

# Epidemiology and Management of Pine Pitch Canker Disease in Europe - a Review

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Bezós, D., Martínez-Alvarez, P., Fernández, M. and Diez, J.J. 2017. Epidemiology and Management of Pine Pitch Canker Disease in Europe - a Review. *Baltic Forestry* 23(1): 279-293.

## Abstract

*Fusarium circinatum* is an ascomycete fungus that causes Pine Pitch Canker (PPC) of pines. The disease is causing damages in forests and nurseries all around the world. In Europe, is present in northern Spain, and also has been detected in Portugal, France and Italy. *Fusarium circinatum* seems to require fresh wounds on trees as infection court. Notwithstanding, the susceptibility of these wounds to infection could decrease significantly with wound age. *Fusarium circinatum* has been reported to be phoretically associated with *P. pubescens* in California. In northern Spain, *T. piniperda* is a major candidate for being an effective vector of *F. circinatum* due to the maturation feeding it practices in the crowns of healthy pines and subsequent overwintering. At present there are no means of controlling PPC disease in adult trees in forest or plantations. However, given the seedborne character of *F. circinatum*, some encouraging results have been obtained by the use of different strategies to reduce the presence of the pathogen in pine seeds. For example hot water treatments (51–52°C for 30 min) were found effective in reducing *F. circinatum* contamination in seeds. Endophytic species which do not cause any damage that could be used for biological control of *Fusarium* spp., have been reported related to pitch canker diseased in *P. radiata* trees. The use of mycoviruses to control fungal diseases of plants could be a promising method when the genetic diversity of the populations of the pathogen is low, for example, when the introduction of the fungus in a region is recent or when sexual reproduction is absent as occurs in PPC. Recently, three different strains of mycoviruses co-infecting a Spanish isolate of *F. circinatum* were found and characterized. More studies are essential to prevent the rapid spread of the disease from southern to northern Europe.

## The pitch canker disease pathogen: *Fusarium circinatum*

*Fusarium circinatum* is an ascomycete fungus belonging to the *Gibberella fujikuroi* clade that causes PPC on pines (Nirenberg and O'Donnell 1998). *Fusarium circinatum* is a seedborne pathogen that can survive both

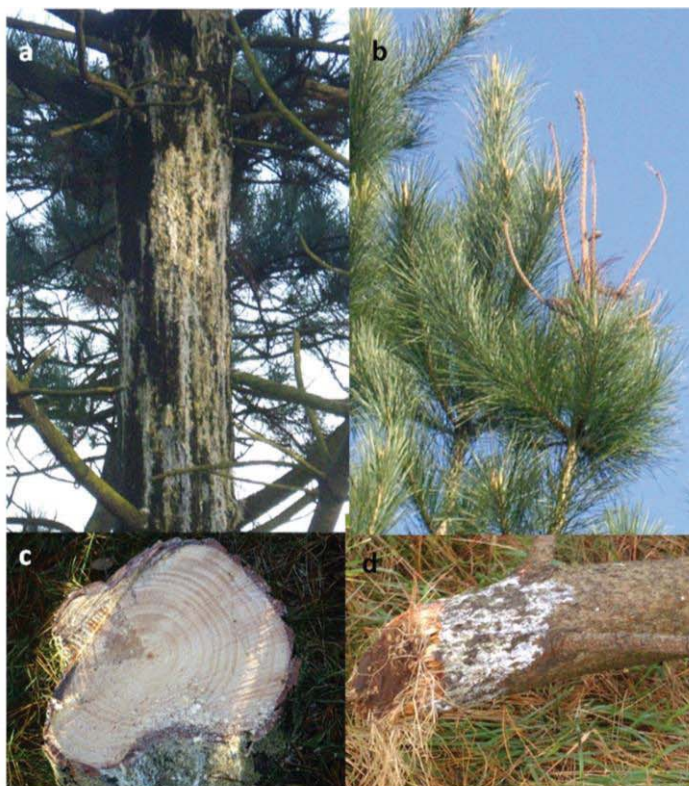
superficially and internally in the seeds, causing seed high mortality rates (Gordon 2011). Seedlings can also show die-back and die due to the girdling, but the main symptoms observed in seedlings are necrosis, chlorosis, wilting of needles, dieback and desiccation of the seedling tip (Viljoen et al. 1994; Martín-Rodrigues et al. 2013). The main symptom of PPC in adult trees is the presence of pitch soaked cankers in trunks and big branches which girdle both

