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Ecology and Epidemiology

Monitoring Spore Dispersal and Early Infections of *Diplocarpon coronariae* Causing Apple Blotch Using Spore Traps and a New qPCR Method

Clémence Boutry,[1] Anne Bohr,[2] Sascha Buchleither,[2] Mathias Ludwig,[1] Thomas Oberhänsli,[1] Lucius Tamm,[1] Hans-Jakob Schärer,[1,†] and Pascale Flury[1,3,†]

[1] Plant Pathology, Research Institute of Organic Agriculture FiBL, Ackerstr. 113, 5070 Frick, Switzerland
[2] Competence Center for Fruit Crops at the Lake of Constance (KOB), Schuhmacherhof 6, 88213 Ravensburg, Germany
[3] Plant Microbe Interactions, University of Basel, Bernoullistrasse 30/32, 4056 Basel, Switzerland
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Abstract

Apple blotch (AB) is a major disease of apple in Asia and recently emerged in Europe and the United States. It is caused by the fungus *Diplocarpon coronariae* (formerly *Marssonina coronaria*; teleomorph: *Diplocarpon mali*) and leads to severe defoliation of apple trees in late summer, resulting in reduced yield and fruit quality. To develop effective disease management strategies, a sound knowledge of the pathogen's biology is crucial. Data on the early phase of disease development are scarce: No data on spore dispersal in Europe are available. We developed a highly sensitive TaqMan qPCR method to quantify *D. coronariae* conidia in spore trap samples. We monitored temporal and spatial dispersal of conidia of *D. coronariae* and the progress of AB in spring and early summer in an extensively managed apple orchard in Switzerland in 2019 and 2020. Our results show that *D. coronariae* overwinters in leaf litter, and spore dispersal and primary infections occur in late April and early May. We provide the first results describing early-season dispersal of conidia of *D. coronariae*, which, combined with the observed disease progress, helps to understand the disease dynamics and will be a basis for improved disease forecast models. Using the new qPCR method, we detected *D. coronariae* in buds, on bark, and on fruit mummies, suggesting that several apple tissues might serve as overwintering habitats for the fungus, in addition to fallen leaves.

Keywords: *Diplocarpon mali*, impaction spore trap, *Malus*, *Marssonina coronaria*, Marssonina leaf blotch, premature leaf fall, rotating-arm spore trap, TaqMan qPCR

Diplocarpon coronariae (Ellis & Davis) Wöhner & Rossman (Crous et al. 2020), formerly *Marssonina coronaria* (Ellis & Davis) Davis, teleomorph *Diplocarpon mali* (Harada & Sawamura), is an ascomycete fungus that causes apple blotch (AB) (Wöhner and Emeriewen 2019). The disease can result in severe tree defoliation, weakened trees, and ultimately decreased yield (Sharma and Thakur 2011) and fruit quality (Park et al. 2013). AB has a significant economic impact, especially in South and East Asia. In South Korea, the loss due to AB is estimated at US$29.79 M (Kwon et al. 2015). In India, AB is emerging as the most destructive disease affecting apple trees, becoming a major constraint to apple cultivation in Himachal Pradesh, an important apple-producing state in the Western Himalayan region (Rather et al. 2017a; Sharma and Gupta 2018). Recently, AB has emerged as an issue in Europe (Wöhner and Emeriewen 2019) and in the United States (Aćimović and Donahue 2018; Khodadadi et al. 2019), especially in low-input orchards, for example, organic orchards and untreated orchards used for juice production (Bohr et al. 2018; Hinrichs-Berger and Müller 2012; Persen et al. 2012). With the rising demand for reduced pesticide residues, the prevalence of AB could increase in conventionally managed apple orchards in the future.

To date, the biology and epidemiology of *D. coronariae* and measures to control AB have been investigated primarily in Japan, China, Korea, and India, where the disease has been an issue since the late 1990s, whereas research on *D. coronariae* and AB in Europe and the United States is nascent (Wöhner and Emeriewen 2019). *D. coronariae* has a hemibiotrophic lifestyle (Horbach et al. 2011; Zhao et al. 2013), and the optimal conditions for infection are temperatures between 20 and 25°C with prolonged leaf wetness (Sastrahidayat and Nirwanto 2016). The minimum leaf wetness period for infection is 8 h at 15°C, and the risk of infection increases with longer leaf wetness periods and increasing temperature (Sharma et al. 2009). During the epidemic in summer, the pathogen reproduces asexually by two-celled conidia. Additionally, in fall, single-celled microconidia (spermatia) are produced and can be found in acervuli together with conidia (Harada et al. 1974; Lindner 2012). Initial infections in spring can be caused by conidia released from acervuli or by ascospores developing in apothecia (Wöhner and Emeriewen 2019). The sexual stage of *D. coronariae* has been described in Japan, China, and India (Gao et al. 2011; Harada et al. 1974; Sharma and Gupta 2018) but not in Europe (Hinrichs-Berger 2015; Wöhner and Emeriewen 2019), the United States, or Korea (Back and Jung 2014). The contribution of the different spore types to initial infections and their mode of dispersal has not been conclusively determined, but wind and splash dispersal have been reported (Dong et al. 2015; Khodadadi et al. 2022; Kim et al. 2019). *D. coronariae* overwinters in fallen leaves (Back and Jung 2014). In addition, buds and twigs have been hypothesized to be overwintering refugia for *D. coronariae* (Wöhner and Emeriewen 2019). However, data on the sources of primary infections and early disease progress are lacking in Europe and the United States.

To predict the risk of primary infections by *D. coronariae* and subsequent outbreaks of AB, it is crucial to understand the spore

†Corresponding authors: H.-J. Schärer; hans-jakob.schaerer@fibl.org, and P. Flury; pascale.flury@unibas.ch

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dispersal pattern of the fungus. Fungal spores can be collected using spore traps combined with subsequent quantification using visual (microscopic) or molecular methods. There are various types of spore traps, and depending on the mode of pathogen dispersal, the research question, or the available resources, certain spore traps are better suited. Spore traps collect spores either passively by gravitational deposition or actively by sampling specific volumes of air and capturing the spores by impaction, impingement, filtration, virtual impaction, cyclone, or electrostatic attraction (West and Kimber 2015). The spores are collected onto a solid surface such as agar on a Petri dish, filter paper, double-sided adhesive tape, petroleum jelly (Vaseline)-coated tape, slides or rods, or electrostatic plastic film, into Eppendorf tubes (Kim et al. 2018), or, more rarely, into a liquid. Spores captured by spore traps have traditionally been identified and quantified by microscopy (Andersen et al. 2009; Sterling et al. 1999). However, microscopy requires a spore-trapping surface that can be examined under a microscope and a trained investigator to identify and count the specific fungal spores accurately. Newer methods combine microscopy with image recognition of spores and machine learning (Basso et al. 2020; Kilin et al. 2019). Laser-based real-time optical particle counters that detect particles in the air are being tested for real-time detection of spores in the air (Basso et al. 2020; Kilin et al. 2019). Besides optical methods, molecular methods are used to quantify fungal spores. Quantitative real-time polymerase chain reaction (qPCR), loop-mediated isothermal amplification (Notomi et al. 2015; Ren et al. 2021), serial analysis of gene expression, and microarray technology (Aslam et al. 2017) are based on quantification of DNA, whereas the enzyme-linked immunosorbent assay (Kennedy et al. 2000) and the fluorescent antibody assay (Schneider et al. 2009) detect fungal proteins via antibodies. In recent years, the qPCR method has become the most frequently used quantitative molecular method to detect and quantify fungal pathogens. It offers a more sensitive and specific quantification compared with microscopy, as it can detect low concentrations of a fungal pathogen against a background of particles and DNA from diverse organisms (Parker et al. 2014) and does not depend on visual identification of a fungus spore.

Information on spore dispersal and observations on disease progress, combined with weather data, can provide a basis for understanding disease dynamics and developing disease forecast models. Forecast models provide a valuable tool for the efficient application of crop protection measures (Agrios 2005; Hardwick 1998). RIMPro (Amsterdam, the Netherlands) developed a disease forecast model for AB in Europe based on information available in the literature from Asia. Recently, *D. coronariae* spore dispersal was investigated in Korea from June to October, employing spore traps and microscopy, and a disease forecast model was developed (Kim et al. 2019). However, the authors did not investigate spore dispersal in the spring, which is a period critical to understanding the primary infection and disease onset and that may be subject to efficacious disease management.

The aim of this study was to gain a better understanding of the early phase of AB disease in spring and summer. Thus, the specific objectives were to (i) develop a qPCR method that allowed for the quantification of *D. coronariae* spores in spore trap samples, (ii) investigate temporal and spatial aspects of *D. coronariae* spore dispersal in relation to development of AB in the field in spring and early summer, and (iii) identify potential sources of primary *D. coronariae* inoculum.

Materials and Methods

Spore traps used in this study

Conidia and ascospores of *D. coronariae* are reported to be dispersed by wind (Dong et al. 2015; Kim et al. 2019), whereas conidia are also reported to be splash-dispersed (Dong et al. 2015). Two trap types, the Mycotrap and rotating-arm spore traps, were used to sample airborne spores in the field (Fig. 1D and E). Additionally, apple bait plants were used as in vivo spore traps (Fig. 1F). A detailed description of the spore traps is provided in Supplementary Materials and Methods S1. Briefly, the Mycotrap (Siegfried et al. 1996) (Fig. 1D) is an impaction spore trap and similar to the Burkard 7-day Recording Volumetric Spore Sampler, which was used to capture airborne conidia of *D. coronariae* in Korea (Kim et al. 2019). The air is sucked in horizontally through a sampling orifice and impacts a trapping surface on a cylinder inside a chamber. The cylinder slowly rotates, completing one turn over 7 days, allowing continuous sampling for the 7-day period. The rotating-arm spore traps (Fig. 1E) were constructed based on the description of Quesada et al. (2018) (Supplementary Materials and Methods S2). The rotating-arm spore traps consisted of two rods connected to a battery-powered motor (Spinart Lightweight Hanging Motor, 30 rpm). Each rod held a microscope slide on which was mounted a strip of Vaseline-coated plastic film (Supplementary Materials and Methods S3) attached with 25-mm foldback clips (Maul, Bad König, Germany). The rotating-arm spore traps were covered with an aluminum shield as rain protection.

To collect splash-dispersed *D. coronariae* conidia in rainwater, funnels (12 cm diameter) were placed in 250-ml Schott bottles to collect rain splash. Approximately 100 ml of rainwater was collected per Schott bottle and filtered through a cellulose acetate filter (25 mm in diameter, 0.8 μm pore size, Sartorius-Stedim, Goettingen, Germany) in polycarbonate filter housings. The filters were subsequently cut into pieces of approximately 3 × 3 mm and stored at −20°C.

Design and evaluation of qPCR primers and probes

The primers and hydrolysis probes designed targeted the nuclear ribosomal internal transcribed spacer (ITS) region between the 18S and 5.8S rDNA of *D. coronariae* using the program Beacon Designer (V8.16, Premier Biosoft, Palo Alto, CA, U.S.A.) with the following parameters: amplicon length between 100 and 200 bp, melting temperature (TM) of primers at 60.0°C ± 1.0°C, and TM of the probe at 10.0°C ± 5.0°C above the TM of the primers (Table 1). The program's default settings were used for maximum ΔG of self-complementarity, 3′-end stability, and percent GC. The specificity of the primers and probes was confirmed in silico using the National Center for Biotechnology Information (NCBI, Bethesda, MD, U.S.A.) Primer-Blast (Ye et al. 2012) by alignment of different ITS gene bank accessions of *D. coronariae*.

The specificity was further tested in vitro with genomic DNA of *D. coronariae* isolates from Switzerland, Korea, and Japan; with apple DNA; and with DNA of different fungi (i.e., fungi within the genus *Diplocarpon*, fungi causing Marssonina diseases on other hosts than apple, and fungi that are common pathogens of apple). Information on the tested isolates is provided in Supplementary Table S1. *D. coronariae* isolates were cultured on potato peptone carrot dextrose agar adapted from Zhao et al. (2010) and containing 100 ml of carrot juice, 39 g of potato dextrose agar (PDA), 10 g of peptone, and 1 liter of double-distilled water. Other pathogens were cultivated on PDA. Fungal DNA from the cultures was extracted using a ZymoBIOMICS Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, U.S.A.) according to the manufacturer's protocol.

