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Identification, characterization and mycotoxigenic ability of *Alternaria* spp. causing core rot of apple fruit in Greece

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ABSTRACT

Alternaria core rot is a major postharvest disease of apple fruit in several countries of the world, including Greece. The study was conducted aiming to identify the disease causal agents at species level, investigate the aggressiveness of *Alternaria* spp. isolates and the susceptibility of different apple varieties and determine the mycotoxigenic potential of *Alternaria* spp. isolates from apple fruit. Seventy-five *Alternaria* spp. isolates obtained from apple fruit showing core rot symptoms were identified as either *Alternaria tenuissima* or *Alternaria arborescens* at frequencies of 89.3 and 11.7%, respectively, based on the sequence of endopolygalacturonase (*EndoPG*) gene. Artificial inoculations of fruit of 4 different varieties (Fuji, Golden Delicious, Granny Smith and Red Delicious) and incubation at two different temperatures (2 and 25 °C) showed that fruit of Fuji variety were the most susceptible and fruit of Golden Delicious the most resistant to both pathogens. In addition, the production of 3 mycotoxins, alternariol (AOH), alternariol monomethyl ether (AME) and tentoxin (TEN) was investigated in 30 isolates of both species. Mycotoxin determination was conducted both *in vitro*, on artificial nutrient medium and *in vivo* on artificially inoculated apple fruit, using a high performance liquid chromatography with diode array detector (HPLC-DAD). The results showed that most of the isolates of both species were able to produce all the 3 metabolites both *in vivo* and *in vitro*. On apple fruit *A. tenuissima* isolates produced more AOH than *A. arborescens* isolates, whereas the latter produced more TEN than the former. Such results indicate that Alternaria core rot represents a major threat of apple fruit production not only due to quantitative yield losses but also for qualitative deterioration of apple by-products.

1. Introduction

Core rot of apple fruit is one of the most important postharvest diseases of apple and is defined as a rot initiating from the loculus that spreads into the fruit mesoderm and leads to either a dry core rot (DCR) or wet core rot (WCR). Fruit affected by DCR are characterized by dark brown, dry and corky tissues. DCR develops slowly and the rot is restricted in the loculus and the mesoderm around the loculus (Shtienberg, 2012). WCR is also characterized by dark brown tissues, however the disease progresses more rapidly and deeply into the mesoderm. External symptoms are not evident until harvest but during storage the rot progresses and after several months, the rot may also be evident on the fruit epidermis. However, in most cases the disease is unnoticed until the fruit is cut open (Shtienberg, 2012). In addition to DCR and WCR, moldy core (MC) can be another type of infection of apple fruit loculus. It is associated with the development of fungal mycelia within the loculus, without invasive penetration into the mesoderm that results in the appearance of the core rot. MC is considered of minor economic importance since it does not affect the fruit qualitative characteristics (Serdani et al., 2002). Infection of the core occurs after the establishment of the disease causal agents on the senescing parts of the blossom and the penetration through the sinus after fruit setting or later stages of fruit development (Combrink et al., 1984; Reuveni et al., 2002). Yield losses due to core rot have been estimated to 3–10% in Israel and 5–8% in South Africa (Combrink et al., 1984; Niem et al., 2007).

The disease is caused by a complex of fungi such as *Fusarium* spp., *Ulocladium* spp., *Cladosporium* spp., *Coniothyrium* spp., *Pezicula* spp., *Mucor* spp., and *Alternaria* spp., with the latter being predominant (Combrink et al., 1985b; Gao et al., 2013; Michailides et al., 1994; Spotts, 1990; Tournas and Uppal Memon, 2009). Early studies on the etiology of *Alternaria* core rot of apple fruit suggested *Alternaria alternata* as the causal agent of the disease (Combrink et al., 1984; Ellis and Barrat, 1983; Reuveni et al., 2002; Spotts, 1990). In these studies identification was made based only on the morphological characteristics of the isolates' cultures and conidia. But it is evident that morphological data are insufficient for taxonomic differentiation due to

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overlapping morphological characters between *A. alternata* and other small-spored *Alternaria* spp. (Pryor and Michailides, 2002; Simmons, 1992). As new molecular tools have been employed in identification and characterization of *Alternaria* spp., more recent studies have shown that several *Alternaria* species such as *Alternaria tenuissima*, *Alternaria infectoria* and *Alternaria arborescens* may be the causal agents of the disease (Gao et al., 2013; Kang et al., 2002; Kou et al., 2014; Serdani et al., 2002).

Alternaria spp. are potential producers of more than 30 secondary metabolites, mostly host-specific or non-specific phytotoxins that play a crucial role in the development of diseases caused by the pathogens. In addition, most of the *Alternaria* species are able to produce mycotoxins with biological activity against several organisms including mammals. Among these metabolites alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), tentoxin (TEN) and tenuazonic acid (TeA) are included (Logrieco et al., 2003). Although the acute toxicity of *Alternaria* mycotoxins is considered to be low in mammals, there is strong evidence that they may be mutagenic and carcinogenic (Logrieco et al., 2009; Ostry, 2008). *Alternaria* mycotoxins have been detected in several foods such as citrus fruit, tomato, tomato products, olives, pepper and wheat (Ostry, 2008). In addition, several *Alternaria* mycotoxins have been detected in apple fruit infected by *Alternaria* spp. or in apple juice concentrates (Andersen et al., 2006; Broggi et al., 2013; Delgado et al., 1998; Prelle et al., 2013; Robiglio and Lopez, 1995; Scott and Kanhere, 2001).

