Monitoring spore dispersal and early infections of *Diplocarpon coronariae* causing apple blotch using selected spore traps and a new qPCR method

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Abstract

Apple blotch (AB) is a major disease of apples in Asia and recently also emerging in Europe and the USA. It is caused by the fungus *Diplocarpon coronariae* (*Dc*) *(formerly: Marssonina coronaria;* teleomorph: *Diplocarpon mali*) and leads to severe defoliation of apple trees in late summer and thus to reduced yield and fruit quality. To develop effective crop protection strategies, a sound knowledge of the pathogen's biology is crucial. However, especially data on the early phase of disease development is scarce, and no data on spore dispersal for Europe is available. In this study, we assessed different spore traps for their capacity to capture *Dc* spores, and we developed a highly sensitive TaqMan qPCR method to quantify *Dc* conidia in spore trap samples. With these tools, we monitored the temporal and spatial spore dispersal and disease progress in spring and early summer in an extensively managed apple orchard in Switzerland in 2019 and 2020. Our results show that *Dc* overwinters in leaf litter and that spore dispersal and primary infections occur already in late April and beginning of May. We provide the first results on early-season spore dispersal of *Dc*, which, combined with the observed disease progress, helps to understand the disease dynamics and improve disease forecast models. Using the new qPCR method, we finally detected *Dc* in buds, on bark and on fruit mummies, suggesting that these apple organs may serve as additional overwintering habitats for the fungus.

Introduction

Diplocarpon coronariae (*Dc*) (Ellis & Davis) Wöhner & Rossman (Crous et al. 2020) formerly *Marssonina coronaria* (Ellis & Davis) Davis; teleomorph *Diplocarpon mali* (Harada & Sawamura) is an ascomycete fungus causing apple blotch (AB) (Wöhner and Emeriewen 2019). The pathogen can lead to severe tree defoliation, weakening the trees and resulting in a decrease in yield (Sharma and Thakur 2011) and fruit quality (Park et al. 2013), as well as spots on fruits. The disease has a significant economic impact, especially in South and East Asia. In South Korea the loss due to AB is estimated at 29.79 M US$ (Kwon et al. 2015) and in India AB is emerging as the most destructive disease affecting apple trees, becoming a major bottleneck in 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 also become a problem in Europe (Wöhner and Emeriewen 2019) and in the USA (Aćimović and Donahue 2018; Khodadadi and Aćimović 2019), especially in orchards with low input of pesticides such as organic orchards and untreated orchards for juice production (Bohr et al. 2018; Hinrichs-Berger and Müller 2012; Persen et al. 2012). With the rising demand of lowering pesticide residues, the relevance of AB may increase also in conventionally managed apple orchards in future years.

Up to the present, knowledge about the biology, epidemiology and disease control of the pathogen mostly comes from Asia, i.e. Japan, China, Korea, and India, where the disease caused problems since the late 1990ies, while research on *Dc* in Europe and the USA is still in its infancy. *Dc* has a hemibiotrophic lifestyle (Horbach et al. 2011; Zhao et al. 2013) and primarily infects mature leaves (Hinrichs-Berger and Müller 2012). The optimal conditions for the infection are temperatures between 20 and 25 °C and a long leaf wetness period (Sastrahidayat and Nirwanto

2016). The minimum leaf wetness period is eight hours at 15 °C, and the risk of infection increases with increasing leaf wetness period and increasing temperature (Sharma et al. 2009). The perfect state of *Dc* has been described so far only in India, Japan, and China (Gao et al. 2011; Harada et al. 1974; Sharma and Gupta 2018), while to date no observation of sexual structures (apothecia, ascospores) are reported from Europe (Hinrichs-Berger 2015; Wöhner and Emeriewen 2019), the USA or Korea (Back and Jung 2014). Therefore, in these regions, the infection is suspected of starting from conidia released by acervuli that develop in overwintered leaves (Back and Jung 2014). Alternatively, buds and twigs have been hypothesized as overwintering places for *Dc* (Wöhner and Emeriewen 2019). However, data on sources of primary infections and early disease progress is missing for Europe and the USA.

The quantification of spores in the air allows assessing the potential for primary infection and subsequent epidemic outbreaks. Moreover, knowledge on spore dispersal combined with disease progress observation and weather data is essential for understanding the disease dynamics and developing disease forecast models. Those, in turn, provide a valuable tool for an efficient application of crop protection measures (Agrios 2005; Hardwick 1998). Recently, *Dc* spore dispersal from June to October was investigated in Korea, and a disease forecast model was developed (Kim et al. 2019). However, this study did not look at spore dispersal in spring, which is critical to understanding the primary infection and disease onset. Moreover, for Europe and the USA data on *Dc* spore dispersal are to our best knowledge missing.

An effective means to monitor a particular pathogen in the air is the use of spore traps. There are various types of spore traps that differ in air sampling flow rate, collection efficiency and sensitivity to spore size, power consumption, length of the sampling period, spore trapping surface and ease of processing the samples, spore identification, and spore viability (Jackson and Bayliss 2011; West and Kimber 2015). Depending on the application area, the research question or the available resources (finances, power availability) one or the other spore trap might be better suited. Spore traps can be categorized into passive and active samplers. Passive (gravitational) sampling spore traps work by passive deposition onto adhesive surfaces such as a petroleum jelly (Vaseline)-coated microscope slide or Vaseline-coated thin glass rods (West and Kimber 2015). Active sampling (non-inertial) spore traps use volumetric methods including collection by impaction, impinging, filtering, virtual impaction, cyclone and electrostatic attraction (West and Kimber 2015). For volumetric impaction spore traps, the air particles impact a solid surface such as a Petri dish, filter paper, a double-sided adhesive tape, petroleum jelly (Vaseline)-coated tape, slides or rods, or electrostatic plastic film. Cyclone samplers direct the air into a collection chamber through a spiraling, swirling flow (Kim et al. 2018). The most commonly used spore traps in phytopathology are the seven-day volumetric spore trap, the cyclone-based spore trap, and the rotating-arm spore trap (more details on spore trap types are are given in Supplementary Materials and Methods).

Spores captured by spore traps have traditionally been identified and quantified by microscopy. However, this requires a spore trapping surface that can be examined under the microscope and a trained investigator to accurately identify the counted morphological fungal structure. Newer methods combine microscopy with image-recognition of spores and machine learning. The accuracy of the spore detection relies on the quality of the image, with a higher accuracy the better the resolution. Multimodal, multiphoton microscopy for example provides 3D images of the spores with high spatial (500 nm) and spectral resolution (32 channels), and has been tested for *Plasmopara viticola* (Basso et al. 2020; Kilin et al. 2019). Furthermore, laser-based real-time optical particle counters (OPC) 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 microscopy, molecular methods based on the detection of fungal DNA or proteins are used to quantify fungal spores. DNA-based methods include the quantitative real-time polymerase chain reaction (qPCR), loop-mediated isothermal amplification (LAMP), (Notomi et al. 2015; Ren et al. 2021), serial analysis of gene expression (SAGE), and microarray technology (Aslam et al. 2017). The enzyme-linked immunosorbent assay (ELISA) (Kennedy et al. 2000), or the fluorescent antibody (FA) assay (Schneider et al. 2009) on the other hand detect fungal proteins by antibodies. The qPCR method is the most frequently used quantitative molecular method to detect and quantify fungal pathogens. It offers a more sensitive and specific quantification than microscopy, as it can detect low concentrations of a fungal pathogen among a background of particles and DNA from diverse organisms (Parker et al. 2014) and does not depend on visual recognition of the fungus. The detection limit of spores from a field sample depends on many factors influencing the sensitivity of the qPCR assay. These factors comprise the DNA extraction efficiency, and the performance of the qPCR, which depends on the quality of the DNA extract (e.g. presence of inhibiting compounds such as phenols, salts, alcohols), the choice of the target gene in the genome (multi-copy vs single-copy), the design of efficient primers, the amplicon lengths, the qPCR reagents, and the equipment and analysis software (Freeman et al. 1999).

The aim of this study was to gain a better understanding of spore dispersal and early disease development of *Dc*. To this end, we monitored AB disease development in a Swiss and

German apple orchard from 2016 to 2020. We evaluated the most suitable spore traps to capture *Dc* spores under experimental conditions, and we developed a highly sensitive qPCR method to quantify *Dc* spores. These tools were used to monitor the temporal and spatial distribution of *Dc* spores in spring and early summer. We detected large amounts of spores already beginning of May and again during the exponential phase of the epidemic. This observation represents to our best knowledge the first report on *Dc* spore flight in Europe. To understand the onset of AB disease, we further investigated potential sources of primary inoculum. The here provided data on spore counts, weather and disease development substantially improves our understanding of the biology of *Dc*. Furthermore, it can be directly used to fight AB disease, e.g. by improving disease forecast models and adapted plant protection measures. Finally, the developed methods represent useful tools for future studies of *Dc*.

Material and Methods

Microorganisms used in this study

Fungal strains used in this study are listed in Supplementary Table S1.

DNA extraction from fungal cultures, spore trap samples and apple leaves

Fungal DNA from in vitro cultures were extracted with the ZymoBIOMICS® Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, USA) according to the manufacturer's recommendations.

We aimed at an optimal DNA extraction efficiency from *Dc* spores in spore trap samples. Therefore, we tested the extraction of known amounts of *Dc* conidia from different spore trap media (plastic film, Vaseline coated plastic film, adhesive tape, 1.5 ml plastic tubes) with different DNA extraction protocols (data not shown) and finally decided to extract *Dc* conidia from Vaseline coated plastic film using the ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit as recommended by the manufacturer but with two modifications: the Bashing Bead® tubes from the Zymo Microprep Kit were replaced by a 2 ml tube with screw cap containing 100 mg of 0.5 mm Zirconia bashing beads (N034.1 Roth, Karlsruhe, DE) and the Bashing Bead™ Buffer volume was reduced from 750 µl to 550 µl. This method was also used to extract spores from uncoated plastic film. Conidia that were directly sampled into a vial, as in the case of the multi-vial cyclone sampler, as well as apple leaves with ambiguous AB symptoms were extracted following a CTAB buffer based protocol (Supplementary Methods).