The qPCR was designed for use with a TaqMan probe, but we also tested whether the primer pair Dc_09 could be used with a DNA intercalating fluorophore (SYBR Green).

qPCR reaction conditions

All primers and probes were synthesized and purified by high-performance liquid chromatography at Microsynth AG (Balgach, Switzerland). Primers and probes (Table 1) were dissolved in a TE dilution buffer (TE Buffer: 10^{-3} mol liter^{-1} Tris, 10^{-5} mol liter^{-1} Na_2EDTA, pH 8.0).

TaqMan-based qPCR reactions consisted of 5 µl of KAPA PROBE FAST (Sigma-Aldrich Chemie AG, Buchs, Switzerland), 1 µl of each primer mix for *D. coronariae* and APA9 (containing primers at a concentration of 3 µM for Dc_09 and 2 µM Africa Cassava Mosaic Virus [ACMV] and the probe at 1 µM each), 0 to 2 µl of double-distilled water, and 1 to 3 µl of template DNA in a total volume of 10 µl. The SYBR-based *D. coronariae*-specific qPCR reactions consisted of 5 µl of KAPA SYBR FAST (Sigma-Aldrich Chemie AG), 1 µl of primer mix (forward and reverse primers at a concentration of 3 µM each), 3 µl of double-distilled water, and 1 µl of template DNA in a total volume of 10 µl. The qPCR assay and data analysis were performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.). The amplification and quantification conditions used were an initial denaturation step of 3 min at 95°C, followed by 39 and 45 cycles of 10 s at 95°C, and 20 s at 60°C for SYBR and TaqMan qPCR, respectively. After each SYBR qPCR, a dissociation curve analysis was performed by gradually increasing the temperature from 65 to 95°C by 0.5°C per cycle.

In specificity tests, a SYBR Green qPCR assay was performed with the ITS primers ITS1F/2R in addition to the *D. coronariae*-specific qPCR, which served as an amplification control detecting all tested fungal species (Table 1). The reactions consisted of 5 µl of KAPA SYBR FAST, 1 µl of primer mix (primers at a concentration of 2 µM each), 3 µl of double-distilled water, and 1 µl of template DNA in a total volume of 10 µl and were subjected to an initial denaturation step of 10 min at 95°C, followed by 39 cycles of 30 s at 95°C, 30 s at 50°C, and a final extension step for 1 min at 72°C.

To visualize the PCR products, 10 µl of the qPCR reaction was loaded on a 2% Tris-acetate-EDTA-buffered agarose gel stained with ROTI GelStainRed (Carl Roth GmbH, Karlsruhe, Germany) and run at 50 V for 60 min. Images were captured under UV using an Azure Biosystems c150 (Azure Biosystems, Dublin, CA, U.S.A.) gel imaging workstation.

DNA extraction from spore trap samples and apple leaves

To extract DNA from *D. coronariae* conidia from Vaseline-coated plastic film (Mycotrap and rotating-arm trap samples, Supplementary Materials and Methods S3), we used a ZymoBIOMICS Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research) following the manufacturer's protocol with two modifications: The Bashing Bead tubes from the Zymo Microprep Kit were replaced by 2-ml

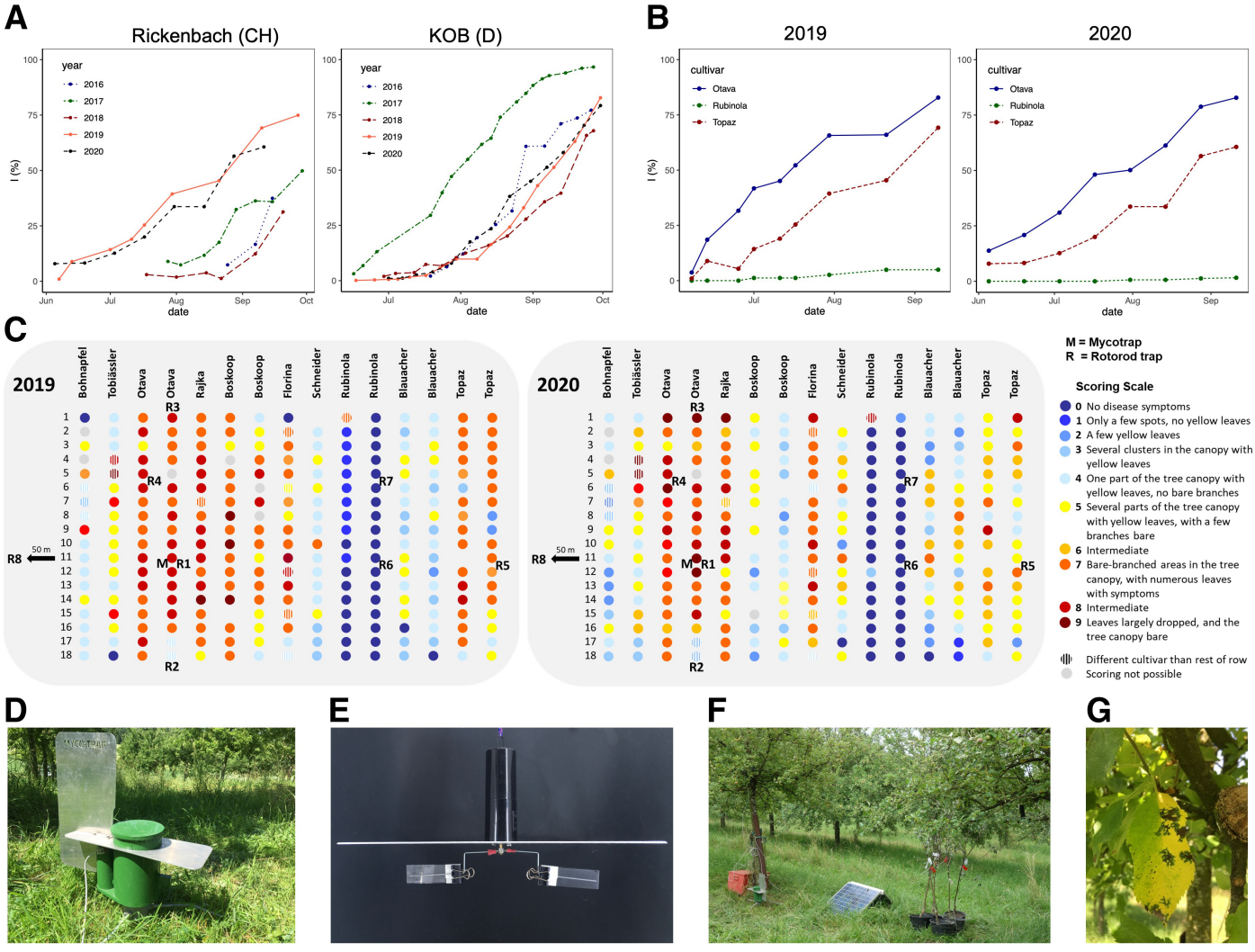

Fig. 1. Apple blotch (AB) development in two apple orchards and the location of the spore traps. **A,** 'Topaz' trees in two orchards (Rickenbach, Switzerland, and Competence Center of Fruit Crops at the Lake of Constance [KOB], Ravensburg, Germany) were regularly assessed for severity of AB from June to October for 5 consecutive years (Rickenbach, 36 trees; KOB, 120 trees). Disease severity is the percent severity according to the McKinney Index (I) (McKinney 1923). **B,** AB development on 'Topaz' (36 trees), 'Rubinola' (35 trees), and 'Otava' (33 trees) in the Rickenbach orchard in 2019 and 2020. Disease severity is the percent severity according to the McKinney Index (I) (McKinney 1923). **C,** AB disease scoring in the Rickenbach orchard in middle of September in 2019 and 2020. The orchard was planted with tree rows of 10 different cultivars. Each dot represents one tree. The color of the dot indicates the disease score of the tree. The placement of the Mycotrap and rotating-arm spore traps is indicated by the letters M and R, respectively. One rotating-arm trap was installed 50 m outside the orchard (R8). **D,** The Mycotrap. **E,** The rotating-arm spore trap. **F,** Potted bait plants. **G,** Leaf with typical AB symptoms.

tubes with screw caps containing 100 mg of 0.5-mm Zirconia bashing beads (N034.1 Carl Roth), and the Bashing Bead Buffer volume was reduced from 750 to 550 µl.

DNA from filter pieces (rain-splash samples) was extracted using a ZymoBIOMICS Quick-DNA Fungal/Bacterial Microprep Kit following the manufacturer's protocol, but adding skim milk to the Bashing Bead Buffer at a final concentration of 2% to prevent the adhesion of free DNA to the filter membrane (Liang and Keeley 2013).

DNA from apple leaves with ambiguous AB symptoms was extracted following a CTAB buffer protocol (described in Supplementary Materials and Methods S4).

qPCR-based quantification of *D. coronariae* spores with a standard curve

The number of conidia corresponding to a given C_q value was calculated using a standard curve based on known quantities of conidia. Conidia suspensions were prepared by stirring AB-symptomatic apple leaves with sporulating *D. coronariae* for 10 min in Volvic natural spring water (Danone S.A., Paris, France), which is routinely used in our lab for the preparation of spore suspensions, and subsequently filtering the spore suspension through a sieve (mesh size 0.1 mm). The spore concentration was assessed using a hemocytometer (0.1 mm depth, 0.0025 mm^2, Neubauer, Paul Marienfeld GmbH, Lauda-Königshofen, Germany). A tenfold dilution series was prepared in double-distilled water containing 0.05% Tween 80 as described by McDevitt et al. (2004), and 10^5 to 10^1 conidia were pipetted to the material used in spore traps (Vaseline-coated plastic film or filter pieces) and let dry. DNA extraction and qPCR were performed as described above. The standard curves were used for the test calibration, the limit of quantification (LOQ), and the calculation of spore numbers based on Cq values.

All spore trap samples were spiked with 10^7 copies of linearized APA9 plasmid (vector pUC19 with an insert ACMV; NCBI GenBank accession number AJ427910) (details in the Supplementary Materials and Methods S5) (Mosimann et al. 2017) as an internal control to account for differences in DNA extraction efficiency between different samples. The APA9 plasmid was quantified together with *D. coronariae* using a multiplexed TaqMan qPCR with the primers and probe for ACMV listed in Table 1. For multiplexing, the single-plex TaqMan qPCR protocol for *D. coronariae* described above was adapted by replacing 1 µl of double-distilled water with 1 µl of primer mix for APA9 (containing primers at a concentration of 2 µM and the probe at 1 µM). The results were used to normalize DNA extraction using the method of Von Felten et al. (2010).

Field sites

D. coronariae spore dispersal and AB epidemiology were investigated in an extensively managed organic apple cider orchard in Rickenbach (Zurich, Switzerland, 47°33′30.5″N 8°47′29.9″E). The site is characterized by a mean annual temperature of 10°C and mean annual rainfall of 980 mm (years 2010 to 2020). The orchard was planted in 2002 with 10 different apple cultivars arranged in 15 tree rows, with 18 apple trees in each row (Fig. 1C), an in-row planting distance of 4 m, and a between-row planting distance of 9 m. In 2019, the orchard was treated with organic fungicides (acidified clay minerals, Myco-Sin at 8 kg/ha, Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland; in a tank mix with sulfur, Netzschwefel Stulln at 4 kg/ha, Biofa AG, Münsingen, Germany) on 25 May. 'Otava', 'Rajka', 'Rubinola', 'Topaz', and 'Boskoop' received additional sulfur with lime (Curatio, Biofa AG) on 11 June. In 2020, the orchard was treated with sulfur and clay on 27 April, 9 May, 22 May (all cultivars except 'Bohnapfel', 'Schneider', and 'Tobiässler'), and 2 June (only Otava, Rajka, Rubinola, and Boskoop). Trees with spore traps were excluded from receiving fungicide treatments in 2019 and 2020. In addition, in 2020, the first tree of each row was left untreated.

