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Secondary metabolite profiles of small-spored *Alternaria* support the new phylogenetic organization of the genus

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ABSTRACT

The group of the small-spored *Alternaria* species is particularly relevant in foods due to its high frequency and wide distribution in different crops. These species are responsible for the accumulation of mycotoxins and bioactive secondary metabolites in food. The taxonomy of the genus has been recently revised with particular attention on them; several morphospecies within this group cannot be segregated by phylogenetic methods, and the most recent classifications proposed to elevate several phylogenetic species-groups to the taxonomic status of section. The purpose of the present study was to compare the new taxonomic revisions in *Alternaria* with secondary metabolite profiles with special focus on sections *Alternaria* and *Infectoriae* and food safety. A total of 360 small-spored *Alternaria* isolates from Argentinean food crops (tomato fruit, pepper fruit, blueberry, apple, wheat grain, walnut, pear, and plum) was morphologically identified to species-group according to Simmons (2007), and their secondary metabolite profile was determined. The isolates belonged to *A. infectoria* sp.-grp. (19), *A. tenuissima* sp.-grp. (262), *A. arborescens* sp.-grp. (40), and *A. alternata* sp.-grp. (7); 32 isolates, presenting characteristics overlapping between the last three groups, were classified as *Alternaria* sp. A high chemical diversity was observed; 78 different metabolites were detected, 31 of them of known chemical structure. The isolates from *A. infectoria* sp.-grp. (=*Alternaria* section *Infectoriae*) presented a specific secondary metabolite profile, different from the other species-groups. Infectopyrones, novae-zelandins and phomapyrones were the most frequent metabolites produced by section *Infectoriae*. Altertoxin-I and alterperylenol were the only compounds that these isolates produced in common with members of section *Alternaria*. None of the well-known *Alternaria* toxins, considered relevant in foods, namely alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), tentoxin (TEN) or altenuene (ALT), were produced by isolates of this section. On the other hand, strains from section *Alternaria* (*A. tenuissima*, *A. arborescens*, and *A. alternata* sp.-grps.) shared a common metabolite profile, indistinguishable from each other. AOH, AME, ALT, TEN, and TeA were the most frequently mycotoxins produced, together with pyrenochaetic acid A and altechromone A. *Alternaria* section *Alternaria* represents a substantial risk in food, since their members in all types of crops are able to produce the toxic metabolites.

1. Introduction

The genus *Alternaria* includes numerous species, widely distributed in the environment, which occur in a range of different habitats. Many of them contaminate crop plants in the field, causing pre-harvest diseases, and are responsible for important economic losses due to yield reduction. Others are saprophytes and cause postharvest spoilage of fruit and grain (Patriarca, 2016). The group of the small-spored *Alternaria* is particularly relevant in foods due to its high frequency and wide distribution in different crops. Its presence is commonly associated with the accumulation of a variety of secondary metabolites, many of which are either mycotoxins or phytotoxins. Others have shown biological activity in different systems and may play important roles in plant pathology and food safety (Andersen et al., 2015). Several species in these groups are responsible for the production of the well-known *Alternaria* mycotoxins, alternariol (AOH), alternariol

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monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), tentoxin (TEN) and altertoxins (ATXs).

The taxonomy of the genus has undergone numerous revisions, with especial attention on the small-spored species closely related to *A. alternata*, which cannot be easily segregated. The traditional methods for identification of *Alternaria* species are primarily based on morphological characteristics of the reproductive structures. The most comprehensive attempt to organize taxa in the genus, based on morphological features, has been made by Emory G. Simmons, who provided a substantial contribution to *Alternaria* taxonomy through several publications. Simmons described more than 270 *Alternaria* morpho-species and introduced the concept of "species-group", a complex of species typified by representative strains, which helps to organize the complexity of the genus (Simmons, 1992, 2007; Simmons and Roberts, 1993). However, the plasticity of morphological features and overlapping of characteristics among closely related species makes this identification method exceedingly laborious and is often restricted to experts in the field. Moreover, the incorrect use of morphological taxonomic keys, or the confusion due to the "morphological plasticity" of the strains, has led to several misidentifications and the consequent association of toxin production to the wrong species.

