers present in internodes showed no significant relationship to pruning. Pruned plots also showed a level of resin trickles significantly higher than unpruned plots (n=50 F=5.064, p=0.029). The NMDS shows the distribution of most pruned plots in the area of the graph where cankers and trickles of resin are present (Figure 2). MRPP test also reflected a relation between health variables and prune (A= 0.0181, p= 0.031).

Regarding symptoms affecting tree crown, like defoliation, dieback and presence of red shoots, it was observed that these variables showed apparently higher mean levels in unpruned plots than in pruned ones (Table 3). Defoliation showed significant differences between unpruned plots and pruned ones (n=50, F=4.209, p=0.045). On the other hand, no significant differences were found for dieback and red shoots (p>0.05).

The non- parametric Kruskal-Wallis test showed that the presence of dead trees was not significantly related to prune (n=50, X^2=0.4774, p=0.489), though higher number of dead trees appeared in unpruned plots (11.1%) than in pruned ones (7.5 %).

Correlation between mean number of cankers per plot and the distance of each plot from the coast showed a significant relationship (r=-0.30, F=4.726 p=0.0347), thus those plots nearest to the coast presented a higher number of cankers. Nonetheless, a significantly higher number of red shoots was observed when the distance from the coast increased (n=50, r=0.64, F=0.108 p=0.743).

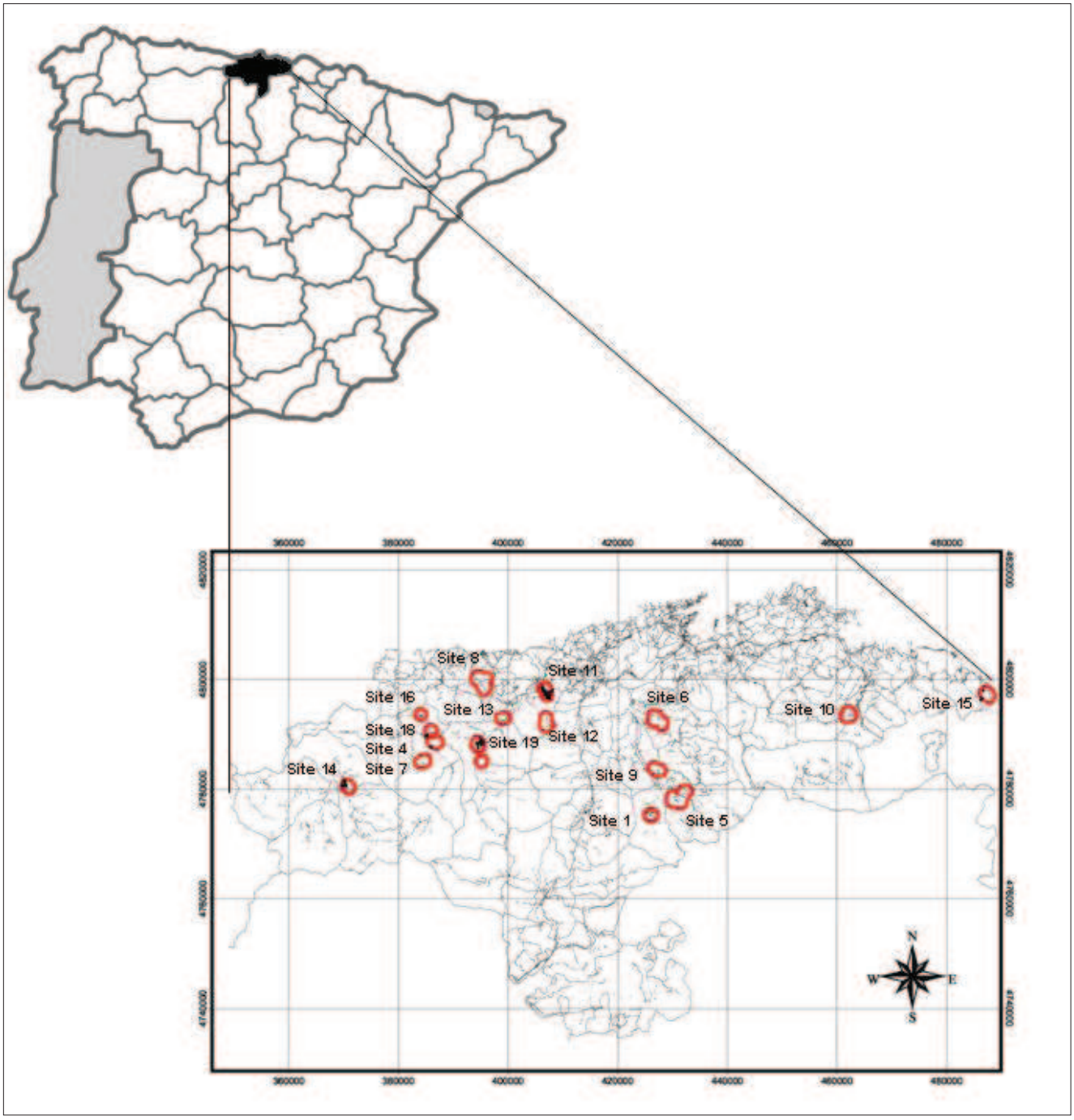

Figure 1. Location of the study sites in Cantabria (Spain).

3.4- Discussion

*Fusarium circinatum´*s capacity for infection seems to depend on the presence of biotic and/or abiotic wounding agents (Gordon, 2006). As noted during this survey, symptoms of PCD that largely appear in the main stem, such as cankers or resin drops, become more frequent in pruned trees. This could indicate that pruning wounds in the trunk have an increased chance of becoming infected by *F. circinatum* as well as increasing the severity of the disease. This is also supported by the relationship found between the number of cankers on whorls and pruning. According to Gordon (2006), mechanical wounds in a PCD infected area sustained infection at a very low rate, and this rate would decrease if the wound size decreases.

Volatiles generated by trees after pruning also in-

Table 1. Localitation of pruned (P) and unpruned (UP) plots.

Plot number	***Municipality***	***Orientation***	***Altitud (m)***	***Coast Dist (Km)***	***P/UP***
1	*Luena*	*S*	*92*	*39.52*	*UP*
2	*Luena*	*E*	*359*	*38.25*	*UP*
3	*Luena*	*N*	*370*	*38.89*	*P*
4	*Rionansa*	*E*	*300*	*36.79*	*P*
5	*Rionansa*	*SW*	*290*	*36.19*	*P*
6	*Rionansa*	*N*	*270*	*31.38*	*P*
7	*Rionansa*	*S*	*268*	*30.09*	*UP*
8	*San Pedro del Romeral*	*SW*	*517*	*30.66*	*P*
9	*San Pedro del Romeral*	*W*	*448*	*30.55*	*P*
10	*Villafufre*	*W*	*413*	*15.28*	*UP*
11	*Villafufre*	*NW*	*476*	*15.48*	*UP*
12	*Villafufre*	*NW*	*487*	*10.26*	*UP*
13	*Rionansa*	*NW*	*600*	*10.52*	*P*
14	*Rionansa*	*SE*	*519*	*9.05*	*P*
15	*Rionansa*	*E*	*322*	*20.93*	*P*
16	*Udías*	*NW*	*269*	*21*	*P*
17	*Udías*	*N*	*130*	*21.24*	*P*
18	*Udías*	*NW*	*219*	*15.14*	*P*
19	*Corvera de Toranzo*	*NW*	*469*	*15.63*	*P*
20	*Corvera de Toranzo*	*NW*	*476*	*14.92*	*UP*
21	*Corvera de Toranzo*	*S*	*359*	*12.9*	*P*
22	*Corvera de Toranzo*	*S*	*359*	*13.35*	*UP*
23	*Ruesga*	*W*	*406*	*12.95*	*P*
24	*Ruesga*	*SE*	*404*	*19.49*	*P*
25	*Ruesga*	*N*	*481*	*20.34*	*P*
26	*Mazcuerras*	*SW*	*147*	*19.3*	*P*
27	*Mazcuerras*	*E*	*170*	*17.08*	*UP*
28	*Mazcuerras*	*NE*	*131*	*16.75*	*P*
29	*Los Corrales de Buelna*	*Flat*	*470*	*17.45*	*UP*
30	*Los Corrales de Buelna*	*SE*	*460*	*23.43*	*UP*
31	*Los Corrales de Buelna*	*SE*	*426*	*22.4*	*P*
32	*Cabezón de la Sal*	*N*	*343*	*23.12*	*P*
33	*Cabezón de la Sal*	*N*	*214*	*15.21*	*P*
34	*Cabezón de la Sal*	*W*	*417*	*12.2*	*UP*
35	*Cillórigo de Liébana*	*NW*	*387*	*15.04*	*P*
36	*Cillórigo de Liébana*	*E*	*374*	*6.61*	*UP*
37	*Cillórigo de Liébana*	*NW*	*370*	*6.17*	*P*
38	*Castro Urdiales*	*N*	*533*	*5.63*	*UP*
39	*Castro Urdiales*	*W*	*297*	*2.94*	*P*
40	*Rionansa*	*S*	*271*	*3.73*	*P*
41	*Rionansa*	*S*	*251*	*20.57*	*P*
42	*Rionansa*	*SE*	*225*	*21.29*	*P*
43	*Cabuérniga*	*S*	*403*	*20.26*	*UP*
44	*Cabuérniga*	*NW*	*376*	*21.52*	*UP*
45	*Cabuérniga*	*S*	*321*	*20.76*	*UP*
46	*Rionansa*	*S*	*285*	*20.49*	*P*
47	*Rionansa*	*W*	*208*	*18.29*	*P*
48	*Cabuérniga*	*N*	*431*	*18.47*	*P*
49	*Cabuérniga*	*E*	*898*	*18.11*	*UP*
50	*Cabuérniga*	*SE*	*313*	*17.08*	*P*

**Meters above sea level *Pruned *Unpruned*

Table 2. ANOVA results for pruning.

Source	*d.f*	*Mean Sq*	*F-value*	*p-value*
Whorl	*1*	*0.93*	*4.256*	*0.044*
Tricles of resin	*1*	*1.09*	*5.064*	*0.029*
Dieback	*1*	*0.005*	*0.023*	*0.878*
Defoliation	*1*	*0.92*	*4.209*	*0.045*
Cankers	*1*	*1.13*	*5.232*	*0.026*
Red shoots	*1*	*0.02*	*0.108*	*0.743*

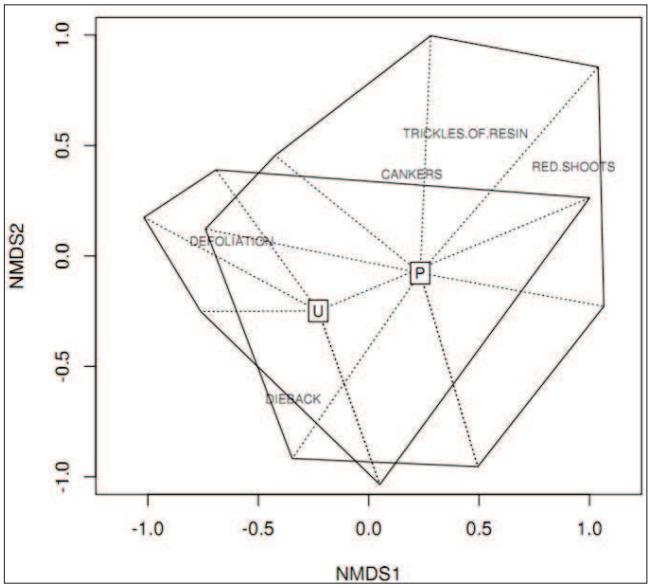

Figure 2. Ordenation diagram diagram NMDS with methodology Ordihull combined with Ordispider. Pruned (P) and Unpruned (UP) plots are ordered in the centroids, forest health variables (Dieback, defoliation, cankers, trickles of resin, red shoots) are also represented.

crease the likelihood of infection, primarily because some insects carrying the fungus feel attracted by these volatiles (Gordon, 2011). Thus, the pine shoot beetle, *Tomicus piniperda*, seemed to attack *P. sylvestris* pruned trees more frequently than unpruned ones (Långström and Hellqvist, 1992). Several bark beetles are also attracted by resin odours from damaged boles after pruning allowing them to later also attack neighbouring healthy trees (Jactel *et al.*, 2009).

Another factor that could possibly increase the disease incidence in relation to pruning could be the lack of disinfections of forestry machinery between and following cuttings, spreading the infection among stands. This is one of the main ways for *Cryphonectria parasitica* (the causal agent of chestnut blight) spread (Gouveia *et al.*, 2001). In order to reduce chestnut blight risk accurate disinfection of pruning tools must be performed. Moreover, pruning should be carried out only during periods of lowest host receptiveness and susceptibility or when infection risk is lower, i.e. when spores inoculum is minimum (Guérin and Robin, 2003). For *C. parasitica*, these two conditions happen in winter. However, the most suitable period for pruning Monterey pine regarding PCD, is not well established. Further studies regarding the F.circinatum cycle and spore dispersal in this region should be conducted to develop a better management of Monterey pine plantations.

The number of dead trees was apparently higher in unpruned plots than in pruned ones, which could be related to the decrease of the quantity of inoculum in the air and surrounding trees due to pruning and wood

Table 3. Mean values of PCD symptoms in pruned and unpruned plots. Variables with the same letter showed no significant differences.

	N cankers/tree	*N crankers on whorl/tree*	*N crankers internode/tree*	*Defoliation level (0-5)/tree*	*Dieback level (0-3)/tree*	*Resin trickle level (0-3)/tree*	*Red shoots (0-3)/tree*
Pruned plots	*0,43+0,39a*	*0,33+0,35a*	*0,10+0,13a*	*0,65+0,61b*	*0,27+0,37a*	*0,28+0,25a*	*0,07+0,15a*
Unpruned plots	*0,21+0,16b*	*0,14+0,14b*	*0,06+0,07a*	*1,14+1,04a*	*0,29+0,19a*	*0,13+0,13b*	*0,09+ 0,14a*
Pruned range	*0,00-1,44*	*0,00-1,44*	*0,00-0,52*	*0,00-2,32*	*0,00-2,00*	*0,00-1,32*	*0,00-0,64*
Unpruned range	*0,00-0,60*	*0,00-0,56*	*0,00-0,24*	*0,00-3,68*	*0,04-0,84*	*0,00-0,56*	*0,00-0,48*

removal. Bernhold *et al.* (2006) reported the effects of clear-cutting and the effect of removing infected slash in *P. sylvestris* stands affected by *G. abietina*, concluding that it reduces the risk of infection but does not eradicate the infection source. Laflamme (1999) demonstrated a reduction in scleroderris canker in red pine (*P. resinosa*) caused by *G. abietina* from 67% to 22% one year following pruning. Furthermore, the effect of pruning apple trees affected by the causal agent of sooty blotch *Gloeodes pomigena* was observed to reduce the incidence and the severity of the disease (Ocamb-Basu *et al.*, 1988). Pruning diseased trees could have different effects depending on the specific behaviour of the pathogen. Thus, for avoiding forest health related problems, Waring and O´Hara (2005) expressed the need of a combined solution among management tools and the knowledge of their effect on each disease. The apparent contradiction between an evident increase in the number of cankers and a decrease in the mortality, both following pruning, requires a detail analysis in further studies.

Another critical component allowing *F. circinatum* to survive and infect is environmental moisture (Gordon, 2006). Stand moisture content can be decreased by pruning through increased light and surface wind speed within the stand (Pollet and Omi, 2002; Jactel *et al.*, 2009), which could reduce successful pathogen survival. To assess the importance of environmental moisture, proximity to the coast and symptomatology were correlated. Thus, it was found that plot distance from the coast is an important factor influencing the disease occurrence, further underscoring the importance of environmental moisture. This influence of the coast, where the environmental conditions are more favourable for the infection (Wingfield *et al.*, 2008), has been previously noted in California (Wikler *et al.*, 2003) with one exception in Sierra Nevada (Vogler *et al.*, 2004). The effect of the coast proximity is also clear in Spain, where more *P. radiata* plantations are affected by the disease on the northern coast. However, experiments carried out by Sakamoto and Gordon (2006) under both controlled and field conditions showed no significant relationship between infection rate and relative humidity

In conclusion, wounds caused by pruning have an increased chance of becoming infected by the pathogen which could increase cankers and deformation. On the other hand, pruning could improve crown aspect decreasing defoliation. Notwithstanding, pruning in Monterey pine diseased plantations is not desirable as a result of stem deformation caused by cankers, making them useless for the wood industry. For better understanding of the impact of pruning and other selvicultural treatments on pitch canker affected trees, further research including tree pruning relative to seasonal and fungal cycle should be done.

Acknowledgements

We thank C. Prieto-Recio, C. Romeralo and L. Botella for their help and useful comments. We thank both reviewers for their suggestions on the manuscript. Milagros Vallejo and Juan Blanco are also acknowledged for allowing this study, which was supported by Cantabria Government and the Ministry of Rural, Marine and Natural Environment project "Etiology, Epidemiology and Control of *Fusarium circinatum*".

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Chapter 4: Fungal communities associated with bark beetles in pine pitch canker affected plantations in northern Spain, with special focus on *Fusarium* species

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Abstract

Fusarium species, as well as other endophytic or pathogenic fungi forming communities, have been reported to be phoretically associated with bark beetle vectors world-wide. This is the case with *Fusarium circinatum*, the causal agent of pitch canker disease (PCD), which currently threatens *Pinus radiata* plantations. The main objective of this work was to study the fungal communities present on bark beetles and in their galleries in PCD diseased stands with special attention to the *Fusarium* species. Funnel traps and logs were placed in a *P. radiata* plot affected by *F. circinatum*. Traps were baited with different attractive compounds: four with pityol and six with ethanol and alpha-pinene. In addition, fresh green shoots with *Tomicus piniperda* feeding galleries were collected from the ground in *P. radiata*-affected plots. The insects and gallery tissues collected were processed and cultured with the aim of isolating and identifying associated fungi. A total of 24 different fungal species were obtained from the bark beetle galleries from logs and shoots while 18 were obtained from the insects' exoskeletons. Ten different *Fusarium* species were isolated from tissue and insects. *Fusarium circinatum* was isolated from the bark beetles´ exoskeletons (1.05 % of the *Pityophthorus pubescens* had *F. circinatum*) and from their galleries (3.5 % of the *T. piniperda* feeding galleries had the pathogen). These results are a look at the fungal communities associated with bark beetles in *P. radiata* stands in northern Spain, suggesting an association between bark beetles and the *Fusarium* species, which is marked by the presence of *F. circinatum* in *T. piniperda* galleries.

Resumen

La asociación entre barrenillos (Coleoptera; Scolytinae) y hongos endófitos o patógenos ha sido descrita en todo el mundo, especialmente su asociación con aquellos hongos pertenecientes al género *Fusarium*. Este sería el caso de *Fusarium circinatum*, el agente causante de la enfermedad del chancro resinoso de los pinos, que supone actualmente una amenaza para las plantaciones de *Pinus radiata* del norte de España. El principal objetivo de este trabajo fue estudiar las comunidades fúngicas presentes tanto en los escolítidos como en sus galerías en plantaciones afectadas por esta enfermedad, prestando especial atención a las especies del género *Fusarium*. Para ello, se dispusieron trampas multiembudo y trozas cebo en una parcela de *P. radiata* afectada por *F. circinatum*. Las trampas multiembudo se cebaron con distintos atrayentes: cuatro de ellas fueron cebadas con (E)-pityol y seis con etanol y alfa-pineno. Además, en parcelas de *P. radiata* afectadas por la enfermedad, se recogieron del suelo ramillos con galería

de alimentación de *Tomicus piniperda*. Con el objetivo de identificar los hongos presentes tanto en el material vegetal como en los insectos, las muestras recogidas se procesaron para su posterior cultivo. Se obtuvieron 24 especies fúngicas de las galerías horadadas por los escolítidos en las trozas y en los ramillos y 18 especies de hongos del exoesqueleto de los insectos. Se identificaron diez especies distintas pertenecientes al género *Fusarium* procedentes de muestras tanto de material vegetal como de insectos. *Fusarium circinatum* fue aislado tanto del cuerpo de los insectos (el 1 % de los *Pityophthorus pubescens* llevaron *F. circinatum*) como de las galerías (el 3.5 % de las galerías de alimentación de *T. piniperda* fueron positivas para el patógeno). Estos resultados suponen un avance en el estudio de las comunidades fúngicas asociadas a los barrenillos en plantaciones de *P. radiata* en el norte de España, sugiriendo la existencia de una asociación entre los escolítidos y los hongos del género *Fusarium* y destacando la presencia de *F. circinatum* en las galerías de *T. piniperda*.

4.1- Introduction

Fungal endophyte species form fungal communities together with saprotrophic and pathogenic species in forests. Knowing the species composition and the factors influencing the presence of different fungal communities is important to understanding the role that fungi play in the regulation of other organisms (Arnold, 2007). Bark beetles are known to be closely associated with fungi worldwide. They are well known for their association with endophytic, plant-pathogenic and entomopathogenic fungi, like *Fusarium* species, which are widespread and abundant in living and dead plants (Teetor-Barsch and Roberts, 1983; Romón *et al.*, 2008).

The genus *Fusarium* includes important plant pathogens affecting both forest and agricultural species (Alves-Santos and Diez, 2012a) because of the production of different types of wall-degrading enzymes (e.g. cellulases, glucanases or glucosidases) and mycotoxins like beauvericin or fumonisins (Mirete *et al.*, 2003; Summerell and Leslie, 2011). Some of the species of this genus, such as the *Fusarium oxysporum* spp. complex, cause disease symptoms in a large number of vegetable crops (Román-Avilés *et al.*, 2011). Bark beetles are known to be closely associated with *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph=*Gibberella circinata*), an ascomycete fungus causing pitch canker disease (PCD) (Nirenberg and O'Donnell, 1998). The main symptom of this disease is the presence of pitch soaked cankers on trunks and big branches in adult trees which can girdle both trunk and branches (Wikler *et al.*, 2003). *Fusarium circinatum* is currently threatening pine plantations and natural stands throughout the world (Wingfield *et al.*, 2008), especially the *Pinus radiata* D. Don, a highly susceptible pine species (Viljoen *et al.*, 1995). Other *Pinus* species like *Pinus pinaster* Ait. and *Pinus sylvestris* L. as well as *Pseudotsuga menziesii* (Gordon *et al.*, 1996; Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007) are also susceptible to the pathogen. In Spain, the disease caused by *F. circinatum* leads to significant ecological and economical loss in forest plantations and nurseries (Landeras *et al.*, 2005).

