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Association levels between *Pityophthorus pubescens* and *Fusarium circinatum* in pitch canker disease affected plantations in northern Spain

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With 2 figures and 1 table

Abstract: *Fusarium circinatum*, the causal agent of pitch canker disease (PCD), poses a threat to *Pinus radiata* plantations due to the presence of bleeding cankers on the trunk that can cause the tree to die. This pathogen has been reported to be phoretically associated with bark beetle species, specifically, with *Pityophthorus* species 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. Specific aims were determined: i) to assess the phoretic association between *P. pubescens* and *F. circinatum*, 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. Funnel traps baited with (E)-pityol were established and twigs from infested trees were sampled to collect insects and plant tissues in PCD affected plots, with the aim of testing the presence of *F. circinatum*. Moreover, an experiment was carried out in nature 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 1.05% and 2% of the insects in the funnel traps during 2010 and 2012 respectively. Regarding 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 those on the trunk. This work affirms a weak association between *P. pubescens* and *F. circinatum* in our study area.

Keywords: *Pinus radiata* – pitch canker disease – twig beetle – phoresy

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1. Introduction

Fusarium circinatum Nirenberg and O'Donnell is an Ascomycete fungus causing pitch canker disease (PCD) on pines (Nirenberg & 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 &Roth 1946) but has also been observed in Haiti (Hepting & Roth 1953), California (McCain et al. 1987), Japan (Muramoto & Dwinell 1990), South Africa (Viljoen et al. 1994), Mexico (Guerra-Santos 1998), Chile (Wingfield et al. 2002), Korea (Cho & Shin 2004), France (EPPO 2004), Spain (Landeras et al. 2005), Italy (Carlucci et al. 2007), Uruguay (Alonso & 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* (Mirb.) Franco (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* (Marsh.), *Hylurgops palliatus* (Gyllenhal), *Ips sexdentatus* (Börner), *Hypothenemus eruditus* (Westwood), *Hylastes attenuatus* Erichson, *Orthotomicus erosus* (Wollaston), (Romón et al. 2007) and *Tomicus piniperda* L. (Bezos et al. 2015). 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 & Hofstetter 2015). The presence of this insect species on the attacked crowns can be observed due to 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 & Hofstetter 2015). This is the case with *Pityophthorus pubescens* which is present 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 & 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.

2. Material 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.

2.1. Funnel traps sampling

Four funnel traps baited with (E)-pityol (Econex) were established in a *P. radiata* plot affected by *F. circinatum* in Vejorís (Cantabria, northern Spain) from July to September 2010 and two were placed from June to September 2011, 2012 and 2013. (E)-pityol is an aggregation pheromone of the genus *Pityophthorus* (Dallara et al. 2000) which specifically in *P. pubescens* attracts both males and females (López et al. 2011). Insects collected during 2010 were plated on *Fusarium* selective media (FSM: 15 g bactone peptone, 1 g KH_2PO_4 monobasic, 0.5 g $MgSO_4\_7H_2O$, 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 & 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.

2.2. Isolation of *Fusarium circinatum* from red twigs

Twigs from *P. pubescens* infested trees were sampled in a pitch canker affected plot located in Cantabria (Spain), in order to collect both insects and plant tissues. With the aim of isolating *F. circinatum,* insects and plant tissues were plated on PDAS culture media as described above.

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 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 (Fig. 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).

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

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 assess 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 in the earlier stages of 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 choice 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).

3. Results

3.1. Funnel traps sampling

A total of 263 specimens of *P. pubescens* were collected from the funnel traps. From the 98 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 1.02% 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 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. The number of collected insects depending on the funnel trap is represented on Table 1.

Table 1. Number of *Pityophthorus pubescens* collected in the different funnel traps (P1, P2, P3 and P4) during the sampling period.

