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Next generation sequencing (NGS) and quantitative PCR (qPCR) as tools to detect *Fusarium circinatum* in different pine species

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TABLE OF CONTENTS

ABSTRACT

Fusarium circinatum, the causal agent of pine pitch canker (PPC) disease, has already been reported worldwide. In Europe, it is considered a quarantine pest (A2) and in Spain it has been reported causing economic damages in *Pinus radiata* and other pine species. In order to detect *F. circinatum* present in samples of different origins, different techniques may be applied such as next generation sequencing (NGS) and quantitative PCR (qPCR). In this aspect, the aim of this study was to evaluate qPCR as a technique to detect *F. circinatum* in stem's samples of five pine species (*P. radiata*, *P. pinaster*, *P. nigra, P. sylvestris* and *P. uncinata*) and to compare the results with data obtained in a ITS-based massive sequencing. The NGS analysis was unable to detect *F. circinatum* in the samples analysed although other *Fusarium* spp. were detected. Quantitative PCR seemed to be affected by inhibitors present in the samples such as polysaccharides and phenolic compounds which in the case of some samples, was overcome by a nested PCR (conventional PCR followed by a qPCR). With the exception of *P. pinaster*, *F. circinatum* was detected in all the other pine species of which samples were recovered. In *P. radiata*, the fungus was detected not only at the inoculation point (15 cm) but also at 65 and 165 cm height, which brings interesting insights about the movement of the pathogen within the plant's tissues.

Keywords: real-time PCR, massive sequencing, *Fusarium circinatum.*

RESUMEN

Fusarium circinatum, el agente causal del chancro resinoso del género *Pinus*, ha sido reportado mundialmente. En Europa es considerado una plaga de cuarentena (A2) y en España causa daños económicos en pinos, siendo la especie más susceptible *Pinus radiata*. Para la detección de *F. circinatum* en muestras de diferentes orígenes, se puede aplicar diferentes técnicas como la secuenciación de nueva generación (NGS) y la PCR cuantitativa (qPCR). En este contexto, el objetivo del trabajo fue evaluar la qPCR como técnica de detección de *F. circinatum* en muestras de tronco de cinco especies de pinos (*Pinus radiata*, *P. pinaster*, *P. nigra*, *P. sylvestris* y *P. uncinata*) y comparar los resultados con los datos obtenidos en secuenciación masiva basada en la región ITS. Con esta última técnica no fue posible detectar *F. circinatum* en las muestras analizadas, sin embargo, otras especies de *Fusarium* sí fueron detectadas. La PCR cuantitativa fue exitosa, aunque en algunas muestras se vió afectada por inhibidores como polisacáridos y compuestos fenólicos, teniendo que recurrir a la PCR anidada (PCR convencional seguida de qPCR). Con excepción de *P. pinaster*, *F. circinatum* fue detectado en todas las especies de *Pinus* de las muestras recogidas. En *P. radiata*, el hongo fue detectado tanto en la zona de inoculación (15cm) como a 65 y 165 cm de altura, lo que nos proporciona una interesante información sobre el movimiento del patógeno en la planta.

Palabras clave: PCR en tiempo real, secuenciación masiva, *Fusarium circinatum.*

1. Introduction

One of the major threats that affect forest ecosystems are the diseases caused by organisms such as the fungi, which may lead to devastating consequences in forests causing a wide range of environmental and economic impacts. Among important pathogens, *Fusarium circinatum* (teleomorph: *Gibberella circinata*), the causal agent of pine pitch canker (PPC) disease, has been globally recognized as one of the most destructive organisms causing extensive tree mortality of *Pinus* species along with *Pseudotsuga menziesii*, both in the field and nursery conditions (Wingfield et al. 2008, Martínez-Álvarez et al. 2014). *Fusarium circinatum* has been reported worldwide including Europe: Spain (Landeras et al. 2005), France (EPPO 2006), Italy (Carlucci et al. 2007) and Portugal (Bragança et al. 2009); America: USA (Hepting & Roth 1946), Haiti (Hepting & Roth 1953), Mexico (Guerra-Santos & Cibrián-Tovar 1998), Chile (Wingfield et al. 2002), Uruguay (Alonso & Bettucci 2009), Colombia (Steenkamp et al. 2012) and Brazil (Pfenning et al. 2014); Asia: Japan (Muramoto & Dwinell 1990) and South Korea (Lee et al. 2000) and Africa: South Africa (Viljoen et al. 1994). Although *Pinus radiata* is the most susceptible species to *F. circinatum*, in Spain, for instance, other species of pine such as *P. nigra*, *P. pinaster*, *P. sylvestris* and *P. uncinata* were also proved to be susceptible to the pathogen (Landeras et al. 2005, Pérez-Sierra et al. 2007, Martínez-Álvarez et al. 2014, Martínez-Álvarez et al. 2016). In the world, at least 60 *Pinus* spp. have been reported to be susceptible to PPC disease (European Food Safety Authority 2010, Bezos et al. 2017).

Trees infected with *F. circinatum* exhibit reduced growth and degradation of timber quality resulting in important economic losses. In adult trees, the main symptom is a bleeding resinous canker on the stem or thick branches (Figure 1) and yellowing of the needles and dieback of the shoots resulting in significant crown dieback (Mullett et al. 2017, UK Forest Research 2017). This aggressive fungus can also cryptically infect pine seeds causing damping-off and wilting in seedlings (Swett et al. 2018). Seeds are one of the main pathways of introduction of *F. circinatum* into new countries and nurseries. However, in natural environments, spores' dispersion through wind and vectors, such as coleopteras of the genera *Ips* and *Pityophthorus* are important ways of dissemination (Elvira-Recuenco et al. 2015, Fourrier et al. 2015). In Spain, for instance, Bezos et al. (2015) have found that *Tomicus piniperda* (Coleoptera; Scolytinae) might act as vector of *F. circinatum* to *P. radiata* trees during its maturation or regeneration feeding on the shoots of healthy trees.

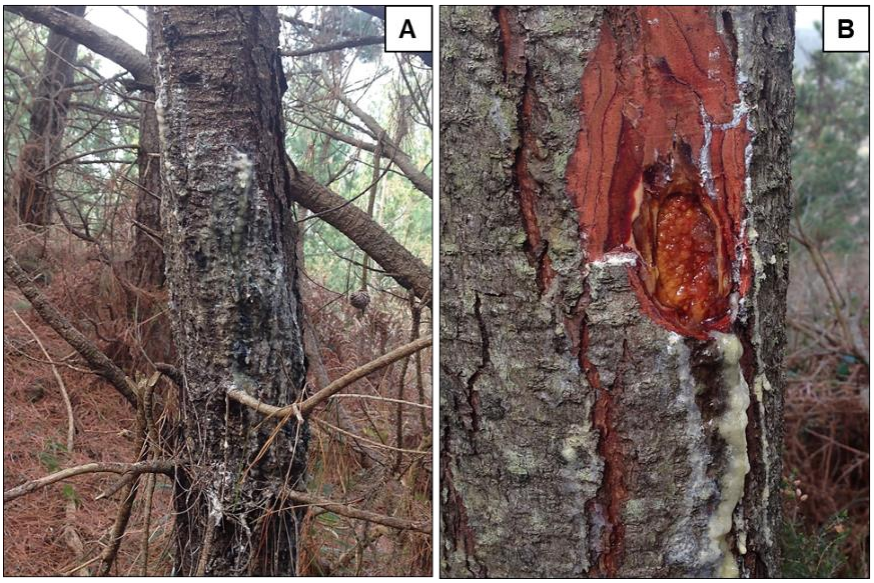

Figure 1. Bleeding canker (A) and necrotic tissue with resinosis in the stem of *Pinus radiata* in Cantabria, Spain (B).

