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**Pathogenicity, molecular characterization and mycotoxigenic potential of *Alternaria* spp. agents of black spots on fruit and leaves of *Pyrus communis* in Italy**

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(Article begins on next page)

1 **Pathogenicity, molecular characterization and mycotoxigenic potential of *Alternaria* spp. agents**  
2 **of black spots on fruit and leaves of *Pyrus communis* in Italy**

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11

12 **Abstract**

13 Brown and black spots, caused by *Stemphylium* and *Alternaria* species, are important fungal diseases  
14 affecting European pear (*Pyrus communis* L.) in orchards. Both fungal genera cause similar  
15 symptoms, which could favour misidentification, but *Alternaria* spp. are increasingly reported due to  
16 the changing climatic conditions. In this study, *Alternaria* spp. were isolated from symptomatic leaves  
17 and fruits of European pear, and their pathogenicity was evaluated on pear fruits from cultivar ‘Abate  
18 Fétel’ and molecular and chemical characterization were performed. Based on Maximum likelihood  
19 phylogenetic analysis, 15 out of 46 isolates were identified as *A. arborescens* species complex  
20 (AASC), 27 as *A. alternata* and four were named *Alternaria* sp. Both species were isolated from  
21 mature fruits and leaves. In pathogenicity assays on pear fruits all isolates reproduced the symptoms  
22 observed in the field, both by wound inoculation and direct penetration. All but one isolates were  
23 produced Alternaria-toxins on European pears, including tenuazonic acid and alternariol (89.1% of  
24 the isolates), alternariol monomethyl ether (89.1%), altertoxin I (80.4%), altenuene (50.0%) and  
25 tentoxin (2.2%). These isolates also produced at least two mycotoxins and 43.5% produced four  
26 mycotoxins, with an average total concentration of the Alternaria-toxins exceeding  $7.58 \times 10^6$  ng/kg.  
27 Our data underline the potential risks for human health related to the high mycotoxin content found  
28 on fruits affected by black spot. This study represents also the first report of AASC as agent of black  
29 spot on European pear in Italy.

30

31 **Keywords:** European pear, black spot, *Alternaria alternata*, *Alternaria arborescens* species complex,  
32 mycotoxin, alternariol.

33

## 34 1. Introduction

35 The estimated global production of pears is around 40 million tonnes/year, and the main producing  
36 country is China with over 16 million tonnes/year. Italy is the third world and first European producer  
37 country with 29,616 ha of cultivated area and a production of 716,821 tonnes/year in 2018  
38 (FAOSTAT, 2020). In terms of production, the most important cultivar worldwide is Williams,  
39 whereas in Europe it is Conference, and in Italy it is Abate Fétel (FreshPlaza, 2020). Pear is highly  
40 appreciated due to its sweet and sour taste, but also for its beneficial role in human nutrition linked  
41 to the richness in polyphenols, flavonoids, vitamins, carotenoids, sugars, organic acids, fibres and  
42 minerals (Savić et al., 2019).

43 Among the most important fungal diseases affecting pear production in orchard, there are brown spot  
44 and black spot, caused by *Stemphylium* and *Alternaria* species, respectively. *Stemphylium vesicarium*  
45 (Cooke) Wint. is an economically relevant species affecting different crops, including pear. Fruits  
46 and leaves can be infected in the orchard causing huge economic losses (Köhl et al., 2013) with a  
47 global incidence between 1 to 10% (Montesinos and Vilardell 1992). *Stemphylium vesicarium* was  
48 reported on European pear (*Pyrus communis* L.) in Italy since the late '70 (Alberoni et al., 2008),  
49 whereas *Alternaria* spp. has been rarely reported. Both fungal genera cause similar symptoms, which  
50 could favour misidentification, but *Alternaria* spp. is increasingly reported due to the changing  
51 climatic conditions, that influence biological, environmental factors and a shift in microbial ecology  
52 (Van de Perre et al., 2015).

53 The genus *Alternaria* comprises ubiquitous species including saprobes and plant pathogens  
54 (Simmons, 2007). Two main species are associated with diseases on pears: *Alternaria gaisen* Nagano  
55 and *A. alternata* (Fr.) Keissl.. *Alternaria* black spot caused by *A. gaisen* on Japanese pear (*Pyrus*  
56 *pyrifolia* (Burm.f.) Nakai) is mainly distributed in Japan and Korea and was first reported in Italy in  
57 1991 with a restricted distribution (EPPO, 2020). Black spots caused by *A. alternata* were reported  
58 in Japan in 1933 and later in Korea, Italy, France, Greece and India on Japanese pear cv. Nijisseiki  
59 (Cavanni and Ponti, 1991; Baudry et al., 1993; Sandeep 2005). *Alternaria alternata* on European pear  
60 was reported in Greece on several cultivars, including cv. Abate Fétel (Thanassolopoulos, 1990). The  
61 pathogen was also reported in Japan on cultivar Le Lectier in 1993 (Tanahashi et al., 2016). During  
62 2012, severe symptoms of *Alternaria* black spot were also reported on leaves and fruits of *Pyrus*  
63 *communis* cv. Abate Fétel in Italy (Gianetti et al., 2013). *Alternaria alternata* is also associated with

64 dead flower buds disease of both European and Japanese pear in different countries (Wenneker et al.,  
65 2019). *Alternaria alternata* is reported to be pathogenic on pears in Asian and American countries,  
66 where it causes Alternaria blotch of apple, and the pathotype causing this symptom is considered a  
67 quarantine pathogen in Europe (Maeno et al., 1984; Tanahashi et al., 2016).

68 The taxonomy of the genus *Alternaria* has undergone different revisions and the current  
69 classification, based on morphological and molecular approaches, divides the genus into 27 sections  
70 (Lawrence et al., 2016). Most of the small-spored *Alternaria* species with concatenated conidia are  
71 grouped in *Alternaria* section *Alternaria*, with almost 60 morphological or host-specific species  
72 (Woudenberg et al. 2013), and *A. gaisen*, *A. alternata* and *A. arborescens* are the most important plant  
73 pathogens within this section. Different molecular approaches have been proposed to identify species  
74 within section *Alternaria*, including random amplified polymorphic DNA, amplified fragment length  
75 polymorphism, selective subtractive hybridisation and sequence characterised amplified genomic  
76 regions (Roberts et al. 2000; Somma et al. 2011; Roberts et al. 2012; Stewart et al. 2013). None of  
77 these approaches resulted in a clear distinction of species inside this section. Later, a multi-gene  
78 phylogeny based on nine gene regions was used in the study of Woudenberg et al. (2015), and 35  
79 morpho-species of *Alternaria* section *Alternaria* were synonymised as *A. alternata*.

80 The genus *Alternaria* is known to produce several secondary metabolites, including mycotoxins, and  
81 the most studied are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN),  
82 tenuazonic acid (TeA), altenuene (ALT), and altertoxins (ATXI, ATXII, and ATXIII) (Escrivá et al.,  
83 2017). The presence of mycotoxins creates issues to food safety and the consumer health (Pose et al.,  
84 2010; Prella et al., 2013). Mycotoxins are classified as non-host specific toxins and recently some  
85 studies reported their role in the pathogenicity of *Alternaria* spp. (Graf et al., 2012; Meena et al.,  
86 2017; Wenderoth et al., 2019). Andersen and Thrane (1996) used a high-performance liquid  
87 chromatography (HPLC) to distinguish small-spored *Alternaria* species from cereals, combined to  
88 morphological and cultural characteristics, whereas Siciliano et al. (2018) used HPLC with tandem  
89 Mass Spectrometry (HPLC-MS/MS) combined with molecular and morphological analyses to  
90 characterize *Alternaria* isolates isolated from basil.

91 The aim of the present work was to identify and characterize *Alternaria* spp. isolated from European  
92 pear in Italy, by evaluating their pathogenicity on fruits of pear cv. Abate Fétel. Molecular and  
93 chemical approaches were used to establish the species occurrence in orchard and to evaluate the  
94 potential risks for human health.

95

## 96 2. Materials and methods

## 97 **Fungal isolates**

98 *Alternaria* spp. samples were collected from pear fruit and leaves showing black spots in seven  
99 orchards of *Pyrus communis* cv. Abate Fétel located in north-western Italy, during August-October  
100 2018 (Table 1). Symptoms on leaves were small and circular, or with irregular margins, brown to  
101 black spots of 2 to 5 mm diameter, often converging to determine a widespread desiccation (Fig 1).  
102 On fruits, circular spots of 1 to 3 mm diameter sometimes surrounded by a reddish halo and centred  
103 on the lenticels were observed (Fig 1). The samples were surface-disinfected with 1% sodium  
104 hypochlorite, washed in sterile deionized water and air dried. Four to five fragments from each  
105 fruit/leaf lesion (black spots) were cut and plated onto Potato Dextrose Agar (PDA, Merck, Germany)  
106 Petri dishes. After 4 days of incubation at 25°C, 46 out of 70 samples (Table 2), selected on the basis  
107 of colony morphology and source of isolation, were maintained as monoconidial cultures in tubes of  
108 PDA and used for the molecular, biological and chemical characterization studies.

## 109 **Micro and macro-morphological observations**

110 For the macro-morphological analysis, all the isolates were plated onto PDA medium and incubated  
111 at  $25 \pm 1$  °C in the dark. Radial growth was measured after 6 days, along two perpendicular lines  
112 intersecting the centre of the plate, where the inoculum plug (3 mm) was positioned. For the micro-  
113 morphology, the isolates were grown onto Potato Carrot Agar (PCA, HiMedia Laboratories, India)  
114 for 20 days and conidia were observed using a Nikon Eclipse 55i microscope at 40× magnification.  
115 The radial growth of cultures derived from twenty conidia per isolate was measured.

## 116 **DNA extraction and molecular analysis**

117 DNA was extracted from mycelium collected on PDA plates incubated at 25 °C in the dark for 8 days  
118 using Omega E.Z.N.A. Fungal DNA Mini Kit (VWR International, USA) according to manufacturer's  
119 instructions. Partial amplification of the RNA polymerase second largest subunit (RPB2), *Alternaria*  
120 major allergen gene (Alt-a1), endopolygalacturonase gene (endoPG) and an anonymous gene region  
121 (OPA10-2) were obtained using the primers and conditions listed in Supplementary Table 1. PCR  
122 was carried out using Taq DNA polymerase kit (Qiagen, Germany) in a total volume of 25 µL  
123 containing 2.5 µL of Qiagen PCR Buffer 10 X, 0.5 µL of MgCl<sub>2</sub>, 0.75 µL of dNTPs (10 mM), 1 µL  
124 of each primer (10 µM), 0.2 µL of Taq DNA polymerase and 20 ng of template DNA. The PCR  
125 products were checked by gel electrophoresis in 1% agarose with 1 µL of GelRed™ (VWR  
126 International) at 100 V/cm for 45 min and purified using QIAquick© PCR purification Kit (Qiagen).  
127 MacroGen, Inc. (The Netherlands) sequenced the amplicons in both directions. The consensus  
128 sequences were created using a DNA Baser program (Heracle BiosoftS.R.L., Romania) and

129 alignment was performed using CLUSTALW through Molecular Evolutionary Genetics Analysis  
130 (MEGA6) software, version 6.0. After cutting the trimmed regions and manual correction, a dataset  
131 of 749 bp for RPB2, 354 bp for Alt-a1, 328 bp for endoPG, and 597 bp for OPA10-2, was obtained.  
132 MEGA version 6 was used to determine the best-fit nucleotide model for each dataset, for the  
133 concatenated dataset and to perform phylogenetic analysis under the Maximum Likelihood (ML)  
134 optimality criterion. All the reference sequences used for phylogeny are reported in **Supplementary**  
135 **Table 2**. All sequences were deposited in GenBank with accession numbers reported in **Table 2**.

