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Characterization of small-spored *Alternaria* from Argentinean crops through a polyphasic approach

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ABSTRACT

Small-spored *Alternaria* have been isolated from a wide variety of food crops, causing both economic losses and human health risk due to the metabolites produced. Their taxonomy has been discussed widely, but no scientific consensus has been established in this field to date. Argentina is a major exporter of agricultural products, so it is essential to thoroughly understand the physiological behaviour of this pathogen in a food safety context. Thus, the objective of this work was to characterize small-spored *Alternaria* spp. obtained from tomato fruits, pepper fruits, wheat grains and blueberries from Argentina by a polyphasic approach involving metabolomic and phylogenetic analyses based on molecular and morphological characters. Morphological analysis divided the population studied into three groups; *A. arborescens* sp.-grp., *A. tenuissima* sp.-grp., and *A. alternata* sp.-grp. However, when these characters were simultaneously analysed with molecular data, no clearly separated groups were obtained. Haplotype network and phylogenetic analysis (both Bayesian and maximum parsimony) of a conserved region yielded the same result, suggesting that all isolates belong to the same species. Furthermore, no correlation could be established between morphological species-groups and a metabolite or group of metabolites synthesized. Thus, the whole set of analyses carried out in the present work supports the hypothesis that these small-spored *Alternaria* isolates from food belong to the same species. Identification at species level through classical morphology or modern molecular techniques does not seem to be a useful tool to predict toxicological risk in food matrices. The detection of any small-spored *Alternaria* from Section *Alternaria* (D.P. Lawr., Gannibal, Peever & B.M. Pryor 2013) in food implies a potential toxicological risk.

1. Introduction

Alternaria is a ubiquitous fungal genus, associated with a wide variety of substrates including seeds, plants, animals, and soil. Due to its capability to colonize plants, either as pathogen or saprophyte, it causes economic losses to several crops worldwide. In Argentina, *Alternaria* spp. have been reported as contaminants of wheat, blueberries, tomato fruit, tomato puree, peaches, apples, sorghum, rice, soybean seeds and citrus fruits (Broggi et al., 2007; Greco et al., 2012; Patriarca et al., 2007; Peres et al., 2003; Pose et al., 2004; Pose et al., 2010; Robiglio and Lopez, 1995; Somma et al., 2011).

Morphological identification at the species level for *Alternaria* is currently based on the taxonomic key proposed by Simmons (2007), which describes 275 species organized in 13 species-groups (sp.-grp.), according to colony morphology on standardized media and conidial chain branching patterns. Subsequent molecular studies have supported many of these groups as monophyletic lineages (Hong et al., 2006; Pryor and Bigelow, 2003; Pryor and Gilbertson, 2000). However, some *Alternaria* morphospecies may vary depending on the culture media, relative humidity and light intensity (Simmons, 1992). In particular, small-spored species closely related to *A. alternata* (members of *A. alternata* sp.-grp., *A. tenuissima* sp.-grp. and *A. arborescens* sp.-grp.) have been thoroughly studied because morphological characters are insufficient for species delimitation since they are strongly influenced by

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small changes in the environment (Andrew et al., 2009). In addition, most characters frequently overlap among these species.

Sequencing of classical conserved regions, which have proved to be useful in the identification of other fungal genera (*e.g.* ITS, mtSSU, mtLSU, β-tubulin, actin, calmodulin), provided no resolution among these taxa (Chou and Wu, 2002; Peever et al., 2004; Pryor and Gilbertson, 2000; Serdani et al., 2002; Stewart et al., 2013). Lately, sequencing of alternative regions has been explored, such as a segment of an endopolygalacturonase (*endoPG*) gene; the *Alternaria* major allergen 1 (*Alt a1*) gene; translation machinery associated protein (*TMA22*); the CDP-diacylglycerol- glycerol-3-phosphate 3-phosphatidyltransferase (*PGS1*); the catalytic subunit of DNA polymerase zeta (*REV3*) and two anonymous noncoding regions, OPA10-2 and OPA1-3 (Andrew et al., 2009; Armitage et al., 2015). In particular, *endoPG* showed variability among species isolated from citrus (Andrew et al., 2009; Peever et al., 2004), and has been used so far to characterize small-spored *Alternaria* from these and other substrates (Armitage et al., 2015; Stewart et al., 2013; Stewart et al., 2014).

Even though *Alternaria* is frequently isolated from Argentinean crops, little is known about the variability and differentiation of its populations in this country. This is important since some species are allergenic and may be opportunistic human pathogens in immunocompromised patients (Armitage et al., 2015). In addition, this genus is well known for its ability to synthesize diverse secondary metabolites, some of them recognized as mycotoxins, such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), and altertoxins I, II, III (ATX-I, -II, -III) (Alexander et al., 2011; Ostry, 2008). Their toxic effects include human haematological disorders, oesophageal cancer and mutagenic activity (Andersen et al., 2015; Logrieco et al., 2009; Ostry, 2008). Other important bioactive compounds produced by *Alternaria* sp. include tentoxin (TEN) and dihydrotentoxin (DHTEN), both with phytotoxic activities; altenuisol (ALS), reported to have toxic effects in mammalian cells *in vitro* and altenusin (ALN), with antibacterial, antifungal and antiparasitic activities (Cota et al., 2008; Lou et al., 2013; Nemecek et al., 2012). In addition, some small-spored *Alternaria* species are able to produce host-specific toxins (HSTs), which are toxic to susceptible plants, such as AM-toxin in apple; AAL-toxins in tomato; AF-toxin in strawberry; and AK-toxin in Japanese pear (Lou et al., 2013; Tsuge et al., 2013).

