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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23 # Abstract

24 Apple blotch (AB) is a major disease of apples in Asia and recently also emerging in Europe
25 and the USA. It is caused by the fungus *Diplocarpon coronariae* (*Dc*) *(formerly: Marssonina*
26 *coronaria;* teleomorph: *Diplocarpon mali*) and leads to severe defoliation of apple trees in late
27 summer and thus to reduced yield and fruit quality. To develop effective crop protection
28 strategies, a sound knowledge of the pathogen's biology is crucial. However, especially data on
29 the early phase of disease development is scarce, and no data on spore dispersal for Europe is
30 available. In this study, we assessed different spore traps for their capacity to capture *Dc* spores,
31 and we developed a highly sensitive TaqMan qPCR method to quantify *Dc* conidia in spore trap
32 samples. With these tools, we monitored the temporal and spatial spore dispersal and disease
33 progress in spring and early summer in an extensively managed apple orchard in Switzerland in
34 2019 and 2020. Our results show that *Dc* overwinters in leaf litter and that spore dispersal and
35 primary infections occur already in late April and beginning of May. We provide the first results
36 on early-season spore dispersal of *Dc*, which, combined with the observed disease progress, helps
37 to understand the disease dynamics and improve disease forecast models. Using the new qPCR
38 method, we finally detected *Dc* in buds, on bark and on fruit mummies, suggesting that these
39 apple organs may serve as additional overwintering habitats for the fungus.

40

41

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

108 been tested for *Plasmopara viticola* (Basso et al. 2020; Kilin et al. 2019). Furthermore, laser-based
109 real-time optical particle counters (OPC) that detect particles in the air are being tested for real-
110 time detection of spores in the air (Basso et al. 2020; Kilin et al. 2019).

111 Besides microscopy, molecular methods based on the detection of fungal DNA or proteins
112 are used to quantify fungal spores. DNA-based methods include the quantitative real-time
113 polymerase chain reaction (qPCR), loop-mediated isothermal amplification (LAMP), (Notomi et al.
114 2015; Ren et al. 2021), serial analysis of gene expression (SAGE), and microarray technology
115 (Aslam et al. 2017). The enzyme-linked immunosorbent assay (ELISA) (Kennedy et al. 2000), or
116 the fluorescent antibody (FA) assay (Schneider et al. 2009) on the other hand detect fungal
117 proteins by antibodies. The qPCR method is the most frequently used quantitative molecular
118 method to detect and quantify fungal pathogens. It offers a more sensitive and specific
119 quantification than microscopy, as it can detect low concentrations of a fungal pathogen among
120 a background of particles and DNA from diverse organisms (Parker et al. 2014) and does not
121 depend on visual recognition of the fungus. The detection limit of spores from a field sample
122 depends on many factors influencing the sensitivity of the qPCR assay. These factors comprise the
123 DNA extraction efficiency, and the performance of the qPCR, which depends on the quality of the
124 DNA extract (e.g. presence of inhibiting compounds such as phenols, salts, alcohols), the choice
125 of the target gene in the genome (multi-copy vs single-copy), the design of efficient primers, the
126 amplicon lengths, the qPCR reagents, and the equipment and analysis software (Freeman et al.
127 1999).

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

130 German apple orchard from 2016 to 2020. We evaluated the most suitable spore traps to capture
131 *Dc* spores under experimental conditions, and we developed a highly sensitive qPCR method to
132 quantify *Dc* spores. These tools were used to monitor the temporal and spatial distribution of *Dc*
133 spores in spring and early summer. We detected large amounts of spores already beginning of
134 May and again during the exponential phase of the epidemic. This observation represents to our
135 best knowledge the first report on *Dc* spore flight in Europe. To understand the onset of AB
136 disease, we further investigated potential sources of primary inoculum. The here provided data
137 on spore counts, weather and disease development substantially improves our understanding of
138 the biology of *Dc*. Furthermore, it can be directly used to fight AB disease, e.g. by improving
139 disease forecast models and adapted plant protection measures. Finally, the developed methods
140 represent useful tools for future studies of *Dc*.
141

142 Material and Methods

143 **Microorganisms used in this study**

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

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

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

149 We aimed at an optimal DNA extraction efficiency from *Dc* spores in spore trap samples.
150 Therefore, we tested the extraction of known amounts of *Dc* conidia from different spore trap
151 media (plastic film, Vaseline coated plastic film, adhesive tape, 1.5 ml plastic tubes) with different
152 DNA extraction protocols (data not shown) and finally decided to extract *Dc* conidia from Vaseline
153 coated plastic film using the ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit as
154 recommended by the manufacturer but with two modifications: the Bashing Bead® tubes from
155 the Zymo Microprep Kit were replaced by a 2 ml tube with screw cap containing 100 mg of 0.5
156 mm Zirconia bashing beads (N034.1 Roth, Karlsruhe, DE) and the Bashing Bead™ Buffer volume
157 was reduced from 750 µl to 550 µl. This method was also used to extract spores from uncoated
158 plastic film. Conidia that were directly sampled into a vial, as in the case of the multi-vial cyclone
159 sampler, as well as apple leaves with ambiguous AB symptoms were extracted following a CTAB
160 buffer based protocol (Supplementary Methods).
161

162 **Primer design and evaluation**

163 We aimed at developing a highly specific and sensitive TaqMan® qPCR assay for *D.*
164 *coronariae*. To this end we designed primers and hydrolysis probes targeting the ITS1 region
165 between 18S and 5.8S rDNA of *D. coronariae* using the program Beacon Designer (V8.16, Premier
166 Biosoft, Palo Alto, CA, USA) with following parameters: amplicon length between 100 and 200 bp,
167 melting temperature (TM) of primers at 60.0°C +/- 1.0°C, and TM of probe at 10.0°C +/- 5.0°C
168 above TM of the primers. Default settings of the program were used for maximal ΔG of self-
169 complementarity, 3'-end stability, and % GC. The specificity of primers and probes was confirmed

170 *in silico* by NCBI Primer-Blast https://www.ncbi.nlm.nih.gov/tools/primer-blast (Ye et al. 2012) by
171 an alignment of different ITS genebank accessions of strains of *Dc* and finally tested in vitro with
172 genomic DNA of different *D. coronariae* strains and other fungal species (Figure 1A,
173 Supplementary Table 2).
174

175 **Conditions of the qPCR reactions**

176 Primers and probes were synthesized and purified by high performance liquid
177 chromatography HPLC at Microsynth AG (Balgach, CH). Primers and probes (Table 1) were
178 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
179 based *Dc* specific qPCR reactions consisted of 5 µl KAPA SYBR FAST (Sigma-Aldrich Chemie AG,
180 Buchs, Switzerland), 1 µl primermix (forward and reverse primers at a concentration of 3 µM
181 each), 3 µl double-distilled water and 1 µl of template DNA in a total volume of 10 µl. TaqMan®
182 based qPCR reactions consisted of 5 µl KAPA PROBE FAST (Sigma-Aldrich Chemie AG, Buchs,
183 Switzerland), 1 µl of each primermix for *Dc* and APA9 (containing primers at a concentration of 3
184 µM and 2 µM for Dc_09 and ACMV, respectively, and the probe at 1 µM each), 0 to 2 µl double-
185 distilled water and 1 to 3 µl of template DNA in a total volume of 10 µl.