Several molecular studies have intended to establish consensus with morphological-based species. Sequencing of "house-hold" genes, such as ITS, mtSSU and mtLSSU, which have been successfully applied in other fungal genera to support morphological classifications, did not show enough variability to become a useful segregation tool in *Alternaria*. Sequences from functional genes (β-tubulin, translation elongation factor α, calmodulin, actin, *etc.*) have provided little resolution among the small-spored *Alternaria* species as well. More recently, a series of studies, based on morphological and molecular data of nuclear protein-coding loci, have proposed significant changes in the taxonomic organization of the genus (Lawrence et al., 2013, 2014; Woudenberg et al., 2013, 2015). Due to the inability to resolve the divergence of small-spored species-groups, it was proposed to elevate several phylogenetic *Alternaria* species-groups to the taxonomic status of section. With the addition of other phylogenetic groups, the genus was reorganized in 27 sections, each with a type specimen (Lawrence et al., 2016). In this new classification, the common food-borne, small-spored *Alternaria* were placed in section *Alternaria*, which comprises *A. alternata*, *A. arborescens*, *A. tenuissima*, among others, and in section *Infectoriae*, which includes *A. infectoria* and related species.

The prevalence of the small-spored *Alternaria* species-groups in a variety of fruits and cereals has been determined worldwide. In Argentina, their presence has been reported in tomato, bell peppers, wheat, blueberries, peaches, apples, grapes, sorghum, rice, soybean seeds and citrus fruits (Broggi et al., 2007; da Cruz Cabral et al., 2017; Greco et al., 2012; Patriarca et al., 2007; Peres et al., 2003; Pose et al., 2004; Pose et al., 2010; Robiglio and Lopez, 1995; Vargas Trinidad et al., 2015). Moreover, the natural occurrence of *Alternaria* toxins has been detected in tomato puree (Terminiello et al., 2006), pepper fruits (da Cruz Cabral et al., 2016), wheat grains (Azcarate et al., 2008), sunflower seeds (Chulze et al., 1995), soybean (Oviedo et al., 2012), fruit juices and wine (Broggi et al., 2013), and in relatively high levels in comparison to those reported elsewhere.

Chemotaxonomy has become an alternative method for identification and classification of fungi. With the advancement of analytical techniques, and availability of high resolution chromatographic and mass spectrometric instruments, the potential of secondary metabolite profiling as a taxonomic tool has increased. It has been used successfully in other mycotoxigenic genera such as *Aspergillus* and *Penicillium* (Frisvad et al., 2008).

On *Alternaria*, chemotaxonomic studies have been made first at species-group level (Andersen et al., 2001, 2002; Andersen and Thrane, 1996) and later at species level (Andersen et al., 2005, 2008), proving only successful in discriminating between the large-spored species. Secondary metabolite profiles of the small-spored *Alternaria* species-groups (*e.g.* the *A. arborescens* and the *A. tenuissima* species-groups), which are common contaminant of food crops seem to have the same plasticity as their morphology. The aim of the present work is to compare the new taxonomic revisions in *Alternaria* with secondary metabolite profiles with special focus on sections *Alternaria* and *Infectoriae* and food safety.

2. Materials and methods

2.1. Fungal strains

A total of 360 fungal isolates belonging to the small-spored *Alternaria* group from Argentinean crops, were analysed in this study; 107 were isolated from black-mould affected tomato fruits (*Solanum lycopersicum*, varieties "Redondo", "Cherry" and "Platense"); 64 from symptomatic bell pepper fruit (black spot) (*Capsicum annuum*); 44 from symptomless blueberries (*Vaccinium angustifolium*; "O'Neal" variety); 45 from symptomatic apples, with either external black spots or mouldy core (*Malus domestica*, "Red Delicious" variety); 94 from symptomless wheat grain (*Triticum aestivum*); 6 from symptomless walnuts (*Juglans regia*, "Criolla" variety); 1 from symptomatic pear (external black spot) (*Pyrus communis* "Williams" variety) and 1 from symptomatic plum (external black spot) (*Prunus salicina*, "Royal Diamond" variety).

Both tomato and pepper fruits were obtained directly from organic producers in La Plata, Buenos Aires province from 2013 to 2014; fruits with typical *Alternaria* lesions (black spots) were selected during sampling. Blueberries were collected from farms in 2015 in Buenos Aires province, apples and pears were provided by producers from Rio Negro province from the 2016 harvest, wheat grains were obtained from production areas known as II-South, IV and V-South (La Pampa and Buenos Aires provinces) in the 2012–2013 harvest, walnut was collected in Catamarca province in 2014, and plum was obtained from local markets in Buenos Aires city in 2015.

