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Spore dispersal patterns of *Fusarium circinatum* on an infected Monterey pine forest in north-western Spain

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Abstract

The airborne inoculum of *Fusarium circinatum*, the fungal pathogen causing Pine Pitch Canker (PPC), is one of the main means of spread of the disease in forest stands and forest nurseries. Since this world-wide known pathogen was introduced in Europe, its biology in this newly infected area still remains scarcely known. To shed more light on this topic, we set an experiment on a naturally PPC infected forest of Monterey pine in Galicia (NW Spain) with the following two goals: (i) to describe the seasonal spore dispersal pattern during one year of regular sampling and (ii) to assess the spatial spore dispersal pattern around the infested plot. Portable rotating arm spore traps were used and complemented with meteorological measurements. The abundance of *F. circinatum* spores in the samples was evaluated by quantitative PCR (qPCR) with hydrolysis probe. The results showed almost permanent occurrence of the air inoculum throughout the whole year, being detected in 27 of the 30 samplings. No clear temporal trends were observed, but higher air inoculum was favoured by previous lower air temperatures and lower leaf wetness. Conversely, neither rainfall nor air humidity seemed to have any significant importance. The spatial spread of the inoculum was noted to be successful up to a distance of 1000 m in the wind direction, even with winds of just 5 m s^{-1}. Our study shows that rotating arm spore traps combined with qPCR may be an efficient tool for *F. circinatum* detection.

Key words: Pine pitch canker, Galicia, spore trap, air sampling, qPCR, seasonal dynamics

Introduction

Fusarium circinatum [1] (teleomorph *Gibberella circinata*) is the causal agent of the disease called Pine Pitch Canker (PPC), which affects up to 60 species of pines and Douglas fir (*Pseudotsuga menziesii)* [2]. In adult trees, the main symptoms of PPC are pitch soaked cankers on the main stem or big lateral branches [3], which may girdle both stem and branches. Roots, shoots, flowers, cones and seeds may result infected as well. PPC is often responsible of a retarded growth of mature trees and massive mortality of saplings in forest nurseries, causing serious economic losses [4].

Originating naturally in Central America [5], *F. circinatum* has nowadays a worldwide distribution. Since it was firstly found out in the USA in 1946 [6], it has been later introduced to Japan [7], South Africa [8], South Korea [9], and Chile [10]. In 2005 it was reported in Spain [11], although it was observed in forest nurseries in the Basque country (Spain) in 1997 [12]. After that, *F. circinatum* rapidly appeared in other European countries, including France [13], Italy [14] and Portugal [15], alarming the European forest authorities. Nowadays, the pathogen is mentioned in the EPPO A2-list as a quarantine organism present in the area of EPPO countries but not widely distributed yet [16]. Although apparently eradicated in Italy and France, it is still present in Portugal and Spain [17].

Transport of infected plant material seems to be the most effective way for PPC introduction, especially for long distances [18]. In forest stands, however, the pathogen can also spread by natural means. *F. circinatum* is a seedborne pathogen that can survive both superficially and internally in seeds [19], favouring the spread of the disease to the following pine generation. Its macro- and microconidia (asexual spores) are also spread by wind, water and insect vectors that infect trees through weather-related injures and wounds associated with insect feeding and pruning [20–23]. Understanding the temporal and spatial spore dispersal is, thus, critical for fine tuning efficient control measures that limit the disease expansion.

The seasonal spore dispersal pattern of *F. circinatum* has been previously investigated in northern California (USA), where more spores were detected in October than in June and July [18]. This pattern was confirmed by the whole year sampling carried out by Garbelotto et al. [24], who detected the highest spore presence in the same area during the cold and wet weather from November to March. In San Francisco, Garbelotto et al. [24] showed the importance of sea fog, which can alleviate the water deficit during dry periods in summer, and enhance the

fungal sporulation. Wingfield et al. [25] emphasizes, however, that the life cycle of the pathogen may largely vary among different geographical areas, host species and particular conditions of forest stands. To our best knowledge, studies exploring the temporal dynamics of spore dispersal in Europe are lacking.

Spatial spread of *F. circinatum* spores in forest stands has only been partly investigated. Although it is not known how far the airborne spores may be dispersed [2], it was concluded that the conidia of *F. circinatum* have limited flight distance potential [2,23,24]. According to Garbelotto et al. [24] its dispersal is little influenced by the wind direction and speed. These authors did not find differences in spore occurrence 100, 200 and 300 m from the infected stand. Anyway, from the epidemiological point of view, long distance transfer of the spores is probably less important due the possibly low viability of the thin walled and hyaline spores [2].