Considering the enormous economic and ecological implications of this disease, in Spain, the Ministry of Agriculture, Fisheries and Food through a Royal Decree published in 2006, has forbidden the plantation of *Pinus* species and *Psedotsuga menziesii* in affected areas of the country (Ministerio de Agricultura Pesca y Alimentación 2006, Martínez-Álvarez et al. 2014). Nevertheless, there is still no strategy of management that has been successfully established for controlling the disease in both adult trees in forest plantations and seedlings in nurseries. Recent studies have investigated the treatment of pine seeds with hot water (Agustí-Brisach et al. 2012); the potential of fungal endophytes (Martínez-Álvarez et al. 2016) and *Trichoderma* spp. (Martínez-Álvarez et al. 2012; Martín-García et al. 2017) as biocontrol agents against *F. circinatum* and a possible hypovirulent effect caused by the presence of viruses in *F. circinatum* strains in Spain (Muñoz-Adalia et al. 2016, Flores-Pacheco et al. 2017). Notwithstanding, prevention of the pathogen introduction and spread in forests and nurseries is still the most recommended strategy to avoid dissemination and progression of the disease.

One of the most important steps before deciding which is the most appropriate strategy to manage a forest disease is the correct detection and identification of the causal agent. In the case of *Fusarium* species, detection and identification has been traditionally performed using morphological data and only in the last decades molecular techniques started to be adopted. Important morphological characteristics include the shape and size of macro and microconidia; color, size and shape of perithecia, as well as colony appearances, pigmentations and growth rates on agar media (European Food Safety Authority 2010).

However, the classical methods of identification of *Fusarium* species based on morphology requires specific skills, knowledge of traditional taxonomy, it is frequently laborious and time-consuming and it can easily result in misidentification (Mirmajlessi et al. 2015).

From the new technologies that may help in the detection and identification of plant pathogens, next generation sequencing (NGS), also known as high-throughput or massive sequencing, is a relatively new technology that has revolutionized genomic research since it allows the sequencing of millions of small fragments of DNA in parallel at the same time. It has as the main advantages the significant cost saving, shorter time to sequence and the higher sequencing accuracy because of deeper achievable coverage (Lin et al. 2012, Behjati & Tarpey 2013). There are a lot of different platforms for NGS which use different technologies, but Illumina is by far the most widely used platform (Cacho et al. 2016).

Illumina sequencing includes four basic steps: library preparation, cluster generation, sequencing and data analysis. The concept behind it is similar to capillary electrophoresis-based sequencing instruments. DNA polymerase catalyses the incorporation of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequential cycles of DNA synthesis. During each cycle, at the point of incorporation, the nucleotides are identified by fluorophore excitation. The critical difference is that, instead of sequencing a single DNA fragment, NGS extends this process across millions of fragments which varies in length from 2 x 150 bp to 2 x 300 bp (depending on the platform) in a massively parallel fashion (Illumina 2018).

Over the past few years, a wide range of polymerase chain reaction (PCR) assays have been developed and applied to the detection of plant pathogens. The emergence of real-time PCR, also known as quantitative polymerase chain reaction (qPCR) (Higuchi et al. 1993) revolutionized the studies on molecular biology and currently is considered one of the best methods for detection of plant pathogens in numerous hosts or environmental samples, since it allows high sensitivity (amplifies short DNA fragments from 70–100 bp) and is very specific in the detection of one or several pathogens in a single assay (Alemu 2014, TermoFisher 2018a). This technique has as the main characteristic the quantification of the DNA or RNA that is being amplified in real time while the amplification occurs, thanks to the non-specific fluorescent DNA dyes and fluorescently labeled oligonucleotide probes that reflects the momentary amount of DNA amplicons in the sample at a specific time based on the intensity of the fluorescent signal (Kralik & Ricchi 2017).

Using a small amount of starting material and combining nucleic acid amplification and detection, qPCR allows for efficiency and eliminates the post-amplification process such as the use of gel electrophoresis, and more importantly it enables the method to be quantitative (Biocompare 2018). Moreover, when compared to the conventional PCR method, qPCR has a wider range of applications including quantification of gene expression, microarray verification, quality control and assay validation, pathogen detection, SNP genotyping, copy number variation, MicroRNA analysis, viral quantification and siRNA/RNAi experiments (TermoFisher 2018b).

Different qPCR protocols have been published to detect *F. circinatum* in different types of samples. Some of them use SYBR Green dye whereas others use a probe-based as the detection dye. While SYBR Green are non-specific dyes which detects any double-stranded DNA generated during the PCR, probe-based detection methods rely on one or more fluorescently labeled probes that are positioned between the two PCR primers. Because the probe is sequence specific, it will only detect the presence of a single amplicon within the reaction (Sigma-Aldrich 2018).

The high sensitivity of qPCR can be exemplified, for instance, with a study of Fourrier et al. (2015) in France, in which individuals of *Ips sexdentatus*, one of the vectors of *F. circinatum*, were artificially contaminated, and the qPCR analysis was able to detect spores of *F. circinatum* to a level of ten conidia per individual and twenty conidia per batch of ten insects, which is below the lowest inoculum amount that occurs in nature, showing the high sensitivity of the technique. Other authors have also used qPCR to detect the presence of airborne inoculum of *F. circinatum* in infested sites in California, USA (Schweigkofler et al. 2004) and Galicia, Spain (Dvorak et al. 2017); the presence of spores in pine seeds (Ioos et al. 2009, Dreaden et al. 2012) and the fluctuation of airborne inoculum in a commercial pine seedling nursery in South Africa (Fourie et al. 2014).

The aims of this study were therefore: i) to compare the information obtained in the NGS and qPCR analyses in regard to the presence of *F. circinatum* and ii) to evaluate the suitability of qPCR as a technique to detect *F. circinatum* in stem's samples of five *Pinus* species.

2. Material and Methods

2.1. Study area and sampling

Samples were collected in a 9-years-old forest stand located in the municipality of Cabezón de la Sal, Province of Cantabria, Spain (Figure 2), composed by thirteen species (Table 1). The species were planted in June of 2009, and with the purpose of testing its susceptibility in field conditions, seedlings were inoculated with *F. circinatum* in November of 2010 (Martínez-Álvarez et al. 2014).

