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ORIGINAL ARTICLE

Temporal and spatial dispersal of *Thekopsora areolata* basidiospores, aeciospores, and urediniospores

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Abstract

Cherry spruce rust causes huge yield losses in Norway spruce seed production in Fennoscandia. The causal agent, *Thekopsora areolata*, has three types of spores that disperse during spring: basidiospores are produced on basidia that grow out from teliospores in overwintered bird cherry leaf litter to infect new pistillate spruce cones, aeciospores are released from old diseased spruce cones to infect bird cherry leaves, and urediniospores are produced from new bird cherry leaves for reinfection. No study has examined the dispersal of *T. areolata* spores, including the basidiospores that cause primary infection in spruce cones. In this study, teliospores of *T. areolata* were germinated in the laboratory and the morphology of basidiospores was described. *T. areolata* spores were sampled in Ultuna, Sweden and Joutsa, Finland with 21 spore traps at each site. Peaks in aeciospores were observed from 11 to 25 May and from 2 to 8 June at the Finnish site, and from 4 to 18 May at the Swedish site. Urediniospores were first observed 2–3 weeks after the peaks in aeciospores and they were mainly distributed within 10 m from the bird cherry trees. Peaks of 1–2 weeks in basidiospore detection coincided with multiple rain events. The basidiospore peak overlapped with the spruce pollen peak in Finland but not in Sweden. The quantities of basidiospores from different spore traps within 100 m from the spore source had no gradient. Information on spatial and temporal spore release is important for making decisions on disease management strategies.

KEYWORDS
cherry spruce rust, *Picea abies*, *Prunus padus*, spore dispersal, spore trap

1 | INTRODUCTION

Understanding the spatial and temporal dynamics of forest pathogen spore dispersal is essential for efficient management strategies against diseases. Information about the distance and time of the spore dispersal of fungal pathogens can assist decisions about the spatial and temporal range of the applications of silvicultural measures or chemical control required. For rust diseases, the dispersal of airborne spores has been investigated in theoretical studies (Aylor, 2003) and experimental field studies (Pfender et al., 2006). Most studies have focused on the dispersal of urediniospores, especially long-distance dispersal of rust diseases such as wheat stem rust (*Puccinia graminis*) (Meyer et al., 2017) and tree diseases such as eucalyptus rust (*Puccinia psidii*) (Lana et al., 2012), because

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urediniospores cause the most extensive damage on these plants. In some other tree rusts, such as white pine blister rust and cherry spruce rust, basidiospores cause the primary infection that leads to heavily damaged trees. Rust basidiospores are considered relatively fragile and they do not spread the disease over long distances (Zhao et al., 2016). In previous studies, the estimates of dispersal distance of basidiospores from white pine blister rust (*Cronartium ribicola*) or comandra rust (*Cronartium comandrae*) varied from 15–18 m to 0.8 km, but under favourable weather conditions it was estimated the basidiospores may disperse up to several kilometres (Jacobi et al., 1993; Van Arsdel, 1965). The estimates of dispersal were mostly based on distance from the basidiospore source to infected trees, possibly due to the lack of identifiable morphological characters of basidiospores for reliable identification in the field; this is also the case for cherry spruce rust. Information on the basidiospore dispersal of cherry spruce rust is needed to aid the establishment of control measures.

Norway spruce (*Picea abies*) is the most important tree species in Fennoscandian forestry and its planting relies on high-quality seeds from seed orchards. Finland and Sweden have large areas of Norway spruce seed orchards, where seeds are produced from clones selected from breeding programmes. However, meeting the demand for spruce seeds is challenging because of the production shortage caused by the irregular flowering of spruce trees (Lundströmer et al., 2020). Diseases such as cherry spruce rust can further reduce the seed yields as infected spruce cones with cherry spruce rust symptoms produce no fertile seeds or poor-quality seeds with a 10-fold reduction in germination rate (Kaitera & Tillman-Sutela, 2014). A severe epidemic of this disease can destroy the entire seed yield (Kaitera, 2013).

Cherry spruce rust is caused by the heteroecious rust fungus *Thekopsora areolata*, with bird cherry (*Prunus padus*) being the most common alternate host (Kaitera et al., 2017, 2019). Infected bird cherry leaves produce reddish-brown angular leaf spots that are limited by veins, and infected spruce cone scales produce reddish-brown to dark brown aecia, which can fully cover the inner side of all the scales. During the 2-year life cycle, *T. areolata* infects a main host and an alternate host and produces five spore types: the overwintered teliospores in bird cherry leaf litter germinate and produce airborne basidiospores to infect spruce pistillate cones in late spring. The mycelia grow in the cones and produce receptive hyphae and spermatia in early to mid-summer, which, after fertilization, can produce aecia with aeciospores in mid to late summer. After overwintering, aeciospores are released from the cones the following year to infect new bird cherry leaves (Kaitera et al., 2009a), on which urediniospores are continuously produced on the abaxial side to reinfect bird cherry leaves under favourable weather conditions. In autumn, teliospores are formed within the leaf epidermal cells to overwinter and thereafter another disease cycle starts in the following spring.

According to the life cycle, the existence of an alternate host is essential for an epidemic of cherry spruce rust, but infected cones can still be found in seed orchards or seed tree stands without *P. padus* in the vicinity (Kaitera, 2013; Kaitera et al., 2009a). It cannot be ruled out that other, previously unknown, host plants for *T. areolata* exist; however, our recent study found no new *Prunus* spp. or other tested wild plant species in Scandinavia that was an alternate host for *T. areolata* (Zhang et al., 2021). Potential long-distance dissemination of *T. areolata* basidiospores may explain the epidemic of cherry spruce rust in the seed orchard in central Finland where the closest *Prunus* spore sources are located several kilometres from the orchard (Kaitera et al., 2009a).

Although their role in a cherry spruce rust epidemic is critical, studies of the basidiospores of *T. areolata* are very limited. Like many other rust fungi, *Thekopsora* spp. basidiospores develop from basidia after teliospore germination (Cummins & Hiratsuka, 2003). In the literature, morphological characters of *Thekopsora* spp. basidiospores that would be useful for taxonomic identification are missing, and the basidia are usually ambiguously described as "basidia external" (Cummins & Hiratsuka, 2003); there is also no available photographic archive of *T. areolata* basidiospores. In addition, epidemiological information about favourable weather conditions for their production, dispersal distance, and temporal abundance are unknown. There is limited information on the susceptible period of the Norway spruce host. Spruce cone scales are open during pollination to receive windborne pollen and so it is possible that *T. areolata* basidiospores enter the spruce cones at or around the same time.

Spore trap techniques with different quantification and sampling methods have been applied to monitor fungal spore load in many studies. Spores can be quantified through counting under the microscope or by quantitative polymerase chain reaction (qPCR) (Duvivier et al., 2016; Garbelotto et al., 2008). Spores of *T. areolata* should be monitored in spring to summer because this is a critical period for the pathogen: basidiospores are produced from *Prunus* leaf litter to infect developing pistillate cones; aeciospores are released from old cones to infect *Prunus* leaves; and subsequently, urediniospores are produced from *Prunus* leaves to reinfect more leaves (Kuprevich & Transchel, 1954). All three windborne spore types may coexist in the air; therefore, spore quantification methods that rely solely on the quantity of DNA in spore trap samples are not suitable for *T. areolata*, because the results cannot provide any spore-type information. Even though it can be time-consuming, identification and counting of spores under a microscope can be carried out with aeciospores and urediniospores of *T. areoloata*, as well as for pollen of *P. abies*. However, this method is not applicable to basidiospores because they lack practical species-specific morphological characteristics for reliable identification.

The spore trap sampling method can be either active or passive. Active sampling instruments such as Burkard spore traps can actively draw in air and capture spores on adhesive tape. This type of spore trap is sensitive but expensive, and thus unaffordable for epidemiology projects that require several replicates (Quesada et al., 2018). Deploying multiple passive spore traps such as filter paper and adhesive slides is easier to achieve. This type of spore trap is commonly used in forest systems (Garbelotto et al., 2008;

Schweigkofler et al., 2004), and trapped spores can be quantified by qPCR or counted under a microscope.