AB epidemiology was further investigated in an organic apple orchard at the Competence Center for Fruit Crops at Lake Constance (Kompetenzzentrum Obstbau Bodensee [KOB], Ravensburg, Germany, 47°46′06.8″N 9°33′18.0″E). The site is characterized by a mean annual temperature of 9.7°C and mean annual rainfall of 908 mm (years 2010 to 2020; data from the weather station in Bavendorf, www.wetter-bw.de). The orchard was planted in 2003 with Topaz on M9 rootstocks with a 3.2 m between-row and 0.8 m in-row planting distance. For monitoring AB, 120 trees in two consecutive rows were regularly assessed. No fungicide treatments were applied to the trees assessed during the entire study period.

Weather data, including rainfall, leaf wetness, and temperature, were obtained from weather stations close to the field sites: the Agrometeo (Agroscope, Nyon, Switzerland; www.agrometeo.ch) weather station "Liebensberg," Zurich, Switzerland (47°53′38.1″N 8°83′73.8″E, altitude 535 m), and the Agrarmeteorologie Baden-Württemberg (Landwirtschaftliches Technologiezentrum Augustenberg, Tübingen, Baden-Württemberg, Germany, www.wetter-bw.de) weather station "Bavendorf" in Germany (47°76′84.4″N 9°55′99.0″E, altitude 481 m), respectively.

Disease assessment in the apple orchard

From 2016 to 2020, the severity of AB was assessed every 1 to 2 weeks from the onset of first symptoms through October by scoring entire trees using a qualitative ordinal scale (0 = no disease symptoms; 1 = only a few spots, no yellow leaves; 2 = a few yellow leaves; 3 = several clusters in the canopy with yellow leaves; 4 = one part of the tree canopy with yellow leaves, but no bare branches; 5 = several parts of the tree canopy with yellow leaves, with a few branches bare; 6 = intermediate; 7 = bare-branched areas in the tree canopy, with numerous leaves with symptoms; 8 = intermediate; 9 = leaves largely dropped, and the tree canopy bare).

The ordinal scores were used to calculate damage (in %) based on the McKinney infection index (I) (McKinney 1923), where I (%) = [sum (class frequency × score of rating class)]/[(total number of ratings) × (maximum grade)] × 100. The scoring scale and the results for 2016 to 2018 have been presented previously in

TABLE 1. Primers used in the qPCR to detect DNA of *Diplocarpon coronariae*

Primer name[a]	Sequence (5′ to 3′)[b]	Mt (°C)[c]	Amplicon size (bp)	Reference
Dc_09_F	GCGTATACCACCCGTGCCTA	60.4	129	This study
Dc_09_R	CTCAGACATCACGTTATTCACACAA	59.4		
Dc_09_P	FAM-CCTACCTCTGTTGCTTTGGCGA-BHQ1	70.0		
ACMV_F	CCACAGACAAGATCCACTCTCC	59.7	86	Mosimann et al. (2017)
ACMV_R	CACTCTACTCAGGTTCCAATCAAAG	57.9		
ACMV_P	ROX-ACAGACAATTCAAGAAGCGAGCCATCC G-BHQ2	62.6		
ITS1F	CTTGGTCATTTAGAGGAAGTAA	53.2	Dependent on target species	Gardes and Bruns 1993; White et al. 1990
ITS2R	GCTGCGTTCTTCATCGATG	57.2		

[a] F, forward primer; R, reverse primer; P, fluorogenic hydrolysis probe.
[b] FAM, 6-carboxyfluorescein; ROX, carboxy-X-rhodamine; BHQ; black whole quencher.
[c] Mt, melt (annealing) temperature.

non-peer-reviewed journals for practitioners (Schärer et al. 2019; Wöhner et al. 2019).

Monitoring spore dispersal in an apple orchard

To investigate dispersal of *D. coronariae* conidia in the field, different spore traps were installed in the Rickenbach orchard from May to July and from March to July in 2019 and 2020, respectively (Supplementary Tables S2 and S3). To monitor the temporal resolution of the spore dispersal, one Mycotrap was placed on the ground in the previous year's AB hotspot in the orchard within a row of Otava trees (Fig. 1C and D). A second Mycotrap was placed in the canopy of an Otava tree (2.5 m aboveground) above the first Mycotrap. We hypothesized that primary spores originating from leaf litter on the ground would be more readily detected by a trap on the ground, whereas a trap in the tree canopy would catch spores released within the tree canopy (e.g., from fruit mummies hanging in the tree canopy or later in the season from diseased leaves). Container-grown, 2-year-old apple trees were used as bait plants and were placed for periods of 5 to 14 days in the orchard and subsequently incubated under rain protection to assess disease development and correlate the presence of spores in the orchard with actual infections (Fig. 1F). In 2019, each series of bait plants consisted of three Topaz, three Gala and three 'Kiku' apple trees. In 2020, each series of bait plants consisted of five Topaz apple trees.

To observe the spatial gradients of airborne spore catches in relation to disease severity in the orchard, seven rotating-arm spore traps were mounted inside and at the edge of the orchard on trees with severe (3 Otava and 1 Topaz tree), mild (1 Schneider tree), and no *D. coronariae* symptoms (2 Rubinola trees) on one of the lowest branches of the apple trees (1.7 m aboveground) and close to the trunk. Moreover, one rotating-arm spore trap was installed at the same height but approximately 50 m beyond the orchard perimeter. The location of the traps is indicated in Figure 1C. In 2019, the traps were installed between 22 May and 5 July. In 2020, sampling was performed over the entire period from March to July. The spore trap samples were subject to the *D. coronariae* TaqMan qPCR assay described above.

To observe the spatial gradients of splash-dispersed *D. coronariae* conidia, 12 Schott bottles each with a funnel were placed within the row directly below trees, as well as in the open area between the rows and outside the orchard. The spore trap positions differed according to the disease severity of nearby trees. Rainwater was collected for 16 h from 24 to 25 September 2020. It was the first rainy period after 7 consecutive days without precipitation. The rainfall registered for the 16-h period was 14 mm.

Precipitation and spore number

To assess whether spore number and precipitation are correlated, a Kendall rank correlation test was performed using R version 4.1.2 (R Core Team 2021), with spore number as the dependent variable and precipitation as the independent variable. Because the origin and amount of the inoculum changed over time, the analysis was performed for each month separately.

Investigating possible sources of primary inoculum

To understand *D. coronariae* spore dispersal, it is essential to know all sources of primary inoculum. We first studied spore dispersal from a sample of overwintered *D. coronariae*-infected leaf litter. In 2019, a Mycotrap was placed in the field on a mesh cage depot filled with heavily *D. coronariae*-infested leaf litter of the cultivar 'Remo' at the KOB site (Supplementary Materials and Methods S6). In this experiment, the clock mechanism of the Mycotrap was removed, and spores were collected constantly on the same piece of plastic film. The plastic film was replaced every 2 weeks from 1 February to 30 June. In 2020, a Mycotrap was placed on a similar leaf litter sample at the Research Institute of Organic Agriculture (FiBL, Frick, Switzerland) and sampled from 1 March to 30 June. The leaf litter was collected in the Rickenbach orchard on 28 February 2020. Spore numbers in the samples were quantified by TaqMan qPCR as previously described.

We hypothesized that besides leaf litter, other inoculum sources (i.e., bark, bud, or fruits) might be sources of inoculum for outbreaks of AB. On 28 February 2020, we collected bark and bud samples from six Otava and six Rubinola trees in the Rickenbach orchard and on 17 March 2020 from six Topaz trees in the KOB orchard. Bark samples were collected as described by Arrigoni et al. (2018). Briefly, bark curls 20 mm long, 5 mm wide, and 1 mm thick were taken using a flame-sterilized scalpel. Each bark sample consisted of a pool of 30 bark curls from the upper trunk and lower branches of a single tree. Ten terminal buds per tree were collected. We further searched for fruit mummies hanging in the trees in the Rickenbach orchard on 28 February 2020. Five pieces of the skin of each mummy (with a diameter of approximately 2 to 4 mm and a thickness of 2 to 5 mm) were pooled. Samples were lyophilized and ground in a mixer mill MM 200 (Retsch GmbH, Haan, Germany) at 30 Hz for 30 s. A sample of 100 mg of the ground powder (or as much as was available) was subject to DNA extraction using a NucleoSpin DNA Stool Kit (Macherey-Nagel, Oensingen, Switzerland). We found only one tree with overwintered leaves hanging in the canopy. This sample was processed the same way but without prior lyophilization. Samples were assessed for the presence of *D. coronariae* DNA by the TaqMan qPCR assay described above.

Finally, to assess whether the identified DNA on bark, buds, and fruit mummies originated from viable and infectious fungal tissues, we inoculated apple leaves in the laboratory using *D. coronariae* PCR-positive bud, bark, and fruit samples. A sample of 400 to 500 mg of fruit, bark, and bud tissue was cut into small pieces and wetted with 3.5 ml of Volvic natural spring water (Danone S.A.) ($n = 3$). Each of the samples was spread on three apple leaves. The experiment was performed twice, once with container-grown Topaz trees and once with Topaz apple seedlings. As controls, three apple leaves were incubated with laboratory-infected apple leaves with *D. coronariae* acervuli (positive control) or with healthy apple leaves (negative control) processed in the same way as described for the bark, bud, and fruit samples. In addition, three leaves were inoculated with pieces of a *D. coronariae*-positive leaf that was found in the tree crown after winter. The plants were incubated at 20°C with a 16/8-h day/night cycle. For the first 4 days, the relative humidity was 100%, followed by 50% for the remainder of the experiment. Symptoms were assessed after 3 weeks. Leaves showing spots or necrosis were tested for *D. coronariae* DNA using the qPCR assay described above.

Investigation of AB on fruit

Apples with symptoms of AB were collected in September of 2020 and 2021 and stored at 4°C until March the following year, when acervuli had developed. The acervuli were examined under a stereo microscope (Leica M205C, Leica Microsystems Switzerland, Heerbrugg, Switzerland). The apple tissue containing acervuli was stained with cotton blue in lactic acid and examined under a Leica DM2000 LED microscope. Images of the apple tissue containing acervuli were captured using a Jenoptic Gryphax Subra camera (Jenoptic AG, Jena, Germany). Conidia formed on cool-stored fruits were tested for their infectivity to apple leaves. To this end, necrotic lesions with acervuli were cut and added to 1 ml of Volvic natural spring water in 2-ml Eppendorf tubes and vortexed for 30 s, and the resulting spore suspension was used to inoculate leaves of six Topaz seedlings. Twelve drops of 5 µl of suspension were pipetted onto two leaves per seedling. The plants were incubated as described above for the infection experiments with bark, bud, and fruit mummies and monitored for the development of AB symptoms. After 1 month, infections by *D. coronariae* were diagnosed visually, and after 3 months, fungal structures were examined by microscopy as described previously.

Results

qPCR quantification of *D. coronariae* conidia

Among the primers tested, primers and probe Dc_09 had the highest amplification efficiency (E = 94.2%, $R^2 = 0.995$), even in comparison to the previously published primer Mc_ITS (Oberhänsli et al. 2014) (E = 83%, $R^2 = 0.88$). Specificity tests for primer Dc_09 revealed sound amplification of DNA from different isolates of *D. coronariae* (Fig. 2A) and did not amplify DNA of other fungal species or apple DNA (Fig. 2A; Supplementary Table S4).

qPCR amplification of the conidial counts for samples from the Vaseline-coated plastic film, which was the spore-trapping surface used for field samples, exhibited a linear response with an efficiency of 89.1% ($R^2 = 0.997$) (mean of seven qPCR runs) (Fig. 2B). The qPCR assay allowed for the consistent detection of as few as 10 conidia; however, the amplification was unreliable with three conidia per sample. Therefore, the LOQ is considered to be 3 to 10 conidia.

