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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

Fungal isolates

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

Micro and macro-morphological observations

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

DNA extraction and molecular analysis

DNA was extracted from mycelium collected on PDA plates incubated at 25 °C in the dark for 8 days using Omega E.Z.N.A. Fungal DNA Mini Kit (VWR International, USA) according to manufacturer's instructions. Partial amplification of the RNA polymerase second largest subunit (RPB2), Alternaria major allergen gene (Alt-a1), endopolygalacturonase gene (endoPG) and an anonymous gene region (OPA10-2) were obtained using the primers and conditions listed in Supplementary Table 1. PCR was carried out using Taq DNA polymerase kit (Qiagen, Germany) in a total volume of 25 µL containing 2.5 µL of Qiagen PCR Buffer 10 X, 0.5 µL of $MgCl_2$, 0.75 µL of dNTPs (10 mM), 1 µL of each primer (10 µM), 0.2 µL of Taq DNA polymerase and 20 ng of template DNA. The PCR products were checked by gel electrophoresis in 1% agarose with 1 µL of GelRed™ (VWR International) at 100 V/cm for 45 min and purified using QIAquick© PCR purification Kit (Qiagen). Macrogen, Inc. (The Netherlands) sequenced the amplicons in both directions. The consensus sequences were created using a DNA Baser program (Heracle BiosoftS.R.L., Romania) and

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

Pathogenicity assays on pear fruits

Two *in vivo* assays were performed to test the pathogenicity of *Alternaria* spp. isolates. Healthy pear fruit cv. Abate Fétel at commercial maturity were surface sterilized with 1% sodium hypochlorite and rinsed in sterile deionized water. The first assay was performed to evaluate the pathogenicity on wounded fruits, where three wounds (2 mm diameter) were made per fruit, and each fruit was inoculated with a monoconidial suspension of $1x10^5$ conidia/mL prepared by growing isolates on Potato Carrot Agar (PCA; PCA, HiMedia Laboratories, India) for 20 days with a photoperiod of 12 h light: 12 h dark. The fruits were maintained at 24 ± 1 °C and exposed to natural daylight. The control pears were prepared as described above but they were treated with sterile deionized water. After 14 days, rot diameters were measured. The experiment was performed twice, with three biological replicates and nine technical replicates per isolate (n=18).

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

***In vivo* mycotoxin production**

Chemicals

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

(purity ≥ 97%) from *Alternaria* spp. were purchased from Fermentek (Jerusalem, Israel), all in crystallized form. A stock solution of 1000 µg/mL was prepared in methanol for each mycotoxin and the working solution of 10 µg/mL was prepared by dilution and mixing the stock solution of each analyte and kept at −20 °C. Standard solutions for HPLC calibration and for additional experiments were prepared daily by diluting the working solution in solvent (CH_3CN:H_2O, 50:50, v/v) or blank matrix. Methanol, acetonitrile (VWR International,) and toluene (Sigma-Aldrich) were HPLC-grade. Ammonium acetate and hydrochloric acid (Sigma-Aldrich) were analytical reagent-grade. Water was obtained from a Milli-Q system (G. Maina, Italy).

Alternaria-toxins extraction from pear fruit

From each pathogenicity assay with wounded and inoculated fruits, two pears (biological replicates) were used to analyse the mycotoxins production *in vivo*. From each fruit, 3 cm-diameter rotten tissues were sampled and homogenized. Two technical replicates (4 g) from each fruit were placed in a centrifuge tube with 20 mL of extraction solution (CH_3OH:CH_3CN:H_2O, 10:45:45, v/v/v) and 500 µL of HCl 2 N. The mixture was shaken for 30 min in an ultrasonic bath and then centrifuged at 4691 g for 15 min. Sample extract was filtered through a Clarify-PP 0.22 µm polypropylene filter (Agela Technologies, China) and transferred to a new centrifuge tube with 10 mL of toluene (twice), vortexed for 1 min and centrifuged at 4691 g for 10 min. The organic phase was evaporated to dryness in a rotary evaporator at 50 °C and the residue dissolved in 500 mL of H_2O:CH_3CN 1:1 for the HPLC-MS/MS analysis. The assay was performed twice, with two biological replicates and four technical replicates (n=8).

