Identification and pathogenicity ~~of groups~~ of *Alternaria* species associated with ~~a recent surge in~~ leaf blotch disease and premature defoliation in French apple orchards

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Abstract

Leaf blotch caused by *Alternaria* spp. is a common disease in apple-producing regions. The disease is usually associated with one phylogenetic species and one species complex, *Alternaria alternata* and *Alternaria arborescens* species complex (*A. arborescens* SC), respectively. Both taxa may include the *Alternaria* apple pathotype, a quarantine or regulated pathogen in several countries. The apple pathotype is ~~characterised~~ characterized by the production of a host-selective toxin~~s~~ (HST~~s~~) which ~~are~~ is involved in pathogenicity towards the apple. A cluster of genes located on conditionally dispensable chromosomes (CDCs) ~~are~~ is involved in the production of ~~these~~ this HST~~s~~ (namely *AMT* in the case of the apple pathotype). Since 2016, leaf blotch and premature tree defoliation attributed to *Alternaria* spp. have been observed in apple-producing regions of central and south-eastern France. Our study aimed to identify the *Alternaria* species involved in apple tree defoliation and assess the presence of the apple pathotype in French orchards. From 2016-2018, 166 isolates were collected and identified by multi-locus sequence typing (MLST). This analysis revealed that all these French isolates belonged to either the *A. arborescens* SC or *A. alternata*. Specific PCR detection targeting three genes located on the CDC did not indicate the presence of the apple pathotype in France. Pathogenicity was assessed under laboratory conditions on detached leaves of Golden Delicious and Gala apple ~~varieties~~ cultivars for a representative subset of 28 *Alternaria* isolates. All the tested isolates were pathogenic on detached leaves of cultivars Golden Delicious and Gala, but no differences were observed between the pathogenicity levels of *A. arborescens* SC and *A. alternata*. However, the results of our pathogenicity test suggest that cultivar Golden Delicious is more susceptible than Gala to Alternaria leaf blotch. Implications in the detection of the *Alternaria*

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50 apple pathotype and the taxonomic assignment of *Alternaria* isolates involved in Alternaria leaf

51 blotch are discussed.

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52 Introduction

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54 *Alternaria* spp. are ubiquitous fungi comprising ~~more than~~ approximately 300 different species

55 (Simmons 2007; Woudenberg et al. 2013). ~~They have~~The genus has different ~~lifestyles (lifestyle~~

56 ~~in fungi is a term related to plant-fungal interactions in the form of endophytic, pathogenic,~~

57 ~~mycorrhizal, and is widely used by mycologists. Please refer to Rai &~~ (Agarkar ,2016; Barelli et

58 al., 2016; Ohm et al, 2012) and can be isolated from a large number of substrates (Thomma

59 2003; Woudenberg et al. 2013). *Alternaria* spp. are major pathogenic fungi in agriculture and the

60 food industry, leading to serious diseases in many economically important crops (Meena et al.

61 2017; Thomma 2003).

62 ~~Several taxa~~Different taxa of *Alternaria* have been associated with Alternaria leaf blotch (ALB)

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63 and Alternaria fruit spot (AFS) diseases (Armitage et al. 2015; Gur et al. 2017; Harteveld et al.

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64 2013b). ALB is ~~characterised~~ characterized by the development of round or irregular brown

65 spots on leaves, bordered by dark brown to purple margins (Rotondo et al. 2012). These

66 symptoms generally start in late spring or early summer, developing to yellowing leaves that can

67 lead to early tree defoliation and a premature fruit drop associated with a reduction in tree vigo~~u~~r

68 and fruit quality over the following years (Harteveld et al. 2013b; Rotondo et al. 2012). ALB

69 may cause up to 80% of defoliation in some susceptible apple cultivars (Filajdić & Sutton 1991)

70 and consequently may drastically decrease fruit yields (Harteveld et al. 2013b; Horlock 2006).

71 Less frequent, AFS is ~~characterised~~ characterized by necrotic spots on the skin of the fruit

72 surrounded by a red halo centered on the lenticels (Harteveld et al. 2013b; Horlock 2006;

73 Rotondo et al. 2012) and in some cases can result in calyx cracking and fruit rot (Gur et al.

74 2017). AFS may consequently downgrade the fruit's value, resulting in a significant financial
75 burden to apple growers (Gur et al. 2017; Harteveld et al. 2014).
76 Both ALB and AFS have been reported in nearly all apple-producing regions of the world
77 (Dickens & Cook 1995; Filajdić & Sutton 1991; Gur et al. 2017; Harteveld et al. 2013b; Kim et
78 al. 1986; Ozgonen & Karaca 2006; Rotondo et al. 2012; Wenneker et al. 2018). ~~Taxa~~ Taxa-
79 causing ALB and AFS are part of the *Alternaria* section *Alternaria* that comprises the small-
80 spored *Alternaria* species. As for the whole genus *Alternaria,* identification of isolates within the
81 section *Alternaria* is challenging due to morphological plasticity and genetic similarity
82 (Armitage et al. 2015; Lawrence et al. 2013; Woudenberg et al. 2013). However, recent
83 advances, especially in multi-gene phylogeny~~,~~ and comparative genomics~~, and transcriptomics~~
84 have allowed the different *Alternaria* sections to be redefined and delineated, with accurate
85 molecular differentiation and identification of isolates (Armitage et al. 2020; Woudenberg et al.
86 2015a). Woudenberg et al. (2015a), for example, have shown that the *Alternaria* section
87 *Alternaria* consists of 11 phylogenetic species and one species complex. The taxonomic
88 implications of this study are major because 35 morphospecies, which could not be distinguished
89 through multi-gene phylogeny, were ~~synonymised~~ synonymized under *Alternaria alternata*
90 (including the ~~morphospecies and~~ important plant pathogens *A. alternata*, *A. tenuissima* and *A.*
91 *citri*).
92 ALB and AFS have been commonly associated with the phylogenetic species *A. alternata* and
93 the *Alternaria arborescens* species complex (*A. arborescens* SC) (Gur et al. 2017; Harteveld et
94 al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018), with both taxa
95 also known as saprophytic and generalist opportunistic pathogens affecting a variety of important
96 crops (Armitage et al. 2015; Thomma 2003). Both *A. alternata* and *A. arborescens* SC may

