Identification and pathogenicity of *Alternaria* species associated with leaf blotch disease and premature defoliation in French apple orchards

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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 characterized by the production of a host-selective toxin (HST) which is involved in pathogenicity towards the apple. A cluster of genes located on conditionally dispensable chromosomes (CDCs) is involved in the production of this HST (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 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* apple pathotype and the taxonomic assignment of *Alternaria* isolates involved in Alternaria leaf blotch are discussed.

1 # Identification and pathogenicity of *Alternaria* species
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3 # defoliation in French apple orchards

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Abstract

29 Leaf blotch caused by *Alternaria* spp. is a common disease in apple-producing regions. The

30 disease is usually associated with one phylogenetic species and one species complex, *Alternaria*

31 *alternata* and *Alternaria arborescens* species complex (*A. arborescens* SC), respectively. Both

32 taxa may include the *Alternaria* apple pathotype, a quarantine or regulated pathogen in several

33 countries. The apple pathotype is characterized by the production of a host-selective toxin (HST)

34 which is involved in pathogenicity towards the apple. A cluster of genes located on conditionally

35 dispensable chromosomes (CDCs) is involved in the production of this HST (namely *AMT* in the

36 case of the apple pathotype). Since 2016, leaf blotch and premature tree defoliation attributed to

37 *Alternaria* spp. have been observed in apple-producing regions of central and south-eastern

38 France. Our study aimed to identify the *Alternaria* species involved in apple tree defoliation and

39 assess the presence of the apple pathotype in French orchards. From 2016-2018, 166 isolates

40 were collected and identified by multi-locus sequence typing (MLST). This analysis revealed

41 that all these French isolates belonged to either the *A. arborescens* SC or *A. alternata*. Specific

42 PCR detection targeting three genes located on the CDC did not indicate the presence of the

43 apple pathotype in France. Pathogenicity was assessed under laboratory conditions on detached

44 leaves of Golden Delicious and Gala apple cultivars for a representative subset of 28 *Alternaria*

45 isolates. All the tested isolates were pathogenic on detached leaves of cultivars Golden Delicious

46 and Gala, but no differences were observed between the pathogenicity levels of *A. arborescens*

47 SC and *A. alternata*. However, the results of our pathogenicity test suggest that cultivar Golden

48 Delicious is more susceptible than Gala to Alternaria leaf blotch. Implications in the detection of

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

50 Alternaria leaf blotch are discussed.

Introduction

52

53 *Alternaria* spp. are ubiquitous fungi comprising approximately 300 different species (Simmons

54 2007; Woudenberg et al. 2013). The genus has different lifestyles and can be isolated from a

55 large number of substrates (Thomma 2003; Woudenberg et al. 2013). *Alternaria* spp. are major

56 pathogenic fungi in agriculture and the food industry, leading to serious diseases in many

57 economically important crops (Meena et al. 2017; Thomma 2003).

58 Different taxa of *Alternaria* have been associated with Alternaria leaf blotch (ALB) and

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

60 2013b). ALB is characterized by the development of round or irregular brown spots on leaves,

61 bordered by dark brown to purple margins (Rotondo et al. 2012). These symptoms generally start

62 in late spring or early summer, developing to yellowing leaves that can lead to early tree

63 defoliation and a premature fruit drop associated with a reduction in tree vigour and fruit quality

64 over the following years (Harteveld et al. 2013b; Rotondo et al. 2012). ALB may cause up to

65 80% of defoliation in some susceptible apple cultivars (Filajdić & Sutton 1991) and

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

67 frequent, AFS is characterized by necrotic spots on the skin of the fruit surrounded by a red halo

68 centered on the lenticels (Harteveld et al. 2013b; Horlock 2006; Rotondo et al. 2012) and in

69 some cases can result in calyx cracking and fruit rot (Gur et al. 2017). AFS may consequently

70 downgrade the fruit's value, resulting in a significant financial burden to apple growers (Gur et

71 al. 2017; Harteveld et al. 2014).

72 Both ALB and AFS have been reported in nearly all apple-producing regions of the world

73 (Dickens & Cook 1995; Filajdić & Sutton 1991; Gur et al. 2017; Harteveld et al. 2013b; Kim et

74 al. 1986; Ozgonen & Karaca 2006; Rotondo et al. 2012; Wenneker et al. 2018). Taxa causing

75   ALB and AFS are part of the *Alternaria* section *Alternaria* that comprises the small-spored

76   *Alternaria* species. As for the whole genus *Alternaria,* identification of isolates within the

77   section *Alternaria* is challenging due to morphological plasticity and genetic similarity

78   (Armitage et al. 2015; Lawrence et al. 2013; Woudenberg et al. 2013). However recent advances,

79   especially in multi-gene phylogeny and comparative genomics, have allowed the different

80   *Alternaria* sections to be redefined and delineated, with accurate molecular differentiation and

81   identification of isolates (Armitage et al. 2020; Woudenberg et al. 2015a). Woudenberg et al.

82   (2015a), for example, have shown that the *Alternaria* section *Alternaria* consists of 11

83   phylogenetic species and one species complex. The taxonomic implications of this study are

84   major because 35 morphospecies, which could not be distinguished through multi-gene

85   phylogeny, were synonymized under *Alternaria alternata* (including the important plant

86   pathogens *A. alternata*, *A. tenuissima* and *A. citri*).

87   ALB and AFS have been commonly associated with the phylogenetic species *A. alternata* and

88   the *Alternaria arborescens* species complex (*A. arborescens* SC) (Gur et al. 2017; Harteveld et

89   al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018), with both taxa

90   also known as saprophytic and generalist opportunistic pathogens affecting a variety of important

91   crops (Armitage et al. 2015; Thomma 2003). Both *A. alternata* and *A. arborescens* SC may

92   include the apple pathotype, which has been recently shown to be polyphyletic (Armitage et al.

93   2020). The apple pathotype, formerly known as *Alternaria mali,* causes significant problems in

94   apple orchards in south-eastern Asia (Li et al. 2019), was responsible for ALB in the south-

95   eastern USA in the early nineties (Filajdić & Sutton 1991) and has been associated with severe

96   AFS in Israel (Gur et al. 2017). It is also listed either as a quarantine or a regulated pathogen in

97   several countries throughout the world (https://gd.eppo.int/).

