Article

Insights into the Phytopathogenicity of *Alternaria alternata* Isolated from Infected Pears (*Pyrus communis*) in Italy: Enzymatic Activity and Production of Non-Host Toxins

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Abstract: *Alternaria alternata* is one of the most devastating phytopathogen fungi. This microorganism causes black spots in many fruits and vegetables worldwide, generating significant post-harvest losses. In this study, an *A. alternata* strain, isolated from infected pears (*Pyrus communis*) harvested in Italy, has been characterized by focusing on its pathogenicity mechanisms. In particular, enzymatic activities potentially involved during the infection have been investigated as their ability to produce toxic metabolites *in vitro*. The fungus showed strong amylase, xylanase and cellulase activities while from its PDB liquid cultures four different toxins have been isolated and identified as altertoxin I (**1**), alteichin (**2**), alternariol (**3**), and alternariol 4-methyl ether (**4**). Only alteichin generated necrotic lesions on pears from the host variety, while compounds **1** and **2** showed moderate or slight necrotic activity on non-host pears. Furthermore, compounds **1-4** exerted severe or moderate necrotic lesions on other non-host fruit (lemon, *Citrus limon*), being concluded they are non-host toxins. Moreover, to understand the strategies used by *A. alternata* in infection dynamics, a multi-infection with the competitor phytopathogen *Botrytis cinerea* has been simulated. The dual culture assay revealed that *B. cinerea* suppresses *A. alternata* growth. On the contrary, *A. alternata* when co-inoculated on pear fruits with *B. cinerea* shows a strong competitive exclusion, inhibiting its growth by 65%. These results suggest a different mechanism of competition between the two pathogens on host pears and *in vitro*.

Keywords: *Alternaria alternata*; pear pathogens; multi-infections; enzymatic activities; mycotoxins; plant necrosis

1. Introduction

Alternaria alternata is a filamentous Ascomycete, with a widespread distribution in nature and occurring on plants as a pathogen and endophyte and in the soil as a saprophyte [1]. The fungus has also been associated with human infections, especially in immunocompromised patients but even more rarely in healthy hosts [2].

As a phytopathogen, this fungus infects diverse plant species of agronomical and ornamental interest, such as potatoes, apples, pears, tomatoes, strawberries, or blueberries. Causing necrotic lesions, the *A. alternata* infections lead to food inedibility and so crop

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losses [3]. *Alternaria* produces a broad arsenal of metabolites that exhibit a variety of biological activities, such as phytotoxic, cytotoxic, and antimicrobial properties. Like many phytopathogenic fungi, *Alternaria* spp. can produce host-selective toxins (HSTs), and this ability has been strictly associated with fungal pathogenesis [4,5]. Moreover, HSTs are essential determinants of host range and specificity in particular plant species or cultivars. For example, some *A. alternata* pathotypes produce AK-toxin causing a black spot on Japanese peat fruit, while others the AAL or AF toxin infecting tomatoes or strawberries, respectively. All the HSTs have different modes of action, causing biochemical and genetic modifications in the host. The AK-toxin, AF-toxin, and AC-toxin can lead to DNA breakage and apoptotic cell death, interrupting plant physiology by mitochondrial oxidative phosphorylation and affecting membrane permeability with a devastating effect on plants.

Since *Alternaria* toxins can be produced in infected food and feed products by pathogenic fungi, they have the potency to exhibit harmful effects on human and animal health [6]. The wide diversity of produced compounds and their potential toxicity has drawn the attention of many researchers, such as chemists, pharmacologists, and plant pathologists. For example, some *Alternaria* metabolites, such as maculosin [7], tenuazonic acid [8], and tentoxin [9], have been proposed and tested as herbicides. In contrast, the porritoxin produced by the endophytic *Alternaria* species has been evaluated as a candidate for cancer chemo-preventive agents [10]. Besides HSTs, *A. alternata* produces non-specific host toxins (NSTs) and cell-wall-degrading enzymes (CWDEs), both required for its full virulence. Although NSTs are thought to contribute to some virulence features, such as the symptom development and in planta pathogen propagation [5], and the CWDEs are probably involved in the first step of plant attacks, as host penetration, the exact pathological roles of NSTs and CWDEs have yet to be well characterized.

This lack of information is due to the primary role of HSTs in fungal pathogenesis, which often masks the functions of NSTs and CWDEs. So, the toxic effects of *A. alternata* metabolites due to NSTs and CWDEs have received minor attention than those reported for HSTs mycotoxins [11]. A wider study of the activity of isolated fungal metabolites can allow the identification of compounds directly related to the pathogenic activity of the fungus, making it possible to create chemo libraries that facilitate the linking of the structure of the compounds with the species that produce it and its effect on host and non-host crops, as well as with biosynthetic features [12]. In this context, our work reports a study focused on NSTs and CWDEs used by an *A. alternata* strain isolated from infected pears in Italy. To this aim, the characterization of hydrolytic enzyme activities of *A. alternata* and the identification of the metabolites produced *in vitro* were performed. Furthermore, the phytotoxic activity of the isolated compounds was evaluated on pear (host and non-host varieties) and lemon fruits [13]. Finally, the competition of *A. alternata* with other pathogens was evaluated to investigate the role of NSTs on co-infections.

2. Materials and Methods

2.1. Fungal Isolation

The fungal phytopathogen strain used in this study was originally isolated from fruits of Abate Fetel pear tree (*Pyrus communis*) showing brown spot disease symptoms, sampled in Terrazzo (Verona, Italy) in 2019. The strain was stored on Potato Dextrose Agar (PDA) plates in the culture collection of Agriges s.r.l., San Salvatore Telesino, Benevento, Italy (40.93345, 14.65799, 401 m.a.s.l.).