include the apple pathotype, which has been recently shown to be polyphyletic (Armitage et al. 2020). The apple pathotype, formerly known as *Alternaria mali,* causes significant problems in apple orchards in south-eastern Asia (Li et al. 2019), was responsible for ALB in the south-eastern USA in the early nineties (Filajdić & Sutton 1991) and has been associated with severe AFS in Israel (Gur et al. 2017). It is also listed either as a quarantine or a regulated pathogen in several countries throughout the world (https://gd.eppo.int/).

In *Alternaria*, pathotypes are ~~characterised~~ characterized by the production of polyketide host-selective toxins (HSTs), which are linked to pathogenicity affecting specific hosts (Tsuge et al. 2013). To date, at least seven pathotypes have been described, each producing a unique HST essential to pathogenicity in apples (*AMT*), Japanese pears *(AKT*), strawberries (*AFT*), tangerines (*ACT*), tomatoes (*AAL*) rough lemons (*ACR*) and tobacco (*AT*) (Tsuge et al. 2013; Wang et al. 2019). The production of these HSTs involves a cluster of genes located on conditionally (or accessory) dispensable chromosomes (CDCs), so named because they are not essential for saprophytic growth and reproduction of pathogens (Hatta et al. 2002; Wang et al. 2019).

In the case of the apple pathotype, at least 17 genes could be involved in the synthesis of *AMT* apple toxin (Harimoto et al. 2007) but so far only four, i.e. *AMT1*, *AMT2*, *AMT3*, and *AMT4*, have been demonstrated to be involved in this process. To date, molecular detection of this pathotype is only possible by PCR targeting one of the genes involved in the production of the *AMT* apple toxin (Armitage et al. 2020; Harimoto et al. 2007; Johnson et al. 2000) or by identifying these genes in the genome of *Alternaria* isolates using bioinformatics (Armitage et al. 2020). However, molecular taxonomic assignment to either *Alternaria alternata* or the *A. arborescens* SC in routine diagnostics requires the construction of multi-gene phylogenies (Armitage et al. 2015; Harteveld et al. 2013b; Rotondo et al. 2012; Woudenberg et al. 2015a).

Indeed, multi-locus sequence typing (MLST) with relevant phylogenetic markers for the *Alternaria* genus has been used to identify *A. alternata* and the *A. arborescens* SC. It has also enabled researchers to understand their association with ALB and AFS in several countries (Armitage et al. 2015; Gur et al. 2017; Harteveld et al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018).

ALB has been observed for years in French orchards without causing serious damage. However, since 2016, significant defoliation in trees infected by *Alternaria* has been reported in ~~four~~ regions of central and south-eastern France. The reported presence of the apple pathotype in northern Italy (Rotondo et al. 2012) has raised serious concerns for both the French plant health authorities and apple growers.

The first objective of this study was to assess whether the upsurge in ALB symptoms observed in French orchards was due to the emergence of the apple pathotype. To identify the pathogens responsible for these unusual cases of defoliation, we conducted MLST analyses to determine the phylogenetic position of the French *Alternaria* isolates. We also assessed the presence of the apple pathotype by PCR tests targeting two genes involved in the production of the *AMT* apple toxin, and a gene found in the apple pathotype CDC that has homolog~~ue~~ genes in the pear and strawberry pathotypes (Armitage et al. 2020). Pathogenicity tests were then carried out and Koch's postulates were assessed on the Gala and Golden Delicious apple cultivars using a representative panel of isolates. Finally, both the phylogenetic position and pathogenicity of the French *Alternaria* isolates were compared with *Alternaria* isolates from different countries and/or isolated from crops other than apples.