Figure 2. Location of the sampling site in the Province of Cantabria (red arrow) (A) and overview of the 9-years-old forest stand (B).

Table 1. List of species present in the studied site and their respective provenances (Modified of Martínez-Álvarez et al. 2014).

Species	Provenance of the seedlings
Abies alba	ESO2 Pirineo Central
Cedrus atlantica	Unidentified
Chamaecyparis lawsoniana	Unidentified
Cupressocyparis leylandii	Unidentified
Picea abies	East Europe
Pinus pinaster	ES08 Meseta castellana
Pinus nigra corsicana	902 Sud-ouest (France)
Pinus radiata	Unidentified
Pinus sylvestris	ES10 Sierra de Guadarrama
Pinus uncinata	ESC Sierra de Gudar
Pseudotsuga menziesii	430 Washington, Randle
Sequoiadendron giganteum	Unidentified
Thuja plicata lobii	Unidentified

From the thirteen species present in the field, five *Pinus* spp. were selected to be sampled including *P. radiata*, *P. pinaster*, *P. sylvestris*, *P. nigra* and *P. uncinata* (Table 2). The sampling methodology consisted in the collection of two pieces of stem's tissue of approximately 3 cm at 15 cm height using a sterilized blade (Figure 3a). In order to investigate the movement of *F. circinatum* in *P. radiata*, the most susceptible species to *F. circinatum* (Ministerio de Agricultura, Pesca y Alimentación 2006; Martínez-Álvarez et al. 2014), samples of stem were collected at four different heights (15, 65, 115 and 165 cm). Additionally, at 200 cm, a twig of approximately 30 cm was also collected. Once the samples were collected, the wounds were covered with a pruning sealer in order to avoid natural contaminations (Figure 3b). Each sample was deposited in an envelope and kept in a cooler at 4℃ in order to be transported to the laboratory.

Besides the stem's samples collected, a piece of four cankers' tissue (approximately 3 cm) present in *P. radiata* trees in a nearby infected stand (Figures 3c and 3d) were also collected in order to be used as positive control. In total, 40 samples were collected (Table 2).

Figure 3. Collection of samples at 15 cm using a sharp blade (A); *P. radiata* after sampling showing the wounds at different heights (B); canker in *P. radiata* exhibiting resinosis (C and D). Photo credit: Pablo Martínez Álvarez.

Table 2. Samples collected showing its respective species and height of recovery.

Code	Species	Sampling height (cm)	Code	Species	Sampling height (cm)
A3800B	*Pinus radiata*	15	A3800V	*Pinus radiata*	115
A3800C	*Pinus radiata*	65	A3800W	*Pinus radiata*	165
A3800D	*Pinus radiata*	115	A3800X	*Pinus radiata*	200
A3800E	*Pinus radiata*	165	A3800Y	*Pinus radiata*	canker
A3800F	*Pinus radiata*	200	A3800Z	*Pinus pinaster*	15
A3800G	*Pinus radiata*	canker	A38010	*Pinus pinaster*	15
A3800H	*Pinus radiata*	15	A38011	*Pinus pinaster*	15
A3800I	*Pinus radiata*	65	A38012	*Pinus pinaster*	15
A3800J	*Pinus radiata*	115	A38013	*Pinus sylvestris*	15
A3800K	*Pinus radiata*	165	A38014	*Pinus sylvestris*	15
A3800L	*Pinus radiata*	200	A38015	*Pinus sylvestris*	15
A3800M	*Pinus radiata*	canker	A38016	*Pinus sylvestris*	15
A3800N	*Pinus radiata*	15	A38017	*Pinus nigra*	15
A3800O	*Pinus radiata*	65	A38018	*Pinus nigra*	15
A3800P	*Pinus radiata*	115	A38019	*Pinus nigra*	15
A3800Q	*Pinus radiata*	165	A3801A	*Pinus nigra*	15
A3800R	*Pinus radiata*	200	A3801B	*Pinus uncinata*	15
A3800S	*Pinus radiata*	canker	A3801C	*Pinus uncinata*	15
A3800T	*Pinus radiata*	15	A3801D	*Pinus uncinata*	15
A3800U	*Pinus radiata*	65	A3801E	*Pinus uncinata*	15

2.2. Processing of the samples in the laboratory and DNA extraction

At the laboratory, samples were cut in small pieces and placed in 2 mL Eppendorf tubes (Figures 4a and 4b) in order to be lyophilised. The lyophilisation process was carried out in a Freeze Dryer Alpha 1-2 LD Plus (Figure 4c) for 48 hours. After lyophilisation, two steel balls-bearing were added in each tube in order to disrupt the cells and prepare the samples for DNA extraction. For this purpose, samples were left for one minute in a Mixer Mill (Model Retsch MM400) in a speed of 13000 rpm and finally it was kept at -20℃ before sending for DNA extraction. The DNA extraction was performed by Biome Makers Company located in Valladolid (Spain) using the PowerSoil® DNA isolation Kit designed for isolating genomic DNA from environmental samples.

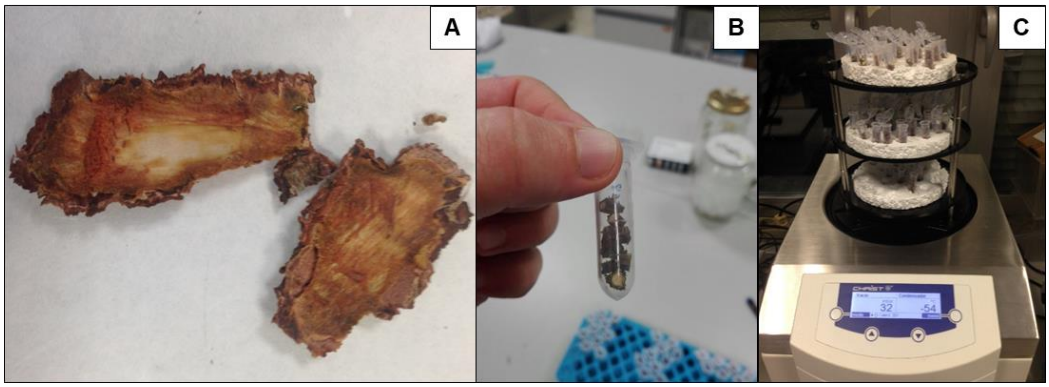

Figure 4. Stem's samples (A); eppendorf tubes containing small pieces of stem prepared for lyophilisation (B) and freeze dryer used for lyophilisation (C).

2.3. Next Generation Sequencing (NGS)

To obtain the NGS data, samples were lyophilised and submitted for DNA extraction and sequencing at Biome Makers, Valladolid. An ITS-based amplicon sequencing technology was used and the analyses performed using WineSeq® platform (Patent publication number: WO2017096385, Biome Makers). DNA from sample was purified by using Dneasy Powerlyzer Powersoil kit (Qiagen). ITS1 region was amplified using WineSeq® custom primers (Patent WO2017096385). Reads were generated using 2 x 301 bp paired-end sequencing with an Illumina MiSeq platform (Illumina, San Diego, CA, USA). A custom bioinformatics pipeline (Patent WO2017096385, Biome Makers) was used to remove adaptor and chimeras. After that, the reads were quality-trimmed and OTU clusters were performed using 97% identity. Taxonomy assignation and abundance estimation were obtained comparing Operational Taxonomic Unit (OTU) clusters obtained with WineSeq® taxonomy database (Patent WO2017096385).