2.2. Morphological Characterization

The phenotypic variant of the fungal isolate was determined by visual inspection. The fungus was grown in PDA broth (Difco, USA) at 28 °C for 7 days, and the Petri plate was photographed. The hyphae and conidia from the fungal cultures were stained with

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lactophenol blue solution (LPCB), Sigma, Saint Louis, MO, USA) and observed under a phase-contrast light microscope using an Olympus BX51 with a 60× objective UPlanF1.

2.3. DNA Extraction and Identification

DNA extraction from fungal mycelium was performed as described by Stirling D. (2003) [14]. Briefly, the fungus was grown in 150 mL of Potato Dextrose Broth (Difco, USA) for 7 days at 28 °C in shaken at 150 rpm. The mycelium was harvested by filtration, and 0.2 g of dry weight was pestled in a mortar and resuspended in 5 mL of CTAB extraction buffer. After incubation at 65°C in a water bath for 30 min, an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 2000 *g* for 10 min at room temperature. Later on, an equal volume of isopropanol was added to the supernatant, and the precipitated DNA was rinsed with 70% ethanol. After air drying, the DNA was resuspended in sterile water overnight at 4°C.

For the identification, the ITS region was sequenced using the forward primer ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and the reverse primer ITS4 (50-TCCTCCGCTTATTGATATGC-30) [15]. The PCR conditions were as follows: 94 °C for 10 min, followed by 30 cycles, 92 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and final synthesis 72 °C for 5 min. The PCR products were separated by agarose gel electrophoresis. The sequence was performed by Bio-Fab research (Rome, Italy), and the resulting ITS sequences were analyzed for homologies to sequences deposited in the GenBank (https://blast.ncbi.nlm.nih.gov). The sequence of the strain was deposited in GenBank as *Alternaria alternata* strain RS (Accession number: OP715870).

2.4. Enzymatic Activities

A. alternata was tested for the extracellular enzyme activities in the chromogenic media as described by Kwon et al. (2007) [16] with some modifications. The preculturing of *A. alternata* was made on PDA (Difco, USA) at 28 °C for 7 days. To detect the fungal extracellular enzyme activity, the precultures were transferred onto the media containing Skimmed Milk Agar (SMA) for the protease activity [17]; the starch from potato (Sigma, USA) for Amylase activity [18]; 0.5% xylan (Megazyme) for Xylanase activity [19] and 0.5% carboxymethylcellulose (CMC) (Sigma, USA) for Cellulase activity [20]. Congo red dye or iodine solution was used for chromogenic reaction to detect the enzymatic activities. After 5 days of incubation at 28 °C, a halo formed around the fungal plug was observed and measured, indicative of the hydrolytic activities.

2.5. Dual Culture Assay

To detect the antifungal activities between pathogen fungi that infect the same host plant (Table 1), the dual culture assay was performed as described by Yasmin et al. (2019) [21] with some modifications. A fungal disc (4 mm) was placed at the margin of the PDA plate and 1 cm of the distance from the antagonist fungal disc (4 mm). The plates were incubated at 28 °C for 7 days. Plates inoculated with the fungus alone were used as negative controls. The assay was replicated three times. The percentage growth inhibition was calculated with the following formula:

$$\% \text{ inhibition} = [1 - (\text{Growth of fungus/control growth})] \times 100$$

Table 1. List of the phytopathogenic fungi used in this study.

Species	Strain	Provenience
Alternaria alternata	RS	Italy
Botrytis cinerea	B05.10	Italy

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2.6. Pathogenicity Tests on Host and Non-Host Pear Fruits

The pathogenicity of the isolate (Table 1) was also tested on pear fruits from two *Pyrus communis* cultivars (Abate Fetel as a global cultivar and Decana del Comizio as an Italian cultivar). The experiment was performed as described by Aung et al. (2020) [22] with some modifications. Briefly, the fruits were surface sterilized by dipping in 70% of EtOH for 2 min and then washed with sterilized distilled water 3 times. The fruits were wounded by a puncher (4 mm), and each strain's mycelia plugs (4 mm) were cut from the edge of 3-day-old colonies and placed on wounded sites. Sterile PDA plugs were used as controls. To detect the antifungal activity between fungi competitors of pear, the same fruit was wounded with two punchers at 1.5 cm of distance, and the fungal plugs were placed. Each treatment was conducted with three replications and repeated three times. The disease development was registered after 4 days.

2.7. Chemical Procedures

^{1}H NMR spectra were recorded in $CDCl_3$, CD_3OD or $(CD_3)_2CO$, also used as internal standards, at 400 MHz on a Bruker (Karlshrue, Germany) spectrometer. ESI mass spectra were recorded using the LC/MS TOF system Agilent 6230B (Agilent Technologies, Milan, Italy), HPLC 1260 Infinity in positive mode. Preparative and analytical TLC was performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.50 and 0.25 mm, respectively) plates (Merck, Darmstadt, Germany), while column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063–0.200 364 mm). The spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Sigma-Aldrich Co. (Milan, Italy) supplied all the reagents and solvents.

2.8. In vitro Fungal Growth, Isolation and Chemical Characterization of Fungal Metabolites

The fungus was grown in PDB (Difco, USA) for 15 days at 28 °C with shaking at 150 rpm. The supernatant was obtained by centrifugation at 7000 *g* for 30 min and filtration using 0.22 µm pore diameter membranes (Whatman, Maidstone, UK).