Materials & Methods

142 **Isolate collection**

143

144 French *Alternaria* spp. isolates (166) were obtained from symptomatic field samples of leaves
145 (156) and fruit (10) collected from ten different apple cultivars or cultivar groups ~~collected~~
146 (Table 1, Supplementary material S1).~~in apple~~Apple orchards ~~in~~ were located in four major
147 apple-producing regions in central and south-eastern France: Auvergne-Rhône-Alpes (~~ARA~~108
148 isolates), Provence-Alpes-Côtes d'Azur (~~PACA~~49 isolates), Occitanie (~~OCC~~8 isolates) and
149 Nouvelle-Aquitaine (~~NA~~1 isolate). The samples were collected over ~~a 3-year period~~3 years
150 (seasons 2016-17, 2017-18, and 2018-19, Table 1, Supplementary material S1). Leaves and fruit
151 surfaces were first disinfected with 70 % ethanol and necrotic spots were excised using a sterile
152 scalpel blade then plated onto Petri dishes containing malt extract agar (Sigma-Aldrich) medium
153 supplemented with chloramphenicol (0.2 g/L) ~~(MALTCH medium)~~. The cultures were incubated
154 for four to seven days at 22 °C with a 12 h alternating dark and light cycling period. A plug of
155 actively growing cultures was then transferred to a malt extract agar medium and incubated
156 under the same conditions as described above. The isolate collection was supplemented with 43
157 *Alternaria* isolates either associated with ALB or AFS from Australia (16), Israel (8), Italy (14),
158 and New Zealand (5). Furthermore, the Food and Environmental Research Agency (FERA)
159 contributed 12 isolates obtained from fruit importations showing AFS and intercepted in the UK
160 (unknown origin). Nine additional ALB or AFS isolates were obtained from the Westerdijk
161 Institute's collection (https://www.wi.knaw.nl). Other *Alternaria* isolates associated with post-
162 harvest apple rot problems in Argentina (4) and South Africa (8) were also included.
163 Additionally, the collection was completed with *Alternaria* isolates from other hosts belonging to
164 different botanical families (19 isolates). Five isolates in the collection were identified as apple

165 pathotype isolates. All the isolates were single-spored ~~prior to~~before analysis. Details of the
166 isolate collection studied in this work are presented in Supplementary material S1.
167
168 **Genomic DNA extraction and loci sequencing**
169 DNA extractions were performed with approximately 0.5 g of *Alternaria* mycelium, scraped
170 from a fresh culture on malt extract, using the NucleoSpin Plant II kit (Macherey Nagel).
171 Mycelium was ground by placing two ~~sterilised~~ sterilized steel beads (3 mm in diameter) in an
172 Eppendorf tube containing mycelium with 400 µL of lysis buffer and 10 µL of RNAse (both
173 provided with the DNA extraction kit). Samples were subsequently ground twice for 60 s at 30
174 Hz in a MM 400 mixer mill (Retch). DNA was extracted according to the manufacturer's
175 instructions. The concentration of the DNA extracts (100 µL final volume) was estimated with a
176 NanoDrop TM 2000 Spectrophotometer (ThermoFisher). All the extracted DNA was stored at -
177 30°C until use. The endopolygalacturonase (EndoPG) and the *Alternaria* major allergen (Alta-1)
178 genes, two loci commonly used in *Alternaria* identification and phylogenetics (Table 2,
179 Armitage et al. 2015; Harteveld et al. 2013b; Lawrence et al. 2013), were sequenced for all the
180 isolates. In addition, the anonymous region OPA 10-2 (Table 2, Rotondo et al. 2011,
181 Woudenberg et al. 2015) was sequenced for a subset of 100 isolates and used to assess putative
182 differences in taxonomic identification by comparison with EndoPG and Alta-1. For PCR
183 amplification of the three loci, the reaction mixtures contained 1X PCR reaction buffer (HGS
184 Diamond Taq, Eurogentec), 2.5 mM $MgCl_2$ (4.0 mM for EndoPG), 4 x 0.25 mM dNTPs, 0.2 µM
185 of forward and reverse primers (Table 2), 1 U of HGS diamond Taq (Eurogentec), 2 µL of DNA
186 extract and molecular grade water to complete up to 25 µL. PCR conditions consisted of an
187 initial denaturation step at 95°C for 10 min, followed by 40 cycles at 94°C for 45 s, annealing

188 temperatures of 57°C for Alta-1, 56°C for EndoPG, and 62°C for OPA 10-2 for 30 s (Table 2),
189 72°C for 1 min and a final extension step at 72°C for 7 min. The GENEWIZ sequencing
190 platform (Leipzig, Germany) was used for bidirectional Sanger sequencing of the amplicons.
191 Consensus sequences were obtained after manual correction using the Geneious R11 program~~me~~.

192 **Phylogenetic analysis**

193

194 The EndoPG, Alta-1, and OPA 10-2 sequence datasets generated in our study were
195 supplemented with data from previous studies. The sequence datasets that enabled taxonomic
196 identification of isolates in these previous studies (Armitage et al. 2015; Gur et al. 2017;
197 Harteveld et al. 2013b; Rotondo et al. 2012; Woudenberg et al. 2015a) were used as a reference
198 in our phylogenetic analysis. As we performed molecular identification using an MLST
199 approach, we decided to use the taxonomy of the *Alternaria* section *Alternaria* proposed by
200 Woudenberg et al. (2015a), which consists of 11 phylogenetic species and one species complex.
201 In other words, we used the *Alternaria alternata* phylogenetic species without including any
202 results of morphospecies (e.g. *A. tenuissima* was taxonomically assigned to the phylogenetic
203 species *A. alternata*), an approach used in other studies that described isolates morphologically
204 (Armitage et al. 2015; Rotondo et al. 2012). DNA sequences were first ~~analysed~~ analyzed with
205 SeaView version 4 (Gouy et al. 2010). These analyses included sequence alignments using
206 MUSCLE (Edgar 2004) and elimination of poorly aligned positions with Gblocks (Talavera &
207 Castresana 2007). MrBayes version 3.2 (Ronquist et al. 2012b) was used for multi-locus
208 phylogeny analysis on concatenated sequences for EndoPG and Alta-1 (two-locus MSLT
209 phylogenetic tree) and EndoPG, Alta-1, and OPA 10-2 (three-locus MLST phylogenetic tree)
210 separately. Runs were performed under the Bayesian MCMC model jumping approach, which
211 provides a convenient alternative to model selection ~~prior to~~before analysis (command lset