98   In *Alternaria*, pathotypes are characterized by the production of polyketide host-selective toxins

99   (HSTs), which are linked to pathogenicity affecting specific hosts (Tsuge et al. 2013). To date, at

100   least seven pathotypes have been described, each producing a unique HST essential to

101   pathogenicity in apples (*AMT*), Japanese pears *(AKT)*, strawberries (*AFT*), tangerines (*ACT*),

102   tomatoes (*AAL*) rough lemons (*ACR*) and tobacco (*AT*) (Tsuge et al. 2013; Wang et al. 2019).

103   The production of these HSTs involves a cluster of genes located on conditionally (or accessory)

104   dispensable chromosomes (CDCs), so named because they are not essential for saprophytic

105   growth and reproduction of pathogens (Hatta et al. 2002; Wang et al. 2019).

106   In the case of the apple pathotype, at least 17 genes could be involved in the synthesis of *AMT*

107   apple toxin (Harimoto et al. 2007) but so far only four, i.e. *AMT1*, *AMT2*, *AMT3* and *AMT4*, have

108   been demonstrated to be involved in this process. To date, molecular detection of this pathotype

109   is only possible by PCR targeting one of the genes involved in the production of the *AMT* apple

110   toxin (Armitage et al. 2020; Harimoto et al. 2007; Johnson et al. 2000) or by identifying these

111   genes in the genome of *Alternaria* isolates using bioinformatics (Armitage et al. 2020). However,

112   molecular taxonomic assignment to either *A. alternata* or the *A. arborescens* SC in routine

113   diagnostics requires the construction of multi-gene phylogenies (Armitage et al. 2015; Harteveld

114   et al. 2013b; Rotondo et al. 2012; Woudenberg et al. 2015a). Indeed, multi-locus sequence

115   typing (MLST) with relevant phylogenetic markers for the *Alternaria* genus has been used to

116   identify *A. alternata* and the *A. arborescens* SC. It has also enabled researchers to understand

117   their association with ALB and AFS in several countries (Armitage et al. 2015; Gur et al. 2017;

118   Harteveld et al. 2013b; Rotondo et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018).

119   ALB has been observed for years in French orchards without causing serious damage. However,

120   since 2016, significant defoliation in trees infected by *Alternaria* has been reported in regions of

121   central and south-eastern France. The reported presence of the apple pathotype in northern Italy

122   (Rotondo et al. 2012) has raised serious concerns for both the French plant health authorities and

123   apple growers.

124   The first objective of this study was to assess whether the upsurge in ALB symptoms observed in

125   French orchards was due to the emergence of the apple pathotype. To identify the pathogens

126   responsible for these unusual cases of defoliation, we conducted MLST analyses to determine the

127   phylogenetic position of the French *Alternaria* isolates. We also assessed the presence of the

128   apple pathotype by PCR tests targeting two genes involved in the production of the *AMT* apple

129   toxin, and a gene found in the apple pathotype CDC that has homologs in the pear and

130   strawberry pathotypes (Armitage et al. 2020). Pathogenicity tests were then carried out and

131   Koch's postulates were assessed on the Gala and Golden Delicious apple cultivars using a

132   representative panel of isolates. Finally, both the phylogenetic position and pathogenicity of the

133   French *Alternaria* isolates were compared with *Alternaria* isolates from different countries

134   and/or isolated from crops other than apple.

135   **Materials & Methods**

136   **Isolate collection**

137

138   French *Alternaria* spp. isolates (166) were obtained from symptomatic field samples of leaves

139   (156) and fruit (10) collected from ten different apple cultivars or cultivar groups (Table 1,

140   Supplementary material S1).Apple orchards were located in four major apple-producing regions

141   in central and south-eastern France: Auvergne-Rhône-Alpes (108 isolates), Provence-Alpes-

142   Côtes d'Azur (49 isolates), Occitanie (8 isolates) and Nouvelle-Aquitaine (1 isolate). The

143   samples were collected over 3 years (seasons 2016-17, 2017-18 and 2018-19, Table 1,

144   Supplementary material S1). Leaves and fruit surfaces were first disinfected with 70 % ethanol

145   and necrotic spots were excised using a sterile scalpel blade then plated onto Petri dishes

146   containing malt extract agar (Sigma-Aldrich) medium supplemented with chloramphenicol (0.2

147   g/L). The cultures were incubated for four to seven days at 22 °C with a 12 h alternating dark and

148   light cycling period. A plug of each actively growing culture was then transferred to a new malt

149   extract agar Petri dish and incubated under the same conditions as described above. The isolate

150   collection was supplemented with 43 *Alternaria* isolates either associated with ALB or AFS

151   from Australia (16), Israel (8), Italy (14) and New Zealand (5). Furthermore, the Food and

152   Environmental Research Agency (FERA) contributed 12 isolates obtained from fruit

153   importations showing AFS and intercepted in the UK (unknown origin). Nine additional ALB or

154   AFS isolates were obtained from the Westerdijk Institute's collection (https://www.wi.knaw.nl).

155   Other *Alternaria* isolates associated with post-harvest apple rot problems in Argentina (4) and

156   South Africa (8) were also included. Additionally, the collection was completed with *Alternaria*

157   isolates from other hosts belonging to different botanical families (19 isolates). Five isolates in

158   the collection were identified as apple pathotype isolates. All the isolates were single-spored

159   before analysis. Details of the isolate collection studied in this work are presented in

160   Supplementary material S1.

161

162   **Genomic DNA extraction and loci sequencing**

163   DNA extractions were performed with approximately 0.5 g of *Alternaria* mycelium, scraped

164   from a fresh culture on malt extract, using the NucleoSpin Plant II kit (Macherey Nagel).

165   Mycelium was ground by placing two sterilized steel beads (3 mm in diameter) in an Eppendorf

166   tube containing mycelium with 400 µL of lysis buffer and 10 µL of RNAse (both provided with

167   the DNA extraction kit). Samples were subsequently ground twice for 60 s at 30 Hz in a MM

168   400 mixer mill (Retch). DNA was extracted according to the manufacturer's instructions. The

169   concentration of the DNA extracts (100 μL final volume) was estimated with a NanoDrop TM

170   2000 Spectrophotometer (ThermoFisher). All the extracted DNA was stored at -30°C until use.

