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Identification and mycotoxigenic capacity of fungi associated with pre- and postharvest fruit rots of pomegranates in Greece and Cyprus

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ABSTRACT

Pre- and postharvest fruit rots of fungal origin are an important burden for the pomegranate industry worldwide, affecting the produce both quantitatively and qualitatively. During 2013, local orchards were surveyed and 280 fungal isolates from Greece (GR) and Cyprus (CY) were collected from pomegranates exhibiting preharvest rot symptoms, and additional 153 isolates were collected postharvest from cold-stored fruit in GR. Molecular identification revealed that preharvest pomegranate fruit rots were caused predominately by species of the genera *Aspergillus* (*Aspergillus niger* and *Aspergillus tubingensis*) and *Alternaria* (*Alternaria alternata*, *Alternaria tenuissima*, and *Alternaria arborescens*). By contrast, postharvest fruit rots were caused mainly by *Botrytis* spp. and to a lesser extent by isolates of *Pilidiella granati* and *Alternaria* spp. Considering that a significant quota of the fungal species found in association with pomegranate fruit rots are known for their mycotoxigenic capacity in other crop systems, their mycotoxin potential was examined. Alternariol (AOH), alternariol monomethyl-ether (AME) and tentoxin (TEN) production was estimated among *Alternaria* isolates, whereas ochratoxin A (OTA) and fumonisin B_2 (FB_2) production was assessed within the black aspergilli identified. Overall in both countries, 89% of the *Alternaria* isolates produced AOH and AME *in vitro*, while TEN was produced only by 43.9%. *In vivo* production of AOH and AME was restricted to 54.2% and 31.6% of the GR and CY isolates, respectively, while none of the isolates produced TEN *in vivo*. Among black aspergilli 21.7% of the GR and 17.8% of the CY isolates produced OTA *in vitro*, while *in vivo* OTA was detected in 8.8% of the isolates from both countries. FB_2 was present *in vitro* in 42.0% of the GR and 22.2% of the CY isolates, while *in vivo* the production was limited to 27.5% and 4.5% of the GR and the CY isolates, respectively. Our data imply that mycotoxigenic *Alternaria* and *Aspergillus* species not only constitute a significant subset of the fungal population associated with pomegranate fruit rots responsible for fruit deterioration, but also pose a potential health risk factor for consumers of pomegranate-based products.

1. Introduction

Pomegranates (*Punica granatum* L.) are deciduous fruit trees originating from Iran to the Himalayas and are cultivated in the Mediterranean basin, Central Asia and countries of North and South America (Fernandez et al., 2014). The intensification of the crop has been relatively new in Greece (GR) and Cyprus (CY), following an accelerating trend with promising prospects. In Greece the cultivated area during the last decade has increased to 4000 ha, with most located in the regions of the Peloponnese and Macedonia. Although the cultivated area is significantly lower in Cyprus, the acreage has increased by more than 50% the last 5 years to 2000 ha.

The rapid expansion of pomegranate throughout the world is due to the great nutritional value of the fruit, with health promoting and dietary benefits. Pomegranates are consumed as fresh fruit, juices, jams, sauces or they are used in food and beverage industry as flavoring and coloring agents (Gil et al., 2000). Pomegranate arils are rich in polyphenols with a high antioxidant activity and consumption of pomegranate fresh fruit or products may prevent cardiovascular diseases, diabetes and prostate cancer (Gil et al., 2000; Johanningsmeier and Harris, 2011).

Pomegranate fruit rots have been shown to be one of the most important factors contributing to yield losses, along with physiological disorders such as chilling injuries, husk scald, weight loss and shrinkage (Selcuk and Erkan, 2014). Fruit decays are caused by various fungal pathogens such as *Alternaria* spp., *Botrytis cinerea*, *Aspergillus niger* and other *Aspergillus* spp., *Colletotrichum gloeosporioides*, *Coniella* spp., *Nematospora* spp., *Pilidiella granati*, *Penicillium* spp. and *Rhizopus* spp. (Palou et al., 2013). Several of these have been reported during the

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last decade as pomegranate fruit rot agents in Greece (Bardas et al., 2009a,b; Tziros et al., 2008; Tziros and Tzavella-Klonari, 2008). The aforementioned pathogens may initiate infections either through injuries and wounds of fruit exocarp or during the blooming period, via the flower parts and the crown (Palou et al., 2013). Infections may remain latent until the onset of fruit maturation and become evident during storage. *Alternaria* spp. and *Botrytis* spp. behave as latent pathogens on pomegranate fruit whereas *Aspergillus* spp., *Penicillium* spp., *P. granati*, etc. behave mainly as wound pathogens.

Fruit decays not only reduce yields quantitatively, but may also deteriorate the quality of the fresh or processed fruit due to mycotoxin production. This is particularly important because contaminated pomegranates designated for processing, especially in concentrated juice, may compromise consumer health safety. Some of the fungal species that have been reported as decay agents of pomegranate fruit such as *A. niger* and *Alternaria alternata* are known mycotoxin producers.

The most common mycotoxins produced by *Alternaria* spp. are alternariol (AOH), alternariol monomethyl-ether (AME), altenuene (ALT), tentoxin (TEN) and tenuazonic acid (TeA) and have been detected in several foodstuffs (Logrieco et al., 2003; Ostry, 2008). 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).