Bark beetles have been reported to be phoretically associated with *Fusarium circinatum* in *P. radiata* plantations in northern Spain, e.g. *Pityophthorus pubescens* (Marsh.), *Ips sexdentatus* (Börner) and *Tomicus piniperda* L. (Romón *et al.*, 2007; Bezos *et al.*, 2013). In California the importance of *Pityophthorus* spp. as main vectors of *F. circinatum* has also been demonstrated (Sakamoto *et al.*, 2007). We hypothesize that different bark beetle species living in these plantations play specific roles in the spread of *Fusarium* spp. The differences in their bioecology could determine the spread of the infections, for example, *Hylastes* spp. feed on roots or trunks of declining trees whereas *T. piniperda* feeds on shoots of healthy crowns (López *et al.*, 2007), and there is a risk of populations increasing to epidemic levels (Raffa and Berryman, 1983), e.g. *I. sexdentatus*.

Fungal communities inhabiting *P. radiata* trees may be another significant factor influencing PCD distribution in Spain. Endophytic species, which do not cause any damage to the host (Arnold, 2007), such as *Trichoderma viride* Bissett, could be used as a biological control of *Fusarium* spp. (Alves-Santos and Diez, 2012b; Martínez-Álvarez *et al.*, 2012), as well as other fungal species like *Diplodia pinea* (Desm.) Kickx which is a latent pathogen in pine trees yet has been associated with PCD in *P. radiata* trees (García-Serna, 2014).

The main objective of this study was to characterize on the fungal communities, with special attention to *Fusarium* species, present on bark beetles and in their galleries in PCD affected stands.

4.2- Materials and Methods

4.2.1- Sample collection

This study was carried out on a *P. radiata* plot affected by *F. circinatum* located in Vejorís (Cantabria,

Spain). Two kinds of traps were set up: piles of bait logs and funnel traps. The bait logs consisted of 6 piles of branches (6.4-16.9cm) and 6 piles of logs from trunks (16-31cm), all of them obtained from healthy *P. radiata* trees. Plant tissue (xylem and phloem) and insects from the breeding galleries were collected weekly from June to October 2010.

On the other hand, four funnel traps baited with (E)-pityol and six baited with ethanol and alpha-pinene (Econex) were arrayed within the plantation. Pityol is an aggregation pheromone of *P. pubescens* that attracts both males and females (López *et al.*, 2011). Insects were collected weekly from June to October 2010.

To better understand the association of *T. piniperda* with *F. circinatum*, fresh fallen green shoots with *T. piniperda* feeding galleries were collected from 25 *P. radiata* plots affected by *F. circinatum*. A total of 285 *P. radiata* fallen shoots with a *T. piniperda* entrance hole and feeding gallery were collected from June to October 2010. Twenty-seven shoots were collected during the summer (from June to August) whereas 258 were collected in autumn (September and October).

4.2.2- Molecular and morphological identification of fungi

Shoots and xylem and phloem from logs were plated on PDAS (potato dextrose agar with 0.3 g/L of streptomycin sulfate) culture medium after surface sterilization (1 min tap water, 1 min ethanol 70 %, 1 min sodium hypochlorite 20 % and 1 min distilled sterilized water). Moreover, a total of 438 bark beetles belonging to 11 different species (Table 1) were plated on *Fusarium* selective media (FSM: 15 g bactone peptone, 1 g KH2PO4 monobasic, 0.5 g MgSO4-7H2O, 20 g agar, 0.2 g of pentachloronitrobenzene (PCNB) and 0.3 g streptomycin sulfate per liter) to avoid bacterial and soil fungi contamination. All specimens were cultured with the exception of those collected from the shoots because they were used for molecular identification.

Fungi isolated from plant material and insects were classified into morphological units, i.e. colonial morphotypes (CMs), on the basis of cultural characteristics (Lacap *et al.*, 2003). Thus, fungi obtained from plant tissues were grouped into 30 CMs according to macromorphological features of the colony growing on the PDAS, whereas those growing on FSM from insects were grouped into 17 different CMs. One isolate from each CM was selected for molecular identification. However, in the case of those that were reddish, orange, yellowish, violet or pinkish, since they most likely belonged to the *Fusarium* genus, 29 isolates were selected. Regarding the identification of *F. circinatum*, 16 isolates were selected on the basis of their macroscopical features for specific molecular identification. Single hyphae cultures were grown prior to molecular identification.

DNA extraction was carried out on the fungal culture following the protocol described by Vainio *et al.*, (1998). Once the DNA was extracted, the polymerase chain reaction (PCR) was run to amplify the Internal Transcribed Spacer (ITS) region of the rDNA with primers ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA- 3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') (Gardes and Bruns, 1993). The thermal cycling program of amplification was: 10 min denaturation at 95ºC followed by 13 cycles of 35 s at 95ºC, 55 s at 55ºC and 45 s at 72ºC; 13 cycles of 35 s at 95ºC, 55 s at 55ºC and 2 min at 72ºC; 9 cycles of 35 s at 95ºC, 55 s at 55ºC and 3 min at 72ºC; and a final elongation 7 min at 72ºC. The PCR product was sent to sequencing (Secugen, Madrid) after purification (NucleoSpin Gel and PCR Clean up, Macherey Nagel). The ITS region sequences were revised with the Geneious Pro 5.6.5 software package for proper searches with Blast in the GenBank database.

Since the ITS region is not a suitable molecular marker for identifying *Fusarium* spp. at the species level, microscopic morphological and morphometric identification was carried out, and specific molecular markers were used. Thus, 29 isolated fungi that, according to their ITS region, belonged to the genus *Fusarium* were plated on Synthetischer Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), both media specifically used for *Fusarium* species identification (Leslie and Summerell, 2006). After 10 to 20 days, SNA diagnostic characters, including shape of the macroconidia, presence or absence of microconidia, shape and mode of aggregation of microconidia, shape of conidiogenous cells and the presence or absence of chlamydospores, were observed. Color and size of the sporodochia were observed in CLA. These samples were amplified with the primers EF1 (forward primer; 5´-ATGGGTAAGGA(A/G)GACAAGAC-3´) and EF2 (reverse primer; 5´-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3´) (O'Donnell *et al.*, 1998) for sequencing the Translation Elongation Factor (alpha-TEF) region, which encodes an essential part of the protein translation machinery and is highly informative at the species level in *Fusarium* (Geiser *et al.*, 2004). PCR reactions were done in vol-

umes of 50 µL: 1 µL template DNA, 1x reaction buffer, 200 mM dNTPs, 0.4 µM forward and reverse primers, 1U of Kapa Taq DNA polymerase. The thermal profile of PCR was one cycle of 10 min at 94ºC followed by 36 cycles of 30 s at 94ºC, 55 s at 62ºC and 1 min at 72ºC and a final 10 min extension at 72ºC (modified from (Pérez-Sierra *et al.*, 2007)). The alpha-TEF region was sequenced and the sequences were corrected with Geneious Pro Software and blasted in the GenBank and *FUSARIUM* ID sequence database.

Multiple sequence alignment (ClustalW) of the alpha-TEF region sequences and evolutionary analyses were conducted with MEGA 6 (Tamura *et al.*, 2013). The phylogenetic tree was built according to the Neighbor-joining statistical method (Tamura *et al.*, 2004). The Bootstrap method (1000 replicates) was used to represent the phylogenetic history of the analyzed taxa (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The rate of variation among sites was modeled with the gamma distribution (Shape parameter=1). All positions containing gaps and missing data were eliminated. *Fusarium equiseti* was used as an outgroup (GenBank accession number: AJ543571.1).

Those fungi morphologically classified as *F. circinatum* were identified with the specific primer pair CIRC-1A (5'-CTTGGCTCGAGAAGGG-3') / CIRC-4A (5'-ACCTACCCTACACCTCTCACT-3') as described by Schweigkofler *et al.* (2004). Electrophoresis was run to observe a diagnostic 360 bp band in 1 % agarose gel 1X TAE buffer (40 mM Tris base, 0.114% glacial acetic acid and 1 mM EDTA (pH=8)) and the gel was stained with 3x GelRedTM solution (Biotium), following the manufacturer instructions.

4.2.3- Molecular identification of insects

Tomicus destruens (Woll.) and *T. piniperda* are morphologically difficult to distinguish although there are some differences between their life cycles (Faccoli, 2006; Gallego and Galian, 2001). In order to confirm the species identity, 17 individuals randomly collected from the feeding galleries were sent to the Department of Animal Biology (University of Murcia) for molecular identification following the protocol described by Gallego and Galian (2001).

4.2.4- Statistical analysis

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effect of insect species from the insect samplings and to test the season of the shoot sampling on fungal species richness. Due to the fact that the data did not meet two of the ANOVA assumptions (normality and homoscedasticity), robust methods were applied (García, 2010). Specifically, one-way fixed-effects ANOVAs were performed under the assumption of non-normality and inequality of variances using the generalized Welch procedure and a 0.2 trimmed mean transformation. The ANOVAs were carried out using the 'Wilcox' Robust Statistics (WRS)' package implemented in the R software environment (R Foundation for Statistical Computing, Vienna, Austria). A paired-sample Wilcoxon test was carried out to check whether fungal species richness varied according to the tissue surveyed, particularly xylem and phloem.

Non-Metric Multidimensional Scaling (NMDS) and Multiple Response Permutation Procedure (MRPP) were carried out with the VEGAN package (Oksanen *et al.*, 2015) implemented in the R software environment in order to analyze the fungal communities associated with i) the insects' bodies ii) the logs, depending on the tissue and the insect species and iii) the shoots, depending on the season. NMDS was conducted using Bray-Curtis as the distance metric and the multivariate ordination was created using the metaMDS results. MRPP was also performed using Bray-Curtis dissimilarity with 1000 permutations.

4.3- Results

A total of 24 different fungal species were obtained from the bark beetle galleries from logs and shoots, while 18 were obtained from the insects' exoskeletons. Bark beetle species collected during this sampling are shown in Table 1.

4.3.1- Fungal communities from insect galleries

Fourteen fungal species from the logs (xylem and phloem) were identified: *F. circinatum*, four other species of *Fusarium*, two species of *Pestalotiopsis*, two *Trichoderma* spp., *Mucor* sp., *Trichoderma harzianum* Rifai, *Diplodia pinea* (Desm.), *Peniophora pini* (Schleich.) Boidin and *Penicillium glabrum* (Wehmer) Westling. Six other fungal species remained unidentified (Table 2). The species richness did not differ depending on the log tissue (xylem and phloem) ($V = 4757.5$, $P = 0.36$).

Regarding the fungal communities associated with the bark beetle galleries, three groups commonly associ-

Table 1. Insect species collected from logs, funnel traps and shoots.

Insect Species	*Total number*	*Logs*	*Ethanol*	*Pityol*	*Shoots*
Ips sexdentatus	*116*	*116*	*0*	*0*	*0*
Pityophthorus pubescens	*97*	*0*	*0*	*97*	*0*
Hylastes attenuatus	*86*	*83*	*2*	*0*	*1*
Orthotomicus erosus	*30*	*30*	*0*	*0*	*0*
Crypturgus mediterraneus	*26*	*26*	*0*	*0*	*0*
Hylastes ater	*25*	*20*	*5*	*0*	*0*
Hylastes angustatus	*23*	*23*	*0*	*0*	*0*
Xyleborinus saxeseni	*22*	*1*	*21*	*0*	*0*
Tomicus piniperda	*19*	*0*	*0*	*0*	*19*
Hylurgops palliatus	*9*	*9*	*0*	*0*	*0*
Xyleborus dispar	*4*	*0*	*4*	*0*	*0*
Hylastes linearis	*1*	*1*	*0*	*0*	*0*
TOTAL	*458*	*309*	*32*	*97*	*20*

ated with the insects were observed: one associated with *Orthotomicus erosus* (Woll.), one associated with those of *I. sexdentatus* and the last one with the three species of *Hylastes* (Figure 1).

Eighteen fungal species were isolated and identified from shoots: *D. pinea*, three species of *Pestalotiopsis*, five species of *Fusarium*, *Gliocadium roseum* Bainier, *T. harzianum*, two other species of *Trichoderma*, *Penicillium glabrum*, *Mucor* sp., *Botritys cinerea*, *Epicoccum nigrum* Link and *Trichoderma atroviride*. However, six fungal species remained unidentified (Table 2).

The species richness of the shoots was significantly

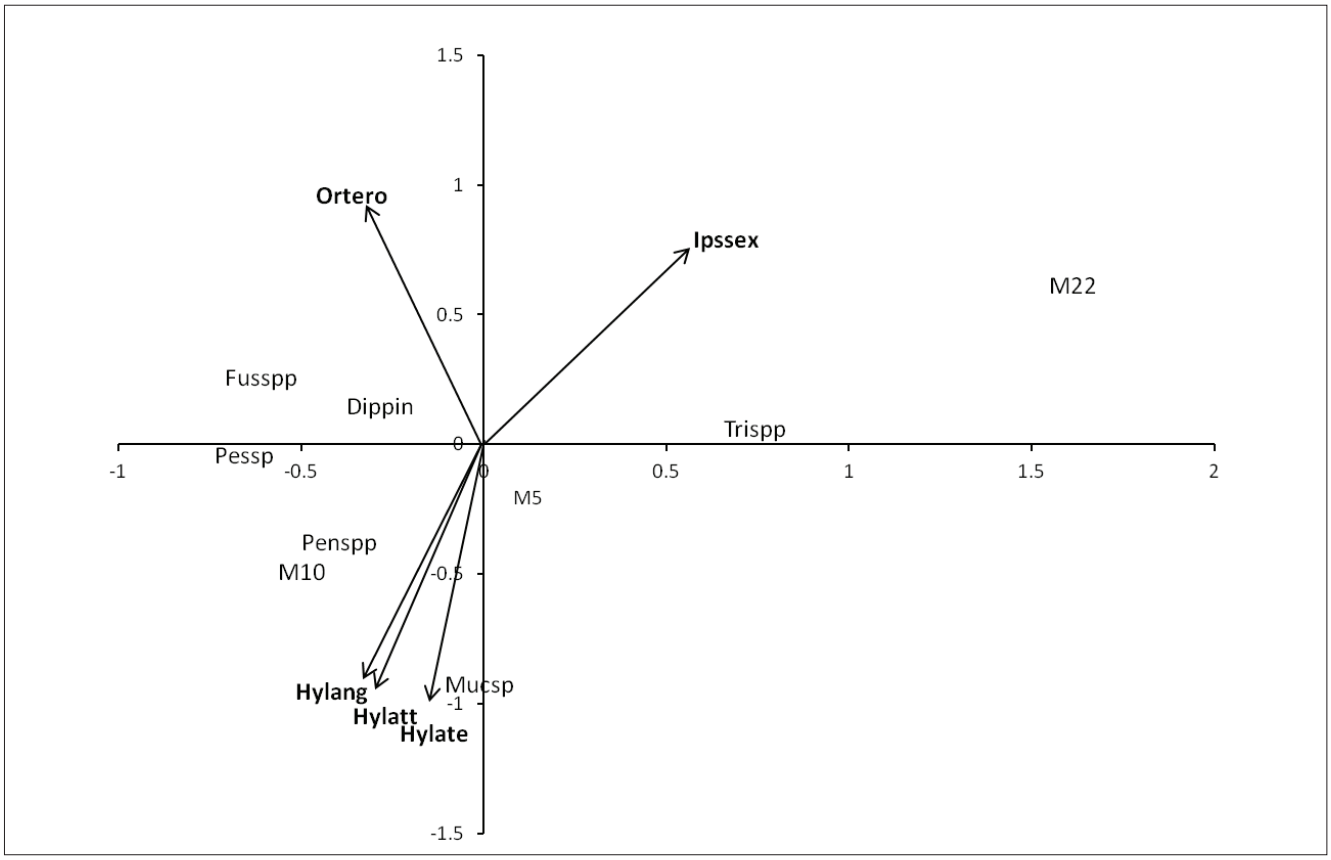

Figure 1. Non-metric multidimensional scaling (NMDS) at insect species level (Ortero=*O. erosus*, Ipssex=*I. sexdentatus*, Hylang=*H. angustatus*, Hylatt=*H. attenuatus*, Hylate=*H. ater*). Fungal species (Fusspp= *Fusarium* spp., Dippin=*D. pini*, Trispp=*Trichoderma* spp., Pessp=*Pestalotopsis* sp., Penspp=*Peniophora* spp., Mucsp=*Mucor* sp.) correspond to those isolated from the insects galleries on the logs. Dissimilarity distance = Bray-Curtis.

Table 2. Percentage of each fungal species isolated from shoots and plant tissue (xylem and phloem) from logs.

Species	**Shoots**	**Logs**												**Accession number**
	Tomicus piniperda	*Ips sexdentatus*		*Hylastes attenuatus*		*Orthotomicus erosus*		*Hylastes ater*		*Hylastes angustatus*		*Hylurgops palliatus*		
		Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	
Diplodia pinea	88.4	62.2	74.1	54.8	67.1	100.0	-	45.0	50.0	84.2	89.4	60.0	100.0	KP900724
Pestalotiopsis sp.	24.5	2.5	5.4	4.11	2.7	-	-	10.0	5.0	21.05	-	-	-	KP900723
Mucor sp.	3.8	0.0	1.8	15.1	6.9	-	-	50.0	15.0	5.26	10.5	-	16.6	KP900722
Trichoderma spp.	9.1	19.3	21.4	11.0	15.0	-	-	15.0	10.0	10.53	15.7	-	-	KP900738
Fusarium spp.	21.05	2.5	9.8	30.1	13.7	100.0	-	40.0	10.5	26.32	15.78	20.0	16.6	
Fusarium circinatum	3.5	-	0.9	-	1.3	-	-	-	-	5.2	5.5	-	-	-
Penicillium glabrum	11.2	3.4	-	16.4	23.3	-	-	15.0	-	26.3	21.0	-	16.6	KP900733
Trichoderma harzianum	11.9	46.2	52.7	20.5	20.5	-	-	5.0	30.0	-	10.5	-	-	KP900736
Trichoderma atroviride	1.4	-	-	-	-	-	-	-	-	-	-	-	-	KP900725
Peniophora sp.	1.4	1.7	-	-	-	-	-	-	-	-	-	-	-	KP900735
Gliocadium roseum	12.2	-	-	-	-	-	-	-	-	-	-	-	-	KP900726
Botriris cinerea	6.3	-	-	-	-	-	-	-	-	-	-	-	-	KP900730
Epicoccum nigrum	1.7	-	-	-	-	-	-	-	-	-	-	-	-	KP900729
M5	11.93	16.0	17.0	27.4	22.0	-	-	30.0	20.0	21.5	21.0	60.0	33.0	-
M10	3.16	0.8	10.7	12.33	4.1	-	100.0	15.0	25.0	10.53	26.0	60.0	-	-
M16	1.4	0.8	-	-	1.4	-	-	-	-	-	-	-	-	-
M17	3.51	2.5	-	2.74	5.5	-	-	-	5.0	10.53	5.3	-	-	-
M22	2.81	3.4	1.8	-	-	-	-	-	-	-	-	-	-	-
M27	8.77	0.8	-	-	-	-	-	-	-	-	-	-	-	-

different depending on the season in which they were collected (summer/autumn) (FWe = 42.1, P < 0.001). Likewise, fungal communities found in *T. piniperda* feeding shoots were statistically different according to the season (summer/autumn) (A= 0.04833, P < 0.001). The presence of *Fusarium* spp. was higher in the plots sampled in autumn and the same pattern was found for *D. pinea* and *P. pini* (Figure 2).

4.3.2- Fungal communities from insect exoskeletons

Eighteen fungal species were obtained from the insects' exoskeletons. *Fusarium circinatum* , other five *Fusarium* spp., *Candida fructicans*, *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels, *Penicillium* sp., *T. atroviride* and *G. roseum* were obtained, together with seven other species that remained unidentified (Table 3).

The species richness on the insects' exoskeletons was significantly different depending on the insect species (FWe = 4.8, P < 0.001) (Figure 3). The fungal communities present on the insects' bodies clustered in two different groups depending on the insect species: one group associated with *Hylastes* spp. and another one with *P. pubescens* (Figure 4).

Fungal communities from the insects collected from logs differed significantly (A = 0.3538, P < 0.001) from those associated with log tissues. Thus two distinct groups of fungi were observed: one associated with the xylem and phloem and the other one related to the insects´ exoskeleton. *Fusarium* spp. were associated with both types of samples, although they appeared to be more closely related to the insects' exoskeleton (Figure 5).