HPLC-MS/MS analysis of Alternaria-toxins

The analysis of Alternaria-toxins was carried out using a 1260 Agilent Technologies system (Agilent, USA) consisting of a binary pump and a vacuum degasser, connected to a Varian autosampler, Model 410 Prostar (Hansen Way, USA), equipped with a 20 µl loop coupled to a Varian 310-MS TQ Mass Spectrometer. The chromatographic column used for LC separation was a Gemini-NX C18 (150 x 3.0 mm, 3.0 µm, Phenomenex, Torrance, CA, USA). Water (solvent A) and acetonitrile (solvent B), both with ammonium acetate 5 mM, were used as mobile phase at a flow rate of 300 µL/min. The initial mobile phase contained 40% B and was held for 2 min, and then the proportion of B was linearly increased to 100% over 6 min and then held for 4 min; finally, the column was returned to initial conditions and equilibrated for 10 min before the next injection. Sample ionization was performed by an electrospray (ESI) ion source operating in negative ion mode and the quantification was carried out performed using multiple reaction monitoring (MRM) using the following transition 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

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 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 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 gas (Ar) pressure was set at 2 mbar for all experiments.

HPLC-MS/MS method validation for Alternaria-toxins

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

Statistical Analysis

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

3. Results

Taxonomic assignment

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

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

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

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

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

Pathogenicity on pear fruits

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

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

A selection of isolates was used to perform the pathogenicity assay on healthy non-wounded fruits. All isolates confirmed to be pathogenic by directly penetrating the fruits (Fig. 5; Supplementary Table 3), with a mean rot diameter of 3.03 ± 0.52 cm. The isolates ALTCER2A and ROS15, isolated from fruit and leaf respectively, showed the highest rot diameter (3.83 ± 0.25 cm).

Alternaria-toxins production on pear

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

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

The most mycotoxigenic isolates were 2AFA (*Alternaria* sp.), F1A (AASC), BR7 (*A. alternata*), ALTFRC (AASC), GB3 (*Alternaria* sp.), DV3 (AASC) and ROS3 (AASC), with a total concentration (all six analytes together) ranging from 20.60 µg/g to 40.57 µg/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 (Thanassoulopoulos, 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

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

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

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

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

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

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

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

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

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

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

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

In conclusion, the presence of different *Alternaria* species pathogenic on European pear suggests that more than one fungal species is responsible for black spot, an important information to plan effective 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[a]	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 [a]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)[a]
MB1A	*A. alternata*	Fruit	1	MT642849	MT642803	MT642895	MT612381	4.03 ± 0.01
MB1B	*A. alternata*	Fruit	1	MT642850	MT642804	MT642896	MT612382	4.18 ± 0.58
ALCER2A	*A. alternata*	Fruit	1	MT642851	MT642805	MT642897	MT612383	3.29 ± 0.95
ALTCER3B	*A. alternata*	Fruit	1	MT642852	MT642806	MT642898	MT612384	4,00 ± 0.40
DV1	*A. alternata*	Leaf	1	MT642853	MT642807	MT642899	MT612385	4.53 ± 0.07
DV2	AASC	Leaf	1	MT642854	MT642808	MT642900	MT612386	3.03 ± 0.18
DV3	*A. alternata*	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	*A. alternata*	Leaf	2	MT642860	MT642814	MT642906	MT612392	4.28 ± 0.13
2AFA	*Alternaria* 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	*A. alternata*	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	*A. alternata*	Leaf	3	MT642867	MT642821	MT642913	MT612399	4.35 ± 0.12
ROS16	*A. alternata*	Leaf	3	MT642869	MT642823	MT642915	MT612401	4.11 ± 0.22
ROS17	*A. alternata*	Leaf	3	MT642868	MT642822	MT642914	MT612400	4.36 ± 0.11
ROA	*A. alternata*	Fruit	3	MT642870	MT642824	MT642916	MT612402	3.38 ± 0.19
ROB	*A. alternata*	Fruit	3	MT642871	MT642825	MT642917	MT612403	4.67 ± 0.64
ROC	*A. alternata*	Fruit	3	MT642872	MT642826	MT642918	MT612404	3.83 ± 0.23