### 136 **Pathogenicity assays on pear fruits**

137 Two *in vivo* assays were performed to test the pathogenicity of *Alternaria* spp. isolates. Healthy pear  
138 fruit cv. Abate Fétel at commercial maturity were surface sterilized with 1% sodium hypochlorite and  
139 rinsed in sterile deionized water. The first assay was performed to evaluate the pathogenicity on  
140 wounded fruits, where three wounds (2 mm diameter) were made per fruit, and each fruit was  
141 inoculated with a monoconidial suspension of  $1 \times 10^5$  conidia/mL prepared by growing isolates on  
142 Potato Carrot Agar (PCA; PCA, HiMedia Laboratories, India) for 20 days with a photoperiod of 12  
143 h light: 12 h dark. The fruits were maintained at  $24 \pm 1$  °C and exposed to natural daylight. The control  
144 pears were prepared as described above but they were treated with sterile deionized water. After 14  
145 days, rot diameters were measured. The experiment was performed twice, with three biological  
146 replicates and nine technical replicates per isolate ( $n=18$ ).

147 A second assay was performed at room temperature to evaluate the pathogenicity by direct  
148 penetration on healthy, non-wounded, fruits, inoculated with three mycelial plugs (5 mm each) per  
149 fruit. Mycelial plugs were obtained by culturing a selection of 17 isolates representative of fungal  
150 species, plant tissue, and orchard, onto Malt Extract Agar (MEA; Sigma-Aldrich, USA) plates  
151 incubated at  $25 \pm 1$  °C in the dark for eight days. The plugs were fixed to the fruits using Parafilm.  
152 The fruits were maintained at  $24 \pm 1$  °C. The control pears were prepared as described above but  
153 using medium plugs of MEA. After 14 days, rot diameters were measured. The experiment was  
154 performed twice, with three biological replicates and nine technical replicates per isolate ( $n=18$ ).

### 155 ***In vivo* mycotoxin production**

#### 156 **Chemicals**

157 Standards of tenuazonic acid (TeA) copper salt from *A. alternata* (purity  $\geq 98\%$ ), alternariol (AOH)  
158 from *Alternaria* spp. (purity  $\geq 94\%$ ), alternariol monomethyl ether (AME) from *A. alternata* (purity  
159  $\geq 98\%$ ) and tentoxin (TEN) from *Alternaria tenuis* (purity  $\geq 99\%$ ) were purchased from Sigma-  
160 Aldrich, whereas altenuene (ALT) from *Alternaria* spp. (purity  $\geq 98\%$ ) and altertoxin I (ATX-I)

161 (purity  $\geq$  97%) from *Alternaria* spp. were purchased from Fermentek (Jerusalem, Israel), all in  
162 crystallized form. A stock solution of 1000  $\mu\text{g}/\text{mL}$  was prepared in methanol for each mycotoxin and  
163 the working solution of 10  $\mu\text{g}/\text{mL}$  was prepared by dilution and mixing the stock solution of each  
164 analyte and kept at  $-20$  °C. Standard solutions for HPLC calibration and for additional experiments  
165 were prepared daily by diluting the working solution in solvent ( $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ , 50:50, v/v) or blank  
166 matrix. Methanol, acetonitrile (VWR International,) and toluene (Sigma-Aldrich) were HPLC-grade.  
167 Ammonium acetate and hydrochloric acid (Sigma-Aldrich) were analytical reagent-grade. Water was  
168 obtained from a Milli-Q system (G. Maina, Italy).

#### 169 **Alternaria-toxins extraction from pear fruit**

170 From each pathogenicity assay with wounded and inoculated fruits, two pears (biological replicates)  
171 were used to analyse the mycotoxins production *in vivo*. From each fruit, 3 cm-diameter rotten tissues  
172 were sampled and homogenized. Two technical replicates (4 g) from each fruit were placed in a  
173 centrifuge tube with 20 mL of extraction solution ( $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}:\text{H}_2\text{O}$ , 10:45:45, v/v/v) and 500  
174  $\mu\text{L}$  of HCl 2 N. The mixture was shaken for 30 min in an ultrasonic bath and then centrifuged at 4691  
175 g for 15 min. Sample extract was filtered through a Clarify-PP 0.22  $\mu\text{m}$  polypropylene filter (Agela  
176 Technologies, China) and transferred to a new centrifuge tube with 10 mL of toluene (twice), vortexed  
177 for 1 min and centrifuged at 4691 g for 10 min. The organic phase was evaporated to dryness in a  
178 rotary evaporator at 50 °C and the residue dissolved in 500 mL of  $\text{H}_2\text{O}:\text{CH}_3\text{CN}$  1:1 for the HPLC-  
179 MS/MS analysis. The assay was performed twice, with two biological replicates and four technical  
180 replicates ( $n=8$ ).

#### 181 **HPLC-MS/MS analysis of Alternaria-toxins**

182 The analysis of Alternaria-toxins was carried out using a 1260 Agilent Technologies system (Agilent,  
183 USA) consisting of a binary pump and a vacuum degasser, connected to a Varian autosampler, Model  
184 410 Prostar (Hansen Way, USA), equipped with a 20  $\mu\text{L}$  loop coupled to a Varian 310-MS TQ Mass  
185 Spectrometer. The chromatographic column used for LC separation was a Gemini-NX C18 (150 x  
186 3.0 mm, 3.0  $\mu\text{m}$ , Phenomenex, Torrance, CA, USA). Water (solvent A) and acetonitrile (solvent B),  
187 both with ammonium acetate 5 mM, were used as mobile phase at a flow rate of 300  $\mu\text{L}/\text{min}$ . The  
188 initial mobile phase contained 40% B and was held for 2 min, and then the proportion of B was  
189 linearly increased to 100% over 6 min and then held for 4 min; finally, the column was returned to  
190 initial conditions and equilibrated for 10 min before the next injection. Sample ionization was  
191 performed by an electrospray (ESI) ion source operating in negative ion mode and the quantification  
192 was carried out performed using multiple reaction monitoring (MRM) using the following transition  
193 reactions:  $m/z$  196 $>$ 139 CE 20 eV and  $m/z$  196 $>$ 112 CE 24 eV for TeA;  $m/z$  257 $>$ 213 CE 22 eV and

194 m/z 257>147 CE 34 eV for AOH; m/z 271>256 CE 22 eV and m/z 271>228 CE 28 eV for AME, m/z  
195 413>271 CE 16 eV and m/z 413>141 CE 18 eV for TEN, m/z 291>229 CE 12 eV and m/z 291>247  
196 CE 20 eV for ALT, m/z 351>297 CE 25 eV and m/z 351>263 CE 35 eV for ATX-I. The collision  
197 gas (Ar) pressure was set at 2 mbar for all experiments.

### 198 **HPLC-MS/MS method validation for Alternaria-toxins**

199 The developed analytical method was evaluated for linearity, limit of detection (LOD), limit of  
200 quantification (LOQ), recovery and matrix effect (ME) for TeA, AOH, AME, TEN, ALT and ATX-  
201 I. These parameters were validated by following the guidelines of EN ISO/IEC 17025:2017 and  
202 performance criteria reported in Commission Regulation (EC) 401/2006. Different concentrations of  
203 mycotoxin standards were analysed to evaluate the linearity of measurements. Calibration standards  
204 were prepared by diluting the working solution in blank matrix. LOD and LOQ were estimated by  
205 the linearity of the calibration curves using spiked matrix samples. The recovery of Alternaria-toxins  
206 was determined at three concentrations in the pear matrix. Blank samples were spiked with standards  
207 of TeA, AOH, AME, TEN and ATX-I at low (50 µg/kg), middle (250 µg/kg) and high concentration  
208 (500 µg/kg) in three replicates, prior to extraction, and after extraction for the ME. The precision of  
209 the method was studied by investigating repeatability and reproducibility of peak area of all  
210 mycotoxins. Repeatability (intra-day precision) was evaluated by measuring 5 parallel injections of  
211 3 replicates within a day. Reproducibility (inter-day precision) was calculated from the data of the  
212 experiment carried out in three consecutive days. Mixed standard solutions of TeA, AOH, AME,  
213 TEN and ATX-I were used at the concentration of 200 µg/L. Evaluation of repeatability and  
214 reproducibility was based on calculating the relative standard deviation (RSD %).

### 215 **Statistical Analysis**

216 The analysis of the differences between the mycotoxins produced by the isolates correlated to the  
217 species was performed using Mann-Whitney two-tailed test using the software IBM SPSS statistics  
218 software Inc. version 24 (Chicago, IL, USA). Mean mycotoxins concentrations were calculated by  
219 using 0 for negative samples. Experimental results are reported as mean ± standard deviation (SD).

220

## 221 **3. Results**

### 222 **Taxonomic assignment**

223 Forty-six isolates of *Alternaria* spp. were collected from pear leaves (23) and fruits (23). Isolates  
224 showed an initially light grey colour that turned into olive to dark green. Colonies were mostly with



225 white margins onto PDA plates (Supplementary Fig. 1), with a mean radial growth of  $3.75 \pm 0.87$  cm  
226 after 7 days growth at  $25 \pm 1$  °C. Hyphae were brown, while conidiophores were light brown. Conidia  
227 were ovoid or ellipsoidal with 1-4 transverse septa and 0-5 longitudinal septa, mean  $20.07 \pm 0.64$  µm  
228 in length and  $11.41 \pm 0.06$  µm in wide (Supplementary Fig. 1). Based on these morphological  
229 observations, the isolates were tentatively identified as *Alternaria* spp. (Simmons, 2007; Woudenberg  
230 et al., 2013).

231 According to the classification reported by Woudenberg et al. (2015) and Lawrence et al. (2016), the  
232 isolates could not be attributed to *Alternaria* section *Alternaria* by morphological observations (Table  
233 2) and a specific expertise is needed to correctly identify based only on morphological characters. An  
234 alternative identification tool in species assignment is represented by molecular analysis.