4.3.3- *Fusarium* spp. from insect galleries

Fusarium species were isolated from both xylem and phloem from logs. A total of eight isolates were identified: two belonged to the *F. oxysporum* spp. complex, one was *Fusarium sporotrichioides* Sherbakoff, two were *Fusarium beomiforme* (Nelson, Toussoun & Burgess), and three belonged to *Fusarium avenaceum* (Fries) Saccardo (Teleomorph=*Gibberella avenacea*)

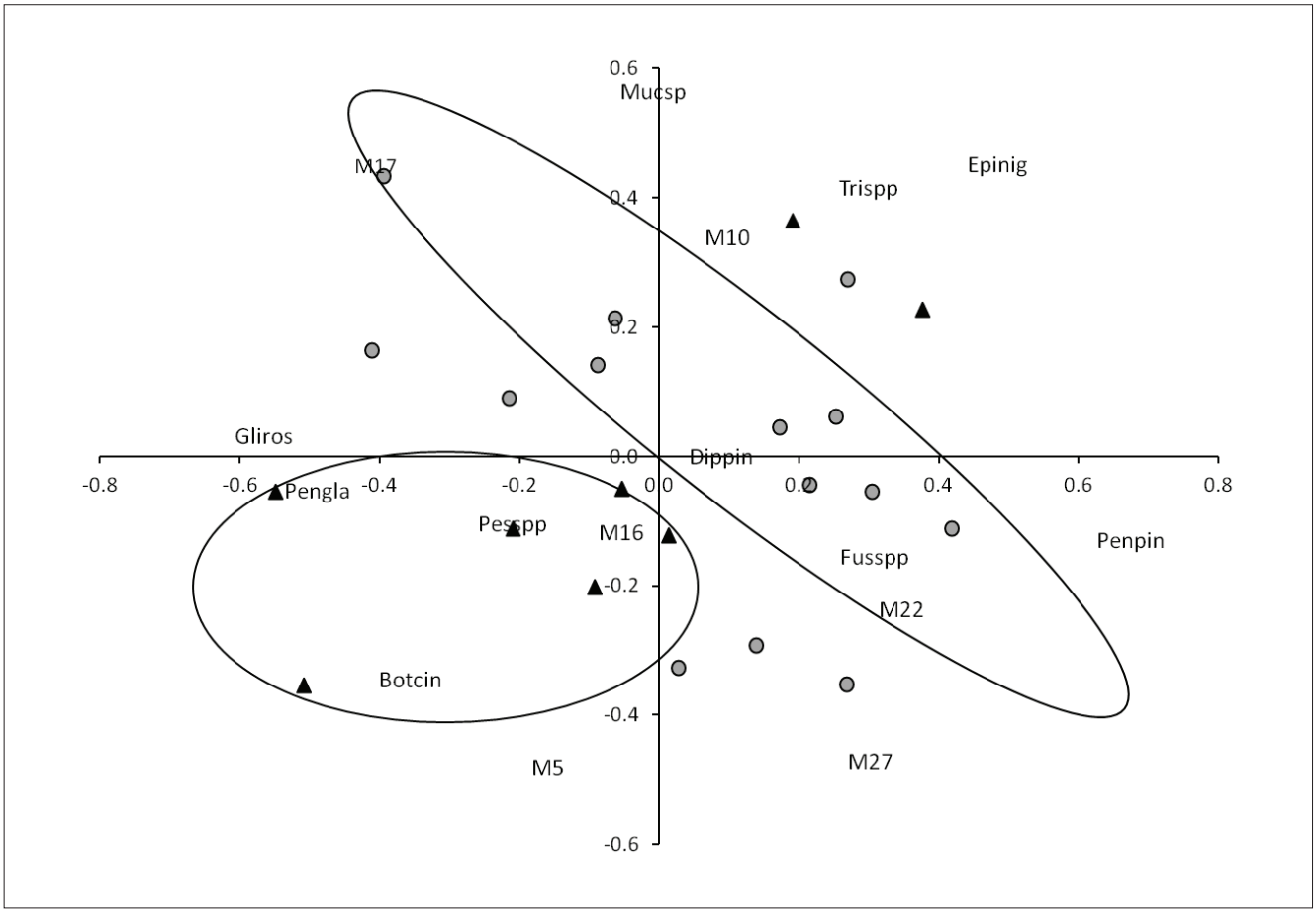

Figure 2. Multivariate analysis for fitting season variables to NMDS (Non-metric multidimensional scaling) ordination plots. Fungal species (Mucsp=*Mucor* sp., Epinig= *E. nigrum*, Trispp=*Trichoderma* spp., Gliros=*G. roseum*, Dippin= *D.pini*, Pengla= *P. glabrum*, Pesspp=*Pestalotiopsis* spp., Fusspp=*Fusarium* spp., Penpin=*P. pini*, Botcin=*B. cinerea*) correspond to those isolated from the galleries on the shoots. Symbols represent the sites (circle = plots sampled during autumn, triangle = plots sampled during summer).

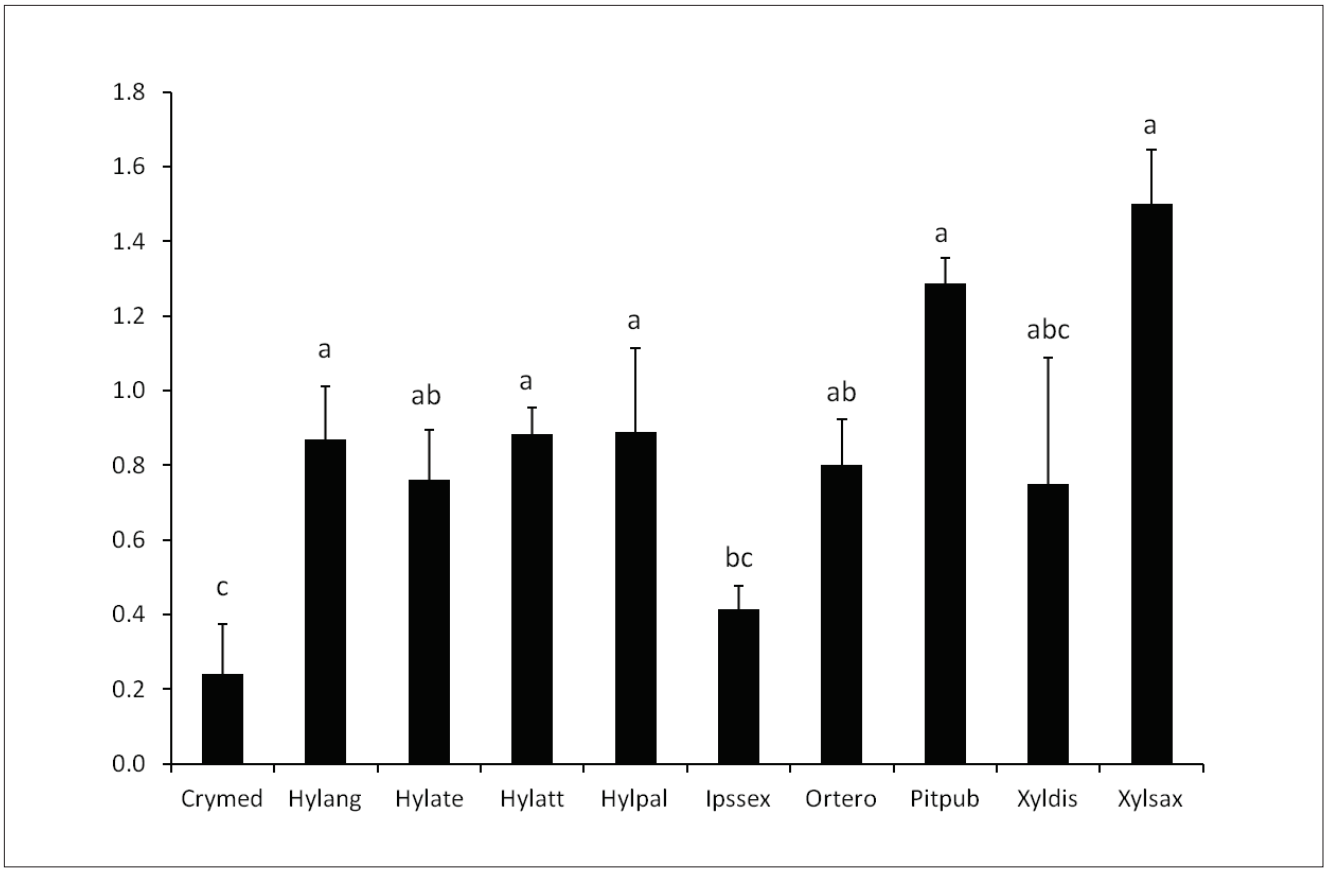

Figure 3. Fungal species richness on the exoskeleton of the different bark beetle species (Crymed=*C. mediterraneus*, Hylang=*H. angustatus*, Hylate=*H. ater,* Hylatt=*H. attenuatus*, Hylpal=*H. palliatus*, Ipssex=*I. sexdentatus*, Ortero=*O. erosus*, Pitpub=*P. pubescens*, Xyldis=*X. dispar*, Xylsax=*X. saxeseni*). Different letters represent significant differences (P<0.05). Error bars represent standard error.

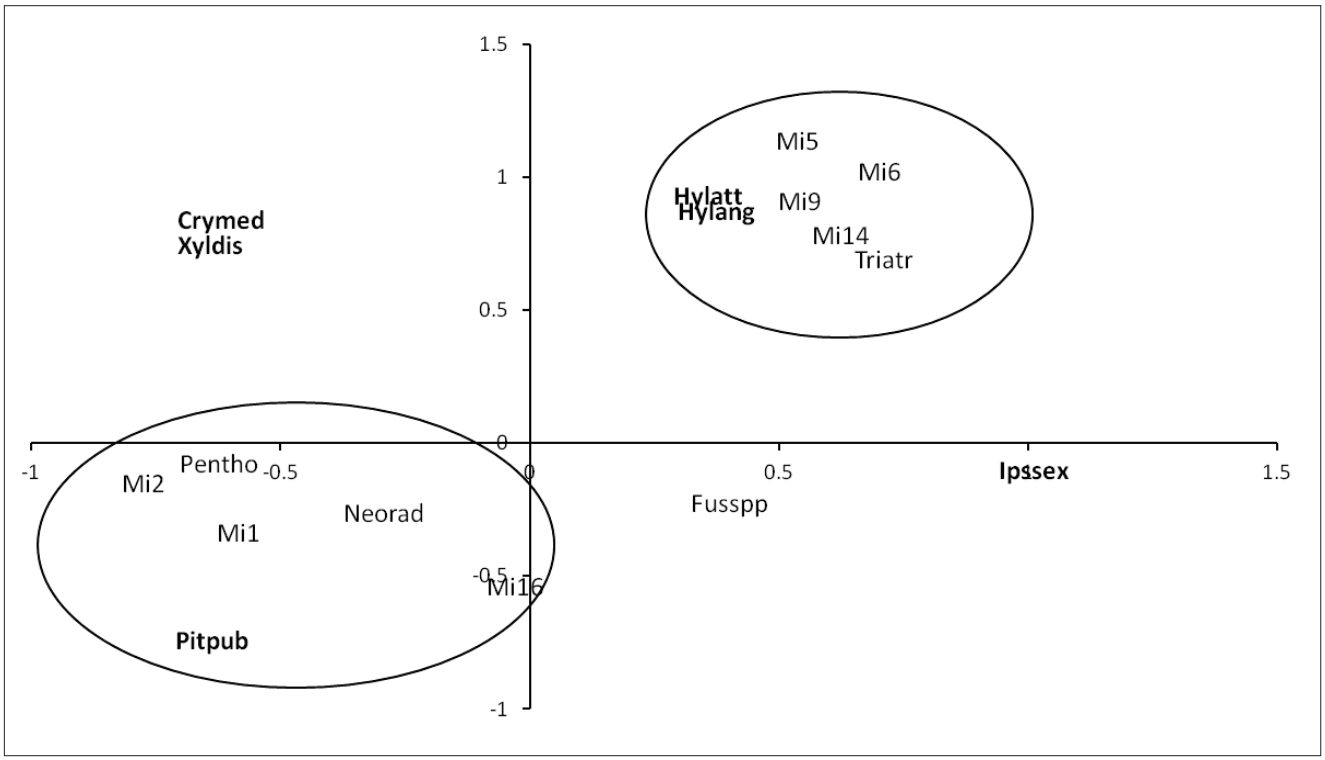

Figure 4. Non-metric multidimensional scaling (NMDS) at insect species level. Fungal species (Triatr=*T. atroviride,* Pentho=*P. thomentosum,* Neorad=*N. radicicola,* Fusspp= *Fusarium* spp.)correspond to those isolated from insects collected from the funnels and the logs (Crymed=*C. mediterraneus,* Hylang=*H. angustatus,* Hylatt=*H. attenuatus,* Ipssex=*I. sexdentatus,* Pitpub=*P. pubescens,* Xyldis=*X. dispar*). Dissimilarity distance = Bray-Curtis.

Table 3. Percentage of fungal species isolated from bark beetles species collected from funnel traps and logs. Mi corresponds to unidentified species.

Species	*Pityophthorus pubescens*	*Ips sexdentatus*	*Hylastes attenuatus*	*Orthotomicus erosus*	*Crypturgus mediterraneus*	*Hylastes ater*	*Hylastes angustatus*	*Xyleborinus saxeseni*	*Hylurgops palliatus*	*Xileborus dispar*	Accesion N.
Candida fructicans	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25	KP900741
Fusarium spp.	20.6	34.48	32.5	56.6	12	64	39.1	31.8	55.5	25	-
Fusarium circinatum	1.05	0.9	1.6	-	-	-	-	-	-	-	-
Gliocadium roseum	-	-	2.3	-	-	-	-	-	-	-	KP900740
Neonectria radicícola	5.1	-	2.3	-	-	-	4.3	22.3	-	-	KP900737
Penicillium sp	16.5	-	-	-	-	-	8.7	4.5	-	-	Kp900731
Trichoderma atroviride	1.03	0.9	3.48	3.3	8	-	4.3	-	-	-	KP900728
Mi1	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25	-
Mi2	1.03	-	11.6	-	-	-	8.7	-	-	-	-
Mi5	-	-	4.6	-	-	8	4.3	-	11.1	-	-
Mi6	1.03	1.7	1.16	-	-	-	-	-	-	-	-
Mi9	1.03	1.7	6.96	-	-	-	8.7	-	-	-	-
Mi14	-	1.7	2.3	6.6	-	4	-	4.5	-	-	-
Mi16	2.06	0	5.81	-	4	-	-	4.5	-	-	-

(Table 4). *Fusarium circinatum* appeared in 0.85 % and 0.43 % of the phloem and xylem samples, respectively. Regarding the galleries of *Hylastes angustatus*, *F. circinatum* appeared in 5.2 % and 5.5 % of the phloem and xylem samples each respectively, whereas the phloem in the *H. attenuatus* galleries only showed a 1.36 % presence of *F. circinatum* in the samples.

Furthermore, in the *T. piniperda* shoot feeding galleries, *Fusarium* species were isolated from 22.1 % of the collected shoots. Eighteen isolates could be identified as belonging to five different *Fusarium* species: one belonged to *Fusarium cortaderiae* (=*Fusarium graminearum* clade), one was *F. sporotrichioides*, three were *F. avenaceum*, three were *Fusarium tricinctum* (Corda) Saccardo (Teleomorph=*Gibberella tricincta*) (Table 4) and ten were *F. circinatum*, corresponding to 3.5 % of the shoot feeding galleries (Table 2).

4.3.4- *Fusarium* spp on insect exoskeletons

A high percentage of *Fusarium* spp. appeared on the insects' exoskeletons, making it apparent that, on the whole, the bark beetle species carried at least one *Fusarium* species (Table 3). In some cases one species was associated with more than one *Fusarium* (Table 4). A total of seven species from the genus *Fusarium* were identified: four isolates were identified as *F. oxysporum* spp. complex, one as *Fusarium anthophilum* (A. Braun) Wollenw, six as *F. avenaceum*, one as *Fusarium sambucinum* Fuckel (Teleomorph= *Gibberella pulicaris*), one as *F. tricinctum* and the last one as

Fusarium konzum (Teleomorph=*Gibberella konza*). Three isolates belonged to *F. circinatum*, one of them isolated from *H. attenuatus* collected from logs, one from *P. pubescens*, and one from *I. sexdentatus*.

Phylogenetic analyses of the alpha-TEF region indicate that each identified *Fusarium* spp. clustered together (Figure 6) regardless of the type of sample (insect, logs or shoots). Moreover, this sequence analyses support the molecular identification since the isolates that were identified as the same species clustered together.

4.4- Discussion

Amongst the fungal species isolated from the log tissue and shoots, we should highlight the presence of *D. pinea*, *Penicillium* spp., *Pestalotiopsis* spp., *Trichoderma* spp. and *Fusarium* spp. because of their pathogenicity, parasitism or potential as control agents. *Diplodia pinea*, for instance, was the species most frequently isolated in the plant tissue samples, although it did not appear on the insects' bodies. This fungus responsible for shoot blight and dieback on pine trees has been previously recorded in association with bark beetles (Whitehill *et al.*, 2007). Other species like *Penicillium* spp, had been previously isolated as saprotrophs in pine species since they rarely occur as endophytes in healthy tissues (Zamora *et al.*, 2008). *Pestalotiopsis* is a ubiquitous genus that acts as an endophyte, saprotrophe and pathogen in different hosts on a worldwide geographical distribution (Jeewon *et al.*, 2004). It was also reported to be found in healthy tissue of pine species in Spain (Zamora *et al.*, 2008), but some species such as *Pestalotiopsis funerea* can cause damping off in other conifers (Bajo *et al.*, 2008). Four CMs were identified as *Trichoderma* spp. in this study, but two of them could not be identified at species level using the ITS region. *Trichoderma harzianum*, *T. atroviride* and *T. viride* have been proposed as effective biological control agents of pathogenic *Fusarium* spp. (Alves-Santos and Diez, 2012a), highlighting the importance of knowing the endophytic species inhabiting *P. radiata* trees. *Trichoderma atroviride* parasitizes a large variety of phytopathogenic fungi due to the production of hydrolytic enzymes, which has led to its use as a biological control agent (Olmedo-Monfil *et al.*, 2002).

Regarding the fungal communities associated with *T. piniperda*-colonized shoots, *D. pinea* was the species most frequently isolated. A seasonal variation was observed in the appearance of different fungal species isolated from shoots. *Fusarium* spp., for example, appeared more in those plots sampled during autumn, which was in accordance with previous results obtained by Bezos *et al.* (2013) in which a higher percentage of *F. circinatum* was isolated during the autumn and winter months.

Bark beetles are known to be associated with endophytic, phytopathogenic and entomopathogenic fungi. In this study 18 species were isolated from the insects' bodies. Among them, the ascomycete yeast, *Candida fructicans*, was identified in nine insect species. *Candida* species have been reported associated with several bark beetles species like entomopathogens, although many yeasts associated with bark beetles play a symbiotic role (Vega and Hofstetter, 2015). *Trichoderma atroviride* appeared on the bodies of six different insect species. In order to evaluate the importance of *Trichoderma* spp. as a *Fusarium* spp. antagonist, it would be necessary to do more in-depth studies of the role of bark beetles in association with these fungal genera. Other fungi, like

Neonectria radicicola, were isolated from four bark beetle species. In Norway, this fungus species has been observed as an endophyte in pine roots without causing any damage (Ndobe, 2012). However, other species of the genus *Neonectria* are known for causing neonectria canker disease on subalpine fir in Denmark (Talgø *et al.*, 2011) and stem cankers on *P. radiata* in Chile (Morales, 2009). The species richness and fungal communities differed according to the bark beetle species. This difference could be due to the presence of specialized structures for carrying spores i.e. mycangia on some species, e.g. *H. ater*, *I. sexdentatus* or *Xyleborinus saxeseni*. Fungal communities were also significantly different on the insects' exoskeletons and in their galleries, although *Fusarium* spp. appeared to be related to both types of samples. This result highligths the important role that bark beetles play in the spreading of *Fusarium* species.

Ten species of *Fusarium* were identified in this study, following both molecular and morphological identification methodologies. They were found to be related to the insects' exoskeletons and their galleries. This is a polyphyletic group that can appear as an endophyte or as a plant pathogen depending on the species and on the plant host. Moreover, these species have a wide geographical distribution, infecting a wide range of organisms worldwide. Several *Fusarium* spp. have a mutualistic association with insects, e.g. *Fusarium solani* (Martius) (teleomorph=*Nectria haematococca*) which has a symbiotic relationship with *Hypothenemus hampei* (Ferrari) while colonizing coffee beans (Morales-Ramos *et al.*, 2000) and with *Xyleborus ferrugineus* (Fabricius) when colonizing dead insects as saprophytes or entomopathogens. In general, those *Fusarium* species associated with beetles are weakly entomopathogenic, although the infection of the pine beetle *Dendroctonus frontalis* (Zimmerman) with *F. solani* resulted in the death of 90 % the insects in 5 days (Teetor-Barsch and Roberts, 1983). In this study, *F. avenaceum* was the most commonly identified species. It was isolated directly from *I. sexdentatus*, *H. attenuatus* and *H. ater* specimens and their galleries. Moreover, it also appeared on *T. piniperda*-infested shoots. *Fusarium avenaceum* has been previously isolated from *P. radiata* in New Zealand, where it was associated with dieback caused by physical injury (Dick and Dobbie, 2002). However, the *F. graminearum* clade and *F. avenaceum* are commonly associated with crops (Satyaprasad *et al.*, 2000) and cause Fusarium head blight (FHB) on wheat (Bottalico and Perrone, 2002). Molecular identification of *Fusarium* species was necessary to distinguish the isolate number 21 (Table 4) whose DNA sequence belonged to *F. avenaceum*, whereas the presence of napiform microconidia in this isolate identified it morphologically as *Fusarium anthophilum*. *Fusarium tricinctum* was isolated from *I. sexdentatus* and from *T. piniperda* feeding galleries. This fungus usually acts as as a saprophyte or weak parasite in Europe and North America (Leslie and Summerell, 2006). The *F. oxysporum* spp. complex was found to be associated with *O. erosus*, *H. attenuatus* and was in the *I. sexdentatus* galleries in logs (both, xylem and phloem). This is a saprophyte and soil pathogen species complex with a wide range of plant hosts divided into many formae specialis depending on its host specificity. *Fusarium sambucinum*, which was isolated from *P. pubescens*, causes dry rot in potatoes (Niemira *et al.*, 1996) but it was also isolated from *P. radiata* in New Zealand and found to be associated with dieback and root rot (Dick and Dobbie, 2002). Other species isolated in this study like *F. sporotrichioides*, *F. konzum* and *F. beomiforme* have not been previously described as plant pathogens or are very weak (Leslie and Summerell, 2006).

