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

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Abstract

Apple blotch (AB) is a major disease of apple in Asia and recently emerged 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 resulting in reduced yield and fruit quality. To develop effective disease management strategies, a sound knowledge of the pathogen's biology is crucial. Data on the early phase of disease development is scarce: no data on spore dispersal in Europe is available. We developed a highly sensitive TaqMan qPCR method to quantify *Dc* conidia in spore trap samples. We monitored temporal and spatial dispersal of conidia of *Dc*, and progress of AB 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 spore dispersal and primary infections occur in late April and early May. We provide the first results describing early-season dispersal of conidia of *Dc*, which, combined with the observed disease progress, helps to understand the disease dynamics and will be a basis for improved disease forecast models. Using the new qPCR method, we detected *Dc* in buds, on bark and fruit mummies, suggesting that several apple tissues may serve as overwintering habitats for the fungus, in addition to fallen leaves.

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 that causes apple blotch (AB) (Wöhner and Emeriewen

2019). The disease can result in severe tree defoliation and weakened trees, and ultimately decreased yield (Sharma and Thakur 2011) and fruit quality (Park et al. 2013). AB has a significant economic impact, especially in South and East Asia. In South Korea, the loss due to AB is estimated at US$ 29.79 M (Kwon et al. 2015). In India, AB is emerging as the most destructive disease affecting apple trees, becoming a major 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 become an issue in Europe (Wöhner and Emeriewen 2019) and in the USA (Aćimović and Donahue 2018; Khodadadi and Aćimović 2019), especially in low-input orchards, for example organic orchards and untreated orchards used for juice production (Bohr et al. 2018; Hinrichs-Berger and Müller 2012; Persen et al. 2012). With the rising demand for reduced pesticide residues, the prevalence of AB may increase in conventionally managed apple orchards in the future.

To date, the biology and epidemiology of *Dc* and measures to control AB have been investigated primarily in Japan, China, Korea, and India, where the disease has been an issue since the late 1990s, while research on *Dc* and AB in Europe and the USA is nascent (Wöhner and Emeriewen 2019). *Dc* has a hemibiotrophic lifestyle (Horbach et al. 2011; Zhao et al. 2013) and the optimal conditions for infection are temperatures between 20 and 25°C and prolonged leaf wetness (Sastrahidayat and Nirwanto 2016). The minimum leaf wetness period for infection is eight hours at 15°C, and the risk of infection increases with increasing leaf wetness period and increasing temperature (Sharma et al. 2009). During the epidemic in summer the pathogen reproduces asexually by two-celled conidia. Additionally, in fall single celled microconidia (spermatia) are produced and can be found in acervuli together with conidia (Harada et al. 1974;

Lindner 2012). Initial infections in spring can be caused by conidia released from acervuli or by ascospores developing in apothecia (Wöhner and Emeriewen 2019). However, the sexual stage of *Dc* has been described only in India, Japan, and China (Gao et al. 2011; Harada et al. 1974; Sharma and Gupta 2018), but not in Europe (Hinrichs-Berger 2015; Wöhner and Emeriewen 2019), the USA or Korea (Back and Jung 2014). The contribution of the different spore types to initial infections and their mode of dispersal has not been conclusively clarified, but wind as well as splash dispersal has been reported (Dong et al. 2015; Khodadadi et al. 2022; Kim et al. 2019). *Dc* overwinters in fallen leaves (Back and Jung 2014). Alternatively, buds and twigs have been hypothesised as overwintering refugia for *Dc* (Wöhner and Emeriewen 2019). However, data on the sources of primary infections and early disease progress is lacking in Europe and the USA.

To predict the risk of primary infections by *Dc* and subsequent outbreaks of AB, it is crucial to understand the spore dispersal pattern of the fungus. Fungal spores can be quantified using spore traps combined with subsequent quantification using visual (microscopic) or molecular methods. There are various types of spore traps, and depending on the mode of pathogen dispersal, the research question or the available resources, certain spore traps are better suited. Spore traps either collect spores passively by gravitational deposition or actively by sampling specific volumes of air and capturing the spores by impaction, impingement, filtration, virtual impaction, cyclone or electrostatic attraction (West and Kimber 2015). The spores are collected either onto a solid surface like agar on a Petri dish, filter paper, a double-sided adhesive tape, petroleum jelly (Vaseline)-coated tape, slides or rods, or electrostatic plastic film, or into Eppendorf tubes (Kim et al. 2018), or more rarely into a liquid. Spores captured by spore traps have traditionally been identified and quantified by microscopy (Andersen et al. 2009; Sterling et

88 al. 1999). However, microscopy requires a spore trapping surface that can be examined under a
89 microscope and a trained investigator to identify and count the specific fungal spores accurately.
90 Newer methods combine microscopy with image recognition of spores and machine learning
91 (Basso et al. 2020; Kilin et al. 2019). Furthermore, laser-based real-time optical particle counters
92 (OPC) that detect particles in the air are being tested for real-time detection of spores in the air
93 (Basso et al. 2020; Kilin et al. 2019). Besides optical methods, molecular methods are used to
94 quantify fungal spores. Quantitative real-time polymerase chain reaction (qPCR), loop-mediated
95 isothermal amplification (LAMP) (Notomi et al. 2015; Ren et al. 2021), serial analysis of gene
96 expression (SAGE), and microarray technology (Aslam et al. 2017) are based on quantification of
97 DNA, while the enzyme-linked immunosorbent assay (ELISA) (Kennedy et al. 2000), and the
98 fluorescent antibody (FA) assay (Schneider et al. 2009) detect fungal proteins via antibodies. In
99 recent years the qPCR method has become the most frequently used quantitative molecular
100 method to detect and quantify fungal pathogens. It offers a more sensitive and specific
101 quantification compared to microscopy, as it can detect low concentrations of a fungal pathogen
102 against a background of particles and DNA from diverse organisms (Parker et al. 2014) and does
103 not depend on visual identification of a fungus spore.

104 Information on spore dispersal along with observations on disease progress and combined
105 with weather data can provide a basis to understand disease dynamics and develop disease
106 forecast models. Forecast models provide a valuable tool for the efficient application of crop
107 protection measures (Agrios 2005; Hardwick 1998). RIMPro (RIMpro, Amsterdam, The
108 Netherlands) developed a first disease forecast model for AB in Europe based on literature
109 information from Asia. Recently, *Dc* spore dispersal was investigated in Korea from June to

110 October, employing spore traps and microscopy, and a disease forecast model was developed
111 (Kim et al. 2019). However, the authors did not investigate spore dispersal in the spring, which is
112 a period critical to understanding the primary infection and disease onset and which may be
113 subject to efficacious disease management.

114 This study aimed to gain a better understanding of the early phase of AB disease in spring
115 and summer. Thus, the specific objectives were to i) develop a qPCR method that allowed the
116 quantification of *Dc* spores in spore trap samples, ii) investigate temporal and spatial aspects of
117 *Dc* spore dispersal in relation to development of AB in the field in spring and early summer, and
118 iii) identify potential sources of primary *Dc* inoculum.

119

120 MATERIALS AND METHODS

121 **Spore traps used in this study.** Conidia and ascospores of *Dc* have been reported to be dispersed
122 by wind (Dong et al. 2015; Kim et al. 2019), while conidia are also reported to be splash-dispersed
123 (Dong et al. 2015). Two trap types, the Mycotrap and the rotating-arm spore traps, were used to
124 sample airborne spores in the field (Figs. 1D, E). Additionally, apple bait plants were used as in
125 vivo spore traps (Fig. 1F). A detailed description of the spore traps is provided in Supplementary
126 Materials and Methods S1. Briefly, the Mycotrap (Siegfried et al. 1996) (Fig. 1D) is an impaction
127 spore trap and similar to the Burkard 7-day Recording Volumetric Spore Sampler, which was used
128 to capture airborne conidia of *Dc* in Korea (Kim et al. 2019). The air is sucked in horizontally
129 through a sampling orifice and impacts a trapping surface on a cylinder inside a chamber. The
130 cylinder slowly rotates, completing one turn over seven days, allowing continuous sampling for
131 the seven day period. The rotating-arm spore traps (Fig. 1E) were constructed based on the

132 description of Quesada et al. (2018) (details in Supplementary Materials and Methods S2). The
133 rotating-arm spore traps consisted of two rods connected to a battery-powered motor (spinart™
134 Lightweight Hanging Motor, 30 rpm). Each rod held a microscope slide on which was mounted a
135 strip of Vaseline coated plastic film (Supplementary Materials and Methods S3) attached with
136 25 mm foldback clips (Maul, Bad König, Germany). The rotating-arm spore traps were covered
137 with an aluminum shield as rain protection.