Black aspergilli (*Aspergillus* section *Nigri*) are considered the main source of ochratoxin A (OTA) contamination in a wide range of food commodities (Battilani et al., 2006a; Juan et al., 2008). OTA has been shown to be nephrotoxic, carcinogenic, genotoxic, immunotoxic and teratogenic, thus the EU has fixed maximum limits for OTA presence in several products. Black aspergilli reported to produce OTA include *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus japonicus*, *Aspergillus aculeatus* and species within the *A. niger* aggregate (*A. niger*, *Aspergillus tubingensis*). Apart from OTA, it was recently shown that strains of black aspergilli (*A. niger* and *Aspergillus welwitchiae*) possess putative fumonisin gene clusters and are able to produce fumonisins (B_2 and B_4) (Frisvad et al., 2011; Mogensen et al., 2010; Susca et al., 2014). Fumonisins are mainly associated with seed rots in cereals and maize caused by several *Fusarium* species and considered to be responsible for harmful effects on the health of humans or animals (Desjardins, 2006).

Although pomegranate is a rapidly expanding crop, information about causal agents of fruit rots is scarce and mainly restricted to reports of the presence of individual diseases in different countries. In addition, although infections of pomegranate fruit by pathogens known for their ability to produce mycotoxins, such as *Alternaria* spp. and *Aspergillus* spp. have been reported in several countries, there is an information deficit about the mycotoxigenic ability of these fungal species on pomegranate fruit. Therefore, this study was initiated aiming to: (i) identify the causal agents and determine the prevalence and incidence of pre- and postharvest fruit rot diseases in two different Mediterranean countries, Greece and Cyprus and (ii) investigate the mycotoxigenic ability of *Alternaria* spp. and *Aspergillus* spp. isolates from pomegranate fruits.

2. Materials and methods

2.1. Fruit sampling

To investigate the etiology of preharvest fruit decays the sampling was conducted in orchards located in 6 and 3 different regions of Greece and Cyprus, respectively (Fig. 1). Fruit were sampled during August–September 2013, just before the harvest. One to seven orchards were sampled in each region depending on the presence of fruit showing rot symptoms. In Greece preharvest fungal isolations were performed from fruit of the cultivars Acco, Ermioni and Wonderful, whereas in Cyprus Acco and Wonderful cultivars were sampled. From each orchard 10–15 fruit showing decay or skin discoloration (redness) were collected and transferred to the laboratory for fungal isolations. In Greece additional samplings were conducted after harvest from packinghouses located in 4 different regions of the country (Fig. 1). In each sampling 10–15 fruits showing decay or skin discoloration were arbitrarily selected from the selection line during the packing operations from November to December 2013. All postharvest isolations were retrieved from pomegranates of the cultivar Wonderful.

2.2. Pathogen isolation and identification

Isolations were carried out from surface-disinfected fruit that had been previously drenched for 1 min in a 1% sodium hypochlorite solution. Fruit samples at the margin of diseased/healthy tissue were removed and transferred to Petri dishes containing Potato Dextrose Agar (PDA) amended with streptomycin sulfate (300 mg/L). Cultures were incubated at 22 °C in the dark for 3 to 5 days and the emerging fungal colonies were transferred to fresh PDA plates to obtain pure cultures. In total 190 and 90 isolates were obtained from fruit showing preharvest rots in Greece and Cyprus, respectively, while an additional 153 isolates came from postharvest fruit rots in Greece (Table 1).

All 433 fungal isolates were preliminarily identified using morphological criteria, such as colony appearance and morphological features of fruiting bodies and spores. Known fruit rot pathogens such as *B. cinerea*, *P. granati*, *Botryosphaeria dothidea* and *Cytospora punicae* were identified to species based on fungal descriptions (Barnett and Hunter, 1998; Palou et al., 2010).

2.3. Identification of Alternaria spp. at species level

Alternaria spp. isolates were identified at species level through a combination of molecular and morphological data. DNA was extracted as described previously (Ntasiou et al., 2015). The endopolygalacturonase (*endoPG*) gene has shown potential to delineate the closely related species within the *A. alternata*-complex and has been used for pathogen identification and phylogenetic analysis (Andrew et al., 2009). The *endoPG* gene from 82 of the collected isolates was amplified using primers PG3 and PG2b (Andrew et al., 2009). Reaction mixtures and conditions, PCR products purification, sequencing and alignment were as described previously (Ntasiou et al., 2015).

The *Alternaria* spp. isolates were further characterized using morphological characteristics based on their sporulation patterns (Andersen et al., 2002; Pryor and Michailides, 2002). Isolates were grown on weak (0.05) PDA and incubated for 7 days at 22 °C under cool fluorescent light (60 μmol/m^2/s, 10:14 h light/dark cycle) after which the sporulation apparatus was examined using a stereomicroscope at 40× magnification. Eight *Alternaria* spp. isolates (two of each *A. alternata*, *Alternaria infectoria*, *Alternaria tenuissima* and *Alternaria arborescens*) were kindly provided by Dr. T. J. Michailides (Kearney Agricultural Research and Extension Center, University of California) and used as reference.

2.4. Identification of Aspergillus spp. at species level

Black aspergilli and in particular those belonging in the *A. niger* aggregate, are difficult to identify based only on morphological differences, thus an ITS-RFLP method, developed by Martinez-Culebras and Ramon (2007), was used. The 5.8S-ITS region was amplified using the primer pair ITS5/ITS4 and the PCR products were digested with the restriction enzymes *Hha*I, *Nla*III and *Rsa*I (Invitrogen, Carlsbad, CA). The digested PCR products from 114 *Aspergillus* spp. isolates were visualized after staining with ethidium bromide under UV light, and were accordingly classified to different species based on the restriction patterns (Martinez-Culebras and Ramon, 2007). ITS-RFLP identification data were further verified on a small subset of 11 isolates using sequence analysis of their calmodulin genes. Amplification conditions and sequencing reactions were set up as previously described (O'Donnell et al., 2000; Varga et al., 2010).