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Endophytic characterization of *Fusarium circinatum* infecting herbaceous plants in a symptomatic Monterey pine plantation

Hernandez-Escribano, L.[1,2], Iturritxa, E.[3], Elvira-Recuenco, M.[1], Berbegal, M.[4], Campos, J.A.[5], Renobales, G.[6], García, I.[7], and Raposo, R.[1,8]

[1] Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Centro de Investigación Forestal (INIA-CIFOR), Ctra. La Coruña, Km.7.5, 28040, Madrid, Spain

[2] Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, 28040-Madrid, Universidad Politécnica de Madrid, Spain

[3] NEIKER, Granja Modelo de Arkaute. Apdo 46. 01080 Vitoria-Gasteiz, Spain

[4] Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera s/n 46022 Valencia, Spain

[5] Universidad del País Vasco UPV/EHU, Apdo.644, 48080 Bilbao, España

[6] Apdo 5039, 01080 Vitoria-Gasteiz, Spain

[7] Arkaia 65, 01193 Arkaia Álava, Spain

[8] Instituto de Gestión Forestal Sostenible (iuFOR), Universidad de Valladolid/INIA, Spain

*Corresponding author.

E-mail address: hernandez.escribano@inia.es

Phone number: +34660804351

Abstract

Fusarium circinatum was recently detected as an endophyte in grasses causing no apparent damage. Our goal was to describe the endophytic colonization of herbaceous host plants growing in a plantation of *Pinus radiata* with symptoms of pitch canker disease, which may act as reservoir of inoculum. We detected the fungus in five species of dicots families (Asteraceae, Lamiaceae, Rosaceae), besides two species of Poaceae. It was found in the aerial part of non-symptomatic hosts, so we describe *F. circinatum* as an endophyte mainly transmitted by spores through the air. However, vertical transmission occurs at least in *Hypochaeris radicata.* SSR markers analyses showed a unique haplotype regardless isolates origin, pine cankers or non-symptomatic herbaceous plants. Thus, the same genotype can adopt a pathogenic or endophytic lifestyle. Non-symptomatic plants can act as reservoir of inoculum: pine seedlings can be infected from senescent tissue of non-symptomatic host colonized by the fungus.

Keywords: *Pinus radiata*, non-symptomatic host range, fungal transmission, pitch canker disease, reservoir of inoculum, population structure.

Introduction

In plant-fungal interaction research, emphasis has been done on plant pathogens and their outcome (Stergiopoulos and Gordon, 2014), as well as on arbuscular mycorrhizae and on endophytes (Zabalgogeazcoa, 2008; Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). Traditionally, a fungus that cause symptoms in the host was defined exclusively as a pathogen, not taking into account the possibility of an endophytic lifestyle in other hosts (i.e. colonizing tissue of living plants without causing symptoms). However, there are a growing number of examples in the literature where a fungal species can be pathogenic to some plants while only endophytic in others. So, *Verticillium dahliae* causes symptoms in at least 400 dicotyledonous species, but it is also an endophyte colonizing asymptomatic monocotyledonous plants (Malcolm *et al.*, 2013). *Colletotrichum gloeosporium* is another example. It causes fruit rot and serious damages in

postharvest (Cannon *et al.*, 2012), but it is also an endophyte of other plants (Rojas *et al.*, 2010). *Fusarium oxysporum* (Gordon and Martyn, 1997; Kuldau et al., 2000), *Phytophthora* spp. (Kroon *et al.*, 2012) and *Rosellinia necatrix* (Takemoto et al., 2014) are other cases reported lately with this duality of endophytic-pathogenic lifestyles. The terms 'symptomatic host plant' and 'non-symptomatic host plant' have been proposed to refer to these two situations in a specific plant-microbe interaction (Malcolm *et al.*, 2013). The former term involves plants showing symptoms of disease, while the latter implies an endophytic relationship between the plant and fungus. The outcome of a plant-microbe interaction depends not only on plant and microbe genotypes but also on other factors that include environmental conditions (Hardoim et al., 2015; Hily et al., 2016; Rai et al., 2016).