235 The dendrogram (Fig. 2) and bootstrap consensus tree (Supplementary Fig. 2), obtained with  
236 phylogenetic ML analysis based on 4 concatenated genes, showed that the isolates clustered in two  
237 groups, each one including the reference species of *Alternaria* section *Alternaria*, with a statistical  
238 support lower than 70%. Fifteen out of 46 isolates grouped together with the CBS references isolates  
239 of *A. arborescens* species complex (AASC), whereas three isolates (GB3, GB4, 2AFA) did not cluster  
240 with any CBS references isolates. The remaining 28 isolates grouped together with the CBS  
241 references isolates of *A. alternata* (Fig. 2). The two species were isolated from both fruits and leaves  
242 and were present in all the sampled orchards except for orchard n° 7, where only *A. alternata* was  
243 found.

244 By considering each gene locus, phylogenetic tree topology and species assignment were different  
245 for some isolates (data not shown). However, the phylogeny based on OPA 10-2 (Fig. 3) was the only  
246 region showing strong bootstrap support for the AASC clade (74% bootstrap), where isolates  
247 clustered with AASC references. Only one isolate (2AFA) did not cluster with the references in the  
248 AASC clade. Furthermore, a second subgroup (bootstrap 84%) with two isolates (GB4 and MS5) did  
249 not cluster with the reference isolates of *A. alternata*. Based on these observations, we decided species  
250 assignment on the congruence between the results of the concatenated dataset and the single OPA 10-  
251 2 dataset. Fifteen isolates were identified as AASC, 27 isolates as *A. alternata* and the four isolates  
252 not clustering with the others (2AFA, GB3, GB4 and MS5) were named *Alternaria* sp..

253

## 254 Pathogenicity on pear fruits

255 All isolates were pathogenic when artificially inoculated on European pear (Table 2), with a mean rot  
256 diameter of  $4.0 \pm 0.59$  cm. The isolate BR3, isolated from fruit, showed the highest rot diameter (4.92

257  $\pm 0.90$  cm), whereas the isolate F1A, isolated from leaf, showed the lowest rot diameter ( $3.01 \pm 0.06$   
258 cm). All isolates inoculated on pears caused the development of black rot (Fig. 4).

259 A selection of isolates was used to perform the pathogenicity assay on healthy non-wounded fruits.  
260 All isolates confirmed to be pathogenic by directly penetrating the fruits (Fig. 5; Supplementary Table  
261 3), with a mean rot diameter of  $3.03 \pm 0.52$  cm. The isolates ALT CER2A and ROS15, isolated from  
262 fruit and leaf respectively, showed the highest rot diameter ( $3.83 \pm 0.25$  cm).

### 263 **Alternaria-toxins production on pear**

264 Six mycotoxins (TeA, AOH, AME, ALT, ATX-I and TEN) were investigated using the external  
265 calibration method and the range of calibration curve was defined for each analyte based on the  
266 amount detected in pear samples. In order to validate the method, some pear samples were analyzed  
267 for the absence of the target mycotoxins to evaluate their natural occurrence. Good linearity was  
268 obtained for every compound ( $R^2 > 0.993$ ). All the calculated recoveries were between 70% and 100%  
269 (Supplementary Table 4), in accordance with the Commission Regulation (EC) No 401/2006 of  
270 February 2006. The matrix mostly influenced TeA and AOH with signal suppression and  
271 enhancement, respectively (Supplementary Table 4). To compensate for the matrix effects on  
272 quantitative results, the calibration curve in blank matrix was built. The LOD and LOQ values of the  
273 six analytes are shown in Supplementary Table 4. The precision of data obtained (intra-day and inter-  
274 day data) at 200  $\mu\text{g/L}$  concentrations of all mycotoxins were within 10.0 RSD %. The retention times  
275 of the toxins were TeA 1.8 min, ALT 4.1 min, TEN 6.1 min, ATX-I 6.5 min, AOH 7.7 min, and AME  
276 9.9 min.

277 All *Alternaria* isolates were analysed for production of the six mycotoxins on pears (Table 3) and  
278 confirmed to be mycotoxin producers, being able to produce at least one analyte. The only exception  
279 is represented by isolate DV2, which did not produce any mycotoxin (Fig. 6). TeA and AOH were  
280 the most frequently produced mycotoxins (both 95.6%), followed by AME, ATX-I, ALT and TEN  
281 (89.1%, 80.4%, 50.0% and 2.2% respectively). About 39% of isolates showed the ability to produce  
282 simultaneously five mycotoxins (TeA, AOH, AME, ALT and ATX-I), but not TEN, produced only  
283 by isolate MS3 (*A. alternata*). Twenty isolates (43.5%) were able to produce four mycotoxins (TeA,  
284 AOH, AME and ALT or ATX-I), whereas six isolates (13%) produced three secondary metabolites.  
285 One isolate (ROC) produced only TeA. We also note that AOH and AME were detected together in  
286 89% of the samples.

287 The most mycotoxigenic isolates were 2AFA (*Alternaria* sp.), F1A (AASC), BR7 (*A. alternata*),  
288 ALTFRC (AASC), GB3 (*Alternaria* sp.), DV3 (AASC) and ROS3 (AASC), with a total concentration  
289 (all six analytes together) ranging from 20.60  $\mu\text{g/g}$  to 40.57  $\mu\text{g/g}$ , whereas the lowest mycotoxigenic

290 isolates were ROC (*A. alternata*), MS6 (*A. alternata*), ALCER2A (*A. alternata*), GB1 (AASC), GBF  
 291 (*A. alternata*) and AL1 (AASC), with a total concentration of less than 1 µg/g. In particular, TeA and  
 292 AME were the most abundant mycotoxins, with concentrations ranging from 0.06 µg/g (MS3) to  
 293 22.02 µg/g (DV3) for TeA and from 0.03 µg/g (ROS17) to 19.71 µg/g (ROS3) for AME. The AOH  
 294 levels varied from 0.04 µg/g (F4B) to 12.0 µg/g (ALTFRC). ALT and ATX-I were on average less  
 295 abundant compared to the other mycotoxins (from 0.006 µg/g to 0.256 µg/g for ALT and from 0.005  
 296 µg/g to 0.11 µg/g for ATX-I). The only isolate that produced TEN on inoculated pears was MS3, with  
 297 a concentration of 0.023 µg/g.

298 Moreover, *A. alternata* isolates showed significantly ( $P < 0.005$ ) different ability to produce AOH and  
 299 ALT compared to AASC isolates, using the Mann-Whitney two-tailed test. For the other mycotoxins  
 300 no significant differences were found ( $P > 0.005$ ).

301

#### 302 4. Discussion

303 *Alternaria*, one of the most common fungal genera, is found in different matrices such as plant tissues,  
 304 agricultural products, soil and the atmosphere (Woudenberg et al., 2013; Nishikawa and Nakashima,  
 305 2020), but few publications reported the presence of *Alternaria* section *Alternaria* as a pathogen on  
 306 European pear in Europe (Thanassouloupoulos, 1990; Gianetto et al., 2013; Wenneker et al., 2019).

307 In this study monitoring was carried out in seven orchards in order to investigate the pathogens  
 308 responsible of black spot on pear fruits cv. Abate Fétel. All orchards investigated showed the presence  
 309 of *Alternaria* spp.. In only one orchard we observed co-occurrence of *Alternaria* spp. and, to a lower  
 310 extent, of *Stemphylium vesicarium* (data not shown). *Stemphylium vesicarium* was the most  
 311 frequently pathogen normally isolated from brown spots on European pear in Italy since the late 1970s  
 312 (Alberoni et al., 2008). Unlike from what has been reported in literature and initially expected, in this  
 313 work we have mostly isolated *Alternaria* spp.. The present work consisted in identifying and  
 314 characterizing *Alternaria* species associated to black spot on European pear, by evaluating their  
 315 pathogenicity on pear fruits cv. Abate Fétel and by using molecular and chemical analysis. As  
 316 described by Peever et al. (2004) and Andrew et al. (2009), most of the species of *Alternaria* section  
 317 *Alternaria* cannot be distinguished using standard housekeeping genes. In the study of Woudenberg  
 318 et al. (2015), nine gene regions, 5.8S nrDNA (ITS), the 18S nrDNA (SSU), the 28S nrDNA (LSU),  
 319 glyceraldehyde-3-phosphate dehydrogenase (gapdh), translation elongation factor 1-alpha (tef1),  
 320 RPB2, Alt-a1, endoPG and OPA10-2, were used to solve the phylogeny of this section, but they  
 321 obtained a Bayesian posterior probability lower than 0.75 for the AASC. The phylogeny obtained  
 322 from our Maximum likelihood concatenated dataset of RPB2, Alt-a1, endoPG and OPA10-2 gene

323 regions for the isolates isolated from European pear in this study provided an unclear species  
324 assignment, with two clades dividing *A. alternata* isolates from AASC isolates, with low bootstrap  
325 value, and a few isolates that did not cluster with any reference isolate. Based on single locus  
326 phylogeny, a different species assignment for some isolates and a low bootstrap value at major  
327 internodes were observed. The only locus showing strong bootstrap support for the AASC clade (74%  
328 bootstrap) was OPA 10-2. Based on these observations, we decided species assignment on the  
329 congruence between the results of the concatenated dataset and the single OPA 10-2 dataset. This  
330 permitted to identify 15 isolates as AASC, 27 as *A. alternata* and four as *Alternaria* sp.. The results  
331 showed the inability of the selected genes to clearly distinguish AASC from *A. alternata*. The  
332 incongruence observed between tree topology and species assignment was similar to the results of  
333 [Woudenberg et al. \(2015\)](#), with a Bayesian Posterior Probabilities, which was lower than 0.75 for the  
334 AASC. The branching topology and low bootstrap support found with ML suggest that *A. alternata*  
335 could be paraphyletic ([DeMers, 2022](#)), although definitive conclusions are not possible at this stage.  
336 Both species were isolated from fruits and leaves and were present in all sampled orchards except  
337 one where only *A. alternata* was found. The presence of both *A. alternata* and AASC was previously  
338 observed in different hosts, such as apple ([Rotondo et al., 2012](#)), pistachio ([Pryor and Michailides,](#)  
339 [2012](#)), basil ([Siciliano et al., 2018](#)), pear ([Wenneker et al., 2019](#)), and citrus ([Garganese et al., 2016](#);  
340 [Aiello et al., 2020](#)).