The prior aim of the present study is to contribute to have a better knowledge of the seasonal spore dispersal pattern of *F. circinatum*, which may help to develop effective control measures of PPC in the European pine forests. Particularly, the objectives of this study were: i) to describe the seasonality of the occurrence of *F. circinatum* spores during a one-year sampling in an infected locality in Galicia (north western Spain) and ii) to investigate the spatial patterns of spore dispersal and the influence of the wind in the spread of the inoculum. To this end we used an active air-sampling trapping system especially designed for fungal spore assessments, but never used before with *F. circinatum*. Quantification of the spores collected in these traps was done by qPCR techniques. Two surveys were conducted, one designed for covering the within annual variation in spore abundance, and one designed for analysing the spatial dispersal around the infested spot.

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Material and methods

Sampling area

The sampling was conducted within a 40 years old forest stand of *Pinus radiata* with some *P. pinaster* close to Ponte Caldelas, Galicia, Spain, 440 – 480 m a.s.l (GPS coordinates of the centre: 42.376249º, -8.478177º). The pine stand covers an area of approximately 7.5 ha and it is isolated from other pine forests due to a large forest fire occurred in 2006. Infection of *F. circinatum* at this stand was confirmed in 2006 by the Regional Forest Service (Xunta de Galicia) and during the progress of the experiment typical symptoms (pitching on the stems) were apparent on many trees.

Spore traps

Actively rotating arm spore traps ROTTRAP 120 (Miloň Dvořák, Boršov nad Vltavou, Czech Republic) were used for all the experiments. The construction of this spore trap is based on the description of Perkins [26] and McCartney et al. [27]. An electric motor rotates 2400 rpm with a 0.8 mm thick U-shaped, square section wire (fig. 1). The impactors – pair of 50 mm long and 200 mm distanced vertical parts of the wire were covered for every sampling with a new double-sided non-woven tape (Tesa SE, Norderstadt, Germany). Covering the front side (according to the direction of rotation) of each impactor, the spore trap provided an impaction area of 80 mm^2. According to equations of Noll [28], the spore traps sample the air with a speed of 120 $L \cdot min^{-1}$ with almost a 100% collecting efficiency for particles bigger than 7.18 µm. Each trap was mounted 1.4 m high and powered by a 12V/19Ah battery.

Figure 1: Running ROTTRAP 120 coated with double-sided tape on the impaction side of the square section wire.

Seasonal dynamics sampling

To follow the seasonal dynamics of the *F. circinatum* inoculum, three spots labelled as A (GPS: 42.378014º, -8.477219º), B (42.376249º, -8.478177º) and C (42.374474º, -8.479641º) were established within the 7.5 ha infested pine stand close to apparently infected trees. In each spot, one spore trap was installed at least two times per month since April 2016 until March 2017, resulting in 30 samplings. In each sampling, the spore traps were running for 48 hours.

Each sampling with the spore trap was accompanied by the sampling of the rain water close to the soil surface. For that a 90 mm Petri dish was left open during the 48 h sampling period. The exposed tapes and 1 mL of rain water were stored in 2 mL microtubes at −20 °C before further processing. The two tapes of each spore traps were mixed and stored together in a single microtube.

Spatial spread sampling

For the analysis of the inoculum spatial spread 11 spore traps were used. The spore traps were installed following a latin cross arrangement with the elongated arm following the forecasted wind direction (see fig. 4). Eight of the spore traps were established 50 and 500 m from the last tree of the infected forest stand in each of the four orthogonal directions. Additionally, in the direction following the wind, two more spots were established at 100 and 1000 m from the last infested tree. Finally, in the middle of the infected stand, the spot B described in the previous section, was also sampled. For each 48h sampling period the forecasted wind direction was previously taken into account to orient the main arm of the experimental design. The wind forecasts were obtained from https://www.windguru.com/ and the real values of wind speed and direction were downloaded after the sampling from the same source and displayed in tables of fig. 4. Data from Pontevedra, 15 km apart from the sampling site and the closest continuously measuring spot at these websites, were used. Four samplings (around one per week) were carried out in September – October 2016.

Meteorological measurement

Meteorological data were recorded every 15 minutes throughout the whole sampling year using an automatic climatic station (AMET, Velké Bílovice, Czech Republic) placed in the spot A. The station, established at 1 m of height, recorded air temperature and humidity,

precipitation and leaf wetness. Leaf wetness was measured as an electric resistance of a filter paper placed between two electrodes.