In this study, a simple spore trap design was used for DNA quantification, as well as spore counting and pollen assessment under the microscope. These methods enabled an estimate of basidiospore loads from the discrepancy between DNA quantity and the number of urediniospores and aeciospores. In other words, if a high quantity of *T. areolata* DNA was detected, but only a low number of aeciospores and urediniospores were counted from the spore trap, this indicated that a large number of basidiospores were deposited on the spore trap. This study aims to enhance our knowledge about spore dispersal and the epidemiology of *T. areolata* by using spore traps deployed in Sweden and Finland. The objectives were to investigate (a) the dispersal distance and peak period of basidiospore release, (b) the dispersal distance and peak period for release of aeciospores and urediniospores, and (c) the weather conditions associated with basidiospore dispersal.

2 | MATERIALS AND METHODS

2.1 | Teliospore germination in the laboratory

P. padus leaf litter infected by *T. areolata* was collected in late October 2019. The leaves were kept in a mesh bag and placed outdoors until April 2020. Subsequently, dry leaves were immersed in distilled water for 2 h, changing the water every 30 min. Then, the leaves were placed adaxial side up in Petri dishes lined with wet paper towels to retain the moisture and were kept at room temperature overnight. After germination, the white mycelia produced on the surface of the telia were collected with a sterilized scalpel for subsequent DNA extraction and qPCR identification as described below. Cross-sections of germinating telia, made by cryostat and microtome, were examined under a microscope after staining with lactophenol cotton blue.

2.2 | Experiment sites and spore trap layout

The field experiments were performed in Ultuna, central Sweden (59°48′32″N, 17°39′30″E), from 13 April to 22 June 2020, and in Joutsa, southern Finland (61°39′28.0″N, 26°16′20.0″E), from 27 April to 29 June 2020. The two sites were selected because there were groups of bird cherry trees that constituted basidiospore and urediniospore sources, and Norway spruces (the aeciospore source) were not in their close vicinity. Meteorological data of daily precipitation and temperature were obtained from LANTMET (http://www.ffe.slu.se/lm/) for the Swedish site and the meteorological grid net provided by the Finnish Meteorological Institute for Natural Resources Institute Finland (not available freely) for the Finnish site.

The experiment site in Ultuna, Sweden, was located close to the Swedish University of Agricultural Sciences campus, in an open field with a row of approximately 10 bird cherry trees in the north–south direction. Other vegetation in the area included wheat, oat, and willow. No lone Norway spruce tree was located within at least 200 m of the bird cherry trees, and the nearest Norway spruce forest was located over 500 m away. *T. areolata* infection had been observed regularly in these bird cherry trees in the previous years (A. Olson and B. Samils, personal observations). A total of 21 spore traps were placed in the field, with sets of three spore traps placed in the centre of the *P. padus* trees (0 m), as well as at 10, 50, and 100 m towards the west direction, and 10, 50, and 100 m towards the east direction.

The experiment site in Joutsa, Finland, was located in a seed orchard (no. 365, Nikkanen et al., 1999) established in a Norway spruce forest. A total of 21 spore traps were placed in the middle of a 1–2 ha cut area in a small valley surrounded by large Norway spruce seed trees. In the centre of the cut area, a group of deciduous trees including bird cherry (*P. padus* subsp. *padus*) trees had been left for nature conservation purposes. A set of three spore traps were placed at the edge of the bird cherry trees (0 m), and at 10, 50, 100 m towards the north direction from the edge, and 10, 50, 100 m towards the east direction. The northward line of spore traps followed the borderline of the Norway spruce seed trees, in an open area. The eastward line was straight towards the forest with seed trees. The eastern 50 m traps were located at the top of the ridge and surrounded by mature spruce trees. The eastern 100 m trap was located further in the forest close to seed trees.

Each spore trap consisted of one filter paper (90 mm in diameter, Grade 1003; Ahlstrom-Munksjö) and one glass slide covered with Vaseline (petroleum gel) (Figure 1a,b). The filter paper was held by a metal net platform fixed on the top of a metal pole, 1–1.2 m above the ground. Each filter paper was treated in advance with 4× TE (Tris-EDTA) buffer according to Garbelotto et al. (2008). The Vaseline-covered slides were prepared according to Quesada et al. (2018). All filter papers and Vaseline slides were changed weekly in the field. Collected slides were stained with lactophenol cotton blue then covered with a 24 × 50 mm cover glass, filter paper samples were rolled up and stored in 50-ml Falcon tubes at −20°C until DNA extraction.

2.3 | Microscopic examination of Vaseline slides

The slides were examined under the microscope with 400× magnification. *T. areolata* aeciospores and urediniospores (Figure 1c,d) were identified based on morphological characters (Kuprevich & Transchel, 1954). The microscope field of view (diameter of 0.6 mm) was moved four times from the left to the right edge of the cover glass to cover 1/10 of the area of the cover glass (24 × 50 mm). The total numbers of *T. areolata* aeciospores and urediniospores captured on the Vaseline slides were estimated as the number counted × 10.

Spruce pollen abundance was assessed to determine the pollination stage of the spruce flowers/cones. To estimate the spruce pollen in the air, five random slides were examined from each batch of 21 slides in Finland and Sweden. The microscope field of view

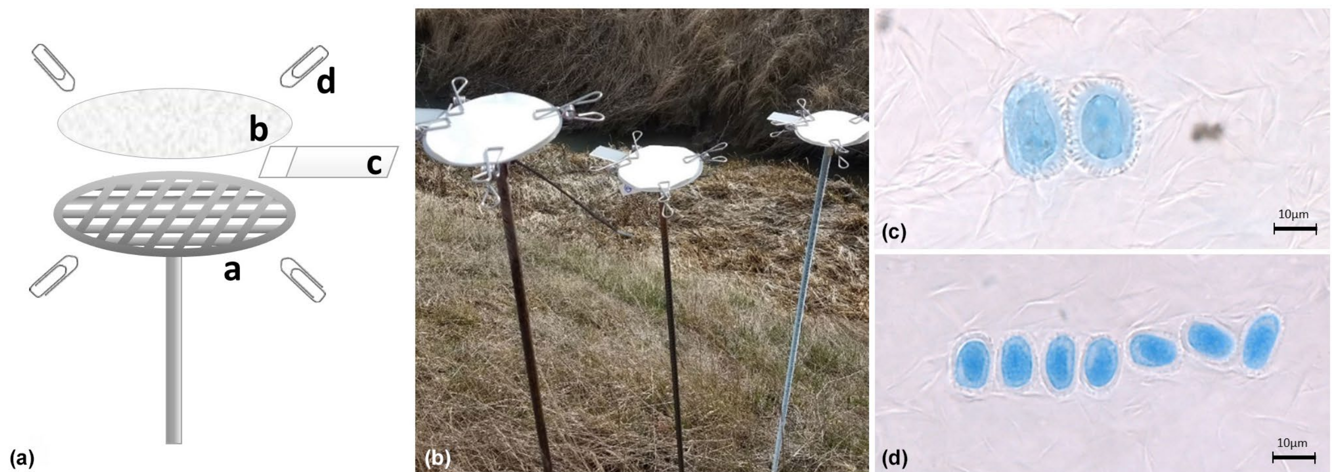

FIGURE 1 Spore trap design and the morphology of *Thekopsora areolata* spores on Vaseline slides. (a) Spore trap assembly: a, metal pole with a frame on top, approximately 1.2 m above the ground; b, filter paper treated with 4 × Tris-EDTA buffer; c, glass slide covered with Vaseline; d, clips to fasten the filter paper and slide. (b) Spore traps deployed in the field. (c) Aeciospores and (d) urediniospores, captured on a Vaseline-covered slide collected from the field and viewed under the microscope. Spores are stained with lactophenol cotton blue and bars represent 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

was moved twice from the left to the right edge of the cover glass to assess the amount of pollen. The average number of pollen grains on each slide was estimated as low (+, 50–500 pollen grains per slide), moderate (++, 501–5000 pollen grains per slide) and high (+++, >5000 pollen grains per slide).

2.4 | Method for quantification of DNA from *T. areolata* spores on filter paper

To confirm the validity of the method for *T. areolata* DNA quantification, standardized filter paper samples and spore suspension samples with known numbers of spores were prepared in the laboratory and processed according to Schweigkofler et al. (2004) with some modifications described below.