Fusarium circinatum is a fungus with this duality in pathogen-endophyte lifestyle. As a pathogen, it is the causal agent of pitch canker disease of Pines (PPC). Symptoms of PPC begin with branch dieback, and as disease progresses sunken cankers appear in stem or branches with abundant production of resin. Multiple infections of branches may happen and then, it causes severe canopy defoliation (Wingfield *et al.*, 2008). As an endophyte, *F. circinatum* colonizes grasses growing under *Pinus radiata* trees with symptoms of PPC (Swett and Gordon, 2012; Swett et al., 2014). Like other fungi mentioned above, *F. circinatum* cannot be distinguished morphologically when isolated from symptomatic or non-symptomatic plants, that is, Pines or grasses (Swett at al., 2014).

The fact that *F. circinatum* colonizes grasses in plantations with symptoms of PPC, arises a relevant question about the potential extension of non-symptomatic hosts serving as reservoirs of inoculum. So far, pathogen survival was studied in pines debris, not taking into account that it could colonize other species than *Pinus* spp. Survival in infected needles and wood fragments of *Pinus radiata* was measured on soil surface over time showing that pathogen can be hardly recovered from debris after two years. *F. circinatum* does not survive in soil either. It was not

isolated from samples of 2-mm-sieved soil taken in plantations with PPC (Serrano *et al.*, 2016). However, *F. circinatum* colonizing non-symptomatic grasses means a new inoculum source that needs to be studied.

Endophytes live at least part of their life cycle within plant tissues (Hardoim et al., 2015; Zabalgogeazcoa I. 2008). They infect new plants by either horizontal or vertical transmission. When a fungus is horizontally transmitted, plant to plant, it emerges to esporulate at plant or senescent tissue (Stone et al., 2004). If fungus is vertically transmitted, which is less frequent than horizontal transmission, it is via host seeds (Hardoim *et al.*, 2015). There is not any evidence of how transmission of *F. circinatum* may happen, and whether it takes place not only among herbaceous plants, but also from plants to pines. The only evidence is that isolates of *F. circinatum* obtained from vegetative tissues of non-symptomatic grasses were pathogenic to artificially inoculated pines, with virulence similar to isolates obtained from symptomatic trees (Swett et al., 2014).

Based on colonization characteristics, Rodriguez et al. (2009) define some classes within each of the two functional groups, i.e. Clavicipitaceous and Non-clavicipitaceous groups, in which endophytes are historically discriminated. We will use those characteristics to describe *F. circinatum* as an endophyte. They are: (1) host range; (2) Plant colonization, i.e. aerial or root tissues; (3) Transmission between hosts, horizontal or vertical; and (4) fungal genetic diversity harbored by colonized plants. *F. circinatum* has been isolated from grasses (Swett et al., 2014, Swett and Gordon, 2012), but we will explore other botanical families and species which are usually found in *P. radiata* plantations in Northern Spain, where this study is going to be done. Population genetic structure of *F. circinatum* was previously studied in Basque Country (NE Spain) with isolates coming from symptomatic trees. Results showed that isolates make up a homogeneous population, with only two VCG, all isolates of mating type 2, and three genotypes distinguished by AFLP markers (Iturritxa *et al.*, 2011). A recent study using eight microsatellites

markers (SSR) to analyze genetically a broader sample, revealed that Spanish population of *F. circinatum* is structured in two distinct groups, with two dominant genotypes, each one in each of those two groups. This study also found only one genotype in the sample of 15 isolates obtained in Basque Country (Berbegal et al., 2013). Therefore, given the clonality of *F. circinatum* in this region and the presence of only one genotype in the area identified by SSR markers, this condition offers a unique opportunity to compare genetically both populations coming from symptomatic and non-symptomatic hosts.

The final objective of this study was to describe and characterize the endophytic association of *Fusarium circinatum* with herbaceous plants growing in a *P. radiata* plantation with PPC in Northern Spain. We explored the non-symptomatic host range, studying if they are isolated from aerial or root tissues, and their type of transmission. When horizontal transmission occurs among plants, we assay if pines may also be infected. Finally, we studied if both subpopulations, one obtained from symptomatic trees and other from non-symptomatic plants, are genetically differentiated.