Isolate	Species	Source	Orchard number	Accession number				*In vivo* pathogenicity assay
				RPB2	endoPG	Alt-a1	OPA 10-2	Rot diameter (cm ± SD)[a]
ROD	*A. alternata*	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	*A. alternata*	Fruit	4	MT642876	MT642830	MT642922	MT612408	4.90 ± 0.42
AL7	AASC	Fruit	4	MT642877	MT642831	MT642923	MT612409	4.71 ± 0.10
BR1	*A. alternata*	Fruit	5	MT642878	MT642832	MT642924	MT612410	4.67 ± 0.59
BR3	*A. alternata*	Fruit	5	MT642879	MT642833	MT642925	MT612411	4.92 ± 0.90
BR5	*A. alternata*	Fruit	5	MT642880	MT642834	MT642926	MT612412	4.44 ± 0.98
BR7	AASC	Fruit	5	MT642881	MT642835	MT642927	MT612413	3.93 ± 0.09
GBB	*A. alternata*	Fruit	6	MT642882	MT642836	MT642928	MT612414	4.01 ± 0.29
GBC	*A. alternata*	Fruit	6	MT642884	MT642838	MT642930	MT612416	3.70 ± 0.49
GBF	*A. alternata*	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	*A. alternata*	Leaf	6	MT642887	MT642841	MT642933	MT612419	4.34 ± 0.15
GB3	*Alternaria* sp.	Leaf	6	MT642888	MT642842	MT642934	MT612420	4.73 ± 0.22
GB4	*Alternaria* sp.	Leaf	6	MT642889	MT642843	MT642935	MT612421	4.29 ± 0.11
GB6	*A. alternata*	Leaf	6	MT642890	MT642844	MT642936	MT612422	4.49 ± 0.41
MS2	*A. alternata*	Leaf	7	MT642891	MT642845	MT642937	MT612423	4.33 ± 0.06
MS3	*A. alternata*	Leaf	7	MT642892	MT642846	MT642938	MT612424	4.65 ± 0.26
MS5	*Alternaria* sp.	Leaf	7	MT642893	MT642847	MT642939	MT612425	4.72 ± 0.02
MS6	*A. alternata*	Leaf	7	MT642894	MT642848	MT642940	MT612426	3.78 ± 0.14