186 The qPCR assay and data analysis were performed with a CFX96 Touch Real-Time PCR
187 Detection System (Biorad, USA). An annealing temperature of 60 °C was found best for primer
188 Dc_09 (assessed with a gradient from 58 to 68 °C tested on two concentrations of *Dc* CH01
189 mycelial DNA using SYBR qPCR, data not shown). The finally used amplification and quantification
190 conditions were: an initial denaturation step of 3 min at 95 °C, followed by 39 and 45 cylces of 10

191 s at 95 °C, and 20 s at 60 °C for SYBR and TaqMan® qPCR, respectively. After each SYBR qPCR, a
192 dissociation curve analysis was performed by gradually increasing the temperature from 65 °C to
193 95 °C with 0.5 °C per cycle.

194 To generally detect fungal DNA a SYBR green qPCR assay was performed with primers
195 ITS1F/2R (Table 1). The reactions consisted of 5 µl KAPA SYBR FAST (Sigma-Aldrich Chemie AG,
196 Buchs, Switzerland), 1 µl primermix (primers at a concentration of 2 µM each), 3 µl double-
197 distilled water and 1 µl of template DNA in a total volume of 10 µl and were subjected to an initial
198 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
199 at 72°C.

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

204

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

206 The number of conidia corresponding to a given C_q value was calculated using a standard
207 curve based on known amounts of conidia. To this end, conidia suspensions were prepared by
208 harvesting conidia from *D. coronariae* CH01 (Supplementary Table 1) isolate, grown on a PPCDA
209 (Zhao et al. 2010) agar plate at room temperature for 22 days, with 50 ml sterile double-distilled
210 water (ELGA®, Purelab Flex 03, Veolia Water Technologies, Celle, Germany) or by stirring apple leaves
211 with sporulating *Dc* for ten minutes in Volvic® water and subsequent filtering of the spore
212 suspension through a sieve (mesh-width approximately 0.1 mm). The spore concentration was

213 assessed by counting the conidia under a microscope using a haemocytometer (0.1 mm depth,
214 0.0025 mm^2, Neubauer®). Ten-fold dilution series of conidia in double-distilled water containing
215 0.05% Tween® 80 as described by Mc Devitt et al. (2004) corresponding 10^5 to 10^1 conidia were
216 prepared and added to the material used in spore traps (Vaseline coated plastic film). DNA
217 extraction and qPCR were performed as described above. The standard curves were used for the
218 test calibration, and the limit of quantification (LOQ), and the calculation of spore numbers out
219 of Cq values.

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

227
228

229 **Spore traps used in this study**

230 Four different types of spore traps were assessed for their ability to capture *Dc* spores
231 under experimental conditions (Table 2) and two of them, the Mycotrap and the rotating-arm
232 spore traps, were used for field sampling (Figure 2D, E). Additionally, apple bait plants were used
233 as in vivo spore traps (Figure 2F). A detailed description on the spore traps is provided in the
234 Supplementary Methods. Briefly, the Mycotrap (Siegfried et al. 1996) (Figure 2D, Table 2) is an

235 impaction spore trap where air is sucked in horizontally through a sampling orifice and impacts
236 on a trapping surface on a cylinder inside a chamber. The cylinder slowly rotates, completing one
237 turn within seven days thus allowing to sample seven days in a row. The high throughput 'jet'
238 spore sampler (Burkard Manufacturing) is a type 'virtual impaction' spore trap with high air
239 throughput based on a settling tower at which base the spores are collected (West and Kimber
240 2015)(Table 2). The cyclone-based spore trap (Burkard Manufacturing) (Table 2) comprises an air
241 intake introducing the airflow tangentially into a glass or metal cylinder (Lacey and West 2006).
242 At the end of the cylinder spores are collected during a period of eight days into eight different
243 detachable 1.5 ml sample vials, by changing the sampling vial position each day. The rotating-arm
244 spore traps (Figure 2E) were constructed based on the description by Quesada et al. (2018)
245 (details in Supplementary Methods). They consisted of two rods connected to a battery powered
246 motor (spinart^{TM} Lightweight Hanging Motor, 30 rpm). Each rod held a microscope slide on which
247 a Vaseline coated plastic film strip was mounted and fixated with 25 mm foldback-clips (Maul,
248 Bad König, Germany). The rotating-arm spore traps were covered with an aluminum shield as rain
249 protection.
250

251 ***Assessment of spore traps in the climate chamber***

252 Spore traps were compared inside a closed climate chamber (33.43 m^3). The experiments
253 included two seven-day volumetric spore traps (Mycotrap), one multi-vial cyclone sampler
254 (cyclone), one high throughput 'jet' spore sampler, and two rotating-arm spore traps.
255 Additionally, apple seedlings were used as bait plants. In a first set of experiments the spore traps

256 were assessed for their capability to capture spores from a conidial suspension of *Dc* sprayed into
257 the air (Supplementary Methods).

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

269

270 ***Field sites***

271 Spore dispersal and AB epidemiology were investigated in an extensively managed organic
272 apple cider orchard in Rickenbach (Zurich, Switzerland) (47°33'30.5"N 8°47'29.9"E). The site is
273 characterized by a mean annual temperature of 10.0 °C and 980 mm of annual rainfall (years 2010
274 to 2020). The orchard was planted in 2002 with different apple cultivars arranged in 15 rows with
275 18 apple trees each (Figure 2C), in a planting distance of 4 m within and 9 m between rows. In
276 2019, the orchard was treated with sulfur and clay (acidified clay minerals, product Myco-Sin® 8
277 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.