2.4. Conventional PCR assay

The protocols for conventional PCR published by Ramsfield et al. (2008) and Schweigkofler et al. (2004) were tested using the original samples as well as dilutions of 1/100 and 1/1000. In addition, the protocol for qPCR published by Ioos et al. (2009) was adapted and tested in a conventional assay.

2.5. Quantitative PCR assay

Quantitative PCR (qPCR) reactions were performed with a QuantStudio 6 Flex Real-Time PCR System (Figure 5) following the methodology described by Ioos et al. (2009).

Amplifications were carried out in 20-µl reaction volume using the FastGene Probe 2X No Rox qPCR Universal Mix (Nippon Genetics). For each sample, the reaction mix included 0.6 µl of respective forward and reverse primers (0.3 µM), 0.2 µl of dual-labeled probe (Table 3) in a concentration of 0.1 µM, 2 µl of template DNA, 10.0 µl of qPCR Universal Mix and 6.6 µl of sterile distilled water (SDW). Quantitative PCR assays were carried out with samples in three different concentrations including: not diluted samples, dilution 1/100 and 1/1000. In addition, a nested PCR was performed amplifying PCR products obtained in a conventional PCR (adapted protocol of Ioos et al. 2009) in a qPCR assay.

The quantitative PCR cycling conditions included an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation for 15 s at 95°C, and annealing-elongation for 55 s at 70°C. The Ct value for each reaction was determined automatically by the software QuantStudio™ 6 Flex Real-Time PCR. Samples that presented a Ct value < 20 followed by an amplification curve with an exponential shape were judged as positive in relation to the presence of *F. circinatum.*

Table 3. Specific primers and probe used for the qPCR for the amplification of *F. circinatum.*

Nucleotide designation	Sequence	Source
FCIR-F	5'-TCGATGTGTCGTCTCTGGAC-3'	Ioos et al. (2009)
FCIR-R	5'-CGATCCTCAAATCGACCAAGA-3'	Ioos et al. (2009)
FCIR-P	5'-/56-FAM/CGAGTCTGGCGGGACTTTGTGC/3BHQ_1/-3'	Ioos et al. (2009)

Figure 5. QuantStudio 6 Flex Real-Time PCR System used for the real-time PCR reactions. Photo credit: Pablo Martínez Álvarez.

3. Results

3.1. Data obtained through next generation sequencing (NGS)

The results of the NGS have shown that *F. circinatum* was not detected in any of the samples analysed (Table 4), however, four species of *Fusarium* (*F. acutatum*, *F. oxysporum*, *F. keratoplasticum*, *F. delphinoides*) and *Fusarium* sp. were revealed by the massive sequencing. *Fusarium* species were detected in all pine species of which samples were collected (*Pinus radiata*, *P. pinaster*, *P. sylvestris*, *P. nigra* and *P. uncinata*). In addition, in three of the four samples collected directly from the cankers of *P. radiata* trees that were exhibiting symptoms of pitch canker disease, *Fusarium* spp. were detected.

Table 4. *Fusarium* species detected through NGS using an ITS-based amplicon sequencing technology (Biome Makers®).

Code	Species	Sampling height (cm)	*Fusarium* species
A3800B	*P. radiata*	15	*F. acutatum, F. oxysporum*
A3800C	*P. radiata*	65	-
A3800D	*P. radiata*	115	-
A3800E	*P. radiata*	165	-
A3800F	*P. radiata*	200	-
A3800G	*P. radiata*	canker	*F. keratoplasticum*
A3800H	*P. radiata*	15	-
A3800I	*P. radiata*	65	*F. acutatum, F. oxysporum*
A3800J	*P. radiata*	115	*F. delphinoides*
A3800K	*P. radiata*	165	-
A3800L	*P. radiata*	200	-
A3800M	*P. radiata*	canker	-
A3800N	*P. radiata*	15	*F. acutatum, F. delphinoides*
A3800O	*P. radiata*	65	*F. keratoplasticum*
A3800P	*P. radiata*	115	*F. acutatum*
A3800Q	*P. radiata*	165	*F. oxysporum*
A3800R	*P. radiata*	200	*F. delphinoides, F. keratoplasticum, Fusarium* sp.
A3800S	*P. radiata*	canker	*F. delphinoides, F.oxysporum*
A3800T	*P. radiata*	15	*F. acutatum, F. keratoplasticum*
A3800U	*P. radiata*	65	-
A3800V	*P. radiata*	115	*F. acutatum, F. delphinoides*
A3800W	*P. radiata*	165	-
A3800X	*P. radiata*	200	-
A3800Y	*P. radiata*	canker	*F. delphinoides*
A3800Z	*P. pinaster*	15	*F. acutatum, F. delphinoides*
A38010	*P. pinaster*	15	*F. acutatum, F. oxysporum*
A38011	*P. pinaster*	15	*F. delphinoides*

A38012	*P. pinaster*	15	*F. delphinoides*
A38013	*P. sylvestris*	15	*F. delphinoides, F. keratoplasticum*
A38014	*P. sylvestris*	15	-
A38015	*P. sylvestris*	15	*F. keratoplasticum*
A38016	*P. sylvestris*	15	-
A38017	*P. nigra*	15	*F. keratoplasticum, F. oxysporum*
A38018	*P. nigra*	15	*F. acutatum*
A38019	*P. nigra*	15	*F. acutatum, F. oxysporum*
A3801A	*P. nigra*	15	*F. acutatum, F. keratoplasticum*
A3801B	*P. uncinata*	15	*F. delphinoides, F. keratoplasticum, F. oxysporum, F. pseudensiforme*
A3801C	*P. uncinata*	15	*F. delphinoides, F. keratoplasticum, F. acutatum, F. oxysporum*
A3801D	*P. uncinata*	15	*F. acutatum, F. keratoplasticum*
A3801E	*P. uncinata*	15	*F. delphinoides, F. keratoplasticum, F. acutatum, F. oxysporum*

3.2. Conventional PCR assay

Amplicons were not observed in any of the protocols tested in a conventional PCR assay (Schweigkofler et al. 2004, Ramsfield et al 2008 and Ioos et al. 2009). Likewise, the different dilutions of the samples tested (1/100 and 1/1000) did not result in amplification for any sample.