trees and branches (Figure 1) (Wikler et al. 2003). Trickle 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. However, on small diameter branches a single infection may be sufficient to cause the death of the branch (Gordon 2011). Dieback symptoms are also common on the crown due to the obstruction of water flow caused by the cankers. The wilting and discoloration of needles, which eventually turn red and finally fall off, is a common symptom of the disease as well (Wingfield et al. 2008). The tree finally dies when cankers girdle the trunk or as a result of the loss of structural integrity at the site of the canker formation. In forest nurseries, *F. circinatum*, can reduce germination of seeds, cause pre- and post-emergence damping-off, the wilting of seedlings, shoot and tip dieback, and finally lead to the death of the established seedlings (Viljoen et al. 1994). The increase in the resin production is due to the increment of number of the 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 death (Martín-Rodríguez et al. 2013). *Fusarium circinatum* also causes growth reduction in adult trees in forest and plantations leading to great economic and ecological losses.

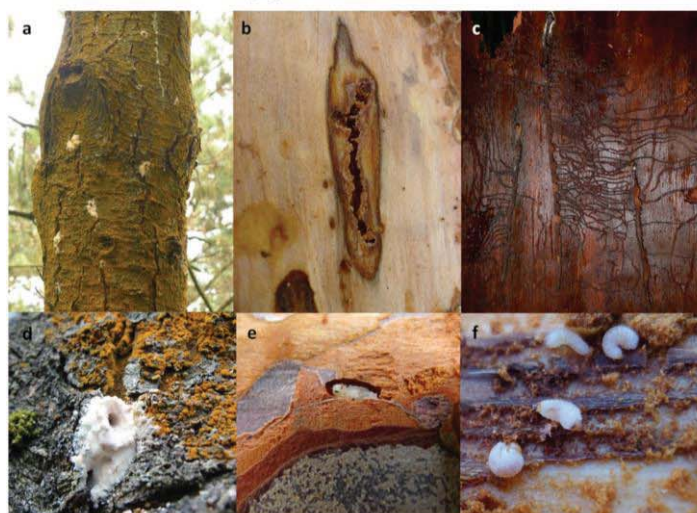
*Fusarium circinatum* has a wide geographical distribution. This pathogen was first reported in North Carolina (Hepting and Roth 1946) on *Pinus virginiana* Mill. in southeastern United States, epidemics occasionally occur and are generally associated with abiotic stress (López-Zamora et al. 2007). Hosts may have co-evolved with the pathogen due to the proximity of this region to the area where the pathogen is said to be endemic. Forty years after its initial detection, the disease was recorded in California affecting landscape pines mainly of the species *P. radiata* D. Don but also *P. muricata* D. Don, *P. pinea* L. and *P. halepensis* Mill. (McCain et al. 1987). Some years later, the hosts and the geographic range of the PPC pathogen increased, affecting native stands of *P. radiata* in the Monterey peninsula and making a transgeneric jump to *Pseudotsuga menziesii* (Mirb.) Franco (Storer et al. 1994). Later, it was also 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 *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. 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 control, 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. The origin of the pathogen introduction in Spain has been deeply studied, showing two significantly differentiated populations regarding all the affected areas (Bergal et al. 2013) and a clonal population within País Vasco (Iturriza et al. 2011). According to the results of this study, the *F. circinatum* isolates detected in the first infected nursery in País Vasco in 1997 (Dwinell et al. 1998) and deposited in the Fusarium Research Center collection (Pennsylvania State University) belong to the most common multilocus genotypes of the two groups, MLG32 (Bergal et al. 2013). Therefore it is probable that one of those introductions occurred in the País Vasco region, and it spread from there to the rest of the regions in northern Spain. The other group of multilocus genotypes is represented by MLG59, which is restricted to the northwest of the country (Bergal et al. 2013). Galicia may be the second place of origin of *F. circinatum*, taking into account that the disease was reported to be in that region in 1996 (MAPA 1996). 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. Since the detection of *F. circinatum* (Landeras et al. 2005), some regions in Spain have stopped planting *P. radiata* due to the ban on using *Pinus* spp. and *P. menziesii* for the reforestation of the affected areas (MAPA 2006). The production of *P. radiata* seedlings in