Primer design and evaluation

We aimed at developing a highly specific and sensitive TaqMan® qPCR assay for *D. coronariae*. To this end we designed primers and hydrolysis probes targeting the ITS1 region between 18S and 5.8S rDNA of *D. coronariae* using the program Beacon Designer (V8.16, Premier Biosoft, Palo Alto, CA, USA) with following parameters: amplicon length between 100 and 200 bp, melting temperature (TM) of primers at 60.0°C +/- 1.0°C, and TM of probe at 10.0°C +/- 5.0°C above TM of the primers. Default settings of the program were used for maximal ΔG of self-complementarity, 3’-end stability, and % GC. The specificity of primers and probes was confirmed *in silico* by NCBI Primer-Blast https://www.ncbi.nlm.nih.gov/tools/primer-blast (Ye et al. 2012) by an alignment of different ITS genebank accessions of strains of *Dc* and finally tested in vitro with genomic DNA of different *D. coronariae* strains and other fungal species (Figure 1A, Supplementary Table 2).

Conditions of the qPCR reactions

Primers and probes were synthesized and purified by high performance liquid chromatography HPLC at Microsynth AG (Balgach, CH). Primers and probes (Table 1) were dissolved in a TE-dilution buffer (TE-Buffer: 10^{-3} mol · l^{-1} Tris, 10^{-5} mol · l^{-1} Na_2EDTA, pH 8.0). SYBR based *Dc* specific qPCR reactions consisted of 5 µl KAPA SYBR FAST (Sigma-Aldrich Chemie AG, Buchs, Switzerland), 1 µl primermix (forward and reverse primers at a concentration of 3 µM each), 3 µl double-distilled water and 1 µl of template DNA in a total volume of 10 µl. TaqMan® based qPCR reactions consisted of 5 µl KAPA PROBE FAST (Sigma-Aldrich Chemie AG, Buchs, Switzerland), 1 µl of each primermix for *Dc* and APA9 (containing primers at a concentration of 3 µM and 2 µM for Dc_09 and ACMV, respectively, and the probe at 1 µM each), 0 to 2 µl double-distilled water and 1 to 3 µl of template DNA in a total volume of 10 µl.

The qPCR assay and data analysis were performed with a CFX96 Touch Real-Time PCR Detection System (Biorad, USA). An annealing temperature of 60 °C was found best for primer Dc_09 (assessed with a gradient from 58 to 68 °C tested on two concentrations of *Dc* CH01 mycelial DNA using SYBR qPCR, data not shown). The finally used amplification and quantification conditions were: an initial denaturation step of 3 min at 95 °C, followed by 39 and 45 cylces 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 °C to 95 °C with 0.5 °C per cycle.

To generally detect fungal DNA a SYBR green qPCR assay was performed with primers ITS1F/2R (Table 1). The reactions consisted of 5 µl KAPA SYBR FAST (Sigma-Aldrich Chemie AG, Buchs, Switzerland), 1 µl primermix (primers at a concentration of 2 µM each), 3 µl 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 °, 30 s at 50 °C, and 1 min at 72°C.

To visualize PCR products, 10 µl of the qPCR reaction were loaded on a 2 % TAE agarose gel stained with ROTI® GelStainRed (Carl Roth GmbH, Karlsruhe, Germany) and run at 50 V for 60 min. Pictures were taken in an Azure Biosystems c150 (Azure Biosystems, Dublin, CA, USA) gel imaging work station.

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 amounts of conidia. To this end, conidia suspensions were prepared by harvesting conidia from *D. coronariae* CH01 (Supplementary Table 1) isolate, grown on a PPCDA (Zhao et al. 2010) agar plate at room temperature for 22 days, with 50 ml sterile double-distilled water (ELGA®, Purelab Flex 03, Veolia Water Technologies, Celle, Germany) or by stirring apple leaves with sporulating *Dc* for ten minutes in Volvic® water and subsequent filtering of the spore suspension through a sieve (mesh-width approximately 0.1 mm). The spore concentration was assessed by counting the conidia under a microscope using a haemocytometer (0.1 mm depth, 0.0025 mm^2, Neubauer®). Ten-fold dilution series of conidia in double-distilled water containing 0.05% Tween® 80 as described by Mc Devitt et al. (2004) corresponding 10^5 to 10^1 conidia were prepared and added to the material used in spore traps (Vaseline coated plastic film). DNA extraction and qPCR were performed as described above. The standard curves were used for the test calibration, and the limit of quantification (LOQ), and the calculation of spore numbers out of Cq values.

All spore trap samples were spiked with 10^7 copies of linearized APA9 plasmid (vector pUC19 with insert of a cassava mosaic virus; GenBank accession number AJ427910) (Supplementary Methods) (Mosimann et al. 2017) as an internal control to account for differences in DNA extraction efficiency between different samples. APA9 plasmid was quantified together with *Dc* using a multiplexed TaqMan® PCR with the primers and probe ACMV listed in Table 1 and the results were used to normalize DNA extraction using the method by Von Felten et al. (2010).

Spore traps used in this study

Four different types of spore traps were assessed for their ability to capture *Dc* spores under experimental conditions (Table 2) and two of them, the Mycotrap and the rotating-arm spore traps, were used for field sampling (Figure 2D, E). Additionally, apple bait plants were used as in vivo spore traps (Figure 2F). A detailed description on the spore traps is provided in the Supplementary Methods. Briefly, the Mycotrap (Siegfried et al. 1996) (Figure 2D, Table 2) is an impaction spore trap where air is sucked in horizontally through a sampling orifice and impacts on a trapping surface on a cylinder inside a chamber. The cylinder slowly rotates, completing one turn within seven days thus allowing to sample seven days in a row. The high throughput 'jet' spore sampler (Burkard Manufacturing) is a type 'virtual impaction' spore trap with high air throughput based on a settling tower at which base the spores are collected (West and Kimber 2015)(Table 2). The cyclone-based spore trap (Burkard Manufacturing) (Table 2) comprises an air intake introducing the airflow tangentially into a glass or metal cylinder (Lacey and West 2006). At the end of the cylinder spores are collected during a period of eight days into eight different detachable 1.5 ml sample vials, by changing the sampling vial position each day. The rotating-arm spore traps (Figure 2E) were constructed based on the description by Quesada et al. (2018) (details in Supplementary Methods). They consisted of two rods connected to a battery powered motor (spinart^{TM} Lightweight Hanging Motor, 30 rpm). Each rod held a microscope slide on which a Vaseline coated plastic film strip was mounted and fixated with 25 mm foldback-clips (Maul, Bad König, Germany). The rotating-arm spore traps were covered with an aluminum shield as rain protection.

Assessment of spore traps in the climate chamber

Spore traps were compared inside a closed climate chamber (33.43 m^3). The experiments included two seven-day volumetric spore traps (Mycotrap), one multi-vial cyclone sampler (cyclone), one high throughput 'jet' spore sampler, and two rotating-arm spore traps. Additionally, apple seedlings were used as bait plants. In a first set of experiments the spore traps were assessed for their capability to capture spores from a conidial suspension of *Dc* sprayed into the air (Supplementary Methods).

A second experiment aimed at simulating spore captures after splash dispersal from *Dc* infected leaf litter. Infected leaves were collected in the field in fall 2018 and were placed outside in mesh bags on the ground on water-permeable ground-cover-foil. After winter, the leaves were dried for 72h at 25 °C and 150 g of dried leaves were used to form an artificial leaf deposit in the climate chamber. The spore traps and bait plants were placed in the climate chamber around the leaf litter deposit with the air suction orifice of the spore traps or the rotating-arms (for rotating-arm spore traps) 30 cm above the leaf litter (details in Supplementary Methods). Water was artificially rained on the setup with a showerhead for four cycles consisting of 2h irrigation followed by 2h without irrigation. A lighting panel (True Daylight-LED, poly klima, Langweld-Foret, Germany) was installed above the leaf deposit. Spore traps were used to catch the spores in the created aerosol. The samples were analyzed by the above described qPCR method.

Field sites

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 10.0 °C and 980 mm of annual rainfall (years 2010 to 2020). The orchard was planted in 2002 with different apple cultivars arranged in 15 rows with 18 apple trees each (Figure 2C), in a planting distance of 4 m within and 9 m between rows. In 2019, the orchard was treated with sulfur and clay (acidified clay minerals, product Myco-Sin® 8 kg/ha company Andermatt Biocontrol Suisse AG, 6146 Grossdietwil, Switzerland; in tank mix with

Sulphur, product "Netzschwefel Stulln" 4 kg/ha, Biofa AG, Münsingen, Germany) on the 25th of May, and the rows of 'Otava', 'Rajka', 'Rubinola', 'Topaz', and 'Boskoop' were additionally treated on the 11th of June with lime sulfur (product Curatio®, Biofa AG, Münsingen, Germany). In 2020, the entire orchard was treated with Myco-Sin® and sulphur on the 27th of April and on the 9th of May. In addition, all trees apart from 'Bohnapfel', 'Schneider' and 'Tobiässler' were treated with the same mixture on the 22nd of May, and 'Otava', 'Rajika', 'Rubinola', and 'Boskoop' were treated again on the 2nd of June. Trees with spore traps were excluded from the fungicide treatments in 2019 and 2020 and in 2020 in addition 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 the Lake of 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 908 mm of annual rainfall over the last ten years. The orchard was planted in 2003 with Topaz on M9 with 3.2 m between and 0.8 m within the rows. For disease monitoring 120 trees of two consecutive rows were regularly scored. No fungicide treatments were applied on these trees during the entire period of the study.