In a recent study aiming to identify the causal agents of postharvest rots of apple fruit in Greece it was found that *Alternaria* spp. was the third most common pathogen after *Penicillium expansum* and *Botrytis cinerea*, whereas in variety Fuji it was found to be the most common pathogen (Konstantinou et al., 2011). Taking into account the increasing significance of this disease, further research was initiated aiming to: i) identify the causal agents of the disease at species level and characterize them molecularly, ii) measure variety susceptibility and aggressiveness of *Alternaria* spp. under different storage temperatures and iii) investigate the ability of *Alternaria* spp. from apple fruit to produce some common *Alternaria* toxins both *in vitro* and *in vivo* on artificially inoculated apple fruit.

2. Materials and methods

2.1. Pathogen isolates

Isolates of *Alternaria* spp. were collected from apple fruit (cv. Golden Delicious, Red Delicious, Fuji and Granny Smith) showing core rot symptoms. The fruits were obtained from packinghouses located in the region of Imathia, northern Greece, during the 2012–13 storage period. Rotted fruit were transferred to the laboratory for isolation of the decay agents. Isolations were carried out from surface-disinfected fruit (they were drenched for 1 min in a 1% sodium hypoclorite solution) by removing small fruit pieces at the margin of diseased/healthy tissue and transferring them to Petri dishes containing acidified Potato Dextrose Agar (Merck, Darmstadt, Germany). Petri dishes were incubated at 22 °C in an incubator with a 12-h photoperiod provided by fluorescent lights. After 3 to 5 days of incubation, the emerging putative *Alternaria* spp. colonies were transferred to fresh PDA medium and incubated under the above mentioned conditions for 1 additional week to induce sporulation. In total 75 single-spore isolates of *Alternaria* spp. were obtained (31, 7, 27 and 10 isolates from Fuji, Red Delicious, Golden Delicious and Granny Smith varieties, respectively) and stored at 4 °C until use.

2.2. DNA isolation

To extract DNA the isolates were grown in Potato Dextrose Broth (Sigma-Aldrich, St. Louis, MO) for 5 days at 25 °C. Then, the mycelium was harvested by filtration, dried, lyophilized, ground to a fine powder using micropestles (Eppendorf International, Wesseling, Germany) and stored at −20 °C until use. DNA was extracted using QIA Puregene Core Kit A (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The concentration of the extracted DNA was measured using a P330 nanophotometer (Implen GmbH, Munich, Germany).

2.3. Species identification and phylogenetic analysis

The endopolygalacturonase (*endoPG*) gene, has shown potential to delineate the closely related species within the *A. alternata*-complex and was used for pathogen identification and phylogenetic analysis (Hong et al., 2005; Andrew et al., 2009). Amplification of *endoPG* gene was conducted using primers PG3 (5′-TACCATGGTTCTTTCCGA-3′) and PG2b (5′-GAGAATTCRCARTCRTCYTGRTT-3′) (Andrew et al., 2009). Reaction mixtures contained 3 μl genomic DNA (50 ng), 0.32 μM of PG3 primer, 1.12 μM of PG2b primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1× PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], and 0.04 units Taq DNA Polymerase and the total volume was adjusted to 25 μl with sterile highly purified H_2O. PCR amplification was conducted using the following conditions: an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. All the PCR reactions were performed in a LabCycler thermal cycler (SensoQuest GmbH, Gottingen, Germany).

PCR products of each isolate were purified using the Qiaquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The purified products were subjected to sequencing in both directions using the forward and the reverse primers. Sequences were aligned using the computer software package Mega 5.05. The sequences obtained were compared with sequences in the National Center for Biotechnology Information database using BlastN 2.2.18.

Phylogenetic analysis of *EndoPG*. Sequences were trimmed to 448 bp to eliminate ambiguous sites and aligned using MAFFT version 6 (Katoh and Toh, 2008). Models of sequence evolution were tested for each alignment and model parameter estimates obtained for each alignment using jModelTest version 0.1 (Posada, 2008). The K80 model was selected for the *EndoPG* data with equal base frequencies, a transition/transversion ratio of 3.05, and equal substitution rates among sites. The rooted *EndoPG* phylogenies were estimated using maximum likelihood with the program PhyML version 3.0 (Guindon and Gascuel, 2003). *Alternaria gaisen* (GenBank AY295033) was used to root the phylogenetic tree.

2.4. Morphological characterization

For all 75 isolates used in the study the morphological characteristics of colony and sporulation apparatus were determined using criteria as described by Pryor and Michailides (2002). To determine the colony characteristics the isolates were grown on PDA and incubated at 22 °C in the darkness for 10 days. At the end of the incubation period the colony diameter, colony color, colony texture, colony margin and the presence of crystals in agar medium were examined. Colony diameter was measured in mm, while the remaining characteristics were assessed visually. For each isolate 3 replicate PDA plates were prepared and diameter values were averaged.

Characterization of sporulation habit was conducted on weak (0.05) PDA (3 replicate plates per isolate). The cultures were incubated for 7 days at 22 °C under cool fluorescent light (60 μmoles/m^2/s, 10:14 h light/dark cycle). At the end of the incubation period the sporulation apparatus was examined using a stereomicroscope at 40× magnification. Conidial size was measured in a ZEISS AxioImager. Z2 microscope using a digital camera (AxioCam MRc 5). The size of 40 conidia per isolate was measured. Eight *Alternaria* spp. isolates (two of each *A. alternata*, *A. infectoria*, *A. tenuissima* and *A. arborescens*) were kindly provided by Dr T. J. Michailides (Kearney Agricultural Research and Extension Center, University of California) and used as reference isolates.