212 applyto= (all) nst=mixed). In model jumping, the Markov Chain Monte Carlo (MCMC) sampler
213 explores different models and weights the results according to the posterior probability of each
214 model (Ronquist et al. 2012a). Four MCMC chains were run using the default heating with tree
215 sampling performed every 5000 generations. Runs were performed for at least 20 million
216 generations, and stopped when the standard deviation of split frequencies was below 0.01
217 (Ronquist et al. 2012a). Homologous sequences of Alta-1 and Endo-PG for *A. brassicicola*
218 (isolate Abra43) were used as an outgroup in all the generated trees. The consensus tree was
219 obtained by using the command sumt. The resulting phylogenetic trees were visualised and
220 annotated with the interactive tree of life (iTOL) online tool (Letunic & Bork 2016). The
221 taxonomic identification of 100 isolates using the concatenated trees EndoPg/Alta-1 and
222 EndoPg/Alta-1/OPA 10-2 was compared with the function tanglegram implemented in
223 DENDROSCOPE 3.2.10 (Huson & Scornavacca 2012). A subset of single-locus sequence data
224 for the corresponding loci was submitted to Genbank (accession nos. MN975269-MN975340,
225 Supplementary material S1).

226 **PCR detection of the Alternaria apple pathotype**

227

228 We searched for the *Alternaria* apple pathotype among all the French isolates by PCR targeting
229 two genes involved in AMT apple toxin biosynthesis — namely *AMT1* and *AMT2* — using
230 primers developed by Johnson et al. (2000) and Harimoto et al. (2007) respectively (Table 2).
231 Additionally, 44 out of these French isolates were also tested by PCR targeting *AMT14*, a gene
232 found in the apple pathotype toxin gene cluster and for which homologous genes also exist in
233 pear and strawberry pathotypes (Armitage et al. 2020). Other non-French isolates were tested by
234 PCR targeting either *AMT1* / *AMT2* or *AMT1* / *AMT2* / *AMT14* genes (27 and 47 isolates
235 respectively, Supplementary material S1). PCRs were performed in 25-µL reaction mixtures

236 containing 1X PCR reaction buffer (HGS Diamond Taq, Eurogentec), 2.5 mM $MgCl_2$, 4 x 0.25
237 mM dNTPs, 0.2 µM of forward and reverse primers (Table 2), 1 U of HGS diamond Taq
238 (Eurogentec), 2 µL of DNA extract and molecular grade water to complete up to 25 µL. PCR
239 conditions comprised an initial 10 min denaturation step at 95°C followed by 40 cycles of a
240 denaturation step at 94°C for 30 s, an annealing step at 65°C for *AMT1*, 57°C for *AMT2*, 66°C
241 for *AMT14* (Table 2), and an extension step at 72°C for 60 s. These cycles were followed by a
242 final extension at 72°C for 7 min. All PCRs were performed in duplicate. Controls were included
243 in all reactions. Positive controls included either gDNA (*Alternaria* apple pathotype isolate
244 LSVM 75) for *AMT14* testing or a plasmid solution of *AMT1* and *AMT2* genes inserted in a
245 vector using the pCR4-TOPO cloning kit (Invitrogen) following the manufacturer's instructions.
246 Negative controls consisted of sterile distilled water (SDW).

247 **Pathogenicity assays and Koch's postulates**

248

249 Twenty-eight isolates were ~~selected~~ used for pathogenicity assays: ten *A. alternata,* 17 *A.*
250 *arborescens* SC and one *A. brassicicola* (Supplementary material S1). Their selection took into
251 account isolation from different apple cultivars (10) and origin (5 countries) (Supplementary
252 material S1). It also covered different clades or subclades of the multilocus sequence typing
253 (MLST) phylogenetic tree constructed with the EndoPG and Alta-1 regions (Figure 1). No apple
254 pathotype isolate could be tested, because no strain in our collection could produce a sufficient
255 amount of conidia in culture, despite several attempts. The *A. brassicicola* isolate Abra43 was
256 included as a non-pathogenic control. Assays were performed on detached apple leaves from ~~the~~
257 ~~cultivars~~ Golden Delicious clone X972 and Gala clone X4712, the most industrially-relevant
258 cultivars in France, representing 45% of the total apple production in the country (AGRESTE
259 2021). For simplicity, both clones will be referred hereafter to as Golden Delicious and Gala.