T. areolata aeciospores and urediniospores were collected from infected Norway spruce cones and bird cherry leaves, respectively, then transferred separately to solutions of 0.2% Tween 20. Spore concentrations of the aecio- and urediniospore suspensions were quantified with a haemocytometer, then adjusted to 1×10^6 spores/ml. Subsequently, the spore suspensions were diluted to 5×10^5, 1×10^5, and 1×10^4 spores/ml. To prepare standard spore suspension samples, 100 μl of spore suspensions of the four different concentrations was transferred to three 1.5-ml tubes each and stored at −20°C until DNA extraction. To prepare standardized filter paper samples, 100 μl of spore suspensions of the four different concentrations were loaded onto filter papers and placed in three 50-ml Falcon tubes. Filter paper samples were stored at room temperature for 7 days before DNA extraction. In summary, three replicates were prepared for each concentration, spore type, and sample type.

To harvest *T. areolata* spores and DNA from the filter paper, 20 ml 3% SDS (sodium dodecyl sulphate) buffer (50 mM Tris at pH 8, 50 mM EDTA, 3% SDS, 1 M NaCl) was added to each Falcon tube. The tubes were incubated in a 65°C water bath for 90 min and vortexed for 5 s every 20 min. After the incubation, the filter paper was removed and 20 ml isopropanol were added to each tube to precipitate the DNA. The samples were left at room temperature overnight, then centrifuged at 7000 × *g* for 10 min. The supernatant was removed as much as possible, then the spores and precipitated DNA were resuspended and transferred to a 1.5-ml centrifuge tube, followed by centrifugation at 9600 × *g* for 10 min to remove further supernatant. Spores and DNA from each filter paper sample were concentrated into about 100 μl of liquid.

DNA from the spore suspension samples and the filter paper samples were extracted with NucleoSpin Soil DNA extraction kit (Macherey-Nagel) according to the manufacturer's manual with the modification of eluting the DNA with 50 μl of ultrapure water. *T. areolata* DNA in each sample was quantified by qPCR, described below, with species-specific primers (Hietala et al., 2008; Zhang et al., 2021). To prepare samples for the standard curve, an 81 bp sequence of the internal transcribed spacer (ITS) region was amplified by standard PCR using *T. areolata* genomic DNA and the same primers. Purified amplicons were quantified with a NanoDrop spectrometer, then the concentration was transformed from ng/μl to ITS copies/μl according to the molecular weight of the 81 bp DNA sequence. Subsequently, the product was serially diluted from 6 to 6×10^6 copies per/μl to construct the qPCR standard curve. In qPCR assays, DNA samples were amplified in 20 μl reaction volumes containing 10 μl SsoFast EvaGreen Supermix (Bio-Rad), 5 μl DNA template, 1 μl each of forward and reverse primers (10 μM), and 3 μl water. PCR cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Total numbers of *T. areolata* ITS copies in the filter paper samples or spore suspension samples were calculated according to the qPCR result using the standard curve.

All filter paper samples from spore traps in the field were processed as described above to calculate *T. areolata* DNA quantity.

2.5 | Data analysis

All data were stored in .csv format using Excel, and the statistical analyses were performed using software R 4.0.2 in R studio v. 1.2.1335.

To verify the efficiency of the DNA extraction method for filter paper samples, linear regression and correlation between *T. areolata* DNA quantity and the number of spores in the laboratory samples were calculated.

The numbers of aeciospores and urediniospores on Vaseline slides and *T. areolata* DNA quantity on filter paper samples were visualized with R package ggplot2. To reveal the discrepancy between DNA quantity and spore quantity, the numbers of aeciospores and urediniospores on the same Vaseline slide were added together as the total spore number. Total spore number and DNA quantities were $\log_{10}$ transformed for statistical analysis. Pearson's correlation coefficient (r) was calculated for the association between total spore number and DNA quantity for each experiment site. Analysis of covariance (ANCOVA) was performed to compare the linear regression lines of different sample collection dates. Linear mixed models were used for ANCOVA, with DNA quantity as the response variable, total spore number as the numerical variable, collection date as categorical variable/treatment, and spore trap distance as random effects. In the post hoc test of multiple comparisons of regression line intercepts, Tukey's honestly significant difference (HSD) test was used. The null hypothesis was that samples from different collection dates had the same regression line, and there were no peaks of basidiospore dispersal that increased the DNA quantity in the filter paper during the sampling period. Samples from collection dates with significant basidiospore dispersal (significantly higher regression intercept) were used in the next ANCOVA to test the effect of spore trap distance on DNA quantity with a linear mixed model, with DNA quantity as the response variable, total spore number as the numerical variable, and collection date as random effects. Spore trap distance was treated as a categorical variable with either seven categories (centre 0 m; 10, 50, and 100 m towards the east; and 10, 50, and 100 m towards the west [Sweden] or north [Finland]) or, alternatively, four categories (0, 10, 50, and 100 m). Tukey's HSD test was used in the post hoc test of multiple comparisons of regression line intercepts. The null hypothesis was that samples from different spore trap distances had the same regression line, and spore trap distance had no effect on the number of collected basidiospores. The alternate hypothesis was that spore traps located at different distances trapped different numbers of basidiospores.

Average daily temperature, number of rainy days, and total precipitation during the sampling interval was calculated based on the daily meteorological data for every 7 days. Total degree-day accumulation at each sampling date was calculated by summing daily temperature from 1 January to each sampling day, using the base threshold of 0°C.

3 | RESULTS

3.1 | Teliospore germination and characterization of *T. areolata* basidiospores

After 2 h rehydration and overnight incubation in moisture, white mycelia could be observed with the naked eye on the bird cherry leaf litter surface where telia were located, but not on surface areas without telia (Figure 2a,b). DNA from mycelial samples collected from three surface areas with germinating telia were tested in the species-specific qPCR assay, and the samples' identities as *T. areolata* were confirmed. When leaf cross-sections were examined, teliospores could be observed within leaf epidermal cells, 13–19 μm high, 11–25 μm across (n = 20), with longitudinal septa (Figure 2c–f). Basidiospores were located outside leaf epidermal cells that contained teliospores; the basidiospores were hyaline, globose or subglobose, 2.5–3.5 μm in diameter (n = 20) (Figure 2c–e). The basidium could not be easily identified during the observations. One central germ pore for the probasidial cell could be found in each teliospore (Figure 2f).

3.2 | Temporal and spatial pattern of aeciospore dispersal

Aeciospores were observed on almost all Vaseline slides from the two experimental sites, and clear peaks in abundance were observed (Figure 3).

In Joutsa, Finland, the highest number of aeciospores were deposited on the slides at the collection dates 18 May, 25 May, and 8 June (Figure 3). Because of the proximity of the spore traps to spruce trees with cones as a source of aeciospores, clusters of aeciospores were occasionally found on the Vaseline slides (data not shown). In all peak periods, aeciospore distribution at different distances from the bird cherry trees showed similar patterns: the highest quantities of aeciospores were counted from the eastern 50 m traps, which were located beside mature spruce trees. Aeciospore quantities had lower variation during the nonpeak periods among all 21 spore traps.

In Ultuna, Sweden, aeciospore release peaked at the collection dates 11 and 18 May, which was 1 week earlier than in Joutsa, Finland. Lower spore release peaks were found on the collection dates 4 May and 1 June (Figure 3). During the peak periods of aeciospore release, aeciospore quantities at different distances showed similar patterns: the number of aeciospores deposited on the slides decreased from the east to the west. Aeciospore counts had lower variation among the 21 spore traps during the nonpeak periods.