DNA extraction

DNA was extracted from all the samples together with an empty microtube as a negative control of extraction. Spores were disrupted and homogenized directly in a Mixer Mill MM400 (Retsch, Haan, Germany), using a mixture of 0.4 g of 0.1 mm balotina beads and 250 µL of 0.1% Nonidet P40—substitute (AppliChem, Darmstadt, Germany). The homogenization was performed for 10 min by 30 Hz. For further processing, the DNEasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's instructions, except of the incubation with AP1 buffer, which was prolonged to 60 min. In the last step, DNA of each sample was eluted only once with 100 µL of preheated elution buffer previously incubated for 10 min.

Quantitative PCR

Direct specific qPCR was performed using a LightCycler ® 480 Instrument II (Roche Diagnostics, Basel, Switzerland), TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers and probe [29] according to the manufacturer's instructions (pre-incubation: 10 min, 95 °C followed by 45 cycles of denaturation: 10 s, 95 °C; annealing: 30 s, 60 °C; extension: 1 s, 72 °C—single acquisition mode). The reaction mixture was as follows: 0.2 µL of each primer (final concentration 400 nM), 0.2 µL of TaqMan probe (200 nM), 5 µL of TaqMan Universal PCR Master Mix, 1.9 µL of sterile deionized water and 3 µL of template DNA.

Every reaction was performed in three technical repetitions together with a positive control, negative control of isolation and also negative control of qPCR reaction containing the master mix without template DNA.

The concentrations of *F. circinatum* DNA in the samples (hereafter CN) were expressed as numbers of copies of the target sequence in 1 µL of template DNA. CNs were determined using a standard curve that was generated by different concentrated aliquots of a plasmid pCR™2.1-TOPO® TA vector (Invitrogen, Carlsbad, CA, USA) with an insert of the target gene (Fragments were amplified with the primers described above). All reactions were performed in a LightCycler ® 480 Software (Roche Diagnostics, Basel, Switzerland).

Statistical methods

Average CN across the three sampling spots (A, B and C) were calculated for every sampling and used for further analyses. Relationships between CN and meteorological variables were tested. Because the analysed variables were not normally distributed even after log-transformation, only non-parametric tests were used. Spearman's correlation was calculated between CN of each sampling and average value of each of the meteorological variables measured 3, 7, 14, 21 and 28 days before the end of the sampling. All the statistical analyses were performed in STATISTICA 12 (StatSoft, Tulsa, OK, USA).

Results

Seasonal spore dispersal

The results of the sampling are shown in fig. 2. In total, *F. circinatum* was detected in 27 out of 30 air samplings carried out over one year. The samplings with no *F. circinatum* detection were noted on May 4, July 6, and September 14. No clear temporal trends were detected. The highest CN ($3.28 \cdot 10^7$) was recorded in July 27, but relative high values of CN were detected across almost all the sampling year. During 12 out of the 30 samplings rain water was collected; six of the water samples were positive. Two negative results of water samples confirmed the absence of inoculum on July 6 and September 14; however, on May 4 the inoculum was detected in the water sample and not in the tape of the spore trap.

Significant correlations between CN and meteorological variables were found only for the air temperature and leaf wetness (fig. 3). The highest correlation ($r = -0.626$, $N = 30$, $p < 0.05$) was found between CN and average air temperature during the 28 days before the end of the sampling period.