341 The pathogenicity assay on wounded pears cv. Abate Fétel at commercial maturity showed that all  
342 isolates of *Alternaria* spp. were pathogenic, with no significant differences in rot development  
343 between isolates isolated from fruits or leaves. A second assay, performed on healthy non-wounded  
344 fruit, confirmed the pathogenicity on a selection of isolates, representative of fungal species, plant  
345 tissue and orchard. The isolates produced a black rot, both on wounded fruit, by conidial inoculation,  
346 and on healthy fruit, by contact with a mycelial plug. This demonstrates their pathogenicity either by  
347 wound colonization or by direct contact with the pear skin, though black spot symptoms could be  
348 obtained on immature fruit. *Alternaria alternata* on European and Japanese pear was previously  
349 reported associated to the dead flower buds disease ([Wenneker et al., 2019](#)). In Greece (1983) and  
350 Italy (2012), severe symptoms of *Alternaria* black spot caused by virulent *A. alternata* isolates were  
351 reported on *Pyrus communis* cv. Abate Fétel, with a disease incidence reaching 80% and 100%,  
352 respectively ([Thanassouloupoulos, 1990](#); [Gianetto et al., 2013](#)), with figures similar to the present  
353 study. During the last years, and in particular during season 2018, high temperature and humidity  
354 during summer, together with high precipitation during spring, were recorded. These changes in the  
355 average climate parameters could favour the infections of *Alternaria* spp. ([Dickinson and Bottomley,](#)  
356 [1980](#)), resulting in more serious and severe symptoms, as already reported by [Reis et al. \(2007\)](#) and

357 Yang et al. (2019). Previously, Gianetto and colleagues (2013) reported similar, but less severe  
358 symptoms in northern Italy regions (Piedmont, Trentino, and South Tyrol). This climatic change, and  
359 in particular the increase in the average temperature, could affect the pre-harvest development of  
360 *Alternaria* spp., as previously demonstrated (Van de Perre et al., 2015).

361 This study represents the first report of *A. arborescens* species complex as agent of black spot on  
362 European pear in Italy, but AASC was previously reported as agent of leaf blotch and premature leaf  
363 drop on apple cv. Golden Delicious in the Netherlands (Wenneker et al., 2018), as agent of dead  
364 flower buds on European pear in the Netherlands (Wenneker et al., 2019), and on *Pyrus* sp. in Austria  
365 (Woudenberg et al., 2015).

366 In our study, no specific toxins were produced by the two species isolated from European pear,  
367 underlining the inability of chemical analyses to differentiate *Alternaria alternata* from AASC. In  
368 particular, TeA and AOH (89.1% of the isolates), AME (89.1%), ATX-I (80.4%), ALT (50.0%) and  
369 TEN (2.2%) were produced. Accordingly to Zwickel et al. (2018), on rice inoculated with the same  
370 species, the main mycotoxins produced were TeA, AOH and AME. Hayashi and colleagues (1990)  
371 reported also the production of ATX-I by *A. alternata* isolates isolated from *Pyrus pyrifolia*.

372 It is well-known that mycotoxin production is influenced by different parameters, such as water  
373 activity ( $a_w$ ), temperature, and carbon and nitrogen sources. Our data showed that TeA, AOH and  
374 AME were the most produced mycotoxins (in frequency and amount) on inoculated pears at  $24 \pm 1$   
375 °C. As reported in literature, the optimal temperature for the production of these secondary metabolite  
376 is 25 °C, either *in vivo* or in synthetic media (Magan et al., 1984; Oviedo et al., 2009; Oviedo et al.,  
377 2010; Meena et al., 2017). Compared to the other *Alternaria*-toxins, ATX-I, ALT and TEN were  
378 produced less in terms of concentration and numbers of isolates able to produce them *in vivo*, different  
379 from the study of Li and colleagues (2001) for *A. alternata* isolates on wheat kernels. The great  
380 amount of carbon sources in pear fruit could have positively influenced the mycotoxin produced, as  
381 already reported by Brzonkalik et al. (2011). Furthermore, Van de Perre et al. (2015) reported that  
382 climate change can have an effect on several factors, including biological and environmental factors  
383 and a shift in microbiological ecology, which are influencing the pre-harvest development of  
384 *Alternaria* spp. and its potential to produce mycotoxins.

385 Currently, no regulatory limits are established by the European Union for *Alternaria* mycotoxins in  
386 food, but European Food Safety Authority (EFSA) classified some secondary metabolites, including  
387 AOH, AME, ATXI, ATXIII, and TeA as mycotoxins potentially harmful to human health, due to  
388 their genotoxic or mutagenic potential (EFSA, 2011; Arcella et al., 2016). In our study, 98% of the  
389 isolates were able to produce at least two mycotoxins and 43.5% produced four mycotoxins with a  
390 mean total concentration exceeding  $7.58 \times 10^6$  ng/kg. There are few or no relevant data on the toxicity

391 of *Alternaria*-toxins, however EFSA (EFSA, 2011) considered appropriate to use the threshold of  
392 toxicological concern (TTC) approach to assess the relative level of concern for these mycotoxins.  
393 For AOH and AME the suggested TTC was 2.5 ng/kg body weight per day (0.15 µg/person per day),  
394 while for TeA and TEN it was 1,500 ng/kg body weight per day (90 µg/person per day). The European  
395 Union, with the recent Commission Recommendation 2022/553, suggested some limits on *Alternaria*-  
396 toxins, with indicative levels for AOH, AME and TEA in certain foods. This document underlines  
397 the need to carry out investigations to identify the factors that influenced the production of these  
398 mycotoxins. In our experiments, the average concentration of the six *Alternaria*-toxins found on  
399 inoculated fruits was higher than the values reported in Commission Recommendation 2022/553 for  
400 the only fruit derived product reported (10 µg/kg AOH, 5 µg/kg AME and 500 µg/kg TeA),  
401 underlining a potential risk for human health.

402 The risks for consumers are related to the consumption of fruit-derived products, such as juices,  
403 nectars or purees. Often, second-choice fruits or fruits apparently healthy but affected by core rot  
404 could be used for the production of fruit-derived products (Moake et al., 2005; Spadaro et al., 2007).  
405 Pear juices and nectars are among the most consumed fruit-based products in Italy, and they are highly  
406 consumed by infants (Spadaro et al., 2008). The above mentioned factors contribute to create a  
407 potential risk for consumers. Gotthardt and colleagues (2019) reported that some apple and pear  
408 products for infants were highly contaminated by AOH, TEN and AME. The presence of AOH, AME,  
409 TeA and TEN was also reported on juices, tomato, cereal based products and sunflower seed during  
410 a European food surveys. Furthermore, in a study of Pan et al. (2017), *A. alternata* was detected on  
411 pear juice underlining the risks for human safety linked to the possible production of mycotoxin.

412 In literature, the main fungal pathogen reported on European pear is *S. vesicarium*, although from the  
413 orchards targeted by this study we were able to isolate this fungal species only in one orchard with a  
414 very low frequency (data not shown).

415 Generally, mycotoxins act as virulence factors, without being essential for the pathogenicity, and only  
416 few studies reported their implication in *Alternaria* spp. pathogenicity (Graf et al., 2012; Meena et  
417 al., 2017). More recently, Wenderoth et al. (2019) demonstrated that the production of AOH was  
418 associated to the virulence and colonization ability of *A. alternata* on tomatoes, citrus and apple. In  
419 our study, all the *Alternaria* spp. isolates isolated from fruits and leaves were virulent and produced  
420 a great amount of AOH and its derivative AME, suggesting that pathogenicity and virulence on  
421 European pear could be linked to the production of these mycotoxin.

422 In conclusion, the presence of different *Alternaria* species pathogenic on European pear suggests that  
423 more than one fungal species is responsible for black spot, an important information to plan effective  
424 management strategies in the field. The production of different mycotoxins also highlights the

425 possible strong effect on human health due to exposure to multiple toxic effects. Furthermore, the  
426 potential involvement of different mycotoxins on pathogenicity should be investigated, including the  
427 study of TeA, which is one of the main mycotoxins produced on pear fruits.

428

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- 601

602 **Table 1** - Orchard number, city, geographical coordinates, crop protection strategy and average  
 603 disease incidence (%) of the orchards of *Pyrus communis* cv. Abate Fétel monitored in northern Italy  
 604 during 2018.

Orchard number	Town	Geographical coordinates		Crop protection <sup>a</sup>	Disease incidence (%)
1	Saluzzo (CN)	44.6597333	7.5682833	Integrated	70%
2	Savigliano (CN)	44.6095556	7.6152500	Integrated	40%
3	Marene (CN)	44.6607222	7.6704444	Integrated	70%
4	Scarnafigi (CN)	44.6730790	7.5735600	Controlled residue	30%
5	Apparizione (CN)	44.6159058	7.5499196	Organic	100%
6	Scarnafigi (CN)	44.6870110	7.5611620	Integrated	10%
7	Scarnafigi (CN)	44.6842722	7.5528306	Organic	70%

605

606 <sup>a</sup>Integrated: copper and metiram before flowering, boscalid, penthiopyrad, fluxapyroxad during  
 607 flowering, tebuconazole, trifloxistrobin and fluazinam during fruit development; Controlled residue:  
 608 copper and metiram before flowering, boscalid, penthiopyrad, fluxapyroxad during flowering,  
 609 fluazinam, copper and sulphur during fruit development; Organic: copper and sulphur throughout the  
 610 cropping season.

611 **Table 2** - Species identification, accession numbers for the loci used for molecular analysis, and mean rot diameter obtained with the pathogenicity  
612 assay with wounded fruits for the isolates isolated from pear.

Isolate	Species	Source	Orchard number	Accession number				In vivo pathogenicity assay	
				RPB2	endoPG	Alt-a1	OPA 10-2	Rot diameter (cm ± SD) <sup>a</sup>	
MB1A	<i>A. alternata</i>	Fruit	1	MT642849	MT642803	MT642895	MT612381	4.03	± 0.01
MB1B	<i>A. alternata</i>	Fruit	1	MT642850	MT642804	MT642896	MT612382	4.18	± 0.58
ALCER2A	<i>A. alternata</i>	Fruit	1	MT642851	MT642805	MT642897	MT612383	3.29	± 0.95
ALTCER3B	<i>A. alternata</i>	Fruit	1	MT642852	MT642806	MT642898	MT612384	4,00	± 0.40
DV1	<i>A. alternata</i>	Leaf	1	MT642853	MT642807	MT642899	MT612385	4.53	± 0.07
DV2	AASC	Leaf	1	MT642854	MT642808	MT642900	MT612386	3.03	± 0.18
DV3	<i>A. alternata</i>	Leaf	1	MT642855	MT642809	MT642901	MT612387	3.95	± 0.19
DV5	AASC	Leaf	1	MT642856	MT642810	MT642902	MT612388	4.49	± 0.60
F1A	AASC	Leaf	2	MT642857	MT642811	MT642903	MT612389	3.01	± 0.05
F2B	AASC	Leaf	2	MT642858	MT642812	MT642904	MT612390	3.53	± 0.46
F4A2	AASC	Leaf	2	MT642859	MT642813	MT642905	MT612391	4.29	± 0.41
F4B	<i>A. alternata</i>	Leaf	2	MT642860	MT642814	MT642906	MT612392	4.28	± 0.13
2AFA	<i>Alternaria</i> sp.	Leaf	2	MT642861	MT642815	MT642907	MT612393	4.03	± 0.24
ALTFRB	AASC	Fruit	2	MT642862	MT642816	MT642908	MT612394	3.67	± 0.28
ALTFRC	AASC	Fruit	2	MT642863	MT642817	MT642909	MT612395	3.75	± 0.26
ALTFRF	<i>A. alternata</i>	Fruit	2	MT642864	MT642818	MT642910	MT612396	4.29	± 0.01
ROS2	AASC	Leaf	3	MT642865	MT642819	MT642911	MT612397	2.73	± 0.14
ROS3	AASC	Leaf	3	MT642866	MT642820	MT642912	MT612398	3.22	± 0.35
ROS15	<i>A. alternata</i>	Leaf	3	MT642867	MT642821	MT642913	MT612399	4.35	± 0.12
ROS16	<i>A. alternata</i>	Leaf	3	MT642869	MT642823	MT642915	MT612401	4.11	± 0.22
ROS17	<i>A. alternata</i>	Leaf	3	MT642868	MT642822	MT642914	MT612400	4.36	± 0.11
ROA	<i>A. alternata</i>	Fruit	3	MT642870	MT642824	MT642916	MT612402	3.38	± 0.19
ROB	<i>A. alternata</i>	Fruit	3	MT642871	MT642825	MT642917	MT612403	4.67	± 0.64
ROC	<i>A. alternata</i>	Fruit	3	MT642872	MT642826	MT642918	MT612404	3.83	± 0.23