171   The endopolygalacturonase (EndoPG) and the *Alternaria* major allergen (Alta-1) genes, two loci

172   commonly used in *Alternaria* identification and phylogenetics (Table 2, Armitage et al. 2015;

173   Harteveld et al. 2013b; Lawrence et al. 2013), were sequenced for all the isolates. In addition, the

174   anonymous region OPA 10-2 (Table 2, Rotondo et al. 2011, Woudenberg et al. 2015) was

175   sequenced for a subset of 100 isolates and used to assess putative differences in taxonomic

176   identification by comparison with EndoPG and Alta-1. For PCR amplification of the three loci,

177   the reaction mixtures contained 1X PCR reaction buffer (HGS Diamond Taq, Eurogentec), 2.5

178   mM $MgCl_2$ (4.0 mM for EndoPG), 4 x 0.25 mM dNTPs, 0.2 μM of forward and reverse primers

179   (Table 2), 1 U of HGS diamond Taq (Eurogentec), 2 μL of DNA extract and molecular grade

180   water to complete up to 25 μL. PCR conditions consisted of an initial denaturation step at 95°C

181   for 10 min, followed by 40 cycles at 94°C for 45 s, annealing temperatures of 57°C for Alta-1,

182   56°C for EndoPG and 62°C for OPA 10-2 for 30 s (Table 2), 72°C for 1 min and a final

183   extension step at 72°C for 7 min. The GENEWIZ sequencing platform (Leipzig, Germany) was

184   used for bidirectional Sanger sequencing of the amplicons. Consensus sequences were obtained

185   after manual correction using the Geneious R11 programme.

186   **Phylogenetic analysis**

187
188   The EndoPG, Alta-1 and OPA 10-2 sequence datasets generated in our study were supplemented

189   with data from previous studies. The sequence datasets that enabled taxonomic identification of

190   isolates in these previous studies (Armitage et al. 2015; Gur et al. 2017; Harteveld et al. 2013b;

191   Rotondo et al. 2012; Woudenberg et al. 2015a) were used as a reference in our phylogenetic

192 analysis. As we performed molecular identification using an MLST approach, we decided to use

193 the taxonomy of the *Alternaria* section *Alternaria* proposed by Woudenberg et al. (2015a),

194 which consists of 11 phylogenetic species and one species complex. In other words, we used the

195 *Alternaria alternata* phylogenetic species without including any results of morphospecies (e.g. *A.*

196 *tenuissima* was taxonomically assigned to the phylogenetic species *A. alternata*), an approach

197 used in other studies that described isolates morphologically (Armitage et al. 2015; Rotondo et

198 al. 2012). DNA sequences were first analyzed with SeaView version 4 (Gouy et al. 2010). These

199 analyses included sequence alignments using MUSCLE (Edgar 2004) and elimination of poorly

200 aligned positions with Gblocks (Talavera & Castresana 2007). MrBayes version 3.2 (Ronquist et

201 al. 2012b) was used for multi-locus phylogeny analysis on concatenated sequences for EndoPG

202 and Alta-1 (two-locus MSLT phylogenetic tree) and EndoPG, Alta-1 and OPA 10-2 (three-locus

203 MLST phylogenetic tree) separately. Runs were performed under the Bayesian MCMC model

204 jumping approach, which provides a convenient alternative to model selection before analysis

205 (command lset applyto= (all) nst=mixed). In model jumping, the Markov Chain Monte Carlo

206 (MCMC) sampler explores di□erent models and weights the results according to the posterior

207 probability of each model (Ronquist et al. 2012a). Four MCMC chains were run using the default

208 heating with tree sampling performed every 5000 generations. Runs were performed for at least

209 20 million generations, and stopped when the standard deviation of split frequencies was below

210 0.01 (Ronquist et al. 2012a). Homologous sequences of Alta-1 and Endo-PG for *A. brassicicola*

211 (isolate Abra43) were used as an outgroup in all the generated trees. The consensus tree was

212 obtained by using the command sumt. The resulting phylogenetic trees were visualised and

213 annotated with the interactive tree of life (iTOL) online tool (Letunic & Bork 2016). The

214 taxonomic identification of 100 isolates using the concatenated trees EndoPg/Alta-1 and

215   EndoPg/Alta-1/OPA 10-2 was compared with the function tanglegram implemented in

216   DENDROSCOPE 3.2.10 (Huson & Scornavacca 2012). A subset of single-locus sequence data

217   for the corresponding loci was submitted to Genbank (accession nos. MN975269-MN975340,

218   Supplementary material S1).

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

220

221   We searched for the *Alternaria* apple pathotype among all the French isolates by PCR targeting

222   two genes involved in AMT apple toxin biosynthesis — namely *AMT1* and *AMT2* — using

223   primers developed by Johnson et al. (2000) and Harimoto et al. (2007) respectively (Table 2).

224   Additionally, 44 out of these French isolates were also tested by PCR targeting *AMT14*, a gene

225   found in the apple pathotype toxin gene cluster and for which homologous genes also exist in

226   pear and strawberry pathotypes (Armitage et al. 2020). Other non-French isolates were tested by

227   PCR targeting either *AMT1* / *AMT2* or *AMT1* / *AMT2* / *AMT14* genes (27 and 47 isolates

228   respectively, Supplementary material S1). PCRs were performed in 25-µL reaction mixtures

229   containing 1X PCR reaction buffer (HGS Diamond Taq, Eurogentec), 2.5 mM $MgCl_2$, 4 x 0.25

230   mM dNTPs, 0.2 µM of forward and reverse primers (Table 2), 1 U of HGS diamond Taq

231   (Eurogentec), 2 µL of DNA extract and molecular grade water to complete up to 25 µL. PCR

232   conditions comprised an initial 10 min denaturation step at 95°C followed by 40 cycles of a

233   denaturation step at 94°C for 30 s, an annealing step at 65°C for *AMT1*, 57°C for *AMT2*, 66°C

234   for *AMT14* (Table 2) and an extension step at 72°C for 60 s. These cycles were followed by a

235   final extension at 72°C for 7 min. All PCRs were performed in duplicate. Controls were included

236   in all reactions. Positive controls included either gDNA (*Alternaria* apple pathotype isolate

237   LSVM 75) for *AMT14* testing or a plasmid solution of *AMT1* and *AMT2* genes inserted in a

238   vector using the pCR4-TOPO cloning kit (Invitrogen) following the manufacturer's instructions.