Portions of contaminated tissue from tomato and pepper fruits, pear and plum were directly transferred onto Dichloran Chloramphenicol Malt Agar (DCMA) plates (Pitt and Hocking, 2009). Whole blueberries were directly plated in the same culture media, as well as halves shelled walnuts. Portions of external contaminated tissue from apples, as well as internal contamination (mouldy core) were placed in DCMA plates. Wheat grains were plated both directly and after surface decontamination (0.4% NaClO for 2 min, followed by rinse with sterile water). *Alternaria* isolation was performed after 5–7 days of incubation at 25 °C and isolates were kept in V8 agar plates for morphological characterization.

2.2. Morphological identification

Morphological identification of *Alternaria* strains was performed according to Simmons (2007). Briefly, isolates were transferred to Potato Carrot Agar (PCA) plates (Samson et al., 2010) at three points, equidistant from the centre, and incubated for seven days at 23 °C under an alternating light cycle consisting of 8 h of cool-white fluorescent daylight and 16 h darkness. On the fifth incubation day, a rectangular block of agar and mycelium of about 0.5 × 2 cm was cut and removed from the edge of one of the colonies at right angles to the radius, to stimulate sporulation and facilitate the observation of sporulation patterns. After discarding the agar block, the plates were further incubated until day 7, when observations were performed. The morphological macroscopic characteristics of the strains (colony diameter, colour, texture, growth and sporulation rings) were observed and the three-dimensional sporulation pattern of the cultures was examined directly on the plates, and in the lateral cut performed in one of the colonies, using a stereo-microscope (100×). Further examination (branching origin and type, length of primary and secondary conidiophores, conidial shapes, sizes, colours, ornamentation, *etc.*) was done at 1000× magnification on slide preparations made by collecting spores from colony surface with transparent adhesive tape mounted in lactic acid. Additionally, all 360 strains were inoculated on Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES) (Samson et al., 2010), without chlortetracycline. The DRYES plates were packed in perforated plastic bags and incubated 7 days in the dark at 25 °C for morphological observation and 7 days more for metabolite extraction (14 days in total). Four representative strains belonging to "small-spored" *Alternaria* species-groups were used for comparison, *A. alternata* EGS 34.016; *A. arborescens* EGS 39.018; *A. infectoria* EGS 43.162; and *A. tenuissima* BA 853. The EGS strains belonged to Emmory G. Simmons collection (Mycological Services, Crawfordsville, IN) and BA strain to the IBT collection at the Department of Biotechnology and Biomedicine, DTU, Denmark. A temporary working collection of the strains was made from agar blocks with conidia from PCA placed in cryo-tubes and kept at 5 °C.

2.3. Secondary metabolite production

The metabolite profiling was done on the 14-day-old DRYES cultures using a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al., 2015). Three agar plugs (6 mm diameter) were cut from the centre of the colonies and placed in a 2 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 60 min. The extract was transferred to a clean vial, evaporated to dryness in a gentle stream of N_2 and re-dissolved in 400 µL methanol HPLC grade. The methanol extract was filtered through a 0.45 µm PTFE filter into a clean 2 mL vial and kept at −18 °C until analysis.

2.4. UHPLC–HRMS analyses

Ultra High-Performance Liquid Chromatography-Diode Array Detection-High Resolution Mass Spectrometry (UHPLC-DAD-HRMS) analysis was done on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA). Sub-samples of 1 µL samples were injected on an Agilent Poroshell 120 phenyl-hexyl column (2.7 µm, 2.1 × 150 mm) kept at 60 °C and separated using a linear gradient at 0.35 mL/min of water-acetonitrile, both buffered with 20 mM formic acid. The gradient started at 10% acetonitrile and was increased to 100% in 15 min, maintained for 2 min, then returned to 10% in 0.1 min and kept there for 3 min before the following run. UV/VIS spectra were collected at wavelengths from 200 to 700 nm.