[a] The values are expressed as the mean ± standard deviation (SD) of two experimental assays, each one with three fruits inoculated at three artificial 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[a]	AOH µg/g ± SD[a]	AME µg/g ± SD[a]	TEN µg/g ± SD[a]	ALT µg/g ± SD[a]	ATX-I µg/g ± SD[a]
2AFA	*Alternaria* sp.	0.14±0.11	7.33±1.11	13.07±4.60	nd[b]	0.06±0.00	nd
AL1	*AASC*	0.31±0.01	0.32±0.04	0.36±0.01	nd	nd	nd
AL2	*AASC*	0.74±0.15	2.50±1.05	1.39±0.40	nd	0.07±0.01	0.01±0.00
AL5	*A. alternata*	0.45±0.00	1.32±0.21	2.14±0.50	nd	nd	0.008±0.00
AL7	*AASC*	nd	1.45±0.63	2.16±0.60	nd	0.06±0.00	0.005±0.00
ALCER2A	*A. alternata*	0.18±0.04	0.26±0.02	0.23±0.04	nd	nd	0.005±0.00
ALTCER3B	*A. alternata*	3.54±0.17	1.89±0.60	0.26±0.19	nd	nd	0.005±0.00
ALTFRB	*AASC*	1.45±0.03	5.17±2.30	4.20±0.60	nd	0.09±0.00	nd
ALTFRC	*AASC*	0.52±0.01	12.0±1.23	9.88±0.70	nd	0.03±0.00	0.011±0.00
ALTFRF	*A. alternata*	0.73±0.08	1.90±0.30	1.82±0.22	nd	nd	0.11±0.03
BR1	*A. alternata*	0.50±0.08	0.32±0.12	0.28±0.16	nd	nd	0.01±0.00
BR3	*A. alternata*	8.04±0.67	1.50±0.32	1.31±0.04	nd	nd	0.09±0.01
BR5	*A. alternata*	1.21±0.12	0.97±0.20	4.38±0.61	nd	0.06±0.00	nd
BR7	*AASC*	6.69±1.79	3.62±0.24	10.50±1.60	nd	0.10±0.00	0.02±0.00
DV1	*A. alternata*	0.12±0.02	0.44±0.02	1.40±0.50	nd	0.02±0.00	0.006±0.01
DV2	*AASC*	nd	nd	nd	nd	nd	nd
DV3	*A. alternata*	22.03±0.30	1.13±0.20	0.62±0.15	nd	0.008±0.00	0.01±0.00
DV5	*AASC*	0.46±0.16	0.36±0.04	0.19±0.07	nd	0.014±0.05	0.01±0.00
F1A	*AASC*	3.52±0.33	5.91±0.14	11.10±1.10	nd	0.23±0.03	0.06±0.00
F2B	*AASC*	0.07±0.01	0.46±0.14	1.07±0.23	nd	0.04±0.00	nd
F4A2	*AASC*	2.4±0.80	2.29±0.21	1.85±0.60	nd	nd	0.01±0.00
F4B	*A. alternata*	0.43±0.02	0.04±0.01	0.71±0.06	nd	nd	0.01±0.00
GB1	*AASC*	0.67±0.05	0.11±0.08	nd	nd	nd	0.02±0.00
GB2	*A. alternata*	3.87±0.34	0.26±0.02	1.54±1.45	nd	0.15±0.02	0.04±0.00
GB3	*Alternaria* sp.	9.38±0.24	7.36±0.54	6.12±0.05	nd	0.256±0.02	0.04±0.00
GB4	*Alternaria* 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[a]	AOH µg/g ± SD[a]	AME µg/g ± SD[a]	TEN µg/g ± SD[a]	ALT µg/g ± SD[a]	ATX-I µg/g ± SD[a]
GB6	*A. alternata*	3.07±0.26	0.23±0.03	0.29±0.05	nd	0.02±0.00	0.01±0.00
GBB	*A. alternata*	2.14±0.02	0.54±0.06	13.20±7.80	nd	0.04±0.01	0.02±0.00
GBC	*A. alternata*	1.42±0.20	4.02±1.40	2.50±0.90	nd	0.02±0.00	0.02±0.00
GBF	*A. alternata*	0.90±0.10	0.05±0.01	nd	nd	nd	0.01±0.00
GBG	*AASC*	0.18±0.05	1.37±0.30	1.20±0.20	nd	0.07±0.00	0.04±0.00
MB1A	*A. alternata*	3.65±0.13	2.38±0.32	1.52±0.30	nd	nd	nd
MB1B	*A. alternata*	8.29±0.15	6.03±2.02	3.47±1.10	nd	0.01±0.00	0.03±0.00
MS2	*A. alternata*	6.84±0.63	0.42±0.30	3.02±0.20	nd	nd	0.01±0.00
MS3	*A. alternata*	0.06±0.02	0.10±0.01	0.20±0.08	0.023±0.00	nd	nd
MS5	*Alternaria* sp.	0.78±0.13	0.35±0.12	0.27±0.02	nd	nd	0.01±0.00
MS6	*A. alternata*	nd	0.25±0.09	0.32±0.06	nd	0.006±0.00	nd
ROA	*A. alternata*	3.03±0.20	0.73±0.13	0.86±0.22	nd	0.008±0.00	0.01±0.00
ROB	*A. alternata*	1.43±0.68	1.02±1.30	nd	nd	nd	0.01±0.00
ROC	*A. alternata*	0.15±0.04	nd	nd	nd	nd	nd
ROD	*A. alternata*	2.07±0.12	0.32±0.01	0.10±0.01	nd	nd	0.02±0.00
ROS15	*A. alternata*	0.73±0.06	0.68±0.50	0.50±0.10	nd	nd	0.01±0.00
ROS16	*A. alternata*	1.14±0.06	0.92±0.10	0.75±0.30	nd	nd	0.04±0.00
ROS17	*A. alternata*	1.18±0.08	0.13±0.05	0.03±0.00	nd	nd	0.007±0.00
ROS2	*AASC*	1.00±0.01	1.01±0.40	0.32±0.07	nd	nd	0.02±0.00
ROS3	*AASC*	10.05±0.03	10.60±0.70	19.70±1.20	nd	0.19±0.01	0.03±0.00

[a] Each value is the average of two experimental assays, each one with two biological replicates and four technical replicates ± SD (n=16).