3.3 | Temporal and spatial pattern of urediniospore dispersal

During the sampling period, more urediniospores were captured on the Vaseline slides in Joutsa, Finland, than in Ultuna, Sweden

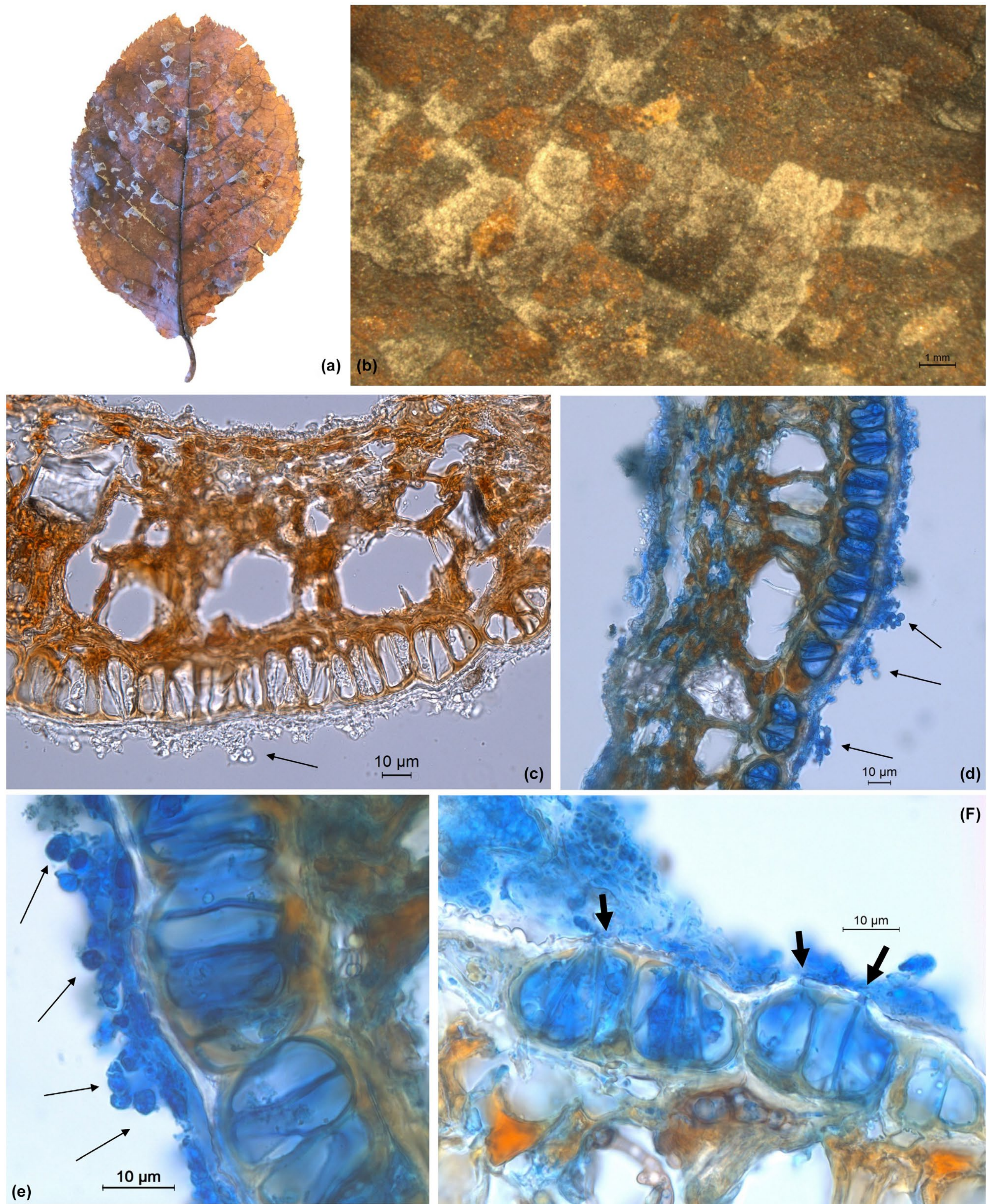

FIGURE 2 Germination of *Thekopsora areolata* teliospores and the microscopic morphology of basidiospores. (a) *Prunus padus* leaf litter with germinating telia. (b) Germinating telia under the dissecting microscope. (c, d) Cross-sections of germinating telia showed teliospores within epidermal cells and basidiospores (thin arrow). Bar = 10 μm. (e, f) Teliospores within epidermal cells and basidiospores (thin arrows). Central germ pores can be seen in the probasidial cells (thick arrows). Bar = 10 μm. In (d–f), slides were stained with lactophenol cotton blue [Colour figure can be viewed at wileyonlinelibrary.com]

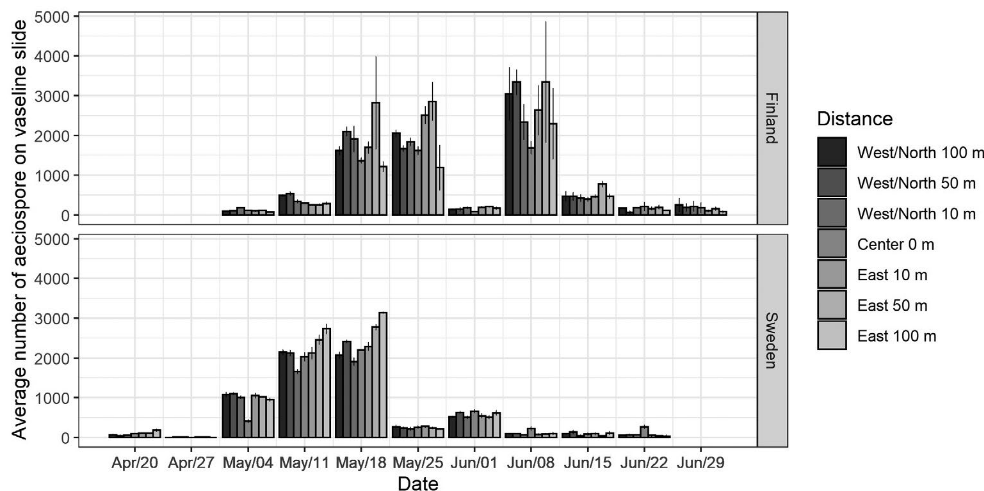

FIGURE 3 Temporal and spatial dispersal of *Thekopsora areolata* aeciospores in Joutsa, Finland, and Ultuna, Sweden. Spores were collected on slides in spore traps placed at varying distances to the west and east (Ultuna) or to the north and east (Joutsa) from the source trees, *Prunus padus*

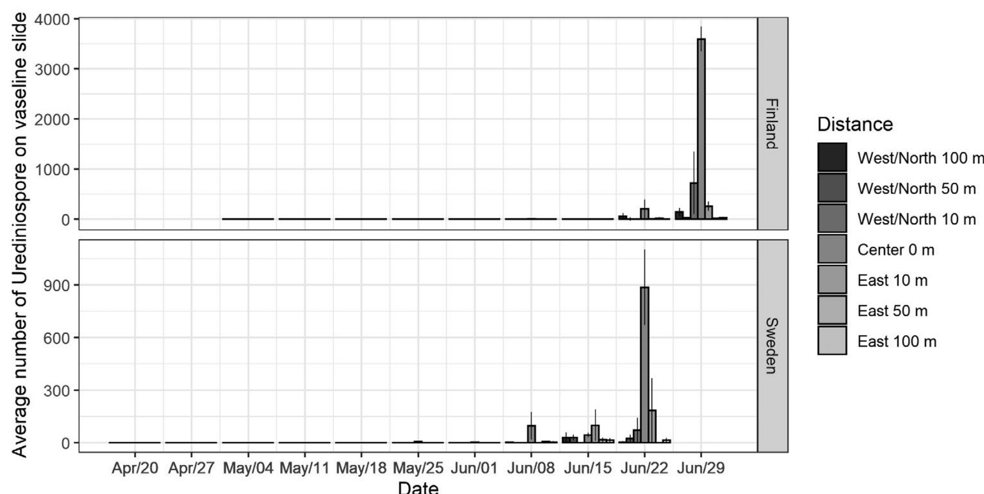

FIGURE 4 Temporal and spatial dispersal of *Thekopsora areolata* urediniospores in Joutsa, Finland, and Ultuna, Sweden. Spores were collected at slides on spore traps placed at varying distances to the west and east (Ultuna) or to the north and east (Joutsa) from the source trees, *Prunus padus*

(Figure 4). The first urediniospore was observed from the 0 m traps collected on 8 June in Joutsa, Finland, 3 weeks after the first aeciospore release peak. The highest number of urediniospores collected during the sampling period was recorded from 23 to 29 June. The highest amounts of urediniospores were always found on slides from the 0 m traps, and urediniospore number decreased significantly as the distances to the source trees increased. Using the average number of urediniospores collected from the 0 m traps as a reference (100%), the average number of urediniospores detected at north and east 10 m was 10.2% during the last sampling week. From samples collected from north and east 100 m traps, 1.7% of urediniospores were found compared to the 0 m reference.