**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



**Figure 3.** 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) maternal 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*



**Figure 4.** *Tomicus piniperda* within shoot feeding galleries: a) *Pinus nigra* and b) *Pinus radiata*



**Figure 6.** *Pinus radiata* attacked by *Pityophthorus pubescens*. a) Reddish twigs, b) detail of the gallery burrowed by *P. pubescens*

forest nurseries was also reduced due to the risk of contamination from the pathogen and the consequent loss in crop and yield.

*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, *Gibberella circinata*, has been produced only 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 differentiation of the hyphae for invading the host tissues, i.e. haustoria or appressoria (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 cytolysis, 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-Rodríguez 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, for the first time, the production of conidiophora orientated towards the hollow cavities of the pith at the moment when the first symptoms of disease appeared.

Until now *F. circinatum* is exclusively a pine species pathogen, although some trees of the species *P. menziesii* were found susceptible to the disease (Storer et al. 1994, Gordon et al. 2006). At least 57 pine species have been reported as being susceptible to the PPC pathogen after observing symptoms of the disease on seedlings or adult trees or performing inoculation experiments (reviewed by Wingfield et al. 2008). However, susceptibility varies among pine species. For example, while species like *P. pinea* or *P. canariensis* seem to be resistant to the

pathogen (Gordon et al. 1998, Iturrity et al., 2013), there is evidence that *P. radiata* is the most susceptible species to the disease (Wingfield et al. 2008). It is also the most widely-planted pine in the world (Critchfield and Elbert 1966), Chile being the country with the most surface planted with this species (more than one and a half million hectares). Other countries with large areas of *P. radiata* plantations are Argentina, Uruguay, South Africa, Australia and New Zealand (Fernández and Sarmiento 2004). *Fusarium circinatum* is still absent from the last two countries, and due to the importance of this pine species there, the occurrence of the disease would have serious economic, ecological, and social impacts. In Spain, around 275 000 hectares are planted with this pine (Fernández and Sarmiento 2004), a relatively small area in comparison with native pines (3.6% of the total area covered by coniferous species). However, due to its fast growth and short rotation time, it provides 25% of the conifer timber in Spain (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).



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



Apart from pines and *P. menziesii*, the susceptibility of a variety of other plant species including trees and herbaceous plants has been tested, with the pathogen failing to infect them (McCain et al. 1987, Wingfield et al., 2008). On the other hand, *F. circinatum* has been found to infect grasses as a symptomless endophyte (Swett and Gordon 2012). This fact makes the eradication of the disease even more difficult in most parts of the world where the pathogen is well-established; grasses may serve as a reservoir of inoculum which, in turn, influences the occurrence of the disease in pine nurseries and plantations (Swett et al. 2014). An intensive sampling must be done to detect the presence of the pathogen in many other plant species in order to improve the management of the disease. Furthermore, susceptibility to PPC disease in other conifer species must be evaluated to find alternatives to *P. radiata* in the areas where the pathogen is already present.

### 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 PPC epidemiology, there are two major aspects that have to be considered: biotic factors and abiotic factors.

#### Abiotic factors

##### a. Forest management:

##### *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).

#### *Tree host selection*

Up to 60 *Pinus* species have been reported to be susceptible to the PPC (Gordon 2006), among them: *P. radiata*, *P. sylvestris* (Landaras et al. 2005), *P. pinaster* (Vivas 2012), *Pinus nigra* Arn. and *Pinus uncinata* Ram. (Martínez-Álvarez et al. 2014a) 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. 2014a). 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 PPC 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 PPC (Gordon 2006).

#### *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 where PPC is destroying the trees, although in Spain Bezos et al. (2012) observed that pruning wounds have an increased chance of becoming infected by the pathogen which could enhance cankers and 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.