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

143

144 **Design and evaluation of qPCR primers and probes.** We aimed at developing a highly specific and
145 sensitive TaqMan® qPCR assay for *Dc*. The primers and hydrolysis probes designed targeted the
146 nuclear ribosomal internal transcribed spacer (ITS1) region between the 18S and 5.8S rDNA of *Dc*
147 using the program Beacon Designer (V8.16, Premier Biosoft, Palo Alto, CA, USA) with the following
148 parameters: amplicon length between 100 and 200 bp, melting temperature (TM) of primers at
149 60.0°C +/- 1.0°C, and TM of the probe at 10.0°C +/- 5.0°C above TM of the primers (Table 1). The
150 program's default settings were used for maximum ΔG of self-complementarity, 3'-end stability,
151 and % GC. The specificity of the primers and probes was confirmed in silico using the National
152 Center for Biotechnology Information (NCBI, Bethesda, MD, USA) Primer-Blast (Ye et al. 2012) by
153 alignment of different ITS gene bank accessions of *Dc.*

154 The specificity was further tested in vitro with genomic DNA of *Dc* isolates from Switzerland, Korea
155 and Japan, with apple DNA, and with DNA of different fungi, i.e. fungi within the genus
156 *Diplocarpon*, fungi causing Marssonina diseases on other hosts than apple, and fungi that are
157 common pathogens of apple. Information on the tested isolates is provided in Supplementary
158 Table S1. *Dc* isolates were cultured on potato peptone carrot dextrose agar (PPCDA) adapted from
159 Zhao et al. (2010) and containing 100 mL carrot juice, 39 g potato dextrose agar, 10 g peptone,
160 and 1 L double-distilled water. Other pathogens were cultivated on potato dextrose agar (PDA).
161 Fungal DNA from the cultures was extracted using a ZymoBIOMICS® Quick-DNA Fungal/Bacterial
162 Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendations.
163 The qPCR was designed for use with a TaqMan® probe, but we also tested whether the
164 primer pair Dc_09 could be used with a DNA intercalating fluorophore (SYBR Green).
165

166 **qPCR reaction conditions.** All primers and probes were synthesized and purified by high-
167 performance liquid chromatography (HPLC) at Microsynth AG (Balgach, Switzerland). Primers and
168 probes (Table 1) were dissolved in a TE-dilution buffer (TE-Buffer: 10^{-3} mol $\cdot L^{-1}$ Tris, 10^{-5} mol $\cdot L^{-1}$
169 Na_2EDTA, pH 8.0).

170 TaqMan® based qPCR reactions consisted of 5 µL KAPA PROBE FAST (Sigma-Aldrich
171 Chemie AG, Buchs, Switzerland), 1 µL of each primermix for *Dc* and APA9 (containing primers at
172 a concentration of 3 µM for Dc_09 and 2 µM Africa Cassava Mosaic Virus (ACMV), and the probe
173 at 1 µM each), 0 to 2 µL double-distilled water and 1 to 3 µL of template DNA in a total volume of
174 10 µL. SYBR based *Dc* specific qPCR reactions consisted of 5 µL KAPA SYBR FAST (Sigma-Aldrich

175 Chemie AG, Buchs, Switzerland), 1 µL primermix (forward and reverse primers at a concentration
176 of 3 µM each), 3 µL double-distilled water and 1 µL of template DNA in a total volume of 10 µL.
177 The qPCR assay and data analysis were performed using a CFX96 Touch Real-Time PCR Detection
178 System (Biorad, Hercules, CA, USA). The amplification and quantification conditions used were an
179 initial denaturation step of 3 min at 95°C, followed by 39 and 45 cycles of 10 s at 95°C, and 20 s
180 at 60°C for SYBR and TaqMan® qPCR, respectively. After each SYBR qPCR, a dissociation curve
181 analysis was performed by gradually increasing the temperature from 65°C to 95°C by 0.5°C per
182 cycle.

183 In specificity tests, a SYBR green qPCR assay was performed with the ITS primers ITS1F/2R
184 in addition to the *Dc* specific qPCR, which served as an amplification control detecting all tested
185 fungal species (Table 1). The reactions consisted of 5 µL KAPA SYBR FAST, 1 µL primermix (primers
186 at a concentration of 2 µM each), 3 µL double-distilled water and 1 µL of template DNA in a total
187 volume of 10 µL and were subjected to an initial denaturation step of 10 min at 95°C, followed by
188 39 cycles of 30 s at 95°, 30 s at 50°C, and a final extension step for 1 min at 72°C.

189 To visualize the PCR products, 10 µL of the qPCR reaction were loaded on a 2% Tris-
190 acetate-EDTA buffered agarose gel stained with ROTI® GelStainRed (Carl Roth GmbH, Karlsruhe,
191 Germany) and run at 50 V for 60 min. Images were captured under u.v. using an Azure Biosystems
192 c150 (Azure Biosystems, Dublin, CA, USA) gel imaging workstation.

193

194 **DNA extraction from spore trap samples and apple leaves.** To extract DNA from *Dc* conidia from
195 Vaseline coated plastic film (Mycotrap and rotating-arm trap samples, Supplementary Materials
196 and Methods S3) we used the ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit (Zymo

197 Research, Irvine, USA) following the manufacturer's protocol with two modifications: the Bashing
198 Bead® tubes from the Zymo Microprep Kit were replaced by 2 mL tubes with screw caps
199 containing 100 mg of 0.5 mm Zirconia bashing beads (N034.1 Roth, Karlsruhe, DE) and the Bashing
200 Bead™ Buffer volume was reduced from 750 µL to 550 µL.

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

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

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

219 numbers based on Cq values.

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

229 **Field sites.** *Dc* spore dispersal and AB epidemiology were investigated in an extensively managed
230 organic apple cider orchard in Rickenbach (Zurich, Switzerland, 47°33'30.5"N 8°47'29.9"E). The
231 site is characterized by a mean annual temperature of 10.0°C and mean annual rainfall of 980 mm
232 (years 2010 to 2020). The orchard was planted in 2002 to ten different apple cultivars arranged
233 in 15 tree rows with 18 apple trees in each row (Fig. 1C) and an in-row planting distance of 4 m
234 and between-row planting distance of 9 m. In 2019, the orchard was treated with organic
235 fungicides (acidified clay minerals, Myco-Sin® at 8 kg/ha, Andermatt Biocontrol Suisse AG, 6146
236 Grossdietwil, Switzerland; in a tank mix with Sulphur, Netzschwefel Stulln at 4 kg/ha, Biofa AG,
237 Münsingen, Germany) on 25 May. The cultivars 'Otava', 'Rajka', 'Rubinola', 'Topaz', and 'Boskoop'
238 received additional sulfur with lime (Curatio®, Biofa AG, Münsingen, Germany) on 11 June. In
239 2020, the orchard was treated with sulfur and clay on 27 April, 9 May, 22 May (all cultivars except

240 ‘Bohnapfel’, ‘Schneider’, and ‘Tobiässler’), and on 2 June (only ‘Otava’, ‘Rajika’, ‘Rubinola’, and
241 ‘Boskoop’). Trees with spore traps were excluded from receiving fungicide treatments in 2019
242 and 2020. In addition, in 2020, the first tree of each row was left untreated.

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

251 Weather data including rainfall, leaf wetness, and temperature were obtained from
252 weather stations close to the field sites: the Agrometeo (Agroscope, Nyon, Switzerland;
253 www.agrometeo.ch) weather station “Liebensberg”, Zurich, Switzerland (47°53'38.1"N
254 8°83'73.8"E, altitude 535 m), and the Agrarmeteorologie Baden-Württemberg
255 (Landwirtschaftliches Technologiezentrum Augustenberg, Tübingen, Baden-Württemberg,
256 Germany, www.wetter-bw.de) weather station “Bavendorf”, Germany (47°76'84.4"N
257 9°55'99.0"E, altitude 481 m).

258 **Disease assessment in the apple orchard.** From 2016 to 2020, the severity of AB was assessed
259 every one to two weeks from the onset of first symptoms through October by scoring entire trees
260 using a qualitative ordinal scale of 0 to 9 (0 = no disease symptoms; 1, only few spots, no yellow

261 leaves; 2, few yellow leaves; 3, several clusters with yellow leaves; 4, one part of the tree with
262 yellow leaves, no bare branches; 5, several parts of the tree with yellow leaves, few branches
263 bare; 6, intermediate; 7, bare-branched areas in the tree, mass of leaves with symptoms; 8
264 intermediate; 9, leaves largely dropped, tree bare).

