

## Detection and quantification of the air inoculum of *Caliciopsis pinea* in a plantation of *Pinus radiata* in Italy

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*Caliciopsis pinea* has been historically described as a secondary pathogen of pines. However, it has recently been associated with severe damages on *Pinus radiata* in Italy. Our study focused on the description of the seasonal spore dispersal of *C. pinea* and its relation to meteorological conditions (temperature, leaf wetness, relative humidity and precipitations). For this experiment one infected *P. radiata* plantation was sampled in Tuscany (Italy). A rotating arm spore trap together with a weather station were installed to sample the aerospora for 24 h every week from May to November 2016. Exposed tapes from spore traps were directly analyzed after DNA extraction by qPCR using specific primers and TaqMan MGB probe. The study shows an irregular occurrence of the inoculum of *C. pinea* throughout the whole sampling period with peak levels in mid-June and early August. The statistical analysis of the DNA and climatic data clearly show the strong influence of precipitation on the spore production of this pathogen. Furthermore, the very low detection limit of the qPCR experiment shows the efficacy and suitability of rotating arm spore traps for early detection of this pathogen.

**Keywords:** *Caliciopsis* Canker, Monterey Pine, Aerospora, qPCR

### Introduction

*Pinus radiata* D. Don represents one of the most successful forest plantation species worldwide. This species has been planted in many parts of the world, such as Australia, Chile, New Zealand, South Africa and others (McDonald & Laacke 1990, Lavery & Mead 1998, Weber 2003). Currently, it represents the most widely planted conifer. Because of its fast development, profitable lumber and pulp production, about 25,000 ha of *P. radiata* were planted in Sardinia and in the peninsular Italy between 1960 and 1980 (Ciancio et al. 2006). As a consequence of forest fires and the effects of climate change in the Mediterranean region, *P. radiata* has been suffering a progressive decline in Italy over the last years. In addition, the sensitivity to frost, the lack of moisture and precipitation during the summer season, and the need for deep rich

and well-drained soils also contributed to its collapse (Ciancio et al. 2006).

Additionally, some pests and diseases may represent a serious threat for *P. radiata* plantations. Among them, *Caliciopsis pinea* Peck, an ascomycetous fungus belonging to the family *Coryneliaceae* and order *Coryneliales* (Fitzpatrick 1942). It infects pine trees of all ages mainly in sandy and well-drained soils in North America (Munck et al. 2016). In eastern USA *C. pinea* may affect *Pinus echinata* Mill, *Pinus pungens* Lamb, *Pinus rigida* Mill, *Pinus virginiana* Mill, *Pinus taeda* L. and *Pinus strobus* L., the white pine, which is suffering a severe damage over the last years (LaFlamme 2004, Munck et al. 2015). *C. pinea* has been observed in Europe on exotic pine species, such as *Pinus pumila* (Pall.) Regel (Rehm 1896), *P. echinata*, *P. pungens*, *P. rigida*, *P. virginiana* and *P. radiata* (Capretti 1978, Lu-

chi et al. 2015), and on native European pines, such as, *Pinus pinaster* Ait., *Pinus pinea* L. and *Pinus halepensis* Mill (Lanier 1965, Delatour 1969). Particularly, in Italy, it was firstly described as causing cortical cankers in young plantations of *P. pinaster* and *Pinus insignis* Dougl. (= *P. radiata* D. Don) (Capretti 1978). The pathogen has been also reported on *Pinus nigra* Dougl. (Capretti 1980).

Symptoms caused by *C. pinea* infection include sharply delimited cankers on trunks and branches crown wilting, defoliation (Ray 1936) and a profuse resin production. Previous studies showed that the cankers did not produce significant damage (Ray 1936, Cram et al. 2009), but other authors have associated the presence of *C. pinea* with top killing cankers on trees (LaFlamme 2004). *C. pinea* produces small, globose, clustered, black pycnidia and stalked perithecia that arise from a stromatic cushion (McCormack 1936, Funk 1963). Black-hair-like fruiting structures persist throughout the year and spores mature in late winter and spring. Spores are disseminated by wind and rain and typically enter through bark lenticels or small insect wounds (Funk 1964, Delatour 1969).