#### **b. 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 PPC development, temperature (20-25°C 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 also 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).

### **Biotic factors**

#### **a. Fungal communities**

Fungal communities inhabiting *P. radiata* trees may be a determinant factor influencing PPC distribution in Spain (Bezos et al. 2013). Fungal communities in forests are formed by endophytes together with saprotrophic and pathogenic species. Knowing the species composition and the factors influencing the presence of different fungal communities is important 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 exploiting 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 PPC 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), but the role of *Penicillium chrysogenum* Link. in association with *F. circinatum* in *P. radiata* resulted in antagonism and induce 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 (Bezos et al. 2013, García-Serna 2014). *Diplodia pinea* is a saprotrophic fungi 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 PPC 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 in vitro when it was introduced as a pioneer (Romón et al. 2008).

#### **b. 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 2007; Fernández et al. 1999a, 1999b).

Bark beetles are associated with several fungal species, in native forests and plantations world-wide, in particular, with endophytic or pathogenic fungi including *F. circinatum* (Lieutier et al. 1989, Jacobs et al. 2004, Kirisits 2004, 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 the insect depends on its ability to overcome tree resistance mechanisms (Christiansen et al. 1987, Långström and Hellqvist 1993, 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 insect 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 PPC 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 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 PPC affected trees is highlighted by its entomopathogenic activity, due to the question less role of bark beetles in *F. circinatum* spreading. These entomopathogenic fungi infecting bark beetles are usually ascomycetes, i.e. *F. 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* (Marshall), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Boerner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* Erichson and *Orthotomicus erosus* (Wollaston) (Romón et al. 2007a, Bezos et al. 2013). 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 2007) during maturation feeding. The population levels may also increase until epidemic levels (Raffa and Berryman 1983) determining 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.

#### *1- Tomicus piniperda* L.

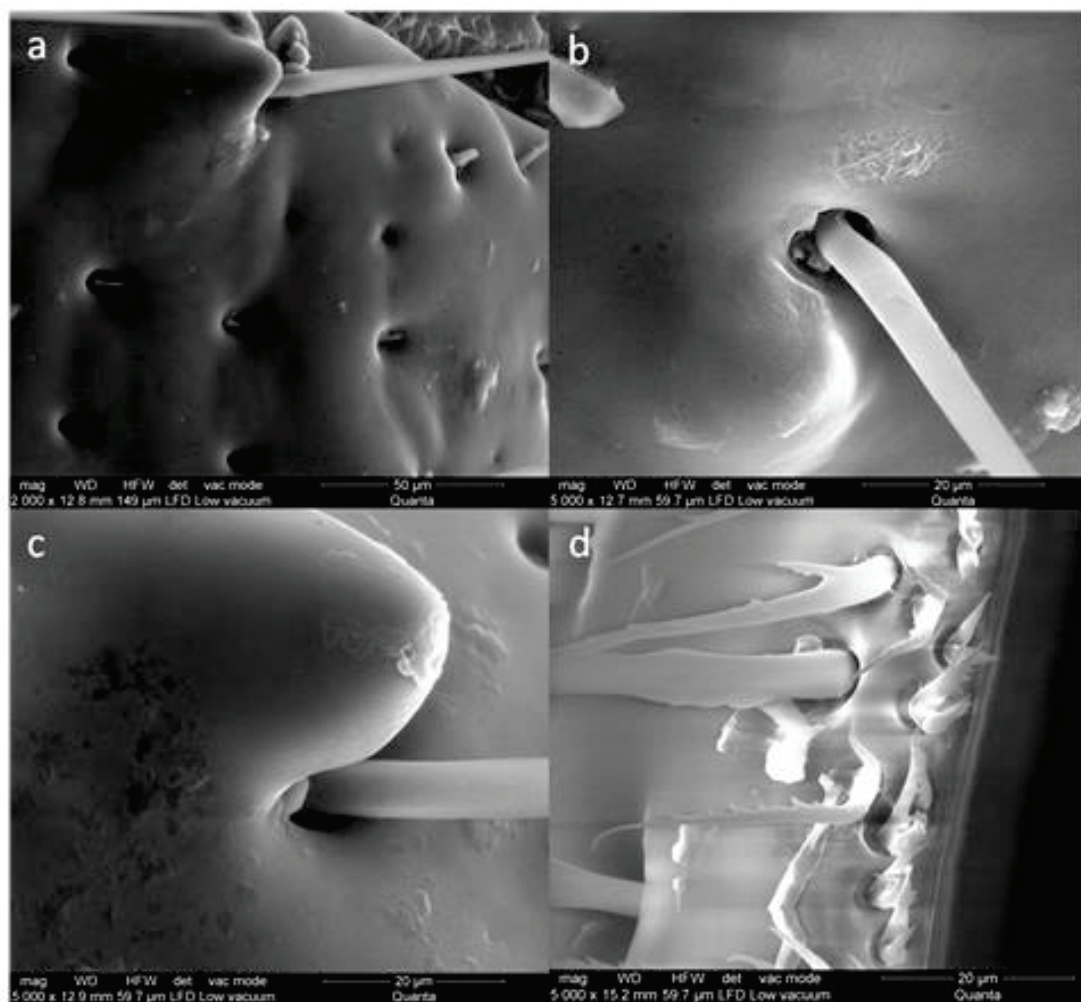
*Tomicus piniperda* is a serious pest affecting pines in Europe, Northern Africa and Asia (Långström 1980, Bouhot et al. 1988; Kirkendall et al. 2008) and in the United States ever since it was introduced in 1992 (McCullough and Smitley 1995). Its main host is *Pinus sylvestris* L. but other pine species are also suitable hosts, as, for example, *P. radiata*. *Tomicus piniperda* is a univoltine species that may present several sister broods. This species can colonize trunks and thick branches of weakened trees where it breeds (Figure 3), 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 4) (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 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, 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 inside the shoots. 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). The association between *T. piniperda* and *F. circinatum* has been demonstrated in northern Spain following Leach's postulates (Leach 1940) where the pathogen appeared on breeding and feeding galleries, as well as on *T. piniperda* exoskeleton (Bezós et al. 2015). 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 susceptible moments for pathogen inoculation.