In Ultuna, Sweden, urediniospores were first observed from 0 m traps on 25 May, 2–3 weeks after the aeciospore release peaks on 4 and 11 May. Similar to the results in Finland, the highest number of urediniospores were counted towards the end of the sampling period. From 16 to 22 June, an average of 10.8% urediniospores were detected at the 10 m traps (west and east), and 0.7% were detected at the 100 m traps (west and east) compared to the 0 m traps (100%).

3.4 | Quantification of DNA from aeciospores and urediniospores on filter paper

To evaluate the efficacy of the spore quantification method by qPCR, ITS copy numbers of aeciospores and urediniospores in suspension and on filter paper were calculated. When the quantities of aeciospores and urediniospores were equal in spore suspensions, similar quantities of *T. areolata* DNA were detected from aeciospore samples and urediniospore samples (Figure 5). The regression analyses of aeciospore and urediniospore numbers against *T. areolata* ITS

copy numbers gave similar results, where the 95% confidence intervals of the two regression lines overlapped, and both of the regression lines had high coefficients of determination, $R^2 = 0.99$.

Compared to spore suspensions, lower DNA quantities were detected from filter paper samples. Filter paper samples with urediniospores released a lower amount of DNA than aeciospore samples. Nevertheless, regression analyses showed strong correlations between spore number and ITS copy number for both aeciospores and urediniospores, with $R^2 = 0.96$ and 0.94, respectively (Figure 5). The results indicated that the DNA quantification protocol was valid, and the *T. areolata* ITS copy number from the qPCR assay could be used to calculate the relative abundance of aeciospores and urediniospores in filter paper samples.

3.5 | Temporal and spatial pattern of *T. areolata* DNA

In Joutsa, Finland, the highest amounts of *T. areolata* DNA were found on filter paper samples collected on 8 June, while other samples had much lower quantities (Figure 6). There was no significant correlation between DNA quantity and distance to the spore source on the bird cherry trees, except among samples collected on 29 June. The highest quantity of *T. areolata* DNA was detected from 0 m spore traps, where the highest amount of urediniospores was captured.

Before 25 May, when urediniospores were first found on the slides in Ultuna, Sweden, the highest amount of *T. areolata* DNA was detected from filter paper collected on 4 and 18 May. After urediniospores were produced, the highest amount of *T. areolata* DNA could always be detected on filter paper from 0 m spore traps, which was consistent with the distribution pattern of urediniospores.

3.6 | Inference of basidiospore dispersal

At both experiment sites, the quantity of *T. areolata* DNA and total spore number had significant positive correlations ($p < 0.01$), with coefficient $r = 0.7212$ and 0.8436 in Joutsa, Finland, and Ultuna, Sweden, respectively (Figure 7).

Analyses of variance (ANOVA) results for the ANCOVA on the effect of collection date is presented in Table 1. The interaction of collection date and spore number was included in the first linear mixed model analysis. The interaction effects were not significant at either Joutsa, Finland, or Ultuna, Sweden ($p > 0.05$), which indicated one uniform

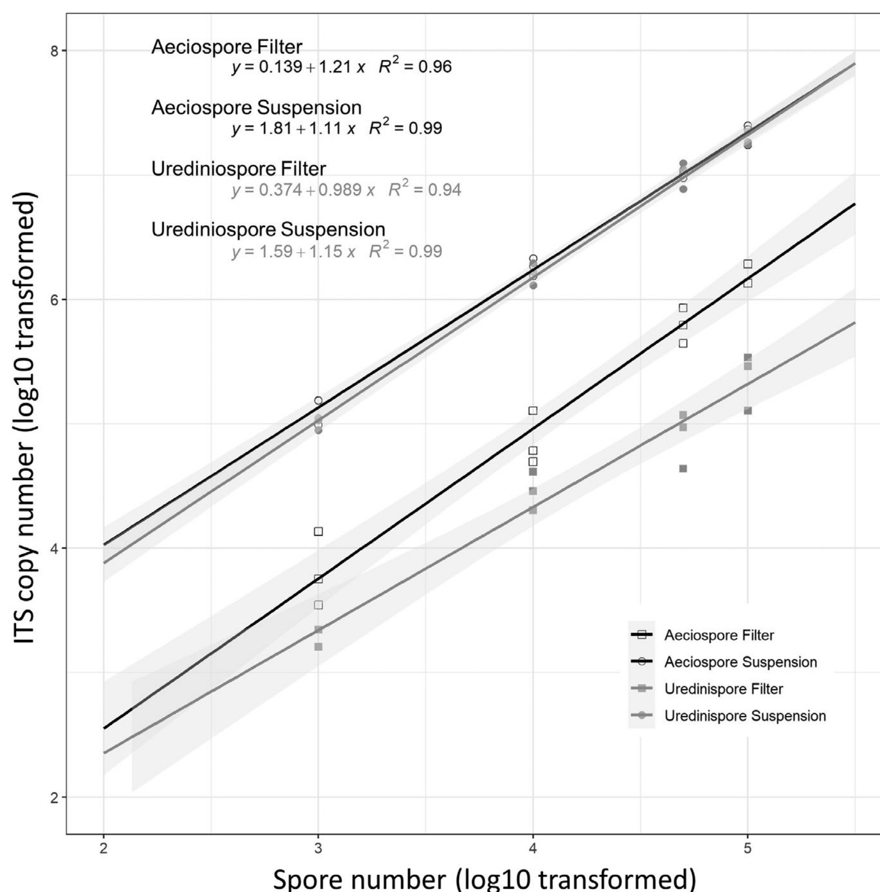

FIGURE 5 Linear regression of the number of aeciospores and urediniospores of *Thekopsora areolata* in prepared samples and the internal transcribed spacer (ITS) copy number determined by quantitative PCR of their extracted DNA. Samples of known numbers of spores were prepared on filter paper (squares) and in spore suspensions (circles)

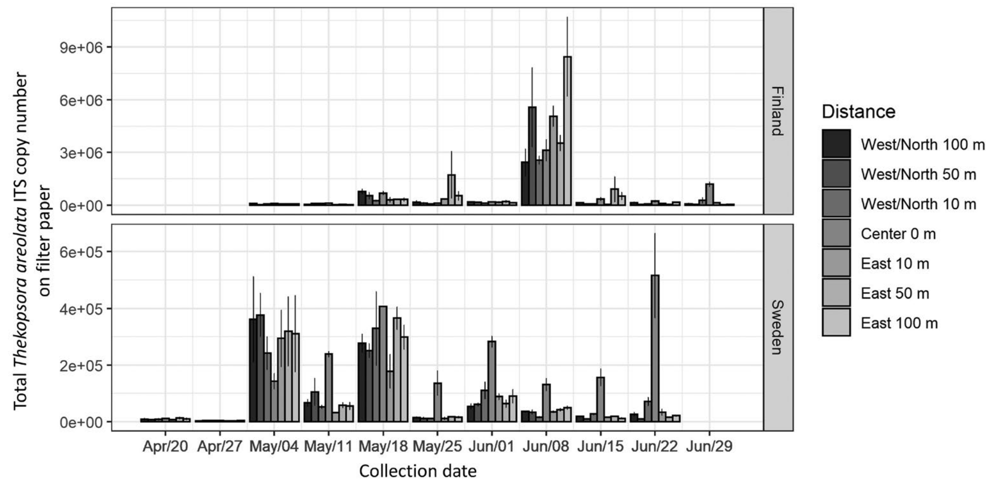

FIGURE 6 Total internal transcribed spacer (ITS) copy number determined by quantitative PCR of *Thekopsora areolata* DNA extracted from spores on filter paper from spore traps in Joutsa, Finland, and Ultuna, Sweden, between 20 April and 29 June. The shades of grey of the bars represent the distance of the trap from the source trees, *Prunus padus*, to the west and east (Ultuna) or to the north and east (Joutsa)