Year/Trap	P1	P2	P3	P4
2010	67	10	8	58
2011	20	23	-	-
2012	34	64	-	-
2013	11	13	-	-

3.2. Isolation of *Fusarium circinatum* from red twigs

A total of 215 specimens were collected from 424 galleries within the twigs in 2012 and 2013. 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). *F. circinatum* was found in 3 galleries collected in the month of July, two of them were engraved in the twig bark and one in the pith, corresponding to 0.75% of the collected samples. In 2013, a total of 24 galleries were collected but no isolate of *F. circinatum* was found within them.

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.

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 Fig. 2.

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 precipitation were 19.2 °C, 76.7% and 22 mm respectively in summer 2012; 11 °C, 73% and 102 mm in autumn 2012; 8.9 °C, 75.3% and 256.9 mm in winter 2013 and 19.9 °C, 77.5% and 20.7 mm in summer 2013 (www.airecantabria.com).

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 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 the two 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 plots was not significantly different at any observation time.

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.

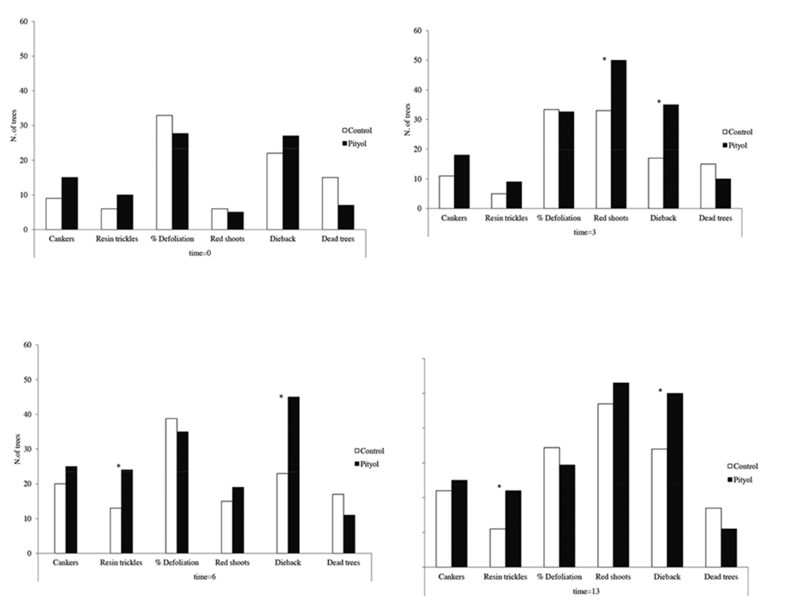

Fig. 2. Number of trees affected by cankers, resin trickles, red shoots, dieback and dead and mean value of defoliation in the pityol baited and unbaited trees for the different observation times.

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 consistent 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, although Romón et al. (2007) found *F. circinatum* in the 25% of the collected insects. However, phoresy rates may vary depending on two factors: the insect species; for example, *F. circinatum* was found on 0–13.69% of *Pityophthorus carmeli* Swaine while it appeared between 0–2% of the *Pityophthorus setosus* Blackman 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 engraved by the insects. Vegetal tissue from the red twigs found in our study area do not show pitch canker disease symptoms. Thus, it is necessary to highlight the importance of the pitch canker infection level of branches. 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). In the case of *P. pubescens* this association has not been observed in *P. radiata* stands in Cantabria. However, other 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. It is also important to highlight that it was observed a difference between the number of twig galleries collected during 2012 (400) and 2013 (24), this fact could be due to the low population levels of this insect species in the study area during the year 2013, making *P. pubescens* a weak vector of *F. circinatum*.

The low phoretic rates found in our study area could be due to the fact that this insect species were not associated with PCD symptomatic branches in our study area, since cankers or resin drops were not observed in the infested branches. Moreover, infested twigs might not be fresh enough for the pathogen development as it happens in the case of *T. piniperda* (Bezos et al. 2015) that feed on fresh shoots (Långström 1982).

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* pheromones 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 suggests that 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 effectiveness 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, 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 dead branches, moreover, the population levels found in our study are not especially high as it was found at endemic levels and not as an epidemic pest.

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