In northern Spain, *T. piniperda* is a major candidate 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) or like *Ophiostoma minus* (Hedgec.) Syd. & P. Syd. (Långström et al. 1993, 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 5).



**Figure 5.** Scanning Electron Microscope pictures of *Tomicus piniperda*'s body structures: a), b) and c) on the elytra, d) at the base of the pronotum



## 2- *Pityophthorus pubescens* (Marshall)

Twig beetles, *Pityophthorus* spp., are phloeophagus and myelophagus species (Vega and Hofstetter 2015). This 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 colonize mainly weak trees with a low economic impact (Vega and Hofstetter 2015). *Pityophthorus pubescens* is present in the Iberian Peninsula that attacks weakly trees. The presence of this insect species on the attacked crowns can be observed by the presence of reddish twigs (Figure 6a). 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 6b) (Sakamoto et al. 2007). Overwintering in *P. pubescens* has been observed to occur on shoots within *T. piniperda* feeding galleries (Balachowsky 1962).

*Fusarium circinatum* has been reported to be phoretically associated with *P. pubescens* in Spain (Bezós et al. 2013, 2016; Romón et al. 2007a). The association of *Pityophthorus* spp. with PPC disease 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 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.

## 3- Other bark beetles in northern Spain

*Ips sexdentatus* 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 2007). Most of this insect's live cycle occurs under the tree bark (Vega and Hofstetter 2015). *Ips sexdentatus* colonize weak and dead *P. radiata* trees in northern Spain, however when population levels increase they can kill healthy trees (Etchebeste and Pajares 2011), although outbreaks usually occur after forest fires or adverse climatic conditions (Gil and Pajares 1986, Fernández and Salgado 1999, Etchebeste et al. 2012).