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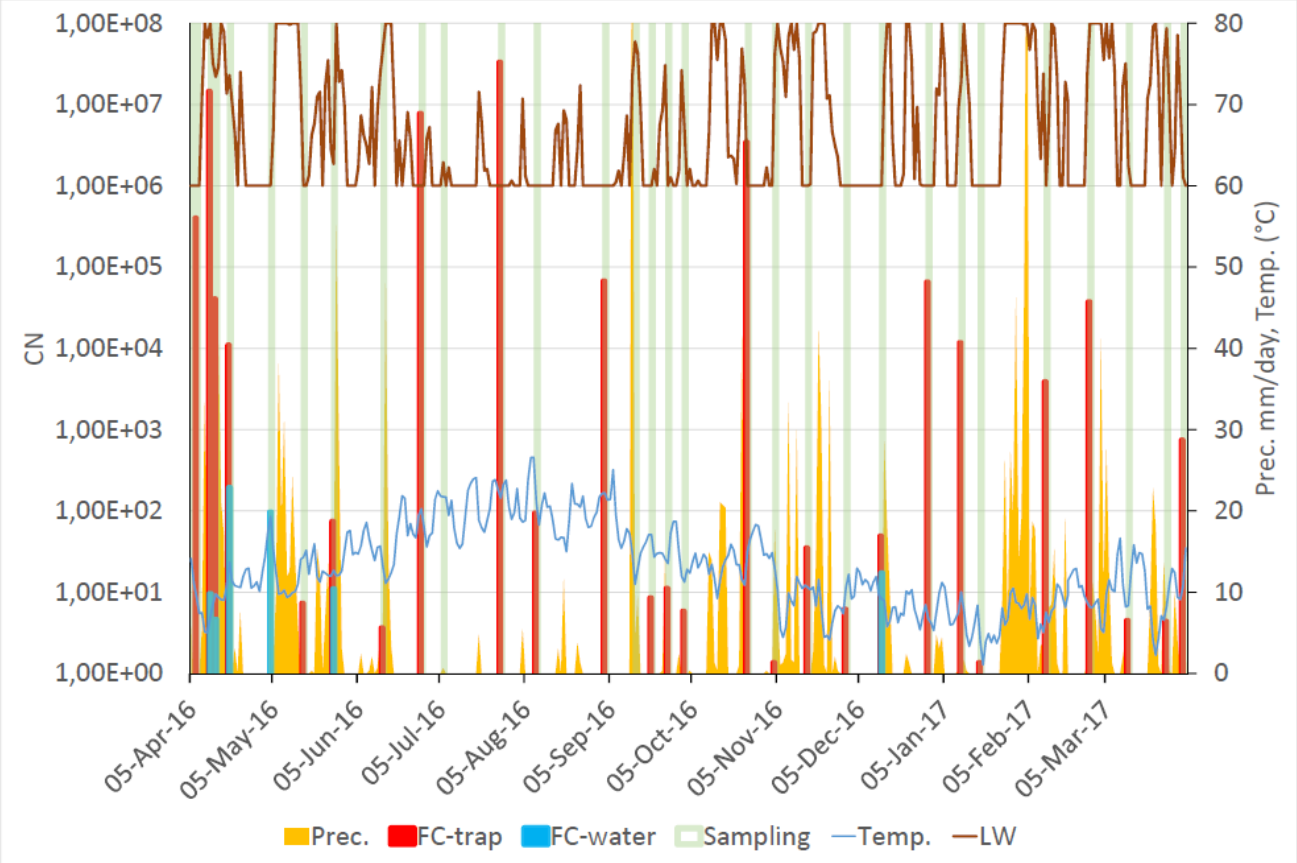

Figure 2: Variation of meteorological variables and abundance of F. circinatum *spores across the whole sampling year. The X-axis shows the progress of time. "FC-trap" values (left y-axis, red columns) display the numbers of copies of the target sequence in 1 µL of template DNA (CN) in a logarithmic scale and reflect the amount of* Fusarim circinatum *inoculum trapped by the rotating arm spore trap. "FC-water" (left y-axis, blue columns) display the CN detected in the samples of rain water. Green columns mark the 30 sampling periods across the whole year. Precipitation ("Prec.", yellow peaks, mm per day), Temperature ("Temp.", blue line, °C) and Leaf wetness ("LW", brown line, scaleless; 60 = dry, 80 = absolutely wet) are shown on an average daily basis and scaled by the right Y-axis.*