The airborne inoculum of forest pathogens is generally underestimated in epidemiological studies. In particular, no information on *C. pinea* sporulation and its relationship with environmental parameters have been studied so far. However, due to the climate changes in the Mediterranean region, the pathogen sporulation and dispersal may be favored, leading to an increase of the disease incidence (Santini &

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**Fig. 1** - Location of the spore traps (A, B, C) and the meteo station (MS) at the sampling site in Carcheri (Tuscany, central Italy).

Ghelardini 2015). As nowadays new spore trapping tools coupled with sensitive molecular methods give the opportunity to efficiently collect and detect airborne fungal inoculum (Brittain et al. 2013), here we propose to analyze the seasonal spore disper-

sal of *C. pinea* in a *P. radiata* plantation, and its relation to local climatic conditions by using a rotating-arm spore trapping method combined with qPCR.

## Materials and methods

### Sampling area

A *P. radiata* plantation in Carcheri (Lastra a Signa, Florence, Italy – 43.715875° N, 11.073649° E) was selected for the aim of this study. The stand is a 2-ha monospecific plantation of *P. radiata*, in a northwest facing slope (Fig. 1). The undergrowth is characterized by some Mediterranean shrub species, including some oaks (*Quercus pubescens*, *Q. cerris*) and other native tree species (*Acer campestre*, *Fraxinus ornus*, *Rosa canina*, *Cornus mas*, *Sorbus estica*, *Crataegus monogyna*, *Equisetum* sp. and *Asparagus officinalis*).

The plantation showed 44.7% of trees affected by *Caliciopsis* cankers with abundance of resin and characteristic fruit bodies from the lesion (Fig. 2a, Fig. 2b). Cankers length ranged around 1-3 cm. The pathogen was detected by isolation on PDA (Luchi et al. 2015) and molecular techniques (Luchi et al. 2018).

### Spore trapping and climate data

The rotating-arm spore traps ROTTRAP 120 (RAST - Fig. 2c) were constructed by Milon Dvůrák (Boršov nad Vltavou, Czech Republic), based on the principles of Perkins & Leighton (1957), developed by Chandelier et al. (2014) and modified by Dvůrák et al. (2017). An electric motor rotates 2400 rpm with a 0.8 mm thick U-shaped, square section wire. The vertical impactors of 50 mm in length distanced at 200 mm were covered for every sampling with a new double-sided non-woven tape (Tesa SE<sup>®</sup>, Norderstadt, Germany). Covering the front side (according to the direction of rotation) of each impactor, the spore trap provided an impactation area of 80 mm<sup>2</sup>. RASTs were sampling the air 1.4 m above the ground at a speed of 120 L min<sup>-1</sup> with almost a 100% collecting efficiency for particles bigger than 7.18 μm. This limiting spherical diameter of particles was calculated using equations and charts published by Noll (1970). In his paper a comprehensive theoretical and experimental testing of rotating-arm spore traps with variable parameters was reported.

The RASTs were placed in three spots (A, B and C – Fig. 1) of the infested forest at a distance of about 100 m each other. The automatic weather station (AMET, Velké Bílovice, Czech Republic) was installed next to the RAST on the spot A (Fig. 1, Fig. 2d).

The RASTs were sampling 24 consecutive hours once a week for a period of 29 continuous weeks from the May 17<sup>th</sup> to November 29<sup>th</sup>, 2016. After each sampling period, the exposed tapes were immediately placed into 2-ml sterile microtubes and stored at -20 °C.

The automatic weather station (Fig. 2d)



**Fig. 2** - (a) *Caliciopsis* cankers on a *P. radiata* trunk; (b) Black-hair-like fruiting bodies of *Caliciopsis pinea* growing from a canker; (c) RAST device; (d) meteorological station.

carried out climatic measurements continuously over the course of the entire sampling period. Variables were recorded every 10 minutes and included air temperature, air relative humidity, dew point, leaf wetness and precipitations. The mean daily temperature, as well as other mean daily values, were calculated as an average of these measurements from midnight to midnight.

#### DNA extraction from the spore trap samples

DNA extractions included negative controls, which were prepared as microtubes with no tape rods.