Fusarium circinatum, the causal agent of PCD, appeared in 3.5 % of the fallen shoots bored by *T. piniperda*. The importance of this association lies in the shoot-feeding maturation behavior of *T. piniperda* in the crowns of healthy pine trees (Lieutier *et al.*, 2015) which could allow for the transmission of the fungal spores into healthy crowns. This insect has also been related to some other highly phytopathogenic fungi, like *Leptographium wingfieldii* Morelet in Europe and North America (Lieutier *et al.*, 1989; Jacobs *et al.*, 2004). They have caused significant economic loss due to the species' blue stain capacity. Brood galleries of *Hylastes* spp. also appeared to be related to *F. circinatum* in this study. These insects have a similar way of interacting with sapwood fungi in several pine species (Reay *et al.*, 2002), including *P. radiata*, although in the present study no ophiostomatoid fungi were found. *Hylastes angustatus* galleries were most frequently associated with *F. circinatum* (5.5 % of xylem samples and 5.2% of phloem samples). But *Hylastes* spp. is a secondary pest, attacking weakened trees and roots, which suggests that they are not able to inoculate healthy trees with the pathogen as it would be the case with *T. piniperda*.

Another bark beetle related to *F. circinatum* was *P. pubescens*, taking into account that 1.05 % of the cap-

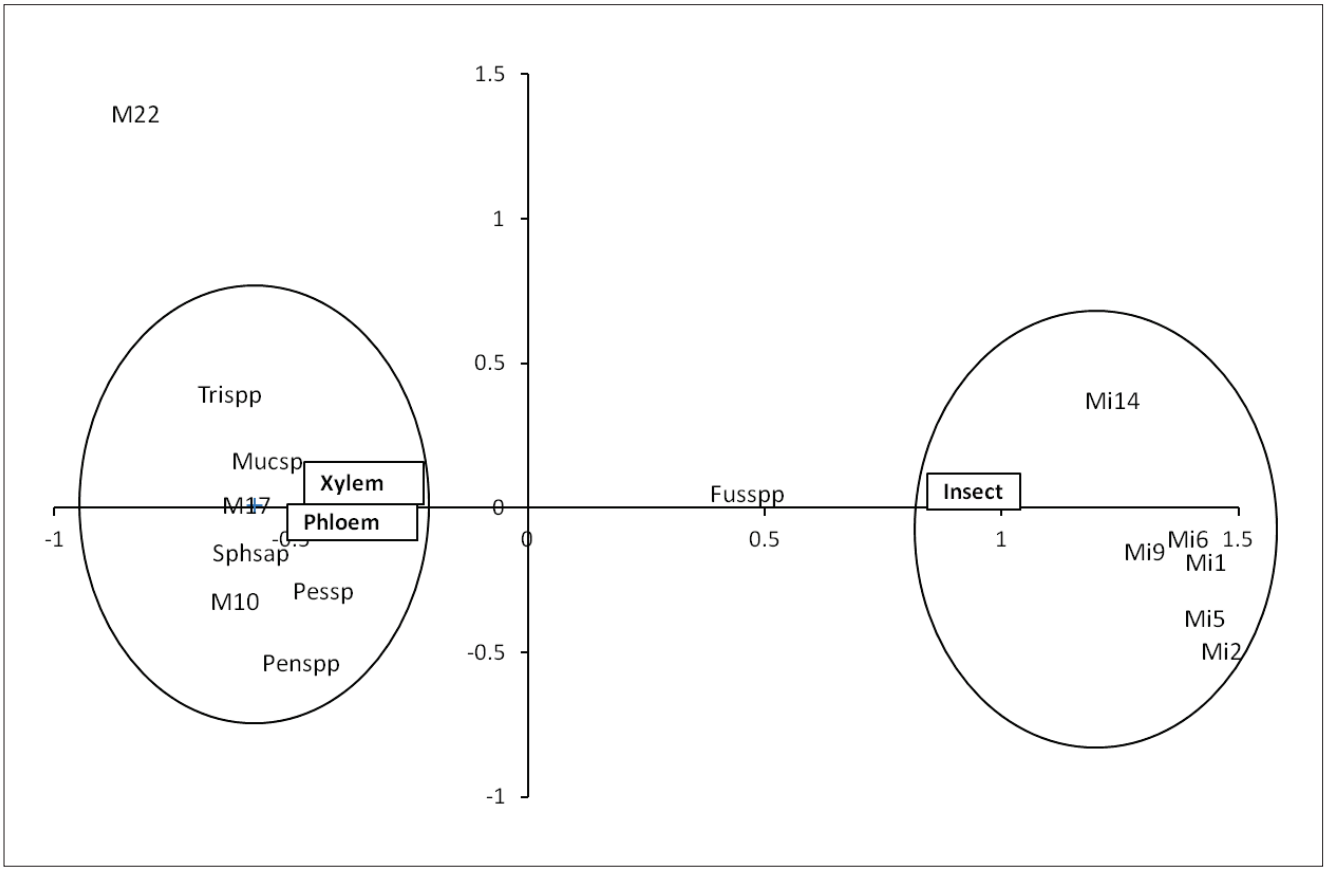

Figure 5. Non-metric multidimensional scaling (NMDS) at sample level. Fungal species (Trispp=*Trichoderma* spp., Mucsp=*Mucor* sp., Sphsap=*S. sapinea*, Pessp=*Pestalotiopsis* sp., Penspp=*Penicillium* spp., Fusspp=*Fusarium* spp.)correspond to those isolated from the logs (xylem and phloem) and from insects collected from the logs. Dissimilarity distance = Bray-Curtis.

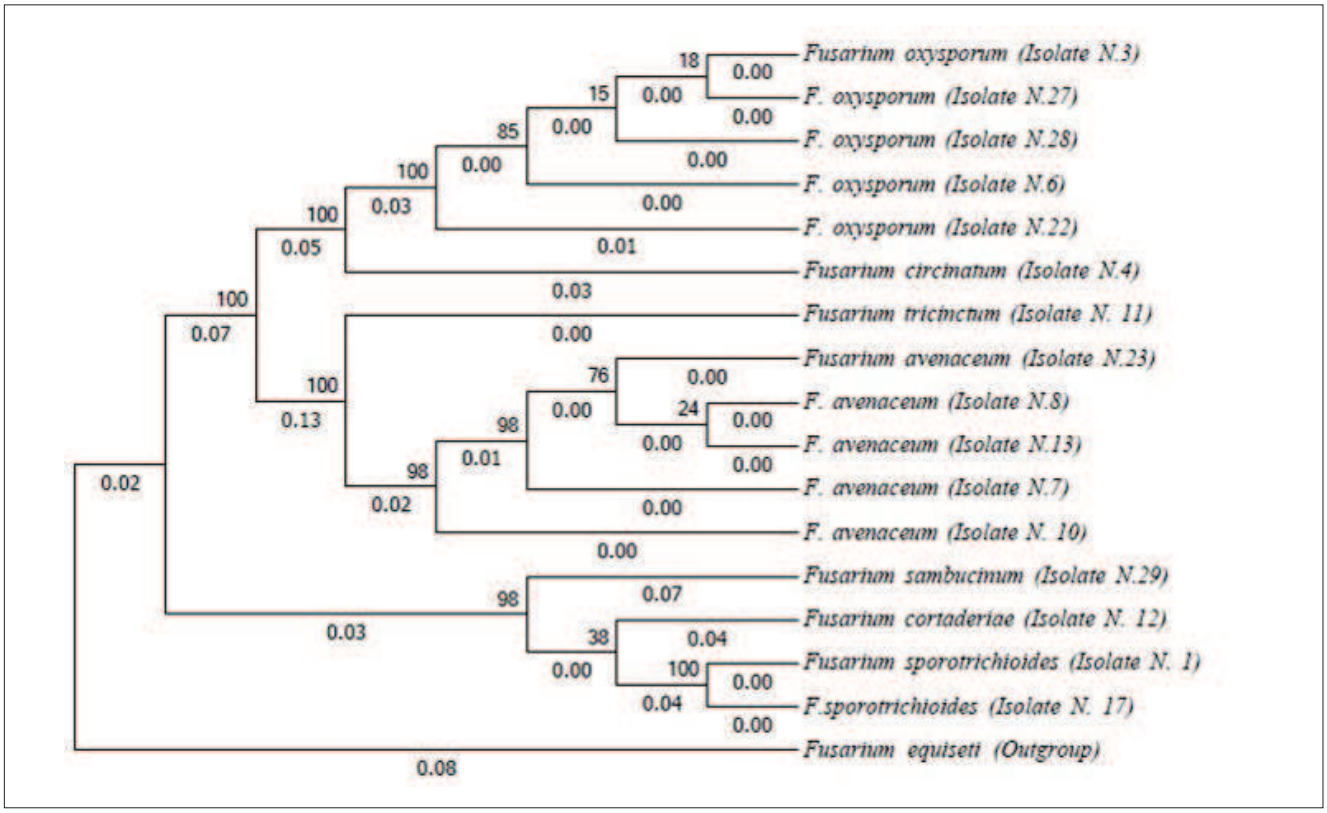

Figure 6. Phylogenetic tree of *Fusarium* spp. inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site.

Table 4. Identified isolates of *Fusarium* species from the different methodologies.

Isolate	Origin	Collected in	ITS región	Morphology	TEF region	ConsensusSpecies	Accesion N TEF
1	Phloem	Logs I.s	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*	KR002044
2	Phloem	Logs H. att	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*	-
3	Phloem	Logs I.s	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002062
4	Phloem	Logs H. ang	-	*F. circinatum*	*F. circinatum*	*F. circinatum*	KR002060
5	Xylem	Logs H. att	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*	-
6	Xylem	Logs H.att	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002050
7	Xylem	Logs H. a	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002057
8	Xylem	Logs H. a	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002049
9	Xylem	Logs Hy. P	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002058
10	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002063
11	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002064
12	Shoot	Shoots T.p.	*Fusarium* sp.	*F. cortaderiae*	*F. cortaderiae*	*F. cortaderiae*	KR002048
13	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002046
14	Shoot	Shoots T.p.	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002051
15	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002054
16	Shoot	Shoots T.p.	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*	KR002047
17	Shoot	Shoots T.p.	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*	-
18	*Hylastes ater*	Logs	*F. lateritium*	*F. avenaceum*	-	*F. avenaceum*	KR002059
19	*Hylastes attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002052
20	*H. attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	-
21	*H. attenuatus*	Logs	*Fusarium* sp.	*F. anthophilum*	*F. avenaceum*	*Fusarium sp.*	KR002055
22	*H. attenuatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002065
23	*Ips sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002056
24	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	KR002046
25	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*	-
26	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. tricinctum*	-	*F. tricinctum*	-
27	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002066
28	*Orthotomicus erosus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	KR002043
29	*Pityophthorus pubescens*	Funnel	*Fusarium* sp.	*F. sambucinum*	*F. sambucinum*	*F. sambucinum*	KR002053
30	*Xyleborinus saxesni*	Funnel	*Fusarium* sp.	*F. konzum*	-	*F. konzum*	-

tured specimens carried the pathogen. The importance of other species of this genus has been noted in several countries. For example, in California the potential role of *Pityophthorus carmeli* Swaine and *Pityophthorus setosus* Blackman in vectoring *F. circinatum* was shown by the wounding behavior of these twig beetles as they tested *P. radiata* branches for their suitability as hosts (Sakamoto *et al.*, 2007). Bonello *et al.* (2001) reported that *Pityophthorus* spp. discriminated between healthy and pitch canker-diseased branches, preferring symptomatic branches due to the increased emission of ethylene. However, *P. pubescens* is a secondary species in our study area affecting small branches from weakened trees (unpublished data), so its role as a vector does not seem to be relevant. On the other hand, *F. circinatum* was present in 0.9 % of the *I. sexdentatus* specimens. *Ips sexdentatus* is a secondary bark beetle but can act as a primary parasite when the population reaches epidemic levels (Etxebeste and Pajares, 2011), and in this situation, it could be able to inoculate healthy trees with the pathogen. In the Basque Country, Spain, 8.57 % of the *I. sexdentatus* analyzed by Romón *et al.* (2007) carried the pathogen. Likewise, in California other species, like *Ips mexicanus* (Hopkins) and *Ips paraconfusus* Lanier, were reported as vectors of the pitch canker fungus (Fox *et al.*, 1991). *Fusarium circinatum* spores may be harboured on bark beetles' exoskeletons when they feed on or breed in pitch canker diseased trees. After that, insects carrying the spores could inoculate healthy pines. Some authors say this association between insects and pathogenic fungi allows them to stimulate trees' resistance (Lieutier *et al.*, 2009). However, as bark beetles at epidemic levels can kill healthy trees without carrying any phytopathogenic fungi, other authors state that insect-pathogen interactions could only benefit the fungus species, helping them expand their range to trees they could never reach without this association (Six and Wingfield, 2011). Bark beetles are also principal wounding agents, contributing to the infections caused by airborne spores even if the insects themselves do not carry the pathogen (Baker and Norris, 1968).

In conclusion, the species richness as well as the fungal communities on the bark beetles' bodies and in their galleries varied depending on different factors such as insect species, kind of tissue and season. Moreover, in this study an association of the *Fusarium* species with bark beetles and their galleries was found. The importance of bark beetles in the distribution of *Fusarium* spp. could be related to the season (as it was observed in this study) and to the population levels, since bark beetles are not primary species in our study area, with the exception of *T. piniperda* because of its maturation feeding on healthy crowns (Långström, 1982). Regarding the spreading of *F. circinatum*, *P. pubescens* and *I. sexdentatus* were associated with the pathogen's spores as well as *H. attenuatus*, *H. angustatus* and T.*pini*perda galleries. Further studies are needed to bring the specific association each bark beetle species has with fungal communities to light, and, especially, to better understand the role of the different Scolytinae species on *Fusarium* spp. distribution.

Acknowledgements

This study was made possible through the project, 'Etiology, Epidemiology and Control of *Fusarium circinatum*', sponsored by the Ministry of Rural, Marine and Natural Environment, and with the support of the Government of Cantabria. We would like to thank Diego Gallego for the molecular insects identification as well as Milagros de Vallejo and Juan Blanco for their help in the establishment of the experiment.

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Chapter 5: The pine shoot beetle *Tomicus piniperda* as a plausible vector of *Fusarium circinatum* in northern Spain

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Annals of Forest Science
DOI: 10.1007/s.13595-015-0515-4

Abstract

Fusarium circinatum, the causal agent of pitch canker disease, currently affects *Pinus radiata* in northern Spain, causing pitch-soaked cankers and tree death. Although several species of the family Scolytinae have been reported as vectors of this pathogen, the role of the pine shoot beetle *Tomicus piniperda* remains unclear. The general objective of this study was to determine whether *T. piniperda* is a vector for the pitch canker pathogen *F. circinatum*. For this purpose, Leach's postulates: (1) an association between *T. piniperda* and trees affected by pitch canker disease; (2) regular visits by *T. piniperda* to healthy *P. radiata* trees; (3) presence of the pathogen on the insect in nature; and (4) transmission of the pathogen to disease-free host material under controlled conditions.

Fresh green shoots with feeding galleries were collected from the ground, breeding galleries were collected from diseased trunks and insects were collected during their dispersion flights. A laboratory experiment was conducted in which specimens of *T. piniperda* were inoculated with the pathogen prior to feeding on shoots. In the field, *T. piniperda* was found to be associated with both diseased and healthy *P. radiata* trees and *F. circinatum* was found to be present, at low rates, on the exoskeleton of *T. piniperda*. In the laboratory experiment evidence of the ability of *T. piniperda* to transfer the pathogen to healthy shoots was found. The study findings indicate *T. piniperda* as a plausible vector of this pathogen. We postulate for the first time a potential relationship between the life cycles of *T. piniperda* and *F. circinatum*.

Resumen

Fusarium circinatum, el agente causante de la enfermedad del chancro resinoso de lo los pinos, amenaza actualmente las plantaciones de *Pinus radiata* en el norte de España. Varias especies de escolítidos han sido descritas como vectores de este patógeno, pero aún se desconoce el papel de *Tomicus piniperda* en la dispersión de esa enfermedad. El objetivo principal de este trabajo fue determinar si *T. piniperda* es posible vector de este patógeno. Para ello, se estudiaron los postulados de Leach: (1) la asociación de *T. piniperda* con árboles afectados por la enfermedad del chancro resinosos; (2) la existencia de visitas regulares de *T. piniperda* a *P. radiata* sanos; (3) la presencia del patógeno en los insectos en la naturaleza; y (4) la transmisión del patógeno a un hospedador libre de la enfermedad bajo condiciones controladas. Se recogieron y procesaron distintos tipos de muestras: ramillos verdes y frescos con galería de alimentación del insecto, galerías de cría provenientes de troncos afectados por la enfermedad y, finalmente,

se capturaron insectos durante su vuelo de dispersión. Además, se llevó a cabo un experimento de laboratorio en el cual se inocularon especímenes *T. piniperda* con *F. circinatum* previamente a que éstos realizaran la alimentación en ramillos. Respecto a los resultados de las muestras recogidas en campo, *T. piniperda* apareció asociado tanto a árboles sanos como a árboles enfermos y la presencia de *F. circinatum* se detectó en un número bajo de especímenes de *T. piniperda.* El experimento de laboratorio también mostró evidencias de la capacidad de *T. piniperda* para transferir el patógeno a ramillos sanos. Estos resultados ponen de manifiesto la posibilidad de que *T. piniperda* sea un vector del patógeno, proponiéndose por primera vez, la existencia de una posible relación entre el ciclo de *T. piniperda* y *F. circinatum.*

5.1- Introduction

Fusarium circinatum Nirenberg and O´Donnell is an ascomycete fungus that causes pitch canker disease (PCD) in pines (Nirenberg and O'Donnell, 1998). The disease is characterized by the formation of large deformed resin-filled cankers, which affect both the trunk and thick branches. The pathogen threatens *Pinus radiata* D. Don plantations and natural forests throughout the world (Martínez-Álvarez *et al.*, 2014 and referneces therein), because of the high susceptibility of this pine species (Viljoen *et al.*, 1995). In Spain, *F. circinatum* was first reported in 2005 (Landeras *et al.*, 2005) although it is suspected that the pathogen has been causing damage since 1997 (Laucirica and Muguruza, 1997).

Fusarium circinatum spores may be dispersed by wind, water, and seedling transport as well as by the activity of insects while excavating their breeding galleries or feeding on the crowns of healthy trees (Storer *et al.*, 2004). Some insects like *Pityophthorus carmeli* Swaine are vectors of the disease in California (Storer *et al.*, 2004), while in the Iberian Peninsula several species of beetles, such as *Pityophthorus pubescens* (Marsham), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Börner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* (Erichson) and *Orthotomicus erosus* (Wollaston), are known to be phoretically associated with the pathogen (Romón *et al.*, 2007a). The widely studied, complex interaction between fungal pathogens and insects is typically considered as a mutual relationship between the vector and the fungus with ecological advantages for both organisms (Paine *et al.*, 1997). For instance, some pathogenic fungi, specifically blue stain fungi, by stimulating tree defence mechanisms could contribute to tree resistance exhaustion and consequently enable beetle and fungi establishment in host tissues (Lieutier *et al.*, 2009). However, as it has been demonstrated that bark beetles can kill trees when no pathogenic fungi are present, other authors have suggested that in this association the fungal pathogenicity may only benefit the fungus rather than the beetle (Six and Wingfield, 2011).

Tomicus piniperda L. (Coleoptera; Scolytinae) is a serious pest that affects pines in Europe, Northern Africa, Asia (Långström, 1982; Bouhot *et al.,* 1988; Kirkendall *et al.,* 2008) and the United States (McCullough and Smitley, 1995). Although the main host is *Pinus sylvestris* L., other pine species are also suitable as hosts, e.g. *P. radiata. Tomicus piniperda* is a univoltine species that may produce several sister broods. The species is considered a secondary pest colonizing trunks and thick branches of weakened trees (Paine *et al.,* 1997). However, emerging young adults, as other *Tomicus* species, perform a maturation feeding on shoots and can act then as a primary species (Långström, 1982; Fernández *et al.,* 1999; Gallego *et al.,* 2008; Lieutier *et al.,* 2015). Each insect penetrates more than one shoot during the feeding phase, especially in the thicker and fresh current-year shoots (Tiberi *et al.,* 2009). They can negatively affect tree growth and structure of healthy trees, cause carbon and nitrogen losses and, in cases of high population densities, death of the tree (López *et al.*, 2007). The fact that *T. piniperda* weakens the host tree after feeding on shoots also increases the number of reproductive niches susceptible to colonization, although shoot damage by *T. piniperda* rarely exceeds 50% (Långström, 1980).

Because of this maturation feeding on the crowns of healthy pines, *T. piniperda* is a strong candidate as an effective vector of *F. circinatum* in the study area. In addition, in southern Europe, *T. piniperda* overwinters within the shoots (Russo, 1946). Thus, in the study area the beetle can remain for 6 to 9 months within the shoots. The possibility that this species is a vector of the fungus is further supported by the fact that the main symptom associated with the presence of *F. circinatum* in shoots excavated by the insects is severe necrosis of the pith (Figure 1). However, little is known about the effectiveness and importance of the life cycle of *Tomicus* species regarding transmission of the pathogen. *Tomicus piniperda* is associated with virulent ophiostomatoid fungi, such as *Leptographium wingfieldii* Morelet in Europe (Lieutier *et al.,* 1989; Jacobs *et al.,* 2004) and

Ophiostoma minus (Hedgc.) Syd. & P. Syd. (Solheim *et al.,* 2001; Jankowiak and Bilanski, 2007).

The general objective of this study was to determine whether the pine shoot beetle *T. piniperda* is a vector for the pitch canker pathogen *F. circinatum.* For this purpose, Leach's postulates (Leach, 1940) were tested: (1) a close, although not necessarily constant, association between *T. piniperda* and trees affected by pitch canker disease; (2) regular visits by *T. piniperda* to healthy *P. radiata* trees; (3) the presence of the pathogen on the insect in nature; and (4) whether *T. piniperda* can successfully transmit the pathogen to disease-free host material under controlled conditions.

5.2- Materials and Methods

Different methods were applied with the aim of testing Leach's postulates. To establish an association between *T. piniperda* and diseased trees (postulate 1), samples were collected from breeding galleries on trees affected by *F. circinatum* for examination. To determine whether *T. piniperda* regularly visits healthy pines (postulate 2), fresh green shoots of *P. radiata* bored by the insect were collected from the ground and analyzed for the presence of the pathogen. To determine whether the pathogen occurs on the insects in nature (postulate 3), funnel traps were placed in a plot affected by pitch canker. Finally, the symptoms of the disease were produced experimentally under controlled conditions (postulate 4) on healthy shoots on which artificially inoculated specimens of *T. piniperda* were fed.