[b] 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 ± 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 ± 1 °C.

637 **Figure 6** - Alternaria-toxin production [µg/g] ± 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 ± 1°C on Potato Dextrose Agar medium. b. Colonies morphology of *A. alternata*
644 isolates after 7 days of incubation at 25 ± 1°C on Potato Dextrose Agar medium. c. Conidial obtained
645 from isolates grown on Potato Carrot Agar medium for 20 days.
646

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.
652

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.
657

658 **Supplementary Table 2 -** References used in this study for the phylogeny of *Alternaria* spp. isolates
659 used in this study.
660

661 **Supplementary Table 3** - Mean rot diameter ± SD obtained with the pathogenicity assay performed
662 by direct penetration using not wounded fruits, with mycelial plugs after 14 days at 24±1°C for
663 representative isolates isolated from pear.
664

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)

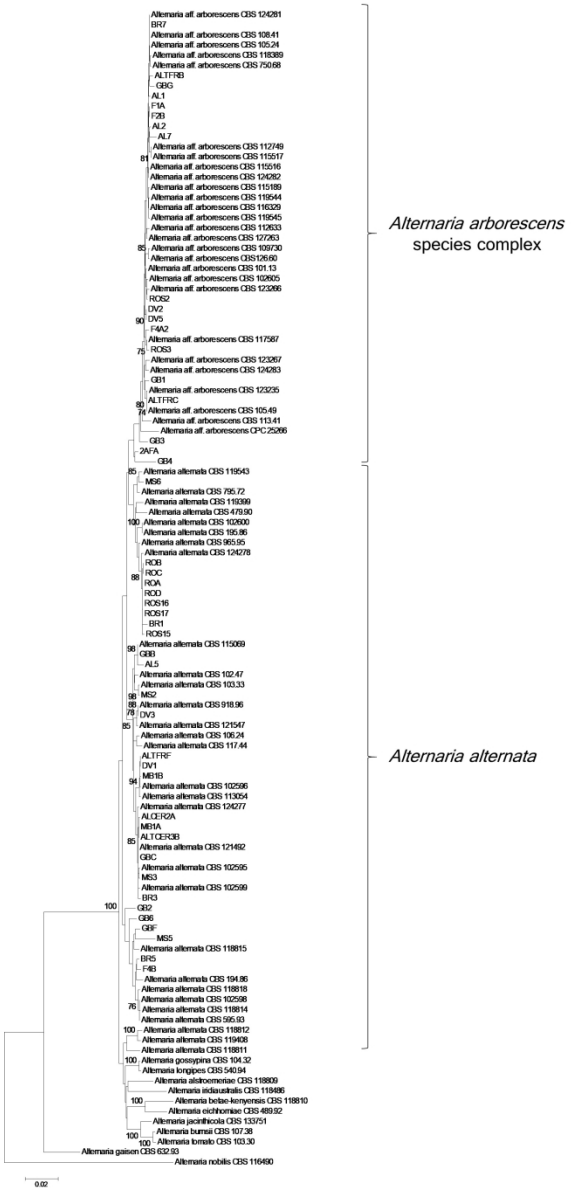

Figure 2 - Phylogenetic tree based on the concatenated RPB2, Alt-a1, endoPG and OPA10-2 sequence datasets. The phylogenetic tree was constructed with Maximum Likelihood analysis using Tamura Nei (TN93) plus Gamma distribution (+G) model and by assuming that a certain fraction of sites are evolutionarily 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)