The association of *Ips* species with fungi has been widely study. Whitehill et al. (2007) found the role of *Ips*

*pini* as a vector of *Diplodia pinea* and several ophiostomatoid species were isolated from *I. sexdentatus*' exoskeleton in the work carried out by Fernández et al. (2004) Romón et al. (2007b) and by Bueno et al. (2010). *Ips sexdentatus* was also found to be phoretically associated with *F. circinatum* in Spain (Romón et al., 2007a; Bezós et al., 2014), with different percentages of specimens carrying the pathogen (0.9%-8.5%). Likewise, the importance of other species like *Ips mexicanus* (Hopkins) and *Ips paraconfusus* Lanier in association with *F. circinatum* has also been observed in PPC affected areas in California, where this 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 other bark beetle species present on pitch canker disease affected stands there are four species from the genus *Hylastes* present on the Iberian Peninsula. They are monogamous, phloeophagus and colonize mainly weak or felled trees (López 2007). *Hylastes ater* (Payk.), *Hylastes angustatus* Herbst 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 the *H. ater* ability of attacking unstressed seedlings more frequently than stressed ones; however the attack to stress seedlings caused substantial girdling-induced mortality. Moreover, *H. ater* has been reported to be associated to several pathogenic fungi i.e. *Ophiostoma* spp. *Leptographium* spp. (Eckhardt et al. 2004) as well as *Fusarium* spp (Romón et al. 2007a, Romón et al. 2014). Bezós et al. (2014) found that the lesser *H. attenuatus* was a carrier, although in low proportion (1.60% of specimens carried spores).

*Orthotomicus erosus* is a polygamous species that generally feed on weak trees, infesting fallen or felled trees, but also attack living trees that suffer from stress due to fire, droughts or diseases (López 2007). Population densities may increase until epidemic levels, what would lead them to overcome food store of weaken 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 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).

## Measures for disease control: Biological control

At present there are no means of controlling PPC disease in adult trees in forest or plantations. However, given the seedborne character of *F. circinatum*, some encouraging results have been obtained by the use of different strategies to reduce the presence of the pathogen in pine seeds. For example hot water treatments (51–52°C for 30 min) were found effective in reducing *F. circinatum* contamination in seeds (Agustí-Brisach et al. 2012, Berbegal et al. 2015). Furthermore, several studies found that hydrogen peroxide is a good disinfectant of the contaminated seeds (Dwinell and Fraedrich 1999, Berbegal et al. 2015). Unfortunately, these methods do not prevent the arrival of infected seeds to the forest nurseries and seedlings to the forest. To reduce the impact of the disease, an integrated management approach is necessary, and, at the same time, the role of biological control is crucial due to the advantages it has over the use of chemicals.

Cook and Baker (1983) defined biological control as the reduction of the amount of inoculum or disease-producing activity of a pathogen produced by or through one or more organisms other than man. The organisms mentioned in the definition are (1) avirulent or hypovirulent strains of the pathogen, (2) the host plant manipulated either genetically, by cultural practices, or with microorganisms toward greater or more effective resistance to the pathogen, and (3) antagonists of the pathogen, i.e. microorganisms that interfere with the survival or disease-producing activities of the pathogen.

Biological control of plant diseases has many advantages when compared to the use of chemicals. For instance, fungicides and bactericides have promoted the development of pathogen strains resistant to chemicals, which has been a problem for at least the last thirty years (Dekker and Georgopoulos 1982). Besides, most chemicals are nonspecific and broad-spectrum in their effect, and they can produce undesirable effects on non-targeted organisms, such as the fungal or bacterial endophytes, which can play an important role as antagonists of the pathogens (Spokes et al. 1981). Humans may be harmed as well, specifically when pesticides are employed in agriculture and potentially enter into the food chain (Kniewald 2003). Another advantage is that longer-term control is achieved because biological control agents (BCAs) act as a host-specific control method continually present and constantly impacting the target pathogen. Thus, although it may be expensive to introduce due to research costs, it can be quite economical in the long term (Cook and Baker 1983). The importance of

biological control methods is even higher in forests where, day after day, chemical use is more and more restricted (EU 2009a).

As explained before, there are several strategies classified as biocontrols in the fight against pathogens. However, here we will focus our attention only on the control of plant pathogens through the use of fungal endophytes and mycoviruses.