The culture filtrate (2 L) was collected (pH 7.5), concentrated under reduced pressure, and extracted with EtOAc (3 x 200 mL). The combined organic extracts were dried with Na_2SO_4 and evaporated under reduced pressure. The recovered residue (254.0 mg) was fractionated by column chromatography on silica gel eluted with a gradient of $CHCl_3$/*i*-PrOH (95:5, 9:1, 7:3, *v*/*v*), yielding eight groups (F1-F8) of homogeneous fractions (F1-F8). The residue of F2 (17.0 mg) was further purified by TLC eluted with $CHCl_3$/*i*-PrOH (95:5, *v*/*v*), giving a pure amorphous solid identified as alternariol 4-methyl ether (4, 6.6 mg). The residue of F5 (26.1 mg) was further purified by TLC eluted with *n*-hexane: EtOAc (4:6, *v*/*v*) yielding a pure amorphous solid identified as alternariol (3, 2.2 mg). The residue of F7 (15.4 mg) was further fractioned by $CHCl_3$/*i*-PrOH (95:5, *v*/*v*), providing 4 fractions (F7A-F7D). The residues of F7A (2.7 mg) and F7B (3.0 mg) were further purified by analytical TLC with $CHCl_3$/*i*-PrOH (99:1, *v*/*v*) yielding two pure amorphous solids identified as altertoxin I (1, 1.1 mg) and alteichin (2, 1.2 mg).

Altertoxin I, also known as dihydroalterperylenol (1): ^{1}H NMR spectrum (Figure S1) agreed with data previously reported [23]; ESIMS (+) *m*/*z*: 353 $[M + H]^+$ (Figure S2), consistent with the molecular formula $C_{20}H_{16}O_6$. $[\alpha]^{25}_D$ +378° (c 0.4, $CHCl_3$); $[\alpha]^{25}_D$ +396° (c 0.39, $CHCl_3$) lit. [24].

Alteichin, also known as alterperylenol (2): ^{1}H NMR spectrum (Figure S3) agreed with data previously reported [25]; ESIMS (+) *m*/*z*: 351 $[M + H]^+$ (Figure S4), consistent with the molecular formula $C_{20}H_{14}O_6$. $[\alpha]^{25}_D$ +672° (c 0.25, acetone); $[\alpha]^{25}_D$ +699° (c 0.26, acetone) lit. [25].

Alternariol (3): ^{1}H NMR spectrum (Figure S5) was in agreement with data previously reported [26,27]. ESIMS (+) *m*/*z*: 259 $[M + H]^+$ (Figure S6), consistent with the molecular formula $C_{14}H_{10}O_5$.

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Alternariol 4-methyl ether, also known as alternariol-9-*O*-methyl ether (4): ^{1}H NMR spectrum (Figure S7) was in agreement with data previously reported [27,28]. ESIMS (+) *m*/*z*: 273 $[M + H]^+$ (Figure S8), consistent with the molecular formula $C_{15}H_{12}O_5$.

2.9. Phytotoxic Activity of the Fungal Metabolites

The phytotoxic activity of compounds 1-4 was assayed on host (*Abate Fetel* pear) and non-host (Decana del Comizio pear, and lemon, *Citrus limon*) fruits at 5 x 10^{-3} M and 2.5 x 10^{-3} M concentrations. Samples were dissolved in DMSO (final concentration: 4%) and diluted in distilled water to reach the desired concentration. The fruit surface was disinfected with sodium hypochlorite (50 mg/mL) and, subsequently, washed three times with distilled water. 20 µL of the solutions containing compounds 1-4 at the two concentrations were released on the fruits. A solution of 4% of DMSO in distilled was used as negative control while a solution of the known phytotoxin *epi*-pyriculol (tested at the same concentrations) was used as a positive control [29]. Then, only for the lemon the drop was punctured three times by the needle of a sterile syringe following the procedure previously reported [30]. The injections were performed at room temperature (i.e., 14–24°C) and the results were daily checked after the inoculation. The experiment was repeated in triplicate and the necrotic lesion development was evaluated using a visual 0–3 scale (0 = no necrosis; 1 = slight necrosis; 2 = intermediate necrosis; 3 = severe necrosis). The effects of the toxins on the fruits were observed up to 10 d.

2.10. Antifungal Activity of the Fungal Metabolites against Botrytis cinerea

The antifungal activity of compounds 1-4 isolated from *A. alternata* was tested against *B. cinerea* as reported in Zdorovenko et al. (2021) [31] with some modifications. The compounds 1-4, and the positive control, the fungicide pentachloronitrobenzene (Sigma-Aldrich), were dissolved in 4% of DMSO at a final concentration of 5 x 10^{-3} M. The sensitivity of *B. cinerea* to these compounds was evaluated on PDA (Difco) as the inhibition of the mycelial radial growth. In brief, 4 mm of diameter of the mycelial plugs were cut from the margin of actively growing 5-day-old colonies and one plug was placed in the center of the Petri dish with the mycelia in contact with the medium. Then, the different compounds were applied separately on the top of each plug. The negative control was obtained by applying 20 µL of 4% of DMSO. The plates were incubated at 28 °C for 5 days. The inhibition of the fungal growth was observed as a decrease of growth compared to the negative control.

2.11. Statistical Analysis

The results of pathogenicity activity analysis are expressed as means of independent experiments ± standard errors (SE). The analysis of variance was carried out by using One-way ANOVA using GraphPad Prism 8 software.