265 The ordinal scores were used to calculate damage (in percent) based on the McKinney
266 Infection Index (I) (McKinney 1923), where I (%) = [sum (class frequency × score of rating
267 class)]/[(total number of ratings) × (maximum grade)] × 100. The scoring scale and the results for
268 2016 to 2018 have been presented previously in non-peer-reviewed journals for practitioners
269 (Schärer et al. 2019; Wöhner et al. 2019).

270 **Monitoring spore dispersal in an apple orchard.** To investigate dispersal of *Dc* conidia in the field,
271 different spore traps were installed in the Rickenbach orchard from May to July and from March
272 to July in 2019 and 2020, respectively. Exact sampling periods for each trap are provided in
273 Supplementary Tables S2 and S3. To monitor the temporal resolution of the spore dispersal, one
274 Mycotrap was placed on the ground in the previous year's AB hotspot in the orchard within a row
275 of 'Otava' trees (Figs. 1C, D). A second Mycotrap was placed in the canopy of an 'Otava' tree (2.5
276 m above ground) above the first Mycotrap. We hypothesized that primary spores originating from
277 leaf litter on the ground might be detected rather by the trap on the ground, while a trap in the
278 tree crown would catch first spores released from other sources. We also hypothesized that later
279 in the season, when the leaves are heavily infected, there would be more spores in the crown
280 than on the ground. Container-grown, two-year-old apple trees were used as bait plants and were
281 placed for periods of five to fourteen days in the orchard and subsequently incubated under rain

282 protection to assess disease development and correlate the presence of spores in the orchard
283 with actual infections (Fig. 1F). In 2019, each series of bait plants consisted of three 'Topaz', three
284 'Gala' and three 'Kiku' apple trees. In 2020, each series of bait plants consisted of five 'Topaz'
285 apple trees.

286 To observe the spatial gradients of airborne spore catches in relation to disease severity
287 in the orchard, seven rotating-arm spore traps were mounted inside and at the edge of the
288 orchard on trees with severe (3 'Otava', 1 'Topaz' tree), mild (1 'Schneider' tree) and no *Dc*
289 symptoms (2 'Rubinola' trees) at one of the lowest branches of the apple trees (1.7 m above
290 ground) and close to the trunk. Moreover, one rotating-arm spore trap was installed at the same
291 height but approximately 50 m outside the orchard perimeter. The exact location of the traps is
292 indicated in Fig. 1C. In 2019, the traps were installed only between 22 Mai and 5 June. However,
293 in 2020, we covered the entire sampling period from March to July. The spore trap samples were
294 analyzed by the TaqMan® qPCR method described above.

295 To observe the spatial gradients of splash-dispersed *Dc* conidia 12 Schott-bottles with a
296 funnel were placed within the row directly below trees as well as between the rows under the
297 open sky and outside the orchard. The spore trap positions differed regarding disease severity of
298 nearby trees. Rainwater was collected for 16 hours from 24 to 25 September 2020. It was the first
299 rainy period after seven consecutive days without precipitation. The rainfall registered for the 16-
300 hour period was 14 mm.

301

302 **Precipitation and spore number**. To assess whether spore number and precipitation are
303 correlated, a Kendall rank correlation test was performed in RStudio version 2021.09.1+372

304 (http://www.rstudio.com) using R version 4.1.2. with the spore number as dependent variable
305 and the precipitation as independent variable. Since the origin and amount of the inoculum
306 changed over time the analysis was performed for each month separately.

307 **Investigating possible sources of primary inoculum.** To understand *Dc* spore dispersal, it is
308 essential to know all sources of primary inoculum. We first studied spore dispersal from a deposit
309 of overwintered *Dc* infected leaf litter. In 2019, a Mycotrap was placed in the field on a mesh cage
310 depot filled with heavily *Dc* infested leaf litter of the variety 'Remo' at the Competence Center for
311 Fruit Crops at the Lake of Constance (KOB) (Supplementary Materials and Methods S6). It sampled
312 for fourteen days on the same piece of plastic film. Consecutive samples were collected (one
313 sample per fourteen days) from 1 February to 30 June. In 2020, a Mycotrap was placed on a similar
314 leaf litter deposit at the Research Institute of Organic Agriculture FiBL (Frick, Switzerland) and
315 sampled from 1 March to 30 June onto Vaseline coated plastic film. The leaf litter for this deposit
316 was collected in the Rickenbach orchard on 28 February 2020. Spore numbers in the samples
317 were quantified by TaqMan® qPCR as previously described.

318 We hypothesized that beside leaf litter other inoculum sources, i.e. bark, bud or fruits,
319 might be relevant for disease outbreaks. On 28 February 2020, we collected bark and bud samples
320 from six 'Otava' and six 'Rubinola' trees in the Rickenbach orchard and on 17 March 2020 from
321 six 'Topaz' trees in the KOB orchard. Bark samples were collected as described by Arrigoni et al.
322 (2018). Briefly, bark curls of 20 mm length, 5 mm width and 1 mm thickness were taken using a
323 flame-sterilized scalpel. Each bark sample consisted of a pool of 30 bark curls from a single tree's
324 upper trunk and lower branches. Ten terminal buds per tree were collected. We further searched

325 for fruit mummies hanging in the trees in the Rickenbach orchard on 28 February 2020. Five pieces
326 of the skin of each mummy (with a diameter of about 2 to 4 mm and a thickness of 2 to 5 mm)
327 were pooled. Samples were lyophilized and ground in a mixer mill MM 200 (Retsch GmbH, Haan,
328 Germany) at 30 Hz for 30 s. A sample of 100 mg of the ground powder (or as much as was
329 available) was subject to DNA extraction using a NucleoSpin™ DNA Stool Kit (Macherey-Nagel,
330 Oensingen, Switzerland). We found only one sample of overwintered leaves hanging in a tree.
331 This sample was processed the same way but without prior lyophilization. Samples were assessed
332 for the presence of *Dc* DNA by the TaqMan® qPCR assay described above.

333 Finally, to assess whether the identified DNA on bark, buds and fruit mummies originates
334 from viable and infectious fungal tissues, we tried to infect apple leaves in the laboratory from *Dc*
335 PCR positive bud, bark, fruit samples. 400 to 500 mg of fruit, bark, and bud tissue were cut into
336 small pieces and wetted with 3.5 mL of Volvic® natural spring water (n=3). Each of the samples
337 was distributed onto three apple leaves. The experiment was performed twice, once with
338 container grown 'Topaz' trees and once with 'Topaz' apple seedlings. As controls, three apple
339 leaves were incubated with laboratory infected apple leaves with *Dc* acervuli (positive control)
340 and healthy apple leaves (negative control) processed the same way as described for bark, bud,
341 and fruit samples. In addition, three leaves were inoculated with pieces of a *Dc* positive leaf that
342 was found in the tree crown after winter. The plants were incubated at 20°C with a 16/ 8 h
343 day/night cycle. The first four days the relative humidity was 100 %, followed by 50 % for the rest
344 of the experiment. Symptoms were assessed after three weeks. Leaves showing any sort of spots
345 or necrosis were tested with the *Dc* qPCR.

346 **Investigation of fruit infections.** Apples with symptoms of AB were collected in September of
347 2020 and 2021 and stored at 4°C until March the following year, when acervuli had developed.
348 The acervuli were examined under a stereo microscope M205C (Leica Microsystems Switzerland,
349 Heerbrugg, Switzerland). The apple tissue containing acervuli were stained with cotton blue in
350 lactic acid and examined with a Leica DM2000 LED microscope (Leica Microsystems Switzerland,
351 Heerbrugg, Switzerland). Images of the apple tissue containing acervuli were captured using a
352 Jenoptic Gryphax Subra camera (Jenoptic AG, Jena, Germany). Conidia formed on cool-stored
353 fruits were tested for their infectivity to apple leaves. To this end, necrotic lesions with acervuli
354 were cut and added to 1 mL Volvic ® natural spring water in 2 mL Eppendorf tubes, vortexed for
355 30 s and the resulting spore suspension was used to inoculate leaves of six 'Topaz' seedlings.
356 Twelve drops of 5 µL suspension were pipetted onto two leaves per seedling. The plants were
357 incubated as described above for infection experiments with bark, bud and fruit mummies.