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

Disease assessment in the apple orchard

Disease severity of AB was assessed every one to two weeks over 2016 to 2020 by scoring entire trees based on a scale from 0 to 9 (0 = no disease symptoms) as described by Schärer et al. (2019) and listed in Figure 2C. These scores were used to calculate damage (in percent) based on the McKinney Index (I) (McKinney 1923), where I (%) = [sum (class frequency × score of rating class)]/[(total number of ratings) × (maximum grade)] × 100.

Monitoring spore dispersal in an apple orchard

To monitor the temporal resolution of the spore dispersal, a Mycotrap and one multi-vial cyclone sampler (only in 2019) were placed in the previous year's disease hotspot of the orchard, within an 'Otava' apple tree row (Figure 2C, D). A second Mycotrap was placed within the tree crown of an 'Otava' tree above the first Mycotrap. Potted two-year old apple trees were used as bait plants and were placed for periods of five to fourteen days in the orchard and subsequently incubated under rain protection to assess disease development (Figure 2F). Each series of bait plants consisted of three 'Topaz', three 'Gala' and three 'Kiku' apple trees, and of five 'Topaz' apple trees in 2019 and 2020, respectively.

To observe the spatial resolution of the spore dispersal, seven rotating-arm spore traps were mounted at different locations within the apple orchard at one of the lowest branches of the apple trees close to the stem. Moreover, one rotating-arm spore trap was installed at the same height, but about 50 m outside the orchard (Figure 2C). Spore trap samples were analyzed by the above described qPCR method.

Monitoring spore dispersal from an infected leaf litter under field conditions

In 2019, a Mycotrap was placed on a leaf litter deposit at the Competence center for fruit crops at the Lake of Constance (KOB) and sampled in consecutive series of fourteen days (sampling on one spot without rotation) from February to June onto plastic film. In 2020, a Mycotrap was placed on a leaf litter deposit at the Research Institute of Organic Agriculture (FiBL, Frick, Switzerland) and sampled from March to June onto Vaseline coated plastic film. Spore numbers in these samples were quantified by qPCR.

Quantification of D. coronariae conidia in rain water

From 24th to 25th of September 2020 rain water was collected by placing 12 Schott-bottles with a funnel within the orchard in Rickenbach for 16 hours. It was the first rainy period after seven consecutive days without precipitation. Amount of rain registered for that period was 14 mm per m^2, approximately 100 ml of rain water 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 in pieces of approximately 3x3 mm and stored at -20 °C.

DNA from filter pieces was extracted using the ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research, Irvine, USA). Skim milk was added to the bashing bead buffer at a final concentration of 2% to prevent adhesion of free DNA to the filter membrane ((Liang and Keeley 2013). Spores were quantified by qPCR based on a standard curve generated with known amounts of *Dc* conidia pipetted onto filter pieces.

Detection of D. coronariae in bark, bud, leaf litter, and fruit mummy samples

On the 28th of February 2020 we collected bark, bud and fruit samples in the Rickenbach orchard. Bark samples were collected as described by Arrigoni et al. (2018). Briefly, bark curls of 20 mm length, 5 mm width and 1 mm thickness were collected using a flame-sterilized scalpel. Each sample consisted of a pool of 30 bark curls of the upper trunk and lower branches of one tree. For bud samples, ten terminal buds per tree were collected. We further searched for fruit mummies hanging in the trees. From those several pieces of the skin (about two to five mm in thickness) were pooled. Samples were lyophilized and processed and ground in a mixer mill MM 200 (Retsch GmbH) at full speed of 30 s^{-1} for 30 s. A portion of 100 mg of powder or as much as available was used for DNA extraction using the NucleoSpin™ DNA Stool Kit (Macherey-Nagel, Oensingen, Switzerland). We only found one sample of overwintered leaves still hanging in a tree. This sample was processed the same way, but without prior lyophilization. Samples were assessed for presence of *Dc* DNA by the above described qPCR assay.

Investigation of fruit infections

Dc infected apples were collected in September 2020 and stored in the fridge until March 2021. By then they had developed acervuli. The acervuli were examined with a stereo microscope M205C (Leica Microsystems Switzerland, Heerbrugg, Switzerland). Sections of apple tissue containing acervuli were stained with cotton blue in lactic acid, and were examined with a Leica DM2000 LED microscope (Leica Microsystems Switzerland, Heerbrugg, Switzerland). Photographs were taken with a Jenoptic Gryphax Subra camera (Jenoptic AG, Jena, Germany).

Results

Development of apple blotch disease in two Central European Apple Orchards

To better understand the epidemics of AB in Central Europe, the disease progression over the season was monitored in two extensively managed apple orchards: one in Germany (KOB field site) consisting of the cultivar 'Topaz' and one in Switzerland (Rickenbach field site) consisting of different cultivars (including two rows of 'Topaz'). We identified the cultivars 'Otava', 'Rajka', 'Florina', and 'Topaz' as highly susceptible (Figure 2B, C). In contrast, 'Bohnapfel', 'Blauacher', 'Tobiässler', and 'Schneider' revealed to be relatively tolerant as represented by low scoring scales at the end of the season (Figure 2C). Only one cultivar, 'Rubinola', showed no to very few AB infections, despite a generally very high disease pressure in the orchard (Figure 2B, C).

The disease progression on 'Topaz' over the season is shown in Figure 2A as percent damage (McKinney-Index). After the appearance of first symptoms with clear evidence for *Dc* (i.e. visible formation of acervuli, yellowing of the leaf), which occurred at the latest in August in both orchards and all years, disease progression was generally characterized by a steady increase of damage. However, large differences in disease severity were observed between years. At KOB, for instance, leaf damage through AB was higher in 2017 compared to other years (Figure 2A). 2017 was the year with most days with rain from June to October, i.e. 78 days compared to 36 to 57 days in the other years (Supplementary Figure 1). At both sites, 2018 was characterized by an exceptionally hot and dry summer (only 36 and 33 days with rain from June to end of September at KOB and Rickenbach, respectively) (Supplementary Figure 1, 2) and lower levels of AB (Figure

2A). At Rickenbach, disease levels were highest in 2019 and 2020, which was not observed at KOB (Figure 2A).

While these results provided valuable information on disease and damage progression and cultivar-specific susceptibilities, the onset of disease and the source of primary infections remained obscure. Therefore, we decided to investigate spore dispersal of *D. coronariae* in combination with symptom development early in the season.

New qPCR allows quantification of D. coronariae conidia

To quantify *Dc* spores in spore trap samples, we developed a new TaqMan qPCR method with the primers and probes Dc_09 (Table 1). Different primers and hydrolysis probes targeting the ITS gene of *Dc* were tested, and among them, primers and probe Dc_09 performed best (E= 94.2 %, R^2= 0.995) also compared to the published primer Mc_ITS (Oberhänsli et al. 2014)(data not shown). Specificity tests for primer Dc_09 revealed a good amplification on DNA of three Swiss, one Japanese and one Korean isolate of *Dc* (Figure 1A). In contrast, no amplification was observed on DNA of other species within the genus *Diplocarpon* (*Diplocarpon mespili*, *Diplocarpon earlianum*, *Diplocarpon rosae)* nor on DNA of the causal agents of other Marssonina diseases (*Gnomonia leptostyla (alternate state: Marssonina juglandis)* and *Drepanopeziza tremulae (alternate state: Marssonina brunnea*) (Figure 1A, Supplementary Table 2). Moreover, Dc_09 did not amplify DNA from healthy apple leaves or other common apple pathogens such as *Venturia inaequalis*, *Monilia fructigena* or *Neofabraea alba* (Supplementary Table 2).

To quantify *Dc* conidia in spore trap samples, we generated standard curves by adding known amounts of conidia to spore trap samples, which allowed the direct conversion of Cq values into spores per sample. The amplification of the qPCR for conidial counts on 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) (average of seven qPCR runs) (Figure 1B). The qPCR assay allowed the consistent detection of as little as ten conidia; however, at three conidia per sample, the amplification became unreliable. The limit of quantification is therefore considered to lie between three and ten conidia.

We further tested whether our primer pair Dc_09 could be used with a DNA intercalating fluorophore (SYBR Green) instead of the use of a hydrolysis probe. The SYBR Green based assay revealed similar sensitivity and specificity as the TaqMan assay (Supplementary Figure 3). However, for our field experiments we used the TaqMan qPCR, since it allowed multiplexing of the *Dc* specific qPCR with the APA9 specific qPCR (plasmid standard), which we used to normalize the *Dc* quantification data with the DNA extraction efficiency.

Evaluation of spore traps under experimental conditions

To identify spore traps suitable to capture *Dc* conidia, we tested different spore traps under controlled conditions. When compared in a climate chamber for their capability to capture spores from a conidial suspension of *Dc* sprayed into the air (Supplementary Methods) all tested spore traps (Mycotrap, High throughput 'jet' spore sampler, Multi-vial cyclone sampler, rotating-arm spore trap) with different spore trapping surfaces (e.g. plastic film and Vaseline coated plastic film) were able to capture spores mostly in the range of thousands to tens of thousands (Supplementary Results). Overall the Mycotrap performed best in these experiments (Supplementary Results). Since catching spores from a conidia suspension sprayed into the air is rather artificial, the spore traps were further tested for their performance to catch conidia that are splash dispersed from an overwintered leaf litter deposit that was artificially irrigated. All spore traps were able to capture spores released by the leaf litter deposit, but much lower numbers than from spore suspensions sprayed into the air (Table 2 and Supplementary Results). Nevertheless, all bait plants placed next to the spore traps developed AB symptoms (Table 2).

Monitoring Dc spore dispersal in the field

Based on the laboratory experiments, we decided to use the Mycotrap and the Multi-vial cyclone sampler to monitor temporal spore dispersal, and rotating-arm spore traps to monitor spatial spore dispersal in the Rickenbach orchard. Since no power connection was available at our field site, we did not include the High throughput 'jet' spore sampler in the field. An overview of spore traps used in the field in 2019 and 2020 is given in Supplementary Table 3 and 4, respectively.