3. Results and Discussion

3.1. Preliminary Characterization

A pure culture fungal isolate on PDA media was obtained from brown spots of Abate Fetel pears (*Pyrus communis*) collected in Terrazzo (Verona, Italy) in 2019. The isolate was identified through morphological analysis and DNA sequencing. On PDA plates (Difco), the mycelium developed air hyphae on grayish-white colonies that turned olivaceous-black in seven days (Figure 1A). At the optical microscope, the conidia appeared ovoid or ellipsoidal, pale brown with a cylindrical beak. Short beaks and slim long septate have been observed, too (Figures 1B and 1C). The mean conidial dimensions were 19.2 × 13.4 µm on PDA. The examined morphological parameters, such as conidia dimensions, conidiophore morphology, number of divisions, and beak structure and colony development, were consistent with the *Alternaria alternata* parameter ranges defined by Simmons (1999) [32], so the fungal isolate was suggested as *A. alternata* (Figure 1). To confirm the

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identity, the ITS region of DNA was amplified revealing the fungus corresponds to *A. alternata* with a percentage of identity of 99.60%.

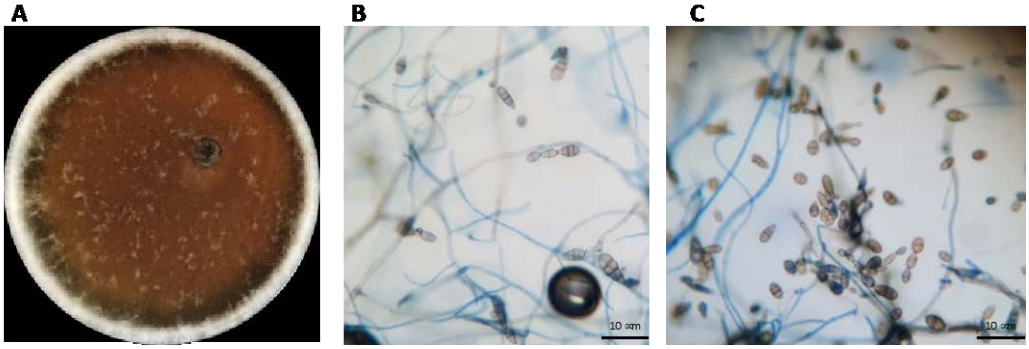

Figure 1. Morphological identification of *Alternaria alternata.* (A) *A. alternata* grown in PDA broth for 7 days at 28 °C. (B and C) Observations under phase-contrast light microscope magnification 60x.

The necrotrophic fungal pathogen *A. alternata* can attack many plant species by producing a broad range of host-selective and non-selective compounds. Cell wall-degrading enzymes (CWDEs) and toxins are the two most common weapons utilized by pathogens to penetrate and colonize their host. CWDEs and non-specific toxins (NSTs) were analyzed to better understand the mechanisms underlying *Alternaria* pathogenesis.

3.2. Enzymatic Activity of A. alternata

Phytopathogenic fungi secrete a broad range of hydrolytic enzymes to break down the complex polysaccharides of the plant cell wall to penetrate the host and develop the disease. Most of the characterized CWDEs belong to glycoside hydrolase, which can hydrolyze glycosides. The hydrolytic activity of the fungus was tested as reported in the Material and Method section. The results show that the fungus has strong amylase and xylanase activities, which are involved in stealth pathogenicity strategies by fungi to avoid host detection and promote the necrosis of the plant tissue surrounding the infected plant areas, respectively [33].

Only a medium cellulase activity was detected, which may be due to the lack the induction of the specific genes as happens during infections. Finally, the fungus did not produce Protease enzymes under tested conditions (Figure 2).

	Enzymatic activities			
Strain	**Protease**	**Amylase**	**Xylanase**	**Cellulase**
A. alternata	-	+++	+++	++

Figure 2. Enzymatic activities produced by *Alternaria alternata*. +: detectable enzyme, -: undetectable enzyme; halo < 5 mm (+); halo 5 mm (++), and halo > 5 mm (+++).

3.3. Dual Culture Assay with Phytopathogenic Fungi Competitors of Pear Fruits

It is possible to observe in nature the presence of multi-infections when a host individual encounters, simultaneously or successively, more than one strain of the same pathogen species and/or of different pathogen species. To examine this possibility, a dual culture assay was performed to investigate the competitive or coexistence activity between *A. alternata* and *Botrytis cinerea* a fungal competitor of pears. The results (Figure 3) show that *B. cinerea* can inhibit *A. alternata* by 44%.

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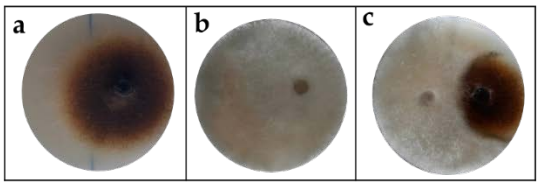
A



B

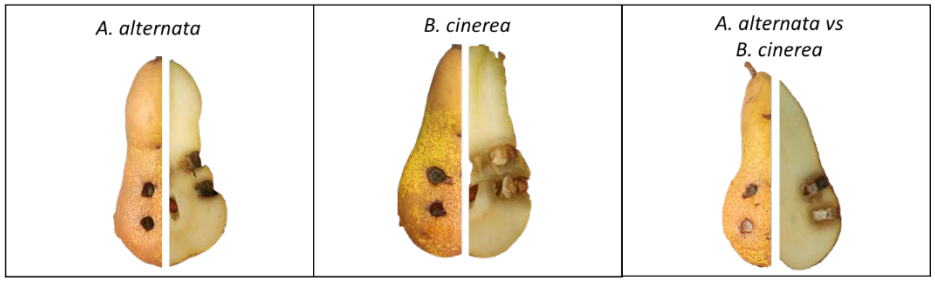
Inhibitor strain	Target strain (% inhibition)
	A. alternata
B. cinerea	44 %

Figure 3. Competitive exclusion between pathogen fungi that infect the same host plant. In the figure A: (a) *Alternaria alternata*; (b) *Botrytis cinerea*; (c) Inhibition plate. The table B: percentage of inhibition of fungal growth.