358

359 **RESULTS**

360 **New qPCR allows quantification of *D. coronariae* conidia.** To quantify *Dc* conidia in spore trap
361 samples, we developed a new TaqMan® qPCR method with the primers and probes Dc_09 (Table
362 1). Different primers and hydrolysis probes targeting the ITS gene of *Dc* were tested. Among them,
363 primers and probe Dc_09 performed best (E= 94.2 %, R^2= 0.995) also when compared to the
364 published primer Mc_ITS (Oberhänsli et al. 2014) (E= 83 %, R^2 = 0.88). Specificity tests for primer
365 Dc_09 revealed sound amplification of DNA from different isolates of *Dc* (Fig. 2A), but neither
366 amplification with DNA of other fungal species nor with apple DNA (Fig. 2A, Supplementary Table

367 S4).

368 The amplification of the qPCR for conidial counts on Vaseline coated plastic film, which
369 was the spore trapping surface used for field samples, exhibited a linear response with an
370 efficiency of 89.1 % (R^2 = 0.997) (average of seven qPCR runs) (Fig. 2B). The qPCR assay allowed
371 the consistent detection of as few as ten conidia; however, the amplification was unreliable with
372 three conidia per sample. Therefore, the limit of quantification is considered to be 3 to 10 conidia.
373 The SYBR Green based hydrolysis probe assay revealed similar sensitivity and specificity as
374 the TaqMan assay (Supplementary Fig. S1). However, we used the TaqMan qPCR for our field
375 experiments since it allowed multiplexing of the *Dc* specific qPCR with the APA9 specific qPCR
376 (plasmid standard), which we used to normalize the *Dc* quantification data with the DNA
377 extraction efficiency.

378 **Apple blotch disease progress in two Central European apple orchards.**

379 At the Rickenbach field site and at the KOB field site disease progression on 'Topaz' started
380 with the appearance of first signs of the pathogen (i.e. visible formation of acervuli) and
381 symptoms of AB (yellowing of the leaf), which varied between years, but occurred at the latest in
382 August in both orchards and all years. Progress of AB was generally characterized by a steady
383 increase in severity. However, differences in disease severity were observed between years. At
384 KOB, for example, AB was more severe in 2017 compared to other years (Fig. 1A). Rainfall was
385 more frequent from June through October in 2017, i.e. 78 days of rainfall compared to between
386 36 and 57 days in the other years (Supplementary Fig. S2). At both sites, 2018 was characterized
387 by an exceptionally hot and dry summer (only 36 and 33 days with rain from June to the end of

388 September at KOB and Rickenbach, respectively) (Supplementary Figs. S2, S3) resulting in lower
389 severity of AB (Fig. 1A). At Rickenbach, AB was most severe in 2019 and 2020; which was not the
390 case at KOB (Fig. 1A).

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

396 **Monitoring dispersal of *Dc* conidia in the field.** In 2017 and 2018, the first clearly visible
397 symptoms on 'Otava' trees developed by mid-June and early July, respectively. Therefore,
398 considering an incubation period of about three weeks (Lee et al. 2011), the first infections were
399 expected to occur towards late May.

400 In 2019, very large numbers of spores were detected by the Mycotrap on the ground
401 during the first half of May (Fig. 3A). Subsequently, spore numbers generally decreased, except
402 for a peak in the first week of June. In May, leaves with spotting indicative of AB were collected
403 and tested for *Dc* by qPCR, revealing the first *Dc* positive leaf on 17 May. The first unambiguous
404 symptoms were observed three weeks later, on 7 June (Fig. 3A). The next spore peak was
405 detected on 24 June after a rainy period. At that time, 'Otava' trees already showed severe leaf
406 yellowing, indicating the emergence of secondary inoculum. In July, large spore numbers were
407 recorded in the air on most days. The second Mycotrap in the crown of an 'Otava' tree detected
408 more spores than the Mycotrap on the ground, except on 24 and 25 June (Supplementary Fig.

409 S4).

410 The first apparent symptoms on bait plants developed between 17 and 22 May (Fig. 3A).
411 From 22 May on, at least one bait plant showed typical AB symptoms of leaf yellowing, except for
412 the period from 3 to 7 June. The most severe leaf yellowing and leaf drop were observed on the
413 bait plants exposed between 7 and 13 June, followed by those exposed between 13 and 19 June.

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

421 A second peak in spore production was detected on 5 June by the spore trap in the tree
422 crown. As in 2019, spores were detected in the air almost every day by both traps from mid-June
423 to the end of July (Figs. 3, 4). Numbers of spores varied, but no significant correlation was found
424 between spore numbers and precipitation (Supplementary Fig. 6). In Mycotrap samples in early
425 June (lower trap: 5 to 19 June, upper trap: 31 May to 9 June), we experienced technical problems
426 resulting in poor DNA extraction efficiency. In cases with signal by the *Dc* qPCR, the low efficiency
427 could be taken into account by normalization with the internal plasmid standard. However, in
428 cases where there was no signal *Dc*, we were unable to conclude whether the zero value was due
429 to no spores or the low efficiency precluding detection. Thus, for some days during the first half
430 of June, *Dc* spores might have been present in the air, although no catch is indicated (Figs. 3B, 4).

431 In 2020, all bait plants placed in the orchard after 22 May developed AB symptoms. From
432 22 May to 5 June, bait plants exhibited approximately 50 to 80% of leaves symptomatic, and after
433 5 June incidence of leaves exhibiting AB exceeded 90% (Supplementary Fig. S5).

434 **Dispersal of conidia of *Dc* with distance from an inoculum source.** Rotating-arm spore traps
435 detected the first spores after 22 May in 2019 and 2020. Spore numbers detected in rotating-arm
436 spore traps were generally lower than those captured in the Mycotrap samples (Table 2, Figs. 3,
437 4). The highest spore numbers were generally detected in traps on 'Otava' trees, which exhibited
438 severe symptoms of AB, while almost no spores were detected in traps on 'Rubinola' trees.
439 Despite being only two rows (18 m) apart from highly diseased trees, the 'Rubinola' trees were
440 virtually free of AB, although some leaf yellowing and premature leaf fall was observed on
441 'Rubinola' trees. The symptoms on 'Rubinola' were distinctly different from typical AB, and when
442 such leaves were tested by qPCR no *Dc* DNA was detectable (n = 7). Furthermore, no spores were
443 detected in the trap 50 m beyond the orchard edge with one exception: during the period from
444 22 May to 3 June 2019 spores of *Dc* were detected under 'Rubinola' trees and outside the orchard.
445 A strong wind event with wind speed up to 37 km/h combined with rain on 21 May may have
446 transported some infected leaves towards the 'Rubinola' trees and outside the orchard explaining
447 these results.

448 Besides the dispersal of *Dc* spores via the air, rainwater splashes might be an important
449 factor for spreading the disease within a tree. Therefore, we collected rainwater below highly
450 infected trees ('Otava', 'Topaz'), below symptomless trees ('Rubinola'), between the rows under
451 the open sky and outside the orchard during a rainy period in September 2020. We detected

452 several hundred spores per mL rainwater below 'Otava' trees, fewer spores between rows, and
453 almost no spores below non-infected 'Rubinola' trees or outside the orchard (Supplementary Fig.
454 S6).

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

463 While all bark and bud samples of 'Rubinola' were negative for *Dc*, the majority of bark
464 and bud samples from 'Topaz' and 'Otava' trees were tested positive for *Dc* (Table 4). Bark
465 samples exhibited high Cq values close to the limit of detection corresponding to low amounts of
466 DNA. Bud samples contained up to 10^2 to 10^4 times higher levels of *Dc* DNA than bark samples.
467 The highest amount of *Dc* DNA was detected in a bud sample of an Otava tree with a Cq value of
468 25.6 (Table 4), which corresponds to the DNA of 10^4 conidia.

469 In spring 2020, four out of five fruit mummies, which were collected from trees with strong
470 AB infestation in 2019, were tested slightly positive (Cq values between 33.65 and 40.38) (Fig. 5C,
471 Supplementary Table S5), while fruit mummies from trees without AB symptoms the previous
472 year were negative (Supplementary Table S5). For comparison, a leaf sample still hanging in a

473 susceptible 'Florina' tree after the winter of 2019 was *Dc* positive (Cq, 25.83).