299 ***Disease assessment in the apple orchard***

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

305

306 ***Monitoring spore dispersal in an apple orchard***

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

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

320

321 ***Monitoring spore dispersal from an infected leaf litter under field conditions***

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

328

329 ***Quantification of D. coronariae conidia in rain water***

330 From 24th to 25th of September 2020 rain water was collected by placing 12 Schott-bottles
331 with a funnel within the orchard in Rickenbach for 16 hours. It was the first rainy period after
332 seven consecutive days without precipitation. Amount of rain registered for that period was 14
333 mm per m^2, approximately 100 ml of rain water was collected per Schott-bottle and filtered
334 through a cellulose acetate filter (25 mm in diameter, 0.8 µm pore size, Sartorius-Stedim,
335 Goettingen, Germany) in polycarbonate filter housings. The filters were subsequently cut in
336 pieces of approximately 3x3 mm and stored at -20 °C.

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

342

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

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

356 ***Investigation of fruit infections***

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

363

364 # Results

365 ***Development of apple blotch disease in two Central European Apple Orchards***

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

374 The disease progression on 'Topaz' over the season is shown in Figure 2A as percent
375 damage (McKinney-Index). After the appearance of first symptoms with clear evidence for *Dc* (i.e.
376 visible formation of acervuli, yellowing of the leaf), which occurred at the latest in August in both
377 orchards and all years, disease progression was generally characterized by a steady increase of
378 damage. However, large differences in disease severity were observed between years. At KOB,
379 for instance, leaf damage through AB was higher in 2017 compared to other years (Figure 2A).
380 2017 was the year with most days with rain from June to October, i.e. 78 days compared to 36 to
381 57 days in the other years (Supplementary Figure 1). At both sites, 2018 was characterized by an
382 exceptionally hot and dry summer (only 36 and 33 days with rain from June to end of September
383 at KOB and Rickenbach, respectively) (Supplementary Figure 1, 2) and lower levels of AB (Figure

384 2A). At Rickenbach, disease levels were highest in 2019 and 2020, which was not observed at KOB
385 (Figure 2A).

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

390 ***New qPCR allows quantification of D. coronariae conidia***

391 To quantify *Dc* spores in spore trap samples, we developed a new TaqMan qPCR method
392 with the primers and probes Dc_09 (Table 1). Different primers and hydrolysis probes targeting
393 the ITS gene of *Dc* were tested, and among them, primers and probe Dc_09 performed best (E=
394 94.2 %, R^2= 0.995) also compared to the published primer Mc_ITS (Oberhänsli et al. 2014)(data
395 not shown). Specificity tests for primer Dc_09 revealed a good amplification on DNA of three
396 Swiss, one Japanese and one Korean isolate of *Dc* (Figure 1A). In contrast, no amplification was
397 observed on DNA of other species within the genus *Diplocarpon* (*Diplocarpon mespili*,
398 *Diplocarpon earlianum*, *Diplocarpon rosae)* nor on DNA of the causal agents of other Marssonina
399 diseases (*Gnomonia leptostyla (alternate state: Marssonina juglandis)* and *Drepanopeziza*
400 *tremulae (alternate state: Marssonina brunnea*) (Figure 1A, Supplementary Table 2). Moreover,
401 Dc_09 did not amplify DNA from healthy apple leaves or other common apple pathogens such as
402 *Venturia inaequalis*, *Monilia fructigena* or *Neofabraea alba* (Supplementary Table 2).

403 To quantify *Dc* conidia in spore trap samples, we generated standard curves by adding
404 known amounts of conidia to spore trap samples, which allowed the direct conversion of Cq

405 values into spores per sample. The amplification of the qPCR for conidial counts on Vaseline
406 coated plastic film, which was the spore trapping surface used for field samples, exhibited a linear
407 response with an efficiency of 89.1 % (R^2 = 0.997) (average of seven qPCR runs) (Figure 1B). The
408 qPCR assay allowed the consistent detection of as little as ten conidia; however, at three conidia
409 per sample, the amplification became unreliable. The limit of quantification is therefore
410 considered to lie between three and ten conidia.

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

417 ***Evaluation of spore traps under experimental conditions***

418 To identify spore traps suitable to capture *Dc* conidia, we tested different spore traps
419 under controlled conditions. When compared in a climate chamber for their capability to capture
420 spores from a conidial suspension of *Dc* sprayed into the air (Supplementary Methods) all tested
421 spore traps (Mycotrap, High throughput 'jet' spore sampler, Multi-vial cyclone sampler, rotating-
422 arm spore trap) with different spore trapping surfaces (e.g. plastic film and Vaseline coated plastic
423 film) were able to capture spores mostly in the range of thousands to tens of thousands
424 (Supplementary Results). Overall the Mycotrap performed best in these experiments
425 (Supplementary Results). Since catching spores from a conidia suspension sprayed into the air is

426 rather artificial, the spore traps were further tested for their performance to catch conidia that
427 are splash dispersed from an overwintered leaf litter deposit that was artificially irrigated. All
428 spore traps were able to capture spores released by the leaf litter deposit, but much lower
429 numbers than from spore suspensions sprayed into the air (Table 2 and Supplementary Results).
430 Nevertheless, all bait plants placed next to the spore traps developed AB symptoms (Table 2).

431 ***Monitoring Dc spore dispersal in the field***

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

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

446 To our surprise, in 2019 very high levels of spores were already detected at the beginning
447 of the experiment during the first half of May (Figure 3A). After that, spore levels generally
448 decreased, apart from a peak around the 4th of June. In May, leaves with suspicious spots were
449 collected and tested for *Dc* by qPCR, revealing the first AB positive leaf on the 17th of May. First
450 unambiguous symptoms were discovered three weeks later on the 7th of June (Figure 3A). A next
451 spore peak was detected on the 24th of June after a rain period. At that time, 'Otava' trees already
452 showed strong leaf yellowing, indicating emergence of secondary inoculum. In July, high spore
453 levels in the air were recorded on most days. All the data shown in Figure 3 are based on captures
454 by the Mycotrap on the ground. The Multi-vial cyclone sampler did not catch significant amounts
455 of *Dc* spores, i.e less than five spores per day on days where the Mycotrap was catching up to 848
456 spores (data not shown). Therefore, we excluded the cyclone sampler from the field trial on the
457 7th of June 2019. From the 7th of June to the 5th of July a second Mycotrap was placed in the tree
458 crown of an 'Otava' apple tree next to the Mycotrap on the ground (Supplementary Figure 4).
459 This trap detected more spores than the Mycotrap on the ground except for the 24th and 25th of
460 June.