3.3. Quantitative PCR assay

A set of different qPCR assays were performed with the original DNA (no dilution applied), diluted DNA (1/100 and 1/1000) and PCR products from a conventional PCR following an adapted protocol of Ioos et al. (2009). Following the criteria established to judge a sample as positive in relation to the presence of *F. circinatum* (Ct < 20 and exponential-shape of the amplification curve), based on the "*Diagnostic Protocol for Regulated Pests, DP22: Fusarium circinatum*", published by IPPC (2017), 15 samples were considered positive for *F. circinatum* (Table 5). The nested PCR resulted in the best amplification curves (Figure 7A and B) and it seems to be a good option in order to have a cleaner DNA and to avoid PCR inhibitors which might be cause of the jagged amplification curves observed in Figure 6A. Although in Figure 6B amplification curves from samples with Ct > 20 is shown, it is evident that the dilution of the samples to 1/100 seems to affect the shape of the curves provided that inhibitors may have less effect in the amplification process and fluorescence emission. When samples were diluted to 1/1000 no amplification was observed (Table 5). Even though in the nested PCR a high number of samples resulted in Ct values > 20, amplification curves were not exponential and thereafter they were judged as negative (Figure 8).

With the exception of *P. pinaster*, *F. circinatum* was detected in all species of which samples were recovered. In the case of *P. radiata*, *F. circinatum* was detected not only at 15 cm height but also at 65 and 165 cm which could be an indicator of the pathogen`s movement within the plant. From the four samples collected directly from cankers in *P. radiata*, in two of them *F. circinatum* was detected, which might raise questions regarding the accuracy of the technique since these samples must have come up as positives.

Table 5. Cycle threshold values obtained in a quantitative polymerase chain reaction (qPCR) with stem´s samples collected from pine trees located in Cantabria region, Spain.

Code	Species	Tree number	Sampling height (cm) or origin	Ct value				Presence of *F. circinatum*
				no dilution	1/100	1/1000	Nested PCR	
A3800B	*P. radiata*	1	15	undet	-	-	undet	absent
A3800C	*P. radiata*	1	65	undet	-	-	>20	absent
A3800D	*P. radiata*	1	115	undet	-	-	>20	absent
A3800E	*P. radiata*	1	165	undet	-	undet	4.265**	**present**
A3800F	*P. radiata*	1	200 (twig)	13.000	undet	-	>20	absent
A3800G	*P. radiata*	21	canker	11.009	undet	undet	5.504*	**present**
A3800H	*P. radiata*	2	15	>20	>20	-	29.633	absent
A3800I	*P. radiata*	2	65	>20	undet	-	11.410	**present**
A3800J	*P. radiata*	2	115	>20	undet	-	>20	absent
A3800K	*P. radiata*	2	165	>20	undet	-	>20	absent
A3800L	*P. radiata*	2	200 (twig)	undet	-	-	>20	absent
A3800M	*P. radiata*	22	canker	>20	>20	-	undet	absent
A3800N	*P. radiata*	3	15	undet	-	-	6.344	**present**
A3800O	*P. radiata*	3	65	>20	undet	-	4.215	**present**
A3800P	*P. radiata*	3	115	undet	-	-	>20	absent
A3800Q	*P. radiata*	3	165	undet	-	-	>20	absent
A3800R	*P. radiata*	3	200 (twig)	undet	-	-	>20	absent
A3800S	*P. radiata*	23	canker	12.428	>20	undet	<4 (undet)	**present**
A3800T	*P. radiata*	4	15	7.638	>20	undet	9.025	**present**
A3800U	*P. radiata*	4	65	undet	-	-	undet	absent
A3800V	*P. radiata*	4	115	>20	undet	-	18.252	absent
A3800W	*P. radiata*	4	165	undet	-	-	9.651	**present**
A3800X	*P. radiata*	4	200 (twig)	undet	-	-	>20	absent
A3800Y	*P. radiata*	24	canker	undet	-	-	undet	absent
A3800Z	*P. pinaster*	5	15	>20	>20	-	undet	absent
A38010	*P. pinaster*	6	15	undet	-	-	>20	absent
A38011	*P. pinaster*	7	15	15.026	>20	undet	undet	absent
A38012	*P. pinaster*	8	15	>20	undet	undet	undet	absent
A38013	*P. sylvestris*	9	15	undet	-	-	undet	absent
A38014	*P. sylvestris*	10	15	>20	undet	-	undet	absent
A38015	*P. sylvestris*	11	15	15.636	undet	undet	11.023**	**present**
A38016	*P. sylvestris*	12	15	>20	undet	-	13.405	**present**
A38017	*P. nigra*	13	15	16.083	undet	undet	17.857	**present**
A38018	*P. nigra*	14	15	16.599	17.623	undet	>20	absent
A38019	*P. nigra*	15	15	>20	undet	-	4.629	**present**
A3801A	*P. nigra*	16	15	undet	-	-	13.407	**present**
A3801B	*P. uncinata*	17	15	undet	-	-	>20	absent

A3801C	*P. uncinata*	18	15	undet	-	-	6.777	**present**
A3801D	*P. uncinata*	19	15	undet	-	-	>20	absent
A3801E	*P. uncinata*	20	15	14.653	undet	-	6.256	**present**
F85	Positive control	-	*F. circinatum* culture	10.868	-	-	-	absent
FCCa6	Positive control	-	*F. circinatum* culture	24.148	>20	>20	< 4 (undet)	**present**
F42	Positive control	-	*F. circinatum* culture	25.622	18.691	-	< 4 (undet)	**present**