Isolate	Species	Source	Orchard number	Accession number				<i>In vivo</i> pathogenicity assay	
				RPB2	endoPG	Alt-a1	OPA 10-2	Rot diameter (cm ± SD) <sup>a</sup>	
ROD	<i>A. alternata</i>	Fruit	3	MT642873	MT642827	MT642919	MT612405	3.95 ± 0.33	
AL1	AASC	Fruit	4	MT642874	MT642828	MT642920	MT612406	4.70 ± 0.31	
AL2	AASC	Fruit	4	MT642875	MT642829	MT642921	MT612407	3.72 ± 0.47	
AL5	<i>A. alternata</i>	Fruit	4	MT642876	MT642830	MT642922	MT612408	4.90 ± 0.42	
AL7	AASC	Fruit	4	MT642877	MT642831	MT642923	MT612409	4.71 ± 0.10	
BR1	<i>A. alternata</i>	Fruit	5	MT642878	MT642832	MT642924	MT612410	4.67 ± 0.59	
BR3	<i>A. alternata</i>	Fruit	5	MT642879	MT642833	MT642925	MT612411	4.92 ± 0.90	
BR5	<i>A. alternata</i>	Fruit	5	MT642880	MT642834	MT642926	MT612412	4.44 ± 0.98	
BR7	AASC	Fruit	5	MT642881	MT642835	MT642927	MT612413	3.93 ± 0.09	
GBB	<i>A. alternata</i>	Fruit	6	MT642882	MT642836	MT642928	MT612414	4.01 ± 0.29	
GBC	<i>A. alternata</i>	Fruit	6	MT642884	MT642838	MT642930	MT612416	3.70 ± 0.49	
GBF	<i>A. alternata</i>	Fruit	6	MT642885	MT642839	MT642931	MT612417	3.97 ± 0.66	
GBG	AASC	Fruit	6	MT642883	MT642837	MT642929	MT612415	3.56 ± 0.50	
GB1	AASC	Leaf	6	MT642886	MT642840	MT642932	MT612418	3.91 ± 0.44	
GB2	<i>A. alternata</i>	Leaf	6	MT642887	MT642841	MT642933	MT612419	4.34 ± 0.15	
GB3	<i>Alternaria</i> sp.	Leaf	6	MT642888	MT642842	MT642934	MT612420	4.73 ± 0.22	
GB4	<i>Alternaria</i> sp.	Leaf	6	MT642889	MT642843	MT642935	MT612421	4.29 ± 0.11	
GB6	<i>A. alternata</i>	Leaf	6	MT642890	MT642844	MT642936	MT612422	4.49 ± 0.41	
MS2	<i>A. alternata</i>	Leaf	7	MT642891	MT642845	MT642937	MT612423	4.33 ± 0.06	
MS3	<i>A. alternata</i>	Leaf	7	MT642892	MT642846	MT642938	MT612424	4.65 ± 0.26	
MS5	<i>Alternaria</i> sp.	Leaf	7	MT642893	MT642847	MT642939	MT612425	4.72 ± 0.02	
MS6	<i>A. alternata</i>	Leaf	7	MT642894	MT642848	MT642940	MT612426	3.78 ± 0.14	

613

614 <sup>a</sup> The values are expressed as the mean ± standard deviation (SD) of two experimental assays, each one with three fruits inoculated at three artificial  
615 wounds ( $n=18$ ).

616 **Table 3** - Isolate name, species and mycotoxin (TeA: Tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether; TEN: tentoxin; ALT:  
617 altenuene; ATX-I: altertoxin I) production *in vivo* for the *Alternaria* isolates isolated from pear.

Isolate	Species	TeA µg/g ± SD <sup>a</sup>	AOH µg/g ± SD <sup>a</sup>	AME µg/g ± SD <sup>a</sup>	TEN µg/g ± SD <sup>a</sup>	ALT µg/g ± SD <sup>a</sup>	ATX-I µg/g ± SD <sup>a</sup>
2AFA	<i>Alternaria</i> sp.	0.14±0.11	7.33±1.11	13.07±4.60	nd <sup>b</sup>	0.06±0.00	nd
AL1	<i>AASC</i>	0.31±0.01	0.32±0.04	0.36±0.01	nd	nd	nd
AL2	<i>AASC</i>	0.74±0.15	2.50±1.05	1.39±0.40	nd	0.07±0.01	0.01±0.00
AL5	<i>A. alternata</i>	0.45±0.00	1.32±0.21	2.14±0.50	nd	nd	0.008±0.00
AL7	<i>AASC</i>	nd	1.45±0.63	2.16±0.60	nd	0.06±0.00	0.005±0.00
ALCER2A	<i>A. alternata</i>	0.18±0.04	0.26±0.02	0.23±0.04	nd	nd	0.005±0.00
ALTCER3B	<i>A. alternata</i>	3.54±0.17	1.89±0.60	0.26±0.19	nd	nd	0.005±0.00
ALTFRB	<i>AASC</i>	1.45±0.03	5.17±2.30	4.20±0.60	nd	0.09±0.00	nd
ALTFRC	<i>AASC</i>	0.52±0.01	12.0±1.23	9.88±0.70	nd	0.03±0.00	0.011±0.00
ALTFRF	<i>A. alternata</i>	0.73±0.08	1.90±0.30	1.82±0.22	nd	nd	0.11±0.03
BR1	<i>A. alternata</i>	0.50±0.08	0.32±0.12	0.28±0.16	nd	nd	0.01±0.00
BR3	<i>A. alternata</i>	8.04±0.67	1.50±0.32	1.31±0.04	nd	nd	0.09±0.01
BR5	<i>A. alternata</i>	1.21±0.12	0.97±0.20	4.38±0.61	nd	0.06±0.00	nd
BR7	<i>AASC</i>	6.69±1.79	3.62±0.24	10.50±1.60	nd	0.10±0.00	0.02±0.00
DV1	<i>A. alternata</i>	0.12±0.02	0.44±0.02	1.40±0.50	nd	0.02±0.00	0.006±0.01
DV2	<i>AASC</i>	nd	nd	nd	nd	nd	nd
DV3	<i>A. alternata</i>	22.03±0.30	1.13±0.20	0.62±0.15	nd	0.008±0.00	0.01±0.00
DV5	<i>AASC</i>	0.46±0.16	0.36±0.04	0.19±0.07	nd	0.014±0.05	0.01±0.00
F1A	<i>AASC</i>	3.52±0.33	5.91±0.14	11.10±1.10	nd	0.23±0.03	0.06±0.00
F2B	<i>AASC</i>	0.07±0.01	0.46±0.14	1.07±0.23	nd	0.04±0.00	nd
F4A2	<i>AASC</i>	2.4±0.80	2.29±0.21	1.85±0.60	nd	nd	0.01±0.00
F4B	<i>A. alternata</i>	0.43±0.02	0.04±0.01	0.71±0.06	nd	nd	0.01±0.00
GB1	<i>AASC</i>	0.67±0.05	0.11±0.08	nd	nd	nd	0.02±0.00
GB2	<i>A. alternata</i>	3.87±0.34	0.26±0.02	1.54±1.45	nd	0.15±0.02	0.04±0.00
GB3	<i>Alternaria</i> sp.	9.38±0.24	7.36±0.54	6.12±0.05	nd	0.256±0.02	0.04±0.00
GB4	<i>Alternaria</i> sp.	7.34±0.00	2.20±0.20	4.78±0.14	nd	0.07±0.00	0.03±0.00



Isolate	Species	TeA µg/g ± SD <sup>a</sup>	AOH µg/g ± SD <sup>a</sup>	AME µg/g ± SD <sup>a</sup>	TEN µg/g ± SD <sup>a</sup>	ALT µg/g ± SD <sup>a</sup>	ATX-I µg/g ± SD <sup>a</sup>
<b>GB6</b>	<i>A. alternata</i>	3.07±0.26	0.23±0.03	0.29±0.05	nd	0.02±0.00	0.01±0.00
<b>GBB</b>	<i>A. alternata</i>	2.14±0.02	0.54±0.06	13.20±7.80	nd	0.04±0.01	0.02±0.00
<b>GBC</b>	<i>A. alternata</i>	1.42±0.20	4.02±1.40	2.50±0.90	nd	0.02±0.00	0.02±0.00
<b>GBF</b>	<i>A. alternata</i>	0.90±0.10	0.05±0.01	nd	nd	nd	0.01±0.00
<b>GBG</b>	<i>AASC</i>	0.18±0.05	1.37±0.30	1.20±0.20	nd	0.07±0.00	0.04±0.00
<b>MB1A</b>	<i>A. alternata</i>	3.65±0.13	2.38±0.32	1.52±0.30	nd	nd	nd
<b>MB1B</b>	<i>A. alternata</i>	8.29±0.15	6.03±2.02	3.47±1.10	nd	0.01±0.00	0.03±0.00
<b>MS2</b>	<i>A. alternata</i>	6.84±0.63	0.42±0.30	3.02±0.20	nd	nd	0.01±0.00
<b>MS3</b>	<i>A. alternata</i>	0.06±0.02	0.10±0.01	0.20±0.08	0.023±0.00	nd	nd
<b>MS5</b>	<i>Alternaria</i> sp.	0.78±0.13	0.35±0.12	0.27±0.02	nd	nd	0.01±0.00
<b>MS6</b>	<i>A. alternata</i>	nd	0.25±0.09	0.32±0.06	nd	0.006±0.00	nd
<b>ROA</b>	<i>A. alternata</i>	3.03±0.20	0.73±0.13	0.86±0.22	nd	0.008±0.00	0.01±0.00
<b>ROB</b>	<i>A. alternata</i>	1.43±0.68	1.02±1.30	nd	nd	nd	0.01±0.00
<b>ROC</b>	<i>A. alternata</i>	0.15±0.04	nd	nd	nd	nd	nd
<b>ROD</b>	<i>A. alternata</i>	2.07±0.12	0.32±0.01	0.10±0.01	nd	nd	0.02±0.00
<b>ROS15</b>	<i>A. alternata</i>	0.73±0.06	0.68±0.50	0.50±0.10	nd	nd	0.01±0.00
<b>ROS16</b>	<i>A. alternata</i>	1.14±0.06	0.92±0.10	0.75±0.30	nd	nd	0.04±0.00
<b>ROS17</b>	<i>A. alternata</i>	1.18±0.08	0.13±0.05	0.03±0.00	nd	nd	0.007±0.00
<b>ROS2</b>	<i>AASC</i>	1.00±0.01	1.01±0.40	0.32±0.07	nd	nd	0.02±0.00
<b>ROS3</b>	<i>AASC</i>	10.05±0.03	10.60±0.70	19.70±1.20	nd	0.19±0.01	0.03±0.00

618 <sup>a</sup> Each value is the average of two experimental assays, each one with two biological replicates and four technical replicates ± SD ( $n=16$ ).