In 2017 and 2018, first clearly visible symptoms on 'Otava' trees appeared by mid-June or beginning of July, respectively. Therefore, the first infections were expected to occur not before the end of May, considering an incubation period of about three weeks. To monitor the early infection period, a Mycotrap and a Multi-vial cyclone sampler were placed on the ground within an 'Otava' tree row from the beginning of May until end of July 2019, capturing spores on a daily basis. In addition, series of bait plants were placed next to the spore traps, replaced periodically and incubated under rain shelter, to be able to correlate the presence of spores to actual infections.

To our surprise, in 2019 very high levels of spores were already detected at the beginning of the experiment during the first half of May (Figure 3A). After that, spore levels generally decreased, apart from a peak around the 4th of June. In May, leaves with suspicious spots were collected and tested for *Dc* by qPCR, revealing the first AB positive leaf on the 17th of May. First unambiguous symptoms were discovered three weeks later on the 7th of June (Figure 3A). A next spore peak was detected on the 24th of June after a rain period. At that time, 'Otava' trees already showed strong leaf yellowing, indicating emergence of secondary inoculum. In July, high spore levels in the air were recorded on most days. All the data shown in Figure 3 are based on captures by the Mycotrap on the ground. The Multi-vial cyclone sampler did not catch significant amounts of *Dc* spores, i.e less than five spores per day on days where the Mycotrap was catching up to 848 spores (data not shown). Therefore, we excluded the cyclone sampler from the field trial on the 7th of June 2019. From the 7th of June to the 5th of July a second Mycotrap was placed in the tree crown of an 'Otava' apple tree next to the Mycotrap on the ground (Supplementary Figure 4). This trap detected more spores than the Mycotrap on the ground except for the 24th and 25th of June.

First apparent symptoms on bait plants were found from the 17th to the 22nd of May (Figure 3A). For all following bait plant series (i.e. from the 22nd of May on), at least one potted tree showed typical leaf yellowing symptoms, except for the period from the 3rd to the 7th of June (Figure 3A). The heaviest leaf yellowing and leaf drop were observed for the bait plants in the period from the 7th to the 13th of June, followed by the period from the 13th to the 19th of June.

In 2020, we started the field experiment already on the 28th of February, to ensure not to miss the first spore flight (Figure 4). We also included one Mycotrap on the ground and one in the tree crown for the entire duration, since we hypothesized that primary spores originating from leaf litter on the ground might be detected rather by a trap on the ground, while a trap in the tree crown would detect high numbers of spores during the epidemic**.**

Very few spores were captured already in March by the lower trap before bud break (Figure 4). However, the first large spore peak was observed on the 5th of May in the lower trap at the end of a rain period that had followed on an extremely dry April (Figure 4, Supplementary Figure 2). The bait plants of this period were also the first ones to develop AB symptoms. The first *Dc* positive leaf based on a PCR test, was found in the orchard on the 8th of May. Though, leaves with clear symptoms, were found on the 22nd of May, which means about two weeks earlier than in 2019. We could confirm by microscopy that by that day the fungus had started to produce conidia on the diseased leaves. A second major spore peak was detected on the 5th of June, this time by the trap in the tree crown. Like in 2019, spores were detected in the air almost every day by both traps from middle of June until end of July also in 2020 (Figure 3, 4). Nevertheless, spore levels varied strongly, with pronounced peaks after wet periods. In Mycotrap samples of early June (lower trap: 5.6.-19.6., upper trap: 31.5.-9.6.) we were facing technical problems leading to low DNA extraction efficiency. While in cases of a Cq output by the qPCR we could take this into account by normalization with the internal plasmid standard, in cases of no signal by the qPCR, we cannot conclude whether there were indeed no spores or whether we were just not able to detect them. Thus, on some days in the first half of June *Dc* spores might have been present in the air, although the graphs in Figure 3B and 4 do not show a spore peak.

In 2020, all bait plants after the 22nd of May developed AB symptoms. While bait plants from the 22nd of May to the 5th of June exhibited about 50 to 80 % symptomatic leaves, on bait plants after the 5th of June over 90 % of leaves became diseased (Supplementary Figure 5).

Beside the dispersal of *Dc* spores via the air, splashes of rainwater might be an important factor for spreading the disease within a tree. Therefore, we collected rainwater below highly infected trees ('Otava', 'Topaz'), below symptomless trees ('Rubinola'), between the rows under the open sky and outside the orchard during a rain period in September 2020. We detected several hundreds of spores per ml rain water below 'Otava' trees, fewer spores between rows, and almost no spores below non-infected 'Rubinola' trees or outside the orchard (Supplementary Figure 6).

D. coronariae spores mainly close to infected trees

Besides the temporal pattern of *Dc* spore dispersal, we were also interested in the spatial distribution of spores within and outside the orchard. Therefore, we placed seven rotating-arm spore traps within the orchard and one outside the orchard as indicated in Figure 2C. In 2019, the traps were installed only between the 22nd of Mai and the 5th of June. However, in 2020, we covered the entire sampling period from beginning of March to end of July. Rotating-arm spore traps detected the first spores only after the 22nd of Mai 2020, thus missing the first large peak on the 5th of Mai. Spore numbers detected in rotating-arm spore traps were generally much smaller than those quantified in samples from Mycotraps (Table 3, Figure 3, 4). Highest spore numbers were generally detected in traps installed on 'Otava' trees, which exhibited very strong AB symptoms, while almost no spores were detected in traps on 'Rubinola' trees. These trees were not infected by *Dc*, although they were only a few rows apart from highly diseased trees. Nevertheless, leaf yellowing and premature leaf fall was also observed on Rubinola trees, but symptoms looked distinctly different from apple blotch and absence of *Dc* infections in leave samples was confirmed by qPCR (data not shown). Furthermore, no spores were detected in the trap about 50 m outside the orchard. An exception for these observations was the period of the 22nd of May until the third of June 2019, where spores were detected under 'Rubinola' trees and outside the orchard.

Origin of primary infections

To understand *Dc* spore dispersal, it is essential to know all sources of primary inoculum. We first studied spore dispersal from a deposit of overwintered *Dc* infected leaf litter at the Competence center for fruit crops at the Lake of Constance (KOB) and the Research Institute of Organic Agriculture (FiBL) in 2019 and 2020, respectively. In 2019, the first spores were released from the leaf litter deposit in the second half of April (Table 4). In 2020, however, few spores were already detected one month earlier. In that year no spores were detected in April, which was characterized by extraordinarily dry weather (Supplementary Figure 1 and 2), but the spores were again released from the leaf litter deposit in May (Table 4). In both years, spores were also detected in the second half of June. Generally, spore numbers detected in these experiments were very low and thus not reliably quantifiable (LOQ = 10 spores).

Since in earlier studies we found no effect of sanitary measures removing leaf litter from the orchard on AB disease development (Buchleither 2019), we hypothesized that other inoculum sources might be relevant for disease outbreak, too. Therefore, we collected bark and bud samples from 'Otava' and 'Rubinola' trees (Rickenbach site) and 'Topaz' trees (KOB site) after winter and tested them for presence of *Dc* by qPCR. While all 'Rubinola' samples, which derived from trees without AB symptoms the year before, were negative for *Dc*, the majority of samples from 'Topaz' and 'Otava' trees were tested positive (Table 5). Bark samples were only slightly positive (high Cq values), but bud samples exhibited relatively high levels of *Dc* DNA (lowest Cq value at 25.6 which corresponds approximately to the DNA of 10^4 conidia)(Table 5).

Furthermore, *Dc* infected fruit mummies might represent sources for primary inoculum. We searched for fruit mummies hanging in the trees after winter. In spring 2020, four out of five fruit mummies, which were collected from trees with strong AB infestation in 2019, were tested slightly positive (Cq between 33.65 and 40.38), while fruit mummies from trees without AB symptoms the previous year were negative (Figure 5C, Supplementary Table 5). For comparison a leaf sample still hanging in a susceptible 'Florina' tree after winter was strongly *Dc* positive (Cq, 25.83).

Finally, to assess whether the identified DNA on bark, buds and fruit mummies originates from viable and infectious fungal tissues, we tried to infect apple leaves in the laboratory by applying wetted pieces of bark, buds, fruit mummies and leaves. However, our attempts did not result in successful infections to this point of time (data not shown). Nevertheless, we could show that *Dc* infected fruits can serve as inoculum for leaf infections after six months of storage. In fall 2020, we detected *Dc* infected 'Otava' fruits in the Rickenbach orchard (Figure 5A). Figure 5A shows AB symptoms on fruits in fall (Figure 5A) and after storage of fruits in the fridge for six months (5B). In spring 2021, conidia formed on these fruits (Figure 5D-I), and those conidia were able to infect leaves of apple seedlings in the laboratory (Supplementary Figure 7).

Discussion

Development of a D. coronariae specific qPCR

To date, *Dc* spores in spore trap samples have been quantified by microscopy (Kim et al. 2019). However, this is labor intensive, and in addition it is often difficult to securely identify a few *Dc* spores in field samples comprising a high diversity of spores and other particles such as pollen and dust. The herein developed qPCR method provides a very sensitive method allowing the quantification of as few as ten conidia per sample, which is in a similar range as methods developed for other fungi (Calderon et al. 2002; Dvořák et al. 2015; Luchi et al. 2013). The qPCR method further allows a diagnosis of AB prior to the development of unambiguous visual symptoms. This is especially useful for detecting very early infections when small necrotic lesions appear that are indistinguishable from symptoms of other origins. In summary, we established a new qPCR method, which allowed to quantify *Dc* spores in spore trap samples, to detect initial infections in the field, and enabled the investigation of *Dc* overwintering in various apple organs.