Materials and methods

Plant material sampling

Non-symptomatic host plants were collected beneath the canopy of a *Pinus radiata* plantation (100 x 55 m of surface) showing symptoms of PPC in Laukiniz (Basque Country, NE Spain) (Table 1). Sampling was initially planned to carry out in three one-meter squares plots randomly marked in the plantation; but this method was discarded when we checked that these plots were only covered with two species (*Agrostis capillaris* and *Pseudarrhenatherum longifolium*). These species were collected at four different points of the plantation chosen arbitrarily. For less abundant species, they were collected where found. Plants were sampled in three different months of year 2016: April, July and August (Table 1). The entire plant was collected and morphologically identified. Cankers from symptomatic pine trees in the same plantation were

sampled to collect an isolate of *F. circinatum* per tree. Vegetal material was stored at 4 ˚C until processed in the following days.

Isolation and identification of *F. circinatum*

For each plant sampled, stem and leaves were separated from root system and from seeds when they were present. Fungal endophytes were isolated following the disinfestation method described by Márquez et al. (2010). Leaves and stems were surface disinfested by immersion in a 20 % commercial bleach with Tween 20 (1 drop / 100 ml) solution during 10 minutes and soaked twice in sterile distilled water. Roots were carefully washed under tap water to remove any soil particle adhered, dipped into 70 % ETOH for 1 minute and following then the same procedure as for leaves and stems. When seeds were present (seeds from six plants of *Pseudarrhenatherum longifolium*, two from *Sonchus oleraceus*, two from *Centaurea debeauxii* and one from *Hypochaeris radicata*) they were briefly rinsed in 70 % ETOH, immersed in 20 % commercial bleach with Tween 20 solution during 3 minutes followed by two sterile distilled water washes. Plant material was aseptically transferred to sterilized filter paper and when dried, cut into 5 mm segments and placed in petri dishes with Fusarium Selective Medium (FSM) (Aegerter and Gordon, 2006) and incubated at 25 ˚C. In the case of seeds, all collected seeds for each plant were mixed, crushed using a sterile stick and placed on FSM too. For further characterization, one isolate from each plant was chosen making up a total sample of 28 isolates. For genetic analysis, isolate obtained from seeds was also included in the sample.

Cankers from symptomatic pine trees of the same plantation were sampled and surface disinfected by immersion in a 30 % commercial bleach solution with Tween 20 for 1 minute and washed twice in sterile distilled water (Pérez-Sierra *et al.*, 2007). Canker wood were cut into 1 cm pieces, placed on FSM and incubated at 25 ˚C. A total of 39 isolates were obtained, one isolate per tree.

For morphological fungal identification, putative colonies of *Fusarium* growing on FSM were transferred to Spezieller Nahrstoffarmer Agar (SNA) medium (Nirenberg, 1981) to confirm the species. Plates were incubated for 7-10 days at 25 ˚C, and then microscopically inspected for the formation of sterile hyphae (circinus), identifier of *F. circinatum* (Nirenberg and O'Donnell, 1998) Monosporic isolates were obtained and conserved on paper (Whatman nº 1) at -20 ˚C until use.

Molecular confirmation of *F. circinatum* was done by PCR using specific primers CIRC1A (CTTGGCTCGAGAAGGG) and CIRC4A (ACCTACCCTACACCTCTCACT) as described by (Schweigkofler et al., 2004) in a final volume per reaction of 25 µl with 1 µl DNA template. DNA was extracted from mycelia growing on PDA using E.Z.N.A. Plant DNA Kit (Omega Biotek), following the manufacturer's instructions and stored at -20 ˚C. PCR amplification was performed in a Veriti 96 well Thermal Cycler (Applied Biosystems), and its product was visualized in a 1% agarose gel (Agarose MS-12, Pronadisa), stained with RedSafe (RedSafe Nucleic Staining Acid Solution, Intron Biotechnology) and visualized under UV light. A 100 bp ladder was used as molecular weight marker (Biotools).

The presumptive identification was confirmed by sequence analysis. PCR amplified fragments were purified with Montage Genomic kit (Millipore Corporation, Bedford, USA) following the manufacturer's instructions and sequenced using CIRC1A primer direction. Resulting sequences were compared with the ones of *Fusarium* species present in the GenBank database (NCBI) by BLAST nucleotide search analysis.