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

466 In 2020, we started the field experiment already on the 28th of February, to ensure not to
467 miss the first spore flight (Figure 4). We also included one Mycotrap on the ground and one in the

468 tree crown for the entire duration, since we hypothesized that primary spores originating from
469 leaf litter on the ground might be detected rather by a trap on the ground, while a trap in the tree
470 crown would detect high numbers of spores during the epidemic**.**

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

488 In 2020, all bait plants after the 22nd of May developed AB symptoms. While bait plants
489 from the 22nd of May to the 5th of June exhibited about 50 to 80 % symptomatic leaves, on bait
490 plants after the 5th of June over 90 % of leaves became diseased (Supplementary Figure 5).
491 Beside the dispersal of *Dc* spores via the air, splashes of rainwater might be an important
492 factor for spreading the disease within a tree. Therefore, we collected rainwater below highly
493 infected trees ('Otava', 'Topaz'), below symptomless trees ('Rubinola'), between the rows under
494 the open sky and outside the orchard during a rain period in September 2020. We detected
495 several hundreds of spores per ml rain water below 'Otava' trees, fewer spores between rows,
496 and almost no spores below non-infected 'Rubinola' trees or outside the orchard (Supplementary
497 Figure 6).

498 ***D. coronariae spores mainly close to infected trees***

499 Besides the temporal pattern of *Dc* spore dispersal, we were also interested in the spatial
500 distribution of spores within and outside the orchard. Therefore, we placed seven rotating-arm spore traps
501 within the orchard and one outside the orchard as indicated in Figure 2C. In 2019, the traps were installed
502 only between the 22nd of Mai and the 5th of June. However, in 2020, we covered the entire sampling period
503 from beginning of March to end of July. Rotating-arm spore traps detected the first spores only after the
504 22nd of Mai 2020, thus missing the first large peak on the 5th of Mai. Spore numbers detected in rotating-
505 arm spore traps were generally much smaller than those quantified in samples from Mycotraps (Table 3,
506 Figure 3, 4). Highest spore numbers were generally detected in traps installed on 'Otava' trees, which
507 exhibited very strong AB symptoms, while almost no spores were detected in traps on 'Rubinola' trees.
508 These trees were not infected by *Dc*, although they were only a few rows apart from highly diseased trees.
509 Nevertheless, leaf yellowing and premature leaf fall was also observed on Rubinola trees, but

510 symptoms looked distinctly different from apple blotch and absence of *Dc* infections in leave
511 samples was confirmed by qPCR (data not shown). Furthermore, no spores were detected in the
512 trap about 50 m outside the orchard. An exception for these observations was the period of the
513 22nd of May until the third of June 2019, where spores were detected under 'Rubinola' trees and
514 outside the orchard.

515 ***Origin of primary infections***

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

526 Since in earlier studies we found no effect of sanitary measures removing leaf litter from
527 the orchard on AB disease development (Buchleither 2019), we hypothesized that other inoculum
528 sources might be relevant for disease outbreak, too. Therefore, we collected bark and bud
529 samples from 'Otava' and 'Rubinola' trees (Rickenbach site) and 'Topaz' trees (KOB site) after
530 winter and tested them for presence of *Dc* by qPCR. While all 'Rubinola' samples, which derived

531 from trees without AB symptoms the year before, were negative for *Dc*, the majority of samples
532 from 'Topaz' and 'Otava' trees were tested positive (Table 5). Bark samples were only slightly
533 positive (high Cq values), but bud samples exhibited relatively high levels of *Dc* DNA (lowest Cq
534 value at 25.6 which corresponds approximately to the DNA of 10^4 conidia)(Table 5).

535 Furthermore, *Dc* infected fruit mummies might represent sources for primary inoculum.
536 We searched for fruit mummies hanging in the trees after winter. In spring 2020, four out of five
537 fruit mummies, which were collected from trees with strong AB infestation in 2019, were tested
538 slightly positive (Cq between 33.65 and 40.38), while fruit mummies from trees without AB
539 symptoms the previous year were negative (Figure 5C, Supplementary Table 5). For comparison
540 a leaf sample still hanging in a susceptible 'Florina' tree after winter was strongly *Dc* positive (Cq,
541 25.83).

542 Finally, to assess whether the identified DNA on bark, buds and fruit mummies originates
543 from viable and infectious fungal tissues, we tried to infect apple leaves in the laboratory by
544 applying wetted pieces of bark, buds, fruit mummies and leaves. However, our attempts did not
545 result in successful infections to this point of time (data not shown). Nevertheless, we could show
546 that *Dc* infected fruits can serve as inoculum for leaf infections after six months of storage. In fall
547 2020, we detected *Dc* infected 'Otava' fruits in the Rickenbach orchard (Figure 5A). Figure 5A
548 shows AB symptoms on fruits in fall (Figure 5A) and after storage of fruits in the fridge for six
549 months (5B). In spring 2021, conidia formed on these fruits (Figure 5D-I), and those conidia were
550 able to infect leaves of apple seedlings in the laboratory (Supplementary Figure 7).

551

552 # Discussion

553 ***Development of a D. coronariae specific qPCR***

554 To date, *Dc* spores in spore trap samples have been quantified by microscopy (Kim et al.
555 2019). However, this is labor intensive, and in addition it is often difficult to securely identify a
556 few *Dc* spores in field samples comprising a high diversity of spores and other particles such as
557 pollen and dust. The herein developed qPCR method provides a very sensitive method allowing
558 the quantification of as few as ten conidia per sample, which is in a similar range as methods
559 developed for other fungi (Calderon et al. 2002; Dvořák et al. 2015; Luchi et al. 2013). The qPCR
560 method further allows a diagnosis of AB prior to the development of unambiguous visual
561 symptoms. This is especially useful for detecting very early infections when small necrotic lesions
562 appear that are indistinguishable from symptoms of other origins. In summary, we established a
563 new qPCR method, which allowed to quantify *Dc* spores in spore trap samples, to detect initial
564 infections in the field, and enabled the investigation of *Dc* overwintering in various apple organs.
565 Nevertheless, qPCR methods also have some disadvantages such as a delay of results due
566 to several processing steps. Moreover, it only allows a statement about the presence of DNA, but
567 not on the type of organ (e.g. mycelium, ascospore, conidia, spermatia) or its viability and
568 infectious potential. Therefore, visual assessments and infection experiments will be needed to
569 understand the form in which *Dc* is present on bark, bud and fruit samples and the role of these
570 organs in the fungal life cycle.