239   Negative controls consisted of sterile distilled water (SDW).

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

241

242   Twenty-eight isolates were used for pathogenicity assays: ten *A. alternata,* 17 *A. arborescens* SC

243   and one *A. brassicicola* (Supplementary material S1). Their selection took into account isolation

244   from different apple cultivars (10) and origin (5 countries) (Supplementary material S1). It also

245   covered different clades or subclades of the MLST phylogenetic tree constructed with the

246   EndoPG and Alta-1 regions (Figure 1). No apple pathotype isolate could be tested, because no

247   strain in our collection could produce a sufficient amount of conidia in culture, despite several

248   attempts. The *A. brassicicola* isolate Abra43 was included as a non-pathogenic control. Assays

249   were performed on detached apple leaves from Golden Delicious clone X972 and Gala clone

250   X4712, the most industrially-relevant cultivars in France, representing 45% of the total apple

251   production in the country (AGRESTE 2021). For simplicity, both clones will be referred

252   hereafter to as Golden Delicious and Gala. Spore suspensions were obtained from isolates grown

253   at 22°C for 21 days on malt extract agar medium under specific light condition cycles (Carvalho

254   et al. 2008): an incubation period of 7 days under a 12 h alternating dark then light cycling

255   period followed by 2 days under an 8 h-UV/16 h dark conditions (UV light-induced by a black

256   fluorescent near UV lamp (Philips/15W, T8-BLB) and a final cycle of 12 days in full darkness.

257   For each isolate, the inoculum was obtained by flooding the culture with 2 mL of SDW before

258   dislodging spores by scraping the plate with an L-shaped spreader. After a filtration step with

259   sterile gauze, the spore suspensions were counted and adjusted to a concentration of 1×10^5

260   conidia/mL with a haemocytometer. Leaves from the third or fourth node were detached from

261   fresh branches of apple saplings grown in a glasshouse. Eight leaves cleaned with 70% ethanol

262   were placed in plastic boxes containing two white absorbent paper towels humidified with SDW

263   and conserved at ambient temperature overnight before leaf inoculations. An experimental

264   replicate consisted of one strain inoculated on five different leaves (placed in five different

265   plastic boxes) per cultivar. Unwounded abaxial leaf surfaces were inoculated at six points with

266   10 µL of conidial suspension. Each plastic box contained a negative control that consisted of one

267   leaf inoculated with SDW. Inoculated leaves were incubated at 20°C for 10 days under an

268   alternating 12 h dark then light cycling period. Each isolate was tested twice in independent

269   experiments. The results from each experiment were analyzed separately at the three data

270   collection times: 4, 7 and 10 days post-inoculation (dpi). Two types of analysis were performed.

271   For data from 4 dpi, a zero-inflated Poisson general linear mixed model (GLMM) was used to

272   assess the number of lesions per leaf. The model included the following explanatory variables:

273   the taxon of the tested isolate (*A. alternata* or *A. arborescens* SC), the apple cultivar (Gala or

274   Golden Delicious), and an experiment repeat variable (each isolate was tested twice in

275   independent experiments). An isolate effect was taken into account as a random variable. For

276   data from 7 and 10 dpi, zero-inflated beta GLMMs were performed. On both 7 and 10 dpi the

277   response variable was the proportion of the diseased leaf area on detached leaves that

278   corresponds to the lesion size. The diseased leaf area proportion (necrosis) was assessed by

279   visual inspection and coded between 0 and 1. The model included the same explanatory and

280   random effect variables used for the 4 dpi data model: taxon, cultivar, repeat and isolate (random

281   effect). All GLMM analyses (on 4, 7 and 10 dpi) took into account only isolates of *A. alternata*

282   and *A. arborescens* SC, as only one *A. brassicicola* isolate was used, which is not enough to be

283   included in the isolate random effect. All the models were run in the R environment (version

284   4.0.3) using the glmmTMB package (Brooks et al. 2017). Excess zeros were in all cases tested

285 with the function testZeroInflation of the DHARMa R package (Hartig 2017), which compares

286 the distribution of expected zeros in the data with the observed zeros. Model residual diagnostics

287 of all models were performed with the DHARMa package. Analysis of variance type II

288 (ANOVA type II) was performed to assess the effect of each explanatory variable. Koch's

289 postulates were assessed by the re-isolation on malt agar extract medium of 23 randomly chosen

290 tested isolates (Supplementary material S1) and the re-sequencing of Alta-1 and EndoPG loci.

291 **Results**

292 **Molecular identification of strains**

293 Concatenation of Alt-a1 and Endo-PG sequences resulted in a 900-bp alignment. This alignment

294 was used for phylogenetic analyses. Depending on the isolate, the number of bases/residues that

295 differed between isolates of *A. alternata* and isolates of other taxa of the *Alternaria* section

296 *Alternaria* included in the analysis (*A. arborescens* SC, *A. gaisen*, *A. longipes*, *A. gossypina* and

297 *A. alstroermeriae*) ranged from 6 to 37. The two-marker phylogenetic tree distinguished two major

298 clades: *A. alternata* and *A. arborescens* SC (Figure 1). The *A. alternata* phylogenetic clade

299 encompassed four subclades, while the *A. arborescens* SC encompassed two. The analysis could

300 also distinguish these two clades from other taxa in the *Alternaria* section *Alternaria*: *A. gaisen*,

301 *A. longipes*, *A. gossypina* and *A. alstroemeriae* (Figure 1). The three concatenated genes (Alta-

302 1/Endo-PG/OPA10-2) resulted in a 1534-bp alignment which included 18–65 differences/residues

303 between *A. alternata* and other taxa of the *Alternaria* section *Alternaria* included in the analysis.

304 As for the two-marker concatenated tree, the phylogenetic analysis using three markers

305 distinguished two major clades, *A. alternata* and *A. arborescens* SC (Supplementary material S2).

306 It could also distinguish these clades from other taxa in the *Alternaria* section *Alternaria* (*A.*

307 *gaisen*, *A. longipes*, *A. gossypina* and *A. alstroemeriae*). The phylogenetic tree pattern was similar

308   to that determined with two loci (i.e. Alta-1 and EndoPG). The *A. alternata* clade was divided into

309   four subclades, whereas the *A. arborescens* SC was separated into two subclades. Adding a third

310   locus to the analysis (OPA 10-2) did not improve the resolution within *A. alternata* and *A.*

311   *arborescens* SC (Supplementary material S2). The two phylogenetic analyses did, however, refine

312   identification: two Australian strains were consequently assigned to *A. alternata* whereas they had

313   previously been assigned to *A. longipes* (BRIP46356 and BRIP46455) by Harteveld et al. (2013b)

314   (Figure 1, Supplementary materiel S1).