For the purpose of spore disruption, we added 0.3 g of 0.1 mm and 0.1 g of 0.5 mm balotina beads and 250 µl of 0.1% Nonidet P40<sup>®</sup>-substitute (AppliChem, Darmstadt, Germany) to each tube. The disruption itself was then accomplished by a Mixer Mill MM400<sup>®</sup> (Retsch, Haan, Germany) activated for 10 min at 30 Hz. Then, the lysis buffer (Dvorák et al. 2015) and 4 µl RNase A (E.Z.N.A.<sup>®</sup> Plant DNA kit, Omega Bio-tek, Norcross, GA, USA) were added to all samples. The mixture was incubated for 60 min at 65 °C with occasional mixing. For further processing, the E.Z.N.A. Plant Mini Kit was used. The elution used 100 µl of preheated buffer after 10 min of incubation at room temperature (Dvorák et al. 2015).

#### qPCR experiments

The primer set Cpin-F (5'-ATTGTCGTCTGA ATTCTGATGCA-3') and Cpin-R (5'-TTCATC GATGCCAGAACCAA-3') and the TaqMan<sup>®</sup> MGB probe (Cpin-Pr) (5'-VIC-AATAAACTTT CAACAACGGATC-MGBNFQ-3') described by Luchi et al. (2018) were used to detect and quantify *C. pinea* by real-time quantitative PCR (qPCR).

DNA extracts from spore trap samples were assayed in MicroAmp Fast 96-well Reaction Plate (0.1 mL) closed with Optical Adhesive, and by using the StepOnePlus<sup>™</sup> Real Time PCR System (Applied Biosystems, Life Science, Foster City, CA, USA). PCR reaction was performed in a 25 µl final volume containing: 12.5 µl TaqMan Universal master mix (Applied Biosystems), 300 nM forward primer (Eurofins MWG Operon, Ebersberg, Germany), 300nM reverse primer (Eurofins MWG Operon, USA), and 200 nM TaqMan MGB probe (Applied Biosystems), 20 ng genomic DNA. Each DNA sample was assayed in duplicate. Two wells, each containing 5µl of sterile water, were used as the no-template control (NTC). Additionally, two wells containing DNA extracted from infected *C. pinea* pine tissue (positive control), and two wells containing DNA from healthy pine tissues (negative control – Luchi et al. 2018) were included. The PCR protocol was 50 °C (2 min), 95 °C (10 min), 50 cycles of 95 °C (30 s), and 60 °C (1 min). Results were analyzed by using a SDS 1.9 Sequence Detection System (Applied Biosystems) after manual adjustment of the baseline and fluorescence

threshold. The standard curve for the absolute quantification of *C. pinea* DNA was generated with 5-fold serial dilutions (ranging from 25 ng/tube to 40 pg/tube) of known concentration of DNA of *C. pinea* (strain IT1, IPSP-CNR collection – Luchi et al. 2018). The amount of fungal DNA from spore trap samples was expressed as pg DNA *C. pinea* / µg of total DNA extracted.

#### Data analysis

Differences in *C. pinea* DNA quantity among different spore traps and sampling periods were tested by the analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test.

Influence of climatic conditions on the occurrence of *C. pinea* inoculum was evaluated by the Random Forest algorithm utilizing conditional inference trees as base learners provided in the "party" package. Variable selection was done by the permutation-based attribute selection algorithm provided in the "Boruta" package. The conditional computation of variable importance accounting for correlated predictors implemented in the "party" package was used as a function to obtain feature importance. The importance of variables that were deemed important by the selection algorithm was then expressed by means of permutation-based mean decrease in accuracy (%IncMSE) using the function "varimp", which calculates the increase in mean squared error (MSE) of predictions when the considered variable is permuted by random shuffling.

The set of predictors that were used as input for the feature importance procedure included values of mean daily temper-

ature, relative humidity, leaf wetness and daily precipitation during the sampling day and every particular day of 40 days before the sampling.

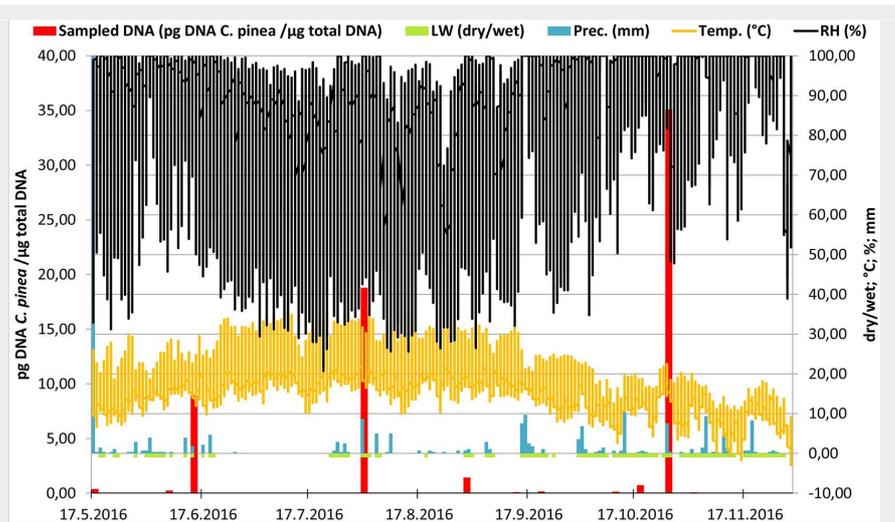
All analyses were carried out by using the R software (R Development Core Team 2008).