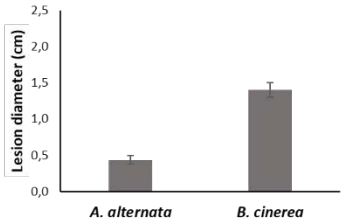
3.4. Pathogenicity Activity of A. alternata in Host Pear Fruits

To analyze the pathogenic effect of *A. alternata* on host pear (Abate Fetel) fruits, 4 mm of the fungal plug was inoculated in the fruits. At the same time, the experiment was also performed for *B. cinerea*. Moreover, a multi-infection with a co-inoculate of these two phytopathogenic fungi on pears fruits was simulated. As shown in Figure 4A, the external appearance of lesions is difficult to appreciate due to the absence of a necrotic area on the tissue. On the contrary, the internal injuries consisted of black or brownish necrosis, which extended from 0.5 to 1.5 cm from the inoculation point (Figure 4B). It also was possible to observe the production of hardening of the tissue at the necrotic patch. When the fungi were inoculated separately, a strong inner lesion was produced by *B. cinerea* as compared to *A. alternata*. Unexpectedly, when the fungi were co-inoculated, *A. alternata* inhibited the fungal growth of *B. cinerea* by about 70% (Figure 4C). This result suggests a different mechanism of competition between the two pathogens on host pears and *in vitro*.

A



B



C

Inhibitor strain	Target strain (% inhibition)
	B. cinerea
A. alternata	70 %

Figure 4. Pathogenicity assays on host Abate Fetel pear fruits. A: Pathogenicity test on pear fruits; B: Detection of lesions on pear fruits. C: Antifungal activity between pear fungi pathogens.

3.5. Production of Fungal Metabolites in vitro

To evaluate the production of fungal metabolites potentially involved in the pathogenicity mechanisms, *A. alternata* was grown *in vitro* as described in the Material and Method section. The EtOAc extract of *A. alternata* culture filtrates was purified following the procedure reported in the same section obtaining four metabolites belonging to two different classes of natural compounds: perylenequinones (compounds 1 and 2), and dibenzopyrones (compounds 3 and 4) (Figure 5) [34].

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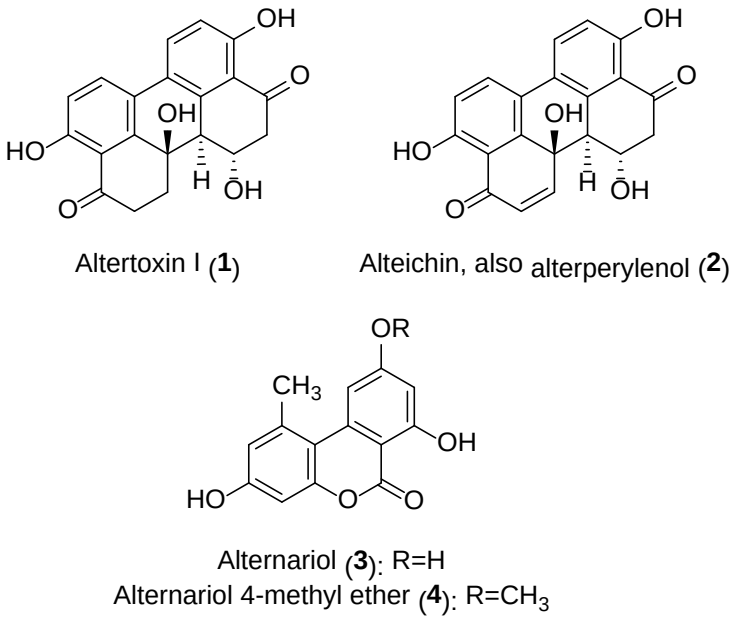

Figure 5. Structure of the isolated metabolites.

The isolated compounds were altertoxin I (1, also known as dihydroalterperylenol), alteichin (2; also known as alterperylenol), alternariol (3) and alternariol 4-methyl ether (4; also known as alternariol-9-*O*-methyl ether). Compound 4 was isolated in a better yield. The ^{1}H NMR spectra and molecular ion peaks obtained for compounds 1 and 2 (Figures S1-S4) indicated that both were perylene derivatives, which are one of the common structural classes of the isolated *Alternaria* toxins [11]. Compound 1 was identified as altertoxin I and compound 2 as alteichin comparing their spectroscopic data (^{1}H NMR) with those already reported in literature [23,25]. Their identity was confirmed by the data obtained from their ESIMS spectra which showed the protonated $[M + H]^+$ ions at *m*/*z* 353 (for compound 1) and 351 (for compound 2). Finally, the specific optical rotation values recorded for compounds 1 and 2 allowed us to unequivocally identify their stereochemistry by comparing them with the values previously reported in literature [24,25]. On the other hand, the spectroscopic (^{1}H NMR) and spectrometric (ESIMS) data obtained for compounds 3 and 4 (Figures S5-S8) indicated that these metabolites were closely related and belong to a class of dibenzopyronederivatives, which is also produced by of *Alternaria* spp. [11,35]. In particular, the ^{1}H NMR spectrum of compound 3 agreed with data previously reported for alternariol (3) [26,27], while that of compound 4 was in agreement with data previously reported for one of its analogues, namely its 4-methyl ether (4) [27,28]. Their identity was also confirmed by the data obtained from their ESIMS spectra which showed the protonated $[M + H]^+$ ions at *m*/*z* 259 (for compound 3) and 273 (for compound 4).