### a. Biological control using fungal endophytes

Many definitions for the term fungal endophytes can be found in scientific literature. One of the most accepted definitions says that fungal endophytes are fungi that are able to infect their hosts without causing visible symptoms of disease (Petrini 1991). Typically, they may be divided into three types: (1) pathogens of another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic fungi; and (3) pathogens that have been rendered non-pathogenic yet are still capable of colonization by selection methods or genetic alteration (Backman and Sikora 2008). Endophytes can produce many benefits for the host plants. For instance, various studies have demonstrated that plants infected with endophytes obtain growth promotion (Barka et al. 2002), resistance to drought stress (Swarthout et al. 2009), tolerance to unsuitable soil conditions (Malinowski et al. 2004), greater access to nutrients (White et al. 1997), and improved defense against herbivorous animals (Carroll 1988) and pathogens (Arnold et al. 2003). Regarding the latter benefit of endophytes, the mechanisms implemented to protect the plant against the infection of plant pathogens can be grouped into: direct effects, indirect effects and ecological effects (Gao et al. 2010). In the case of direct effects, endophytes directly suppress pathogens by producing antibiotics (Richardson et al. 2014) or secreting lytic enzymes (Tripathi et al. 2008). Induction of plant resistance, stimulation of the plant's secondary metabolites and promotion of plant growth and physiology all are indirect effects that the endophytes have on the plant to help it reduce damage caused by pathogens. Finally, examples of ecological effects are the occupation of an ecological niche, as well as hyperparasitism and predation (Gao et al. 2010).

One group of fungi stands out among the endophytes because of their potential as a biological control agent of plant diseases: genus *Trichoderma* (Howell 2003). This group is well known and worldwide in occurrence. One of the most salient characteristics of the group is their ability to parasitize other fungi (Weindling 1932), but they also produce antibiotic substances that are inhibitory to many plant pathogens (Howell and Stipanovic 1983). However,



the principal mechanism in the biocontrol process of *Trichoderma* spp. is the competition for space and nutrients in the rhizosphere (Howell 2003). The growth of these fungi is not restricted to the soil and plant roots, but rather they are also able to colonize the phloem and even the sapwood of trees (Jankowiak 2006). To understand the potential of this group of fungi as BCAs of phytopathogenic fungi, it is important to point out that 90% of the applications performed to control plant diseases have been carried out with different strains of the genus *Trichoderma* (Benítez et al. 2004). There are many examples of *Trichoderma* spp. employed in the successful control of different plant diseases (Latunde-Dada, 1993, Abdullah et al. 2008, Ruano-Rosa et al. 2010) which, in some cases, are caused by pathogens of the genus *Fusarium* (Sivan et al. 1987, Bernal-Vicente et al., 2009, Basak and Basak 2011). *Trichoderma* spp. have potential not only to control fungal pathogens but also bacteria (Phupiewkham et al. 2015).

Regarding tree diseases, the most well-known example of biocontrol is the one carried out by *Phlebiopsis gigantea* (Fr.) Jülich against *Heterobasidion annosum* (Fr.) Bref, considered the most harmful forest pathogen in economic terms in the Northern Hemisphere (Woodward et al. 1998). However, the importance of endophytes as BCAs is not restricted to forests but also extends to nurseries (Capieau et al. 2004). The production of seedlings carrying antagonistic endophytes to the most aggressive pathogens may be the future of biocontrol in forest pathology.

Regarding PPC disease, some experiments have been performed to reduce the impact of the pathogen using fungal endophytes. Antagonistic interactions between *F. circinatum* and the fungal species *Penicillium chrysogenum* Link. and *Fusarium lateritium* Ness. were observed in an experiment performed in the lab by Romón et al. (2008). In this experiment, seedlings emerged from *Pinus radiata* surface-sterilized seeds were coated with 0.5 mL of a 900 000 conidia/mL suspension of *Penicillium chrysogenum*, *F. lateritium* and control treatment in soil simultaneous infected with 0, 450 000, 4500, or 45 pitch canker fungus conidia inoculum levels. Similarly, Soria et al. (2012) showed that two endophytic bacteria (*Bacillus subtilis* Cohn and *Burkholderia* sp.) were antagonists to the pitch canker pathogen. In another experiment, the potential use of *Trichoderma* spp. and *Clonostachys* spp. strains to control *F. circinatum* on *P. radiata* seedlings was evaluated by (Moraga-Suazo et al. 2011). One of the strains of *Clonostachys* sp. tested significantly increased the survival of *P. radiata* seedlings, but no effect was observed with the *Trichoderma* strains. The same conclusions were reached in previous studies in which *Trichoderma* spp. were tested as