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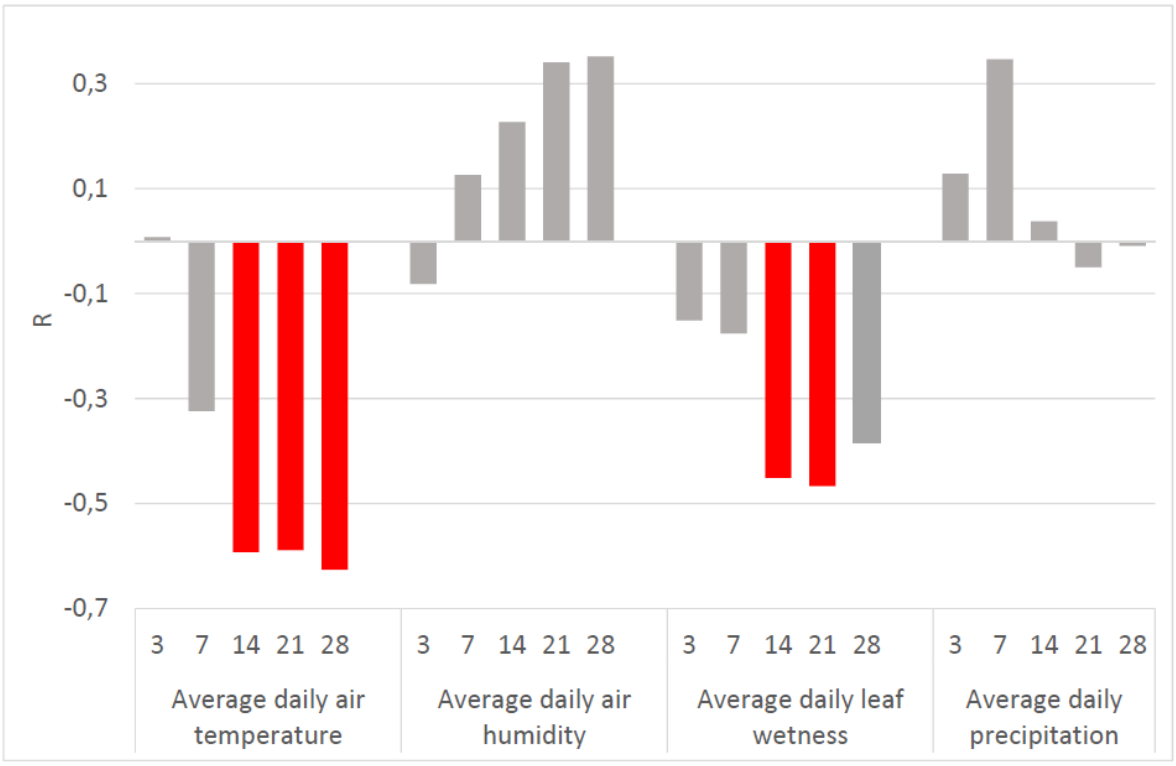

Figure 3: Spearmann correlation coefficients between the amount of the detected inoculum in the air samples (CN) and meteorological variables (air temperature, air humidity, leaf wetness and precipitation). *For each meteorological variable, daily values were averaged across the 3, 7, 14, 21 and 28 days before the end of each spore sampling. Significant correlation coeficients are displayed in red.*

Spatial spore dispersal

All the four samplings showed positive detection of *F. circinatum*, although for each sampling the amount of spores was very variable across the 11 traps, and, in average, the inoculum levels were fairly low. The highest CN detected was $6.48 \cdot 10^2$, recorded during the first sampling in the spot sited 100 m apart of the infested stand following the wind direction.

The CN values of the spatial dispersal study are displayed in figure 4, which shows a map of the sampled area including the arrangement of the sampling spots, wind direction and speed. During all the four samplings the wind speed never exceeded 5 m s^{-1} and the direction was very variable. In the first sampling the wind was blowing 1 – 5 m s^{-1} to the SE and E and highly positive detections were recorded 100 and 1000 m from the infected stand in the SE direction. Another positive, but lower value was noted 50 m from the stand in the opposite direction. The second sampling showed a little bit ambiguous results. The wind was changing and blowing to SW and SE directions with a speed of 5 m s^{-1}. Positive values were recorded in all the

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directions, except at NE, 1000 m apart from the stand. The same wind speeds and directions were also measured during the third sampling. In this case the detected CN values were very low, but 500 m opposite to the wind direction (NE and NW) the inoculum detection was negative. The last sampling was characterised by a very low wind speed, around 3 m s^{-1} and changes of directions to E and W. In this case, the inoculum was detected only in close distances to the infected stand and 500 m in the NE direction. All the CN values were very low (up to 10.9). Altogether, the results indicated no clear spatial patterns, and no evident relations with the main wind direction. Nevertheless, traps sited 1000 m apart of the infested stand along the predominating wind direction were positive in three out of the four samplings, indicating that light breezes of just 5 m s^{-1} can promote spore dispersal up to distances of at least 1000 m.