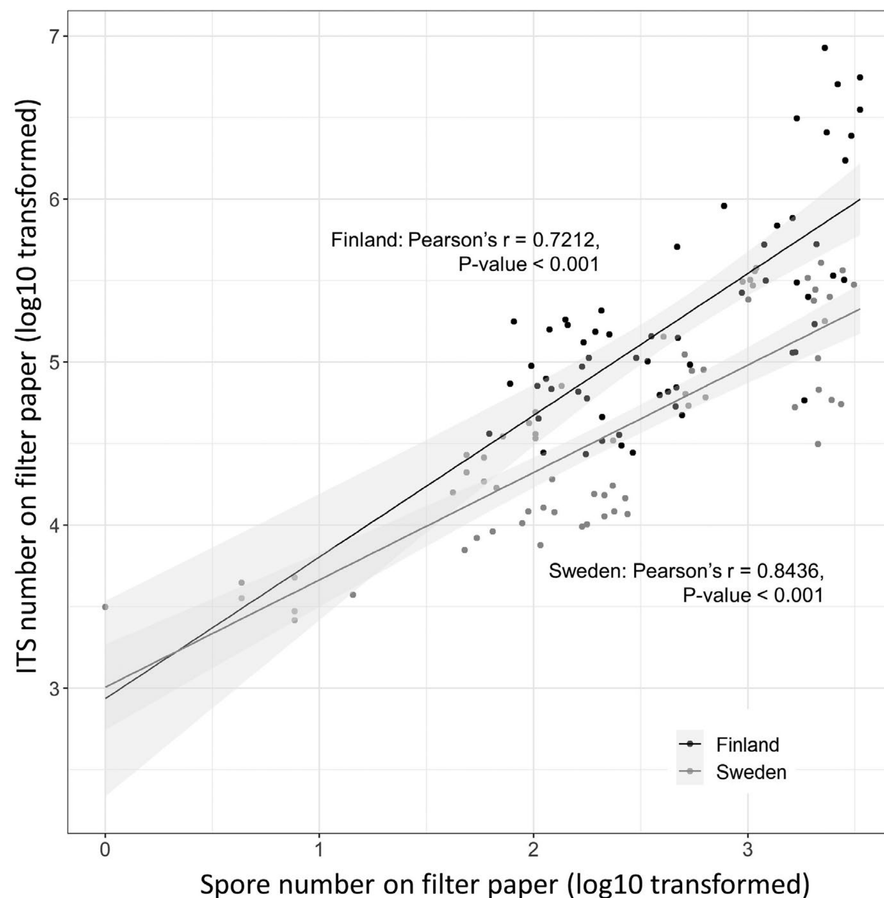

FIGURE 7 Correlation of total number of *Thekopsora areolata* aeciospores and urediniospores on Vaseline slides and *T. areolata* internal transcribed spacer (ITS) copy number determined by quantitative PCR of DNA extracted from spores on filter paper, both collected from spore traps in the field in Joutsa, Finland, and Ultuna, Sweden

slope estimate for the regression lines of different collection dates. In the analyses without the interaction effects, the significant collection date effect ($p < 0.001$) suggested different intercepts of regression lines among groups (Table 1). Therefore, the null hypothesis was rejected, and it was confirmed there were peaks in basidiospore dispersal during the sampling collection period at both experiment sites.

TABLE 1 Analysis of covariance of the effect of collection date on the amount of *Thekopsora areolata* DNA extracted from spores, with linear mixed model

	Sweden				Finland			
Model	*df*	Den *df*	*F*	*p*	*df*	Den *df*	*F*	*p*
DNA quantity ~ Spore number + Collection date + interaction, random = spore trap distance								
Intercept	1	39	26,893.840	<0.0001	1	39	12,404.397	<0.0001
Spore number	1	39	772.584	<0.0001	1	39	162.296	<0.0001
Collection date	9	39	26.965	<0.0001	8	39	11.506	<0.0001
Interaction	9	39	1.614	0.145	8	39	1.448	0.208
DNA quantity ~ Spore number + Collection date, random = spore trap distance								
Intercept	1	48	48366.29	<0.0001	1	47	9866.836	<0.0001
Spore number	1	48	655.34	<0.0001	1	47	153.211	<0.0001
Collection date	9	48	23.50	<0.0001	8	47	10.829	<0.0001

TABLE 2 Analysis of covariance of the effect of collection date in 2020 on the amount of *Thekopsora areolata* DNA extracted from spores, with linear mixed model

	Sweden				Finland			
Coefficient	Estimate	*SE*	*t*	*p*	Estimate	*SE*	*t*	*p*
Intercept	3.666	0.235	15.575	<0.0001	3.156	0.531	5.944	<0.0001
Spore number	0.158	0.117	1.346	0.184	0.810	0.254	3.193	0.002
27 Apr	−0.232	0.167	−1.390	0.170	—	—	—	—
4 May	1.315	0.150	8.786	<0.0001	—	—	—	—
11 May	0.659	0.187	3.518	0.001	−0.458	0.196	−2.339	0.024
18 May	1.266	0.191	6.616	<0.0001	−0.157	0.340	−0.462	0.646
25 May	0.091	0.107	0.853	0.397	−0.431	0.347	−1.240	0.221
1 Jun	0.778	0.133	5.839	<0.0001	0.278	0.157	1.769	0.083
8 Jun	0.556	0.093	5.994	<0.0001	0.687	0.379	1.813	0.076
15 Jun	0.221	0.093	2.361	0.022	−0.126	0.228	−0.552	0.583
22 Jun	0.418	0.093	4.499	<0.0001	−0.020	0.162	−0.125	0.901
29 Jun	—	—	—	—	−0.270	0.188	−1.433	0.158

Note: Model: DNA quantity ~ Spore number + Collection date, random = spore trap distance.

The results of the multiple comparisons and the post hoc test of regression line intercepts are presented in Table 2 and Figure 8. In Joutsa, Finland, the regression line of samples collected on 8 June had the highest estimated intercept. The peak of aeciospore dispersal was on collection dates 18 and 25 May and 8 June, with similar numbers of aeciospore counts from the slides (Figure 4). However, *T. areolata* DNA recovered from the samples collected on 8 June was significantly higher than other collected dates (Figure 6). Because only limited numbers of urediniospores were observed among these samples, we infer that the week between 2 and 8 June was most probably the dispersal peak of basidiospores.

In Ultuna Sweden, the regression line of samples collected on 4 and 18 May had the highest intercept ($p < 0.05$; Figure 8). The aeciospore dispersal peak was on collection dates 11 and 18 May, when similar numbers of aeciospores were counted from the slides (Figure 3). Higher quantities of *T. areolata* DNA were recovered from the samples collected on 4 and 18 May than on 11 May (Figure 6). Therefore, the weeks between 28 April and 4 May and between 12 and 18 May were most probably the basidiospore dispersal peak periods at Ultuna, Sweden.

Data collected on 1 and 8 June in Joutsa, Finland, and 4 and 18 May in Ultuna, Sweden, were used in the ANCOVA of *T. areolata* DNA quantity from spore traps at different distances (Table 3). The interaction effects of spore number and distance were not significant, which indicated one uniform slope estimate for the regression lines of different spore trap distance. Both the ANCOVA and the multiple comparison result showed that the spore trap distance factor was not significant in any location, that is, there was no significant increase or decrease of *T. areolata* DNA quantity detected from spore traps with increased distance from the bird cherry as spore source. The result indicates that no basidiospore gradient was found within 100 m in this experiment setting. Data were also analysed