Nevertheless, qPCR methods also have some disadvantages such as a delay of results due to several processing steps. Moreover, it only allows a statement about the presence of DNA, but not on the type of organ (e.g. mycelium, ascospore, conidia, spermatia) or its viability and infectious potential. Therefore, visual assessments and infection experiments will be needed to understand the form in which *Dc* is present on bark, bud and fruit samples and the role of these organs in the fungal life cycle.

Comparison of spore traps

To optimize the chance of catching *Dc* spores in the field, we assessed different spore traps for their spore trapping capacity, but also for their reliability, manageability, and suitability for a field application. All tested spore traps were able to capture *Dc* spores from artificial conidia aerosol, created by a sprayed spore suspension, and also from an artificially irrigated leaf litter deposit. The latter further showed that conidia are discharged from the leaf litter into the air by intervals of heavy rainfall and the experimental setup allowed to compare different spore traps under close to natural conditions. Automatic volumetric spore traps are often regarded as the standard for sampling fungal spores in the field and have been used extensively to study the temporal variation in fungal spore concentrations in many different crops (McCartney et al. 1997). However, for qPCR analysis of spore trap samples, the multi-vial cyclone sampler, collecting spores directly into plastic vials, would have been more convenient. Unfortunately, this trap detected the least spores from the irrigated leaf litter deposit (Table 2) and in the field, it caught substantially less spores than the Mycotrap and was therefore excluded from further experiments.

Our self-constructed battery-powered low-cost rotating-arm spore traps (similar to Quesada et al. (2018)) represented a reasonable compromise between the protecting shield size, the length of the arms, the weight, the applicability in the field and the energy consumption as well as the feasibility and financial investment. Rotating-arm spore traps could be built for less than twenty USD per spore trap, making it affordable to have a sufficiently large number of spore traps to refine the spatial resolution of spore dispersal. A disadvantage of our rotating-arm spore traps was their low weather-resistance leading to technical issues, e.g. power failure due to rust.

The rotating-arm spore traps performed only slightly worse than the Mycotrap in the laboratory, but they captured substantially less spores in the field. Thus, the rotating-arm sporetraps are rather useful during the late phase of the epidemic, but not sensitive enough to detect low amounts of primary spores.

April and Mai: primary infections and the start of the epidemic

To identify the time-point of primary AB infections, we combined data on airborne spore catches on symptom development both in the field and on consecutive series of potted apple trees as bait plants brought into and back from field to the growth chamber every second week. In the first three study years (2016-2018), clear AB symptoms were observed the earliest in June. However, testing leaves with ambiguous necrotic spots by qPCR in 2019 and 2020 revealed that first leaves of highly susceptible varieties were already infected in May (17^{th} of May 2019 and 8^{th} of Mai 2020). Most probably these infections were caused by spores released in late April or early Mai. Indeed, in both years, first significant spore peaks were detected in the orchard in early May. In 2019, spore traps were only installed on the 9^{th} of Mai 2019. Thus, it is possible and regarding the weather data even likely that spores were already present one or two weeks earlier. In 2020, the experiment was started at the end of February, and still the first large spore peak was detected on the 5^{th} of May by the Mycotrap on the ground. We believe that these spores were the ones causing the first infections, since the first bait plant series in the field, which developed symptoms was the one from 24^{th} of April to 8^{th} of May 2020.

Moreover, spore traps above leaf litter deposits were able to catch *Dc* spores at the end of April and beginning of May in both study years. Nevertheless, very few spores were detected above the leaf litter deposit and in the field already in March. Since these spores were detected before bud break, we believe that they were not relevant for primary leaf infections. Our qPCR method does not allow to distinguish between conidia and ascospores. Since *Dc* apothecia have not yet been observed in Europe (Wöhner and Emeriewen 2019), we assume that we captured conidia. Nevertheless, the relevance of ascospores as primary inoculum in Europe should be subject for further research. A recent population genetic study suggested a mixed sexual and asexual reproduction of *Dc* in Europe, although *Dc* populations in Europe are genetically homogenous and clonal, and dominated by a few multi-locus genotypes (Oberhänsli et al. 2021).

The first spore peaks were observed at the end of or shortly after rainy periods, whereas no spores were detected during dry periods before rain events, e.g. in April 2020. Thus, rain might be required for the release of *Dc* conidia from leaf litter and acervuli might need some wetting time before releasing the conidia.

Based on the presented lines of evidence, it can be assumed that primary infections with *Dc* occur in Central Europe in late April or early May after periods of rain, which is earlier than assumed to date (Sutton et al. 2014). Moreover, our spore catches in March indicate that *Dc* spore discharge can even occur before April and, depending on weather conditions and phenological stage of the host, infections might be possible. Knowing the exact timing of primary infection can be very valuable for successful prevention of AB epidemics. While protection strategies against AB in Europe are currently focused on the summer months (Hinrichs-Berger 2015), prevention of primary infections in spring might be an important additional step to optimize the management strategy in the future.

Leaf litter and other sources of primary inoculum

In addition to the timing of the primary infections, we were also interested in the origin of the primary inoculum. Several studies report that conidia inside acervuli are overwintering on fallen infected leaves (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). The conidia are released in a water film on the leaf surface and are then dispersed by rain splashes onto apple leaves (Hinrichs-Berger 2015). Our experiment with an artificially irrigated leaf litter deposit nicely simulated this process. It demonstrated, that irrigation of an overwintered leaf litter results in *Dc* conidia in the air, as confirmed by spore traps, and these conidia can cause infections on bait plants nearby. This confirms that infected leaf litter can serve as source of primary infections. Leaf litter on the ground as source of primary inoculum could also explain the observation that the disease starts in the lower tree crown and then progresses upwards (Sharma and Gautam 1997). Furthermore, it is in accordance with our finding that in May spores were mainly found in the lower Mycotrap and not in the tree crown.

Nevertheless, small amounts of *Dc* DNA were also detectable on fruit mummies still hanging in the trees by the end of February and acervuli with infectious conidia were produced on *Dc* infected fruits after overwintering in the fridge. Moreover, considerable amounts of *Dc* DNA were detected on buds, and small amounts of *Dc* DNA was found on bark of trees with previous *Dc* infection. All these organs are reported sites for overwintering of other apple pathogens, for instance *Monilia* spp. on fruit mummies, *Podosphaera leucotricha* in buds or *Neofabraea* spp. on bark (Sutton et al. 2014). To date, it is unclear in which form (e.g. mycelium, spores) *Dc* is present on bark and buds and whether the fungal structures are infectious. The fact that we have not yet succeed in producing leaf infections from bud, bark or fruit mummy tissues may have been for technical reasons, and the role of these apple organs in the disease cycle of *Dc* definitely requires further investigation. It was also speculated that *Dc* present on buds and wood in propagation material exchanged between nurseries across Europe, could be one of the reasons for the rapid spread of this disease into many European apple production areas within just one decade after its first detection in Northern Italy (Oberhänsli et al. 2021). In any case, our finding that conidia produced on fruits after six months of storage in the fridge are able to infect apple leaves provide a first indication that fruit-derived conidia might, similarly to conidia produced on leaf litter, represent sources of primary inoculum at least on a local scale. Moreover, our description of fruit infections is one of the first reports of its kind and adds to our very limited knowledge on the role of fruit infections with *Dc* (Wöhner and Emeriewen 2019).

June and July: secondary infections and the exponential phase

By the end of May (2020) or beginning of June (2019) first leaves with sporulating *Dc* were observed, presumably initiating the secondary phase of infection. In contrast to the first major spore peak in May 2020, the second major spore peak (5th of June) was not detected by the Mycotrap on the ground, but by the one situated in the tree crown. Based on these results, we hypothesize that spores in early May originated from overwintered fallen leaves, causing higher spore counts in the lower trap, while in June the first spores were discharged from newly infected leaves in the tree crown, leading to higher spore counts in the upper trap. In line with this, rotating-arm spore traps, which were also installed in the tree crown, only started to capture spores from the end of May onwards.

From June on, damage caused by AB steadily increased in the orchard (Figure 2). From middle of June until the end of the experiment high numbers of spores could be detected irrespective of rain periods, which is in contrast to our observation on early spore peaks, which especially in 2020 occurred always at the end of rain periods and this is also in contrast to observations by Kim et al. (2019). They investigated spore *Dc* spore dispersal from June to October 2013 and 2015 in Korea and reported more spores during and two days after rain periods compared to dry periods (Kim et al. 2019). In that study disease incidence and total spore counts were much lower than in our field site, which might explain parts of the difference. Based on our results, one could hypothesize that primary spores need water to ripen and discharge, while secondary spores are released also on dry days. Lab experiments indicate that after landing on a susceptible leaf, *Dc* conidia are able to survive for several days under dry conditions, still causing successful infections once the leaf becomes wet (our unpublished data). This suggests that after beginning of secondary spore formation every period of leaf wetness represents a risk for infections.

The development of AB is strongly affected by environmental factors (Li et al. 2011). Rainfall and the following relative humidity are positively correlated with the disease severity in the summer months (Rather et al. 2017b; Sastrahidayat and Nirwanto 2016; Sharma and Sharma 2005). The minimum leaf wetness period for infection under controlled conditions is 8 h at 15 °C and decreases to 4 h for temperatures between 20 and 25 °C (Sharma et al. 2009). Moreover, the incubation period can greatly vary depending on the weather conditions. At a temperature range of 15-25 °C and seven 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 one to four weeks after they were returned from the orchard, with shorter incubation period for bait plants placed in the orchard later in the season (June, July) compared to the beginning of the season (May). In 2020, first necrotic spots as well as clear symptoms were observed earlier and within a shorter interval than in 2019. This faster disease progression in late spring 2020, was probably due to higher May temperatures compared to 2019.

Spatial spore distribution: concentration around highly infected trees

Rotating-arm spore traps distributed in the orchard, revealed high spore concentrations in diseased trees, but no or very few spores in trees without symptoms, only a few rows apart. This could indicate that the spores are not dispersed over large distances. However, the generally small spore counts in rotating-arm traps compared to the Mycotrap also shows that rotating-arm traps were much less efficient in capturing spores in the field. Thus, low amounts of spores might still be transported by wind currents over larger distances. Beside wind dispersal, dispersal by rain water might substantially contribute to spread of *Dc* within the tree crown, as indicated by the very high spore counts in rain water below highly symptomatic trees (Supplementary Figure 6).