619 <sup>b</sup> nd: not detected.

620 **Figures captions**

621 **Figure 1** - Symptoms of *Alternaria* black spot on *Pyrus communis* cv. Abate Fétel fruit and leaves.

622 **Figure 2** - Phylogenetic tree based on the concatenated RPB2, Alt-a1, endoPG and OPA10-2  
623 sequence datasets. The phylogenetic tree was constructed with Maximum Likelihood analysis using  
624 Tamura Nei (TN93) plus Gamma distribution (+G) model and by assuming that a certain fraction of  
625 sites are evolutionarily invariable (+I). The numbers at the major nodes indicate the bootstrap value  
626 from 1,000 bootstrapped datasets. Branches with bootstrap values lower than 70% are not shown.  
627 Evolutionary analyses were conducted using MEGA, version 6.

628 **Figure 3** - Phylogenetic tree based on the OPA 10-2 sequences. The phylogenetic tree was  
629 constructed with Maximum Likelihood analysis using Kimura 2-parameter plus Gamma distribution  
630 model. The numbers at the major nodes indicate the bootstrap value from 1,000 bootstrapped datasets.  
631 Branches with bootstrap values lower than 70% are not shown. Evolutionary analyses were conducted  
632 using MEGA, version 6.

633 **Figure 4** - Symptoms of *Alternaria* black rot on wounded *Pyrus communis* cv. Abate Fétel fruit  
634 inoculated with *Alternaria* species after 20 days at  $24 \pm 1$  °C.

635 **Figure 5** - Symptoms of *Alternaria* black rot on healthy un-wounded *Pyrus communis* cv. Abate Fétel  
636 fruit inoculated with *Alternaria* species after 14 days at  $24 \pm 1$  °C.

637 **Figure 6** - *Alternaria*-toxin production [ $\mu\text{g/g}$ ]  $\pm$  SD by the 46 *Alternaria* spp. isolates inoculated on  
638 *Pyrus communis* cv. Abate Fétel. Each value is the average ( $n=16$ ) of two experimental assays, each  
639 one with two biological replicates and four technical replicates.

640 **e-Xtra Figures captions**

641 **Supplementary Figure 1** - Morphology of some *Alternaria* spp. isolates isolated from fruits and  
642 leaves of *Pyrus communis* cv. Abate Fétel. a. Colonies morphology of AASC after 7 days of  
643 incubation at  $25 \pm 1^\circ\text{C}$  on Potato Dextrose Agar medium. b. Colonies morphology of *A. alternata*  
644 isolates after 7 days of incubation at  $25 \pm 1^\circ\text{C}$  on Potato Dextrose Agar medium. c. Conidial obtained  
645 from isolates grown on Potato Carrot Agar medium for 20 days.

647 **Supplementary Figure 2** - Bootstrap consensus tree based on the concatenated RPB2, Alt-a1,  
648 endoPG and OPA10-2 sequence datasets. The phylogenetic tree was constructed with Maximum  
649 Likelihood analysis using Tamura Nei (TN93) plus Gamma distribution (+G) model and by assuming  
650 that a certain fraction of sites are evolutionarily invariable (+I). Evolutionary analyses were conducted  
651 using MEGA, version 6.

653 **e-Xtra Table titles**

654 **Supplementary Table 1** - Information for the primers used for the phylogenetic analysis of *Alternaria*  
655 isolates used in this study: locus name, primer name, sequence, and references for the primers and  
656 the amplification protocols used.

658 **Supplementary Table 2** - References used in this study for the phylogeny of *Alternaria* spp. isolates  
659 used in this study.

661 **Supplementary Table 3** - Mean rot diameter  $\pm$  SD obtained with the pathogenicity assay performed  
662 by direct penetration using not wounded fruits, with mycelial plugs after 14 days at  $24 \pm 1^\circ\text{C}$  for  
663 representative isolates isolated from pear.

665 **Supplementary Table 4** - Validation parameters for the six *Alternaria* toxins in pear matrix.

666



Symptoms of Alternaria black spot on *Pyrus communis* cv. Abate Fétel fruit and leaves.

254x140mm (300 x 300 DPI)

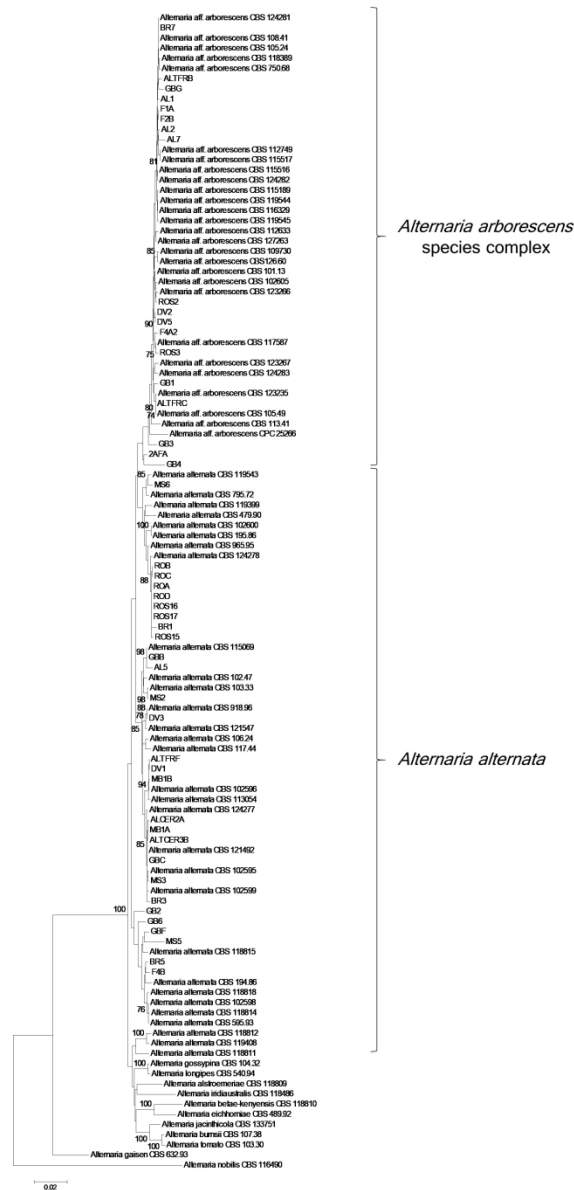


Figure 2 - Phylogenetic tree based on the concatenated RPB2, Alt-a1, endoPG and OPA10-2 sequence datasets. The phylogenetic tree was constructed with Maximum Likelihood analysis using Tamura Nei (TN93) plus Gamma distribution (+G) model and by assuming that a certain fraction of sites are evolutionarily invariable (+I). The numbers at the major nodes indicate the bootstrap value from 1,000 bootstrapped datasets. Branches with bootstrap values lower than 70% are not shown. Evolutionary analyses were conducted using MEGA, version 6.

190x338mm (300 x 300 DPI)

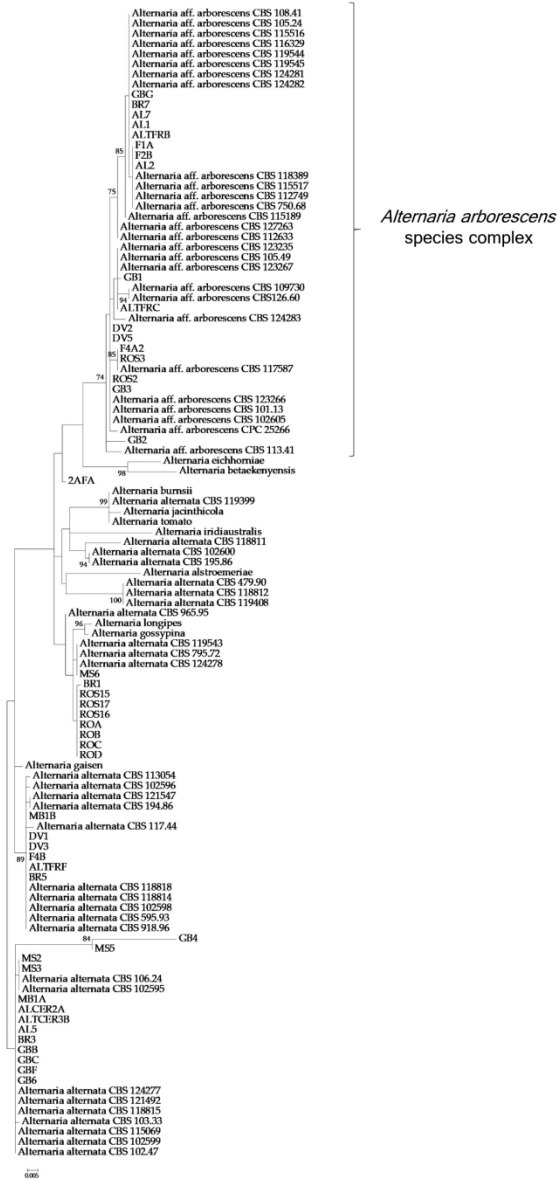


Figure 3 - Phylogenetic tree based on the OPA 10-2 sequences. The phylogenetic tree was constructed with Maximum Likelihood analysis using Kimura 2-parameter plus Gamma distribution model. The numbers at the major nodes indicate the bootstrap value from 1,000 bootstrapped datasets. Branches with bootstrap values lower than 70% are not shown. Evolutionary analyses were conducted using MEGA, version 6.

190x338mm (300 x 300 DPI)



Figure 4 - Symptoms of *Alternaria* black rot on wounded *Pyrus communis* cv. Abate Fétel fruit inoculated with *Alternaria* species after 20 days at  $24 \pm 1$  °C.

165x80mm (300 x 300 DPI)



Figure 5 - Symptoms of *Alternaria* black rot on healthy un-wounded *Pyrus communis* cv. Abate Fétel fruit inoculated with *Alternaria* species after 14 days at  $24 \pm 1$  °C.