BCAs of the disease (Dumroese et al. 1988; Mitchell et al. 2004, Martínez-Álvarez et al. 2012). Recently, two fungal isolates belonging to the species *Chaetomium aureum* and *Alternaria* sp. reduced the micelial growth in dual cultures, and the symptoms caused by *F. circinatum* on *P. radiata* seedlings planted in the field, indicating that they may therefore be suitable for use as BCAs of the disease (Martínez-Álvarez et al. 2016).

#### **b. Biological control using mycoviruses**

Mycoviruses or fungal viruses are widespread in all major taxa of fungi. Most of them have dsRNA (double-stranded RNA) genomes, although an increasing number of positive or negative ssRNA (single-stranded RNA) and ssDNA (single-stranded DNA) viruses have been isolated and characterized in recent years (Ghabrial et al. 2015). Mycoviruses are, in general, associated with latent infections in their hosts (Ghabrial and Suzuki 2009). However, in some cases they induce different symptoms such as changes in growth, colour, sporulation, and sometimes enhancement (hypervirulence) or attenuation of fungal virulence (hypovirulence) (Ghabrial and Suzuki 2009, Pearson et al. 2009). Hypovirulence in the host's physiology is the most important reason why plant pathologists are interested in mycoviruses and why mycoviruses can be used as BCAs.

The mycovirus that dominates in the context of plant pathology is the *Cryphonectria hypovirus 1* (CHV1) (Heiniger and Rigling 1994), which has been successfully used as a BCA for the chestnut blight pathogen throughout Europe (Robin and Heiniger 2001, Turchetti et al. 2008, Zamora et al. 2014). This hypovirus reduces mycelial growth and sporulation of *Cryphonectria parasitica*. It also produces changes in the morphology and colour of the fungus colony (Rigling et al. 1989, Peever et al. 2000). After being infected, the pathogen is only capable of forming superficial healing cankers on stems, allowing trees to survive after the attack (Nuss 1992).

Among mycoviruses, one genus only found in fungi stands out due to its importance in the biological control of plant diseases. This is the genus, *Mitovirus*, which belongs to the family *Narnaviridae*, a group in which the members have the simplest genomes of any autonomous RNA virus (Ghabrial et al. 2015). Putative members of the genus *Mitovirus* are located and translated in the mitochondria (Polashock and Hillman 1994), where they mostly occur as dsRNA replicative forms (Ghabrial 1998). In some cases they exhibit phenotypic changes and cause hypovirulence in major plant pathogens, such as *Botrytis cinerea* (Wu et al.

2010), *Ophiostoma novo-ulmi* (Rogers et al. 1987) or *Sclerotinia homoeocarpa* (Deng et al. 2003).

Recently, three different strains of mycoviruses co-infecting a Spanish isolate of *F. circinatum* were found and characterized (Martínez-Álvarez et al. 2014b). They belonged to two novel species of the genus Mitovirus, *Fusarium circinatum* mitovirus 1 (FcMV1) and *Fusarium circinatum* mitovirus 2 (FcMV2) and are common among the isolates of the PPC pathogen in northern Spain (Vainio et al. 2015).

The use of mycoviruses to control fungal diseases of plants could be a promising method when the genetic diversity of the populations of the pathogen is low, for example, when the introduction of the fungus in a region is recent or when sexual reproduction is absent. This is the case with the pitch canker pathogen in Spain (Bergal et al. 2013). However there is still a long way to go in the development of a biological control tool using mycoviruses, and it requires first finding a virus producing hypovirulence then finding out the limitations in its transmission.

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