571 ***Comparison of spore traps***

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

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

593 The rotating-arm spore traps performed only slightly worse than the Mycotrap in the laboratory,
594 but they captured substantially less spores in the field. Thus, the rotating-arm sporetraps are
595 rather useful during the late phase of the epidemic, but not sensitive enough to detect low
596 amounts of primary spores.
597

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

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

613 Moreover, spore traps above leaf litter deposits were able to catch *Dc* spores at the end
614 of April and beginning of May in both study years. Nevertheless, very few spores were detected

615 above the leaf litter deposit and in the field already in March. Since these spores were detected
616 before bud break, we believe that they were not relevant for primary leaf infections. Our qPCR
617 method does not allow to distinguish between conidia and ascospores. Since *Dc* apothecia have
618 not yet been observed in Europe (Wöhner and Emeriewen 2019), we assume that we captured
619 conidia. Nevertheless, the relevance of ascospores as primary inoculum in Europe should be
620 subject for further research. A recent population genetic study suggested a mixed sexual and
621 asexual reproduction of *Dc* in Europe, although *Dc* populations in Europe are genetically
622 homogenous and clonal, and dominated by a few multi-locus genotypes (Oberhänsli et al. 2021).
623 The first spore peaks were observed at the end of or shortly after rainy periods, whereas no
624 spores were detected during dry periods before rain events, e.g. in April 2020. Thus, rain might
625 be required for the release of *Dc* conidia from leaf litter and acervuli might need some wetting
626 time before releasing the conidia.
627 Based on the presented lines of evidence, it can be assumed that primary infections with
628 *Dc* occur in Central Europe in late April or early May after periods of rain, which is earlier than
629 assumed to date (Sutton et al. 2014). Moreover, our spore catches in March indicate that *Dc* spore
630 discharge can even occur before April and, depending on weather conditions and phenological
631 stage of the host, infections might be possible. Knowing the exact timing of primary infection can
632 be very valuable for successful prevention of AB epidemics. While protection strategies against
633 AB in Europe are currently focused on the summer months (Hinrichs-Berger 2015), prevention of
634 primary infections in spring might be an important additional step to optimize the management
635 strategy in the future.

636 ***Leaf litter and other sources of primary inoculum***

637 In addition to the timing of the primary infections, we were also interested in the origin of the
638 primary inoculum. Several studies report that conidia inside acervuli are overwintering on fallen
639 infected leaves (Back et al. 2015; Dong et al. 2015; Gao et al. 2011; Goyal et al. 2018; Lee et al.
640 2011; Sastrahidayat and Nirwanto 2016; Sharma et al. 2009). The conidia are released in a water
641 film on the leaf surface and are then dispersed by rain splashes onto apple leaves (Hinrichs-Berger
642 2015). Our experiment with an artificially irrigated leaf litter deposit nicely simulated this process.
643 It demonstrated, that irrigation of an overwintered leaf litter results in *Dc* conidia in the air, as
644 confirmed by spore traps, and these conidia can cause infections on bait plants nearby. This
645 confirms that infected leaf litter can serve as source of primary infections. Leaf litter on the
646 ground as source of primary inoculum could also explain the observation that the disease starts
647 in the lower tree crown and then progresses upwards (Sharma and Gautam 1997). Furthermore,
648 it is in accordance with our finding that in May spores were mainly found in the lower Mycotrap
649 and not in the tree crown.

650 Nevertheless, small amounts of *Dc* DNA were also detectable on fruit mummies still hanging
651 in the trees by the end of February and acervuli with infectious conidia were produced on *Dc*
652 infected fruits after overwintering in the fridge. Moreover, considerable amounts of *Dc* DNA were
653 detected on buds, and small amounts of *Dc* DNA was found on bark of trees with previous *Dc*
654 infection. All these organs are reported sites for overwintering of other apple pathogens, for
655 instance *Monilia* spp. on fruit mummies, *Podosphaera leucotricha* in buds or *Neofabraea* spp. on
656 bark (Sutton et al. 2014). To date, it is unclear in which form (e.g. mycelium, spores) *Dc* is present
657 on bark and buds and whether the fungal structures are infectious. The fact that we have not yet

658 succeed in producing leaf infections from bud, bark or fruit mummy tissues may have been for
659 technical reasons, and the role of these apple organs in the disease cycle of *Dc* definitely requires
660 further investigation. It was also speculated that *Dc* present on buds and wood in propagation
661 material exchanged between nurseries across Europe, could be one of the reasons for the rapid
662 spread of this disease into many European apple production areas within just one decade after
663 its first detection in Northern Italy (Oberhänsli et al. 2021). In any case, our finding that conidia
664 produced on fruits after six months of storage in the fridge are able to infect apple leaves provide
665 a first indication that fruit-derived conidia might, similarly to conidia produced on leaf litter,
666 represent sources of primary inoculum at least on a local scale. Moreover, our description of fruit
667 infections is one of the first reports of its kind and adds to our very limited knowledge on the role
668 of fruit infections with *Dc* (Wöhner and Emeriewen 2019).
669

670 ***June and July: secondary infections and the exponential phase***

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

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

693 The development of AB is strongly affected by environmental factors (Li et al. 2011).
694 Rainfall and the following relative humidity are positively correlated with the disease severity in
695 the summer months (Rather et al. 2017b; Sastrahidayat and Nirwanto 2016; Sharma and Sharma
696 2005). The minimum leaf wetness period for infection under controlled conditions is 8 h at 15 °C
697 and decreases to 4 h for temperatures between 20 and 25 °C (Sharma et al. 2009). Moreover, the

698 incubation period can greatly vary depending on the weather conditions. At a temperature range
699 of 15-25 °C and seven days of leaf wetness, the incubation period is 10 to 20 days (Harada et al.
700 1974). In our study, the bait plants showed symptoms within one to four weeks after they were
701 returned from the orchard, with shorter incubation period for bait plants placed in the orchard
702 later in the season (June, July) compared to the beginning of the season (May). In 2020, first
703 necrotic spots as well as clear symptoms were observed earlier and within a shorter interval than
704 in 2019. This faster disease progression in late spring 2020, was probably due to higher May
705 temperatures compared to 2019.