474 Attempts to infect apple leaves in the laboratory by applying wetted pieces of bark, buds,
475 fruit mummies and leaves from previously AB symptomatic trees did not result in successful
476 infections (data not shown). But *Dc* infected fruits can serve as an inoculum source for infection
477 of leaves after six months of storage. In fall 2020 and 2021, *Dc* infections were observed on 'Otava'
478 fruits in the Rickenbach orchard (Fig. 5A). In the following spring, after storing fruits at 4°C for six
479 months, conidia formed (Figs. 5B, D-I), and the conidia infected the leaves of apple seedlings in
480 the laboratory (Supplementary Fig. S7).

481

482 DISCUSSION

483 **Development of a *D. coronariae* specific qPCR.** To date, spore trap catches of *Dc* conidia have
484 been quantified using microscopy (Kim et al. 2019). However, microscopy is labor intensive and
485 identification of the *Dc* spores in field samples may be challenging. We developed a qPCR method
486 to provide a very sensitive means for quantification of as few as ten conidia per sample, which is
487 in a similar range to qPCRs developed for other fungi (Calderon et al. 2002; Dvořák et al. 2015;
488 Luchi et al. 2013). The qPCR method further allows diagnosis of
489 AB prior to developing unambiguous visual symptoms. Early diagnosis is especially useful when
490 small necrotic lesions appear that are indistinguishable from symptoms from other causes. In
491 summary, we established a new qPCR method, which allowed the quantification of *Dc* spores in
492 spore trap samples, and that can be used to detect initial infections in the field, and that enabled
493 the identification of *Dc* DNA in various apple organs.

494 qPCR, though it requires several processing steps, is a rather fast method and allows the
495 processing of many samples concurrently. However, it has the disadvantage to only allow
496 conclusions regarding the presence of DNA, but not regarding the fungal organ (e.g. mycelium,
497 ascospore, conidia, spermatia) or its viability and infectious potential. Therefore, visual
498 assessments and infection experiments are needed to understand how *Dc* exists on bark, bud and
499 fruit samples and the role of the tree organs in the fungal life cycle.

500 **Timing of primary infections.** To determine the timing of primary infections by *Dc*, we combined
501 data on airborne spore catches with symptom development both in the field and on a consecutive
502 series of exposed bait plants. In the first three study years (2016-2018), clear AB symptoms were
503 observed, the earliest showing in June. However, testing leaves with ambiguous necrotic spots by
504 qPCR in 2019 and 2020 revealed that the first leaves of highly susceptible varieties were already
505 infected with *Dc* in May (17 May 2019 and 8 May 2020). The infections were most likely caused
506 by spores released in late April or early May. The first spore peaks were detected in the orchard
507 in early May in both 2019 and 2020. In 2019, spore traps were installed on 9 May 2019. Thus, it is
508 possible that spores were already present one or two weeks earlier. In 2020, the experiment was
509 initiated at the end of February, and the first clear spore peak was detected by the Mycotrap on
510 the ground on 5 May. We conclude that the spores collected in early May likely caused the first
511 infections in the field since the first bait plants in the field that developed symptoms were
512 exposed from 24 April to 8 May 2020.

513 In line with the results of the spore traps in the orchard, the spore traps located above leaf
514 litter caught conidia of *Dc* at the end of April and early May in both study years. The few spores

515 detected above the leaf litter and in the field in March 2020 were released before bud break, and
516 we, therefore, conclude that they were irrelevant to primary leaf infections.

517 Our qPCR method does not distinguish between conidia and ascospores, but apothecia of
518 *Dc* have not yet been observed in Europe (Wöhner and Emeriewen 2019), indicating it is unlikely
519 they were the spore type captured. Although not yet observed, the importance of ascospores as
520 primary inoculum in Europe should be subject to further research. A recent population genetic
521 study suggested a mixed sexual and asexual reproduction of *Dc* in Europe, although *Dc*
522 populations in Europe are genetically homogenous, clonal and dominated by a few multi-locus
523 genotypes (Oberhänsli et al. 2021).

524 The first spore peaks of the season were observed at the end of, or shortly after rainy
525 periods, whereas no spores were detected during dry periods before rain events, e.g. April 2020.
526 The result indicates that rain might be required for the release of conidia of *Dc* from leaf litter,
527 and acervuli might need a wetting period prior to release of conidia. This hypothesis may explain
528 the slightly negative correlation found for May 2019 between daily spore catches and
529 precipitation (Supplementary Table S6).

530 Based on our results, primary infections of apple with *Dc* occur in Central Europe in late
531 April or early May after periods of rain, which is earlier than was previously assumed (Sutton et
532 al. 2014). Moreover, the spore catches in March indicate that *Dc* spore discharge may occur
533 before April and, depending on weather conditions and phenological stage of the host, infections
534 could be possible. Knowing the timing of primary infections can be valuable as a basis for
535 management actions to preventing epidemics of AB. While protection strategies against AB in
536 Europe are currently focused on the summer months (Hinrichs-Berger 2015), prevention of

537 primary infections in the early spring might be an important additional step for more efficacious
538 management strategies in the future.

539 **Leaf litter and other sources of primary inoculum.** In addition to the timing of primary infections,
540 we were also interested in the origin of the primary inoculum. Several studies report that conidia
541 inside acervuli overwinter in leaf litter (Back et al. 2015; Dong et al. 2015; Gao et al. 2011; Goyal
542 et al. 2018; Lee et al. 2011; Sastrahidayat and Nirwanto 2016; Sharma et al. 2009). This is in
543 accordance with our finding of *Dc* spores above leave litter deposits in spring and also with the
544 observation that in May, most spores were captured in the Mycotrap on the ground, and not in
545 the Mycotrap in the tree canopy.

546 Small quantities of *Dc* DNA were detectable on fruit mummies hanging in the trees at the end
547 of February, and acervuli with infectious conidia were produced on *Dc* infected fruits after
548 overwintering at 4°C. Moreover, considerable quantities of *Dc* DNA were detected on buds, and
549 small amounts of *Dc* DNA were found on the bark of trees that had AB the previous season. All
550 these organs are reported as overwintering sites for other apple pathogens, including *Monilia*
551 spp. on fruit mummies, *Podosphaera leucotricha* in buds and *Neofabraea* spp. on bark (Sutton et
552 al. 2014). Moreover, *D. rosae*, a close relative of *Dc* causing black spot disease on roses,
553 overwinters, in addition to leaf litter, on bud scales and stems (Cook 1981). To date, it is unclear
554 what the survival structures of *Dc* on bark and buds are (e.g. mycelium, spores), and whether they
555 are infectious. The fact that we could not cause leaf infections from bud, bark or fruit mummy
556 tissues may be due to technical issues, and the role of the various apple tree organs for
557 overwintering of *Dc* requires further investigation. *Dc* present on buds and wood in propagation

558 material could be exchanged between nurseries across Europe and may explain the rapid spread
559 of this disease to many European apple production areas since its first detection in Northern Italy
560 (Oberhänsli et al. 2021). Our observation that conidia produced on fruits after six months in
561 storage at 4°C are able to infect apple leaves provides an indication that fruit-derived conidia
562 might represent an additional source of primary inoculum at least on a local scale. Moreover, our
563 description of fruit infection adds to our very limited knowledge regarding the role of fruit
564 infections in the epidemiology of AB (Wöhner and Emeriewen 2019).

565 **Timing of secondary infections and epidemic development.** By the end of May (2020) or the
566 beginning of June (2019), the first leaves that were producing acervuli and conidia of *Dc* were
567 observed, initiating the secondary phase of infection. In contrast to the first major peak of conidia
568 captured in May 2020, the second major peak (5 June) was not detected by the Mycotrap on the
569 ground but by the Mycotrap situated in the tree canopy. We hypothesize that the conidia
570 produced in early May originated from overwintered leaf litter, resulting in higher spore counts
571 in the Mycotrap on the ground. But in June, the conidia originated from the recently infected
572 leaves in the tree canopy, resulting in a higher spore count in the Mycotrap situated in the tree
573 canopy. In support of the hypothesis, the rotating-arm spore traps in the tree crown did not
574 capture conidia before the end of May.