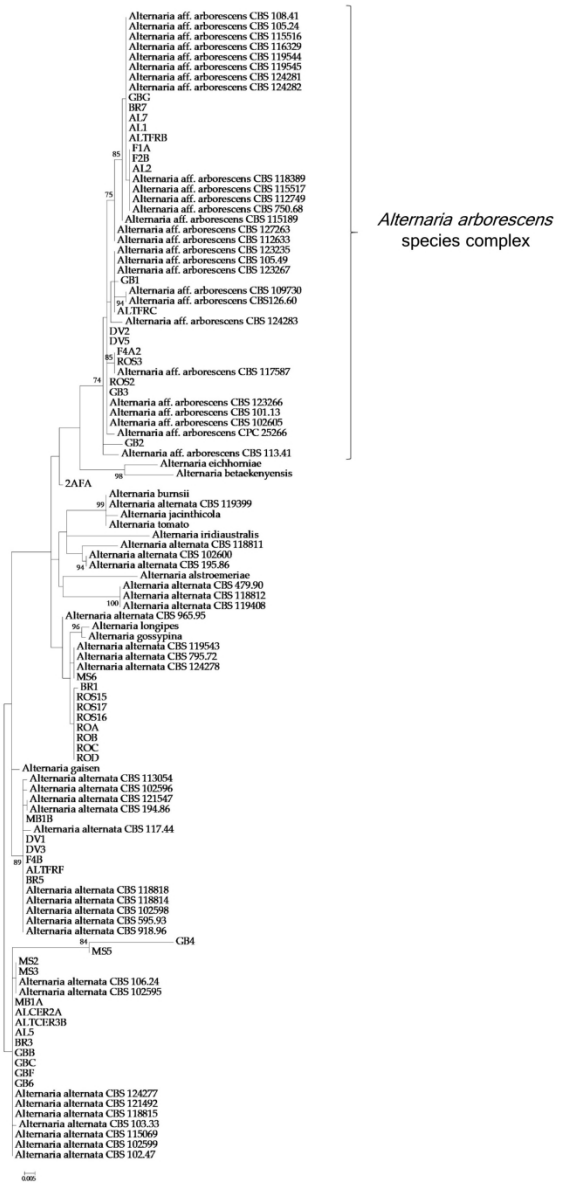

Figure 3 - Phylogenetic tree based on the OPA 10-2 sequences. The phylogenetic tree was constructed with Maximum Likelihood analysis using Kimura 2-parameter plus Gamma distribution model. The numbers at the major nodes indicate the bootstrap value from 1,000 bootstrapped datasets. Branches with bootstrap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

190x338mm (300 x 300 DPI)

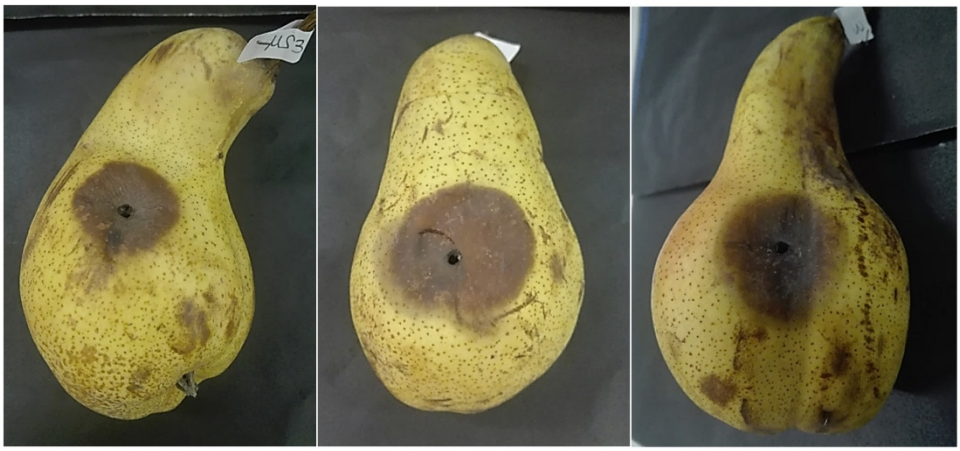

Figure 4 - Symptoms of *Alternaria* black rot on wounded *Pyrus communis* cv. Abate Fétel fruit inoculated with Alternaria species after 20 days at 24 ± 1 °C.