Molecular identification of insects was carried out,

Figure 1. a) *Pinus radiata* shoot with *Tomicus piniperda* feeding gallery, necrotic pith and green tissue, b) and c) details of *F. circinatum* structures growing on the necrotic pith.

because of the possible sympatry between *T. piniperda* and *Tomicus destruens* in the study area (Gallego *et al.*, 2004) and because of the complexity of identifying some morphological characters or even their absence in some individuals (Faccoli, 2006). For this purpose, the DNA was extracted from nine randomly selected insects, with the Biotools extraction kit (Speedtools Tissue DNA Extraction Kit). Samples were taken from the insects' heads to prevent contamination due to fungi and nematodes present in the digestive tract and elytra. The ITS2 fragment was amplified by PCR with the ITS3 (5.8S region) and ITS4 primers (28S region). PCR was performed in a reaction volume of 25 µl, and the cycling programme was 5 min at 96 °C followed by 35 cycles of 30 s at 96 °C, 1 min at 50 °C and 1 min at 72 °C with a final elongation 10 min at 72 °C. The PCR product was purified and digested with Hinc II restriction enzyme overnight at 37 ° C. The digestion product was observed by agarose gel electrophoresis (MetaPhor) (Gallego and Galian, 2001).

5.2.1- Association between *T. piniperda* and diseased trees

With the aim of studying the association between *T. piniperda* and diseased trees, breeding galleries were collected from a *P. radiata* plot affected by PCD in Santibañez (Cabezón de la Sal, Cantabria) (Martínez-Álvarez *et al.*, 2012). Ten trees with pitch cankers on the trunks were sampled in 2014. Tree bark showing signs of the presence of *T. piniperda* next to the cankers was removed, with an axe, and analyzed in the laboratory along with plant tissue collected from breeding galleries. The gallery tissue was plated on potato dextrose agar (PDA Scharlau) modified by the addition of 0.6 g of streptomycin sulphate (Fluka Analytical) per 39g of PDA (PDAS), after superficial sterilization of the samples by submergence in 100 ml each of four different liquids (1 min tap water, 1 min 70 % ethanol, 1 min sodium hypochlorite 2 %, 1min sterile distilled water). The material from five of the breeding galleries from which *F. circinatum* had been isolated by plating on PDAS was placed on wet chambers for microscopic observation of the structure of the pathogen. With the aim of determining whether *F. circinatum* was present on breeding specimens of *T. piniperda* on diseased trunks, insects at different developmental stages were collected from galleries (15 parental adults, 32 F1 adults, 23 pupae and 58 larvae). The insect material was then plated on PDAS media in accordance with the methodology described by Ambourn *et al.* (2006) with some modifications; each

insect was placed in a 1.5 ml micro tube with 200 µl of 1 % Tween 80 and sonicated for 5 seconds. One hundred µl of the solution was plated on PDAS and extended on the medium with a sterile loop. *Fusarium circinatum* colonies were identified by their morphology following the method described by Leslie and Summerell (2006) for culturing mycelia on SNA (Synthetischer Nährstoffärmer Agar). The typical structures of this fungus (i.e. oval microconidia, mono and polyphialides, coiled sterile hyphae and absence of clamidiospores) were observed on SNA. The differences between the different insect stages collected from the breeding galleries in relation to presence of *F. circinatum* were analyzed by Fisher's exact test (SPSS software).

5.2.2- Association between *T. piniperda* and healthy trees

The association between *T. piniperda* and healthy crowns of pine trees has been studied by different authors (Långström, 1982; Lieutier *et al.*, 2015). In the present study, we aimed to demonstrate the capacity of *T. piniperda* to infest symptom-free green crowns of *P. radiata* trees in plots affected by pitch canker. Between April 2011 and June 2012, 954 fresh green fallen shoots bored by *T. piniperda* were collected in six *P. radiata* plots affected by *F. circinatum* in Cantabria (Cabezón de la Sal, Udías, Rionansa and Santiurde de Toranzo) (Martínez-Álvarez *et al.*, 2012). Sampling was carried out for 1 hour every 15 days, and up to 100 shoots were collected monthly. As the length of the feeding gallery may be related to the time that the insect spends within the shoot, the presence of *F. circinatum* may be influenced by the gallery length, which was therefore measured in each shoot. The section of each shoot burrowed by the insect was plated on PDAS medium, after surface sterilization of the sample by submergence in 100 ml of four different solutions (1 min tap water, 1 min 70 % ethanol, 1 min sodium hypochlorite 2 %, 1min sterile distilled water). To establish any possible influence of the season on the presence of the pathogen inside the shoots galleries, a Chi-square test was conducted. To analyze the influence of the gallery length on the presence of the pathogen, a logistic regression was carried out.

To identify the part of the shoots where the pathogen was most abundant, and to determine the development of the pathogen within the shoot, three different areas were identified (gallery, necrotic pith and transition zone) (Figure 1). The tissue collected from each area was subsequently plated on PDAS. This analysis was carried out with the 200 fallen shoots infested with *T. piniperda* that were collected during January and February 2012. The part of the shoot where the insect bores to make its feeding gallery was considered as the "gallery"; the adjacent part with brown pith was considered the "necrotic pith"; and the part of the shoot where the pith is still green and healthy was classified as the "transition zone". A total of 482 gallery samples, 248 necrotic pith and 65 transition zone samples were analyzed. A Chi-square test was carried out to evaluate the differences in the number of positive *F. circinatum* samples found in the gallery relative to the necrotic pith, since the transition zone did not contain a sufficient number of positive isolates for inclusion in the analysis.

To detect the presence of the pathogen on the insects' exoskeletons, 44 specimens found inside the shoots galleries were processed according to the method described above.

5.2.3- Presence of *F. circinatum* on *T. piniperda* in nature

In the periods comprising May-October 2012, February-September 2013, and May-August 2014, two sliding funnel traps (Econex) were baited with ethanol and alpha-pinene (Econex) in a plot affected by *F. circinatum* (Cabezón de la Sal, Cantabria). Captured specimens were collected weekly. In order to avoid catching beetle predators, mesh grids (5mm) were placed over the funnels (Martín *et al.*, 2013). To determine the presence of spores attached to the body of *T. piniperda*, the specimens were cultured on PDAS as described above. In addition, between February and September 2013, two additional sliding traps were baited with ethanol and alpha-pinene and placed in a disease-free plot of *P. sylvestris* located in San Miguel de Aguayo (Cantabria).

5.2.4- Transmission assay under controlled conditions

In March 2012, ten logs of *P. sylvestris* naturally attacked by *T. piniperda* and free of *F. circinatum* were collected in San Miguel de Aguayo (Cantabria). The logs (50 cm in length and 15 cm in diameter) were placed in emergence boxes in the laboratory to collect both reemerging parental and emerging F1 progeny. In order to confirm that the insects obtained from logs collected in the field were free of *F. circinatum*, reemerging parents (25 females and 40 males) and 50 young specimens (25 males and 25 females) that emerged from the *P. sylvestris* logs were analyzed for the presence of *F. circinatum*, as described above. The F1 insects emerging from the logs were experimentally infected with *F.*

circinatum for subsequent testing of the insect's ability to infect healthy *P. radiata* shoots. Thus, the insects were allowed to walk for different durations (1 min, 10 min, 30 min and 60 min) on PDAS plates completely covered by *F. circinatum* mycelium. The four Shoot+Insect+Mycelium (SIM) treatments tested were designated SIMa = 1´, SIMb = 10´, SIMc = 30´, SIMd = 60´. For these treatments, each insect was placed in a sterile glass jar (20.5 cm in high and 11 cm in diameter) with a current fresh *F. circinatum*-free shoot, of length 20 cm, to enable it to carry out its maturation feeding and infect the shoot with the pathogen. Twenty insects and 20 shoots were used for each treatment (Table 1). Prior to this experiment, a test was conducted to determine the optimal number of days required for the fungus to develop inside the shoots in which the insect had burrowed, by leaving the insects and shoots in the jars for 5, 10 and 20 days. The results confirmed that ten days was the optimum length of time for the insect to carry out maturation feeding and to transfer the pathogen without growth of saprophytic fungi.

Positive controls without insects, Shoot+Mycelium (SMC), were prepared by boring the shoots with a 5mm cork-borer and placing an agar disc with the pathogen in direct contact with the pith. Finally, a control treatment, Shoot+Insect (SIC), was established by placing *F. circinatum*-free insects in individual jars to feed on healthy shoots. Ten days after the beginning of each treatment, the length of the feeding gallery made by the insect and the length of the necrosis caused by the pathogen in the pith was recorded in each shoot. In the SMC treatment, the length of the necrotic pith was also measured. At the end of the trial, 25 % of the shoots (five shoots per treatment, 30 in total) were randomly selected for plating on PDAS, after superficial sterilization of the material, in order to isolate the pathogen. The above-described method was used to process 25 % of the insects.

Data from the inoculation assay were analyzed by a one way ANOVA to test the influence of the treatment on the gallery length and on the necrosis length, followed by a post-hoc analysis (Tukey's test).

All statistical analyses were carried out with SPSS software.

To confirm the effectiveness of the method of infecting the insects with the fungus, 40 young specimens of *T. piniperda* from the F1 generation (10 insects per treatment) were infected via contact with mycelium for four different : Mycelium+Insect (MI): MIa= 1´, MIb= 10´, MIc= 30´, MId=60´ and the processed samples were subsequently plated on *Fusarium* Selective Medium (FSM).

Fusarium circinatum colonies were identified morphologically by culture on SNA medium.

Moreover, to determine where the pathogen is harbored by the insect, twenty specimens of *T. piniperda* were inoculated. For this purpose, the insects walked on a plate with mycelium for two different durations (Treatments: A = 1 min, B = 10 min). The insect samples were then frozen at -20 °C and sent to the National Research Center on Human Evolution (Burgos, Spain) for processing by scanning electron microscopy to identify the most common sites of adhesion of the spores to the insect exoskeleton. The insect samples were coated with gold film (10 nm) and examined in a FEI Quanta 650 scanning electron microscope.

5.3- Results

The insect specimens were identified as *Tomicus piniperda* by detection of the restriction pattern of the

Table 1. Different experimental infection treatments with insects and/or shoots

Name	*Materials*	*N.*
SIMa	*Shoot+Insect+Mycelium 1min*	*20*
SIMb	*Shoot+Insect+Mycelium 10min*	*20*
SIMc	*Shoot+Insect+Mycelium 30min*	*20*
SIMd	*Shoot+Insect+Mycelium 60min*	*20*
SIC	*Shoot+Insect=Control*	*20*
MIa	*Insect+Mycelium 1min*	*10*
MIb	*Insect+Mycelium 10min*	*10*
MIc	*Insect+Mycelium 30min*	*10*
MId	*Insect+Mycelium 60min*	*10*
SMC	*Shoot+Mycelium (5 mm Ø)=Positive Control*	*20*

ITS2 region after enzymatic digestion with HincII, i.e. two bands of around 221 and 339 bp (Gallego and Galian, 2001).

5.3.1- Association between *T. piniperda* and diseased trees

In 2014, a total of 118 beetle specimens were collected between May and July from 10 trees with symptoms of PCD. *Fusarium circinatum* was isolated at all the development stages although the differences between stages were not statistically significant: 33 % of the parental adults, 15 % of the F1 adults, 13 % of the pupae and 15 % of the larvae. A total of 121 breeding galleries were sampled in 2014 from symptomatic trees, and 16 % of them gave rise to *F. circinatum* colonies when plated on PDAS. The *F. circinatum* structures were observed on all samples placed in wet chambers.

5.3.2- Association between *T. piniperda* and healthy trees

Fusarium circinatum was isolated from 12 % of the 571 shoots collected from the ground between April and December 2011. No *F. circinatum*-infected shoots were found in May or June, whereas maximum numbers were reached in November and December (21 % and 29 % respectively). Regarding the 383 shoots collected between January and June 2012, 10 % were positive for *F. circinatum*, with maximum values for the months of January and February (26 % and 8 % respectively), whereas no positive shoots were observed in May and June (Figure 2). The presence of *F. circinatum* was not significantly affected by season in which they were collected (df=3, X2=46.389, p=0.059), although most specimens were found in winter (64 out of 236, 27 %) followed by autumn (16 out of 236, 7 %) and spring (6 out of 74, 3 %). The presence of *F. circinatum* was not significantly influenced by the length of gallery length excavated by the insects (df=1, F=3.579, p=0.059). The mean length of the gallery was longer in shoots in which *F. circinatum* was detected (2.4 cm) than in shoots that tested negative for the pathogen (2 cm).

In the three different areas of the 200 shoots analyzed (484 gallery samples, 244 necrotic pith samples and 65 transition zone samples), 10 % of the galleries, 5 % of the cultivated necrotic pith and 1 % of the transition zones were positive for *F. circinatum*. The rate of infection was significantly different in the gallery and the necrotic pith (df=1, X2= 5.361, p= 0.021).

During the entire sampling period, a total of 44 insects were found inside the shoot feeding galleries. *Fusarium circinatum* was isolated from 1 specimen collected in November. During the winter period, the occupation rate (% of shoots occupied by an insect) of the

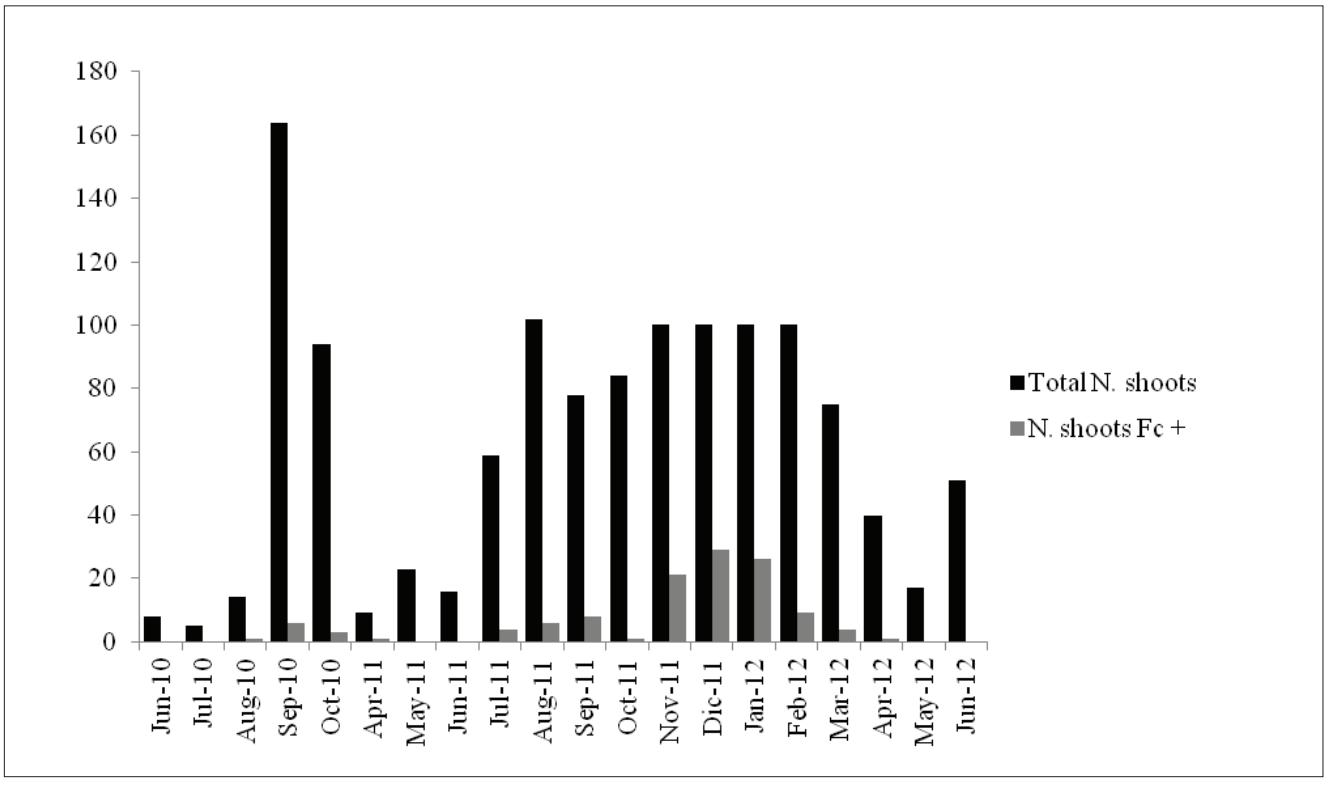

Figure 2. Percentage of *Pinus radiata* shoots collected in the field from which *Fusarium circinatum* was isolated at each sampling period.

galleries was 5 %, while in the summer it was 2 %, in the spring, 5 %, and in autumn, 6 %.

5.3.3- Presence of *F. circinatum* on *T. piniperda* in nature

The total numbers of individual *T. piniperda* collected from funnel traps were 6, 74 and 3 in respectively 2012, 2013 and 2014. Of the 74 specimens collected in 2013, 69 % were captured in March, 23 % in April, 8 % in May and 0 % in June. *Fusarium circinatum* was isolated from 2 of the insects captured in 2012, in May and August, and from 1 of those collected in May 2014. *Fusarium circinatum* was not isolated from any of the specimens captured in 2013. In the funnel traps placed in a *P. sylvestris* plot free of the disease in 2013, a total of 65 beetles were collected: 5 % in March, 90 % April, 5 % May and 0 % in June. None of these specimens carried the pathogen.

5.3.4- Transmission assay under controlled conditions

All re-emerged parents and offspring obtained from the *P. sylvestris* logs from San Miguel de Aguayo were free of *F. circinatum*. The rate of reisolation of *F. circinatum* from inoculated shoots and insects varied depending on the treatment. In the plant tissue in treatments SIMa, SIMb, SIMc and SIMd, *F. circinatum* was found in 60 %, 60 %, 40 % and 20 % of the shoots respectively, whereas in the SM treatment it was present in 100 % of the shoots. The pathogen was reisolated in 60 %, 60 %, 40 % and 40 % of the inoculated insects feeding on shoots in treatments SIMa, SIMb, SIMc and SIMd, respectively. However, the rate of reisolation was higher in the four treatments MIa, MIb, MIc and MId (80 %, 90 %, 60 % and 60 %, respectively).

In the inoculation assay, there were no significant differences between the treatments (SIMa, SIMb, SIMc, SIMd and SIC) in relation to gallery length (df=4, F=1.133, p=0.347) (Figure 3). However, there were significant differences between SIM treatments (SIMa, SIMb, SIMc and SIMd) and SM, but not among SIMa, SIMb, SIMc and SIMd treatments in relation to the length of the necrotic area (Figure 3).

The electron micrographs revealed the presence of *F. circinatum* structures on the insects' bodies in both inoculation treatments (A=1 min and B=10 min). Microconidia and phialides were clearly observed (Figures 4 and 5 respectively).

5.4- Discussion

This study aimed to confirm whether *T. piniperda* is a likely vector of *F. circinatum* in *P. radiata* plantations in northern Spain, on the basis of Leach's postulates (Leach, 1940). The hypothesis was confirmed by the following observations: an association between the insect and trees affected by pitch canker disease (postulate 1); an association between the insect and healthy *P. radiata* trees (postulate 2); the presence of the pathogen on the exoskeleton of *T. piniperda* specimens in nature (postulate 3); and the capacity of *T. piniperda* to transmit the disease to healthy host material under certain controlled conditions (postulate 4).

The association between *T. piniperda* and *P. radiata* trees affected by pitch canker disease was observed during field sampling in 2014. Breeding galleries (and the insects they contained) collected from symptomatic trees were positive for *F. circinatum*. This may indicate that the insect was already infected with the pathogen when excavating the breeding gallery or that the bark where the insects made the galleries was already infected with *F. circinatum*. The probability of *T. piniperda* being contaminated with the pathogen would be increased by the insects excavating their breeding galleries in diseased trees. Microscopic examination (of samples in wet chambers) revealed *F. circinatum* structures growing in the breeding galleries, suggesting that breeding galleries provide suitable conditions for fungal fruiting.

Regarding the second postulate, the association between *T. piniperda* and healthy crowns of pine trees has been intensively studied by several authors (Långström, 1982; Lieutier *et al.*, 2015). In this study, 11 % of the 957 fresh shoots collected from green crowns were positive for *F. circinatum*, and the fungus appeared in a higher proportion inside the feeding galleries than in the surrounding areas. This may indicate the role of *T. piniperda* as a wounding agent, as *F. circinatum* requires a wound to penetrate and infect the tree (Dwinell *et al.*, 1985). However, the finding may also indicate that *T. piniperda* infects the shoots with the pathogen during feeding on shoots. The fact that *T. piniperda* attacks symptomless green crowns in plots affected by pitch canker may increase the incidence of the disease.