The SYBR Green-based hydrolysis probe assay revealed similar sensitivity and specificity to the TaqMan assay (Supplementary Fig. S1). However, we used the TaqMan qPCR for our field experiments because it enabled multiplexing of the *D. coronariae*-specific qPCR with the APA9-specific qPCR (plasmid standard), which we used to normalize the *D. coronariae* quantification data with the DNA extraction efficiency.

Apple blotch disease progress in two Central European apple orchards

At the Rickenbach and KOB field sites, disease progress on Topaz was first observed as the appearance of signs of the pathogen (i.e., visible formation of acervuli) and symptoms of AB (yellowing of the leaf) (Fig. 1G), the timing of which varied between years but occurred at the latest in August in both orchards and all years. Progress of AB was generally characterized by a steady increase in severity. However, differences in disease severity were observed between years. At KOB, for example, AB was more severe in 2017 compared with the other years (Fig. 1A). Rainfall was more frequent from June through October in 2017 (i.e., 78 days of rainfall compared with 36 to 57 days of rainfall in the other years; Supplementary Fig. S2). At both sites, 2018 was characterized by an exceptionally hot and dry summer (only 36 and 33 days with rain from June to the end of September at KOB and Rickenbach, respectively) (Supplementary Figs. S2 and S3), resulting in lower severity of AB (Fig. 1A). At Rickenbach, AB was most severe in 2019 and 2020, which was not the case at KOB (Fig. 1A).

At the Rickenbach site, we identified Otava, Rajka, Florina, and Topaz as apple cultivars highly susceptible to AB (Fig. 1B and C). In contrast, Bohnapfel, Blauacher, Tobiässler, and Schneider were relatively tolerant, with low AB severity, even at the end of the season (Fig. 1C). Rubinola had no AB or very low severity, despite a high inoculum pressure in the orchard (Fig. 1B and C).

Monitoring dispersal of *D. coronariae* conidia in the field

In 2017 and 2018, the first clearly visible symptoms of AB on Otava trees developed by mid-June and early July, respectively. Therefore, considering an incubation period of about 3 weeks (Lee et al. 2011), the first infections were expected to occur in late May.

In 2019, large numbers of spores were detected by the Mycotrap on the ground from 9 to 17 May (Fig. 3A), but because spore traps were only installed on 9 May in that year, it is possible that spores were present even earlier. Subsequently, spore numbers generally decreased, except for a peak in the first week of June. In May, leaves with spotting indicative of AB were collected and tested for *D. coronariae* by qPCR, revealing the first *D. coronariae*-positive leaf on 17 May. The first unambiguous symptoms were observed 3 weeks later, on 7 June (Fig. 3A). The next spore peak was detected on 24 June after a rainy period. At that time, Otava trees already showed severe leaf yellowing, indicating the emergence of secondary inoculum. In July, large spore numbers were recorded in the air on most days. The second Mycotrap in the crown of an Otava tree detected more spores than the Mycotrap on the ground, except on 24 and 25 June (Supplementary Fig. S4).

The first apparent symptoms on bait plants developed between 17 and 22 May (Fig. 3A). From 22 May onward, at least one bait plant showed typical AB symptoms of leaf yellowing, except for

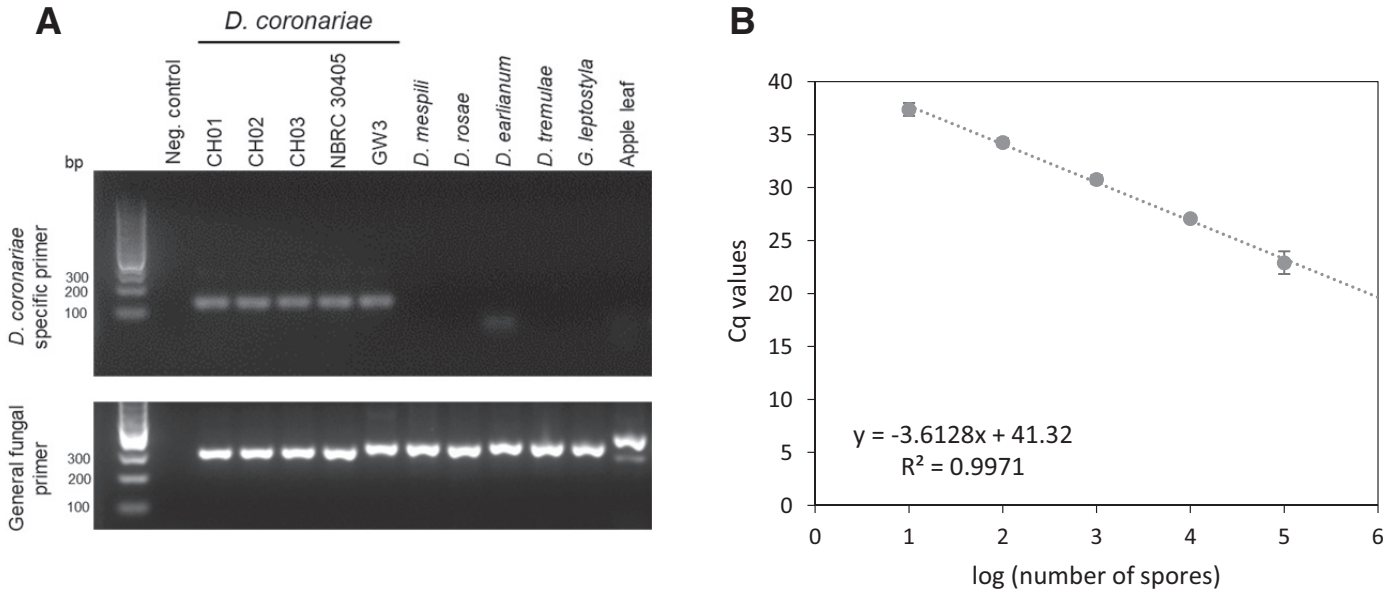

Fig. 2. *Diplocarpon coronariae*-specific qPCR. **A,** Specificity test of primer Dc_09. A PCR was performed with DNA from different *D. coronariae* isolates, related *Diplocarpon* species (*D. mespili*, *D. rosae*, *D. earlianum*), and DNA of other pathogens causing Marssonina diseases (*Drepanopeziza tremulae* and *Gnomonia leptostyla*). The image shows a 2% TAE agarose gel stained with ROTI GelStainRed (the ladder marker is a peqGOLD O'range 100-bp DNA Ladder; peqlab). **B,** In vivo standard curve for calculation of spore numbers. A volume corresponding to 10^5, 10^4, 10^3, 10^2, and 10^1 conidia was pipetted onto a Vaseline-coated plastic film, and DNA was extracted with the same method as was used for the Mycotrap and rotating-arm spore traps (ZymoBIOMICS Quick-DNA Fungal/Bacterial Microprep Kit). Samples were spiked with an APA9 plasmid for normalization of DNA extraction, and normalized Cq values are depicted. Error bars represent the standard deviation of the mean of seven qPCR runs. The mean efficiency was 89.1%.

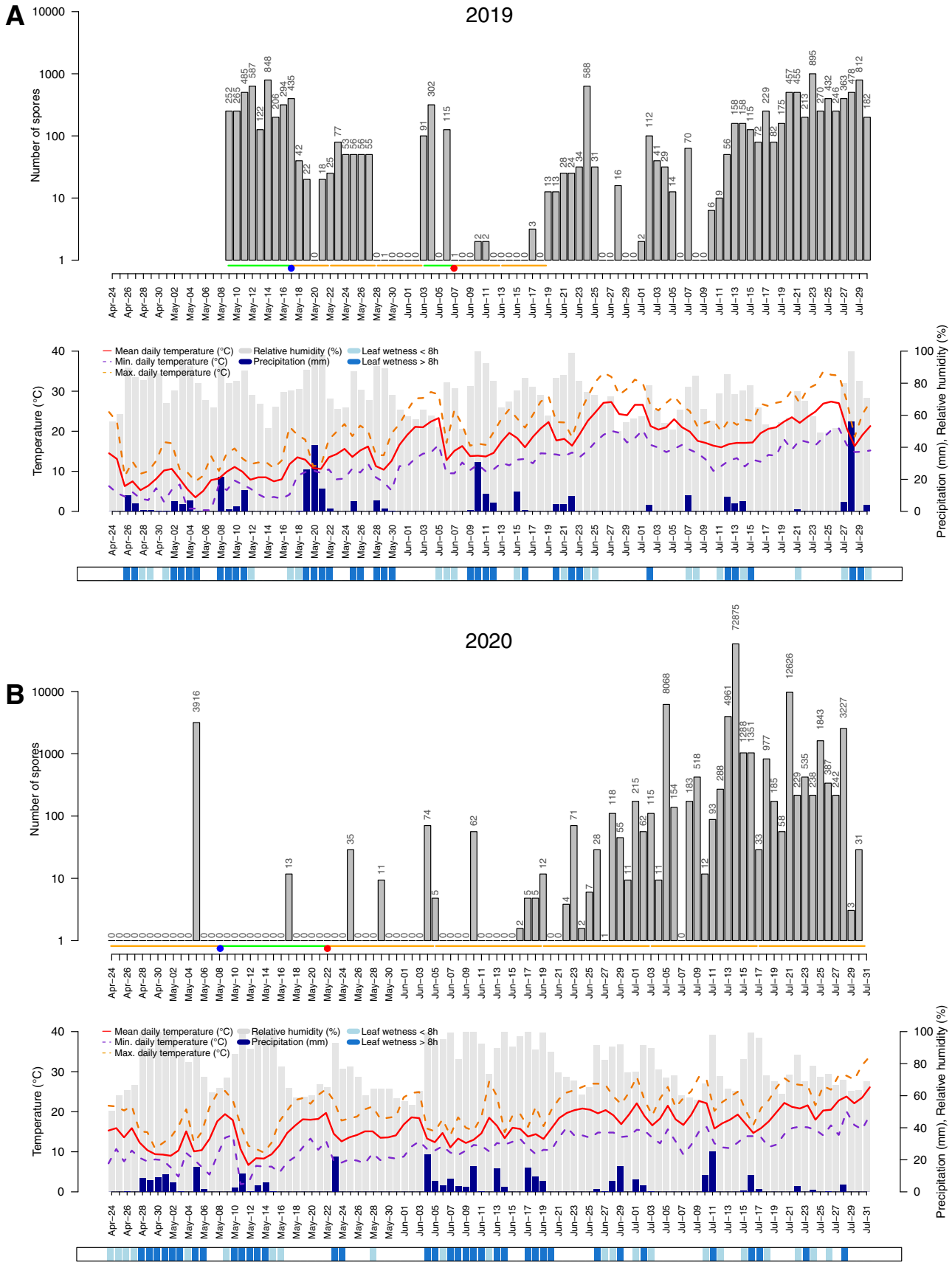

Fig. 3. *Diplocarpon coronariae* spore dispersal in **A,** 2019 and **B,** 2020 at the Rickenbach site in Switzerland. Daily spore numbers of *D. coronariae* captured by a Mycotrap placed within a row of 'Otava' apple trees. The number of spores was calculated based on normalized Cq values from the TaqMan qPCR assay with primer Dc_09 using a standard curve with known numbers of conidia. The green and yellow lines indicate the periods when a bait plant was exposed in the orchard next to the Mycotrap and whether the bait plant developed apple blotch (AB) symptoms (yellow) or not (green). The blue circles indicate days where first leaves with ambiguous AB symptoms tested positive for *D. coronariae*. The red circles indicate first unambiguous AB symptoms in the orchard. Weather data were obtained from Agrometeo (location "Liebensberg") including temperature (°C), relative humidity (%), precipitation (mm), and leaf wetness (light blue, <8 h; dark blue, >8 h).

during the period from 3 to 7 June. The most severe leaf yellowing and leaf drop were observed on the bait plants exposed between 7 and 13 June, followed by those exposed between 13 and 19 June.

In 2020, very few spores were captured by the trap on the ground before bud break in March (Fig. 4). The first peak in spore production was captured on 5 May by the trap on the ground at the end of a rainy period that had followed an extremely dry April (Figs. 3B and 4; Supplementary Fig. S2). The bait plants standing in the orchard from 24 April to 8 May were the first to develop AB symptoms. The first *D. coronariae*-positive leaf based on a qPCR test was on 8 May. Leaves with clear symptoms and production of conidia were observed on 22 May 2020, which was approximately 2 weeks earlier than observed in 2019.