*Sample diluted 1/100

**Sample diluted 1/10

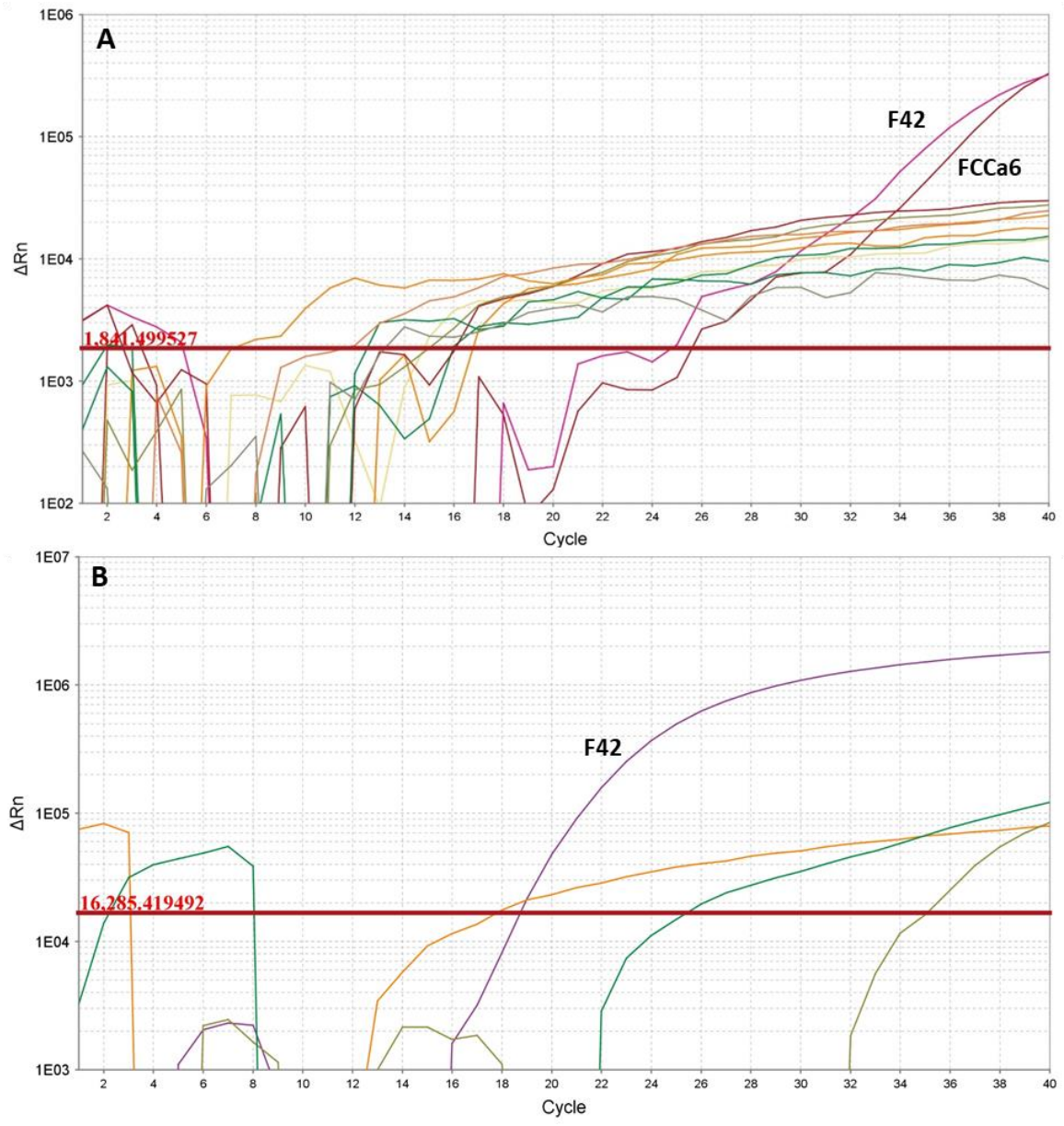

Figure 6. Amplification curves (logarithmic representation) generated in a qPCR assay (Ioos et al, 2009) with DNA extracted from *Pinus* spp. Graph A includes only curves from samples (no dilution applied) that resulted in a Ct value < 20. Graph B includes samples (Dilution 1/100) that resulted in amplification curves with no jagged pattern. FCCa6 and F42 are

positive controls (DNA extracted from pure cultures of *F. circinatum*). Target threshold was generated automatically by the Software QuantStudio Real-Time PCR v.1.2.

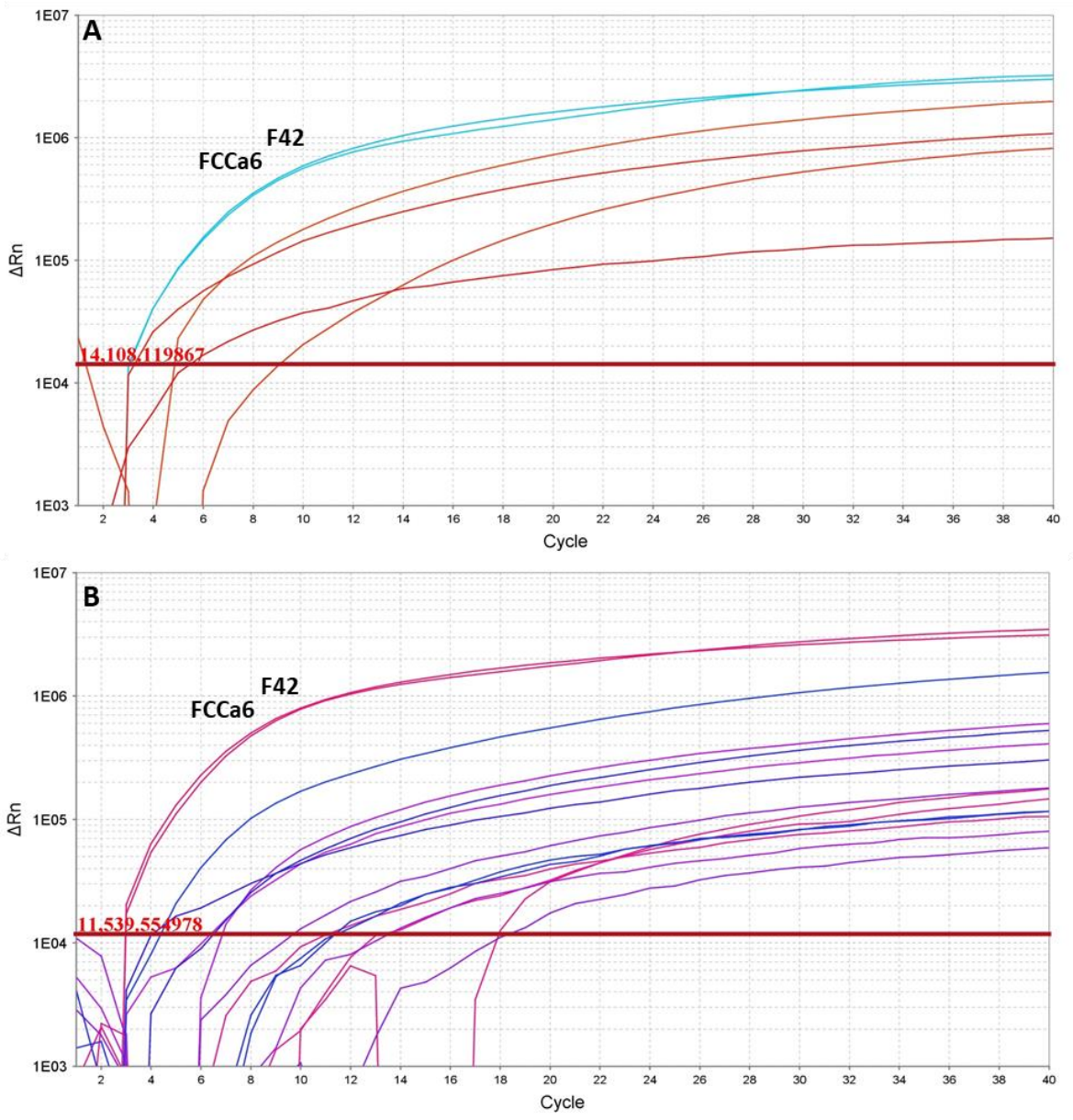

Figure 7. Amplification curves (logarithmic representation) generated in a qPCR (Ioos et al, 2009) with PCR products from a conventional assay (adapted protocol of Ioos et al. 2009). Graphs A refers to a first trial (only 8 samples included) and graph B refers to a second trial (32 samples included). Graphs A and B includes only curves from samples that resulted in a Ct value < 20. FCCa6 and F42 are positive controls (DNA extracted from pure cultures of *F. circinatum*). Target threshold was generated automatically by the Software QuantStudio Real-Time PCR v.1.2.