## Results and discussion

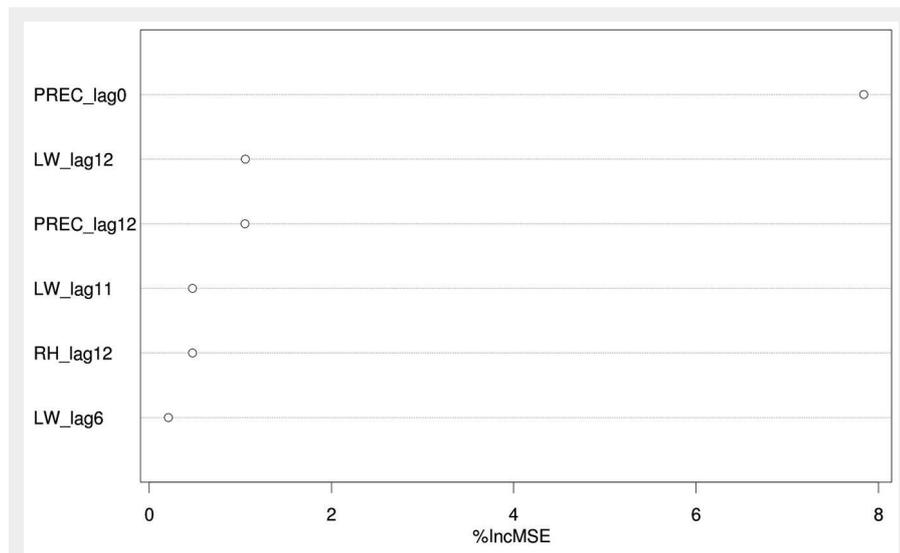
### Occurrence of the air inoculum of *C. pinea*

Our study shows that *C. pinea* inoculum is present in aerobiological samples collected in a *P. radiata* plantation from May to November 2016 (throughout the entire sampling period). No significant differences ( $p = 0.07$ ) of *C. pinea* DNA collected among the traps placed in three different spots (A, B, C) were observed. A continuous *C. pinea* inoculum production has been recorded over the whole sampling period (May–November). Despite no significant differences ( $p = 0.824$ ) were observed among different months, three outstanding peaks of spore production were recorded (mid-June, early August and late October – Fig. 3).

Although *C. pinea* triggered damages on *P. strobus* in the 1930s and some epidemiological studies were conducted in North America (Ray 1936, McCormack 1936), it has arisen scientific attention only in 2009 as a result of unexpected serious damages on *P. strobus* forests throughout several north-eastern US states (Munck et al. 2015, 2016). In Europe, it is even less studied since it was traditionally pointed as a secondary pathogen (Delatour 1969) related to stressed trees in poor plantations (Cappretti 1980). *C. pinea* has been reported in



**Fig. 3** - Representation of the meteorological variables in relation with the amount of airborne inoculum of *C. pinea* detected over the whole sampling period. The x-axis shows the progress of time. Left y-axis displays the template DNA quantity (pg) detected in 1 µg of total extracted DNA in a logarithmic scale and reflects the amount of *C. pinea* inoculum trapped by the rotating arm spore trap (red columns). Daily range of precipitation ("Prec.": light blue bars, mm per day), temperature ("Temp.": orange line, °C), leaf wetness ("LW": green line, scaleless; 1 = dry, 0 = absolutely wet) and relative humidity ("RH", black line, %) are shown on an average daily basis and scaled by the right y-axis.



**Fig. 4** - Importance (%incMSE, permutation-based mean decrease in accuracy derived from the Random Forest algorithm) of predictors significantly accounting for the amount of airborne *C. pinea* spores. (PREC): precipitation (mm); (LW): leaf wetness (1=dry; 0=wet); (RH): relative humidity (%); (\_lagX): X<sup>th</sup> days before sampling.