Altertoxin I (1), alternariol (3) and alternariol 4-methyl ether (4) are among the most common mycotoxins produced by *Alternaria* that can be found as contaminants of foodstuffs [35,36]. Alteichin (2) or alternariol (3) were also described as reddish pigments [37]. Compounds 1 and 2 are perylenequinones, a family of natural products whose structure is characterized by a pentacyclic conjugated chromophore that provides light-induced biological activity [38]. The biological activities of perylenequinones have been wider studied in the pharmacological field. 85% of the already-known perylenequinones have been discovered from fungal sources, though they have been also found in plants and animals [39]. Altertoxin I (1) and alteichin (2) are produced by different *Alternaria* species, including fungal pathogens, and phytotoxic activities have been reported for both compounds [35,40,41]. Alternariol (3) and alternariol 4-methyl ether (4) aredibenzopyrone derivatives,

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tricyclic aromatic compounds commonly produced by *Alternaria* species [35]. These compounds are mycotoxins that are frequent contaminants in foodstuffs, though it may be noted that the toxicity of alternariols for humans and animals is considered low [42,43]. There are several reports in the literature that have focused their interest on pharmacological activities and biosynthetic aspects of compounds 3 and 4, but also some studies have proven activities of agronomic interest for both toxins [42,44,45].

3.6. Pathogenicity Activity of A. alternata in Non-Host Pear Fruits

Since the studies on the toxins secreted by *A. alternata* detected the presence of unselective toxins, the same experiment performed in paragraph 3.4 was replied in non-host pear fruits, using the Italian cultivar of pear fruits Decana del Comizio. As shown in Figure 6A, the external appearance of lesions is visible as a black area to *A. alternata* and brownish to *B. cinerea*. The external injuries extended from 0.5 to 2 cm from the inoculation point (Figure 6B). Also, in this case, *B. cinerea* results more phytotoxic than *A. alternata*. The co-inoculation of both fungi on the pear fruits confirms the previous data observed in Figure 4. *A. alternata* in the presence of *B. cinerea* showed a competitive exclusion during the co-inoculation, inhibiting the growth of *B. cinerea* by about 65 % (Figure 6C).

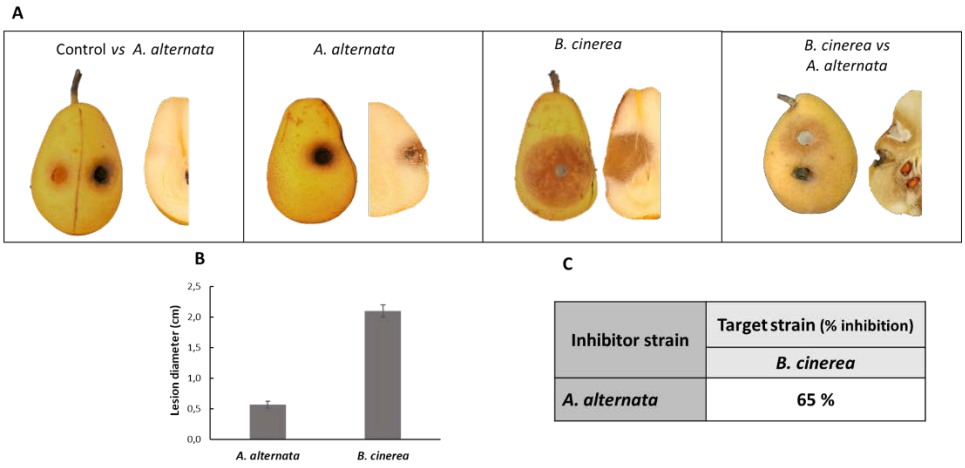

Figure 6. Pathogenicity assays on non-host Decana del Comizio pear fruits. A: Pathogenicity test on pear fruits; B: Detection of lesions on pear fruits. C: Antifungal activity between pear fungi pathogens.

3.7. Phytotoxic Activity of the Isolated Compounds

The phytotoxic activity of the isolated compounds (1-4) was tested against two pear species (Abate Fetel, host; and Decana del Comizio, non-host), and lemon (non-host) fruits, together with the known phytotoxin *epi*-pyriculol [29] as a positive control, at 5 x 10^{-3} M and 2.5 x 10^{-3} M. Different activity levels were obtained according to the fruit at 5 x 10^{-3} M, while no activity was observed for compounds 1-4 at 2.5 x 10^{-3} M. Observations were daily made for 10 days, though no significant changes were observed from the third day. Positive control always showed necrosis, and null effects were always observed for the negative control. The results obtained using a concentration of 5 x 10^{-3} M are reported in Figure 7 and in Table 2.