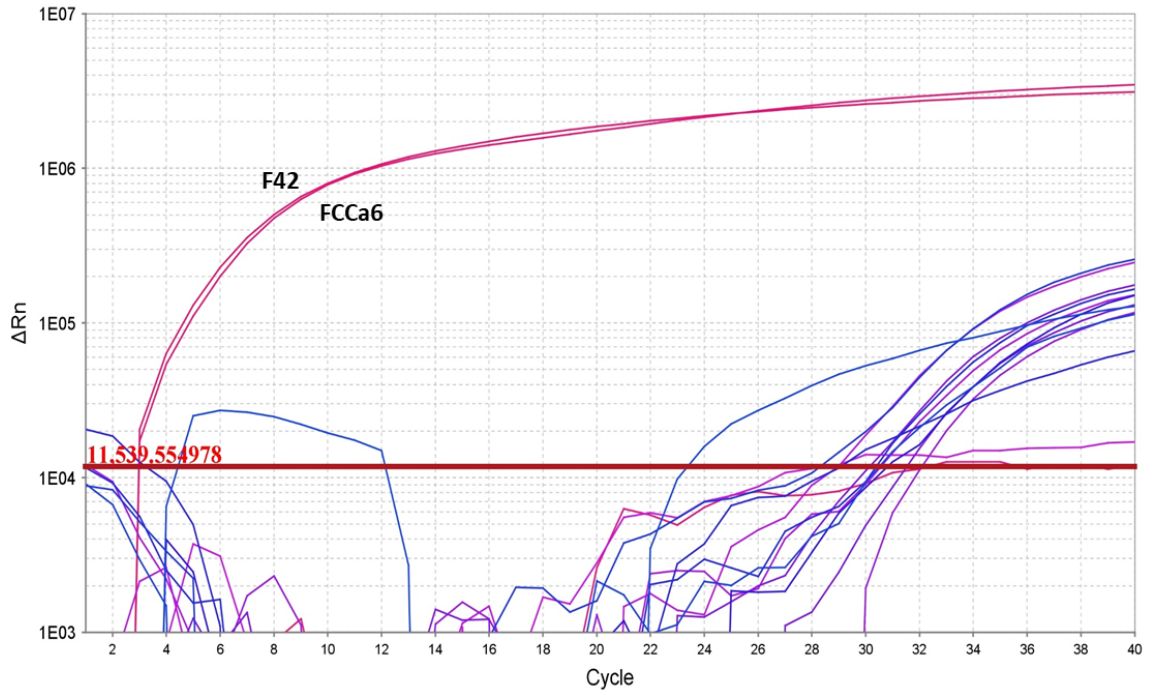

Figure 8. Amplification curves (logarithmic representation) generated in a qPCR with PCR products from a conventional assay. Only curves from samples that resulted in a Ct value > 20 were included. FCCa6 and F42 are positive controls (DNA extracted from pure cultures of *F. circinatum*). Target threshold was generated automatically by the Software QuantStudio Real-Time PCR v.1.2.

4. Discussion

In the recent years, the identification and detection of plant pathogens through traditional methods such as growing the target organism in culture media, has been put aside when compared to the enormous amount of molecular techniques currently available. In spite of the fact that it provides important information, the detection and identification of fungi based exclusively on morphology might result in misidentification since it requires specific skills and knowledge of traditional taxonomy (Mirmajlessi et al. 2015). For instance, in *F. circinatum*, the presence of sterile sinuous hyphae has been used as a morphological characteristic which separate this species from other closely related species (IPPC 2017), nevertheless, Mullett et al. (2017) have shown that non-coiled sterile hyphae can also be present in *F. circinatum* isolates which makes this characteristic not as unique of *F. circinatum* as previously believed. Following this aspect, the main goal of this study was to detect the presence of *F. circinatum* in pine trees using qPCR: a technique that provides a quick answer, is highly sensitive and specific. Additional data obtained through NGS (Next Generation Sequencing) and conventional PCR were also taken into account and contrasted with qPCR results to enrich the discussion herein presented.

The data presented in this study obtained in the massive sequencing using ITS primers have shown the absence of *F. circinatum* in the samples analysed, including those collected directly from cankers (Table 4). The universal barcode ITS is very useful for fungi in general, however, for *Fusarium* species it should not be used alone since it is not sufficiently polymorphic for species that are closely related, including some *Fusarium* species (IPPC 2017, Raja et al. 2017). Another point regarding the NGS data is that the Illumina MiSeq Platform used in this study, does not yet allow for obtaining the entire ITS region (ITS1, 5.8S, and ITS2). The longest reads available through this platform are 2 x 300 base pairs (Illumina 2018) which allows for the simultaneous sequencing of ITS1 and ITS2 of most species, however without the possibility to overlap these reads in the 5.8S region. In this sense, the reads are not sufficiently long to contain a variation that is necessary to separate species, especially those that are closely related such as *Fusarium* spp. Other molecular markers may be used to identify *Fusarium* species with a high level of certainty. For instance, protein-coding genes such as the translation elongation factor 1-α, the largest RNA polymerase II B-subunit (RPB1), second largest RNA polymerase II B-subunit (RPB2) and beta-tubulin are very useful for this purpose (Balint et al. 2014).

On the other hand, Schweigkofler et al. (2004) have shown that the intergenic spacer region (IGS) is also useful for identification of *F. circinatum* with primer pairs CIRC1A-

CIRC4A amplifying a DNA fragment of 360-base pairs. Although the protocol described by Schweigkofler et al. (2004) is applied for amplification of DNA extracted from both pure culture and plant tissue, in this study, after many attempts we did not succeed in amplifying DNA extracted directly from the plants through a conventional PCR using this protocol as well as the protocols of Ramsfield et al. (2008) and Ioos et al. (2009). This could be related to the low concentration of *F. circinatum* DNA present in the samples which makes difficult the observation of amplicons in an agarose gel.

Quantitative PCR has become a widely used and efficient tool for detection of *F. circinatum* in samples from different sources mainly due to its specificity and high sensitivity. This technique has already been used to detect *F. circinatum* in a range of different samples including individuals of *Ips sexdentatus*, a vector of *F. circinatum* (Fourrier et al. 2015), in pine seeds (Ioos et al. 2009, Dreaden et al. 2012) and in airborne spore traps (Schweigkofler et al. 2004, Fourie et al. 2014). In this study, the protocol described by Ioos et al. (2009) to detect *F. circinatum* in pine seeds through a dual-labeled probe chemistry was followed. Its methodology, as well as the methodology described in Fourrier et al. (2015), consider that a DNA amplification followed by a Ct value <40 is the criterion to judge a sample as positive regarding the presence of *F. circinatum*. Nevertheless, no information is mentioned in relation to the amplification curve generated. In this regard, the "*Diagnostic Protocol for Regulated Pests, DP22: Fusarium circinatum*", published by IPPC (2017) states that a sample is considered positive if it produces a Ct value <40, provided that the amplification curve has an exponential shape. The pattern of the amplification curve is an important criterion to consider since in the case of some samples, Ct values were generated, but it was actually related to inconsistent peaks of fluorescence that crossed the threshold and therefore resulting in Ct values.