A second peak in spore production was detected on 5 June by the spore trap in the tree crown. As in 2019, spores were detected in the air almost every day by both traps from mid-June to the end of July (Figs. 3 and 4). Numbers of spores varied, but no significant correlation was found between spore numbers and precipitation (Supplementary Table S5). In Mycotrap samples in early June (lower trap: 5 to 19 June, upper trap: 31 May to 9 June), we experienced technical problems, resulting in poor DNA extraction efficiency. For samples in which DNA amplification by qPCR was successful, the low efficiency could be considered by normalization with the internal plasmid standard. However, for samples in which amplification failed (i.e., no Cq value was obtained), we were unable to conclude whether no spores were present or whether the low DNA extraction efficiency precluded detection. Thus, for some days during the first half of June, *D. coronariae* spores might have been present in the air, although no catch was indicated (Figs. 3B and 4).

In 2020, all bait plants placed in the orchard after 22 May developed AB symptoms. From 22 May to 5 June, bait plants exhibited approximately 50 to 80% of leaves being symptomatic, and after 5 June, incidence of leaves exhibiting AB exceeded 90% (Supplementary Fig. S5).

Dispersal of *D. coronariae* conidia with distance from an inoculum source

Rotating-arm spore traps detected the first spores after 22 May in 2019 and 2020. Spore numbers detected by rotating-arm spore traps were generally lower than those captured in the Mycotrap samples (Table 2; Figs. 3 and 4). The highest spore numbers were generally detected in traps on Otava trees, which exhibited severe symptoms of AB, whereas almost no spores were detected in traps on Rubinola trees. Despite being only two rows (18 m) apart from severely diseased trees, the Rubinola trees were virtually free of AB, although some leaf yellowing and premature leaf fall were observed on the Rubinola trees. The symptoms on Rubinola were distinctly different from typical AB, and when the leaves were tested by qPCR, no *D. coronariae* DNA was detectable ($n = 7$). Furthermore, no spores were detected in the trap 50 m beyond the orchard edge, with one exception: During the period from 22 May to 3 June 2019, spores of *D. coronariae* were detected under Rubinola trees and outside the orchard. A strong wind event with wind speeds up to 37 km/h combined with rain on 21 May possibly transported some infected leaves toward the Rubinola trees and outside the orchard, explaining the observed dispersal.

Besides the dispersal of *D. coronariae* spores via the air, rain splash might be an important factor for dispersing spores within a tree canopy. Therefore, we collected rainwater below severely diseased trees (Otava and Topaz), below symptomless trees (Rubinola), between the rows, and outside the orchard during a rainy period in September 2020. We detected several hundred spores per milliliter of rainwater below Otava trees, fewer spores between rows, and almost no spores below noninfected Rubinola trees or outside the orchard (Supplementary Fig. S6).

Origin of primary infections

At KOB in 2019, the first spores were released from a deposit of overwintered leaf litter in the second half of April (Table 3). At FiBL in 2020, however, a few spores were detected above a leaf litter deposit in the second half of March. In 2020, no spores were detected in April at FiBL, which was characterized by extraordinarily dry weather (Supplementary Fig. S3), but the spores were again released from the leaf litter deposit during May (Table 3). Spores were detected in the second half of June at KOB and FiBL. Generally, spore numbers detected in the experiments were very low and thus not reliably quantifiable (LOQ = 10 spores).

Whereas all bark and bud samples of Rubinola were negative for *D. coronariae*, the majority of bark and bud samples from Topaz

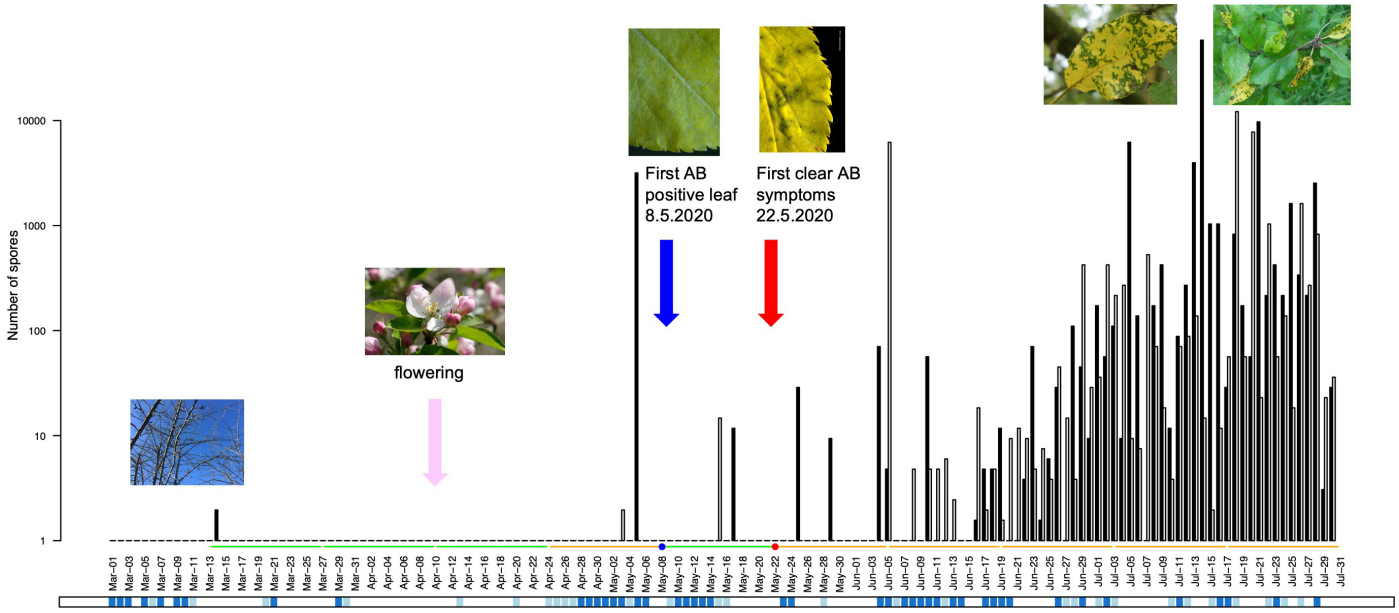

Fig. 4. *Diplocarpon coronariae* spore dispersal on the ground and in the tree crown in 2020 at Rickenbach in Switzerland. Daily spore numbers of *D. coronariae* captured by the Mycotraps placed on the ground within a row of 'Otava' apple trees (black bars) and within the tree canopy (gray bars). The number of spores was calculated based on normalized Cq values from a TaqMan qPCR assay with primer Dc_09 using a standard curve with known amounts of conidia. The green and yellow lines indicate the periods where a bait plant was exposed in the orchard next to the Mycotrap and whether the bait trap plants developed apple blotch symptoms (yellow) or not (green). Estimated duration of leaf wetness is indicated as light blue (<8 h) and dark blue (>8 h) bands.

and Otava trees tested positive for *D. coronariae* (Table 4). Bark samples exhibited high Cq values close to the limit of detection corresponding to low amounts of DNA. Bud samples contained up to 10^2 to 10^4 times higher levels of *D. coronariae* DNA than bark samples. The highest amount of *D. coronariae* DNA was detected in a bud sample of an Otava tree with a Cq value of 25.6 (Table 4), which corresponds to the DNA of 10^4 conidia.

In spring 2020, four out of five fruit mummies, which were collected from trees with severe AB in 2019, tested positive (with marginal Cq values between 33.65 and 40.38) (Fig. 5C; Supplementary Table S6), whereas fruit mummies from trees without AB symptoms the previous year were negative (Supplementary Table S6). For comparison, an overwintered leaf sample in a susceptible Florina tree after the winter of 2019 was *D. coronariae* positive (Cq, 25.83).

Attempts to infect apple leaves in the laboratory with wetted bark, buds, fruit mummies, and leaves from previously AB symptomatic trees was not successful (data not shown). However, *D. coronariae*-infected fruit can serve as an inoculum source for infection of leaves after 6 months of storage. In fall 2020 and 2021, symptoms of AB were observed on Otava fruits in the Rickenbach orchard (Fig. 5A). In the following spring, after storing fruits at 4°C for 6 months, conidia formed (Fig. 5B and D to I), and the conidia infected the leaves of apple seedlings in the laboratory (Supplementary Fig. S7).

Discussion

To date, spore trap catches of *D. coronariae* conidia have been quantified using microscopy (Kim et al. 2019). However, microscopy is labor intensive, and identification of the *D. coronariae* spores in field samples can be challenging. We developed a qPCR method to provide a specific and sensitive means for quantification of as few as 10 conidia per sample, which is in a similar range to qPCRs developed for other fungi (Calderon et al. 2002; Dvořák et al. 2015; Luchi et al. 2013). The qPCR method enables diagnosis of AB prior to the host developing unambiguous visual symptoms. Early diagnosis is especially useful when small necrotic lesions appear that are indistinguishable from symptoms from other causes. Furthermore, the qPCR method to detect and quantify conidia of *D. coronariae* can be combined with the quantification of other apple pathogens (e.g., *Venturia inaequalis*; Meitz-Hopkins et al. 2014) in spore trap samples in future studies.

Although qPCR requires sample processing, it is a relatively fast method and allows for the concurrent processing of many samples. However, the disadvantage is that a positive detection provides no information regarding the fungal organ (e.g., mycelium, ascospore, conidia, spermatia) or its viability and infectious potential. Therefore, visual assessments and infection experiments are needed to understand how *D. coronariae* persists on bark, bud, and fruit samples and the role of the tree organs in the fungal life cycle.

To determine the timing of primary infections by *D. coronariae*, we combined data on airborne spore catches with symptom development both in the field and on a consecutive series of exposed bait plants. In Europe, symptoms of AB were thought to not appear before June (Hinrichs-Berger 2015), but in Asia, first infections can occur as leaves unfurl (Dong et al. 2015; Takahashi and Sawamura 2014). With our qPCR method, we were able to diagnose *D. coronariae* infections in leaves with ambiguous necrotic spots collected in the orchard on 17 May 2019 and 8 May 2020. Moreover, the first bait plants that developed symptoms were exposed in the field from 24 April to 8 May 2020. These observations propose early May as the time point of first infections. Indeed, the first relevant spore peaks were detected in the orchard in early May in both 2019 and 2020, and the spore traps located above leaf litter caught conidia of *D. coronariae* at the end of April and early May in both study years. Taken together, we conclude that the spores collected in early May likely caused the first infections in the field. Thus, the start of disease development in Europe is similar to that in Asia (Dong et al. 2015; Gao et al. 2011; Sharma and Gupta 2018). This knowledge about possible spring infections with *D. coronariae* will be important for the development of crop protection measures in Europe.

D. coronariae is reported to overwinter in leaf litter inside acervuli (Back et al. 2015; Dong et al. 2015; Gao et al. 2011; Goyal et al. 2018; Lee et al. 2011; Sastrahidayat and Nirwanto 2016; Sharma et al. 2009). In accordance with this, we captured *D. coronariae* spores above leaf litter deposits in spring, with the first spores

TABLE 3. The number of conidia of *Diplocarpon coronariae* collected in a Mycotrap spore trap above leaf litter on an orchard floor in 2019 and 2020[a]

Sample period	KOB (2019)	FiBL (2020)
15/02–01/03	0	–
01/03–15/03	0	0
15/03–01/04	0	14
01/04–15/04	0	0
15/04–29/04	<10	0
29/04–16/15	<10	<10
16/05–04/06	0	<10
04/06–17/06	0	0
17/06–25/06	<10	<10

[a] Spore trap samples were from a Mycotrap installed above a *D. coronariae*-infected leaf litter at the Competence Center for Fruit Crops at Lake Constance (KOB) in Ravensburg-Bavendorf (Germany) and at the Research Institute of Organic Agriculture (FiBL) in Frick (Switzerland) in 2019 and 2020, respectively. *D. coronariae* DNA was quantified by a TaqMan qPCR using the primers and probe Dc_09, and spore numbers were calculated based on a standard curve with known amounts of conidia. 0, no spores detected; <10, *D. coronariae* DNA detected but below the limit of quantification.