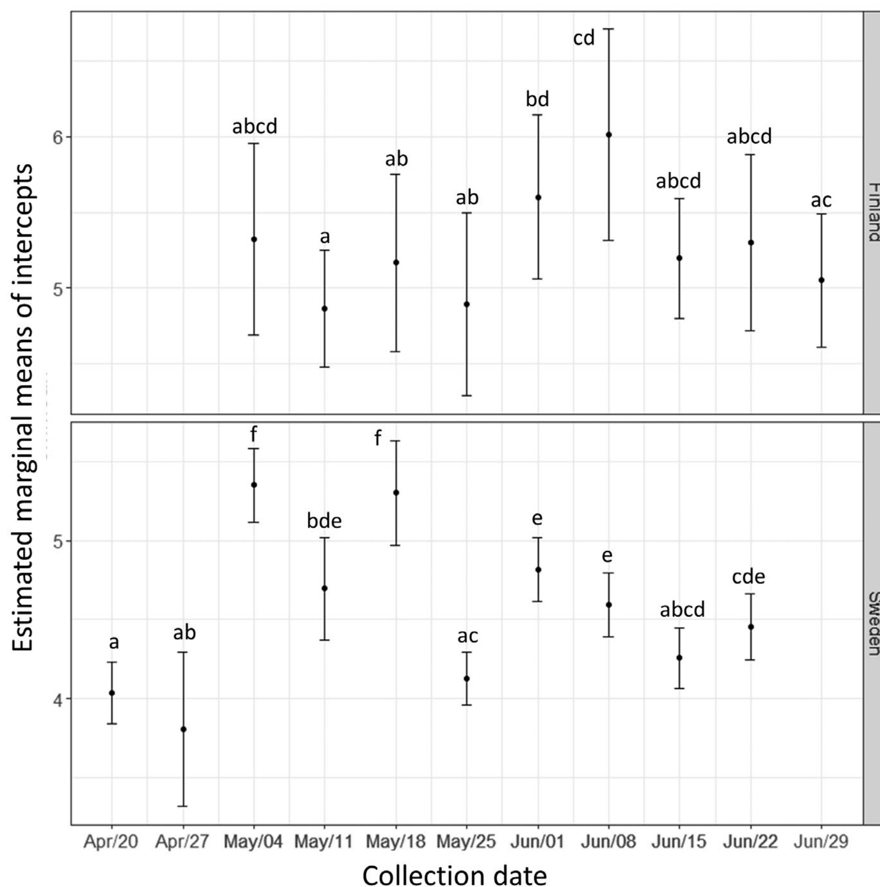

FIGURE 8 Multiple comparisons of intercepts of the collection date effect on the quantity of *Thekopsora areolata* DNA extracted from spores collected in the field in Joutsa, Finland, and Ultuna, Sweden. A linear mixed model was used. Intercepts that were not significantly different from each other ($p > 0.05$) are assigned with the same letter

with spore trap distance treated as a categorical variable with seven categories to investigate the influence of spore trap directions. The spore trap distance factor was not significant in any location. The statistic result is included in the supplementary information (Table S1).

3.7 | Weather conditions and abundance of spruce pollen during the spore dispersal peaks

Because the sampling was discontinued before the termination of urediniospore release, the association between urediniospore production and weather conditions is not discussed here. During the weeks with aeciospore peaks, frequent rainfall and high volumes of precipitation were recorded at both Joutsa, Finland, and Ultuna, Sweden, except for 19 to 25 May in Joutsa, Finland (Table 4). When the first inferred basidiospore peaks appeared, the degree-day accumulations were 447.9–484.7°C in Joutsa, Finland, and 442.0–537.5°C in Ultuna, Sweden. Multiple rain events, 4–6 days per week, always occurred during the peaks. The Norway spruce pollen and basidiospore dispersal peak appeared in the same week in Joutsa, Finland. In Ultuna, Sweden, basidiospore dispersal peaks occurred before the peak of pollen dispersal.

4 | DISCUSSION

In Norway spruce seed orchards, *T. areolata* basidiospores cause primary infection in cones and major seed yield losses, but the production and dispersal of basidiospores are seldom studied. Basidiospores of *T. areolata* are produced from basidia emerging from germinating subepidermal teliospores in leaf litter. Germination of teliospores can usually be induced by spraying with water (Yu et al., 2001) and high humidity (Moricca & Ragazzi, 2001). The protocol we describe here can regularly induce teliospore germination after samples have overwintered in the field. The method can be used in studies of spruce cone inoculation and molecular biology of the teliospore germination process. The examination of *T. areolata* basidiospores affirmed that it is difficult to quantify basidiospores based on morphology, especially from field samples that often contain spores from other fungal species.

We used the discrepancy between microscopic spore counts and DNA quantity to infer the presence of basidiospores. The rationale behind this was that aeciospores, urediniospores, and basidiospores are the only airborne structures of *T. areolata* that can be captured by spore traps. Because the laboratory test showed that the quantity of *T. areolata* DNA positively correlates with the number of aeciospores and urediniospores on filter paper, the excess DNA from

TABLE 3 Analysis of covariance and multiple comparisons of regression line of the effect of spore trap distance on amount of *Thekopsora areolata* DNA, with a linear mixed model; model: DNA quantity ~ Spore number + spore trap distance, random = Collection date

	Sweden				Finland			
	df	Den *df*	*F*	*p*	*df*	Den *df*	*F*	*p*
Intercept	1	34	15,978.721	<0.0001	1	36	52.203	<0.0001
Spore number	1	34	1.574	0.218	1	36	0.341	0.563
Distance	3	34	0.205	0.892	3	36	0.073	0.974
	Sweden				**Finland**			
Coefficient	**Estimate**	***SE***	***t***	***p***	**Estimate**	***SE***	***t***	***p***
Multiple comparisons of regression line intercepts								
Intercept	4.7240	0.5659	8.3474	<0.0001	6.2392	1.0176	6.1313	<0.0001
Spore number	0.2002	0.1878	1.0662	0.294	−0.1535	0.2308	−0.6647	0.510
10 m	0.0206	0.1422	0.1450	0.886	0.0580	0.1401	0.4140	0.681
50 m	0.0970	0.1452	0.6651	0.510	0.0339	0.2176	0.1560	0.877
100 m	0.0404	0.1429	0.2828	0.779	0.0665	0.1447	0.4595	0.649

Note: Spore trap distance is treated as a categorical variable with four categories (0, 10, 50, and 100 m).

field samples with high quantities of DNA is presumably from basidiospores. Therefore, our results show that dispersal peaks of *T. areolata* basidiospores were during 2 to 8 June in Joutsa, Finland, and 28 April to 4 May and 12 to 18 May in Ultuna, Sweden. There were multiple rain events during the peaks in both locations (Table 4; Figure S1) and, probably because of the slower degree-day accumulation (Table 4; Figure S2), the basidiospore dispersal time was later in Finland than in Sweden. The association between multiple rain events and a basidiospore dispersal peak was also observed in our pilot study in 2019, Ultuna, Sweden (Figure S3; Table S2). This result is consistent with other teliospore germination studies: frequent rain events and high degree-day accumulations often have positive effects on teliospore germination and basidiospore production, such as in *Melampsora pinitorqua* (Desprez-Loustau et al., 1998).

The association between the cone development stages and its susceptibility to *T. areolata* basidiospores is unknown. Spruce female cone scales are closed before pollination and then opened during pollination to receive windborne pollen. Differential cell proliferation and expansion after pollination causes the closure of the scales again to protect the seeds (Leslie & Losada, 2019). In the field, the basidiospore peak occurred before the pollen peak in Ultuna, Sweden, while the two peaks were concurrent in Joutsa, Finland. We hypothesize that the pollination stage in Norway spruce, when the cone scales are open to receive pollen, could be the stage most susceptible to *T. areolata* basidiospores. Consequently, unsynchronized peaks in basidiospore and pollen release may lead to a lower infection rate, but this hypothesis needs further investigation.

During the basidiospore peaks, similar amounts of *T. areolata* DNA were recovered from spore traps that were deployed 0 or 100 m away from the bird cherry trees in both locations. This result suggests that a significant number of basidiospores can disperse to at least 100 m away from the source. The lack of a detectable gradient might be explained by a shallow spore dispersal gradient, meaning that the basidiospores in the air might not decrease substantially over the first 100 m from the source. The basidiospores are small and may be carried by air movements for a longer time than larger spores (e.g., aeciospores and urediniospores) due to less downward drift caused by gravity. Movement due to diffusion has been suggested to be more important than movement due to gravity for spores below a critical spore size (Stockmarr et al., 2007). Background deposition from more distant bird cherry trees may also reduce the deposition gradient from the basidiospore source under investigation. Another contributing factor to the lack of a dispersal gradient might be the redistribution of bird cherry leaves by wind after leaf fall. If leaves with teliospores were spread by wind over a larger area around the bird cherry trees, it would reduce the detection of a gradient of basidiospores along the investigated distance. Basidiospores of rust are considered to spread diseases only within short distances because they are sensitive to ultraviolet light and dry conditions (Zhao et al., 2016). However, in some Norway spruce seed orchards in Finland that lacked bird cherry within at least 1 km, *T. areolata* still caused epidemics (Kaitera et al., 2009a); this suggests that these seed orchards might have received viable basidiospores from more distant sources. Short- and long-distance dispersal of *T. areolata* basidiospores under different conditions requires more investigation. Incidences of comandra blister rust (*C. comandrae*) in lodgepole pine have been shown to depend on long-distance dispersal, and were influenced by landform and airflow pattern as well as host and pathogen population distribution (Jacobi et al., 1993). A disease gradient of 2%–4% over the first 10 km and <0.5% beyond 10 km from the inoculum source was observed but with large variation between study areas, which might be explained by the differences