Conclusions

Based on our results we hypothesize the following disease cycle for *Dc* in Central Europe (Figure 6). The fungus mainly overwinters in infected fallen leaves. However, bark and especially buds and fruit mummies might be alternative sources of primary inoculum, since we detected *Dc* DNA on these organs after winter in previously *Dc* infected trees. Few spores might be released already in March, but primary infections start in late April or early May, depending on the weather conditions. In our field sites secondary spores developed in late May or early June, thus, within three to four weeks after primary infections. While in May, spore peaks were mainly found at the end or after periods of rain, spore load in the air was generally high from middle of June to end of July. Thus, every wet period in summer represents a risk for infections. The disease spreads by wind and rainstorms over larger distances, for the spread of *Dc* within one tree or two neighboring trees, rain splashes might be more relevant.

In summary, we developed a sensitive and specific method to detect *Dc* spores in the air, which can be combined with the quantification of other apple pathogens in spore trap samples in the future. Our data significantly adds to our understanding of AB disease, especially on early spore dispersal and infections. This can help to improve disease forecast models for AB and direct disease prevention in the field. Regarding the disease management, more research is needed to test whether preventing the primary infections could reduce the disease development over the season and thus fungicide treatments in summer. Finally, presence of *Dc* DNA on bark, buds, and fruit mummies indicates that the role of these organs for overwintering and for the anthropogenic dispersal of the pathogen on propagation material should be investigated in more detail.

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Table 1. Primers used in the present study.

Primer name	Label	Sequence (5' to 3')	Mt (°C)	Amplicon size (bp)	Reference
Dc primer 09	Dc_09_F	GCG TAT ACC ACC CGT GCC TA	60.4		This study
	Dc_09_R	CTC AGA CAT CAC GTT ATT CAC ACA A	59.4	129	
	Dc_09_P	FAM-CCT ACC TCT GTT GCT TTG GCG A-BHQ1	70.0		
ACMV primer	ACMV_F	CCA CAG ACA AGA TCC ACT CTC C	59.7		Mosimann et al. (2017)
	ACMV_R	CAC TCT ACT CAG GTT CCA ATC AAA G	57.9	86	
	ACMV_P	ROX-ACA GAC AAT TCA AGA AGC GAG CCA TCC G-BHQ2	62.6		
General ITS Primer	ITS1F	CTTGGTCATTTAGAGGAAGTAA	53.2	Dependent on target species	(Gardes and Bruns 1993; White et al. 1990)
	ITS2R	GCTGCGTTCTTCATCGATG	57.2		

F denotes forward primer, R reverse primer and P fluorogenic hydrolysis probe. FAM = 6-Carboxyfluorescein, ROX = Carboxy-X-rhodamine, BHQ = black whole quencher. Mt = melt (annealing) temperature.

Table 2. Assessment of the spore traps with a *D. coronariae* infected leaf litter deposit artificially irrigated.

Spore trap	**Type of spore trap**	**Producer**	**Spore trapping surface**	**Air sam-pling rate**	**Powered by**	**C_q TaqMan**	**C_q APA TaqMan**	**Norm. C_q TaqMan**	**Number of conidia**	**Apple blotch symptoms**
Mycotrap	Volumetric impaction spore trap	Self-construction	Vaseline coated plastic film (Rotation of the drum 40 mm/d, intake slit 15 x 3 mm)	22 l/min	12 V/100 Ah car battery	35.99	17.29	36.48	31	-
Multi-vial cyclone sampler	Volumetric cyclone-based spore trap	Burkard Manufacturing Co Ltd, Hertfordshire, UK)	Eight 1.5 ml plastic vial	16.5 l/min	12 V/ 100 Ah car battery	39.85	16.26	41.77	< 10	-
Rotating-arm spore trap	Volumetric impaction spore trap	Self-construction, details in Supplementary Materials	Vaseline coated plastic film (25 x 40) mm	24 l/min (*)	1.5 V battery (Type D LR20)	35.62	17.36	35.96	44	-
High throughput 'jet' spore sampler	Volumetric impaction spore trap	Burkard Manufacturing Co Ltd, Hertfordshire, UK)	Vaseline coated plastic film, sampling chamber Ø13 cm	100 l/min	main power (220 V)	35.10	17.37	35.42	64	-
Bait plants			-					-	-	12 /12

The spore traps were installed around resp. above a deposit of D. coronariae infected leaves (dry weight 150 g) in a climate chamber. The leaf litter deposit was artificially irrigated with a shower head for four cycles of 2h rain (30 L/h) and 2h no rain. The spore traps were sampling for the whole duration of the trial (17 h). Spores were quantified by TaqMan qPCR using Mc Primer_09.
The air sampling rate was measured empirically for the Mycotrap and the high throughput 'jet' spore sampler, calculated for the rotating-arm spore trap, and taken from the manufacturer's specifications for the multi-vial cyclone sampler.
(*) Air sampling rate per rod

Table 3. Spore numbers detected in rotating-arm spore traps

cultivar	Trap Nr.	dates (2019)				dates (2020)						
		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	2412	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

Trap Nr. 2 was installed below a 'Schneider' tree, which is standing at the edge of an 'Otava' row. The location of the traps within the orchard is depicted in Figure 2C.

For 2019 the full sampling period is shown. In 2020 Rotorod traps were sampling from the 28th of February until the 31st of July. Since first spores were only detected after the 22nd of May, data for early series are not shown.

Table 4. Number of *D. coronariae* spores detected above a leaf litter deposit

Month	KOB (2019)	Frick (2020)
2nd half of February	0	-
1st half of March	0	0
2nd half of March	0	14
1st half of April	0	0
2nd half of April	<10	0
1st half of May	<10	<10
2nd half of May	0	<10
1st half of June	0	0
2nd half of June	<10	<10

Spore trap samples from a Mycotrap at KOB in Ravensburg-Bavendorf (Germany) and at FiBL in Frick (Switzerland) above a *D. coronaria*e infected leaf litter deposit. TaqMan qPCR using the primers Dc_09.
0 = no spores detected
< 10 = *D. coronariae* DNA detected, but below limit of quantification

Table 5. After winter *D. coronariae* can be detected on bark and in buds of apple trees that had apple blotch disease in the previous year (Otava and Topaz trees).

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

Samples were collected in the Rickenbach orchard on the 28th of February and on the 13th of March 2020 and at KOB on the 17th of March 2020.

ND = not detected

Cq values depicted result from a TaqMan qPCR with primer Dc_09. APA9 plasmid was used to normalize for DNA extraction efficiency.

Figure captions

Figure 1. *Diplocarpon coronariae* specific qPCR. **A)** Specificity test of Primer Dc_09. A PCR was performed with DNA of different *D. coronariae* strains, related *Diplocarpon* species (*D. mespili*, *D. rosae*, *D. earlianum*), and with DNA of further pathogens causing Marssonina diseases (*Drepanopeziza tremulae*, *Gnomonia leptostyla*). 2 % TAE agaraose gel stained with ROTI® GelStainRed, marker: peqGOLD O'range 100bp 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 the same way as from the Mycotrap and rotating arm spore trap samples, i.e. using ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit. Samples were spiked with 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 at 89.1 %.

Figure 2. Apple blotch (AB) disease development in two apple orchards and location of spore traps. A) Topaz trees in two orchards (Rickenbach, Switzerland and KOB, Germany) were regularly scored for AB disease development from June to October for five consecutive years according to a scale from zero to nine depicted in (C) (Rickenbach, 36 trees; KOB, 120 trees). B) AB disease development on Topaz (36 trees), Rubinola (35 trees), Otava (33 trees) in the Rickenbach orchard in 2019 and 2020. A), B) Disease severity was calculated as percent damage according to the McKinney Index (I) (McKinney 1923). C) Plan of the Rickenbach orchard and disease assessment by middle of September 2019 and 2020. The orchard is planted with rows of ten different cultivars. Each dot represents one tree. The placement of Mycotrap and rotating-arm spore traps

is indicated by the letters M and R, respectively. One rotating-arm trap was installed about 50 m outside the orchard (R8). D, E, F) Pictures of spore traps used in this study. D) Mycotrap, E) rotating-arm spore trap, F) potted bait plants. G) Leaf with typical AB symptoms.

Figure 3. *Diplocarpon coronariae* spore dispersal in 2019 (left) and 2020 (right). Top: Daily spore numbers of *D. coronariae* (*Dc*) captured by a Mycotrap, which was placed within an 'Otava' apple tree row. The number of spores was calculated based on normalized Cq values from TaqMan qPCR with primer Dc_09 using a standard curve with known amounts of conidia. The green and the yellow lines indicate the periods where a bait plant set was standing in the orchard next to the Mycotrap and whether they developed AB symptoms (yellow) or not (green). The two blue circles indicate days where first leaves with ambiguous AB symptoms have been tested positive for *Dc*. The red circles indicate first clear AB symptoms in the orchard. **Bottom:** weather data (Agrometeo, location "Liebensberg") including temperature (°C), relative humidity (%), precipitation (mm), and leaf wetness (light blue, <8h; dark blue, >8h)

Figure 4. ***Diplocarpon coronariae* spore dispersal on the ground and in the tree crown in 2020.** Daily spore numbers of *D. coronariae* captured by Mycotraps placed within an 'Otava' apple tree row on the ground and within the tree crown. The number of spores was calculated based on normalized Cq values from TaqMan qPCR with primer Dc_09 using a standard curve with known amounts of conidia. The green and the yellow lines indicate the periods where a bait plant set was standing in the orchard next to the Mycotrap and whether they developed apple blotch

symptoms (yellow) or not (green). Estimated duration of leaf wetness is indicated as light blue (<8h) and dark blue (>8h) bands.