In regard to the interpretation of qPCR results, there are still a lot of debate mainly about the reliability of Ct values that are close to the end of a qPCR run (Chandelier et al. 2010, Grosdidier et al. 2017). The problem involving misinterpretation of qPCR is that false negative results may lead to the introduction of pathogens in areas in which the disease is still not present and false positives may result into inappropriate destruction of plant material, or ban on trade (unpublished data, Ioos et al.). In this aspect, only Ct values < 20 followed by an amplification curve with an exponential shape were considered to judge a sample positive for *F. circinatum* in this study.

When a first qPCR assay was carried out with the original samples (no dilution applied), nine samples resulted in Ct values < 20 (Table 5). Nevertheless, the amplification

curves associated to these samples had a jagged pattern and no exponential shape was observed (Figure 6A) and therefore it was considered negative for *F. circinatum*. When samples were diluted to 1/100, only one sample resulted in Ct < 20, however, the shape of the amplification curves was improved and no inconsistence in fluorescence emission was observed in three samples (Figure 6B). In an attempt to amplify DNA diluted to 1/1000 through qPCR, no Ct value was generated for any sample and in the case of FCCa6 (pure culture of *F. circinatum*) used as control, the dilution resulted in a Ct > 20. Although DNA dilution may enhance PCR efficiency helping to remove PCR inhibitors present in the samples which inhibit polymerase activity for amplification of the target DNA as well as the binding of the probe to the DNA strand (Wilson 1997, Phister & Mills 2013), high dilutions such as 1/1000, may decrease the sensitivity of the technique which may lead to false negatives results (Demeke & Jenkins 2010).

PCR inhibitors are one of the main problems affecting DNA amplification. Potential PCR inhibitors may be originated from the tissue of which DNA has been extracted as well as from the purification method applied and from the plastics used during sample preparation (TermoFisher 2018b). In the case of DNA extracted from plant tissues, the main inhibitors are polysaccharides, polyphenols, pectin and xylan. Whereas phenolic compounds are known to degrade the DNA polymerases, polysaccharides can disturb the enzymatic process by mimicking the structure of the nucleic acid (Schrader et al. 2012). Depending on the extraction protocol used, these substances may be co-extracted and thereafter affecting the PCR (Wei et al. 2008, Schrader et al. 2012). The appropriate protocol for DNA extraction is an important step to overcome inhibitors and it has to consider the origin of the samples of which the nucleic acid will be extracted (Schrader et al. 2012). Commercially available kits for nucleic acid purification and PCR, generally use a lot of strategies to remove inhibitors and to increase the efficiency of PCR enzymes. Several methods have been developed for removal of specific classes of inhibitors such as polysaccharides and polyphenols which are commonly present in plants. Demeke & Jenkins (2010) mentioned a polyvinylpyrrolidone (PVP) method for DNA extraction from samples containing a high amount of polyphenolic and polysaccharides, which may be the case of the samples utilised in this study collected from *Pinus* species. It has also been reported that substances that are present in plants such as berries and tomatoes might be responsible to inhibition occurring in quantitative PCR assays using TaqMan probes (Love et al. 2008). This could be the case of this study, in which plant compounds might have affected the probe to bind to the DNA strand and therefore resulting in a deficient emission of fluorescence signals which finally led to irregular amplification curves (Figure 6A).

In order to overcome PCR inhibitors, a nested PCR was undertaken to produce a cleaner DNA, containing a lower concentration of inhibitors which afterwards could be used in a qPCR assay. PCR products originated from a conventional PCR using the primers of Ioos et al (2009) were used in the qPCR. Although amplified DNA obtained in the conventional assay was not observed in agarose gel (probably due to its low concentration), when it was used in a qPCR, it resulted in more efficient amplification curves with exponential shape (Figures 7A and B). Considering the criteria previously established to judge a sample as positive, from the 40 samples tested, 15 were considered positive being *F. circinatum* detected in four of the five species inoculated in 2010 (*P. radiata*, *P. sylvestris*, *P. nigra* and *P. uncinata*). The only species in which *F. circinatum* was not detected was *P. pinaster*, although this species has been reported as one of the most susceptible to this pathogen (Pérez-Sierra et al. 2007, Iturritxa et al. 2013, Martínez-Álvarez et al. 2014) and it was possible to detect some PPC symptoms in one of the trees of this species during the sample collection.

From the four samples collected directly from cankers in *P. radiata*, in two of them *F. circinatum* was detected, which raise questions regarding the accuracy of the technique since *F. circinatum* was undoubtedly present in those samples. In fact, those samples were collected in order to have a positive control in the experiment. In the case of *P. pinaster* samples, considering the criteria established to consider a sample positive, none of them were assumed as positive. However, when a qPCR assay was run with samples not diluted, Ct values were generated for three out of four samples but it was associated to jagged amplification curves which brings us doubts about its accuracy.

In the case of *P. radiata*, probably the most susceptible species to *F. circinatum*, it is noteworthy that the fungus was detected not only near the inoculation area (15 cm) but also in higher parts of the trees such as at 65 and 165 cm, which was the case of trees number one and four. The absence of *F. circinatum* in samples collected at 115 cm might be associated to an insufficient sampling or to a low concentration of *F. circinatum* DNA in the samples, which makes the detection through qPCR more difficult, even though this technique is highly sensitive. Regarding the movement of *F. circinatum* within the plant tissues, it has been shown by Martín-Rodrigues et al. (2015) that in seedlings of radiata pine, the fungus can spread from the roots to the aerial parts of the plant probably via two pathways: phloem and xylem through the tracheids. In fact, the presence of *F. circinatum* in higher parts of the tree could be associated to symptoms of resinosis observed in some *P. radiata* trees of which samples were recovered. Although the movement of *F. circinatum* within seedlings´ tissues has been already reported in literature (Martín-Rodrigues et al.

2015), to our knowledge this is the first time it has been detected through qPCR in higher parts of *P. radiata* trees.

5. Conclusions

- The NGS analysis using ITS primers was not able to detect *F. circinatum* in any sample although five different species of *Fusarium* were revealed through this technique. Protein-coding genes may be the best option in regard to the identification of *Fusarium* at species level based on sequencing analyses;

- Quantitative PCR appears as a good tool to detect *F. circinatum* in plant samples however PCR reaction may be affected by inhibitors present in the samples such as polysaccharides and phenolic compounds, which might have an influence in the binding of the probe to the DNA strand. Nested PCR might be a good solution to overcome inhibitors and to produce better amplification curves;

- *F. circinatum* was detected in fifteen of the forty samples tested. From the five pine species of which samples were recovered, only in *P. pinaster* the pathogen was not detected;

- In the case of *P. radiata*, it seems that the pathogen is distributed within the plant tissues since it was detected in samples collected at 15, 65 and 165 cm height. The majority of the positive samples were collected at 15 cm, near the area of which inoculation was done seven years ago.

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