260 Spore suspensions were obtained from isolates grown at 22°C for 21 days on malt extract agar
261 ~~MALTCH~~ medium under specific light condition cycles (Carvalho et al. 2008): an incubation
262 period of 7 days under a 12 h alternating dark then light cycling period followed by 2 days under
263 an 8 h-UV/16 h dark conditions (UV ~~light~~ light-induced by a black fluorescent near UV lamp
264 (Philips/15W, T8-BLB) and a final cycle of 12 days in full darkness. For each isolate, the
265 inoculum was obtained by flooding the culture with 2 mL of SDW before dislodging spores by
266 scraping the plate with an L-shaped spreader. After a filtration step with ~~a~~ sterile gauze, the spore
267 suspensions were counted and adjusted to a concentration of $1x10^5$ conidia/mL with a
268 haemocytometer. Leaves from the third or fourth node were detached from fresh branches of
269 apple saplings grown in a glasshouse. Eight leaves cleaned with 70% ethanol were placed in
270 plastic boxes containing two white absorbent paper towels humidified with SDW and conserved
271 at ambient temperature overnight ~~prior to~~before leaf inoculations. An experimental replicate
272 consisted of one strain inoculated on five different leaves (placed in five different plastic boxes)
273 per cultivar. Unwounded abaxial leaf surfaces were inoculated at six points with 10 µL of
274 conidial suspension. Each plastic box contained a negative control that consisted of one leaf
275 inoculated with SDW. Inoculated leaves were incubated at 20°C for 10 days under an alternating
276 12 h dark then light cycling period. Each isolate was tested twice in independent experiments.
277 The results from each experiment were ~~analysed~~ analyzed separately at the three data collection
278 times: 4, 7, and 10 days post-inoculation (dpi). Two types of analysis were performed. For data
279 from 4 dpi, a zero-inflated Poisson general linear mixed model (GLMM) was used to assess the
280 number of lesions per leaf. The model included the following explanatory variables: a taxon of
281 the tested isolate (*A. alternata* or *A. arborescens* SC), the apple ~~leaf variety~~ cultivar (Gala or
282 Golden Delicious), and an experiment repeat variable (each isolate was tested twice in

independent experiments). An isolate effect was taken into account as a random variable. For data from 7 and 10 dpi, zero-inflated beta GLMMs were performed. On both 7 and 10 dpi the response variable was the proportion of the diseased leaf area on detached leaves that corresponds to the lesion size. The diseased leaf area proportion (necrosis) was assessed by visual inspection and coded between 0 and 1. The model included the same explanatory and random effect variables used for the 4 dpi data model: taxon, ~~variety~~cultivar, repeat and isolate (random effect). All GLMM analyses (on 4, 7, and 10 dpi) took into account only isolates of *A. alternata* and *A. arborescens* SC, as only one *A. brassicicola* isolate was used, which is not enough to be included in the isolate random effect. All the models were run in the R environment (version 4.0.3) using the glmmTMB package (Brooks et al. 2017). Excess zeros were in all cases tested with the function testZeroInflation of the DHARMa R package (Hartig 2017), which compares the distribution of expected zeros in the data with the observed zeros. Model residual diagnostics of all models were performed with the DHARMa package. Analysis of variance type II (ANOVA type II) analyses ~~were~~ was performed to assess the effect of each explanatory variable. Koch's postulates were assessed by the re-isolation on ~~MALTCH~~ malt agar extract medium of 23 randomly chosen tested isolates (Supplementary material S1) and the re-sequencing of Alta-1 and EndoPG loci.

Results

Molecular identification of strains

Concatenation of Alt-a1 and Endo-PG sequences resulted in a 900-bp alignment. ~~Concatenated Alta-1 and Endo-PG sequences resulted in a length of 900 bp; these sequences~~This alignment ~~were~~ was used for phylogenetic analyses. Depending on the isolate, the number of bases/residues that differed between isolates of *A. alternata* and isolates of other taxa of the *Alternaria* section

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307 *Alternaria* included in the analysis (*A. arborescens* SC, *A. gaisen*, *A. longipes*, *A. gossypina* and
308 *A. alstroermeriae*) ranged from 6 to 37. The two-marker phylogenetic tree distinguished two major
309 clades: *A. alternata* and *A. arborescens* SC (Figure 1). The *A. alternata* phylogenetic clade
310 encompassed four subclades, while the *A. arborescens* SC encompassed two. The analysis could
311 also distinguish these two clades from other taxa in the *Alternaria* section *Alternaria*: *A. gaisen*,
312 *A. longipes*, *A. gossypina* and *A. alstroemeriae* (Figure 1). The three concatenated genes (Alta-
313 1/Endo-PG/OPA10-2) resulted in a 1534-bp ~~sequence length~~ alignment which included 18–65
314 differences/residues between *A. alternata* and other taxa of the *Alternaria* section *Alternaria*
315 included in the analysis. As for the two-marker concatenated tree, the phylogenetic analysis using
316 three markers distinguished two major clades, *A. alternata* and *A. arborescens* SC (Supplementary
317 material S2). It could also distinguish these clades from other taxa in the *Alternaria* section
318 *Alternaria* (*A. gaisen*, *A. longipes*, *A. gossypina* and *A. alstroemeriae*). The phylogenetic tree
319 pattern was similar to that determined with ~~only~~ two loci (i.e. Alta-1 and EndoPG). The *A.*
320 *alternata* clade was divided into four subclades, whereas the *A. arborescens* SC was separated into
321 two subclades. Adding a third locus to the analysis (OPA 10-2) did not improve the resolution
322 within *A. alternata* and *A. arborescens* SC (Supplementary material S2). The two phylogenetic
323 analyses did, however, refine identification: two Australian strains were consequently assigned to
324 *A. alternata* whereas they had previously been assigned to *A. longipes* (BRIP46356 and
325 BRIP46455) by Harteveld et al. (2013b) (Figure 1, Supplementary materiel S1).