Mating type determination

Idiomorphs MAT-1 or MAT-2 were determined for each isolate. A multiplex PCR assay was performed using primers MAT1p1 (AGAAACTGACTGATACATCAAGGGG) – MAT1p3 (TCATAAGAAGTGTTGAAGGAATCACAG) and GcHMG1 (CTTTACCGTAAGGAGCGTCACCAT) - GcHMG2 (TGATCCGCCATCTGCTTGTAGAGT) for alleles MAT-1 y MAT-2, respectively, as described by Wallace and Cover (2000) and Schweigkofler et al. (2004). A final volume of 25 µl

with 1 µl of DNA template per reaction was used following amplification conditions described by Berbegal et al. (2012). PCR products were visualized in a 1.5 % agarose gel under UV light. A 100 bp ladder was used as molecular weight marker. MAT-1 isolates amplify a 380 bp fragment, while MAT-2 produce a 190 bp amplicon.

Genotype diversity

The whole number of isolates (68 isolates) were used for genotype analyses, 29 obtained from non-symptomatic herbaceous hosts (28 from each plant and one from seeds) (Table 1) and 39 from *Pinus radiata* cankers. A reference isolate in representation of the haplotype of Basque Country (Isolate PV1) (Berbegal et al. 2012) and a negative control with no DNA were also included. Primers for eight SSR loci (FCM-2, FCM-4, FCM-6, FCM-7, FCM-19, FCM-24, FCM-25 and FCM-26) previously characterized and selected for reproducible polymorphism were used (Berbegal et al., 2013). Fluorescent labelling for each SSR locus was performed in one reaction using three primers (Schuelke, 2000): SSR forward primer with M13 tail (TGTAAAACGACGGCCAGT) at 5' end, SSR reverse primer, and the universal fluorochrome 6-carboxy-fluorescein (FAM) labelled M13 primer. PCR reaction was performed as described by Berbegal et al. (2013) in a final volume of 10 µl. Amplifications were conducted in 96 well plates and PCR products were purified by adding ExoSAP-IT PCR Product Cleanup (Affimetrix) following the manufacturer's instructions. PCR products were sized by capillary electrophoresis (Macrogen Europe Lab). Allele calls were performed using Geneious R10 10.0.9 Software.

When polymorphism for SSR loci was detected, confirmation was done by sequencing the PCR product of that specific SSR locus. To confirm the polymorphic variation, sequences of amplified loci were aligned and compared to the sequence of others isolates with no polymorphism, using Sequencher 5.0 software.

Pathogenicity tests

Pathogenicity test was conducted with all isolates obtained from non-symptomatic herbaceous hosts and one known virulent strain of *Fusarium circinatum* representative of the fungal population analysed in the Basque Country (Isolate CECT20759) (Iturritxa et al., 2011) as positive control. Two-year-old *Pinus radiata* commercial seedlings were acclimatized in a greenhouse during 1 week at 20 – 22 ˚C and a photoperiod of 12h light / 12h darkness. Each isolate was cultured on PDA for 7-10 days in darkness. Mycelium of each isolate was scrapped off the agar plate with a sterile needle and inoculated by wound in the pine stem parallel to the stem axis (Elvira-Recuenco *et al.*, 2014). The wound was made approximately at 10 cm above soil level. A sterile needle without inoculum was used to wound plants as negative control. Plants were covered with a plastic bag during 24 h in order to maintain high humidity and favour fungal infection. Five seedlings were randomly inoculated per each isolate. Lesion length was measured at the 5th post-inoculation week. Re-isolation of the pathogen from 2 plants of each isolate on FSM and SNA were conducted to confirm that inoculated isolates were responsible for the lesions observed. One way ANOVA with Tukey's comparison test was performed using Statgraphics Centurion to compare lesion lengths.

Horizontal transmission from herbaceous plants to pines under controlled conditions

A qualitative assay was performed to evaluate if pine seedling colonization is viable by inoculum coming from non-symptomatic herbaceous hosts but infected by *F. circinatum*. To do this, remaining aerial plant segments of those herbaceous plants from which the fungus was previously isolated, were surface disinfested following the same procedure as before and cut into 1 cm length fragments. These fragments were scattered over a sterile peat substrate (autoclaved for 1 hour, three days in a row). Total 60 *Pinus radiata* seeds were sown in two boxes of 13 x 10 cm containing the substrate described above, and other 30 pine seeds were sown in a box with no inoculum to serve as control. The boxes were incubated at 25 ˚C with a photoperiod 12 h light/12 h darkness and irrigated periodically. Seedlings were observed during

one month, and sampled when they showed symptoms of damping-off or at the end of the experiment. Pine seedlings were carefully washed with tap water to eliminate any substrate particles adhered and then immersed in 70 % ETOH solution for 30 s, 20 % commercial bleach with Tween 20 (1 drop / 100 µl) solution during 2 minutes, followed by two washes in sterile distilled water (Swett et al., 2016). Dry and disinfected pine material were cut into 5-mm segments and cultured on FSM. *F. circinatum* cultures grown in these medium were transferred to SNA for morphological confirmation.