165x57mm (300 x 300 DPI)



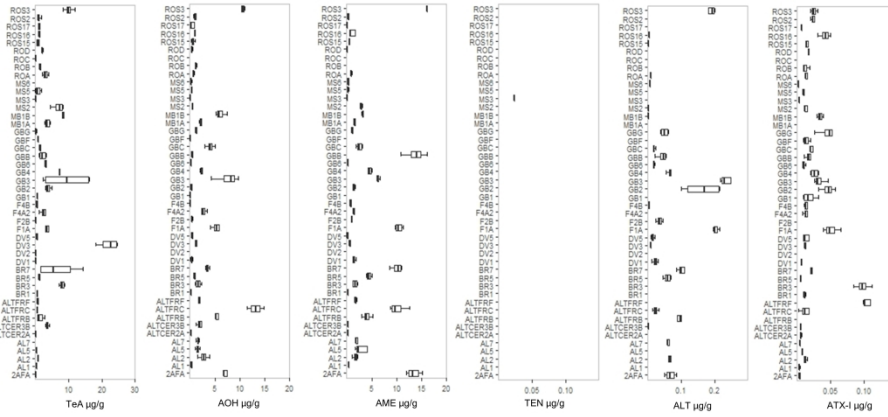


Figure 6 – Alternaria-toxin production [ $\mu\text{g/g}$ ]  $\pm$  SD by the 46 *Alternaria* spp. isolates inoculated on *Pyrus communis* cv. Abate Fétel. Each value is the average ( $n=16$ ) of two experimental assays, each one with two biological replicates and four technical replicates.

447x225mm (300 x 300 DPI)

## Supplementary Tables

**Supplementary Table 1** - Information for the primers used for the phylogenetic analysis of *Alternaria* isolates used in this study: locus name, primer name, sequence, and references for the primers and the amplification protocols used.

<b>Locus</b>	<b>Primers</b>	<b>Primer sequence</b>	<b>Primer reference</b>	<b>Amplification reference</b>
<b>endoPG</b>	PG3	TACCATGGTTCTTTCCGA	Isshiki <i>et al.</i> , 1997, 2001	Andrew <i>et al.</i> , 2009
	PG2b	GAGAATTCRCARTCRTCYTGRTT		
<b>OPA 10-2</b>	OPA 10-2R	GATTCGCAGCAGGGAAACTA	Andrew <i>et al.</i> , 2009	Andrew <i>et al.</i> , 2009
	OPA 10-2L	TCGCAGTAAGACACA TTCTACG		
<b>RPB2</b>	RPB2-5F2	GAYGAYMGWGATCAYTTYGG	Sung <i>et al.</i> , 2007	Woudenberg <i>et al.</i> , 2014
	fRPB2-7cR	CCCATRGCTTGYTTRCCCAT	Liu <i>et al.</i> , 1999	
<b>Alt-a1</b>	Alt-for	ATGCAGTTCACCACCATCGC	Hong <i>et al.</i> , 2005	Woudenberg <i>et al.</i> , 2014
	Alt-rev	ACGAGGGTGAYGTAGGCGTC		

**Supplementary Table 2** - References used in this study for the phylogeny of *Alternaria* spp. isolates used in this study.

Species	Strain designation	RPB2	endoPG	AltA1	OPA 10-2
<i>Alternaria alternata</i>	CBS 106.24; E.G.S. 38.029; ATCC 13963 ( <i>A. mali</i> )	KP124766	AY295020	KP123847	JQ800620
<i>Alternaria alternata</i>	CBS 103.33; E.G.S. 35.182; IHEM 3319 ( <i>A. soliaegyptiaca</i> )	KP124770	KP123999	KP123852	KP124607
<i>Alternaria alternata</i>	CBS 117.44; E.G.S. 06.190; VKM F-1870 ( <i>A. godetiae</i> )	KP124772	KP124001	KP123854	KP124609
<i>Alternaria alternata</i>	CBS 102.47; E.G.S. 02.062 ( <i>A. citri</i> )	KP124773	KP124002	KP123855	KP124610
<i>Alternaria alternata</i>	CBS 795.72; ATCC 24127; IHEM 3789	KP124778	KP124009	KP123862	KP124616
<i>Alternaria alternata</i>	CBS 194.86; E.G.S. 04.090; QM 1347 ( <i>A. pulvinifungicola</i> )	KP124784	KP124016	KP123869	KP124623
<i>Alternaria alternata</i>	CBS 195.86; E.G.S. 36.172; DAOM 185214 ( <i>A. angustiovoidea</i> )	KP124785	KP124017	JQ646398	KP124624
<i>Alternaria alternata</i>	CBS 479.90; E.G.S. 29.028 ( <i>A. pellucida</i> )	KP124787	KP124019	KP123870	KP124626
<i>Alternaria alternata</i>	CBS 595.93 ( <i>A. rhadina</i> )	KP124787	KP124019	KP123870	KP124626
<i>Alternaria alternata</i>	CBS 965.95; IMI 289679 ( <i>A. tenuissima</i> )	KP124791	KP124023	KP123872	KP124629
<i>Alternaria alternata</i>	CBS 918.96; E.G.S. 34.015; IMI 255532 ( <i>A. tenuissima</i> )	KC584435	KP124026	AY563302	KP124633
<i>Alternaria alternata</i>	CBS 102595; E.G.S. 45.100 ( <i>A. limoniasperae</i> )	KC584408	KP124029	AY563306	KP124636
<i>Alternaria alternata</i>	CBS 102596; E.G.S. 45.090 ( <i>A. citrimacularis</i> )	KP124796	KP124030	KP123877	KP124637
<i>Alternaria alternata</i>	CBS 102598; E.G.S. 46.141 ( <i>A. citriarbusti</i> )	KP124797	KP124031	KP123878	KP124638
<i>Alternaria alternata</i>	CBS 102599; E.G.S. 44.166 ( <i>A. turkisafria</i> )	KP124798	KP124032	KP123879	KP124639
<i>Alternaria alternata</i>	CBS 102600; E.G.S. 39.181; ATCC 38963 ( <i>A. toxicogenica</i> )	KP124799	KP124033	KP123880	KP124640
<i>Alternaria alternata</i>	CBS 113054; CPC 4263 ( <i>A. tenuissima</i> )	KP124814	KP124047	KP123894	KP124656
<i>Alternaria alternata</i>	CBS 115069; CPC 4254 ( <i>A. tenuissima</i> )	KP124815	KP124048	KP123895	KP124657
<i>Alternaria alternata</i>	CBS 118811; E.G.S. 35.158 ( <i>A. brassicinae</i> )	KP124824	KP124057	KP123904	KP124667
<i>Alternaria alternata</i>	CBS 118812; E.G.S. 37.050 ( <i>A. daucifolii</i> )	KC584393	KP124058	KP123905	KP124668
<i>Alternaria alternata</i>	CBS 118814; E.G.S. 44.048 ( <i>A. tomatocola</i> )	KP124825	KP124059	KP123906	KP124669
<i>Alternaria alternata</i>	CBS 118815; E.G.S. 51.132 ( <i>A. tomatocola</i> )	KP124826	KP124060	KP123907	KP124670
<i>Alternaria alternata</i>	CBS 118818; E.G.S. 31.032 ( <i>A. vaccinii</i> )	KP124827	KP124061	KP123908	KP124671
<i>Alternaria alternata</i>	CBS 119115	KP124828	KP124062	KP123909	na
<i>Alternaria alternata</i>	CBS 119399; E.G.S. 39.189 ( <i>A. postmessia</i> )	KP124829	KP124063	KP123910	KP124672
<i>Alternaria alternata</i>	CBS 119408; E.G.S. 40.140 ( <i>A. herbiphorbicola</i> )	KP124830	KP124064	JQ646410	KP124673
<i>Alternaria alternata</i>	CBS 119543; E.G.S. 12.160 ( <i>A. citricancri</i> )	KP124831	KP124065	KP123911	KP124674
<i>Alternaria alternata</i>	CBS 121492; HSAUP0207 ( <i>Ulocladium cucumis</i> )	KP124840	KP124074	KP123918	KP124683
<i>Alternaria alternata</i>	CBS 121547; E.G.S. 50.048 ( <i>A. yali-inficiens</i> )	KP124842	KP124076	KP123920	KP124685

Species	Strain designation	RPB2	endoPG	AltA1	OPA 10-2
<i>Alternaria alternata</i>	CBS 124277 ( <i>A. tenuissima</i> )	KP124843	KP124077	KP123921	KP124686
<i>Alternaria alternata</i>	CBS 124278 ( <i>A. tenuissima</i> )	KP124844	KP124078	KP123922	KP124687
<i>Alternaria arborescens</i> SC	CBS 101.13; E.G.S. 07.022; QM1765 ( <i>A. geophila</i> )	KP124862	KP124096	KP123940	KP124705
<i>Alternaria arborescens</i> SC	CBS 105.24; IHEM 3123 ( <i>A. alternata</i> )	KP124863	KP124097	KP123941	KP124706
<i>Alternaria arborescens</i> SC	CBS 108.41; E.G.S. 44.087; ATCC 11892 ( <i>A. alternata</i> )	KP124864	KP124098	KP123942	KP124707
<i>Alternaria arborescens</i> SC	CBS 113.41; IHEM 3318 ( <i>A. alternata</i> )	KP124865	KP124099	KP123943	KP124708
<i>Alternaria arborescens</i> SC	CBS 105.49 ( <i>A. alternata</i> )	KP124866	KP124100	KP123944	KP124709
<i>Alternaria arborescens</i> SC	CBS 126.60; IMI 081622 ( <i>A. maritima</i> )	KP124867	KP124101	JQ646390	KP124710
<i>Alternaria arborescens</i> SC	CBS 750.68; LCP 68.1989 ( <i>A. tenuissima</i> )	KP124868	KP124102	KP123945	KP124711
<i>Alternaria arborescens</i> SC	CBS 102605; E.G.S. 39.128 ( <i>A. arborescens</i> )	KC584377	AY295028	AY563303	KP124712
<i>Alternaria arborescens</i> SC	CBS 109730 ( <i>A. arborescens</i> )	KP124869	KP124103	KP123946	KP124713
<i>Alternaria arborescens</i> SC	CBS 112633; CPC 4244 ( <i>A. arborescens</i> )	KP124870	KP124104	KP123947	KP124714
<i>Alternaria arborescens</i> SC	CBS 112749; CPC 4245 ( <i>A. arborescens</i> )	KP124871	KP124105	KP123948	KP124715
<i>Alternaria arborescens</i> SC	CBS 115189; CPC 4345 ( <i>A. arborescens</i> )	KP124872	KP124106	KP123949	KP124716
<i>Alternaria arborescens</i> SC	CBS 115516; CPC 4247 ( <i>A. arborescens</i> )	KP124873	KP124107	KP123950	KP124717
<i>Alternaria arborescens</i> SC	CBS 115517; CPC 4246 ( <i>A. arborescens</i> )	KP124874	KP124108	KP123951	KP124718
<i>Alternaria arborescens</i> SC	CBS 116329 ( <i>A. alternata</i> )	KP124875	KP124109	KP123952	KP124719
<i>Alternaria arborescens</i> SC	CBS 117587 ( <i>A. alternata</i> )	KP124876	KP124110	KP123953	KP124720
<i>Alternaria arborescens</i> SC	CBS 118389; E.G.S. 90.131 ( <i>A. gaisen</i> )	KP124877	KP124111	KP123954	KP124721
<i>Alternaria arborescens</i> SC	CBS 119544; E.G.S. 43.072 ( <i>A. cerealis</i> )	KP124878	KP124112	KP123955	KP124722
<i>Alternaria arborescens</i> SC	CBS 119545; E.G.S. 48.130 ( <i>A. senecionicola</i> )	KP124879	KP124113	KP123956	KP124723
<i>Alternaria arborescens</i> SC	CBS 123235 ( <i>A. alternata</i> )	KP124880	KP124114	KP123957	KP124724
<i>Alternaria arborescens</i> SC	CBS 123266 ( <i>A. alternata</i> )	KP124881	KP124115	KP123958	KP124725
<i>Alternaria arborescens</i> SC	CBS 123267 ( <i>A. alternata</i> )	KP124882	KP124116	KP123959	KP124726
<i>Alternaria arborescens</i> SC	CBS 124274 ( <i>A. arborescens</i> )	na	KP124117	KP123960	KP124727
<i>Alternaria arborescens</i> SC	CBS 124281 ( <i>A. arborescens</i> )	KP124883	KP124118	KP123961	KP124728
<i>Alternaria arborescens</i> SC	CBS 124282 ( <i>A. arborescens</i> )	KP124884	KP124119	KP123962	KP124729
<i>Alternaria arborescens</i> SC	CBS 124283 ( <i>A. tenuissima</i> )	KP124885	KP124120	KP123963	KP124730
<i>Alternaria arborescens</i> SC	CBS 127263 ( <i>A. alternata</i> )	KP124886	KP124121	KP123964	KP124731
<i>Alternaria arborescens</i> SC	CPC 25266	KP124887	KP124122	KP123965	KP124732
<i>Alternaria tomato</i>	CBS 103.30	KP124915	KP124151	KP123991	KP124762