TABLE 2. Numbers of conidia of *Diplocarpon coronariae* captured using rotating-arm spore traps in an apple orchard in Switzerland[a]

		Date (2019)[b]				Date (2020)[c]						
Cultivar	Trap number	22/5–3/6	3/6–13/6	13/6–25/6	25/6–5/7	24/4–8/5	8/5–22/5	22/5–5/6	5/6–19/6	19/6–3/7	3/7–17/7	17/7–31/7
Otava	1	7	0	335	558	0	0	70	0	0	611	41
Schneider	2	0	0	681	17	0	0	0	13	0	1	2
Otava	3	0	33	70	9	0	0	0	0	13	260	106
Otava	4	364	46	786	2,412	0	0	49	16	207	171	114
Topaz	5	27	0	0	17	0	0	0	22	0	0	251
Rubinola	6	29	0	0	2	0	0	0	0	0	1	10
Rubinola	7	57	0	0	0	0	0	0	0	0	0	7
Outside	8	22	0	0	0	0	0	0	0	0	0	0

[a] Rotating-arm spore traps were distributed within an apple orchard (Rickenbach, Switzerland) comprising 15 rows planted with different cultivars. Traps were placed in a low branch of the apple trees (about 1.7 m). Trap No. 2 was installed below a 'Schneider' tree, which was at the edge of an 'Otava' tree row. The location of the traps within the orchard is depicted in Figure 1C. DNA of *D. coronariae* in the spore trap samples was quantified by TaqMan qPCR using primers and probe Dc_09, and spore numbers were calculated based on a standard curve with known amounts of *D. coronariae* conidia.

[b] For 2019, the full sampling period is shown.

[c] In 2020, Rotorod traps were used for sampling from 28 February to 31 July. Because first spores were detected after 22 May, data for early series are not shown.

in the field being captured by the Mycotrap on the ground, and not in the Mycotrap in the tree canopy. Thus, leaf litter is affirmed as an important source of primary inoculum. However, additional inoculum sources such as buds and twigs might be alternative sites for overwintering of *D. coronariae* (Wöhner and Emeriewen 2019). The detection of *D. coronariae* DNA on buds and bark by our qPCR assay provides the first evidence for a role of these organs in overwintering of *D. coronariae* and emphasizes the importance of more research to determine sources of inoculum. Moreover, small quantities of *D. coronariae* DNA were detectable on fruit mummies hanging in the tree canopies at the end of February, and acervuli with infectious conidia were produced on *D. coronariae*-infected fruits after overwintering at 4°C. These organs are reported as overwintering sites for other apple pathogens, including *Monilia* spp. on fruit mummies, *Podosphaera leucotricha* in buds, and *Neofabraea* spp. on bark (Takahashi and Sawamura 2014). *D. rosae*, a close relative of *D. coronariae* causing black spot disease on roses, overwinters on bud scales and stems, in addition to in leaf litter (Cook 1981). To date, it is unclear what the survival structures of *D. coronariae* on bark and buds are (e.g., mycelium, spores) and whether they are infectious. The fact that we could not cause leaf infections from bud, bark, or fruit mummy tissues could be due to technical issues. *D. coronariae* present on buds and wood in propagation material could be exchanged between nurseries across Europe and might explain

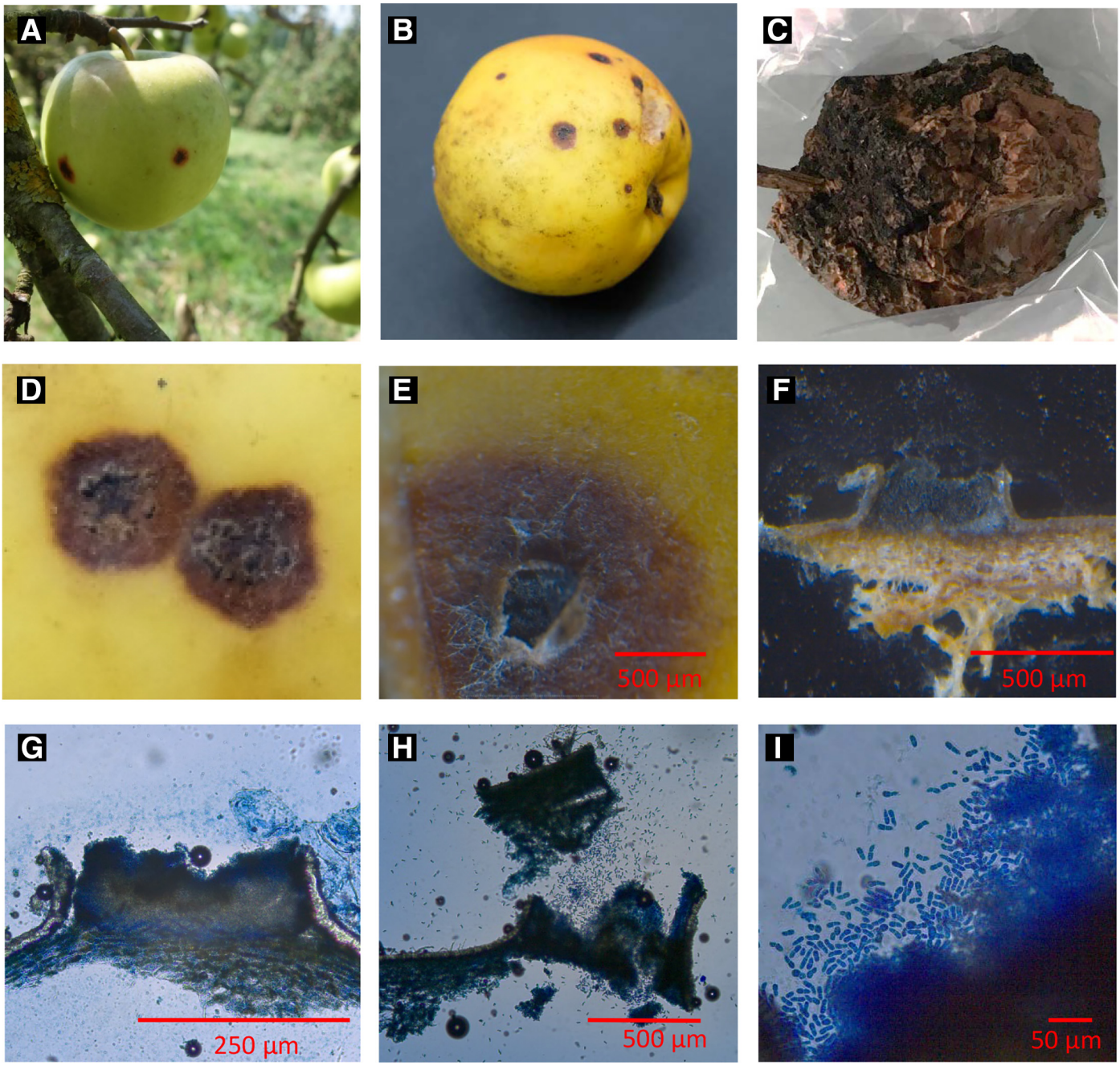

Fig. 5. Apple fruit infection with *Diplocarpon coronariae*. **A,** 'Otava' apple with apple blotch (AB) symptoms hanging in a tree in September 2020 (Rickenbach, Zurich, Switzerland). **B,** *D. coronariae*-infected Otava apple after 6 months of storage at 4°C. **C,** 'Florina' fruit mummy found hanging in a tree in February 2020 and tested positive for *D. coronariae* by TaqMan qPCR. **D and E,** Acervuli and conidia developed on Otava apple with symptoms of AB collected in the field in September 2019 and stored at 4°C for 6 months. **F and G,** Longitudinal section through an acervulus. **H,** Conidia released from an acervulus. **I,** *D. coronariae* conidia. Images E and F were captured using a stereo microscope M205C (Leica Microsystems Switzerland, Heerbrugg, Switzerland). Samples in images G to I were stained with cotton blue in lactic acid and images captured using a Leica DM2000 LED microscope (Leica Microsystems Switzerland).

the rapid spread of AB to many European apple-production areas since its first detection in Northern Italy (Oberhänsli et al. 2021). Our observation that conidia produced on fruits after 6 months in storage at 4°C can infect apple leaves provides an indication that fruit-derived conidia might represent an additional source of primary inoculum, at least on a local scale. Moreover, our description of fruit infection adds to our very limited knowledge regarding the role of fruit infections in the epidemiology of AB (Wöhner and Emeriewen 2019).

In Asia, ascospores as well as conidia have been reported to cause primary infections (Dong et al. 2015; Gao et al. 2011; Goyal et al. 2018; Sharma and Gupta 2018). Our qPCR method does not distinguish between conidia and ascospores, but apothecia of *D. coronariae* have not been observed in Europe (Wöhner and Emeriewen 2019), indicating that it is unlikely that they were the spore type captured. Although not yet observed, the importance of ascospores as a primary inoculum in Europe should be subject to further research. A recent population genetic study suggested a mixed sexual and asexual reproduction of *D. coronariae* in Europe, although *D. coronariae* populations in Europe are genetically homogenous, clonal, and dominated by a few multi-locus genotypes (Oberhänsli et al. 2021). The mating system of *D. coronariae* has not been elucidated, but mating type genes in a Chinese isolate indicate that *D. coronariae* possesses the genetic potential for heterothallism (Cheng et al. 2021). Whether both mating types occur in Europe is unknown.

The first peaks in spore production were observed at the end of, or shortly after, rainy periods, whereas no spores were detected during dry periods before rain events (e.g., April 2020). This implies that rain is required for dispersal of conidia of *D. coronariae* from leaf litter, which supports results from Dong et al. (2015) indicating that asexual spores are splash-dispersed. The acervuli might need a wetting period prior to release of conidia, which could explain the weak negative correlation between daily spore catches and precipitation in May 2019 (Supplementary Table S5).

By the end of May (2020) or the beginning of June (2019), the first leaves producing acervuli and conidia of *D. coronariae* were observed, initiating the secondary phase of infection. In contrast to the first major peak of conidia captured in May 2020, the second major peak (5 June) was not detected by the Mycotrap on the ground but by the Mycotrap situated in the tree canopy. We hypothesize that in June, the conidia originated from the recently infected leaves in the tree canopy, resulting in a higher spore count in the Mycotrap situated in the tree canopy. In support of the hypothesis, the rotating-arm spore traps in the tree crown did not capture conidia before the end of May.