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

316

317   Isolates from France were identified as either *A. arborescens* SC (91 isolates, 55%) or *A. alternata*

318   (75 isolates, 45%) based on the taxonomic identification with the Alta-1 and EndoPG markers. No

319   changes in the taxonomic identification were observed for the subset of isolates with sequences of

320   Alta-1, EndoPG and OPA 10-2 markers (Supplementary material S1). The distribution of isolates

321   differed according to the cultivar (Table 1). It was observed that *A. arborescens* SC isolates were

322   more frequent on Gala and Golden Delicious cultivars, whereas *A. alternata* isolates were more

323   frequent on cultivars Reinette grise du Canada, Dalinette and Crimson. On Braeburn and Pink lady

324   cultivars, there was a similar number of isolates of *A. arborescens* SC and *A. alternata*. On Garance

325   and GoldRush (Coop38cov) cultivars, too few isolates were recovered to make any comparison

326   (Table 1).

327   **Screening for the *Alternaria* apple pathotype**

328

329   None of the 166 French isolates were identified as the apple pathotype by PCR tests targeting the

330   *AMT* apple toxin (*AMT1*, *AMT2*) or cross-pathotype (*AMT14*) loci (Supplementary material S1).

331   All five apple pathotype reference isolates behaved as expected and yielded positive results for

332   *AMT1* and *AMT2* PCR tests and also for the tests targeting *AMT14,* which is common to apple,

333   pear and strawberry pathotypes (Supplementary material S1). However, four isolates formerly

334   identified as apple pathotype in earlier studies in Italy (Rotondo et al. 2012) and Israel (Gur et al.

335   2017) gave negative PCR results for the three loci in our conditions, thus overturning their

336   identification (Supplementary material S1).

337

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

339   At 4 dpi, 27 isolates — including the negative control *A. brassicicola* — were able to induce at

340   least one necrotic spot on detached leaves of apple cultivars Golden Delicious and Gala. The only

341   exception was an *A. arborescens* SC isolate (16_489b3a) that did not induce necrotic spots on

342   detached Gala cultivar leaves. The zero-inflated Poisson GLMM used to assess the number of

343   lesions per leaf on 4 dpi, indicated a significant effect of the experiment repetition (Type II Wald;

344   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

345   cultivar (Type II Wald; χ^2=0.06; df=1; p=0.810) (Figure 2). All 28 tested isolates were able to

346   induce leaf blotch after 7 dpi on Golden Delicious and Gala. On 7 dpi, the zero-inflated beta

347   GLMM used to assess the proportion of the diseased leaf area indicated a significant effect of the

348   experiment repetition (Type II Wald; χ^2=84.013; df=2; p<0.001) and the apple cultivar (Type II

349   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)

350   (Figure 3a). On 10 dpi the zero-inflated beta GLMM indicated a significant effect of the

351   experiment repetition (Type II Wald; χ^2=91.82; df=2; p<2.2e^{-16}) and the apple cultivar (Type II

352   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)

353   (Figure 3b). On 7 and 10 dpi, leaves of the Golden Delicious cultivar were more susceptible than

354   those of Gala, as measured by the proportion of the diseased leaf area (Figure 3). Raw

355   measurements at 4, 7 and 10 dpi are presented in the supplementary material S4 section. Finally,

356   the identity of 23 of these strains was confirmed by re-isolating and sequencing (Alta-1 and

357   EndoPG loci), fulfilling Koch's postulates (Supplementary material S1). No disease symptoms

358   were observed on leaves inoculated with water. Examples of the results from the pathogenicity

359   tests are shown in the supplementary material S5 section.

360

361   Discussion

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

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

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

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

366   and *AMT14* (Armitage et al. 2020) — which characterizes isolates of the apple pathotype. Our

367   results showed that this pathogen was not present in France within the sampled regions and

368   years. To date, molecular detection of the apple pathotype has only been possible by PCR tests

369   that target genes present in the CDC. These targets are associated with secondary metabolite

370   clusters involved in the production of the apple pathotype host-specific toxin *AMT* (Armitage et

371   al. 2020; Harimoto et al. 2007; Johnson et al. 2000). Although all our apple pathotype reference

372   strains gave positive results using the three markers, the Italian and Israeli strains previously

373   identified as apple pathotype by PCR based on the amplification of a modified PCR test targeting

374   *AMT1* (Rotondo et al. 2012) and *AMT3* (Gur et al. 2017) gave negative results in our study with

375   tests targeting loci *AMT1*, *AMT2* and *AMT14*. In the case of the Israeli strains, the primers

376   targeting *AMT3* (Harimoto et al. 2007), showed unsatisfactory results in our preliminary tests as

377   several unexpected bands appeared after gel electrophoresis of the PCR product (data not shown)

378   and these primers were discarded for subsequent molecular tests. The results obtained with the

379   Italian strains are more difficult to explain because the initial study of Rotondo et al. (2012)

380   performed several confirmation tests (including sequencing of the products). One hypothesis that

381   may explain the difficulty in amplifying these loci is the occurrence of partial or total

382   chromosomal loss in isolates. This phenomenon has previously been reported in the apple

383   pathotype by Johnson et al. (2001) and is due to chromosomal instability in culture. To avoid this

384   problem, in the analysis of French isolates, our tests targeting *AMT1*, *AMT2* and *AMT14* were

385   performed right after isolation, avoiding several subculturing cycles. However, in all the cases

386   where the presence of *AMT1*, *AMT2* and *AMT14* was assessed (in the five reference isolates;

387   Supplementary material S1), all three gene-specific PCR assays gave positive results. Based on

388   the results of our study, we suggest that the apple pathotype should be detected from pure

389   cultures by using at least two or more of the existing molecular tests to target *AMT1* and *AMT2*,

390   which is a good option if the objective is to specifically detect the apple pathotype. By

391   optimizing and validating current tools or developing new molecular tests, it might be possible to

392   detect diseases *in planta* from symptomatic leaves, which could avoid isolate subculturing cycles

393   while minimizing the risk of chromosomal loss.

394

395   **Co-existence of *Alternaria alternata* and the *Alternaria arborescens* species complex in**

396   **French orchards**

397   The second objective of this study was to identify *Alternaria* species or groups associated with

398   *Alternaria* leaf blotch (ALB) and *Alternaria* fruit spot (AFS) in French orchards. The

399   phylogenetic trees generated after using MLST clearly showed that these diseases are caused by

400   two phylogenetic clades: *A. alternata* and *A. arborescens* SC, regardless of the apple cultivar.