The season in which the samples were collected did not affect the presence of *F. circinatum* inside *T. piniperda* feeding galleries on fresh green shoots, although November, December and January were the months with more isolation rates. Gallery length did not signifi-

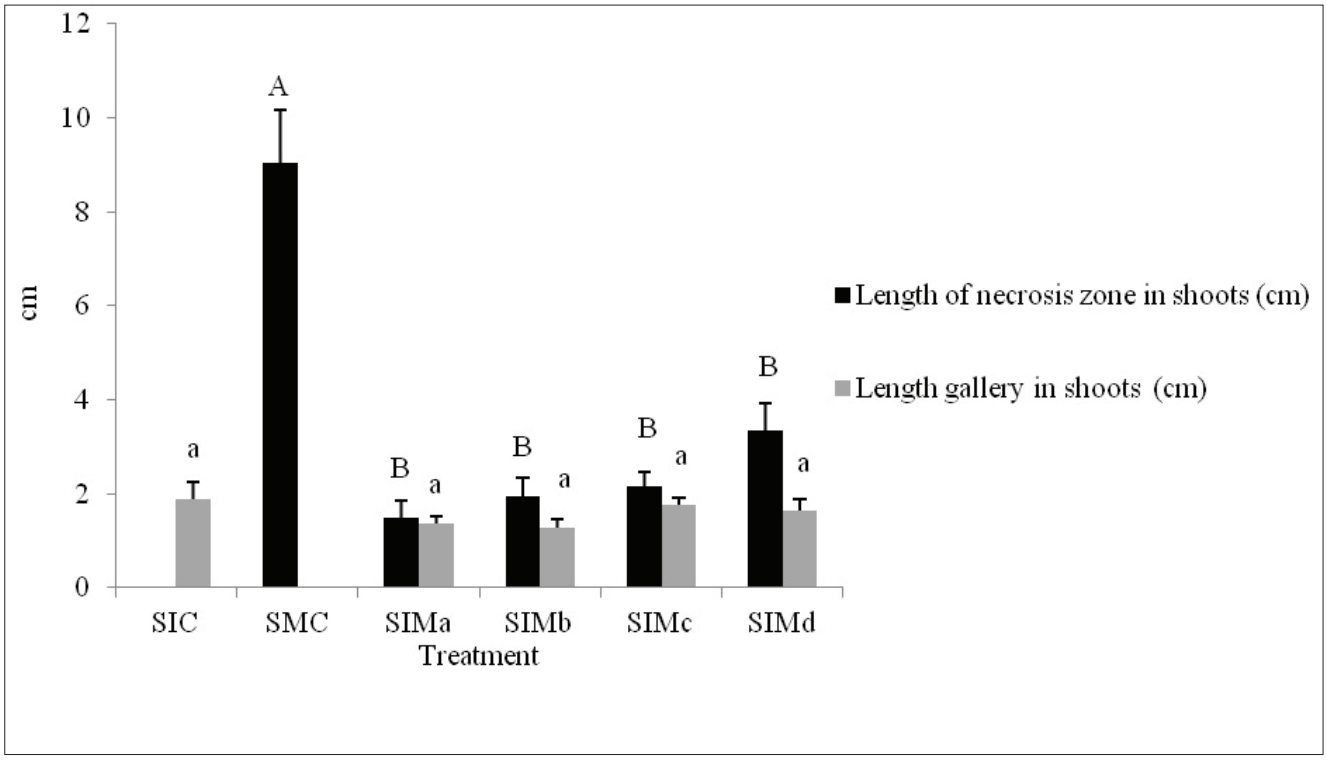

Figure 3. Mean length of necrotic zone and mean length of the gallery by the insects in the shoots in the experimental infection assay. Different letters represent significant differences (capital letters indicate length of necrotic zone and lower case letters indicate gallery length). Error bars represent standard error.

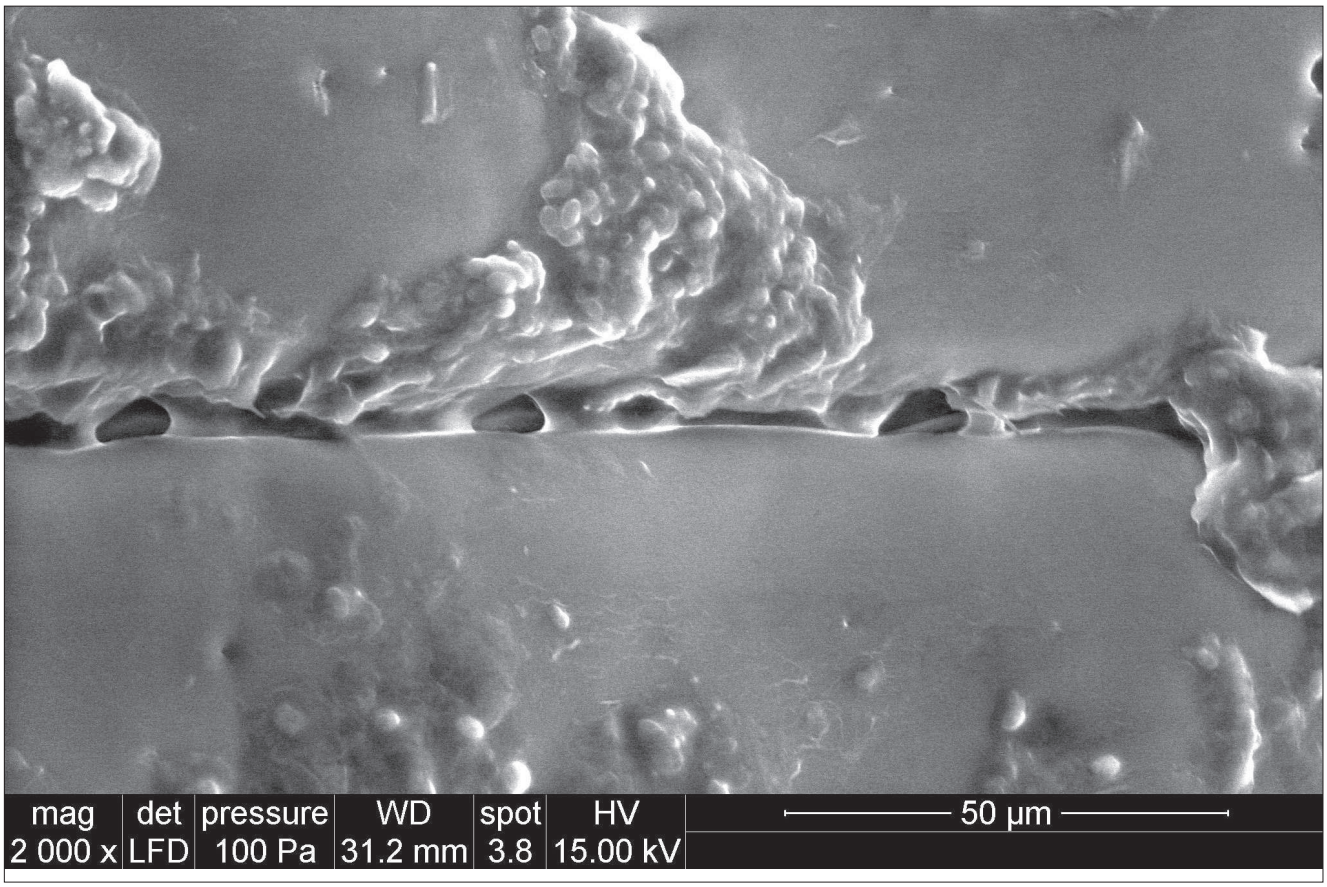

Figure 4. *Tomicus piniperda* elytra with microconidia of *Fusarium circinatum.*

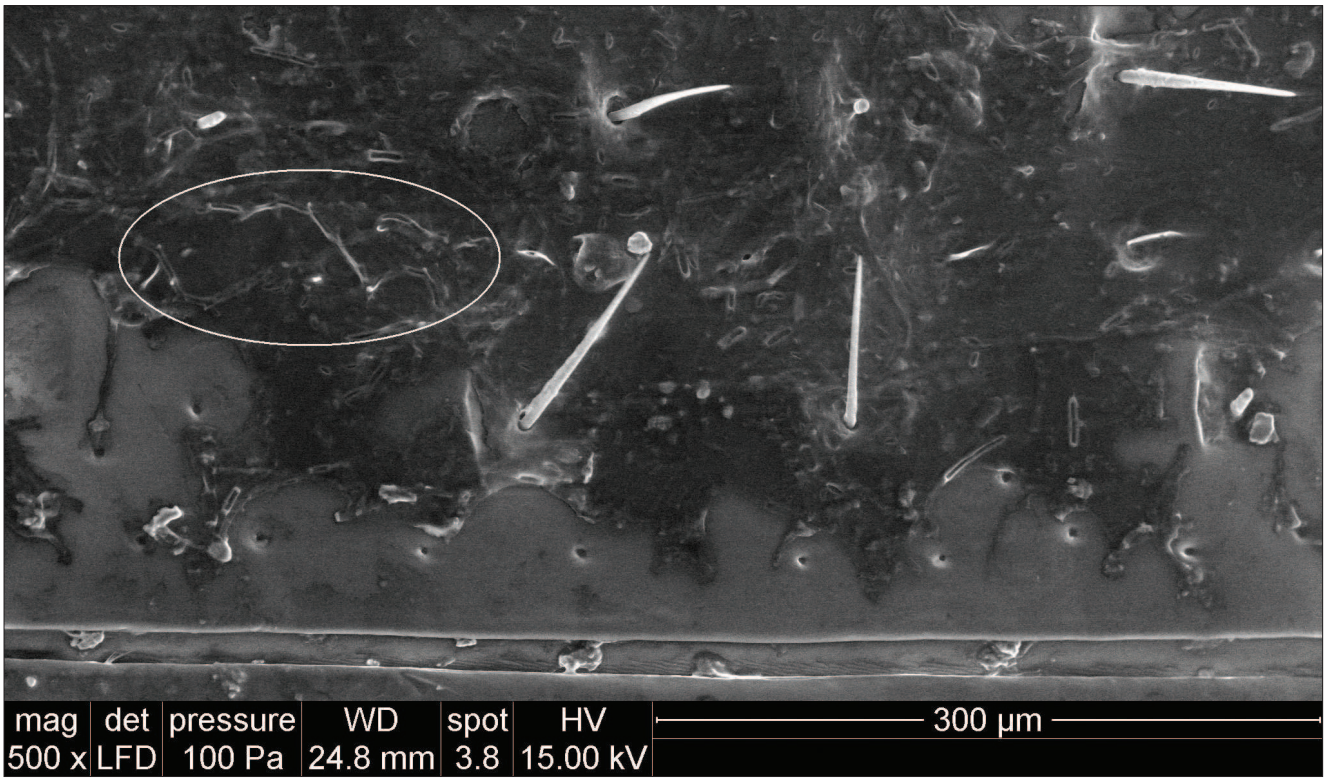

Figure 5. *Tomicus piniperda* elytra with phialides of *Fusarium circinatum.*

cantly influence the presence of the pathogen, although it is related to the time that the insect spends inside the shoot. This sampling was carried out as a first approach to determine the factors influencing the distribution of *F. circinatum* on the feeding galleries of *T. piniperda* on healthy crowns.

The presence of the pathogen on the insect in nature was tested by collecting insects during their dispersion flight in plots affected by PCD. The *T. piniperda* population was found to carry an inoculum of *F. circinatum* during its dispersion flight. The observed phoresy rates obtained in this work, i.e. 4 % in three years, are consistent with those determined by Whitehill *et al.* (2007) in a study of the role of *Ips pini* as a vector of *Sphaeropsis sapinea.* However, it is low in comparison with the rates observed in other bark beetle-pathogenic fungi systems, e.g. in a study carried out in California, 17 % of *Pityophthorus* spp. were found to be carrying *F. circinatum* (McNee *et al.*, 2002), and *Ips sexdentatus* was associated with *Ophiostoma ips* in Romón *et al.* (2007b). *Tomicus piniperda* is known to be associated with other species of pathogenic fungi worldwide (Jacobs *et al.*, 2004; Kirisits, 2004), e.g. *Ophiostoma minus,* which appears at a very low and variable frequency. Lieutier *et al.* (2009) described this bark beetle as being capable of exhausting tree defenses but very loosely associated with fungi.

In this study, we demonstrated the ability of the insect to infect the shoots with the pathogen during shoot maturation feeding under certain conditions. The presence of the pathogen was confirmed in a high percentage of the shoots from the mycelium inoculation assay (60 %-20 %). These results are not consistent with those obtained during the field sampling. This may be related to the method of inoculation, highlighting the need to determine how the insect is loaded with *F. circinatum* spores in nature. The insect may become contaminated with spores from zones of the tree where the humidity and temperature conditions are suitable for the mycelium and conidiophores, although it is not known how many spores are harboured on the insect exoskeleton. The low phoresy rates observed in the field sampling relative to the experimentally established rates may be explained by the fact that insect flight did not take place in the laboratory experiment. In the laboratory study, the length of time that the insect was in contact with the mycelium did not influence either the length of necrosis on the shoot or the gallery length.

The scanning electron micrographs showed microconidia on the elytra (Figure 4), whereas the absence of

macroconidia was due to the inoculation methodology, as these spores do not grow easily on PDA. Moreover, other *F. circinatum* structures were observed on the insects' exoskeletons, such as the phyalides, the structures that produce microconidia (Figure 5). Although *T. piniperda* does not have specific structures for transporting fungus like mycangia (Paine *et al.*, 1997), the spores may be stored in other body locations, as in other bark beetles. For example, *Hypothenemus hampei* transports *Fusarium solani* spores at the base of its asperites (Morales-Ramos *et al.*, 2000). No regular distribution pattern of *F. circinatum* spores on the *T. piniperda* exoskeleton was observed. This may be due to the fact that insects were artificially inoculated, what could have condition the distribution of the fungi on the beetle. Moreover, in this inoculation assay insects did not take flight before feeding on the shoot (or before SEM). In nature, maturation flight may change the distribution pattern of the spores on the insects' exoskeletons, or even lead to loss of spores, before reaching the shoots.

Previous studies have reported the presence of several bark beetle species in plots affected by pitch canker, e.g. *I. sexdentatus*, *O. erosus*, *P. pubescens*, *H. palliatus* and *H. attenuatus* (Bezos *et al.*, 2013; Romón *et al.*, 2008). The fact that *T. piniperda* performs its maturation feeding on healthy shoots (Långström, 1982) may be critical for the success of this insect species as a potentially important vector of this disease compared with other secondary bark beetles species reported in northern Spain as insect vectors of *F. circinatum* (Romón *et al.*, 2007a). Bark beetle species such as *I. sexdentatus* and *P. pubescens* are phoretically associated with *F. circinatum* according to these authors; however, they do not attack healthy trees at endemic state.

The behaviour of *T. piniperda* throughout its life cycle may enable dispersion of *F. circinatum* (Figure 6). The tree that the insects colonize for breeding may be affected by pitch canker and consequently be loaded with the fungal spores or mycelium growing under the bark of the diseased tree, in the same way as occurs with Dutch elm disease (Webber, 2008). After the breeding period, both reemerging parents (P) and the young emerging adults (F1) fly to the crowns of healthy pines for regeneration and maturation feeding, respectively. The insects would thus transfer the fungus to healthy current shoots, enabling it to grow inside the feeding gallery and along the pith. As the insects feed on several shoots (Kirkendall *et al.*, 2008), they can thus spread the disease along the tree crown. Moreover, the conditions inside the shoot may be suitable for growth of the pathogen, which may then reinfect the insects before they leave to feed on another healthy shoot. After the maturation feeding, the F1 individuals overwinter inside the shoots while the fungal structures are growing inside the gallery. After regeneration feeding, the reemerging parents fly to new sister brood establishments and can disperse the fungus to other non-affected trees and return to the crowns of healthy trees for regeneration feeding once again. This is supported by the fact that reemerging parents were found inside the feeding shoots during March, April and May (in 2011 and 2012).

The study findings indicate that *T. piniperda* is probably a vector of *F. circinatum*, according to Leach´s postulates (Leach, 1940). The presence of *F. circinatum* in the galleries and on the bodies of the insects throughout the lifecycle shows that *T. piniperda* may transport the pathogen and later introduce it into healthy trees, under the bark and inside the shoots. The shoots are most likely to become infected with the pathogen during maturation feeding and overwintering. Here we describe, for the first time, the cycle relating *F. circinatum* and *T. piniperda*; however, further studies are required for a better understanding of the relationship between the life cycles of the insect and the pathogen.

Acknowledgments

This research was financially supported by the Spanish Ministry of the Environment and Rural and Marine Affairs project 'Etiology, Epidemiology and Control of *Fusarium circinatum*' and by Cantabria Government project 'Control of pitch canker of pines in Cantabria region'. We want to deeply thank Milagros de Vallejo, Juan Blanco and the technical staff at the 'Dirección General de Montes y Conservación de la Naturaleza (Government of Cantabria)' for their invaluable help in the field work. We thank Silvia Gonzalez, from the National Research Center on Human Evolution (Burgos, Spain), for her work in the SEM pictures. Silvia Bezos is also acknowledged for her accurate work in the cycle drawing. Fernando M. Alves-Santos is thankful for the help with the insect identification. Sarah Kathryn Fox, Nathan Brenville and Christine Francis are acknowledged for the manuscripts revisions.

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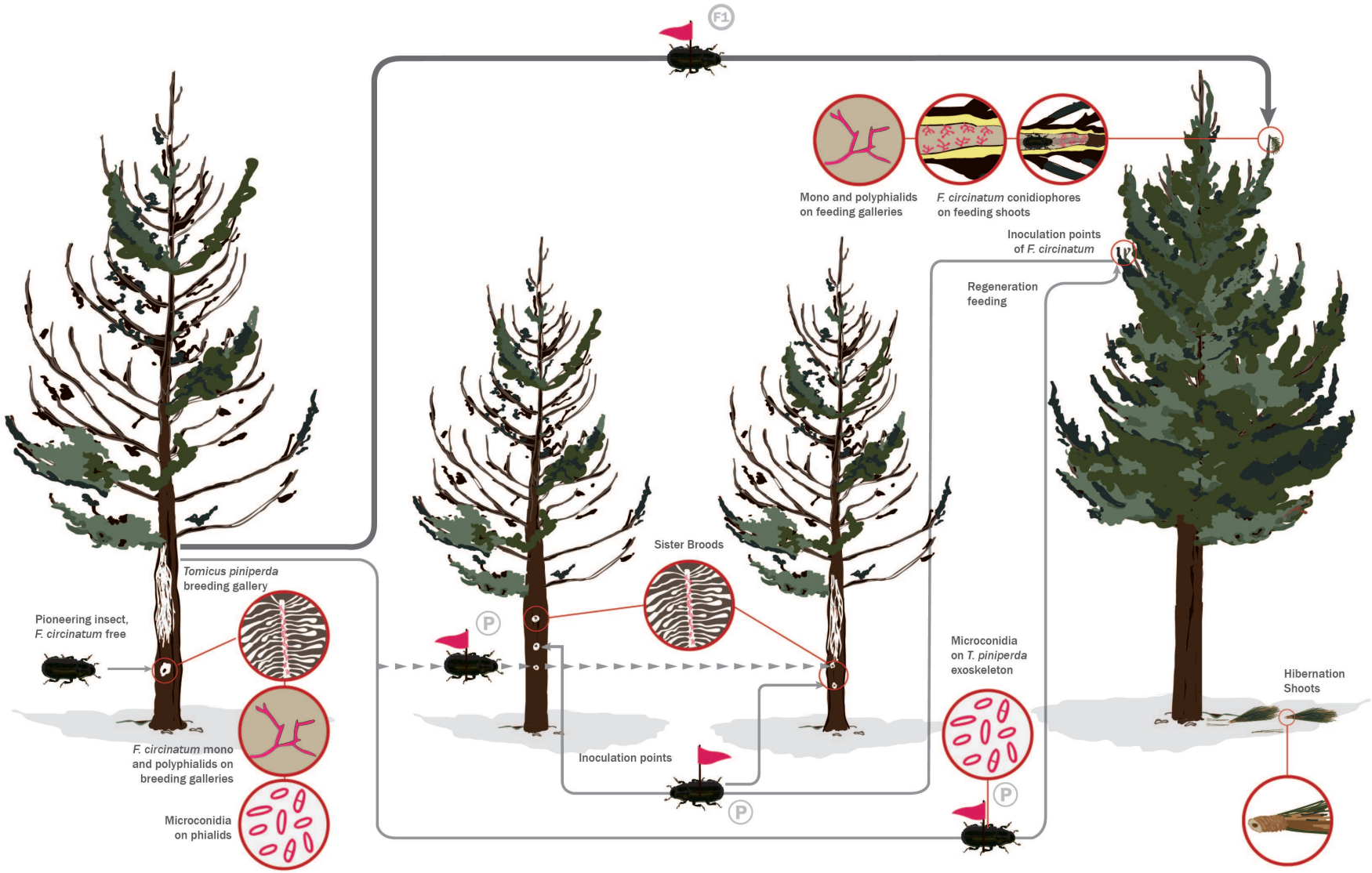

Figure 6. Dispersion of pitch canker disease during the *Tomicus piniperda* life cycle. Flags indicate individuals that are conceivably carrying *Fusarium circinatum* spores. P: parental adults, F1: young adults. Thick dark grey line: F1 maturation flight. Thin light grey line: P regeneration flight to crowns, followed by dispersion flight; dashed light grey line: P dispersion.

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Chapter 6: Association levels between *Pityophthorus pubescens* and *Fusarium circinatum* in PCD affected plantations in northern Spain

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Abstract

Fusarium circinatum, the causal agent of pitch canker disease (PCD), is a threat to *Pinus radiata* plantations due to the high susceptibility of this pine species. The main symptom of this disease is the presence of bleeding cankers that can cause the tree to die. This pathogen has been reported to be phoretically associated with several bark beetle species worldwide, specifically, *Pityophthorus* spp. (Coleoptera; Scolytinae) has been described as vectors in California. *Pityophthorus pubescens* is a secondary pest, attacking weak trees or broken branches in healthy trees. The aim of this study was to know the association between *P. pubescens* and *F. circinatum* in PCD affected plantations in northern Spain. Three specific aims were determined: i) to assess the phoretic association of *P. pubescens* with the pitch canker pathogen, ii) to study the presence of *F. circinatum* in *P. pubescens* infested twigs and iii) to evaluate whether PCD damages were enhanced in (E)-pityol baited *P. radiata* trees. For these purposes, funnel traps baited with (E)-pityol were established and twigs from infested trees were sampled to collect both, insects and plant tissues in pitch canker affected plots. Insects and vegetal tissues were plated on culture media with the aim of isolating *F. circinatum*. Moreover, an experiment was carried out under natural conditions in which *P. radiata* trees were baited with (E)-pityol and PCD symptoms were evaluated 4 times during one year. A total of 263 specimens were collected from funnel traps between June and September 2010, 2011, 2012 and 2013. Moreover, 215 specimens were collected from 424 galleries within the twigs in 2012 and 2013. The pathogen appeared on 1 % and 2 % of the collected insects in the funnel traps during 2010 and 2012 respectively. In the collected twigs, *F. circinatum* was found in 3 galleries, whilst results of the baiting experimentation showed symptoms in the crown were more influenced by (E)-pityol than symptoms on the trunk. This work afirms a weak association of *P. pubescens* with *F. circinatum* in our study area.