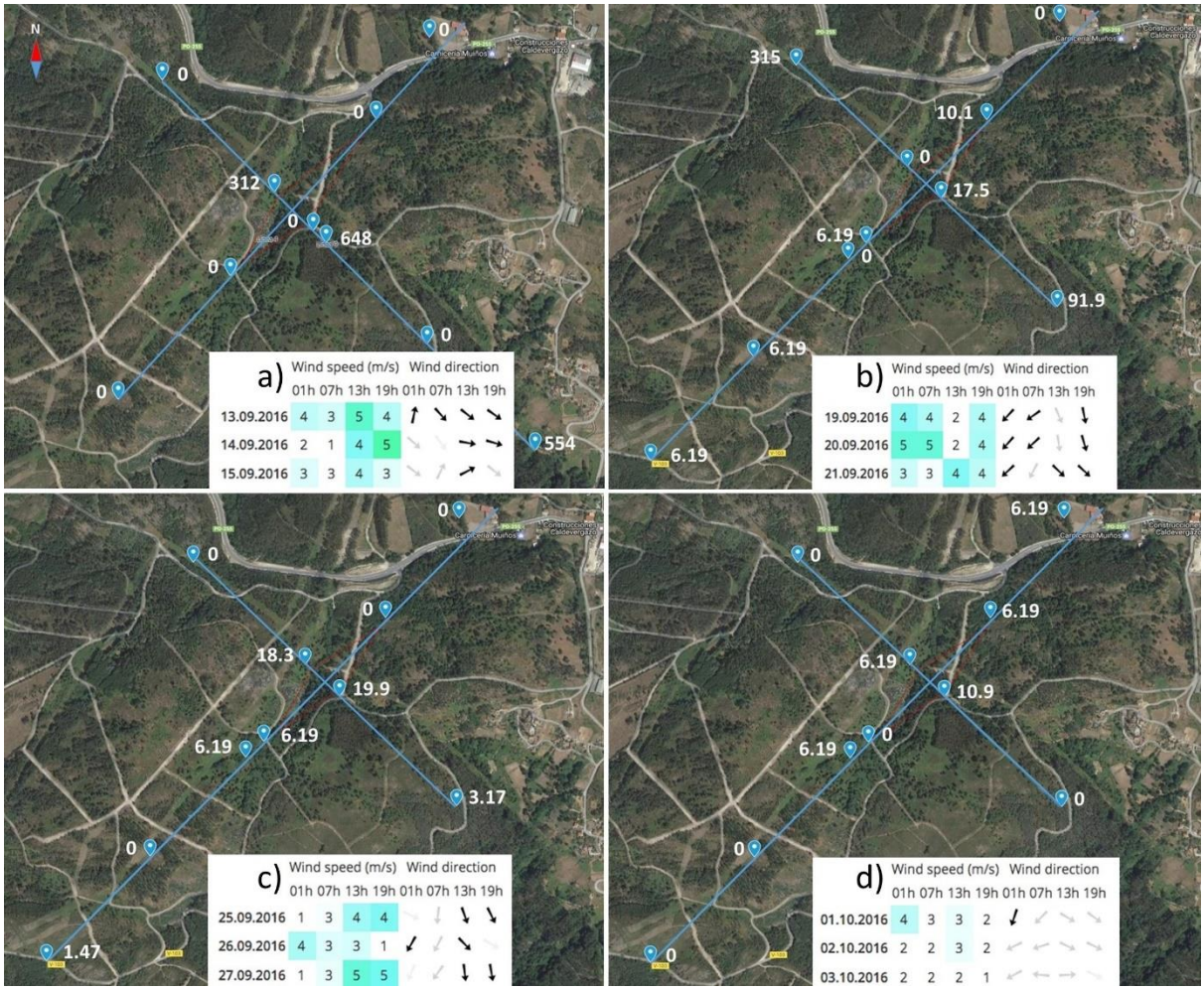

Figure 4. Results of the F. circinatum *spore trapping for analysing the spatial patterns of inoculum dispersal. The four panels correspond to the four samplings carried out (September 13-15th (a), September 19-21th (b), September 25-27 th (c), October 1-3th (d)). For each sampling period, traps (blue drops) were established following a latin cross arrangement (blue line) with the longest line following the forecasted wind direction (NW in a, and NE in b, c, and d) (see more details in the main text). Within each panel the white number show the copies of the target sequence in 1 ul of template DNA (CN) detected by the rotating arm spore traps. The red dashed line connects the crosses of the four axes with the border of the infected stand (i.e. the limit of the infested stand in each orthogonal direction). Tables at the bottom of each panel reflect the real wind speed and direction measured during each sampling period.*

Discussion

Results from the present paper indicate that *F. circinatum* can disperse in Galicia (NW Spain) over the whole year. Air sampling by the rotating arm spore traps detected the presence of *F. circinatum* spores in almost all of the 30 samplings across all seasons, but no clear temporal patterns were observed. Abundance of *F. circinatum* spores was, however, significantly related to the preceding weather conditions, with mean air temperature and mean leaf wetness negatively affecting the dispersal of the fungus. Spatial patterns of spore dispersal were also poorly defined, with no clear evidences of the effect of the predominating winds. Nevertheless, results presented here indicated that light breezes of just 5 m s^{-1} can promote spore dispersal up to distances of at least 1000 m. Altogether, results from the present study adds light to the understanding of the natural dispersal of *F. circinatum* from infested stands in Europe and can contribute to fine tune appropriate methods to prevent the spread of this important pine disease.