575 From June onwards, damage caused by AB steadily increased in the orchard (Fig. 1A, B).
576 From the middle of June to the end of the experiment, high numbers of spores were detected
577 irrespective of rain events, and no correlation was found between daily precipitation and caught
578 spores in June and July for both years (Supplementary Table S6). This contrasts with our

observation of the early spore peaks, which occurred at the end of the rain periods and the observations by Kim et al. (2019). They investigated dispersal of conidia of *Dc* from June to October 2013 and 2015 in Korea and reported spore dispersal peaks during and two days after rain events compared to dry periods. In that study, disease incidence and total spore counts were much lower than we observed at our field site, which might explain some of the differences. Based on our results, we hypothesize that primary inoculum may require water to ripen and discharge, while secondary inoculum are also released on dry days. Lab experiments indicate that *Dc* conidia can survive for several days under dry conditions after landing on a susceptible leaf, and cause infection once the leaf becomes wet (Clémence Boutry et al., unpublished data). If so, all secondary inoculum result in risk of infection whenever leaf wetness occurs.

The development of AB is affected by environmental factors (Li et al. 2011). Rainfall and the subsequent 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). Under controlled conditions, the minimum leaf wetness period for infection is 8 h at 15°C and decreases to 4 h with temperatures between 20 and 25°C (Sharma et al. 2009). Moreover, the incubation period can vary depending on the weather conditions. At a temperature range of 15 to 25°C and 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 return from the orchard, with a 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, the first necrotic spots indicative of AB and the more developed symptoms were observed earlier and more rapidly than in 2019. The faster disease progression in late spring 2020 was likely due to higher May

601 temperatures compared to the temperatures in 2019.

602 **Spore dispersal in relation to distance from an inoculum source.** Rotating-arm spore traps placed
603 in the orchard revealed high spore concentrations in AB-diseased trees, but no or few spores in
604 trees without symptoms. The observation could indicate that conidia of *Dc* are not dispersed over
605 large distances. However, as noted there were generally low spore counts captured by the
606 rotating-arm traps compared to the Mycotrap, indicating that the rotating-arm traps were much
607 less efficient in capturing spores in the field (so spores might have been there but the lower
608 sensitivity of the trap precluded detection). Thus, wind might transport some spores over larger
609 distances than we observed. Besides wind dispersal, dispersal in rain splash may contribute to the
610 spread of *Dc* within the tree canopy, as indicated by the very high spore counts in rainwater below
611 highly symptomatic trees (Supplementary Fig. S6).

612

613 ***Dc* life cycle.** Based on our results we contend that in Europe, the fungus overwinters primarily in
614 infected leaf litter (Fig. 6). However, bark, buds and fruit mummies might be alternative sources
615 of primary inoculum since we detected *Dc* DNA on these organs after winter in previously AB-
616 symptomatic trees. A few spores appear to be released in March, but primary inoculum infections
617 start in late April or early May, depending on the weather conditions. Secondary inoculum spores
618 developed in late May or early June in our field sites, which was within three to four weeks of the
619 primary infections. In May, spore peaks were mainly found at the end of, or after periods of rain;
620 whereas spore load in the air was generally high from the middle of June to the end of July. Thus,
621 every wet period in summer represents a risk for infection. The exact mode of pathogen dispersal

622 has not yet been conclusively determined. Conidia are probably dispersed by wind and rain splash
623 over larger distances; for the spread of *Dc* within a tree canopy or between neighboring tree
624 canopies, rain splash could be a major dispersal agent.
625
626 In summary, we developed a sensitive and specific qPCR method to detect and quantify
627 conidia of *Dc* captured in spore traps, which can be combined with the quantification of other
628 apple pathogens, e.g. *Venturia inaequalis* (Meitz-Hopkins et al. 2014), in spore trap samples from
629 apple orchards in the future. Our data contributes to our understanding of AB, especially the
630 timing of early spore dispersal and infections. The information can help to improve disease
631 forecast models for AB, such as the one by RIMpro (RIMpro, Amsterdam, The Netherlands), and
632 provide a basis for early disease management. However, more research is needed to test whether
633 preventing primary infections will reduce disease progress over the season, thereby reducing the
634 need for fungicide applications in the summer. Finally, the presence of *Dc* DNA on bark, buds and
635 fruit mummies indicates that the role of overwintering inoculum on these organs and the
636 anthropogenic dispersal of the pathogen on propagation material should be further investigated.
637

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815 apple leaves by *Diplocarpon mali*. European Journal of Plant Pathology 136:41-49.
816

817
818 TABLE 1. Primers used in the qPCR to detect DNA of *Diplocarpon coronariae*.

Primer name[a]	**Sequence (5' to 3')**[b]	**Mt (°C)**[c]	**Amplicon size (bp)**	**Reference**
Dc_09_F	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_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		
ITS1F	CTTGGTCATTTAGAGGAAGTAA	53.2	Dependent on target species	(Gardes and Bruns 1993; White et al. 1990)
ITS2R	GCTGCGTTCTTCATCGATG	57.2		

819 [a] F, forward primer; R, reverse primer; P, fluorogenic hydrolysis probe.
820 [b] FAM, 6-Carboxyfluorescein; ROX, Carboxy-X-rhodamine; BHQ; black whole quencher
821 [c] Mt, melt (annealing) temperature.
822
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TABLE 2. Numbers of conidia of *Diplocarpon coronariae* captured using rotating-arm spore traps in an apple orchard in Switzerland.[a]

Cultivar	Trap number	Dates (2019)[b]				Dates (2020)[c]						
		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

[a] Rotating-arm spore traps were distributed within an apple orchard (Rickenbach, Switzerland) comprising 15 rows planted with different cultivars. Traps were placed in a low branch of the apple trees (about 1.7 m). Trap Nr. 2 was installed below a 'Schneider' tree, which was at the edge of an 'Otava' tree row. The location of the traps within the orchard is depicted in Fig. 1C. DNA of *D. coronariae* in the spore trap samples was quantified by TaqMan qPCR using primers and probe Dc_09 and spore numbers were calculated based on a standard curve with known amounts of *D. coronariae* conidia.

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

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

840
841 TABLE 3. The number of conidia of *Diplocarpon coronariae* collected in a Mycotrap spore trap above leaf litter on an
842 orchard floor in 2019 and 2020.[a]