Results

Based on morphological and molecular analyses, 28 isolates of *F. circinatum* were recovered from 165 non-symptomatic plants sampled from a *P. radiata* plantation (Table 1). These plants were identified as being of 16 different species (Table 1), from which *F. circinatum* was isolated in 7 species included in several plant families: *Agrostis capillaris* (Poaceae)*, Pseudarrhenatherum longifolium* (Poaceae)*, Centaurea debeauxii* (Asteraceae)*, Teucrium scorodonia* (Lamiaceae)*, Sonchus oleraceus* (Asteraceae)*, Rubus ulmifolius* (Rosaceae) and *Hypochaeris radicata* (Asteraceae). The fungus was recovered from leaves and stem and was never obtained from the root system. Seeds were processed when present and only in that plant of the *Hypochaeris radicata* species the fungus was isolated, thus vertical transmission was confirmed at least in this species (Table 1).

Two species were infected in high proportion although sampled in a low number. They were *Sonchus oleraceus*, which was the species most infected (67 %) with 4 infected plants out of 6 (Figure 1), followed by *Teucrium scorodonia* (40 %) with 2 infected plants out of 5 (Figure 1). *Hypochaeris radicata* plants had the lowest frequency of infection (9 %). The most abundant species in the plantation were grasses (Poaceae) with a total of 60 plants sampled for *P. longifolium* and 30 plants for *A. capillaris*. Despite being the most abundant during the three months of sampling, the number of infected plants from which *F. circinatum* was recovered was

lower. Only 20 % and 18 % of plants were infected for *A. capillaris* and *P. longifolium* (Figure 1), respectively. *F. circinatum* was not recovered from species *Crocosmia crocosmiiflora, Anthoxanthum odoratum, Taraxacum officinalis, Luzula multiflora, Holcus mollis, Brachypodium rupestre, Dactylis glomerata, Rumex acetosa* and *Cortaderia selloana*. *Fusarium circinatum* isolated from cankers of symptomatic pine trees were also confirmed by morphological and molecular methods.

Figure 1: Number of infected plants over the total sampled for each species where *Fusarium circinatum* was detected.

Regarding determination of fungal mating types, results showed that all isolates, from either pine cankers or non-symptomatic herbaceous plants, were exclusively MAT-2.

Analysis of genotype diversity by SSR markers revealed that all isolates were identical among them and they did not show polymorphism for any SSR loci. Neither do haplotypes were differentiated on the basis of their origin, symptomatic or non-symptomatic hosts. All isolates presented only a peak size of 178, 153, 251, 190, 173, 124, 204 and 244 bp resulted for the SSR loci FCM-2, FCM-4, FCM-6, FCM-7, FCM-19, FCM-24, FCM-25 and FCM-26, respectively. No peak was detected in the negative controls. The exception to this genetic uniformity was due to one isolate coming from a canker, which showed polymorphism for FCM25 SSR locus. The SSR peak had a size of 199 bp instead of 204 bp. Sequentiation of this amplicon showed that it had one repetition less (AGACA) than the two other isolates used for comparison.

All *F. circinatum* isolates from non-symptomatic plants resulted pathogenic to *P. radiata*. Lesion size measured at the 5^{th} week after inoculation ranged from 2.4 ± 0.75 to 4.5 ± 0.45 cm, and the mean lesion caused by the pathogenic reference isolate was 3.5 ± 1.02 cm. No lesions were developed in the non-inoculated plants. No significant differences were found in lesion length among isolates (p-value=0.6837). *F. circinatum* was successfully recovered from the sampled inoculated pines and its identity was morphologically confirmed.