Figure 5. Fruit infections with *Diplocarpon coronariae (Dc)*. A) 'Otava' apple with apple blotch symptoms hanging in a tree in September 2020 (Rickenbach, Zurich, Switzerland). B) *Dc* infected 'Otava' apple after 6 months of storage in the fridge. C. 'Florina' fruit mummy found hanging in a tree in February 2020, and tested positive for *Dc* by TaqMan qPCR. D-I) Acervuli and conidia developed on *Dc* infected 'Otava' apple collected in the field in September 2019 and stored in the fridge for six months. F-G) Longitudinal section through an acervulus. H) Conidia released from an acervulus. I) *Dc* conidia. D-F) Pictures were taken with a stereo microscope M205C (Leica Microsystems Switzerland, Heerbrugg, Switzerland) G-I) Samples were stained with cotton blue in lactic acid and pictures were taken with a Leica DM2000 LED microscope (Leica Microsystems Switzerland, Heerbrugg, Switzerland).

Figure 6. Infection cycle of *Diplocarpon coronariae* (*Dc*) in Central Europe.

Based on former knowledge and the herein presented data, we hypothesize that primary spores (since no ascospores were reported in Europe to date, presumably conidia) are released in early spring and cause first infections between end of April and middle of May depending on the weather conditions. Without crop protection, secondary spores have to be expected from end of May onwards. The epidemic spread of *Dc* leads to massive tree defoliation and in highly infested orchard also to infections of the fruits. The fungus overwinters on leaf litter, but is also found on fruit mummies, in buds and on bark after winter. These organs might thus represent alternative organs for overwintering. Green, primary infections; yellow, secondary infections; blue, overwintering.

Figure 1

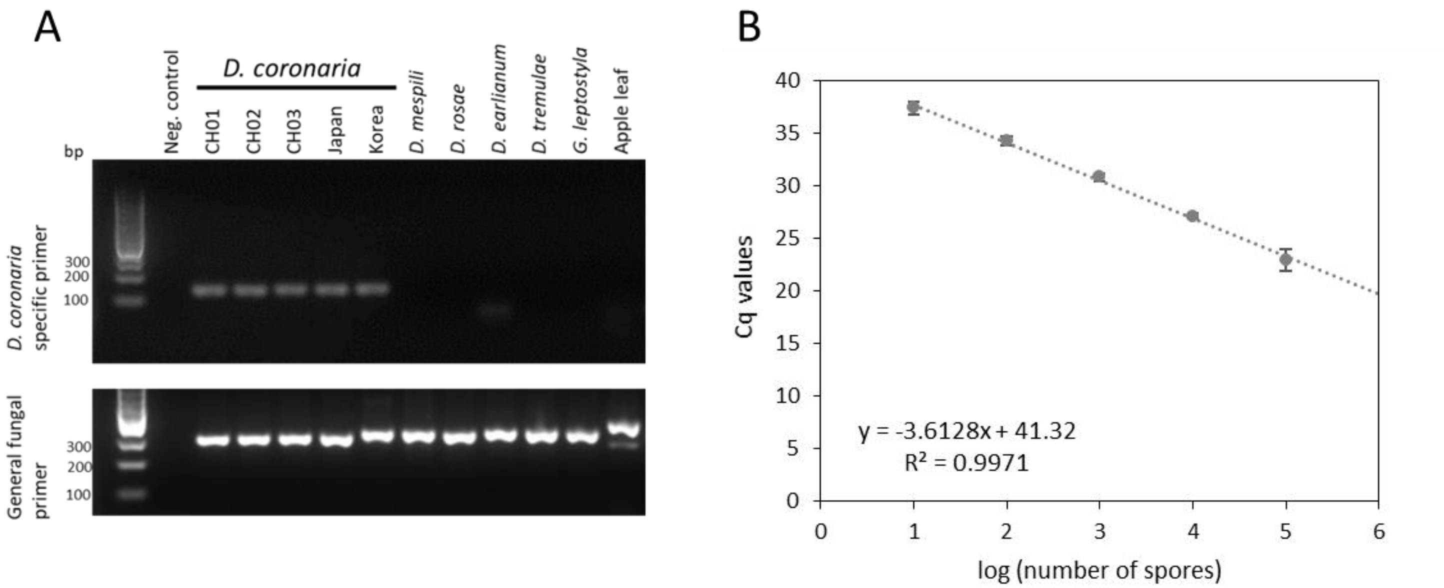

A
D. coronaria
Neg. control
CH01
CH02
CH03
Japan
Korea
D. mespili
D. rosae
D. earlianum
D. tremulae
G. leptostyla
Apple leaf
bp
300
200
100
D. coronaria specific primer
General fungal primer
B
Cq values
log (number of spores)
y = -3.6128x + 41.32
R² = 0.9971
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6

Figure 2

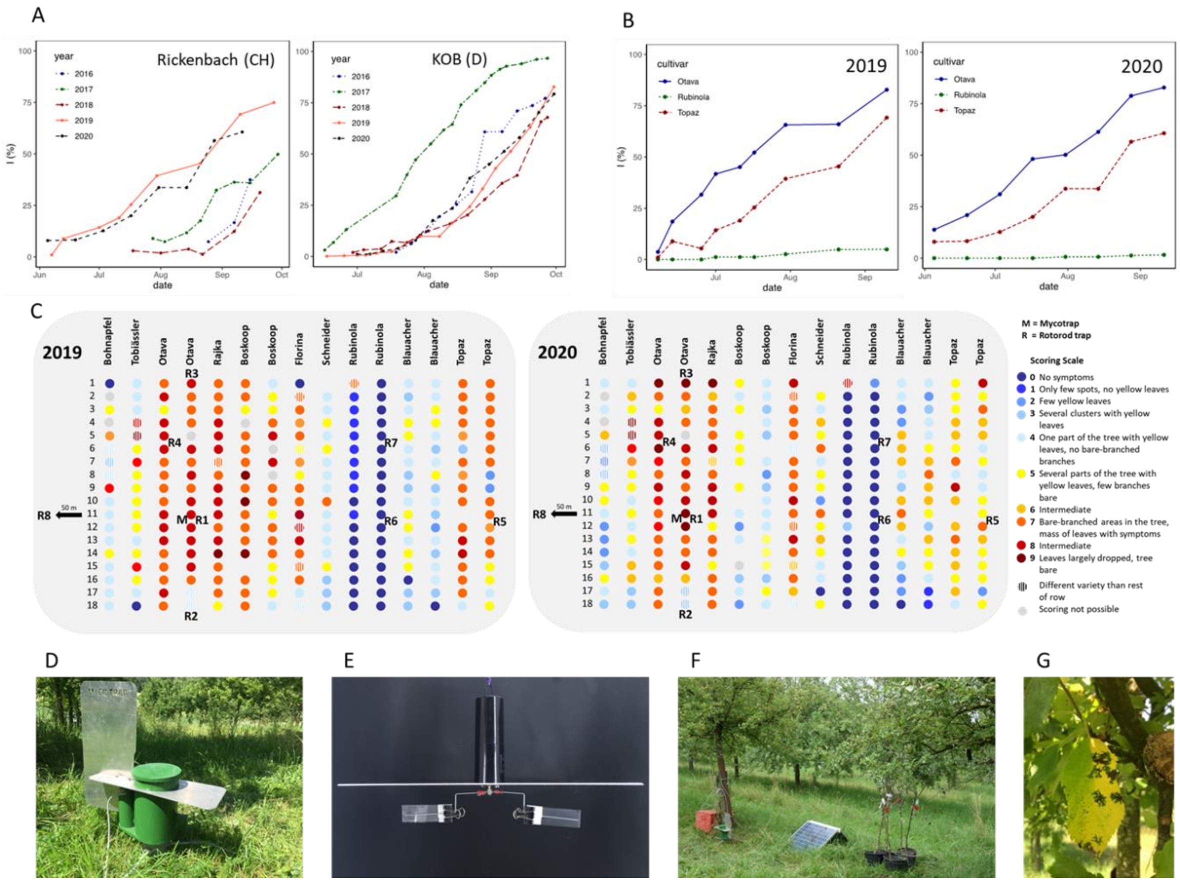

A
Rickenbach (CH)
KOB (D)
year
2016
2017
2018
2019
2020
I (%)
date
B
cultivar
Otava
Rubinola
Topaz
2019
2020
C
2019
2020
Bohnapfel
Tobiässler
Otava
Rajka
Boskoop
Florina
Schneider
Rubinola
Blauacher
Topaz
M = Mycotrap
R = Rotorod trap
Scoring Scale
0 No symptoms
1 Only few spots, no yellow leaves
2 Few yellow leaves
3 Several clusters with yellow leaves
4 One part of the tree with yellow leaves, no bare-branched branches
5 Several parts of the tree with yellow leaves, few branches bare
6 Intermediate
7 Bare-branched areas in the tree, mass of leaves with symptoms
8 Intermediate
9 Leaves largely dropped, tree bare
Different variety than rest of row
Scoring not possible
D
E
F
G