401   Our results also showed that both taxa may co-exist in the same orchard. These results confirm

402   that these two *Alternaria* taxa are the major cause of ALB and AFS in regions of the world

403   where these diseases have been studied so far (Gur et al. 2017; Harteveld et al. 2013b; Rotondo

404   et al. 2012; Toome-Heller et al. 2018; Wenneker et al. 2018). In addition, our results suggest that

405   sequencing two loci, i.e. Alta-1 and EndoPG, is enough to be able to distinguish *Alternaria*

406   isolates involved in these diseases. Firstly, these two loci enable the two major phylogenetic

407   clades — *A. alternata* and *A. arborescens* SC — to be distinguished. Secondly, the loci also

408   clearly distinguish these two clades from other *Alternaria* taxa within the *Alternaria* section.

409   Including the OPA 10-2 locus did not substantially improve the molecular identification of the

410   strains.

411

412   ***Alternaria alternata* and the *Alternaria arborescens* SC are responsible for defoliation in**

413   **French apple orchards**

414   We showed that the isolates collected from necrotic leaves were able to produce symptoms on

415   detached apple leaves of cultivars Gala and Golden Delicious. The latter cultivar was more

416   susceptible under our conditions, as shown by measurements of the diseased leaf area after 7 and

417   10 dpi, a quantitative trait generally used to measure pathogen aggressiveness. Gala and Golden

418   Delicious were used for pathogenicity tests since they are the most important cultivars in France.

419   Although these cultivars are considered as relatively "susceptible" to ALB and AFS (Filajdić &

420   Sutton 1991; Harteveld et al. 2014; Rotondo et al. 2012), earlier studies have shown that there is

421   little, or no cultivar specificity in *Alternaria* taxa causing ALB and AFS, at least for the most

422   economically important apple cultivars used worldwide (Filajdić & Sutton 1991; Harteveld et al.

423   2014; Li et al. 2019). Management of the disease may involve resistant cultivars. However,

424 further research involving more cultivars would be required to assess potential cultivar-

425 specificity among *Alternaria* taxa causing ALB and AFS. The selection of disease-resistant

426 cultivars should rely upon studies combining data collected from the field and from trials under

427 controlled conditions (Li et al. 2019).

428 Our results also showed that the entire subset of *Alternaria* isolated from apple leaves or fruit

429 fulfilled Koch's postulates. The pathogenicity tests showed that there are no significant

430 differences between isolates of *A. alternata* and *A. arborescens* SC as assessed by the number of

431 lesions per leaf on 4 dpi or the proportion of the diseased leaf area on 4 and 7 dpi. Both results

432 are in agreement with previous studies that suggest that pathogenicity may be isolate-dependent

433 rather than species-dependent (Harteveld et al. 2014; Rotondo et al. 2012). One of the limits of

434 our study is that we could not assess the pathogenicity of any of the reference apple pathotype

435 strains because too few spores could be obtained during cultivation. It is important to highlight,

436 however, that previous studies comparing the pathogenicity of apple pathotype isolates with

437 other *Alternaria* isolates in apples have shown discrepant results: while Armitage et al. (2020)

438 showed that apple pathotype strains were significantly more pathogenic than other isolates that

439 do not carry CDCs, Rotondo et al. (2012) did not observe any difference in levels of

440 pathogenicity between apple pathotype isolates and other *Alternaria* isolated from apple leaves

441 or fruit. Unexpectedly, we observed symptoms on apple leaves inoculated by *A. brassicicola,*

442 which has never been reported as pathogenic on apples to our knowledge. These results suggest

443 that *Alternaria* isolates from other *Alternaria* sections that do not carry CDCs involved in the

444 production of HTS may also cause ALB symptoms under controlled conditions. This is probably

445 associated with the production of nonspecific Alternaria toxins that can affect many plants

446 regardless of whether they are or are not a host of the pathogen (Tsuge et al. 2013). However, as

447   shown here and elsewhere, under natural conditions only small-spore Alternaria (*Alternaria*

448   section *Alternaria*) have so far been described as apple pathogens causing ALB and AFS. Recent

449   genomic resources, including the genome of *A. brassicicola* (Belmas et al. 2018) and isolates of

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

451   analysis that may clarify these pathogenicity mechanisms.

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

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

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

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

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

457   1992; Harteveld et al. 2013a; Kim et al. 1986). Potential changes in these two parameters, or

458   other climatic factors, should be studied in greater depth in the French regions concerned by

459   AFS. Another hypothesis is the introduction of more virulent strains. This could occur by the

460   long-distance movement of spores carried by wind currents that may have transported *Alternaria*

461   air inoculum into apple orchards from sources in other apple-producing regions (Fernández-

462   Rodríguez et al. 2015; Woudenberg et al. 2015b). Finally, the emergence of fungicide resistance

463   among strains should not be ruled out, considering that apple orchards are treated intensely with

464   fungicides, mainly used to control apple scab caused by *Venturia inaequalis*, which also

465   contributes to the control of ALB and AFS (Horlock 2006).

466

467   **Conclusions**

468   Since 2016, Alternaria leaf blotch and premature defoliation attributed to *Alternaria* spp. have

469   been observed in apple-producing regions in central and south-eastern France. The emergence of

470   the *Alternaria* apple pathotype was suspected following its observation in northern Italy. The

471 presence of the apple pathotype in French orchards was therefore assessed by a specific PCR

472 targeting three genes located on conditionally dispensable chromosomes across a large collection

473 of *Alternaria* isolates. Our results showed that the *Alternaria* apple pathotype was not present.

474 Taxonomic identification of these isolates, assessed by multi-locus sequence typing and

475 construction of phylogenetic trees, indicates that Alternaria leaf blotch in France is associated

476 with isolates of *A. alternaria* and *A. arborescens* SC. Pathogenicity tests of a subsample of

477 isolates demonstrated that they were all able to induce necrotic symptoms on detached apple

478 leaves of the cultivars Gala and Golden Delicious. Our results also showed that there are no

479 significant differences in levels of pathogenicity between isolates of *A. alternata* and *A.*

480 *arborescens* SC. Our controlled pathogenicity tests do suggest, however, that cultivar Golden

481 Delicious is more susceptible to Alternaria leaf blotch. In the future, genetic and epidemiological

482 approaches are required to clarify why Alternaria leaf blotch events have increased in frequency

483 and severity in some regions of France.