Rotating arm spore traps – efficient tool for the detection of Fusarium circinatum

Garbelotto et al. [24] firstly applied a passive air sampling system using filter paper to collect deposited spores of *F. circinatum.* These authors suggested, however, the use of active air sampling spore traps to get more precise results in aerobiological studies of the inoculum of *F. circinatum*, especially for the study of its spatial spore dispersal. Here, we used for the first time rotating arm spore traps that actively sampled the *F. circinatum* spores increasing the amount of air sampled per unit of time.

According to the amount of positive results of the whole year sampling shown in our study, the rotating arm spore traps combined with qPCR can be pointed out as a reliable and efficient detection tool of *F. circinatum* air inoculum. This type of air samplers was successfully used in a number of studies focused on the detection of fungal pathogens' airborne inoculum. Recently, it was used by Chandelier et al. [30] and Dvořák et al. [31] for the detection of Ash dieback pathogen, and by Choudhury et al. [32] for powdery mildew on spinach. The use of rotating arm spore traps is often limited by their disability to impact very small particles [33,34]. Too small particles can pass around the impactor being blown by the air pillow built by the rotation of the impactor [34]. Using the Noll´s equation [28], ROTTRAP 120 was calculated to be reliably efficient for particles of spheric diameter of at least 7.18 µm. Microconidia of *F. circinatum* are known to have in average 9.7 x 3.2 µm and macroconidia 38.2 x 3.6 µm [1]. Therefore, part of the inoculum might not be sampled. Particularly, those

microconidia facing the impactor by its narrow side might be blown around, so ROTTRAP 120 may be underestimating the real amount of *F. circinatum* spores. Despite this disadvantage, the spore traps were able to catch the inoculum in 90 % of the whole year samplings, reaching notable concentrations of *F. circinatum* inoculum (up to CN of 10^7).

Seasonal spore dispersal

The constant occurrence of the inoculum of *F. circinatum* (27 positive samplings out of 30) confirms that *F. circinatum* persists in the area of Galicia with a notable level throughout the whole year. Similar results with constant presence of *F. circinatum* along the whole year, were obtained in other areas such as northern California [24] and South Africa [35]. This finding means that *F. circinatum* in Galicia can develop the infection at any time of the year whenever suitable environmental conditions and susceptible hosts are available for spore germination, as it was expected from the calculation of models of spread [36,37].

The amount of inoculum expressed as CN varied during the year, oscillating between low and high values, but no clear temporal trends were observed. The most striking difference was noted in July, when the sampling at the beginning of July was negative and the sampling at the end of the month showed positive result with the highest value of the year. Similar patterns were shown by Garbelotto et al. [24] in San Francisco in July-August. However, results of the present study are not reliably comparable with that of Garbelotto et al. [24] due to the length of the sampling time, which was two weeks and continuous in the American study and 48 h twice a month in ours.

Effect of the meteorological conditions

The influence of the weather on the inoculum occurrence was estimated via correlation between the meteorological variables and the CN. The meteorological variables were included as averages across certain amount of days before the end of each sampling.

Results indicated no significant correlations between the CN and the preceding precipitation (3, 7, 14, 21 and 28 days before sampling), neither with the air humidity. These results disagree with those reported by Garbelotto et al. [24], who found a positive relation between the precipitation and the trapping frequency. A possible explanation of this incongruence may be related to the contrasting mechanisms of the spore trapping used in the two studies. The spore traps used here sample the particles occurring directly in the air by impaction on the adhesive

surface, while the passive spore traps used by Garbelotto et al. [24] and other researchers [18,35] sample the spores passively deposited on a filter paper. Passive deposition may be supported by rainfall, during which the rain drops trap the air inoculum and deliver it onto the surface of the filter paper. Therefore, that type of air sampling may overestimate the amount of inoculum in the air during the rain, favouring the positive correlation between precipitation and trapping frequency. Accordingly, half of our water rain samples were positive, evidencing the ability of the raindrops to collect the spores of *F. circinatum.*