326 **Identification of *Alternaria* isolates causing ALB in France**

327

328 Isolates from France were identified as either *A. arborescens* SC (91 isolates, 55%) or *A. alternata*
329 (75 isolates, 45%) based on the taxonomic identification with the Alta-1 and EndoPG markers. No
330 changes in the taxonomic identification were observed for the subset of isolates with sequences of

331 Alta-1, EndoPG, and OPA 10-2 markers (Supplementary material S1). The distribution of isolates
332 differed according to the cultivar (Table 1). It was observed that *A. arborescens* SC isolates were
333 more frequent on Gala and Golden Delicious ~~varieties~~cultivars, whereas *A. alternata* isolates were
334 more frequent on cultivars Reinette grise du Canada, Dalinette and Crimson. On Braeburn and
335 Pink lady cultivars, there was a similar number of isolates of *A. arborescens* SC and *A. alternata*.
336 On Garance and GoldRush (Coop38cov) ~~varieties~~cultivars, too few isolates were recovered to
337 make any comparison (Table 1).

338 **Screening for the *Alternaria* apple pathotype**

339

340 None of the 166 French isolates were identified as the apple pathotype by PCR tests targeting the
341 *AMT* apple toxin (*AMT1*, *AMT2*) or cross-pathotype (*AMT14*) loci (Supplementary material S1).
342 All five apple pathotype reference isolates behaved as expected and yielded positive results for
343 *AMT1* and *AMT2* PCR tests and also for the tests targeting *AMT14,* which is common to apple,
344 pear, and strawberry pathotypes (Supplementary material S1). However, four isolates formerly
345 identified as apple pathotype in earlier studies in Italy (Rotondo et al. 2012) and Israel (Gur et al.
346 2017) gave negative PCR results for the three loci in our conditions, thus overturning their
347 identification (Supplementary material S1).

348

349 **Pathogenicity assays and Koch's postulates**

350 On 4 dpi, 27 isolates — including the negative control *A. brassicicola* — were able to induce at
351 least one necrotic spot on detached leaves of apple ~~varieties~~ cultivars Golden Delicious and Gala.
352 The only exception was an *A. arborescens* SC isolate (16_489b3a) that did not induce necrotic
353 spots on detached Gala cultivar leaves. The zero-inflated Poisson GLMM used to assess the
354 number of lesions per leaf on 4 dpi, indicated a significant effect of the experiment repetition (Type

II Wald; χ^2=17.75; df=2; p=0.00014) but not of the taxa (Type II Wald; χ^2=2.44; df=1; p=0.118) or the apple ~~variety~~ cultivar (Type II Wald; χ^2=0.06; df=1; p=0.810) (Figure 2). All 28 tested isolates were able to induce leaf blotch after 7 dpi on Golden Delicious and Gala. On 7 dpi, the zero-inflated beta GLMM used to assess the proportion of the diseased leaf area indicated a significant effect of the experiment repetition (Type II Wald; χ^2=84.013; df=2; p<0.001) and the apple ~~variety~~ cultivar (Type II Wald; χ^2=17.296; df=1; p=3.199e^{-5}), but not of the taxa (Type II Wald; χ^2=0.001; df=1; p=0.97) (Figure 3a). On 10 dpi the zero-inflated beta GLMM indicated a significant effect of the experiment repetition (Type II Wald; χ^2=91.82; df=2; p<2.2e^{-16}) and the apple ~~variety~~ cultivar (Type II Wald; χ^2=11.80; df=1; p=0.0006), but not of the taxa (Type II Wald; χ^2=0.06; df=1; p=0.801) (Figure 3b). On 7 and 10 dpi, leaves of the Golden Delicious ~~variety~~ cultivar were more susceptible than those of Gala, as measured by the proportion of the diseased leaf area (Figure 3). Raw measurements at 4, 7, and 10 dpi are presented in the supplementary material S4 section. Finally, the identity of 23 of these strains was confirmed by re-isolating and sequencing (Alta-1 and EndoPG loci), fulfilling Koch's postulates (Supplementary material S1). No disease symptoms were observed on leaves inoculated with water. ~~Examples of the results from the pathogenicity tests~~~~Some examples of the carried pathogenicity tests are shown in the~~ (S~~s~~upplementary material S5 section).