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485

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Distribution of *Alternaria* isolates obtained from French orchards from years 2016-2019.

The table shows the apple cultivar, the number of samples, the taxa (*Alternaria arborescens* SC or *Alternaria alternata*) and the co-occurrence of the isolates in the same orchard. The samples were identified by sequencing EndoPg and Alta-1. a: cultivars Galastar and Royal Gala included, b: cultivar Reinette grise du Canada (Canada), c: cultivar Crimson Crisp included and d: cultivar Rosy Glow included.

1 **Table 1.** Distribution of *Alternaria* isolates obtained from French orchards from years 2016-2019.

2 The table shows the apple cultivar, the number of samples, the taxa (*Alternaria arborescens* SC or

3 *Alternaria alternata*) and the co-occurrence of the isolates in the same orchard. The samples were

4 identified by sequencing EndoPg and Alta-1. a: cultivars Galastar and Royal Gala included, b:

5 cultivar Reinette grise du Canada (Canada), c: cultivar Crimson Crisp included and d: cultivar

6 Rosy Glow included.

Cultivar	No. of samples (including fruit samples)	*A. arborescens* species complex	*A. alternata*	Co-occurrence/sample (including fruit samples)
Braeburn	2	4	4	1
Gala[a]	13 (2)	16	8	3
Golden Delicious	10	39	10	5
Canada[b]	9	14	17	3
Dalinette	4	5	12	4
Crimson[c]	4	2	8	1
Belchard	1	1	1	1
Pink Lady[d]	7 (3)	9	10	4 (2)
Garance	1	0	2	0
GoldRush	1	1	3	1
Total	**52 (5)**	**91**	**75**	**23**

Characteristics of primer pairs used in this study for multi-locus sequence typing (MSLT) identification of isolates and specific PCR.

1   **Table 2.** Characteristics of primer pairs used in this study for multi-locus sequence typing (MSLT) identification of isolates and specific

2   PCR.

Locus / function	Primer	Primer sequence (5'-3')	Reference	Annealing temperature (°C)	Amplicon length (pb)
Alta-1 / Alternaria major allergen 1	Alt-for	ATGCAGTTCACCACCATCGC	(Hong et al. 2005)	57	472
	Alt-rev	ACGAGGGTGAYGTAGGCGTC			
EndoPG / Endopolygalacturonase	PG3	TACCATGGTTCTTTCCGA	(Andrew et al. 2009)	56	464
	PG2b	GAGAATTCRCARTCRTCYTGRTT			
OPA 10-2 / Anonymous noncoding region	OPA10-2L	TCGCAGTAAGACACATTCTACG	(Andrew et al. 2009)	62	634
	OPA10-2R	GATTCGCAGCAGGGAAACTA			
AMT-1 / Non-ribosomal peptide synthethase	LinF1	TATCGCCTGGCCACCTACGC	Johnson et al. (2000)	65	496
	LinR	TGGCCACGACAACCCACATA			
AMT-2 / Aldo-keto reductase	AMT2-f2	GTTGCAGAATCGCAAACTCA	(Roberts et al. 2012)	57	653
	AMT2-r2	GGCTCTTGGTCTCAAATCCA			
AMT-14 / Unknown function	AMT14-EMR-F	TTTCTGCAACGGCGKCGCTT	Armitage et al. (2015)		
	AMT14-EMR-R	TGAGGAGTYAGACCRGRCGC		66	436

Figure 1

Bayesian phylogenetic tree of Alta-1 and EndoPg markers. The tree was constructed with sequences of 352 *Alternaria* isolates (261 sequences were generated in this study).

The color legend refers to the Bayesian posterior probabilities of the tree nodes. *Alternaria alternata* isolates are shown in blue. The *Alternaria arborescens* SC isolates are shown in red. Isolates from other taxonomic groups of the *Alternaria* section *Alternaria* are represented in orange (*Alternaria alstroemeriae*), purple (*Alternaria gaisen*), brown (*Alternaria longipes*) and grey (*Alternaria gossypina*). Isolates used in pathogenicity tests are highlighted in a yellow background.