<b>Species</b>	<b>Strain designation</b>	<b>RPB2</b>	<b>endoPG</b>	<b>AltA1</b>	<b>OPA 10-2</b>
<i>Alternaria longipes</i>	CBS 113.35	KP124910	KP124145	KP123986	KP124756
<i>Alternaria jacinthicola</i>	CBS 133751; MUCL 53159	KP124108	KP124143	KP123984	KP124754
<i>Alternaria iridiaustralis</i>	CBS 118486; E.G.S. 43.014	KP124905	KP124140	KP123981	KP124751
<i>Alternaria eichhorniae</i>	CBS 489.92; ATCC 22255 ATCC 46777; IMI 121518	KP124895	KP124130	KP121973	KP124740
<i>Alternaria burnsii</i>	CBS 107.38; E.G.S. 06.185	KP124889	KP124124	KP23967	KP124734
<i>Alternaria betae-kenyensis</i>	CBS 118810; E.G.S. 49.159; IMI 385709	KP124888	KP124123	KP123966	KP124733
<i>Alternaria alstroemeriae</i>	CBS 118808; E.G.S. 50.116	KP124764	KP123993	KP123845	KP124691
<i>Alternaria gossypina</i>	CBS 104.32	KP124900	KP124135	JQ646395	KP124746
<i>Alternaria nobilis</i>	AC1	LC476798	LC480952	LC481624	na

na: sequence not available.

**Supplementary Table 3** - Mean rot diameter  $\pm$  SD obtained with the pathogenicity assay performed by direct penetration using not wounded fruits, with mycelial plugs after 14 days at  $24\pm 1^\circ\text{C}$  for representative isolates isolated from pear.

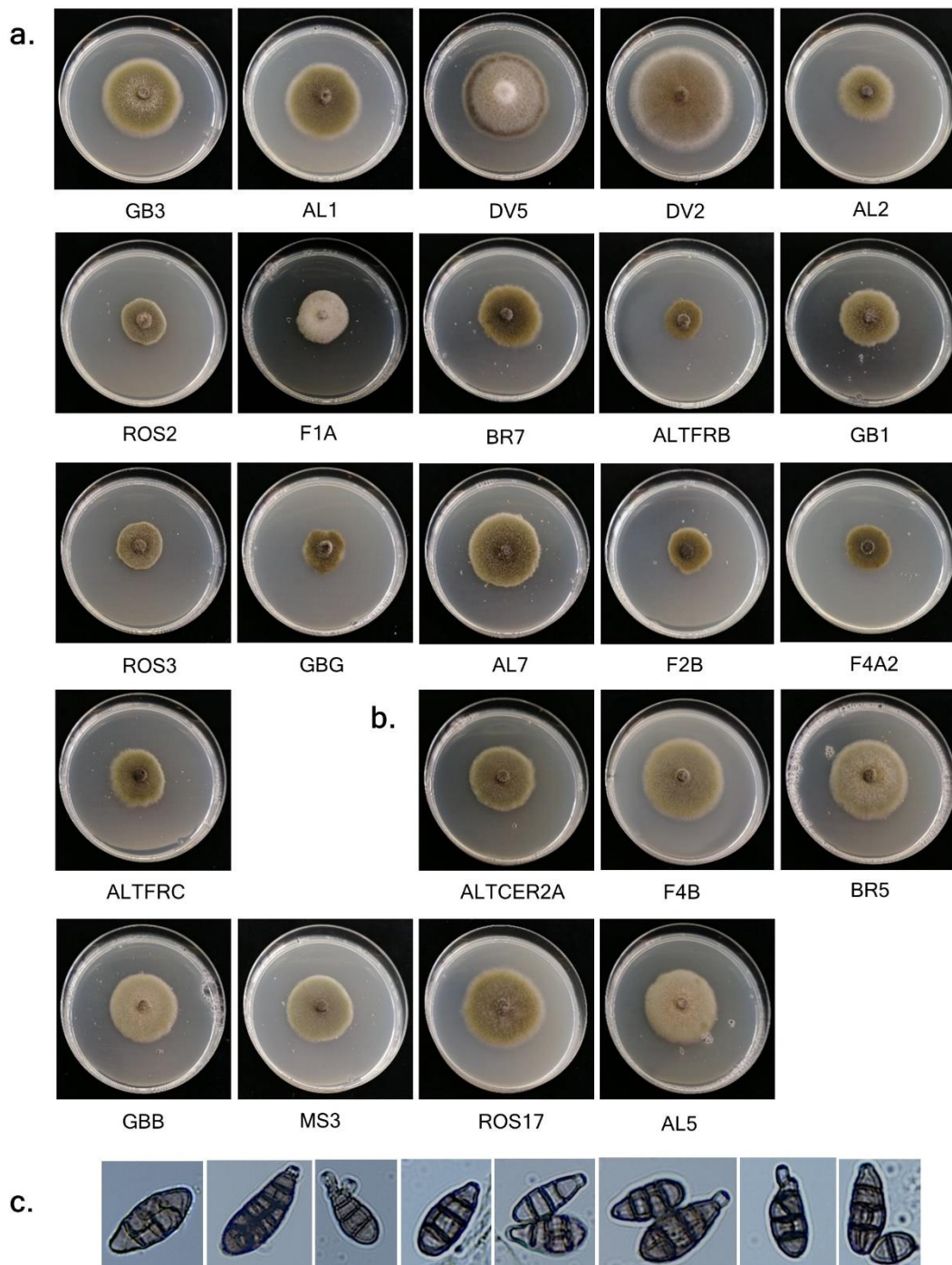
Isolate	Species	Source	Orchard number	<i>In vivo</i> pathogenicity assay	
				Rot diameter (cm) $\pm$ SD	
MB1B	<i>A. alternata</i>	Fruit	1	2.90	$\pm 0.14$
ALCER2A	<i>A. alternata</i>	Fruit	1	3.83	$\pm 0.46$
DV3	<i>A. alternata</i>	Leaf	1	2.70	$\pm 0.07$
F2B	AASC	Leaf	2	2.95	$\pm 0.07$
F4A2	AASC	Leaf	2	3.10	$\pm 0.35$
F4B	<i>A. alternata</i>	Leaf	2	2.15	$\pm 0.21$
ALTFRB	AASC	Fruit	2	2.68	$\pm 0.18$
ALTFRC	AASC	Fruit	2	2.98	$\pm 0.11$
ALTFRF	<i>A. alternata</i>	Fruit	2	2.15	$\pm 0.14$
ROS2	AASC	Leaf	3	2.68	$\pm 0.18$
ROS15	<i>A. alternata</i>	Leaf	3	3.83	$\pm 0.04$
ROS16	<i>A. alternata</i>	Leaf	3	3.65	$\pm 0.07$
AL1	AASC	Fruit	4	3.40	$\pm 0.28$
AL7	AASC	Fruit	4	2.55	$\pm 0.64$
BR5	<i>A. alternata</i>	Fruit	5	3.05	$\pm 0.42$
GB1	AASC	Leaf	6	3.48	$\pm 0.69$
MS6	<i>A. alternata</i>	Leaf	7	3.40	$\pm 0.14$

**Supplementary Table 4** - Validation parameters for the six *Alternaria* toxins in pear matrix.

Analyte	Recovery %	ME %	LOD ng/g	LOQ ng/g	R <sup>2</sup>	Inter-day (RDS %)	Inter-day (RDS %)
TeA	86.8±4.48	39.8	18.1	60.5	0.9977	5.2	7.8
AOH	93.1±13.2	153.9	1.9	6.5	0.9960	6.5	7.1
AME	92.5±7.8	125.5	3.4	11.2	0.9941	2.7	4.0
TEN	78.53±9.7	69.2	8.5	28.4	0.9994	6.4	9.3
ALT	88.7±4.6	120.8	3.3	11.1	0.9962	6.6	7.3
ATX-I	82.3±6.1	77.7	4.2	13.8	0.9938	7.0	9.1

## Supplementary Figures

**Supplementary Figure 1** - Morphology of some *Alternaria* spp. isolates isolated from fruits and leaves of *Pyrus communis* cv. Abate Fétel. a. Colonies morphology of AASC after 7 days of incubation at  $25 \pm 1^\circ\text{C}$  on Potato Dextrose Agar medium. b. Colonies morphology of *A. alternata* isolates after 7 days of incubation at  $25 \pm 1^\circ\text{C}$  on Potato Dextrose Agar medium. c. Conidial obtained from isolates grown on Potato Carrot Agar medium for 20 days.





**Supplementary Figure 2** - Bootstrap consensus tree based on the concatenated RPB2, Alt-a1, endoPG and OPA10-2 sequence datasets. The phylogenetic tree was constructed with Maximum Likelihood analysis using Tamura Nei (TN93) plus Gamma distribution (+G) model and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Evolutionary analyses were conducted using MEGA, version 6.