Discussion

~~Severe symptoms of apple leaf blotch (ALB) reported in French orchards since 2016 raised questions about the identity of the *Alternaria* spp. responsible for unprecedented early defoliation in orchards. Our principal objectives were to verify whether the *Alternaria* apple~~

377 ~~pathotype could have emerged in France, to accurately identify the *Alternaria* taxa involved in~~

378 ~~such defoliation, and to assess the pathogenicity of tested isolates~~

379

380 **The *Alternaria* apple pathotype was not found in French orchards**

381 We firstly checked whether we were witnessing the emergence of the apple pathotype in French

382 orchards. We did this through PCR assays targeting three genes located in the conditionally

383 dispensable chromosome (CDC) — *AMT1* (Johnson et al. 2000), *AMT2* (Harimoto et al. 2008),

384 and *AMT14* (Armitage et al. 2020) — which ~~characterises~~ characterizes isolates of the apple

385 pathotype. Our results showed that this pathogen was not present in France within the sampled

386 regions and years. To date, molecular detection of the apple pathotype has only been possible by

387 PCR tests that target genes present in the CDC. These targets are associated with secondary

388 metabolite clusters involved in the production of the apple pathotype ~~host~~ host-specific toxin

389 *AMT* (Armitage et al. 2020; Harimoto et al. 2007; Johnson et al. 2000). Although all our apple

390 pathotype reference strains gave positive results using the three markers, the Italian and Israeli

391 strains previously identified as apple pathotype by PCR based on the amplification of a modified

392 PCR test targeting *AMT1* (Rotondo et al. 2012) and *AMT3* (Gur et al. 2017) gave negative results

393 in our study with tests targeting loci *AMT1*, *AMT2* and *AMT14*. In the case of the Israeli strains,

394 the primers targeting *AMT3* (Harimoto et al. 2007), showed unsatisfactory results in our

395 preliminary tests as several unexpected bands appeared after gel electrophoresis of the PCR

396 product (data not shown) and these primers were discarded for subsequent molecular tests. The

397 results obtained with the Italian strains are more difficult to explain because the initial study of

398 Rotondo et al. (2012) performed several confirmation tests (including sequencing of the

399 products). One hypothesis that may explain the difficulty in amplifying these loci is the

400 occurrence of partial or total chromosomal loss in isolates. This phenomenon has previously
401 been reported in the apple pathotype by Johnson et al. (2001) and is due to chromosomal
402 instability in culture. To avoid this problem, in the analysis of French isolates, our tests targeting
403 *AMT1*, *AMT2*, and *AMT14* were performed right after isolation, avoiding several subculturing
404 cycles. However, in all the cases where the presence of *AMT1*, *AMT2*, and *AMT14* was assessed
405 (in the five reference isolates; Supplementary material S1), all three gene-specific PCR assays
406 gave positive results. Based on the results of our study, we suggest that the apple pathotype
407 should be detected from pure cultures by using at least two or more of the existing molecular
408 tests to target *AMT1* and *AMT2*, which is a good option if the objective is to specifically detect
409 the apple pathotype. By optimising and validating current tools or developing new molecular
410 tests, it might be possible to detect diseases *in planta* from symptomatic leaves, which could
411 avoid isolate subculturing cycles while minimising the risk of chromosomal loss.
412
413 **Co-existence of *Alternaria alternata* and the *Alternaria arborescens* species complex in**
414 **French orchards**
415 The second objective of this study was to identify *Alternaria* species or groups associated with
416 *Alternaria* leaf blotch (ALB) and *Alternaria* fruit spot (AFS) in French orchards. The
417 phylogenetic trees generated after using MLST clearly showed that these diseases are caused by
418 two phylogenetic clades: *A. alternata* and *A. arborescens* SC, regardless of the apple cultivar.
419 Our results also showed that both taxa may co-exist in the same orchard. These results confirm
420 that these two *Alternaria* taxa are the major cause of ALB and AFS in regions of the world
421 where these diseases have been studied so far (Gur et al. 2017; Harteveld et al. 2013b; Rotondo
422 et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018). In addition, our results suggest that

423 sequencing two loci, i.e. Alta-1 and EndoPG, is enough to be able to distinguish *Alternaria*
424 isolates involved in these diseases. Firstly, these two loci enable the two major phylogenetic
425 clades — *A. alternata* and *A. arborescens* SC — to be distinguished. Secondly, the loci also
426 clearly distinguish these two clades from other *Alternaria* taxa within the *Alternaria* section.
427 Including the OPA 10-2 locus did not substantially improve the molecular identification of the
428 strains.
429

430 ***Alternaria alternata* and the *Alternaria arborescens* SC are responsible for defoliation in**
431 **French apple orchards**

432 We showed that all the ~~strains isolated~~isolates collected from necrotic leaves were able to
433 produce symptoms on detached apple leaves of ~~varieties~~ cultivars Gala and Golden Delicious.
434 The latter ~~variety~~ cultivar was more susceptible under our conditions, as shown by measurements
435 of the diseased leaf area after 7 and 10 dpi, a quantitative trait generally used to measure
436 pathogen aggressiveness. Gala and Golden were used for pathogenicity tests since they are the
437 most important cultivars in France. Although these cultivars are considered as relatively
438 “susceptible” to ALB and AFS (Filajdić & Sutton 1991; Harteveld et al. 2014; Rotondo et al.
439 2012), earlier studies have shown that there is little, or no cultivar specificity in *Alternaria* taxa
440 causing ALB and AFS, at least for the most economically important apple cultivars used
441 worldwide (Filajdić & Sutton 1991; Harteveld et al. 2014; Li et al. 2019). Management of the
442 disease may involve resistant cultivars. However, further research involving more cultivars~~,~~
443 would be required to assess potential cultivar-~~-~~specificity among *Alternaria* taxa causing ALB
444 and AFS. The selection of disease-resistant cultivars should rely on studies combining data
445 collected from the field and ~~from~~ trials under controlled conditions (Li et al. 2019).