TABLE 4 Average daily temperature, degree-day accumulation, number of rainy days, total precipitation, and indication of peaks in numbers of *Thekopsora areolata* spores and pollen during each sampling interval in Sweden and Finland

Location	Collection date	Average daily temperature (°C)[a]	Total degree-day accumulation (°C)[b]	No. of rainy days[a]	Total precipitation (mm)[a]	Aeciospore peak	Basidiospore peak	Urediniospore peak	Pollen peak
Ultuna, Sweden	20 Apr	7.0	390.2	2	1.2				
	27 Apr	8.2	447.9	1	4.5				
	4 May	5.3	484.7	4	14.5	++	+++		
	11 May	8.1	541.2	1	11.6	+++			
	18 May	5.6	580.5	6	9.6	+++	+++		+
	25 May	10.0	650.5	1	3.4				+
	1 Jun	14.0	748.2	1	7.1				+++
	8 Jun	14.3	848.5	2	11.8				+++
	15 Jun	17.3	969.5	0	0				+++
	22 Jun	18.4	1098.3	2	28.5			+++	+
Joutsa, Finland	4 May	4.8	199.4	3	2.7[c]				
	11 May	6.8	246.7	3	19.9[c]				
	18 May	4.3	277.1	4	18.9	+++			
	25 May	9.8	345.6	2	1.6	+++			
	1 Jun	13.8	442.0	0	0				++
	8 Jun	13.6	537.5	4	18.2	+++	+++		+++
	15 Jun	17.4	659.3	1	4.6				+++
	22 Jun	19.1	793.2	1	5.1				++
	29 Jun	21.4	943.3	3	15.4			+++	+

[a]Data were calculated based on the daily temperature or precipitation within 7 days.

[b]Total degree-day accumulation was calculated by summing daily temperature from 1 January to each sampling day, using the base threshold of 0°C.

[c]Both rain and snow were recorded as precipitation.

in topography. Variations in the basidiospore dispersal range have been reported in other rust species such as *C. ribicola*. The effective dispersal distance of basidiospores, represented by disease incidence in pine, is usually limited to a few hundred metres (Buchanan & Kimmey, 1938). The distance of local dispersal is limited by factors such as the settling of condensation droplets with basidiospores and the loss of viability of the basidiospores, but, with specific landscape and microclimate, *C. ribicola* basidiospores could disperse over long distances (Zambino, 2010). For example, nocturnal air currents generated in the Great Lakes area in the United States could pick up basidiospores from *Ribes* growing on the lake shore, move them across the body of water, and deposit them 16–27 km away from the source (Dahir & Carlson, 2001; Van Arsdel, 1965). Airplane sampling of *Gymnosporangium juniperi-virginianae* basidiospores and viability tests showed that, under the right environmental conditions (temperature and humidity), the spores can travel several miles and stay viable for many days (MacLachlan, 1935).

The aeciospores of *T. areolata* are produced in spruce cones in autumn and released in spring (Kaitera et al., 2009a). Spruce cones with aecia can stay in the canopy and spread viable aeciospores for up to 4 years (Kaitera & Tillman-Sutela, 2014). In the present study, we could detect low numbers of aeciospores in both locations over the whole sampling period, but distinct peaks in numbers were also found. The number of aeciospores counted was higher in the seed orchard (Joutsa) than in an open area (Ultuna); however, during the highest peak of aeciospore dispersal, the number of spores in the air was also surprisingly high in the open area. The aeciospore peaks are likely to be induced by rain. During the sampling period (Table S2), there was significant rainfall during most peaks of aeciospore numbers, but daily observations of the association between rain and aeciospore release are needed to confirm this effect. It is possible that rain may help the rupture of aecia walls. For example, the outer cell walls are thicker than the inner cell walls of aecia peridial cells of *Puccinia graminis*; hence, water absorption can cause greater expansion of inner than outer walls, leading to wall rupture and spore discharge (Heath, 1979). Because the spore traps were not deployed according to the distance from aeciospore source in this study, our data are not optimal for analysis of the spatial pattern of aeciospore dispersal. Nonetheless, similar spatial dispersal patterns were found on different dates when there were peaks in aeciospore release. In Ultuna, Sweden, no spruce tree was located within 200 m from the 0 m spore trap, and the nearest spruce forest is over 500 m away. Therefore, the aeciospores on slides and infections on bird cherry leaves suggested that viable *T. areolata* aeciospores could disperse several hundred metres. In other studies, steep disease gradients have been observed within 152 m of the aeciospore source for western gall rust (*Cronartium harknessii*) (Schmidt et al., 1982), but the long "tail end" of spore distribution still resulted in long-distance dispersal at low concentrations (Hamelin et al., 2005). In addition, even migration over hundreds of kilometres has been reported (Nagarajan & Singh, 1990).

In both experiment sites, urediniospores were first observed 2–3 weeks after the peaks in aeciospores. This time range corresponded with laboratory observations by Kaitera et al. (2019), where it took 2 weeks for the bird cherry leaves to produce urediniospores after aeciospore inoculation under optimal conditions. In Ultuna, Sweden, bird cherry leaves were already developed and a low number of aeciospores were observed on 20 April, but urediniospores were not observed until 25 May. Thus, the urediniospore production in Ultuna, Sweden, resulted from the peaks in aeciospore release rather than the availability of bird cherry leaves. In Joutsa, Finland, due to cold weather, bird cherry leaves were either not yet developed or poorly developed during the aeciospore peaks of mid- to late May (J. Kaitera, personal observations); on 11 May, a 2 cm layer of snow covered the spore traps. The high number of urediniospores collected on 29 June probably resulted from the peak in aeciospore release from 2 to 8 June.

The number of airborne spores generally decreases rapidly as distance from the spore source increases. For example, only 16% and 0.6% of *Blumeria graminis* conidia can be detected at 50 and 200 m, respectively, compared to the number at 0.5 m from the spore source (Hovmøller, 1996). In our study, compared to the number of spores collected at 0 m (100%), the average number of urediniospores collected from 10 m spore traps in both directions was 10.8% in Ultuna, Sweden, and 10.2% in Joutsa, Finland. The amount of urediniospores dropped further to 0.7% at 100 m in Ultuna, Sweden, and to 1.7% in Joutsa, Finland. In other words, *T. areolata* urediniospores mainly deposit within 10 m from the spore source and only a low number of spores may spread beyond 100 m.

When using filter paper as passive spore traps, trapped spores need to be washed off and then homogenized before DNA extraction (Schweigkofler et al., 2004). Our result showed that the sensitivity of this method is low as the amount of *T. areolata* DNA detected from filter paper was only about 1/10 of that detected from spore suspensions with the same number of spores. However, with the high sensitivity of qPCR, this method is still suitable for detection of spores deposited on filter paper. In this study, qPCR results were precise among filter paper samples, with R^2 = 0.94 and 0.96 for urediniospores and aeciospores, respectively. Thus, the results could be used to examine the relative abundance of target spores trapped on filter paper.

Aeciospores and urediniospores of *T. areolata* only infect bird cherry and so the key to protection of spruce cones is to prevent the infection caused by basidiospores. Current disease control is by silvicultural methods, such as removing bird cherry from the vicinity of a spruce seed orchard. Our results indicated that basidiospores from bird cherry within 100 m of the seed orchard could certainly reach the spruce seed trees. A general Norwegian recommendation is to remove bird cherry trees within at least 400 m from the Christmas tree nurseries (Talgø & Stensvand, 2020). In Finland, several kilometres are required as a safe distance (J. Kaitera, unpublished data; Kaitera et al., 2009a). Cone bagging can interrupt the basidiospore infection, though it may also hinder cone development (Kaitera et al., 2009b). If sheltering of cones were used as a protection method, cones should be bagged before the basidiospore peak that is associated with multiple rainy days. Chemical applications may be feasible

for needle rusts in conifer nurseries and Christmas tree plantations (Hansen, 1997). In the case of cherry spruce rust, the optimal application time of contact fungicides is difficult to find because of the wash-off by frequent rain. Systemic fungicides would be more effective to control this disease.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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