Italy since several years as the causal agent of necrosis and cankers on stems and branches on *P. pinea*, *P. nigra* and *P. radiata* (Capretti 1978, 1980, Luchi et al. 2015). A possible reason for the lack of knowledge on this pathogen is that the identification of *Caliciopsis* canker symptoms become more challenging as tree size increases (Munck et al. 2015, 2016). Moreover, *C. pinea* symptoms, such as pitching blackened, rough, resinosis and sunken bark, are similar to (and may be confused with) those caused by other important pine pathogens like *Fusarium circinatum*, *Diplodia pinea* or *Diplodia scrobiculata*. The capabil-

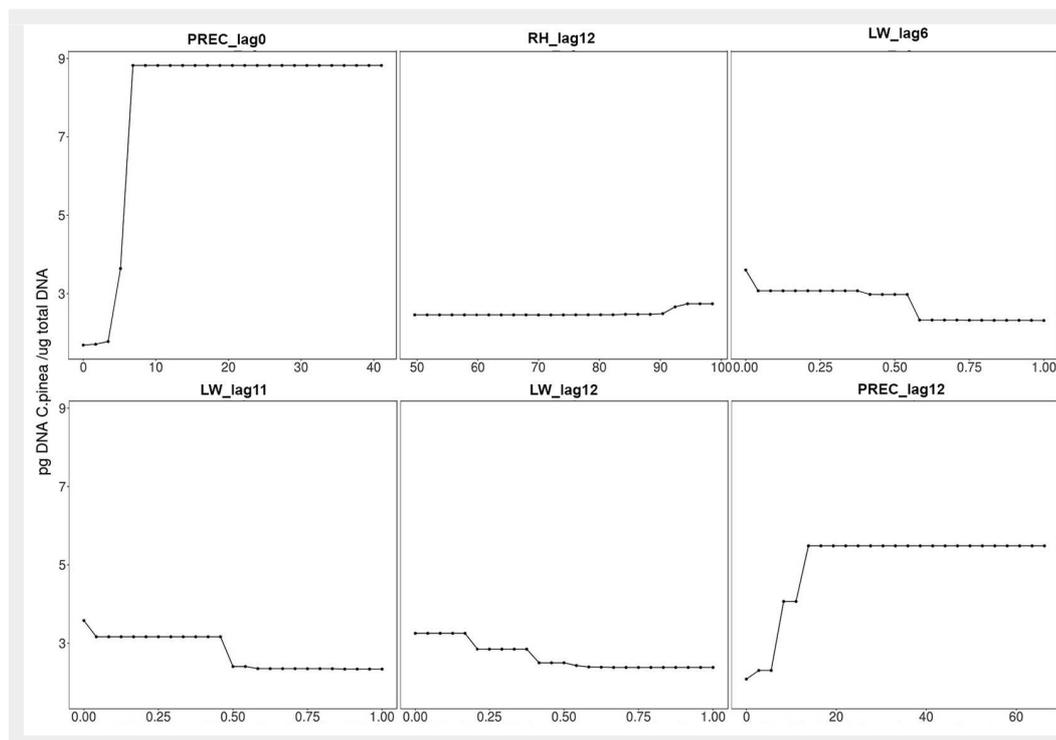
ity of *C. pinea* to play a combined role in fungal infections on pines has been observed in 2006 on *P. strobus* trees in Bath County, VA (USA), where *C. pinea* was isolated together with *D. scrobiculata* from cankers and lesions of branches and stems (Cram et al. 2009). Likewise, it was collected in 2010 from a *P. radiata* sample in Spain, where both *C. pinea* and *F. circinatum* occurred on the same stem (Capretti et al. 2013). However, in our study *F. circinatum* was never detected from any of the conspicuous tissue explants made from symptomatic *P. radiata* tissues collected in the sampling area (data not shown). Con-

versely, in Carcheri the characteristic fruiting bodies of the *C. pinea* were observed on small cankers on primary and secondary branches of *P. nigra* twigs affected by *D. pinea* (Aglietti 2015).