Resumen

Fusarium circinatum es el hongo causante de la enfermedad del chancro resinoso de los pinos y supone actualmente una amenaza para las plantaciones de *Pinus radiata* debido a la alta susceptibilidad de esta especie. El principal síntoma de esta enfermedad es la presencia de chancros resinosos en el tronco que pueden causar la muerte del árbol. Varias especies de escolítidos se han descrito como vectores de este patógeno, como es el caso de las especies del género *Pityophthorus* (Coleptera; Scolotinae) en California. *Pityophthorus pubescens* es una plaga secundaria que ataca a árboles debilitados o ramas rotas de árboles sanos. El objetivo de este trabajo es estudiar la asociación entre *P. pubescens* y *F. circi-*

natum en plantaciones afectadas por la enfermedad del chancro resinoso en el norte de España. Se establecieron tres objetivos específicos: i) estudiar la asociación forética entre *P. pubescens* y *F. circinatum*, ii) analizar la presencia de *F. circinatum* en ramillas atacadas por *P. pubescens*, iii) evaluar los daños causados por la enfermedad del chancro resinoso en árboles cebados con (E)-pityol. Para ello, se recogieron insectos mediante trampas multiembudo cebadas con (E)-pityol y ramillas atacadas por el insecto en parcelas afectadas por la enfermedad. Tanto los insectos como el material vegetal recogidos se cultivaron en medio patata dextrosa agar con el objetivo de aislar *F. circinatum*. Además se llevó a cabo un experimento en campo, en el cual se cebaron árboles afectados por la enfermedad con (E)-pityol y se evaluaron los daños causados en cuatro momentos diferentes a lo largo de un año. Se capturaron un total de 263 especímenes en las trampas multiembudo durante los meses de junio a septiembre de 2010, 2011, 2012 y 2013. Además, se recogieron 215 especímenes de un total de 424 galerías en el interior de las ramillas en 2012 y 2013. El patógeno fue aislado del 1 % y 2 % de los insectos durante los años 2010 y 2012, respectivamente. En relación con las ramillas recogidas, *F. circinatum* se aisló en tres galerías. Los resultados del experimento con árboles cebados mostraron una mayor influencia del atrayente sobre los síntomas de la copa que sobre los del tronco. Este trabajo muestra una asociación leve entre *P. pubescens* y *F. circinatum* en nuestra área de estudio.

6.1- Introduction

Fusarium circinatum Nirenberg and O´Donnell is an ascomycete fungus causing pitch canker disease (PCD) on pines (Nirenberg and O'Donnell, 1998). The main symptom of this disease in adult trees is the presence of pitch soaked cankers which girdle both trees and branches (Wikler *et al.*, 2003). Trickles of resin can also be found on the trunks of diseased trees. The disease can affect the crown when suitable wounds are available for infection (Gordon *et al.*, 2001), causing dieback that can lead to tree death, since a single infection on small diameter branches may be sufficient to cause the death of the branch (Gordon, 2011). This pathogen was first reported in North Carolina (Hepting and Roth, 1946) but has also been observed in Haiti (Hepting and Roth, 1953), California (McCain *et al.*, 1987), Japan (Muramoto and Dwinell, 1990), South Africa (Viljoen *et al.*, 1994), Mexico (Guerra-Santos, 1998), Chile (Wingfield *et al.*, 2002), Korea (Cho and Shin, 2004), France (EPPO, 2004), Spain (Landeras *et al.*, 2005), Italy (Carlucci *et al.*, 2007), Uruguay (Alonso and Bettucci, 2009), Portugal (Bragança *et al.*, 2009), Colombia (Steenkamp *et al.*, 2012) and more recently in Brazil (Pfenning *et al.*, 2014). Pitch canker disease poses a threat to pine plantations and natural stands throughout the world (Wingfield *et al.*, 2008), especially *Pinus radiata* D. Don plantations due to the high susceptibility of this pine species (Viljoen *et al.*, 1995). However other *Pinus* species like *Pinus pinaster* Ait. and *Pinus sylvestris* L. (Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007) as well as *Pseudotsuga menziesii* (Gordon *et al.*, 1996) are susceptible to the pathogen.

Fusarium circinatum has been reported to be phoretically associated to several bark beetles species in *P. radiata* plantations in northern Spain, e.g. *Pityophthorus pubescens* (Marsham), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Boerner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* Erichson, *Orthotomicus erosus* (Wollaston) (Romón *et al.*, 2007) and *Tomicus piniperda* L. (Bezos *et al.*, 2013). This association has also been observed in other PCD affected areas, for example, California, where the importance of *Pityophthorus* spp. as the main vectors of *F. circinatum* has been demonstrated (Sakamoto *et al.*, 2007). Bonello *et al.* (2001) reported the ability of *Pityophthorus* spp. to discriminate between healthy and pitch canker diseased branches, preferring symptomatic branches due to the increasing ethylene emission. *Pityophthorus* spp. present different phoretic rates (% of insects carrying the pathogen) depending on the species and on the type of plant tissue (symptomatic or asymptomatic). For example, Romón *et al.* (2007) isolated *F. circinatum* from the 25 % of the *P. pubescens* specimens collected in Spain, but in California the association rates of *Pityophthorus* spp. with the PCD pathogen ranged between 0 % in asymptomatic branches to 17 % in the symptomatic ones (McNee *et al.*, 2002).

Pityophthorus species are phloeophagous and myelophagous species (Vega and Hofstetter, 2015). The presence of this insect species on the attacked crowns can be inferred by the presence of reddish twigs, as they construct their galleries in the phloem or in the pith of small branches of the host tree (Sakamoto *et al.*, 2007). Most species on this genus are secondary species with a low economic impact (Vega and Hofstetter, 2015). This is the case with *P. pubescens* which is pres-

ent in the Iberian Peninsula attacking weakened trees (López *et al.*, 2007). This bark beetle species is widely distributed in Europe, living in several *Pinus* species such as *Pinus pinea* L., *P. pinaster* and *P. radiata* (Gil and Pajares, 1986).

The main objective of this work was to evaluate the association of *P. pubescens* with *F. circinatum* in PCD affected stands in Cantabria (northern Spain). For this purpose three specific aims were established: i) to assess the phoretic association of *P. pubescens* with the pitch canker pathogen, ii) to study the presence of *F. circinatum* in *P. pubescens* infested red twigs and iii) to evaluate whether PCD damages were enhanced in (E)-pityol baited *P. radiata* trees in natural conditions.

6.2- Materials and Methods

The present work consisted of three samplings: one in which *P. pubescens* specimens were collected with funnel traps baited with pityol, another in which both insects and galleries were collected from *P. pubescens* infested *P. radiata* twigs and, finally, a PCD damages evaluation was carried out in a *P. radiata* stand regarding the presence or absence of pityol.

6.2.1- Funnel traps sampling

Funnel traps baited with (E)-pityol (Econex) were established in a *P. radiata* plot affected by *F. circinatum* in Vejorís (Cantabria, northern Spain) from June to September 2010, 2011, 2012 and 2013 and a total of 263 beetles were collected. Insects collected during 2010 were plated on *Fusarium* selective media (FSM: 15 g bactone peptone, 1 g KH2PO4 monobasic, 0.5 g MgSO4-7H2O, 20 g agar, 0.2 g of pentachloronitrobenzene (PCNB) and 0.3 g streptomycin sulfate per 1 L of distilled water). However, insects collected in 2011, 2012 and 2013 were plated on potato dextrose agar (PDA Scharlau) modified by adding 0.6 g of streptomycin sulfate (Fluka Analytical) per 39 g of PDA (PDAS) media in accordance with the methodology described by Ambourn *et al.* (2006) with some modifications. Hence, each insect was introduced into a 1.5 ml micro tube with 200 µl of Tween 80 1% and sonicated for 5 seconds and 100 µl of the solution was plated in medium PDAS and extended on the media plates with a sterile loop.

Fusarium circinatum colonies were identified by their morphology as described by Leslie and Summerell (2006). The mycelium was cultured on SNA (Synthetischer Nährstoffärmer Agar) and the typical structures of this fungus (i.e. oval microconidia, mono and polyphialides, coiled sterile hyphae and absence of clamidiospores) were observed.

6.2.2- Isolation of *F. circinatum* from red twigs

Twigs with galleries of *P. pubescens* were sampled in a pitch canker affected plot of *P. radiata* located in Cantabria (Spain), in order to collect both insects and plant tissues. Thus, 215 specimens were collected from 424 galleries within the twigs in 2012 and 2013. With the aim of isolating *F. circinatum*, insects and plant tissues were plated on PDAS culture media as described above.

6.2.3- Evaluation of baited trees in natural conditions

The aim of this experiment was to assess whether the addition of (E)-pityol, and consequently the increased presence of *P. pubescens*, affected the incidence of the PCD in a *P. radiata* stand in nature. For this purpose one *P. radiata* stand affected by *F. circinatum* located in Vejoris (Cantabria) was selected and six plots were evaluated; three of them were baited with the attractant (BP1, BP2 and BP3) and three of them were established as control treatment (BC1, BC2 and BC3). Each plot consisted of 24 trees with a capsule of (E)-pityol in BP1, BP2 and BP3 with at least 50 m space between them. Thus, a total of 144 trees were measured and evaluated, considering dendrometric and forest health variables. Specifically, regarding the dendrometric variables, tree diameter and total height were measured at the beginning of the sampling in July 2012 whilst the forest health variables included presence or absence of cankers, presence of trickles of resin outside the cankers, presence of red shoots in the crown dieback (Figure 1), percentage of defoliation and tree mortality. These variables were also measured at the beginning of the sampling in July 2012 (time=0) and subsequently in September 2012 (time=3 months), February 2013 (time=6 months) and August 2013 (time=13 months).

The selected trees had an average of 35.1 cm in diameter and 24.5 m height in baited points and 33.2 cm in diameter and 24.6 m height in unbaited points. The mean temperature, relative humidity (RH), and mm of total precipitation were 19.2 ºC, 76.7 % and 22 mm respectively in summer 2012; 11ºC, 73 % and 102 mm in autumn 2012; 8.9ºC, 75.3% and 256.9 mm in winter 2013 and 19.9ºC, 77.5 % and 20.7 mm in summer 2013 (www.airecantabria.com).

Figure 1. Pitch canker disease symptoms evaluated on pityol baited trees under natural conditions: a) canker, b) trickles of resin, c) red shoots, d) dieback.

6.2.4- Statistical analyses

With the aim of knowing the health conditions of the selected trees at the beginning of the sampling, Pearson's Chi-squared test with Yates' continuity correction was performed to test whether significant differences occurred at time=0 between baited and unbaited trees regarding presence and absence of cankers, trickles of resin, red shoots, dieback and tree death. The Chi square test was also used for testing the differences in the number of trees affected by the pitch canker symptoms (cankers, trickles of resin, red shoots, dieback and tree death) depending on the treatment (control/pityol) for the subsequent observations (time=3,time=6, time=13).

Due to the fact that the defoliation data did not meet the normality and homoscedasticity assumptions, robust methods were applied (García, 2010). Hence, the Mann Whitney test was run to asses the differences in defoliation between treatments at time=0, time=3, time=6 and time=13.

Since the probability of occurrence of a symptom (P) at a determinate time could not only depend on the presence of the attractant but also on the previous tree health conditions, a multinomial logit model was performed. In order to check how the treatment (pityol/control) and the health conditions of the trees during the process (time=0, time=3 and time=6) affected the forest health variables at the end of the sampling (time=13), the variables at different observation times were included in this model.

In order to check the effect of tree vigour on the PCD symptoms, dendrometric variables (diameter and height) were also included in the multinomial analyses, but this time the choices of variables were: the increment on the presence of cankers, resin trickles, red shoots, dieback, defoliation and tree death from the beginning of the observations (time=0) to the end (time=13).

All statistical analyses were performed in the R software environment (R Core Team, 2013).

6.3- Results

6.3.1- Phoretic association of *F. circinatum* with *P. pubescens*

From the 97 insects collected from the funnel traps in 2010 (49 in July, 38 in August and 11 in September) only one of them collected in August tested positive for *F. circinatum*, corresponding to 1.05 % of the insects collected during this year. A total of 43 individuals were collected in 2011 (22 in June, 9 in July, 10 in August, 2 in September), and of them no specimen tested positive for the pathogen. Regarding the sampling in 2012, a total of 98 insects were collected (66 in June, 27 in July and 5 in August) and two specimens tested positive for *F. circinatum* in June, corresponding to 2.04 % of the insects collected. In 2013, 25 individuals of *P. pubescens* were collected (8 in June, 13 in July, 3 in August and 1 in September) and no isolates of *F. circinatum* were obtained in this case (Table 1).

Regarding the insects collected within the red twigs, in 2012 a total of 197 *P. pubescens* specimens were collected (100 in July and 97 in August), whereas in 2013, 18 insects were found within the galleries (1 in June and 17 in July). *F. circinatum* was not found on these insects' exoskeleton (Table 1).

6.3.2- Isolation of *F. circinatum* from red twigs

Regarding the 400 galleries collected from red twigs in 2012, 200 belonged to the pith (100 collected in July and 100 in August) and 200 belonged to the twig bark (100 collected in July and 100 in August). *Fusarium circinatum* was isolated from 3 galleries collected in the month of July, two of them were burrowed in the twig bark and one was burrowed in the pith, correspond-

Table 1. Phoresy rates during the sampling period in *Pityophthorus pubescens* specimens collected from funnel traps and red twigs on *Pinus radiata* plots affected by *Fusarium circinatum.*

	Insects from funnel traps	*Insects from red twigs*
Year	*Phoresy %(n)*	*Phoresy %(n)*
2010	*1.05 (97)*	-
2011	*0 (43)*	-
2012	*2.04 (98)*	*0 (197)*
2013	*0 (25)*	*0 (18)*
Total	*2.14 (263)*	*0 (215)*

ing to 0.75 % of the collected samples. In 2013, a total of 24 galleries were collected but no isolate of *F. circinatum* was obtained from them.

6.3.3- Evaluation of baited trees in natural conditions

The number of trees affected by each one of the pitch canker symptoms mentioned above and the mean percentage of defoliation in baited (pityol) and unbaited (control) trees at the different observation times is represented in Figure 2.

The Chi square test indicated that there were no significant differences on the evaluated variables between pityol and control treatments at the beginning of the observation (time=0). Thus, the presence of cankers in the baited points did not differ significantly from the control treatment points (p=0.26). Likewise, the presence of trickles of resin on the trunk was not significantly different (p=0.42). Regarding the forest health variables affecting the tree crown (presence of red shoots, dieback and defoliation) no significant differences were found between treatments (p=0.83; p=0.71 and p=0.49, respectively). Regarding the symptoms at time=3, the Chi-square test showed no significant differences between baited and unbaited trees when the symptoms present on the trunk were analyzed: cankers (p=0.21) and trickles of resin (p=0.39). In regard to those symptoms present in the crown, significant differences were found in the presence of red shoots (p=0.02) and dieback (p<0.01), the number of trees with symptomatic crowns in the baited points being higher, but not the defoliation levels (p=0.55). At the observation time =6, trickles of resin and dieback appeared in a significantly higher number of trees in baited trees than in unbaited ones (p=0.05 and p<0.01, respectively). The rest of the studied variables were not found to be significantly different between pityol and control treatments. At the observation time =13, the number of trees with trickles of resin and dieback continued to be significantly higher in pityol than in control points (p=0.04 and p=0.02, respectively). The rest of the studied variables were not found to be significantly different between pityol and control treatments. Moreover, the number of dead trees between baited and unbaited trees was not significantly different at any observation time: time=0, time=3, time=6 and time=13.

The multinomial logit model showed that red shoots at time=13 were significantly influenced by the presence of red shoots at time=3 (p<0.01; P = 93.7 %), but not by the treatment (pityol or control). Regarding the dieback at time=13, the multinomial logit model showed that it was significantly affected by the presence of dieback at time=0 (p< 0.01; P=52.5 %) and at time=6 (p<0.01; P=47.5 %), but the presence of the attractant did not influence the process during the complete observation period (p=0.37). The presence of trickles of resin at the end of the observation period (time=13) was significantly affected by the trickles of resin present at time=6 (p=0.01; P=59.9 %) and by the presence of pityol throughout the complete observation period (p=0.02; P=35.6 %). As the multinomial logit model showed, the rest of the variables (number of cankers, defoliation and mortality) were not significantly influenced at the end of the observation (time=13) by the presence or absence of pityol, nor by the forest health conditions observed in previous evaluations. Finally, tree height and diameter did not significantly alter the increment in the presence of the evaluated variables.

6.4- Discussion

Regarding the phoretical association between *P. pubescens* and *F. circinatum* in the present study, it ranged between 0 % and 2.04 %. These results are con-

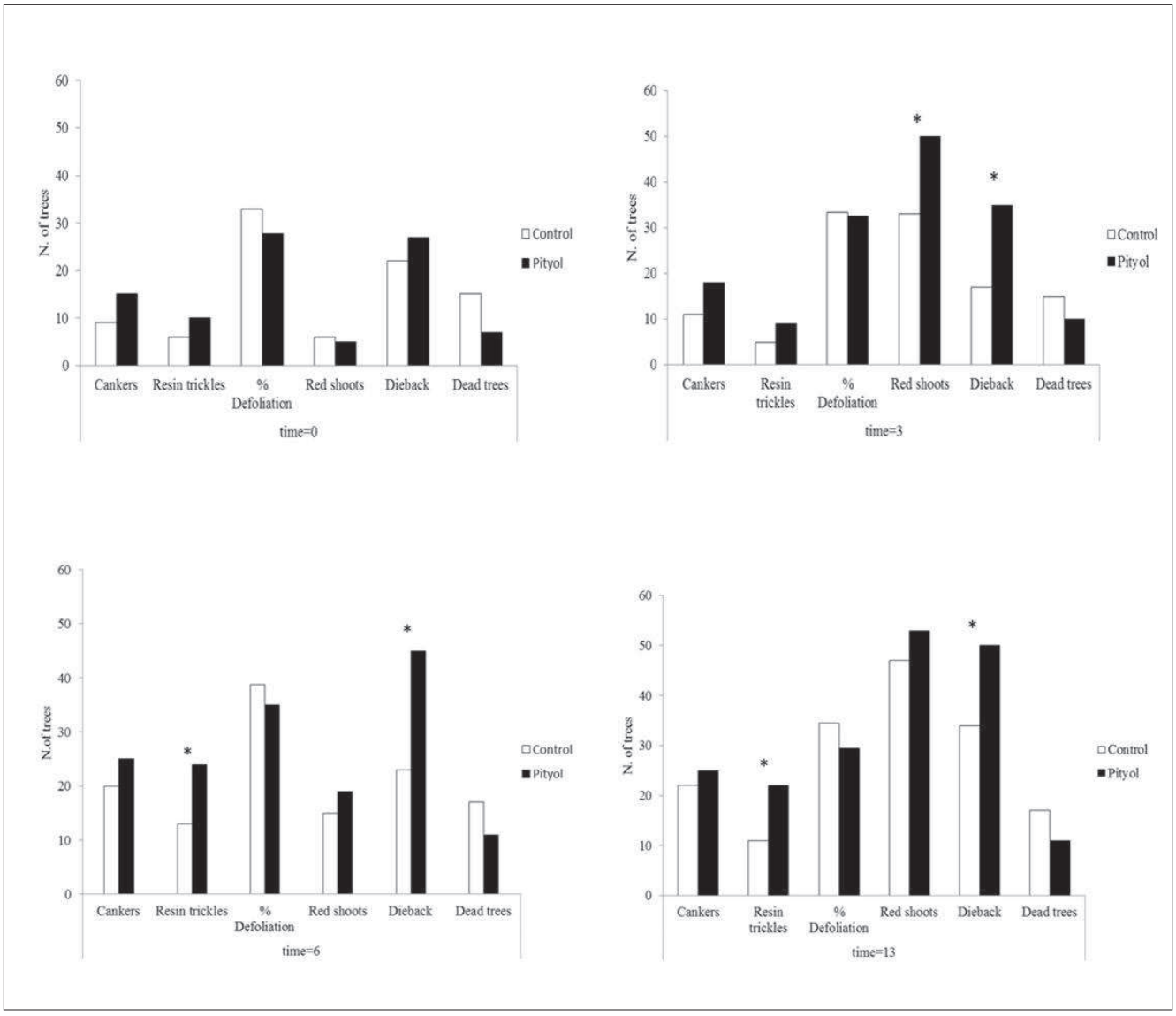

Figure 2. Number of trees affected by cankers, resin trickles, red shoots, dieback and dead and mean value of defoliation in the pityol baited and unbaited trees for the different observation times. * indicate significant differences between treatments.

sistent with those obtained for other *Pityophthorus* species as *F. circinatum* phoretic agents, for example, the work carried out by Erbilgin *et al.* (2005) in which 0 % of the specimens carried the pathogen or those obtained by Dallara (1997) in which 2.5 % of the analyzed insects carried the pathogen. However, phoresy rates may vary depending on two factors: the insect species; for example, *F. circinatum* was found on 0-13.69 % of *P. carmeli* while it appeared between 0-2 % of the *P. setosus* in Monterey Peninsula in California (Storer *et al.*, 2004), and on relative humidity (RH). Hence, Sakamoto *et al.* (2007) found that the efficiency of *Pityophthorus* species in *F. circinatum* infection is affected by RH, thus more pitch canker lesions per tree appeared when plants were in contact with *P. setosus* at a 100% of RH than when kept at ambient RH of 49-82 %. In our study area, the RH ranged between 72-77 % during summer 2012 and summer 2013.