From June onward, the AB epidemic steadily increased in the orchard (Fig. 1A and B). From mid-June to the end of the experiment, large numbers of spores were detected irrespective of rain events, and no correlation was found between daily precipitation and spores caught in June and July for either year (Supplementary Table S5). This contrasts with our observation of the early spore peaks, which

TABLE 4. Detection of *Diplocarpon coronariae* on the bark and in the buds of apple trees during winter (the apple trees had symptoms of apple blotch the previous summer)

Apple organ	Field site[a]	Apple cultivar[b]	Tree number[c]	Normalized C_q value[d]
Bark	KOB	Topaz	18–17	38.7
Bark	KOB	Topaz	18–25	41.1
Bark	KOB	Topaz	18–37	ND
Bark	KOB	Topaz	18–38	42.3
Bark	KOB	Topaz	18–39	42.0
Bark	KOB	Topaz	18–40	ND
Bark	Rickenbach	Otava	4–10	41.1
Bark	Rickenbach	Otava	4–11	ND
Bark	Rickenbach	Otava	4–13	39.9
Bark	Rickenbach	Otava	4–14	40.71
Bark	Rickenbach	Otava	4–15	38.10
Bark	Rickenbach	Otava	4–16	38.30
Bark	Rickenbach	Rubinola	10–4	ND
Bark	Rickenbach	Rubinola	10–5	ND
Bark	Rickenbach	Rubinola	10–6	ND
Bark	Rickenbach	Rubinola	10–7	ND
Bark	Rickenbach	Rubinola	10–8	ND
Bark	Rickenbach	Rubinola	10–9	ND
Bud	KOB	Topaz	18–17	ND
Bud	KOB	Topaz	18–25	36.3
Bud	KOB	Topaz	18–37	28.0
Bud	KOB	Topaz	18–38	31.4
Bud	KOB	Topaz	18–39	36.1
Bud	KOB	Topaz	18–40	37.5
Bud	Rickenbach	Otava	4–10	33.8
Bud	Rickenbach	Otava	4–11	ND
Bud	Rickenbach	Otava	4–13	41.6
Bud	Rickenbach	Otava	4–14	33.8
Bud	Rickenbach	Otava	4–15	25.6
Bud	Rickenbach	Otava	4–16	30.7
Bud	Rickenbach	Rubinola	10–4	ND
Bud	Rickenbach	Rubinola	10–5	ND
Bud	Rickenbach	Rubinola	10–6	ND
Bud	Rickenbach	Rubinola	10–7	ND
Bud	Rickenbach	Rubinola	10–8	ND
Bud	Rickenbach	Rubinola	10–9	ND

[a] Bud and bark samples were collected from apple trees in the Rickenbach orchard (Switzerland) on 28 February and on 13 March 2020 and from trees in the Competence Center for Fruit Crops at Lake Constance (KOB) orchard (Germany) on 17 March 2020.
[b] 'Topaz' and 'Otava' trees were heavily diseased with apple blotch in 2019, whereas 'Rubinola' trees showed no symptoms.
[c] The tree number indicates the position of the tree within the orchard and consists of row number and tree number within row.
[d] Cq (quantification cycle) values result from a TaqMan qPCR with primers and probe Dc_09. The APA9 plasmid was used to normalize for DNA extraction efficiency. ND, not detected.

occurred at the end of the rainy periods, and contrasts with the observations by Kim et al. (2019). In Korea, spore-dispersal peaks occurred during and 2 days after rain events. In Korea, disease incidence and total spore counts were lower than we observed at our field site, which might explain some of the differences. Based on our results, we hypothesize that primary inoculum might require water to ripen and discharge, whereas secondary inoculum might also be released on dry days. Lab experiments indicate that *D. coronariae* conidia can survive for several days under dry conditions and still cause infection (Clémence Boutry et al., *unpublished data*). If so, all secondary inoculum poses a risk of infection when leaf wetness occurs, even after a dry period.

The development of AB is affected by environmental factors (Li et al. 2011). Rainfall and the subsequent high relative humidity are positively correlated with severity of AB in the summer months (Rather et al. 2017b; Sastrahidayat and Nirwanto 2016; Sharma and Sharma 2005). Under controlled conditions, the minimum leaf wetness period for infection is 8 h at 15°C and decreases to 4 h with temperatures between 20 and 25°C (Sharma et al. 2009). Moreover, the incubation period can vary depending on the weather conditions. At a temperature range of 15 to 25°C and 7 days of leaf wetness, the incubation period is 10 to 20 days (Harada et al. 1974). In our study, the bait plants showed symptoms within 1 to 4 weeks after return from the orchard, with a shorter incubation period for bait plants placed in the orchard later in the season (June, July) compared with the beginning of the season (May). In 2020, the first necrotic spots indicative of AB and the more advanced symptoms were observed earlier and more rapidly than in 2019. The faster disease progression in late spring 2020 was likely due to higher May temperatures compared with the temperatures in 2019.

The mode of *D. coronariae* dispersal has not been conclusively determined, and nothing is known about the spatial dispersal of *D. coronariae.* Rotating-arm spore traps placed in the orchard revealed high spore concentrations in AB-diseased trees but no or few spores in trees without symptoms. We found very high spore counts in rainwater below severely diseased trees but not in traps between rows (Supplementary Fig. S6). Similarly, Khodadadi et al. (2022) detected large numbers of *D. coronariae* spores in rainwater traps from non-fungicide-treated orchards but not in samples from nearby forests. These findings indicate that conidia of *D. coronariae* are not dispersed over large distances, which would fit with a splash-dispersed mode of dispersal as described by Dong et al. (2015). However, as noted, there were generally low spore counts captured by the rotating-arm traps compared with the Mycotrap, indicating that the rotating-arm traps were much less efficient in capturing spores in the field (so spores might have been there, but the lower sensitivity of the trap precluded detection). Thus, wind-blown rain might transport some conidia over larger distances than we observed. The role of wind-dispersed ascospores (if produced in Europe) in long-distance dispersal of *D. coronariae*, and the impact of human-assisted dispersal, needs further research.

Based on our results, we hypothesize a life cycle for *D. coronariae* in Europe (Fig. 6). We contend that in Europe, as in Japan (Harada et al. 1974), China (Gao et al. 2011), Korea (Back and Jung 2014), and India (Sharma and Gupta 2018), the fungus overwinters primarily in infected leaf litter. However, bark, buds, and fruit mummies might be alternative sources of primary inoculum. First infections occur in late April or early May, depending on the weather conditions, and secondary inoculum sources develop in late May or early June, within 3 to 4 weeks of the primary infections occurring. In May, spore peaks were mainly found at the end of, or after, periods of rain, whereas spore load in the air was generally high from the middle of June to the end of July. Thus, every wet period in summer represents a higher risk for infection.

Our results contribute to the understanding of AB epidemiology, especially the timing of early spore dispersal and infection in Europe. The information can help improve disease forecast models, including the model developed by RIMpro, and provide a basis for early disease management. Whereas protection strategies against AB in Europe are currently focused on the summer months (Hinrichs-Berger 2015), prevention of primary infections in the early spring could be an important additional step for more efficacious management strategies in the future, reducing the need for fungicide applications in the summer.

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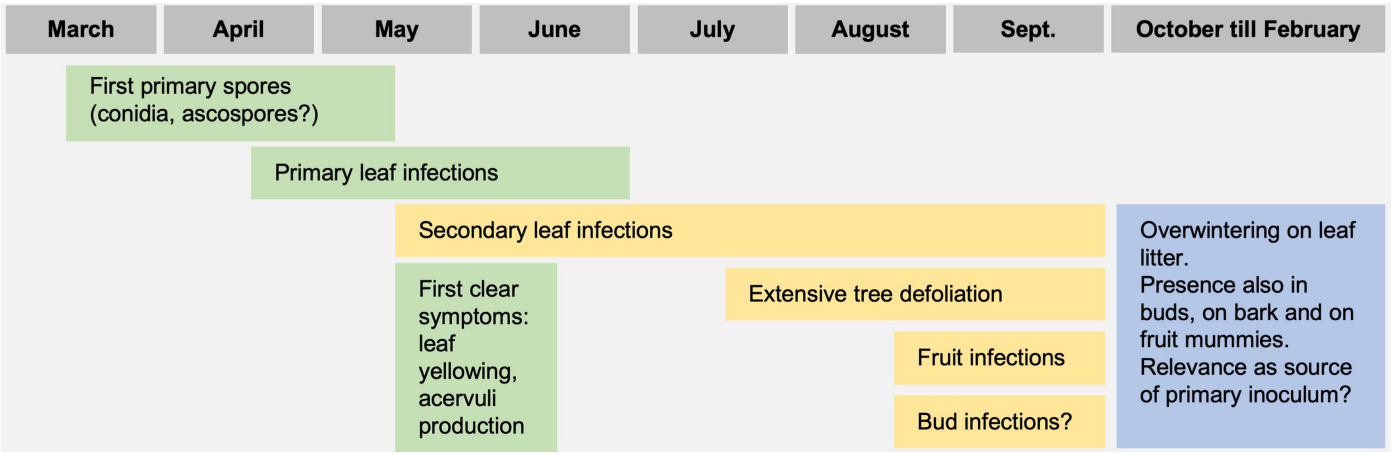

Fig. 6. The proposed life cycle of *Diplocarpon coronariae* in Central Europe. Based on previous knowledge and the data we present in the current study, we hypothesize that primary spores (conidia because no ascospores have been reported in Europe) are released in early spring and cause the first infections between the end of April and the middle of May depending on the weather conditions. Without crop protection, secondary spore inoculum is produced from the end of May onward. The epidemic of apple blotch (AB) spreads and leads to tree defoliation and infection of fruit in severely diseased orchards. The fungus overwinters on leaf litter but is also found on fruit mummies, in buds, and on bark after winter. The fruit mummies, buds, and bark might represent alternative organs for overwintering of *D. coronariae*. Green, primary infections; yellow, secondary infections; and blue, overwintering.

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Literature Cited

Aćimović, S. G., and Donahue, D. 2018. Marssonina leaf blotch of apple. Tree Fruit News. 6:1-4.

Agrios, G. N. 2005. Plant Pathology. 5th edition. Elsevier Academic Press, Amsterdam, the Netherlands.

Andersen, G. L., Frisch, A. S., Kellogg, C. A., Levetin, E., Lighthart, B., and Paterno, D. 2009. Aeromicrobiology/air quality. Pages 11-26 in: Encyclopedia of Microbiology (3rd edition). M. Schaechter, ed. Elsevier, Amsterdam, the Netherlands.

Arrigoni, E., Antonielli, L., Pindo, M., Pertot, I., and Perazzolli, M. 2018. Tissue age and plant genotype affect the microbiota of apple and pear bark. Microbiol. Res. 211:57-68.

Aslam, S., Tahir, A., Aslam, M. F., Alam, M. W., Shedayi, A. A., and Sadia, S. 2017. Recent advances in molecular techniques for the identification of phytopathogenic fungi–a mini review. J. Plant Interact. 12:493-504.

Back, C.-G., and Jung, H.-Y. 2014. Biological characterization of *Marssonina coronaria* infecting apple trees in Korea. Kor. J. Mycol. 42:183-190.

Back, C.-G., Lee, S.-Y., Kang, I.-K., Yoon, T.-M., and Jung, H.-Y. 2015. Occurrence and analysis of apple blotch-like symptoms on apple leaves. Kor. J. Hort. Sci. Technol. 33:429-434.

Basso, T., Tran, M., Pini, V., Schnée, S., Dubuis, P.-H., Kasparian, J., and Wolf, J.-P. 2020. Détection des spores de mildiou et d'oïdium en temps réel à haute résolution spatiale. Revue Suisse Aboriculture, Viticulture, Horticulture 52:344-349.

Bohr, A., Buchleither, S., Hechinger, M., and Mayr, U. 2018. Symptom occurrence and disease management of Marssonina blotch. 18th International Conference on Organic Fruit-Growing, 19-21 February 2018, Hohenheim, Germany.

Calderon, C., Ward, E., Freeman, J., and McCartney, A. 2002. Detection of airborne fungal spores sampled by rotating-arm and Hirst-type spore traps using polymerase chain reaction assays. J. Aerosol Sci. 33:283-296.

Cheng, Q., Chen, J., and Zhao, L. 2021. Draft genome sequence of *Marssonina coronaria*, causal agent of apple blotch, and comparisons with the *Marssonina brunnea* and *Marssonina rosae* genomes. PLoS One 16:1-17.

Cook, R. T. A. 1981. Overwintering of *Diplocarpon rosae* at Wisley. Trans. Br. mycol. Soc. 77:549-556.

Crous, P. W., Wingfield, M. J., Schumacher, R. K., Akulov, A., Bulgakov, T. S., Carnegie, A. J., Jurjevic, Z., Decock, C., Denman, S., Lombard, L., Lawrence, D. P., Stack, A. J., Gordon, T. R., Bostock, R. M., Burgess, T., Summerell, B. A., Taylor, P. W. J., Edwards, J., Hou, L. W., Cai, L., Rossman, A. Y., Wohner, T., Allen, W. C., Castlebury, L. A., Visagie, C. M., and Groenewald, J. Z. 2020. New and interesting fungi. 3. Fungal Syst. Evol. 6: 157-231.

Dong, X.-L., Gao, Y.-E., Li, B.-H., Yong, D.-J., Wang, C.-X., Li, G.-f., and Li, B.-D. 2015. Epidemic dynamics of apple Marssonina leaf blotch over whole growth season in the central area of Shandong Peninsula. Scientia Agricultura Sinica 48:479-487.