The air temperature was determined as the most significantly correlated factor with *F. circinatum* spore abundance. The strongest correlation was found for the average of temperatures of the previous 28 days ($R = -0.63$, $N = 30$, $p < 0.05$), although the last 14 ($R = -0.59$, $N = 30$, $p < 0.05$) and 21 ($R = -0.59$, $N = 30$, $p < 0.05$) were also significant. These negative correlation coefficients may be due to the pathogen's demand of lower temperatures or/and the limiting effect of extreme high temperatures for developing fruiting structures (phialides and sporodochia), followed by conidial production in the next 14 – 28 days. The preferences of *F. circinatum's* inoculum for lower temperatures were also pointed out by Garbelotto et al. [24], who observed a higher amount of inoculum during the coldest period of the year from November to March. These authors also suggested that the minimum temperature of sporulation is around 0°C. Such temperatures are rare in the region of Galicia. During our monitoring the minimal temperature dropped below zero only few hours during five days. The lowest recorded temperature during the whole year was -3.4°C, which was recorded on January 19 during one of the samplings with a positive but extremely low CN = 1.36. This value was the lowest CN detected through the whole year, apart of the three zero values. Therefore, our results also confirm that too low temperatures are a limiting factor of *F. circinatum* sporulation. The negative influence of temperature on spore production is apparently opposite to the ability of spores to germinate. Inman et al. [38] revealed that at 10°C the germination of spores is limited to around 10%, being more than 70% at 15°C and more than 90% at 20°C.

Leaf wetness (LW) was calculated as the second most correlated meteorological variable with *F. circinatum* spore abundance. Leaf wetness is an important variable which specifically affects the fungal growth and fructification of *F. circinatum* on the plant surfaces [31,39,40]. This variable was significantly correlated with precipitations ($R = 0.83$, $N = 337$, $p < 0.05$) and apart of the wetness caused by rainfall, it also records the occurrence of dew, condensation of the fog, etc. As occurred with the air temperature, its effect was negative and delayed two (R

= -0.45, N = 30, $p < 0.05$) to three ($R = -0.47$, $N = 30$, $p < 0.05$) weeks before sampling. The presence of water on the host surface seems, thus, to be not favourable for the fructification structures' development. Again, conditions for sporulation appear to be opposite to those needed for spore germination [41], creating a putative obstacle for the disease spread.

The correlation analysis carried out in the present study can be biased by some other disturbing factors related to the construction of the spore trap. Particularly, the impactors with the adhesive tape are not protected from the rain and, therefore, an amount of particles already trapped may be washed out by rain showers. However, according to the results of the correlation analyses, this seems to be not happening, as there was not a significant correlation of the CN and the precipitations of past 3 days (including the duration of the sampling).

Spatial spore dispersal

Unfortunately, the inoculum levels in the air during the period of this survey were very low in comparison with the rest of the whole year samplings. Nevertheless, results allowed to infer some conclusions about the spatial spread of the inoculum.

Although the relationship between spore trapping and the predominant wind was not very clear, the influence of the wind speed was apparent above 5 m s^{-1}. In case of such winds, the spores were transferred at least over distances of 1000 m, and detected in amounts similar to those at 50 m distance. A similar result was found by Garbelotto et al. [24], who did not find significant differences between trapping frequencies 100, 200 and 300 m from the putative source of inoculum. These authors did not confirm any differences in the inoculum levels due to the wind direction. They explained this lack of relationship by the long duration of the sampling period (two weeks per sampling), which makes the result insensitive to short-term wind direction changes. Neither in our work we can provide any statistical support of our observations due to the changing wind direction, the low amount of inoculum detected during the sampling periods, and the low wind speed in some of the samplings. Additionally, the geomorphology of the sampled area is very diverse, which leads to particular local air currents spreading the air inoculum, and the data of the wind direction and speed were downloaded for a locality 15 km apart.

Conclusion

Monterey pine forests in Galicia are strongly endangered by the natural spread of *F. circinatum*. Results presented here indicated that air inoculum was available throughout the whole year, with only a very few exceptions. Lower temperatures and less humidity on the host surface caused by vertical and horizontal precipitations are more suitable for the pathogen to develop higher amounts of inoculum. Masses of inoculum can be transferred by winds of just 5 $m \cdot s^{-1}$ for distances of at least one kilometre. Rotating arm spore traps combined with qPCR detection are a reliable method for detection of *F. circinatum* spores.

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Author contributions: M.D. conceived the study, designed and performed the field experiments, extracted DNA, analysed data and wrote the article; P.J. performed the field experiment; L.B. conceived the study and wrote the article; G.R. optimized and performed the qPCR analysis; R.Z. proposed the experimental area, performed the field surveys and wrote the article.

Conflicts of interest: The authors declare no conflict of interest.

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