*Influence of climatic conditions on the occurrence of C. pinea*

The load of *C. pinea* spores in the air proved to be mostly influenced by precipitation during the sampling day and, to a lesser extent, by the amount of precipitation, leaf wetness and relative humidity of the 12<sup>th</sup> day before sampling (i.e., spore trapping), leaf wetness of the 11<sup>th</sup> day before and relative humidity of the 6<sup>th</sup> day before. The importance of these variables (i.e., permutation-based mean decrease in accuracy) is shown in decreasing order in Fig. 4. All other variables were deemed not important by the algorithm used. Partial dependence plots, i.e., the dependence of *C. pinea* DNA amount on the significant attributes accounting for all other tested features, are displayed in Fig. 5. These results may be explained by considering the chain of events in terms of climatic conditions, fructification and sporulation. Indeed, climatic conditions may influence the formation of fruiting bodies, which has a direct impact on the number of spores produced, but they also affect sporulation, which may take place several days after the formation of fruiting bodies. This can explain the significant effect of both lagged (11-12 days before sampling) and non-lagged (like PREC\_lag0) climatic variables on the *Caliciopsis pinea* air inoculum observed in this study.

Other studies have pointed out the influence of precipitations on the inoculum occurrence of other forest pathogens, such



**Fig. 5** - Partial dependence plots for significant variables. (PREC): precipitation (mm); (LW): leaf wetness (1=dry; 0=wet); (RH): relative humidity (%); (\_lagX): X<sup>th</sup> days before sampling.

as *F. circinatum* (Garbelotto et al. 2008), *Dothistroma septosporum* (Woods et al. 2016) and *Hymenoscyphus fraxineus* (Cermáková et al. 2017, Dvorák et al. 2015). In the latter case, a significantly higher concentration of spores was detected in wetter than in drier localities in the Czech Republic (Cermáková et al. 2017) and the highest correlation was found between relative humidity and *H. fraxineus* air inoculum records with an approximately 13-day lag (Cermáková et al. 2017, Dvorák et al. 2015). In the present study the highest correlation (75.96%) of *C. pinea* air inoculum was found with the relative humidity recorded 11 days before sampling.

#### Efficiency of rotating arm spore traps and qPCR for the detection of *C. pinea*

The rotating arm spore traps combined with qPCR assay allowed an efficient and suitable method for early detection of this pathogen showing an extreme sensitivity up to  $7.74 \times 10^{-4}$  pg DNA *C. pinea* /  $\mu\text{g}$  of total DNA extracted on 11-12<sup>TH</sup> July, 2016 (Fig. 3). This type of spore trap has been previously utilized for the detection and quantification of the air inoculum of other important forest pathogens, such as *H. fraxineus* in Belgium (Chandelier et al. 2014) and the Czech Republic (Dvorák et al. 2017), and more recently for the detection of *F. circinatum* in a plantation of *P. radiata* in Galicia, Spain (Dvorák et al. 2017). Using the Noll's equation (Noll 1970), ROTTRAP 120 was calculated to be almost 100% efficient for particles of spherical diameter of at least 7.18  $\mu\text{m}$  (Dvorák et al. 2017). The chiefly ellipsoidal ascospores of *C. pinea* are  $5\text{-}6 \times 3\text{-}3.5 \mu\text{m}$  (Fitzpatrick 1942, Lanier 1965), and the spermatia 2.5-3.5  $\mu\text{m}$  in length. Regarding the theoretical calculation of Noll (1970), particles of such size are trapped by ROTTRAP 120 with the efficiency of 50 % and 13 %, respectively. Consequently, the detected inoculum of *C. pinea* might be strongly underestimated and the actual air-borne spores' amount might be higher. The *C. pinea* inoculum was collected in 25 out of the 29 sampling days, demonstrating that the inoculum production of this pathogen covers a large part of the vegetative season.

#### Conclusions

The efficiency and suitability of rotating arm spore traps combined with the sensitivity of qPCR allowed the detection of *C. pinea* inoculum in the pine stand during the sampling period. The results here obtained improve our understanding of *C. pinea* epidemiology showing that the period of 12 days before the spore occurrence, which is influenced by higher relative humidity and precipitations, may enhance the fruiting body development. Later, immediate release of spores in mature fruiting bodies is triggered by rain. This approach could be applied for an efficient and effective management and control measures against this pathogen in pinewood stands.

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#### Author contributions

LB, MD and NL conceived of the study; AB, ALP, NL, MD and LB performed the experiments; TK and LB analysed the data; LB, MD, AS and NL wrote the manuscript.

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