Dvořák, M., Rotková, G., and Botella, L. 2015. Detection of airborne inoculum of *Hymenoscyphus fraxineus* and *H. albidus* during seasonal fluctuations associated with absence of apothecia. Forests 7:1.

Gao, Y.-E., Li, B.-H., Dong, X.-L., Wang, C.-X., Li, G.-F., and Li, B.-D. 2011. Effects of temperature and moisture on sporulation of *Diplocarpon mali* on overwintered apple leaves. Scientia Agricultura Sinica 44:1367-1374.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Goyal, A., Sharma, J. N., and Phurailatpam, S. 2018. Studies on the mode of survival of *Diplocarpon mali* causing premature leaf fall of apple. Int. J. Curr. Microbiol. Appl. Sci. 7:2465-2474.

Harada, Y., Sawamura, K., and Konno, K. 1974. *Diplocarpon mali*, sp. nov., the perfect state of apple blotch fungus *Marssonina coronaria*. Ann. Phytopathol. Soc. Jpn. 40:412-418.

Hardwick, N. V. 1998. Disease forecasting. Pages 207-230 in: The epidemiology of plant diseases. D. G. Jones, ed. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Hinrichs-Berger, J. 2015. Die Marssonina-Blattfallkrankheit des Apfels. Pages 72-74 in: Obstbau.

Hinrichs-Berger, J., and Müller, G. 2012. Vorzeitiger Blattfall an Apfelbäumen in Baden-Württemberg durch Befall mit *Marssonina coronaria*. Pages 71-71 in: 58. Deutsche Pflanzenschutztagung Julius-Kühn-Archiv, Braunschweig, DE.

Horbach, R., Navarro-Quesada, A. R., Knogge, W., and Deising, H. B. 2011. When and how to kill a plant cell: Infection strategies of plant pathogenic fungi. J. Plant Physiol. 168:51-62.

Kennedy, R., Wakeham, A. J., Byrne, K. G., Meyer, U. M., and Dewey, F. M. 2000. A new method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. Appl. Environ. Microbiol. 66:2996-3003.

Khodadadi, F., Martin, P. L., Donahue, D. J., Peter, K. A., and Acimovic, S. G. 2022. Characterization of an emerging disease: Apple blotch caused by *Diplocarpon coronariae* (syn. *Marssonina coronaria*) in the Mid-Atlantic United States. Plant Dis. 106:1803-1817.

Khodadadi, F., Peter, K. A., Donahue, D., and Aćimović, S. G. 2019. Marssonina blotch of apple caused by *Marssonina coronaria* in New York. Page 114 in: Plant Health 2019 – American Phytopathological Society Annual Meeting, Cleveland, OH.

Kilin, V., Pini, V., Kasparian, J., Gros, S., and Wolf, J. P. 2019. Real-time and spatially resolved assessment of pathogens in crops for site-specific pesticide reduction strategies. BIO Web Conf. 15:1-3.

Kim, H. S., Jo, J. H., Kang, W. S., Do, Y. S., Lee, D. H., Ahn, M. I., Park, J. H., and Park, E. W. 2019. MBCAST: A forecast model for Marssonina blotch of apple in Korea. Plant Pathol. J. 35:585-597.

Kim, K. H., Kabir, E., and Jahan, S. A. 2018. Airborne bioaerosols and their impact on human health. J. Environ. Sci. (China) 67:23-35.

Kwon, D., Kim, S., Kim, Y., Son, M., Kim, K., An, D., and Kim, B. 2015. An empirical assessment of the economic damage caused by apple Marssonina blotch and pear scab outbreaks in Korea. Sustainability 7: 16588-16598.

Lee, D.-H., Back, C.-G., Win, N. K. K., Choi, K.-H., Kim, K.-M., Kang, I.-K., Choi, C., Yoon, T.-M., Uhm, J. Y., and Jung, H.-Y. 2011. Biological characterization of *Marssonina coronaria* associated with apple blotch disease. Mycobiology 39:200-205.

Li, J., Gou, L.-X., H, X.-M., Ren, F.-P., Wei, J.-F., and An, D.-R. 2011. Effects of climate factors on the *Marssonina coronaria* epidemic in Shaanxi Province and related prediction Models. Chin. J. Appl. Ecol. 22:268-272.

Liang, Z., and Keeley, A. 2013. Filtration recovery of extracellular DNA from environmental water samples. Environ. Sci. Technol. 47:9324-9331.

Lindner, L. 2012. Die Marssonina-Blattfleckenkrankheit jetzt auch in Südtirol. Pages 66-68 in: Obstbau Weinbau.

Luchi, N., Ghelardini, L., Belbahri, L., Quartier, M., and Santini, A. 2013. Rapid detection of *Ceratocystis platani* inoculum by quantitative real-time PCR assay. Appl. Environ. Microbiol. 79:5394-5404.

McDevitt, J. J., Lees, P. S. J., Merz, W. G., and Schwab, K. J. 2004. Development of a method to detect and quantify *Aspergillus fumigatus* conidia by quantitative PCR for environmental air samples. Mycopathologia 158: 325-335.

McKinney, H. H. 1923. Influence of soil temperature and moisture on infection of wheat seedlings with *Helminthosporium sativum*. J. Agric. Res. 26:195-217.

Meitz-Hopkins, J. C., G., v. D. S., Koopman, T. A., Bahramisharif, A., and Lennox, C. L. 2014. A method to monitor airborne *Venturia inaequalis* ascospores using volumetric spore traps and quantitative PCR. Eur. J. Plant Pathol. 140:527-541.

Mosimann, C., Oberhänsli, T., Ziegler, D., Nassal, D., Kandeler, E., Boller, T., Mäder, P., and Thonar, C. 2017. Tracing of two *Pseudomonas* strains in the root and rhizoplane of maize, as related to their plant growth-promoting effect in contrasting soils. Front. Microbiol. 7:1-14.

Notomi, T., Mori, Y., Tomita, N., and Kanda, H. 2015. Loop-mediated isothermal amplification (LAMP): Principle, features, and future prospects. J. Microbiol. 53:1-5.

Oberhänsli, T., Dalbosco, A., Leschenne, V., Bohr, A., Buchleither, S., Tamm, L., Wille, L., Aćimović, S. G., Khodadadi, F., Ryu, Y.-H., Studer, B., Schärer, H.-J., and Patocchi, A. 2021. Multiplexed SSR marker analysis of *Diplocarpon coronariae* reveals clonality within samples from Middle Europe and genetic distance from Asian and North American isolates. CABI Agric. Biosci. 2:21.

Oberhänsli, T., Vorley, T., Tamm, L., and Schärer, H. 2014. Development of a quantitative PCR for improved detection of *Marssonina coronaria* in field samples. Pages 187-190 in: Ecofruit. 16th International Conference on Organic Fruit-Growing Conference Fördergemeinschaft Ökologischer Obstbau e.V., Hohenheim, Germany.

Park, M. Y., Sagong, D. H., Kweon, H. J., Do, Y. S., Song, Y. Y., and Lee, D. H. 2013. Influence of seasonal incidence and defoliation degree of Marssonina blotch on fruit quality and shoot growth of 'Fuji'/M.9 Apple Tree. Kor. J. Hortic. Sci. 31:523-530.

Parker, M. L., McDonald, M. R., and Boland, G. J. 2014. Evaluation of air sampling and detection methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 98:32-42.

Persen, U., Steffek, R., Freiding, C., and Bedlan, G. 2012. Erstnachweis von *Diplocarpon mali* an *Malus domestica* in Österreich. J. für Kulturpflanzen 64:168-170.

Quesada, T., Hughes, J., Smith, K., Shin, K., James, P., and Smith, J. 2018. A low-cost spore trap allows collection and real-time PCR quantification of airborne *Fusarium circinatum* spores. Forests 9:586.

R Core Team. 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rather, S. A., Bhat, M. A., and Khan, N. A. 2017b. Screening of *Malus* genotypes against Marssonina leaf blotch. J. Pharmacogn. Phytochem. 6:134-136.

Rather, S. A., Bhat, M. A., and Mohiddin, F. A. 2017a. Influence of weather factors on development of Marssonina leaf blotch of apple caused heterosis in cowpea for yield and quality. Indian J. Ecol. 44:2494.

Ren, W., Liu, N., and Li, B. 2021. Development and application of a LAMP method for rapid detection of apple blotch caused by *Marssonina coronaria*. Crop Prot. 141:105452.

Sastrahidayat, I. R., and Nirwanto, H. 2016. Marssonina leaf blotch on the apple orchard in Batu. Indonesia. AGRIVITA J. Agric. Sci. 38:204-212.

Schärer, H.-J., Bohr, A., and Wöhner, T. 2019. Anfälligkeit von Apfelsorten gegenüber der Blattfallkrankheit *Marssonina coronaria*. SZOW 19: 8-12.

Schneider, R. W., Haudenshiled, J. S., Hartman, G. L., and Mahaffee, W. F. 2009. Development and use of fluorescent antibody and qPCR protocols for the electrostatic spore trap. (Abstr.) Phytopathology 99:S115.

Sharma, J. N., and Thakur, V. S. 2011. Epidemic outbreak of apple blotch disease: Epidemiology and management in Himachal Pradesh. Indian Phytopathol. 63:141-144.

Sharma, J. N., Thakur, V. S., Mohan, J., Paul Khurana, S. M., and Sharma, S. 2009. Epidemiology of Marssonina blotch (*Marssonina coronaria*) of apple in India. Indian Phytopathol. 62:348-359.

Sharma, P. K., and Sharma, J. N. 2005. Studies on morphology and physiology of *Marssonina coronaria* causing premature leaf fall in apple. Pages 9-17 in: Integrated Plant Disease Management Challenging Problems in Horticultural and Forest Pathology. R. C. Sharma and J. N. Sharma, eds. Scientific Publishers, Jodhpur, India.

Sharma, U., and Gupta, B. 2018. First report of *Diplocarpon mali*, the perfect state of apple blotch fungus *Marssonina coronaria* from India. J. Krishi Vigyan 7:239.

Siegfried, W., Holliger, E., and Meier, H. 1996. Prognose des Rotbrenners und des Falschen Rebenmehltaus. SZOW 132:373-374.

Sterling, M., Rogers, C., and Levetin, E. 1999. An evaluation of two methods used for microscopic analysis of airborne fungal spore concentrations from the. Burkard Spore Trap. Aerobiologia 15:9-18.

Takahashi, S., and Sawamura, K. 2014. Marssonina blotch. Pages 46-47 in: Compendium of Apple and Pear Diseases and Pests. T. B. Sutton, H. S. Aldwinckle, A. M. Agnello, and J. F. Walgenbach, eds. American Phytopathology Society, St. Paul, MN.

Von Felten, A., Defago, G., and Maurhofer, M. 2010. Quantification of *Pseudomonas fluorescens* strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-specific real-time PCR unaffected by the variability of DNA extraction efficiency. J. Microbiol. Methods 81:108-115.

West, J., and Kimber, R. 2015. Innovations in air sampling to detect plant pathogens. Ann. Appl. Biol. 166:4-17.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfland, J. J. Sninsky, and T. J. White, eds. Academic Press, London.

Wöhner, T., Bohr, A., and Schärer, H.-J. 2019. Marssonina-Anfälligkeit von Apfelsorten. Obstau 3:172-178.

Wöhner, T., and Emeriewen, O. F. 2019. Apple blotch disease (*Marssonina coronaria* (Ellis & Davis) Davis) - Review and research prospects. Eur. J. Plant Pathol. 153:657-669.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinf. 13:134.

Zhao, H., Han, Q. M., Wang, J., Gao, X. N., Xiao, C.-L., Liu, J., and Huang, L. L. 2013. Cytology of infection of apple leaves by *Diplocarpon mali*. Eur. J. Plant Pathol. 136:41-49.

Zhao, H., Huang, L., Xiao, C. L., Liu, J., Wei, J., and Gao, X. 2010. Influence of culture media and environmental factors on mycelial growth and conidial production of *Diplocarpon mali*. Lett. Appl. Microbiol. 50:639-644.