706 ***Spatial spore distribution: concentration around highly infected trees***

707 Rotating-arm spore traps distributed in the orchard, revealed high spore concentrations
708 in diseased trees, but no or very few spores in trees without symptoms, only a few rows apart.
709 This could indicate that the spores are not dispersed over large distances. However, the generally
710 small spore counts in rotating-arm traps compared to the Mycotrap also shows that rotating-arm
711 traps were much less efficient in capturing spores in the field. Thus, low amounts of spores might
712 still be transported by wind currents over larger distances. Beside wind dispersal, dispersal by rain
713 water might substantially contribute to spread of *Dc* within the tree crown, as indicated by the
714 very high spore counts in rain water below highly symptomatic trees (Supplementary Figure 6).
715

716 # Conclusions

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

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

737

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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	129	This study
	Dc_09_R	CTC AGA CAT CAC GTT ATT CAC ACA A	59.4		
	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	86	Mosimann et al. (2017)
	ACMV_R	CAC TCT ACT CAG GTT CCA ATC AAA G	57.9		
	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.

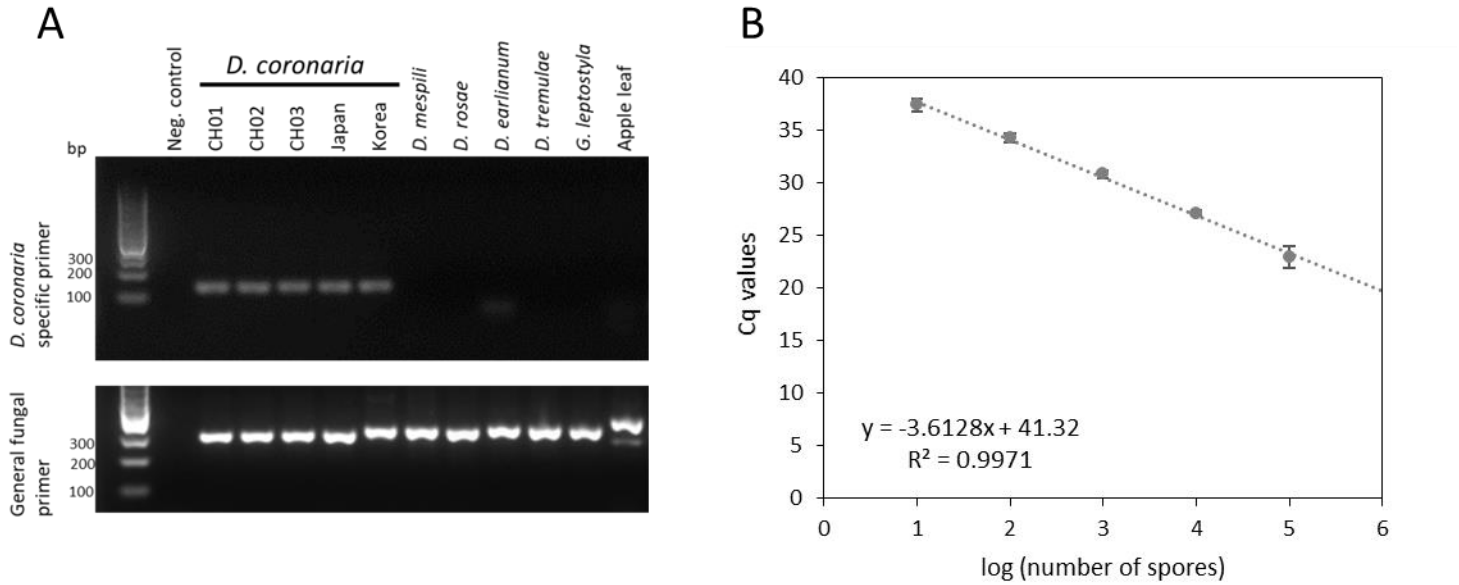
Figure 5. Fruit infections with *Diplocarpon coronariae (Dc)*. A) 'Otava' apple with apple blotch symptoms hanging in a tree in September 2020 (Rickenbach, Zurich, Switzerland). B) *Dc* infected 'Otava' apple after 6 months of storage in the fridge. C. 'Florina' fruit mummy found hanging in a tree in 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
Neg. control
D. coronaria
CH01
CH02
CH03
Japan
Korea
D. mespili
D. rosae
D. earlianum
D. tremulae
G. leptostyla
Apple leaf
bp
D. coronaria specific primer
300
200
100
General fungal primer
300
200
100
B
Cq values
log (number of spores)
y = -3.6128x + 41.32
R² = 0.9971
0
5
10
15
20
25
30
35
40
0
1
2
3
4
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6