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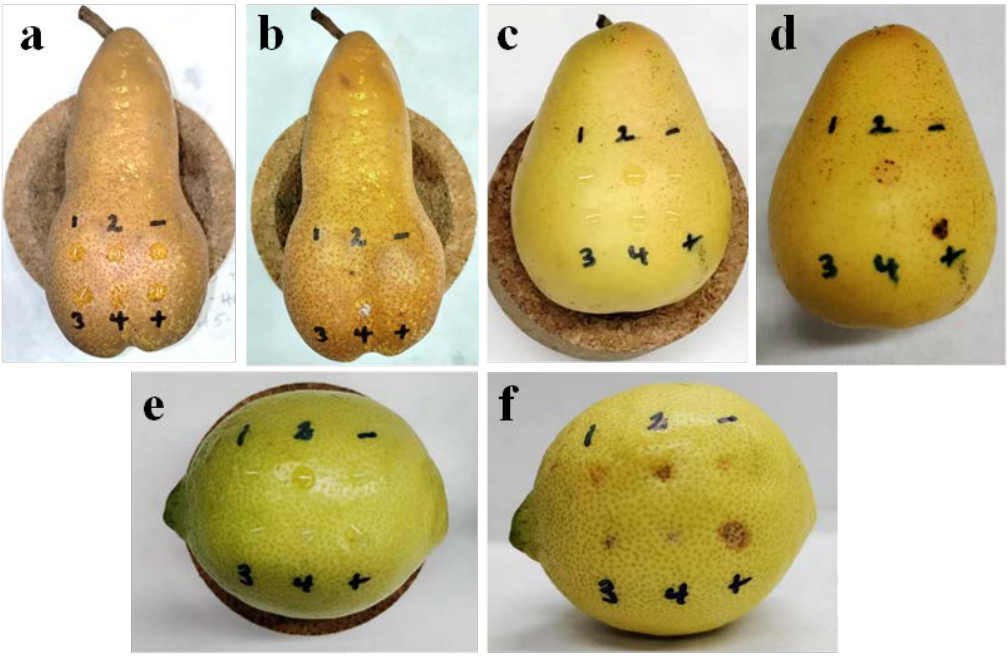

Figure 7. Necrotic effects produced by compounds 1-4 (tested at a concentration of 5 x 10^{-3} M), the positive control *epi*-pyriculol (+) (tested at a concentration of 5 x 10^{-3} M), and the negative control (-) (a solution of 4% of DMSO in distilled water), on Abate Fetel pears after 0 (a) and 3 (b) days; on Decana del Comizio pears after 0 (c) and 3 (d) days; and on lemon fruits after 0 (e) and 3 days (f).

Table 2. Phytotoxic activity of compounds **1-4**, and the positive (*epi*-pyriculol) and negative controls, on pear and lemon samples after 3 days at the concentration of 5 x 10^{-3} M**.**

Compound	Necrosis intensity[1] on Abate Fetel pears	Necrosis intensity[1] on Decana del Comizio pears	Necrosis intensity[1] on lemon fruits
Altertoxin I (**1**)	0	1	2
Alteichin (**2**)	1	2	3
Alternariol (**3**)	0	0	3
Alternariol 4-methyl ether (**4**)	0	0	2
Positive control	1	3	3
Negative control	0	0	0

[1] Intensity of necrosis is reported as: 3, severe necrosis; 2, intermediate necrosis; 1, slight necrosis; 0, no necrosis.

As shown in Figure 7, visible necrotic effects were observed after treatment when tested at 5 x 10^{-3} M. After 24h of application, the most active compounds caused mild effects, reaching the maximum level of activity on the third day, as shown in Figure 7 and Table 2. On pear, both on Abate Fetel and Decana del Comizio fruits, after 1 day of treatment alteichin (2) was the only compound showing necrosis, at a slight level, and increasing this symptom to intermediate necrosis in the case of the non-host pear after 3 days (Figures 7B and 7D). Altertoxin I (1) also showed activity (slight) after 3 days (Figure 7D) but only on the non-host pear variety (Decana del Comizio). Thus, necrotic activity was only generated by the tested perylenequinones (1 and/or 2), being the dibenzopyrones 3 and 4 totally inactive. Though no necrotic lesions were observed after treatment with compound 4 on pear fruits, necrosis activity on pear leaves was reported for this compound [45].

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On lemon samples, compounds 1-4 showed a remarked higher necrotic activity. This improved activity would be also in agreement with the minor changes in the appearance described for the infected area in pears fruits during the early stages of the infection with *A. alternata* [46]. After 1 or 2 days, compounds 2 and 3 showed intermediate necrosis, and compounds 1 and 4 at slight level. After 3 days, the effects of the activity of all the compounds increased, with compounds 2 and 3 showing severe necrotic effects comparable to those of the positive control (Figure 7F). From the structural point of view, on lemon samples an improved activity between perylenequinones and dibenzopyrones was not observed. It must be highlighted that alteichin (2) showed the highest necrotic effects, as also found on pear samples. Considering the chemical differences between compounds 1 and 2, the results obtained in our study suggest the importance of the α,β-unsaturated ketone group present in compound 2. In fact, the absence of this moiety in compound 1 causes a reduction of the phytotoxic activity. It is interesting to note that compound 2 caused necrotic lesions in a previous report on tomato, sunflower, Canada thistle, wheat, and barley leaves [47], and moderate necrotic areas were also found on corn and soybean as well [40]. The other tested perylenequinone (altertoxin I, 1), like in the study herein presented, also showed selectivity in a previous study that showed remarked necrotic lesions on corn, though no significant lesions for other species like soybean, crabgrass or timothy [40]. It may be noted that phytotoxic effects like necrosis could be directly related with the pro-oxidant properties of the compound [48], so it is should be remarked the pro-oxidant activity previously suggested for compound 1 [49]. Perylenequinones 1 and 2 also exerted phytotoxic activity against seedling growth of lettuce and amaranth (*Amaranthus retroflexus* L.) [41].

Regarding the dibenzopyronederivatives alternariol (3) and alternariol 4-methyl ether (4), the higher necrotic effects showed by compound 3 could be explained by the hydroxyl group placed in the position where compound 4 presents a methoxy group, hinting that polar groups could have a positive effect on the necrosis-inducing activity, due to the capacity of forming H-bonds [50]. For both compounds, found herein as necrotic-causing toxins on lemon fruits, but inactive on pear fruits (host and non-host species), other previous studies reported activities of interest. Pro-oxidative activity was described for compound 3 [51], and it would be also worth highlighting its inhibitory activity against the root growth of *Pennisetum alopecuroides* and *A. retroflexus* when tested at 1000 µg/mL [44]. For compound 4, necrosis on pear leaves, chlorosis on tobacco leaves, and inhibition of the electron transport chain in isolated spinach chloroplasts would support the toxic potential of this toxin produced by *A. alternata* [42,45].