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

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

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

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

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

Figures

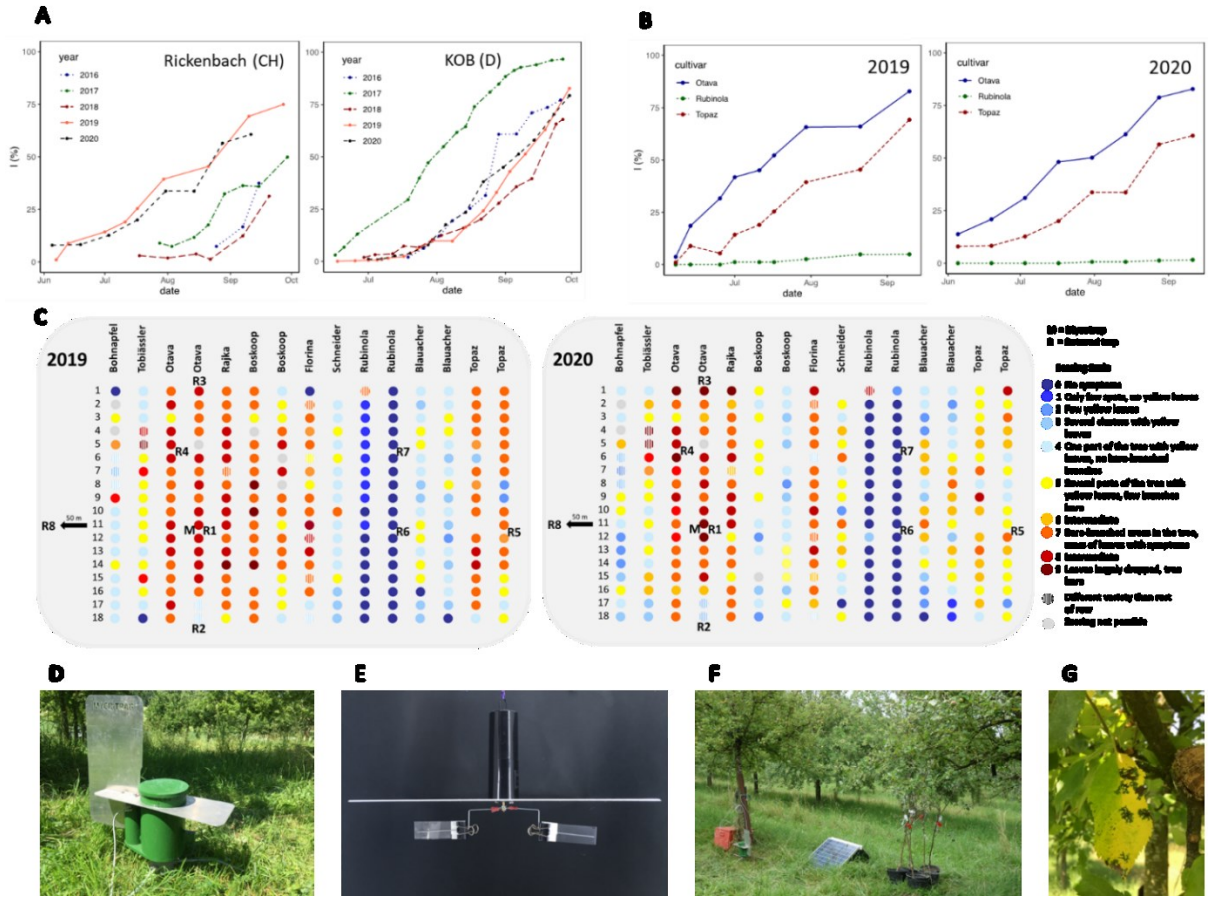

Fig. 1. Apple blotch (AB) development in two apple orchards and the location of the spore traps. **A, B)** AB disease severity as percent damage according to the McKinney Index (I) (McKinney 1923). **A)** 'Topaz' trees in two orchards (Rickenbach, Switzerland and KOB, Germany) were regularly scored for severity of AB from June to October for five consecutive years (Rickenbach, 36 trees; KOB, 120 trees). **B)** AB development on 'Topaz' (36 trees), 'Rubinola' (35 trees), and 'Otava' (33 trees) in the Rickenbach orchard in 2019 and 2020. **C)** AB disease scoring in the Rickenbach orchard by middle of September 2019 and 2020. The orchard is planted with tree rows of ten different cultivars. Each dot represents one tree. The color of the dot indicates the disease score of the tree. The placement of the Mycotrap and rotating-arm spore traps is indicated by the

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

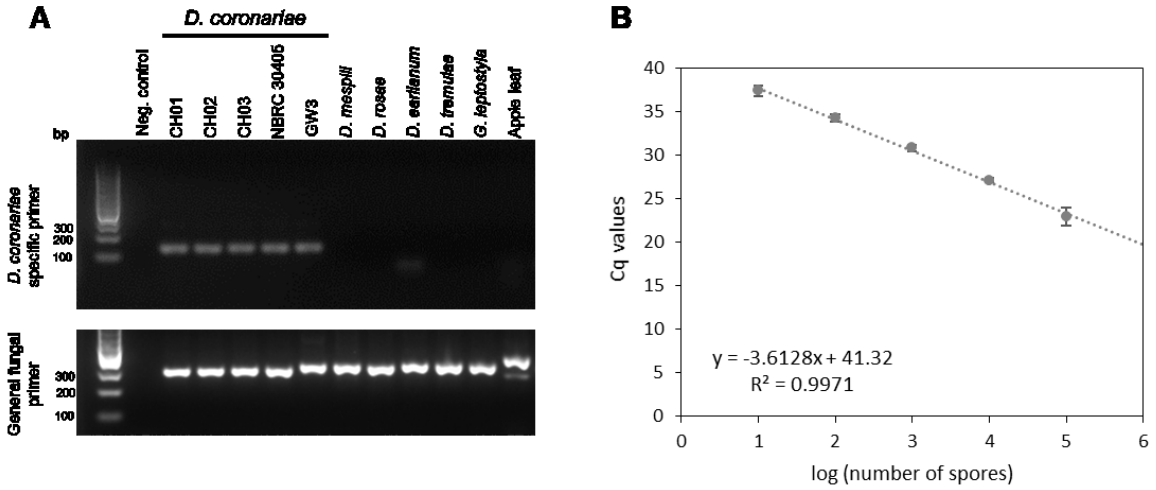

Fig. 2. *Diplocarpon coronariae* specific qPCR. **A)** Specificity test of Primer Dc_09. A PCR was performed with DNA from different *D. coronariae* isolates, related *Diplocarpon* species (*D. mespili*, *D. rosae*, *D. earlianum*), and with DNA of other pathogens causing Marssonina diseases (*Drepanopeziza tremulae* and *Gnomonia leptostyla*). A 2 % TAE agaraose gel stained with ROTI® GelStainRed (the ladder marker is a 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 was done from the Mycotrap and rotating arm spore traps, i.e. using a ZymoBIOMICS® Quick-DNA Fungal/Bacterial Microprep Kit. Samples were spiked with an APA9 plasmid for normalization of DNA extraction and normalized Cq values are depicted. Error bars represent the standard deviation of the mean of seven qPCR runs. The mean efficiency was 89.1 %.

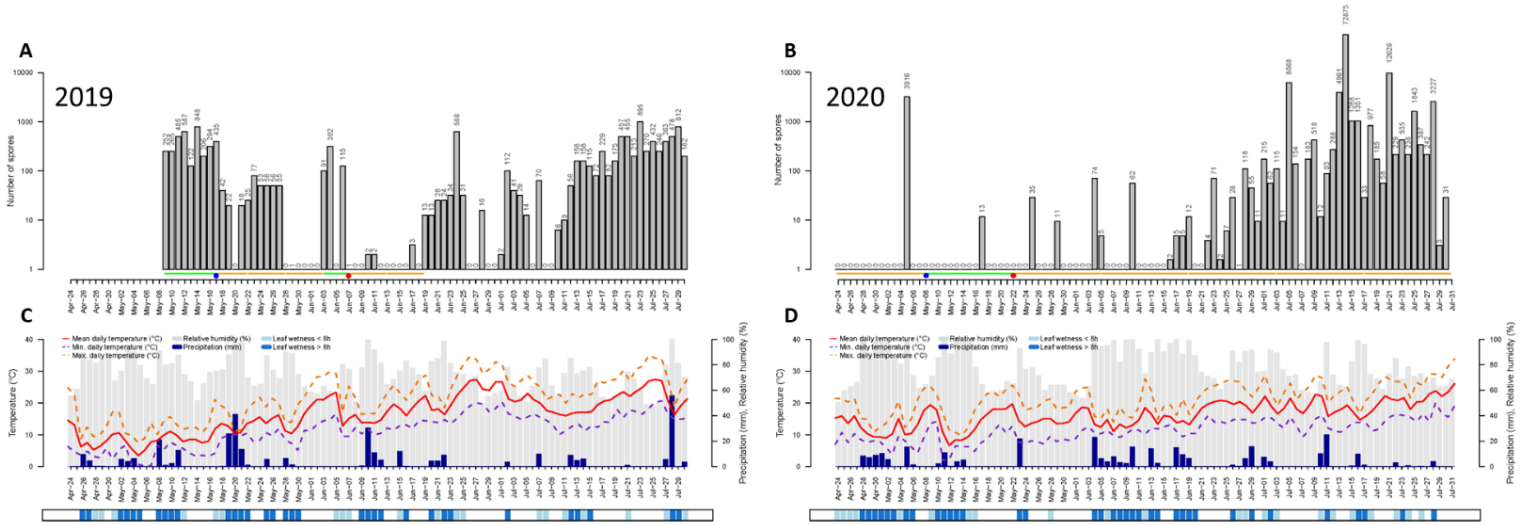

Fig. 3. *Diplocarpon coronariae* (*Dc*) spore dispersal in 2019 **(A)** and 2020 **(B)**.Daily spore numbers of *Dc* captured by a Mycotrap placed within an 'Otava' tree row of apple trees. The number of spores was calculated based on normalized Cq values from the TaqMan qPCR assay with primer Dc_09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