Regarding the presence of *F. circinatum* on the insects' exoskeletons when captured within the galleries, no isolate of *F. circinatum* was obtained. However, the pathogen was isolated from 0.75 % of the galleries burrowed by the insects. Vegetal tissue from the red twigs found in our study area do not show pitch canker disease symptoms. McNee *et al.* (2002) found that 0 % of the *Pityophthorus* specimens from asymptomatic branches carried the pathogen whereas 17 % from symptomatic branches were phoretically associated with the fungus. The presence of the pathogen within the insects' galleries could indicate that the insect acted as wounding or as transmission agents. Likewise, the presence of the pathogen within the breeding and feeding galleries could be

a way for the next generation of insects to be inoculated with the pathogen spores, although experimental work on this issue should be carried out under controlled conditions for more accurate conclusions. The importance of the role of the association between an insect and a pathogen depends also on the association between insect and host tree, requiring frequent visitation of the insect to a healthy host suitable for inoculation (Leach, 1940). Species like *P. carmeli* and *P. setosus* in California have shown an exploratory tasting behaviour that occurs when the insects taste several twigs before definitively choosing the one for feeding (Storer *et al.*, 2004). This behaviour increases the chances of these species acting as wounding or transmission agents of the pitch canker pathogen, although in the case of *P. pubescens* this association has not been observed in *P. radiata* stands in Cantabria.

The experimentation in which baited and unbaited trees were evaluated under natural conditions showed the effect of pityol in several disease symptoms. Thus, the Chi-square test showed that the number of trees affected by red shoots and dieback were significantly higher in baited points at time=3, 6 and 13. It is important to highlight that the increase in the number of trees with the symptoms affecting the crown in baited points was first observed 3 months after the baiting, but also in subsequent visits (time=6 and time=13). However, the number of trees with trickles of resin on the trunk was significantly higher from time=6 and in subsequent observations but not before, which could suggest that the first symptoms of PCD on the trunk may not be observed until 6 months from the insects attack. Since the presence of cankers did not vary significantly between treatments at any observation time, we can conclude that this symptom would need more than one year from the insects attack to be noticed. Other studies have been made on this issue in California where *Pityophthorus* spp. are considered important vectors of the pitch canker pathogen. Storer *et al.*, (2004) demonstrated that PCD infections under controlled conditions were more probable when trees were baited with *P. setosus* feromones than when unbaited. However, Sakamoto *et al.* (2007) did not find significant differences between treatments (pityol baited vs unbaited) regarding *F. circinatum* damages when carrying out the experiment under natural conditions.

Multinomial regression was performed with the objective of establishing how the parameters of plant health variables at observation time=13 depended on the variables observed during the observation process (time= 0, 3 and 6). From the multinomial regression results we can conclude that the presence of red shoots at time=13 (summer 2013) were dependent on the results observed at time=3 (autumn 2012). Hence, the presence of red shoots in the crowns of the trees in our study area during summer may be influenced by the presence of this symptom the previous autumn, suggesting an insects' attack at the end of the previous summer. Dieback at time=13 (summer 2013) was also affected by the previous crown conditions during summer 2012, indicating that trees that have dieback one summer are more probably affected by this symptom the next summer, regardless of the effect of the attractant. Trickles of resin at time=6 (winter 2013) significantly affected the presence of this symptom at the end of the observation in summer 2013, which could indicate that the trickles of resin that appear during winter remain on the tree trunk until the next summer.

In conclusion, *P. pubescens* has a weak association with *F. circinatum* in our study area due to the low phoresy rates and the low presence of the pathogen within the insects' galleries. This bark beetle species is a secondary pest that attacks broken and dying branches, moreover, the population levels found in our study were not especially high as it was found at endemic levels and not as an epidemic pest.

Acknowledgements

This research was financially supported by the Spanish Ministry of the Environment and Rural and Marine Affairs project 'Etiology, Epidemiology and Control of *Fusarium circinatum*' and by Cantabria Government project 'Control of pitch canker of pines in Cantabria region'. We want to deeply thank Milagros de Vallejo, Juan Blanco and the technical staff at the 'Dirección General de Montes y Conservación de la Naturaleza (Government of Cantabria)' for their invaluable help. We also thank Ruben Valles and Wilson Lara for his guidance and support with the statistical analyses. Nathan Brenville is also acknowledged for the writing revision.

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Chapter 7: General discussion

In the present work, four studies were carried out with the aim of knowing the factors affecting the epidemiology of *Fusarium circinatum* in northern Spain. Thus, different studies including abiotic and biotic factors influencing the dispersion, incidence and severity of the disease were performed.

Fusarium circinatum's capacity for infection seems to depend on the presence of biotic and/or abiotic wounding agents (Gordon, 2006). In the present study, pruning was shown to affect the PCD symptoms, thus those symptoms that largely appear in the main stem, such as cankers or resin drops, become more frequent in pruned trees. This could indicate that pruning wounds in the trunk increase the chance of trees becoming infected by *F. circinatum* as well as increasing the severity of the disease. This is also supported by the relationship found between the number of cankers on whorls and pruning. According to Gordon (2006), mechanical wounds in a PCD infected area sustained infection at a very low rate, and this rate would decrease if the wound size decreases. Volatiles generated by trees after pruning also increase the likelihood of infection, primarily because some insects carrying the fungus feel attracted by these volatiles (Gordon, 2011). Several bark beetles are also attracted by resin odours from damaged boles after pruning allowing them to later also attack neighbouring healthy trees (Jactel *et al.*, 2009).

Another abiotic and critical component allowing *F. circinatum* to survive and infect is environmental moisture (Gordon, 2006). Stand moisture content can be decreased by pruning through increased light and surface wind speed within the stand (Jactel *et al.*, 2009; Pollet and Omi, 2002), which could reduce successful pathogen survival. To assess the importance of environmental moisture in our study area, proximity to the coast and PCD symptoms were correlated. Thus, it was found that plot distance from the coast is an important factor influencing the disease occurrence, further underscoring the importance of environmental moisture. The influence of the coast, where the environmental conditions are more favourable for the infection (Wingfield *et al.*, 2008), has been previously noted in California (Wikler *et al.*, 2003), with one exception in Sierra Nevada (Vogler *et al.*, 2004).

Regarding the biotic factors, the presence of fungal communities associated with bark beetles in pine pitch canker affected *P. radiata* trees was evaluated, since bark beetles are known to be associated with endophytic, phytopathogenic and entomopathogenic fungi. Amongst the fungal species isolated from the insects galleries, we should highlight the presence of *D. pinea*, *Penicillium* spp., *Pestalotiopsis* spp., *Trichoderma* spp. and *Fusarium* spp. due to their pathogenicity, parasitism or potential as control agents. In this study 18 species were isolated from the insects' bodies. The species richness and fungal communities differed according to the bark beetle species. This difference could be due to the presence of specialized structures for carrying spores i.e. mycangia on some species, e.g. *H. ater*, *I. sexdentatus* or *X. saxeseni.* Most fungal communities were also significantly different on the insects' exoskeletons and in their galleries, although *Fusarium* spp. appeared to be related to both types of samples. This result could give us a better idea of the important role bark beetles play in the spreading of *Fusarium* species. Ten species of *Fusarium* were identified in this study, following both molecular and morphological identification methodologies, being *F. avenaceum* the most commonly identified species. It was isolated directly from *I. sexdentatus*, *H. attenuatus* and *H. ater* specimens and their galleries. Moreover, it

also appeared on *T. piniperda*-infested shoots. *Fusarium avenaceum* has been previously isolated from *P. radiata* in New Zealand, where it was associated with dieback caused by physical injury (Dick and Dobbie, 2002). *Fusarium tricinctum* was isolated from *I. sexdentatus* and from *T. piniperda* feeding galleries. This fungus usually acts as a saprophyte or weak parasite in Europe and North America (Leslie and Summerell, 2006). The *F. oxysporum* spp. complex was found to be associated with *O. erosus, H. attenuatus* and also with the *I. sexdentatus* galleries (both, xylem and phloem). This is a saprophyte and soil pathogen species complex with a wide range of plant hosts divided into many formae specialis depending on its host specificity. *Fusarium sambucinum*, which was isolated from *P. pubescens*, causes dry rot in potatoes (Niemira *et al.*, 1996) but it was also isolated from *P. radiata* in New Zealand where it was found to be associated with dieback and root rot (Dick and Dobbie, 2002). Other species isolated in this study like *F. sporotrichioides, F. konzum* and *F. beomiforme* have not been previously described as plant pathogens or are very weak parasites (Leslie and Summerell, 2006).

Previous studies have reported the presence of several bark beetle species on pitch canker affected plots, e.g. *I. sexdentatus, O. erosus, P. pubescens, H. palliatus* or H.attenuatus (Romón *et al.*, 2008). Bark beetle species such as *I. sexdentatus* or *P. pubescens*, are phoretically associated with *F. circinatum* according to these authors, however they do not attack healthy trees at endemic level. In the present study, *F. circinatum* was present in 0.9 % of the *I. sexdentatus* specimens. *Ips sexdentatus* is a secondary bark beetle but can act as a primary parasite when the population reaches epidemic levels (Etxebeste and Pajares, 2011), and in this situation, it could be able to inoculate healthy trees with the pathogen. In the Basque Country 8.57 % of the *I. sexdentatus* analyzed by Romón *et al.* (2007a) carried the pathogen. Likewise, in California other *Ips* species, like *I. mexicanus* (Hopkins) and *I. paraconfusus* Lanier, were reported as vectors of the pitch canker fungus (Fox *et al.*, 1991). Another bark beetle related to *F. circinatum* was *P. pubescens*, taking into account that 1.05 % of the captured specimens of *P. pubescens* carried the pathogen. On the other hand, the role of *T. piniperda* in the disease transmission could be more important than other secondary bark beetles species reported on in northern Spain as insect vectors of *F. circinatum* (Romón *et al.*, 2007a). *Fusarium circinatum* appeared in 3.5 % of the fallen shoots bored by *T. piniperda* in summer 2010.

After this previous findings, we focused our efforts on studying the importance of the roles of two of the bark beetles species found to be associated with *F. circinatum* in Cantabria: *T. piniperda*, due to its association with *F. circinatum* and healthy trees, and *P. pubescens*, due to the importance of this genus in *F. circinatum* transmission in California (Storer *et al.*, 2004). Thus, we firstly aimed at demonstrating that *T. piniperda* is a likely vector of *F. circinatum* in *P. radiata* plantations in northern Spain on the basis of Leach's postulates (Leach, 1940): an association of the insect with pitch canker diseased trees (postulate 1); an association of the insect with healthy *P. radiata* trees (postulate 2); the presence of the pathogen on the exoskeleton of *T. piniperda* specimens in nature (postulate 3) and *T. piniperda*'s capacity for transmitting the disease to healthy host material under certain controlled conditions (postulate 4).

The association of *T. piniperda* with pitch canker diseased *P. radiata* trees (postulate 1) was observed during the field sampling in 2014. Breeding galleries, and insects within them, collected from symptomatic trees, were positive for *F. circinatum*. This could mean that the insect was already infected with the pathogen when burrowing into the breeding gallery or that the bark where the insect tunneled its gallery was already infected by *F. circinatum* when the insect began its tunneling activity. The fact that *T. piniperda* burrows its breeding galleries into diseased trees increases the chances of insects' contamination with the pathogen. Regarding the second postulate, *T. piniperda* association with healthy crowns of pine trees has been intensively studied by several authors like Långström (1982) or Lieutier *et al.* (2015). In this study, 954 fresh green shoots with *T. piniperda* feeding gallery were collected from the ground. Although these shoots belonged to green crowns, 11.42 % of them were positive for *F. circinatum*. This result could show the role of *T. piniperda* as a wounding agent, since *F. circinatum* requires a wound to penetrate and infect the tree (Dwinell *et al.*, 1985), but this fact could also indicate that *T. piniperda* is inoculating the pathogen when feeding on shoots. Overwintering of *T. piniperda* on the shoots of healthy crowns is a determinant factor for highlighting the association of this bark beetle with healthy *P. radiata* trees. The presence of the pathogen on the insect in nature (postulate 3) was tested by collecting insects during their dispersion flight in PCD affected plots and it was found that *T. piniperda* population carried inoculum of *F. circinatum* during its dispersion flight. The phoresy rates (% of individu-

als carrying the pathogen) obtained in this work (3.6 % in average of three years) are consistent with those obtained by Whitehill *et al.* (2007) regarding the role of *I. pini* as a vector of *S. sapinea.* However, this phoresy rate could be considered low if compared with other bark beetle-pathogenic fungi systems e.g.: *Pityophthorus* spp. in California carried the fungus in a 17 % of the insects (McNee *et al.*, 2002) and *I. sexdentatus* which was associated with *O. ips* in a 69.5 % of the samples analized by Romón *et al.* (2007b). Finally, the laboratory experiment performed in this study evidenced the ability of the insect for inoculating the pathogen when shoot maturation feeding occurs under certain conditions (postulate 4). The presence of the pathogen was confirmed in a high percentage in the shoots from the mycelium inoculation assay, varying from 60 % to 20 % depending on the treatment. The differences between the percentages of *F. circinatum* infected shoots in the field and in laboratory may be related with the inoculation methodology, highlighting the need of knowing in which way the insect is loaded with *F. circinatum* spores in nature. The most likely option seems to be the contamination from zones of the tree where the mycelium and conidiophores have suitable humidity and temperature conditions, but the amount of spores that are harbored on the insect exoskeleton remains unknown.

The behavior of *T. piniperda* throughout its life cycle could allow *F. circinatum* dispersion. Thus, during a tree attack for breeding, insects can infest a pitch canker affected tree and consequently pick up spores in the same way that happens with Dutch elm disease (Webber, 2008). After that, they could be loaded with the fungal spores or mycelium that grew under the bark of diseased trees when completing their subcortical life stage. After breeding, both reemerging parents and the young emerging adults fly to the crowns of nearby pines for regeneration and maturation feeding, respectively. Then, when they go to feed on shoots, they would transfer the pathogen to healthy current shoots, allowing it to grow inside the feeding gallery and along the pith. As the insects feed on several shoots (Kirkendall *et al.*, 2008), they can disperse the disease along the pine crown. In the case of F1, after the maturation feeding, they remain inside the shoots for overwintering (between 6-9 months in our study area) while the fungal structures are growing inside the gallery. Regarding the reemerging parents, after regeneration feeding, they fly to new sister brood establishments and can disperse the fungus to other non-affected PCD trees and go back to the crowns of healthy trees for regeneration feeding once again. This is supported by the finding of reemerging parents inside the feeding shoots during March, April and May in 2011 and 2012.

In regard to the role of *P. pubescens* in *F. circinatum* dispersion, it was evaluated by performing three different samplings: one in which *P. pubescens* specimens were collected with funnel traps baited with pityol, another one in which both insects and galleries were collected from *P. pubescens* infested *P. radiata* twigs and, finally, a PCD damages evaluation was carried out in a *P. radiata* stand regarding the presence or absence of (E)-pityol, the insect aggregation pheromone.

Phoretical association between *P. pubescens* and *F. circinatum* ranged between 0 % and 2.04 % in the present study. This results are consistent with those obtained in other works studying *Pityophthorus* species as *F. circinatum* phoretic agents, as for example the work carried out by Erbilgin *et al.* (2005) in which 0 % of the *Pityophthorus* specimens carried the pathogen or those obtained by Dallara (1997) in which 2.5 % of the analyzed insects carried the pathogen. However, phoretic rates vary depending on the insect species; for example, *F. circinatum* was found on the 0-13.69 % of *P. carmeli* while it appeared between the 0-2 % of the *P. setosus* in Monterey Peninsula in California (Storer *et al.*, 2004). Regarding the presence of *F. circinatum* on the insects' exoskeleton when captured within the galleries, no isolate of *F. circinatum* was obtained. However, the pathogen was isolated from the 0.75 % of the galleries burrowed by the insect. Vegetal tissue from the insects galleries found in our study area presented red needles, although no pitch canker disease symptoms were observed. Thus, it is necessary to highlight the importance of the pitch canker infection level of branches, as McNee *et al.* (2002) found that 0 % of the insects obtained from asymptomatic branches carried the pathogen but 17 % of *Pityophthorus* specimens collected from symptomatic branches were phoretically associated with the fungus. The presence of the pathogen within the insects' galleries could indicate that the insect acted as wounding or as transmission agents.

The experimentation in which baited and unbaited trees were evaluated under natural conditions showed the effect of pityol in several disease symptoms. Thus, the number of trees affected by those symptoms in the crown (red shoots and dieback) was significantly higher in baited points at time=3, 6 and 13. Other studies have been made on this issue in California where *Pityophtho-*

rus spp. are considered important vectors of the pitch canker pathogen. Thus, Storer *et al.* (2004) demonstrated that PCD infections under controlled conditions were more probable when trees were baited with *P. setosus* feromones than when unbaited. However, Sakamoto *et al.* (2007) did not found significant differences between treatments (pityol baited and unbaited) regarding *F. circinatum* damages when carrying the experiment under natural conditions.

Multinomial regression was performed with the objective of knowing how the parameters of plant health variables changed along the observation process (time= 0, 3 and 6) affecting at observation time=13. From these results we can conclude that the presence of red shoots at time=13, concurring with the summer 2013, were dependant on the results observed at time=3, autumn 2012. Hence, the presence of red shoots in the crowns of the trees in our study area during summer may be influenced by the presence of this symptom the previous autumn, suggesting an insect attack at the end of the previous summer. Dieback at time=13 was affected by the crown conditions during the previous summer, indicating that trees that have dieback one summer are more probably affected by this symptom the next summer, regardless the effect of the attractant. Trickles of resin at time=6, winter 2013, significantly affected the presence of this symptom at the end of the observation in summer 2013 and pityol was influencing this process, what could indicate that the presence of trickles of resin that appear during winter remain on the tree trunk until the next summer.

In short, our outcomes evidenced an influence of several abiotic and biotic factors on *F. circinatum* epidemiology. Pruning was shown to have a determinant role on the presence of cankers on the tree trunks. Moreover, biotic factors, like the presence bark beetles on pitch canker affected trees resulted to have a chief association with fungal communities, especially with *Fusarium* spp.. Regarding the role of bark beetles on *F. circinatum* spreading, *T. piniperda*, which feeds on healthy shoots, is suggested to be more important in *F. circinatum* transmission than *P. pubescens* in our study area. However, further studies taking into account these factors are necessary in order to achieve a more proper disease management.

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Chapter 8: Conclusions

1) Pruning wounds have an increased chance of becoming infected by *F. circinatum* which could enhance the formation of cankers and trunk deformation. Notwithstanding, pruning in Monterey pine diseased plantations is not desirable as a result of stem deformation caused by cankers, making them useless for the wood industry.

2) Fungal communities found associated with the pitch canker affected plots of *P. radiata* consisted on 24 different fungal species obtained from the bark beetle´s galleries from logs and shoots while 18 were obtained directly from the insects' exoskeletons. Specifically it was found an association of the *Fusarium* species with bark beetles and their galleries, highlighting that *F. circinatum* spores were present on *Pityophthorus pubescens* and *Ips sexdentatus* exoskeletons as well as on *Hylastes attenuatus, H. angustatus* and *Tomicus piniperda* galleries.

3) This study raises the participation of *T. piniperda* as a vector of *F. circinatum* in our study area, following Leach´s postulates (Leach 1940): 1) *T. piniperda* appeared associate with fresh green tree crowns in the study area, 2) The presence of *F. circinatum* on breeding and feeding galleries was evidenced by the results obtained during the field sampling, 3) *F. circinatum* appeared on the *T. piniperda´*s exoskeleton while breeding, feeding and during dispersion flight, 4) the laboratory experiment evidenced that this insect can introduce the pathogen into the shoots of healthy trees. That indicates that this species could transport the pathogen and later introduce it both under the bark and into the pith of the shoots. Maturation feeding and overwintering within the shoots are the most probable moments for pathogen inoculation.

4) *Pityophthorus pubescens* has a weak association with *F. circinatum* in our study area evidenced by the low phoretic rates and the low presence of the pathogen within the insects' galleries together with the fact that it is a secondary pest that attacks broken and weakened branches. Moreover, population levels are not especially high as it was found at endemic level. The role of bark beetles species as *T. piniperda* that feed on healthy shoots is suggested to be more important in PCD transmission than *P. pubescens.*

Chapter 9: Conclusiones

1) Las heridas de poda aumentan el riesgo de infección por *F. circinatum*, lo que incrementaría la presencia de chancros y de deformación en el tronco. Por lo que la poda no es recomendable en plantaciones afectadas por la enfermedad, ya que la deformación causada por los chancros haría los troncos inservibles en la industria maderera.

2) Las comunidades fúngicas asociadas a árboles de *P. radiata* afectados por el chancro resinoso estuvieron formadas por un total de 24 especies provenientes de galerías de escolítidos, además se aislaron 18 especies del exoesqueleto de los insectos. Concretamente, en este estudio se identificaron diez especies de *Fusarium* asociadas a escolítidos y sus galerías, destacando la presencia de *F. circinatum* en los exoesqueletos de *Pityophthorus pubescens* e *Ips sexdentatus,* así como en las galerías de *Hylastes attenuatus*, *H. angustatus* y *Tomicus piniperda.*

3) Este trabajo pone de manifiesto la posibilidad de que *T. piniperda* sea un vector de *F. circinatum* en nuestro área de estudio. Según los postulados de Leach (Leach, 1940): 1) *T. piniperda* apareció asociado a copas verdes en las parcelas estudiadas, 2) *F. circinatum* apareció asociado a las galerías de cría y de alimentación en las muestras recogidas en campo, 3) *F. circinatum* fue aislado del exoesqueleto de *T. piniperda* durante la cría, la alimentación en ramillos y el vuelo de dispersión y 4) el experimento de laboratorio evidenció la capacidad de *T. piniperda* de introducir el patógeno en ramillos sanos. Esto indica que esta especie podría transportar el patógeno y más tarde inocularlo tanto bajo la corteza como en el interior de los ramillos de copa. La alimentación de maduración y la invernación son las etapas del ciclo más susceptibles para la inoculación del patógeno.

4) *Pityophthorus pubescens* mostró una asociación débil con *F. circinatum* en nuestro área de estudio, evidenciado por las bajas tasas de foresía y por la baja presencia del patógeno en el interior de las galerías, junto con el hecho de que es una plaga secundaria que ataca ramas rotas y debilitadas. Además, los niveles poblacionales encontrados no fueron remarcablemente altos indicando que la población se encontraba a niveles endémicos. El papel de *T. piniperda* como vector de *F. circinatum* parece ser más importante en nuestra área de estudio que el de *P. pubescens*, debido a la alimentación que el primero lleva a cabo en árboles sanos.


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