3.8. Antagonistic Effect of Compounds 1-4 Against B. cinerea

The unselective toxins (1-4) isolated from *A. alternata* were tested for their antifungal activities to investigate their involvement in inhibiting the growth of *B. cinerea* observed in the previous experiments. As positive control, the commercial fungicide pentachloronitrobenzene (PCNB) was employed. The concentrations tested are those used in the experiment represented in Figure 8.

As shown in Figures 8A and 8B, when the fungus is treated with different toxins, the growth is slowed. In particular, toxins 2 and 4 decreased the radial growth of the fungus. It is possible to observe that the mycelium assumes a circular form, and the hyphae show slow outward growth. Since the radiating hyphae make it easy to move nutrients quickly around the growing mycelium, this could explain the difference observed in plates treated with toxins than to the control (untreated). Instead, the commercial fungicide PCNB completely inhibited the growth of *B. cinerea.*

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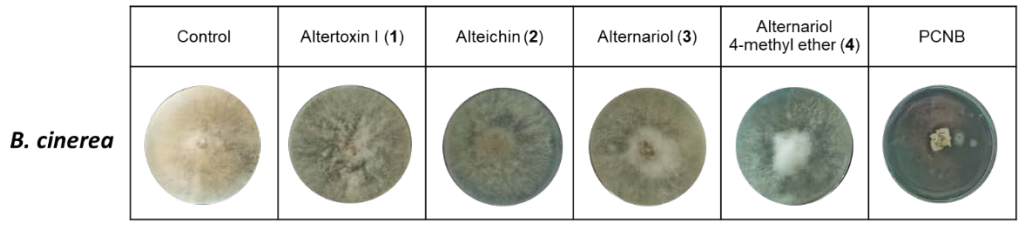
A



B

	Altertoxin I (**1**)	Alteichin (**2**)	Alternariol (**3**)	Alternariol 4-methyl ether (**4**)	PCNB
A. alternata	+	++	+	++	-

Figure 8. *In vitro* antagonistic effect of compounds isolated from *Alternaria alternata*. A: representative photographs of antifungal activity against *Botrytis cinerea* of compounds 1-4 isolated from *A. alternata* compared with the positive control pentachloronitrobenzene (PCNB) at a final concentration of 5 x 10^{-3} M. B: evaluation of inhibition growth: +: low inhibition; ++: medium inhibition; +++: high inhibition; -: no growth.

5. Conclusions

The morphological observation of mycelium and the amplification of a fragment of rDNA (including ITS1 and ITS2 and the 5.8S rDNA gene) of a phytopathogenic fungus, isolated from infected pear fruits in Italy, allowed us to identify the species as *Alternaria alternata*. The fungus showed strong amylase, xylanase and cellulase activities. Moreover, its PDB liquid culture led to the isolation of the four compounds which were identified as altertoxin I (1), alteichin (2), alternariol (3) and alternariol 4-methyl ether (4). When tested on pears (host and non-host varieties) and lemon fruits the compounds showed a different level of activity. In particular, only compound 2 generated visible necrotic effects on host pears, while on the non-host pear variety, the perylenequinones (1 and 2) showed moderate-slight necrotic activity. All the compounds (1-4) exerted severe or moderate necrotic lesions on lemon fruits. These results showed that compounds 1-4 are non-host-selective toxins and that compound 2 is the most active one.

Furthermore, to understand the strategies used by *A. alternata* in infection dynamics, a multi-infection with the competitor phytopathogen *Botrytis cinerea* has been simulated. Our results demonstrated that the co-infection of *A. alternata* with fungal competitor *B. cinerea* probably depends on available nutrients. When simulated *in vitro* dual culture assay using the PDA broth, *B. cinerea* inhibits *A. alternata* growth. On the contrary, when replicated in the experiment that simulates the same ecological niche (host and non-host pear fruits), *A. alternata* showed a competitive exclusion that inhibits the fungal growth of *B. cinerea* by 70 and 65%, respectively. The experiment demonstrated that the interaction between different phytopathogens sharing similar ecological niches results in competition, and the most virulent fungus have an intra-host competitive advantage that leads to the exclusion of less virulent fungi. Probably the mechanisms used by *A. alternata* to infect plants and in the defense against other pathogens competitors are based on the synergism between enzymatic activities and the secretion of the non-host toxin. In fact, compounds 2 and 4 have been shown a key role in the mechanism used by *A. alternata* to decrease *B. cinerea* growth. These toxins slow down the extension of the fungal hyphae which is beneficial for the uptake of nutrients.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: ^{1}H NMR spectrum (400 MHz) of altertoxin I (1) in $CDCl_3$; Figure S2: ESI MS spectrum of altertoxin I (1) recorded in positive mode; Figure S3: ^{1}H NMR spectrum (400 MHz) of alteichin (2) in $CDCl_3$; Figure S4: ESI MS spectrum of alteichin (2) recorded in

positive mode; ^{1}H NMR spectrum (400 MHz) of alternariol (3) in CD_3OD; ESI MS spectrum of alternariol (3) recorded in positive mode; ^{1}H NMR spectrum (400 MHz) of alternariol 4-methyl ether (4) in $(CD_3)_2CO$; ESI MS spectrum of alternariol 4-methyl ether (4) recorded in positive mode.

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