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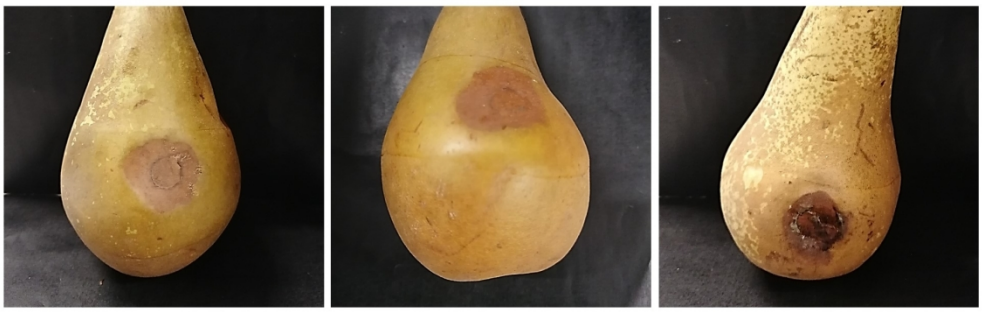


Figure 5 - Symptoms of *Alternaria* black rot on healthy un-wounded *Pyrus communis* cv. Abate Fétel fruit inoculated with Alternaria species after 14 days at 24 ± 1 °C.

165x57mm (300 x 300 DPI)

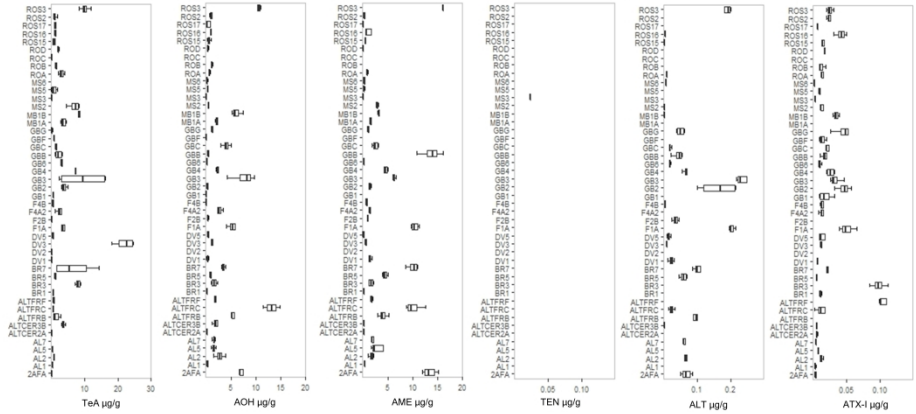


Figure 6 – Alternaria-toxin production [µg/g] ± SD by the 46 *Alternaria* spp. isolates inoculated on *Pyrus communis* cv. Abate Fétel. Each value is the average (n=16) of two experimental assays, each one with two biological replicates and four technical replicates.

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.

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

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

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

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

na: sequence not available.

Supplementary Table 3 - Mean rot diameter ± SD obtained with the pathogenicity assay performed by direct penetration using not wounded fruits, with mycelial plugs after 14 days at 24±1°C for representative isolates isolated from pear.