Figure 2

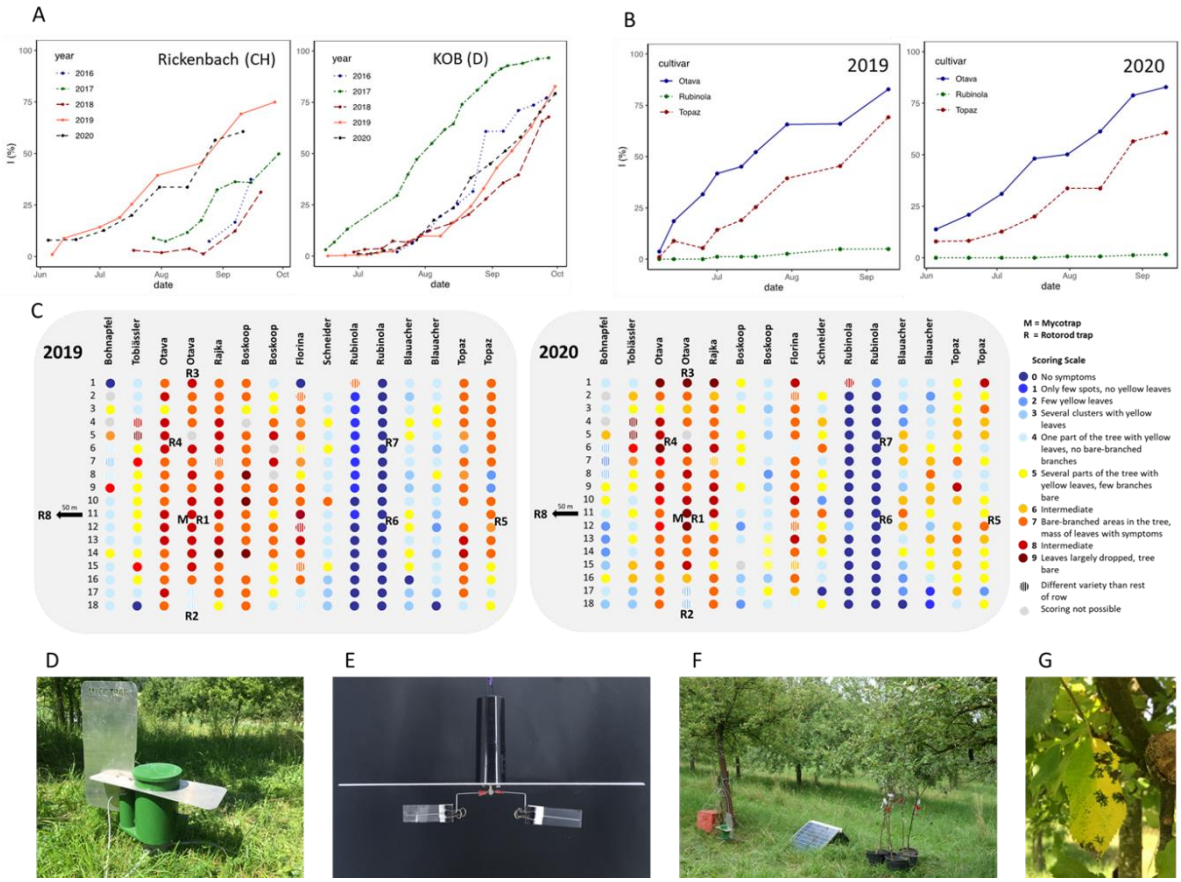

A
Rickenbach (CH)
KOB (D)
year
2016
2017
2018
2019
2020
I (%)
date
B
2019
2020
cultivar
Otava
Rubinola
Topaz
C
2019
2020
Bohnapfel
Tobiässler
Otava
Otava
Rajka
Boskoop
Boskoop
Florina
Schneider
Rubinola
Rubinola
Blauacher
Blauacher
Topaz
Topaz
R1
R2
R3
R4
R5
R6
R7
R8
M
50 m
M = Mycotrap
R = Rotorod trap
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

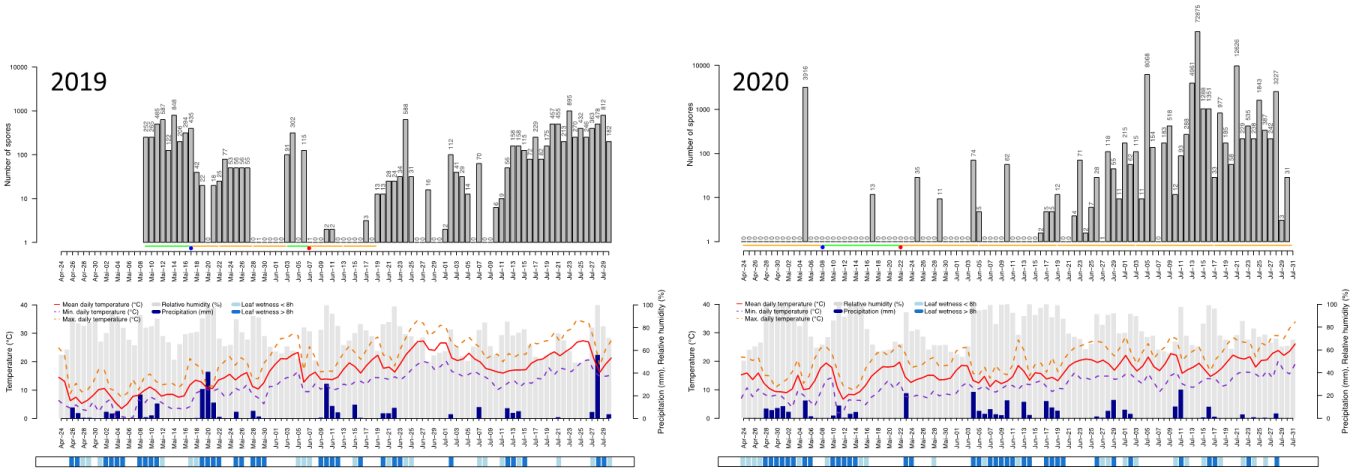

2019
2020
Number of spores
Temperature (°C)
Precipitation (mm), Relative humidity (%)
Mean daily temperature (°C)
Min. daily temperature (°C)
Max. daily temperature (°C)
Relative humidity (%)
Precipitation (mm)
Leaf wetness < 8h
Leaf wetness > 8h

Figure 4

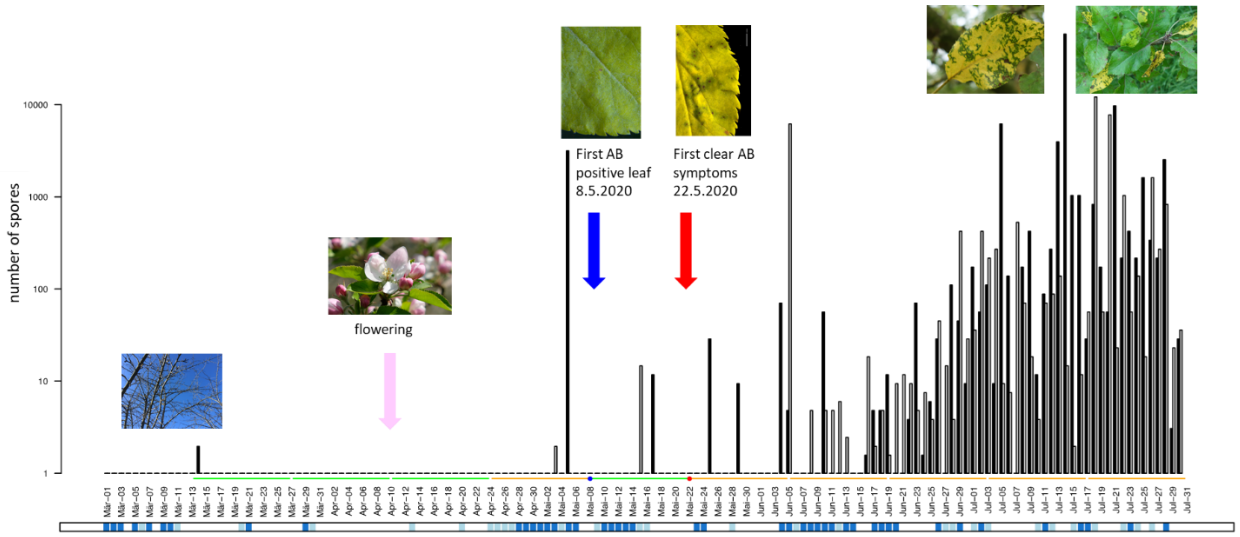

number of spores
10000
1000
100
10
1
flowering
First AB positive leaf 8.5.2020
First clear AB symptoms 22.5.2020