Most Argentinean *Alternaria* isolates from food belong to small-spored *Alternaria* groups, with high metabolomic potential, implying a consequent toxicological risk for consumers. Considering Argentina is a major exporter of agricultural products worldwide, it is essential to thoroughly understand the physiological behaviour of this pathogen in a food safety context. Moreover, the European Food Safety Authority recently published a report on *Alternaria* toxins, considering their toxicokinetics, natural occurrence, and influence of food and feed processing in order to discuss the establishment of guideline limits (Alexander et al., 2011).

The aim of this work was to characterize small-spored *Alternaria* spp. isolates obtained from edible parts of crops of agronomical importance in Argentina using a polyphasic approach, involving metabolomic and phylogenetic analyses based on molecular and morphological characters. These data are crucial to the development of control strategies related to pest management and accumulation of toxic metabolites in foods.

2. Materials and methods

2.1. Fungal strains

Forty-five *Alternaria* strains were isolated from four Argentinean crops during the period 2010–2013. Fourteen strains were obtained from symptomatic tomato fruit (*Lycopersicon esculentum*, "T"), 14 from symptomatic red pepper (*Capsicum annuum*, "P"), and two from symptomless blueberries of the O'Neal variety (*Vaccinium angustifolium*; "B"), all collected from organic producers in La Plata, Buenos Aires province. The remaining 15 isolates were obtained from symptomless wheat grains (*Triticum aestivum*, "W") cultivated in the Argentinean wheat production area known as V-South (La Pampa and South West Buenos Aires provinces). Isolation was performed in DCMA (Dichloran Cloramphenicol Malt Agar) plates after 5–7 days of incubation at 25 °C. *Alternaria* isolates were kept in V8 agar plates. Three representative strains were used in this study: *A. alternata* EGS 34016, *A. tenuissima* EGS 34015, and *A. arborescens* EGS 39128, for comparison purposes.

2.2. Morphological characterization

Traditional morphological classification of *Alternaria* strains was performed according to Simmons (2007). Briefly, isolates were inoculated in Potato Carrot Agar (PCA) plates and incubated for seven days at 25 °C under an alternating light cycle consisting of 8 h of cool-white fluorescent daylight and 16 h of darkness. The three-dimensional sporulation pattern of the cultures was examined directly on the plates using a stereo-microscope (× 80). Further examination (length of primary and secondary conidiophores, secondary conidiophores shape, conidial shapes, sizes, colours and ornamentation) was done at × 400 magnification on slide preparations made by collecting spores from colony surface with transparent adhesive tape mounted in lactic acid. Colony characteristics (*e.g.* color, texture and diameter) were recorded from plates after the incubation period. The complete list of morphological characters registered is shown in Table S1.

2.3. Secondary metabolite production

For metabolite profiling, Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES, Samson et al., 2010) plates were inoculated at three points and incubated 14 days at 25 °C in darkness. Extraction was carried out on a micro-scale using a modified method for *Alternaria* metabolites (Andersen et al., 2005). Three agar plugs were cut from the centre of the three colonies and the nine plugs were placed in a 4 mL vial. Then 1 mL ethyl acetate containing 1% formic acid (vol/vol) was added to each vial and the plugs were extracted by sonication for 30 min. The extract was transferred to a clean 2 mL vial, evaporated to dryness in a gentle stream of N_2 and re-dissolved in 400 μL methanol. The methanol extract was filtered through a 0.45 μm PTFE filter into a clean 2 mL vial and kept at − 18 °C prior to HPLC analysis.

Analyses were performed using ultra-high-performance liquid chromatography (UHPLC) with a diode array detector (DAD) and high-resolution (HR) maXis HD QTOF mass spectrometer (MS) (Bruker Daltonics, Bremen, Germany) equipped with an ESI source and connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, USA) equipped with a Kinetex 2.6-μm C_{18}, 100 mm × 2.1 mm column (Phenomenex, Torrance, CA). A linear water-acetonitrile gradient was used (buffered with 20 mM formic acid) starting from 10% (vol/vol) acetonitrile and increased to 100% in 10 min, maintained for 3 min before returning to the starting conditions. MS was performed in ESI^+ and ESI^- in the scan range *m/z* 100–1000, with a mass accuracy < 1.5 ppm. UV/VIS spectra were collected at wavelengths from 200 to 700 nm. Data processing was performed using DataAnalysis 4.2 and TargetAnalysis 1.3 (Bruker Daltonics) by the aggressive dereplication approach (Klitgaard et al., 2014). For this study, a database of 678 known and putative compounds from *Alternaria*, *Lewia*, *Ulocladium* and other related genera were used, tentatively identifying them based on accurate mass (deviation < 1.5 ppm) and isotopic pattern (isotope fit < 50) and UV/Vis data (Klitgaard et al., 2014). For compounds not available as reference standards MS/HRMS were further conducted to match fragmentations with the molecular structure (Andersen et al., 2015; Nielsen and Larsen, 2015). All major peaks (observed in the BP chromatograms) not tentatively identified by the approach were added to the search list as unknown compounds for mapping. All major peaks (known and unknown) for the 48 extracts, corresponding to 45 wild