Horizontal transmission from non-symptomatic plants to pines in controlled conditions

Results show that non-symptomatic herbaceous hosts segments fallen on the soil but infected by *F. circinatum* may act as inoculum reservoir. Pine seedlings resulted infected with *F. circinatum* when growing in peat soil with senescent infected plant tissue on it. In the first and second box the 80 % and 87 % over emerged plants developed disease symptoms, respectively, while those emerged seedlings grown in sterile peat did not show any disease symptom. When plating symptomatic pine seedlings on FSM all of them resulted infected by the fungus and no pathogen was detected in seedlings growing in a sterile soil. *F. circinatum* was detected generally in the collar region of pine seedlings. Regarding emergence, in the first box emerged only the 63 % of seedlings referred to the control (24 seedlings out of 30) while in the second box, 96 % of seedlings emerged.

Discussion

We confirm the presence of *F. circinatum* in alternative non-symptomatic hosts to pines in Spain, as reported before in South Africa and California (Swett and Gordon, 2012; Swett et al., 2014). Furthermore, we report for the first time the presence of *F. circinatum* in plants of families different from Poaceae, grasses where initially the fungus was found. Besides grasses, families reported here are the dicotyledoneae Asteraceae, Rosaceae and Lamiaceae, and this finding implies that the non-symptomatic host range is broader than initially thought. Endophytic association of *F. circinatum* with grasses was explained, at least in part, on the basis that this fungus is phylogenetically close to other *Fusarium* species that are able to colonize grasses, such as *F. subglutinans* which is besides interfertile with *F. circinatum* (Desjardins et al., 2000). However, the fact that the fungus is found in other plant families not close to grasses suggest that this endophytic association is related to the capacity of *F. circinatum* of adopting different lifestyles other than the parasitism when colonizing their hosts. There are other reports that include some examples of fungi with different lifestyles according to hosts: *Colletotrichum*

magna which causes anthracnose in cucurbit plants, but it colonizes non-cucurbit species in an endophytic lifestyle (Kogel et al., 2006); Verticillium dahliae, pathogenic in dicots while endophytic in monocots (Malcolm *et al.*, 2013); *Fusarium oxysporum* a pathogen that can colonize root system and move through the cortical tissue of susceptible host causing wilt disease in many economically important crops (Gordon and Martyn, 1997) and at the same time is one of the fungi that commonly appears as endophyte in other plants (Kuldau et al., 2000). On the other hand, some fungi known as endophytes can behave as pathogens under changed environmental conditions (Delaye et al., 2013). For example, the same strain of *Leptosphaeria maculans* was found in completely asymptomatic *Arabidopsis thaliana* plants under natural conditions, but the fungus become necrotrophic in a more stressful situation (Junker et al., 2012). Similarly, *Diplodia mutila*, endophyte living within tropical palm, can cause necrosis under high light intensity (Álvarez-Loayza *et al.*, 2011). An endophyte usually colonizes the intercellular spaces of the tissues of its hosts without inducing symptoms (Hiruma *et al.*, 2016; Garrido-Jurado *et al.*, 2017). Studies show that *Fusarium circinatum* is able to colonize the intercellular spaces of pine roots, and only the first symptoms appear when the fungus reaches the vascular (Martín-Rodrigues *et al.*, 2015; Swett et al., 2016). It is possible that colonization and invasion of *F. circinatum* in herbaceous plants is limited to intercellular space without causing symptoms.

The outcome of a plant-microbe interaction depends on many factors that include plant and microbe genotypes and environmental conditions (Hardoim et al., 2015; Hily et al., 2016; Redman et al., 2001). Given that we have detected a unique haplotype (with only one exception) in the fungus population collected either from symptomatic pines or non-symptomatic herbaceous plants, our study shows that the same genotype of *F. circinatum* can have a lifestyle as endophyte or as pathogen. This situation, where host plays no role in the haplotype distribution of the fungus population, has been also reported for the population of *Lasiodiplodia theobromae*, where the same haplotypes were found in all host species (Mohali et al., 2005). Moreover, in the case of *F. circinatum*, it is highly possible that the outcome of the plant-fungus

interaction relies mainly on the plant genotype since plants and pines are growing in a common environment.