Figure 5

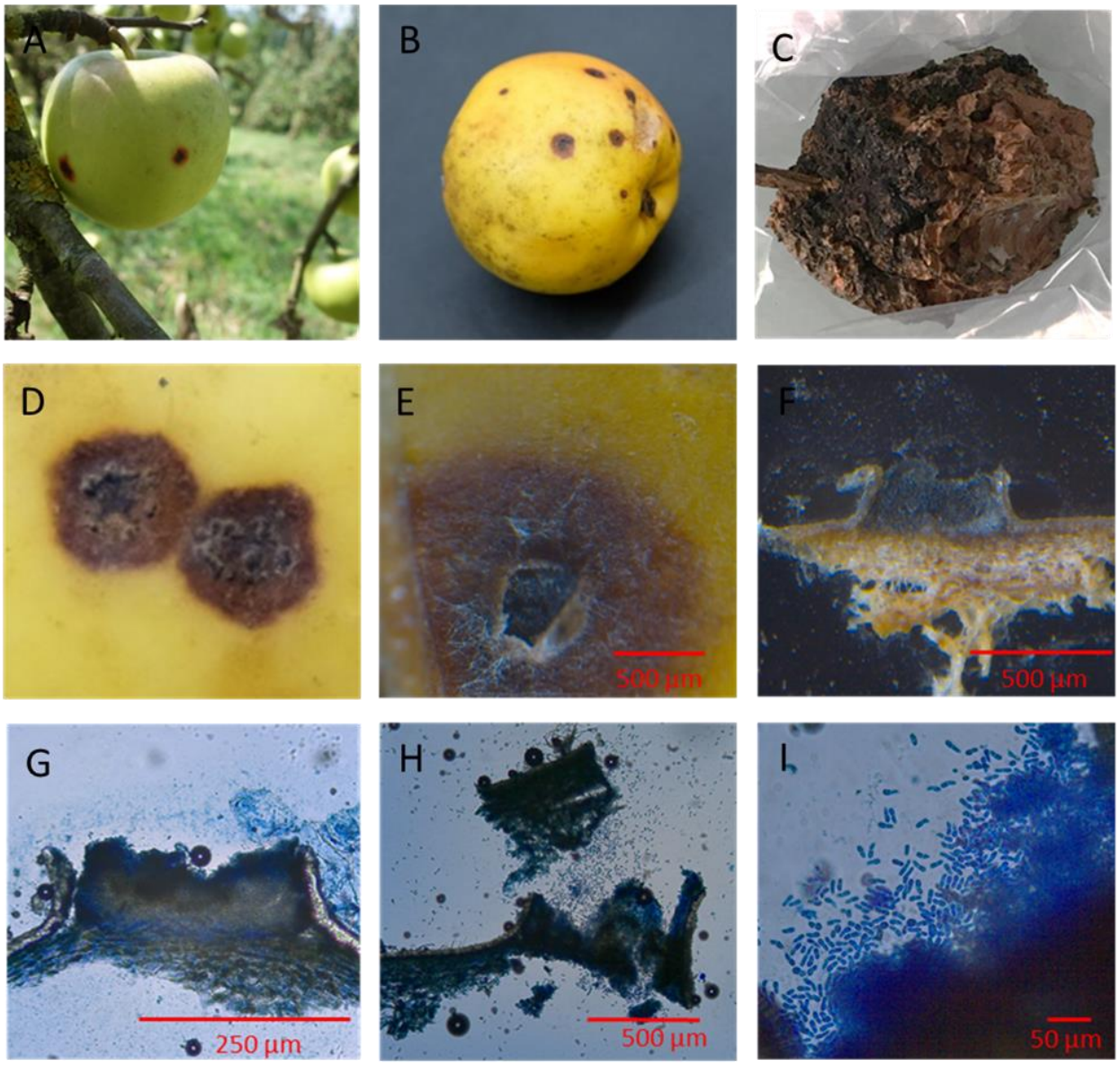
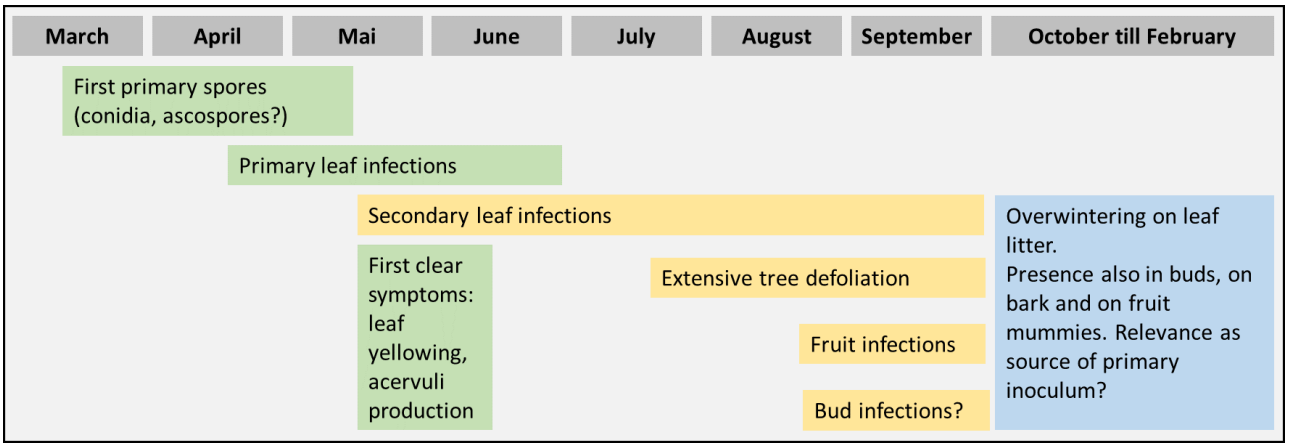


Figure 6


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?