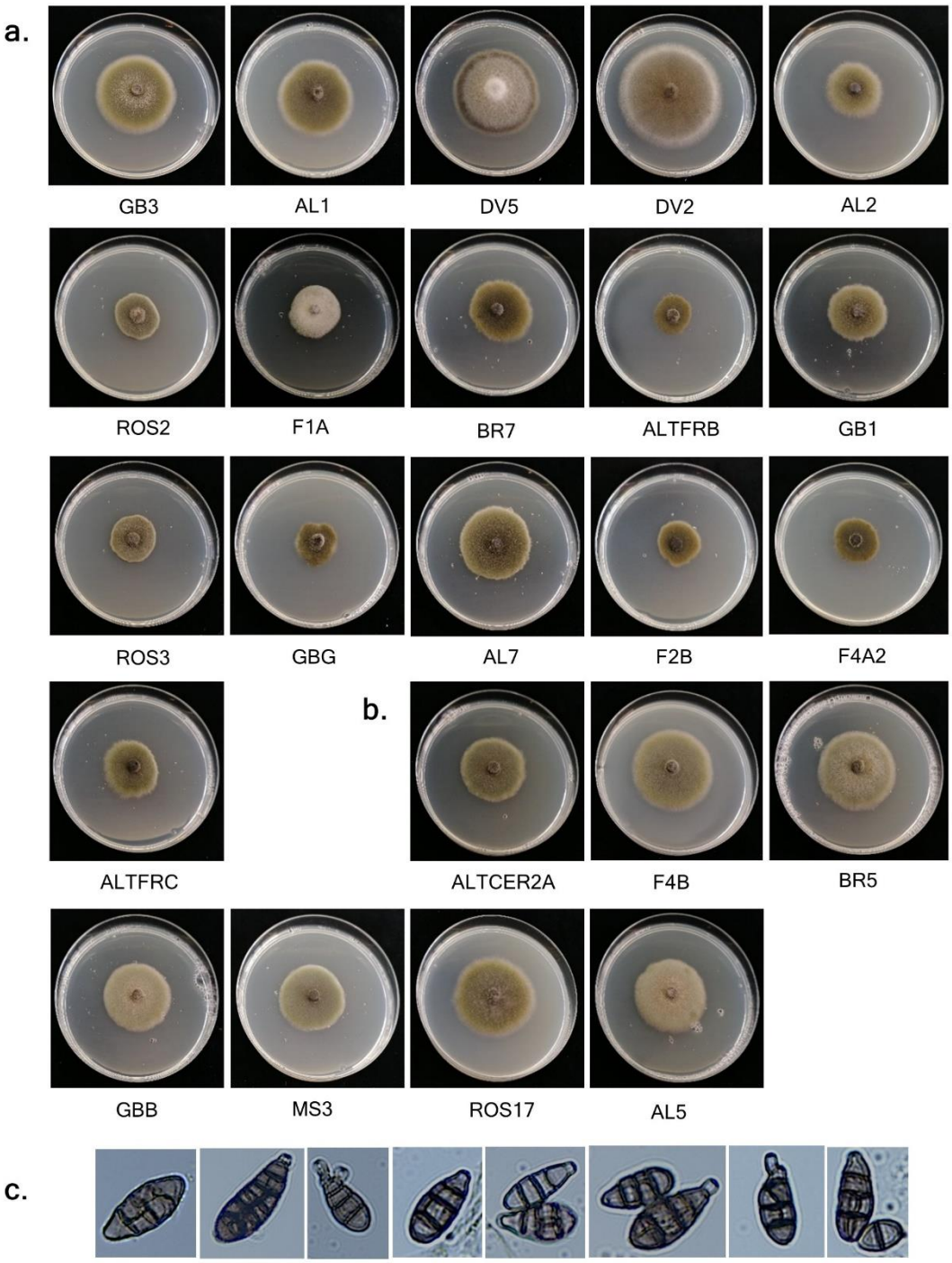
Isolate	**Species**	**Source**	**Orchard number**	***In vivo* pathogenicity assay**
				Rot diameter (cm) ± SD
MB1B	*A. alternata*	Fruit	1	2.90 ± 0.14
ALCER2A	*A. alternata*	Fruit	1	3.83 ± 0.46
DV3	*A. alternata*	Leaf	1	2.70 ± 0.07
F2B	AASC	Leaf	2	2.95 ± 0.07
F4A2	AASC	Leaf	2	3.10 ± 0.35
F4B	*A. alternata*	Leaf	2	2.15 ± 0.21
ALTFRB	AASC	Fruit	2	2.68 ± 0.18
ALTFRC	AASC	Fruit	2	2.98 ± 0.11
ALTFRF	*A. alternata*	Fruit	2	2.15 ± 0.14
ROS2	AASC	Leaf	3	2.68 ± 0.18
ROS15	*A. alternata*	Leaf	3	3.83 ± 0.04
ROS16	*A. alternata*	Leaf	3	3.65 ± 0.07
AL1	AASC	Fruit	4	3.40 ± 0.28
AL7	AASC	Fruit	4	2.55 ± 0.64
BR5	*A. alternata*	Fruit	5	3.05 ± 0.42
GB1	AASC	Leaf	6	3.48 ± 0.69
MS6	*A. alternata*	Leaf	7	3.40 ± 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^2	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 ± 1°C on Potato Dextrose Agar medium. b. Colonies morphology of *A. alternata* isolates after 7 days of incubation at 25 ± 1°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.