Based on our results we describe *F. circinatum* as a foliar endophyte in non *Pinus* sp, mainly transmitted by spores that colonize the aerial part plant. We have only isolated the fungus from leaves and/or stem, but never from roots. This corroborates our previous results in the sense that *F. circinatum* was not found in the soil sampled from different plantations with symptoms of PPC (Serrano *et al.*, 2016). Composition of endophyte population is partially shaped by the environment where it originates (Hardoim *et al.*, 2015), that is, the air for the foliar endophytes and the soil for root endophytes. Therefore, our results indicate that non-symptomatic hosts are colonized by spores from the air, but we cannot determine whether those spores are coming from tree cankers, from other floor plants or from both. Many endophytes spread horizontally from plant to plant. This is the case of *Epichloë* (anamorph *Neotyphodium*), endophyte of several grasses. The fungus produces epiphyllous conidia on the leaf surface spreading horizontally from plant to plant (White et al., 1996). *Neotyphodium* is another example of dissemination from *Poa ampla,* but in this case conidia are spread by air currents containing water (Tadych *et al.*, 2007).

Vertical transmission of *F. circinatum* is also possible. We isolated the fungus from seeds of one plant of *Hypochaeris radicata,* likely colonizing the inner seed and not its coat, (it was surface disinfested before plating seeds). Endophytic fungus can be transmitted vertically via seed, like the case of *Epichloë festuca*, common symbiont in *Festuca* and *Lolium*. The fungus has the ability to live inside above ground host tissue and disseminate via seeds (Gundel et al., 2009; Schardl et al., 2001).

F. circinatum is transmitted in pines by spores either through the air or via insects (Wingfield et al., 2008). Our assay has shown that pine seedlings can be infected from senescent tissue of non-symptomatic plants colonized by the fungus. Some herbaceous plants sampled here are annual or biannual, so when plants die senescent tissue fall on the soil. Other plants are perennial and

when a new leaf has been stablished, the older and lowermost leaves often senesce (Moore and Moser, 1995). Sporulation of endophytes is frequent to occur in this way, when infected host tissue dies (Sánchez Márquez et al., 2007; Zabalgogeazcoa, 2008), so we showed that non-symptomatic plants may constitute an inoculum source in the plantation or even in new plantations. Nowadays it is mandatory a minimum of two years lag before a new plantation of pines can be done in Spain. The importance that non-symptomatic plants may have as inoculum reservoir needs to be quantified, since frequency of infected plants and inoculum density may decrease in absence of symptomatic hosts.

But non-symptomatic hosts may act not only as a reservoir of inoculum but also as a reservoir of genetic diversity of the fungus. However, this is not the case even here that the sampling is much more intense (62 isolates from a plantation of 400 m2). We confirm the genetic homogeneity of *F. circinatum* population in this Northern region: one common haplotype, the same that was detected in a previous analysis (Berbegal et al., 2013); with only allele MAT-2 detected, in agreement with other studies (Iturritxa *et al.*, 2011; Berbegal et al., 2013) that only detected this mating type.

Several studies attribute ecological functions to endophytes in plants they are colonizing. These studies refer specially an increase in resistance or tolerance to plant stress, either of biotic or abiotic condition (Redman *et al.*, 2011; Hardoim *et al.*, 2015). Increase in tolerance to water stress (Ravel *et al.*, 1997; Bae *et al.*, 2009), in resistance to diseases and herbivory are reported (Siegel *et al.*, 1990; Herre *et al.*, 2007). But there are other studies which do not find any ecological function (at least so far) for the endophytes (commensalism). We do not know the role that *F. circinatum* may have in these plants, but when causing no symptoms (cryptic infections) in pines (Evira-Recuenco et al., 2015; Martín-Rodrigues et al., 2013) it increases growth rate of seedlings (Swett and Gordon, 2017). It also increases resistance to posterior infections with the pathogen (Swett and Gordon, 2017).

Finally, a brief comment about the method we used to isolate endophytic fungi. We used an intensive method for superficial disinfestation of plant parts before plating them on selective growth media. It is possible that using shorter times of disinfestation like those used in previous works (Swett et al., 2014), frequency of colonization by *F. circinatum* could be higher than we found here or even that new species could be reported.

Our findings evidence the potential risk of spread of *Fusarium circinatum* across non-symptomatic species that serve as a reservoir of inoculum and provide insights into the epidemiology of the pitch canker disease. Further studies trying to determine the role of *Fusarium circinatum* as an endophyte is needed in order to have a more comprehensive understanding of evolution, dynamics and ecology of plant-fungi interaction.

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