Tree scale: 0.01
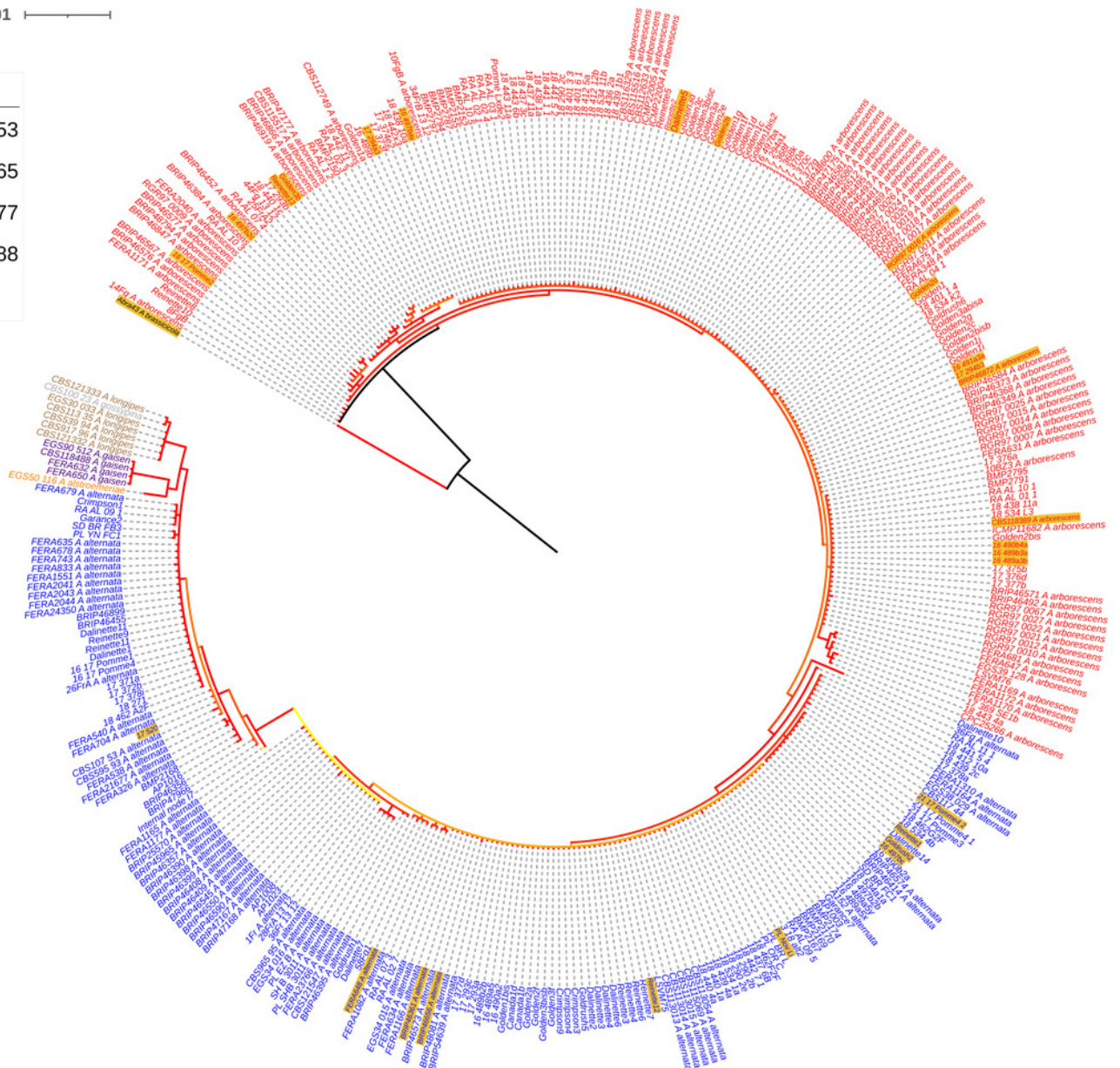
Figure 2

Mean number of leaf lesions per cultivar (Gala in red and Golden Delicious in blue) 4 days post-inoculation (4 dpi).

Results are reported for isolates of *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola*, which were identified by multi-locus sequence typing (MLST). Pathogenicity tests were performed on unwounded abaxial leaf surfaces with six separate point inoculations of 10 μl of *Alternaria* conidial suspensions (concentration of 1x105 conidia/μL). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. No differences were observed between isolates of *Alternaria alternata* and *Alternaria arborescens* SC or the apple cultivar (Golden Delicious and Gala). A significant effect of the experiment repetition (Type II Wald; $\chi^2=17.75$; $p<0.001$) was observed.
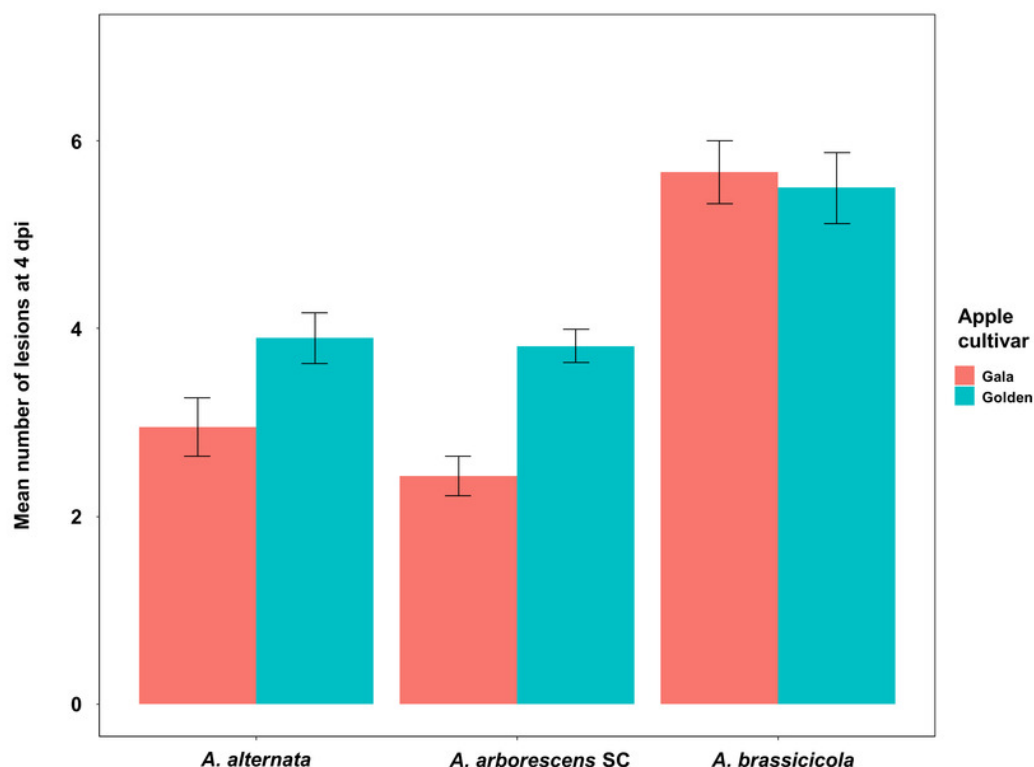
Figure 3

Mean proportion of the diseased leaf area per variety 7 and 10 days post-inoculation (7, 10 dpi) reported for *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola*.

a. Mean proportion of the diseased leaf area per cultivar (Gala in red and Golden Delicious in blue) 7 days post-inoculation (7 dpi) reported for isolates of *Alternaria alternata*, *Alternaria arborescens* SC and *Alternaria brassicicola*, identified by multi-locus sequence typing (MLST). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. The results showed that on 7 dpi, leaves of the Golden Delicious cultivar were more susceptible than leaves of the Gala cultivar (Type II Wald; $\chi^2=17.296$; $p<0.001$). No differences were observed between isolates of *Alternaria alternata* and *Alternaria arborescens* SC. A significant effect of the experiment repetition (Type II Wald; $\chi^2=84.013$; $p<0.001$) was also observed. b. Mean proportion of the diseased leaf area per cultivar (Gala in red and Golden Delicious in blue) 10 days after inoculation (10 dpi) reported for isolates of *Alternaria alternata*, *Alternaria arborescens* and *Alternaria brassicicola*, identified by multi-locus sequence typing (MLST). Statistical tests were only performed on *Alternaria alternata* and *Alternaria arborescens* SC. The results showed that on 10 dpi, leaves of the Golden Delicious cultivar were more susceptible than leaves of the Gala cultivar (Type II Wald; $\chi^2=11.80$; $p<0.001$). No differences were observed between isolates of *Alternaria alternata* and *Alternaria arborescens* SC. A significant effect of the experiment repetition (Type II Wald; $\chi^2=91.82$; $p<0.001$) was also observed.