Figure 3

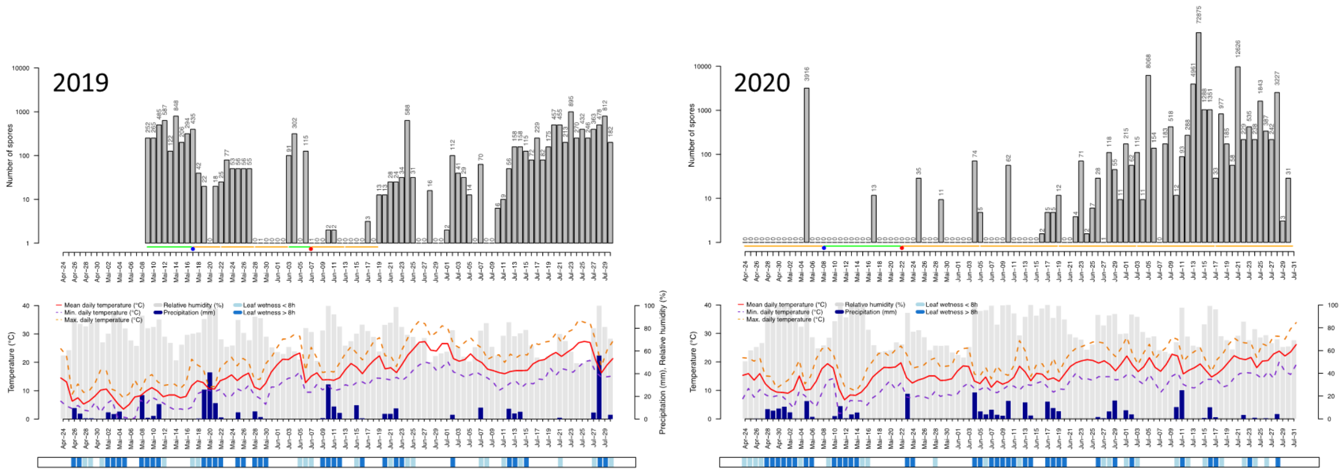

2019
2020
Number of spores
Temperature (°C)
Precipitation (mm), Relative humidity (%)
Mean daily temperature (°C)
Min. daily temperature (°C)
Max. daily temperature (°C)
Relative humidity (%)
Precipitation (mm)
Leaf wetness < 8h
Leaf wetness > 8h

Figure 4

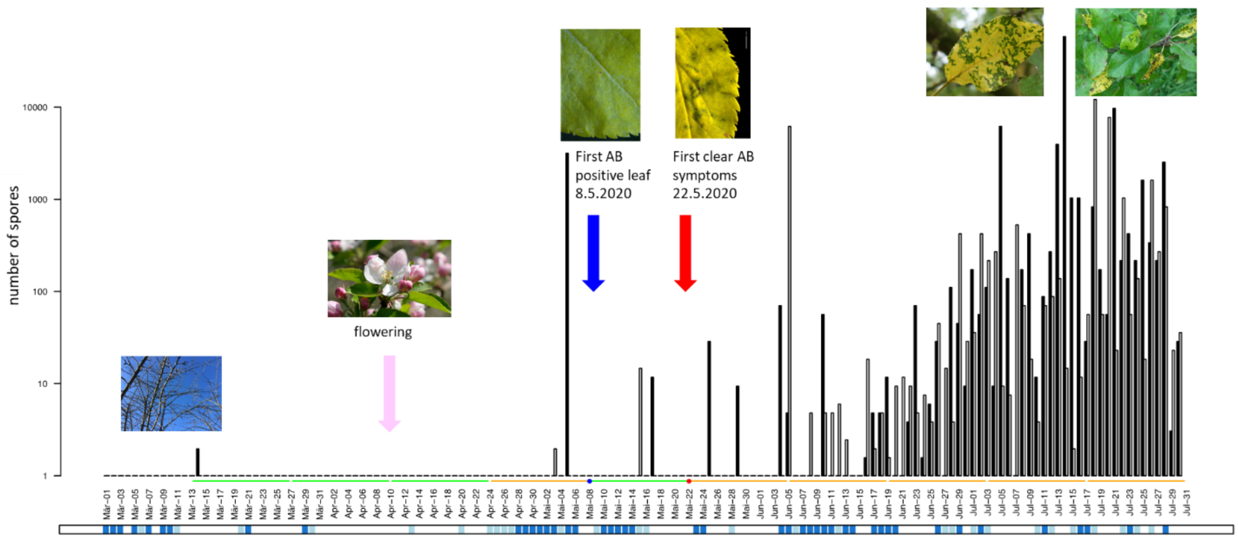

number of spores
10000
1000
100
10
1
flowering
First AB
positive leaf
8.5.2020
First clear AB
symptoms
22.5.2020

Figure 5

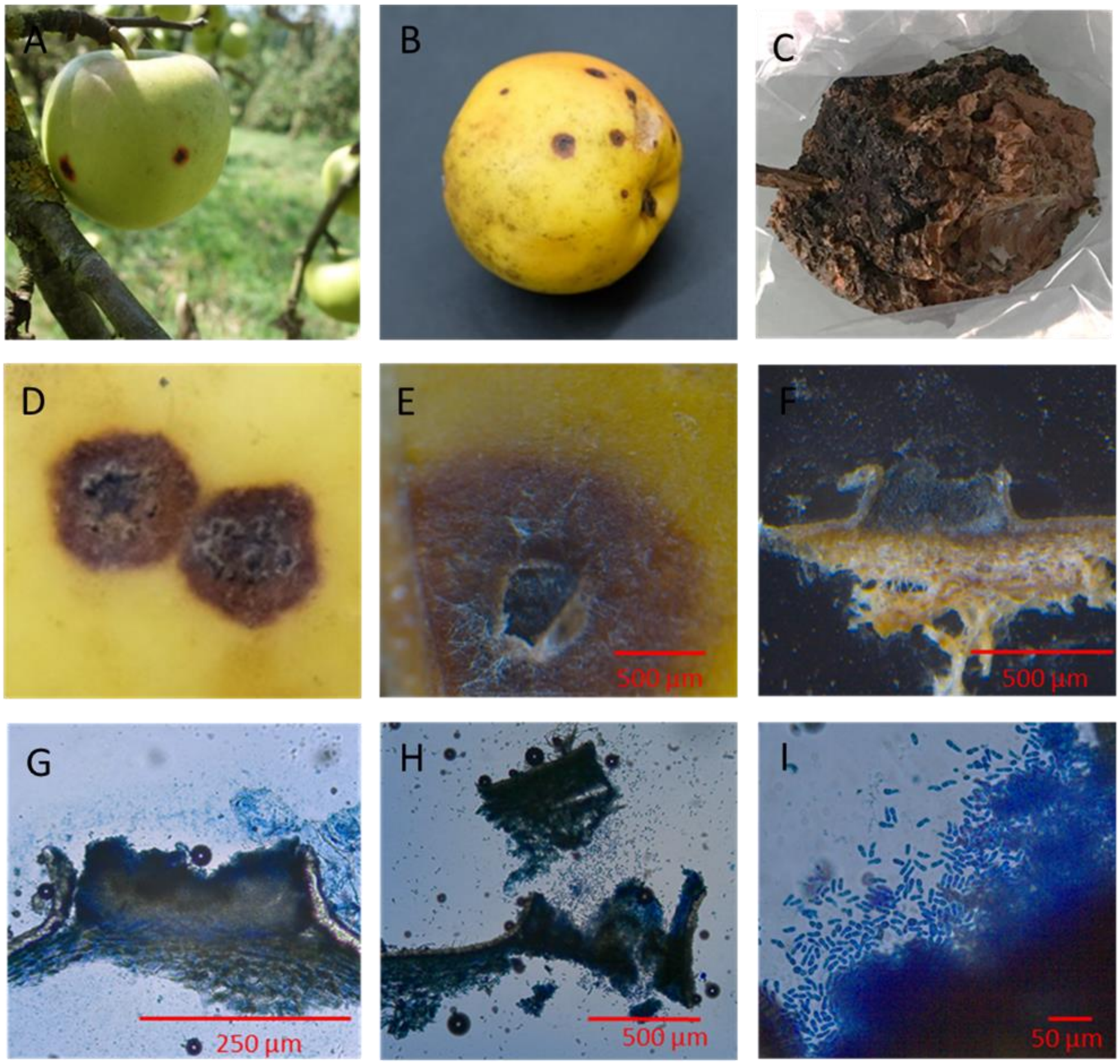
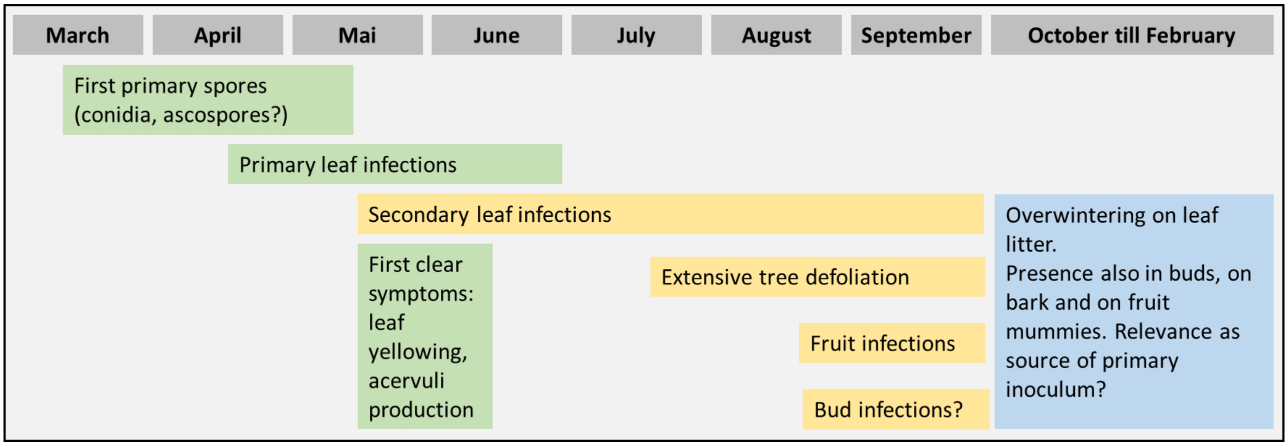


Figure 6


March
April
Mai
June
July
August
September
October till February
First primary spores (conidia, ascospores?)
Primary leaf infections
Secondary leaf infections
First clear symptoms: leaf yellowing, acervuli production
Extensive tree defoliation
Fruit infections
Bud infections?
Overwintering on leaf litter.
Presence also in buds, on bark and on fruit mummies. Relevance as source of primary inoculum?