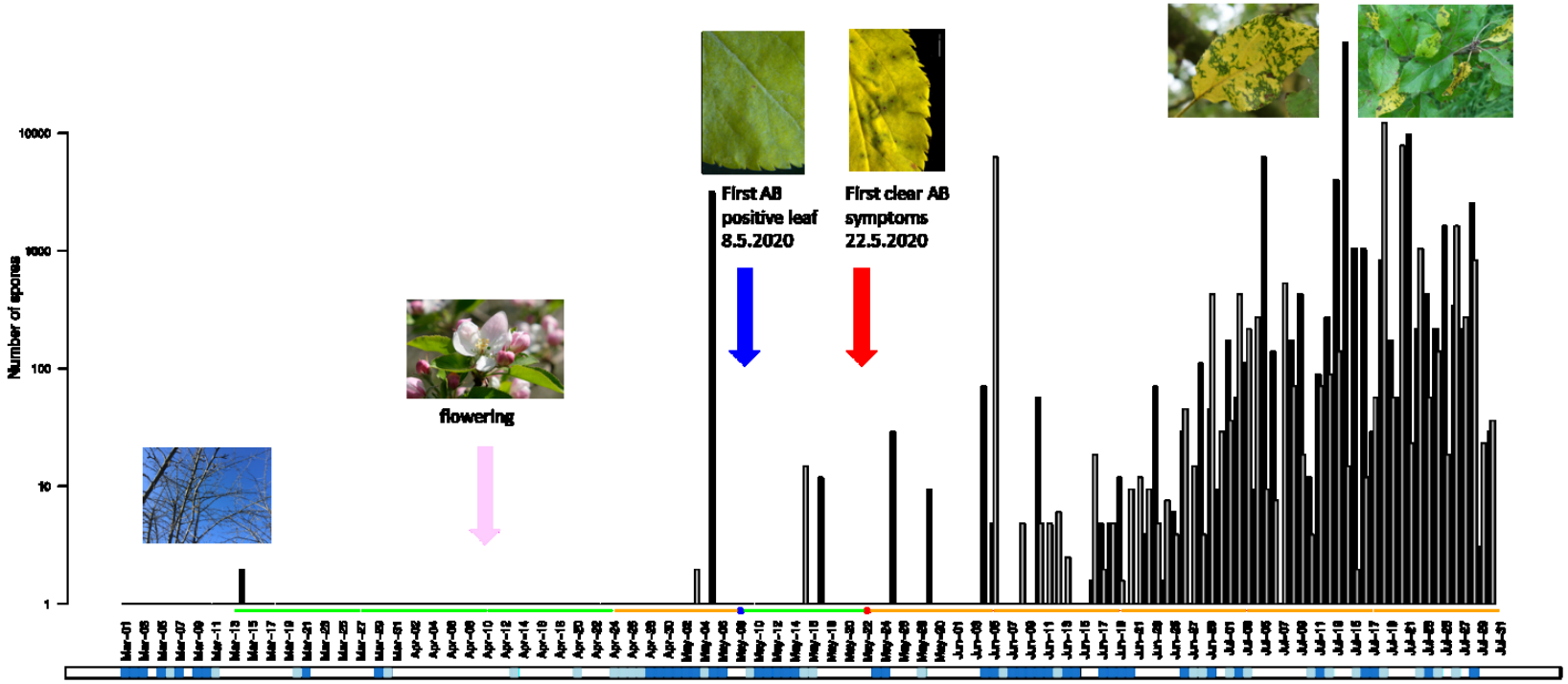

Fig. 4. *Diplocarpon coronariae* (*Dc*) spore dispersal on the ground and in the tree crown in 2020. Daily spore numbers of *Dc* captured by the Mycotraps placed on the ground within an 'Otava' tree row of apple trees (black bars) and within the tree canopy (grey bars). The number of spores was calculated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

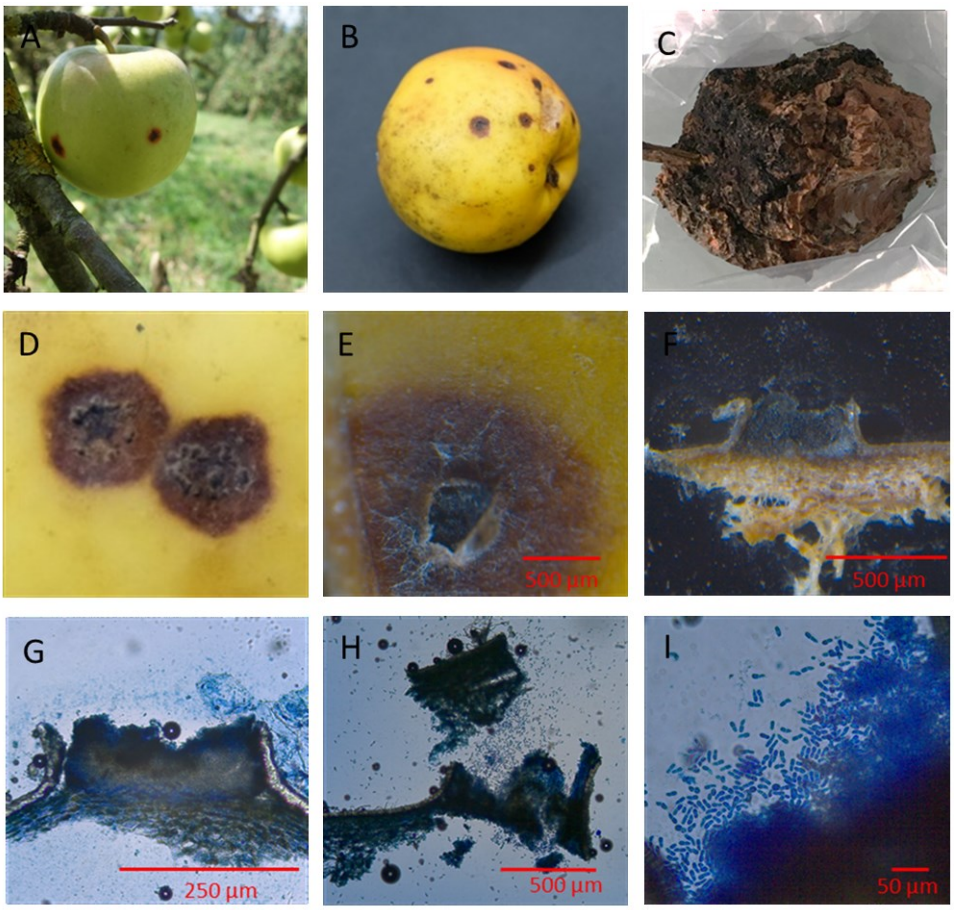

Fig. 5. Fruit infections with *Diplocarpon coronariae (Dc)*. A) 'Otava' apple with apple blotch symptoms hanging in a tree in September 2020 (Rickenbach, Zurich, Switzerland). B) *Dc* infected 'Otava' apple after 6 months of storage at 4°C. C) 'Florina' fruit mummy found hanging in a tree in February 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 at 4°C 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).

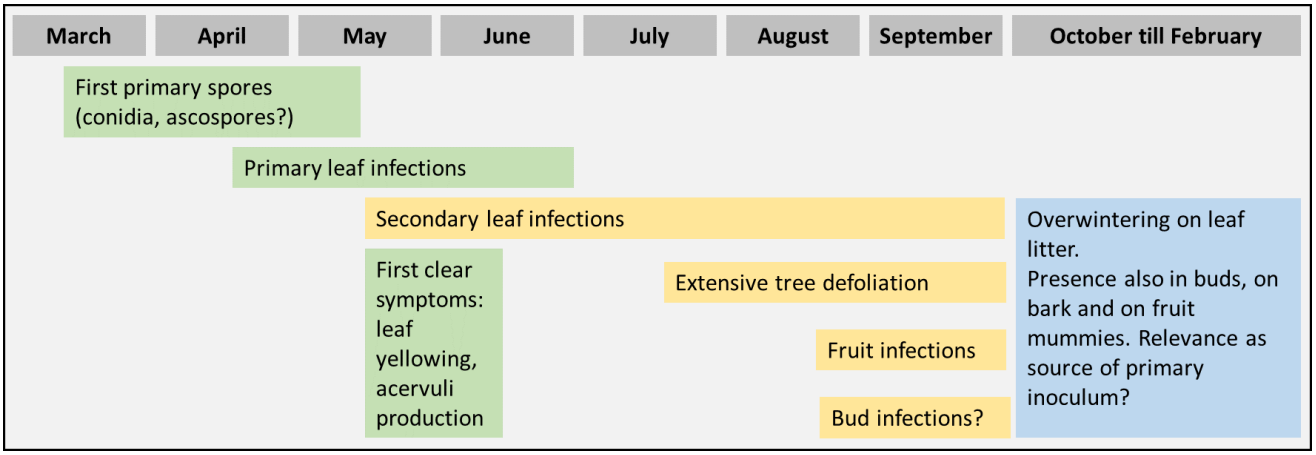

Fig. 6. The proposed infection cycle of *Diplocarpon coronariae* (*Dc*) in Central Europe. Based on previous knowledge and the data we presented in the current study, we hypothesize primary spores (conidia since no ascospores have been reported in Europe to date) are released in early spring and cause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