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446 Our results also showed that the entire subset of *Alternaria* isolated from apple leaves or fruit
447 fulfilled Koch's postulates. The pathogenicity tests showed that there are no significant
448 differences between isolates of *A. alternata* and *A. arborescens* SC as assessed by the number of
449 lesions per leaf on 4 dpi or the proportion of the diseased leaf area on 4 and 7 dpi. Both results
450 are in agreement with previous studies that suggest that pathogenicity may be isolate-dependent
451 rather than species-dependent (Harteveld et al. 2014; Rotondo et al. 2012). One of the limits of
452 our study is that we could not assess the pathogenicity of any of the reference apple pathotype
453 strains because too few spores could be obtained during cultivation. It is important to highlight,
454 however, that previous studies comparing the pathogenicity of apple pathotype isolates with
455 other *Alternaria* isolates in apples have shown discrepant results: while Armitage et al. (2020)
456 showed that apple pathotype strains were significantly more pathogenic than other isolates that
457 do not carry CDCs, Rotondo et al. (2012) did not observe any difference in levels of
458 pathogenicity between apple pathotype isolates and other *Alternaria* isolated from apple leaves
459 or fruit. Unexpectedly, we observed symptoms on apple leaves inoculated by *A. brassicicola,*
460 which has never been reported as pathogenic on apples to our knowledge. These results suggest
461 that *Alternaria* isolates from other *Alternaria* sections that do not carry CDCs involved in the
462 production of HTS may also cause ALB symptoms under controlled conditions. This is probably
463 associated with the production of nonspecific Alternaria toxins that can affect many plants
464 regardless of whether they are or are not a host of the pathogen (Tsuge et al. 2013). However, as
465 shown here and elsewhere, under natural conditions, only small-spore Alternaria (*Alternaria*
466 section *Alternaria*) have so far been described as apple pathogens causing ALB and AFS. Recent
467 genomic resources, including the genome of *A. brassicicola* (Belmas et al. 2018) and isolates of

468 *Alternaria* involved in AFS and ALB (Armitage et al. 2020) will allow comparative genomics

469 analysis that may clarify these pathogenicity mechanisms.

470 Finally, this study identified the *Alternaria* taxa involved in ALB and AFS in France~~,~~ but did not

471 determine the cause of the increased severity in these diseases over recent years (e.g.

472 introduction of the apple pathotype). However, alternative explanations may be suggested based

473 on previous epidemiological studies. Firstly, it seems that the disease develops better in

474 relatively hot (between > 20°C and 30°C) and rainy weather (Bhat et al. 2015; Filajdić & Sutton

475 1992; Harteveld et al. 2013a; Kim et al. 1986). Potential changes in these two parameters, or

476 other climatic factors, should be studied in greater depth in the French regions. ~~concerned in~~

477 ~~order to draw conclusions.~~ Another hypothesis is the introduction of more virulent strains. This

478 could occur by the long-distance movement of spores carried by wind currents that may have

479 transported *Alternaria* air inoculum into apple orchards from sources in other apple-producing

480 regions (Fernández-Rodríguez et al. 2015; Woudenberg et al. 2015b). Finally, the emergence of

481 fungicide resistance among strains should not be ruled out, considering that apple orchards are

482 treated intensely with fungicides, mainly used to control apple scab caused by *Venturia*

483 *inaequalis*, which also contributes to the control of ALB and AFS (Horlock 2006).

484

485 **Conclusions**

486 Since 2016, ~~apple~~ Alternaria leaf blotch and premature defoliation ~~and fruit tree defoliation~~

487 attributed to *Alternaria* spp. have been observed in apple-producing regions in central and south-

488 eastern France. The emergence of the *Alternaria* apple pathotype was suspected following its

489 observation in northern Italy. The presence of the apple pathotype in French orchards was

490 therefore assessed by a specific PCR targeting three genes located on conditionally dispensable

491 chromosomes across a large collection of ~~166~~ *Alternaria* isolates ~~from different varieties and~~

492 ~~production areas during the 2016-2018 period~~. Our results showed that the *Alternaria* apple
493 pathotype was not present. Taxonomic identification of these isolates, assessed by multi-locus
494 sequence typing and construction of phylogenetic trees, indicates that ~~apple leaf blotch and fruit~~
495 ~~tree defoliation~~Alternaria leaf blotch in France is ~~are~~ associated with isolates of *A. alternaria* and
496 *A. arborescens* SC. Pathogenicity tests of a subsample of isolates demonstrated that they were all
497 able to induce necrotic symptoms on detached apple leaves of the ~~varieties~~ cultivars Gala and
498 Golden Delicious. Our results also showed that there are no significant differences in levels of
499 pathogenicity between isolates of *A. alternata* and *A. arborescens* SC. Our controlled
500 pathogenicity tests do suggest, however, that cultivar Golden Delicious is more susceptible to
501 Alternaria leaf blotch. In the future, genetic and epidemiological approaches are required to
502 clarify why Alternaria leaf blotch events have increased in frequency and severity in some
503 regions of France.

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505

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