MS detection was made on an Agilent 6545 QTOF MS system equipped with Agilent Dual Jet Stream electrospray ion source. Mass spectra were recorded in both ESI^+ and ESI^- (separate runs) in the range *m/z* 85–1700, with an acquisition rate of 10 spectra/s. Hexakis (2,2,3,3-tetrafluoropropoxy) phosphazene (Apollo Scientific Ltd.) was used as lock mass. Automated data-dependent acquisition MS/HRMS analysis was performed for ions detected in the full scan above 50,000, applying fixed CID energies of 10, 20, and 40 eV with a maximum of three selected precursor ions per cycle, as described in detail in Kildgaard et al. (2014).

HRMS data were then analysed in three separate ways (Nielsen and Larsen, 2015). First, full-scan HRMS data were analysed by aggressive dereplication for the $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M + H-H_2O]^+$, $[M-H]^-$, and $[M + HCOO]^-$ adducts of all known elemental compositions described from *Alternaria* and related genera, verifying the elemental composition by mass accuracy, isotopic ratios, and isotopic spacing. UV–Vis data were used for identity confirmation. Then, the MS/HRMS spectra matched against the in-house library (approx. 2000 compounds) using the Agilent MassHunter PCDL manager. Finally, all peaks identified were mapped in all samples using Agilent MassHunter Quantitative Analysis software, and the most intense ion (± 5 ppm) was used as relative quantification ion. Reference standards of alternariol, alternariol monomethyl ether, tentoxin, altertoxin-II, altenusin, and tenuazonic acid were acquired from Sigma-Aldrich (Steinheim, Germany), while altertoxin-I and 4-hydroxy alternariol were acquired from Cayman (Ann Arbor, MI, USA). Alterperylenol, pyrenochaetic acid A, altechromone A, tetramic acids, alterperylenol, 3-hydroxyalternariol 5-*O*-methyl ether, altenuisol, altersetin, dihydrotentoxin, alternarienonic acid, altertoxin-III, dehydrocurvularin, and dehydroaltenusin were obtained as compound library with microgram quantities in 50–95% purity from AnalytiCon Discovery GmbH (Potsdam, Germany). Infectopyrones and the novae-zelandines were available form earlier studies in our lab (Christensen et al., 2005; Larsen et al., 2003).

2.5. Data treatment

All major peaks for the 360 extracts from Argentinean isolates and the four reference strains were subsequently ordered in a binary data matrix in Microsoft Excel. Statistical analysis was performed using the software IBM SPSS Statistics v.24, through a multiple correspondence analysis (MCA) considering 31 metabolites as principal variables and both sp.-grp. and substrate as supplementary variables.

3. Results

3.1. Morphological characterization

The morphological identification of the strains to species-group level (according to Simmons, 2007) is summarised in Table 1. The main micro- and macroscopic characteristics corresponding to each sp.-grp. are shown in Figs. 1 and 2.

Nineteen isolates, all of them from wheat, were classified as *A. infectoria* sp.-grp. The three-dimensional pattern of sporulation observed for these strains was highly branched, characterized for short primary conidiophores, with new conidial chains emerging at different lengths along the conidiophore length (Fig. 1a and b). Conidia were ob-pyriform to ob-clavate, light grey, and smooth-walled. Secondary conidiophores were conspicuous, originating from the conidial apex, of variable length, and bearing several conidiogenous loci, from which new chains were generated by geniculation. Colonies in PCA were light grey, and the lightest in comparison with the rest of the sp.-grps. The appearance of the colonies in DRYES was a very distinctive characteristic of these isolates, being light grey to white, with cottony texture (Fig. 2a and b).

The vast majority of the isolates (262 out of 360) presented a sporulation pattern correspondent to the *A. tenuissima* sp.-grp. (Table 1). This group was characterized by a sporulation pattern

Table 1
Alternaria species-groups isolated from food crops in Argentina as identified through morphological identification.

Species-group	Tomato	Wheat	Pepper	Apple	Blueberry	Walnut	Plum	Pear	Total
A. tenuissima	65	64	48	38	42	4	–	1	262
A. arborescens	29	4	3	3	–	–	1	–	40
A. infectoria	–	19	–	–	–	–	–	–	19
A. alternata	1	1	4	–	1	–	–	–	7
Alternaria sp.	11	6	9	3	1	2	–	–	32
Total	106	94	64	44	44	6	1	1	360

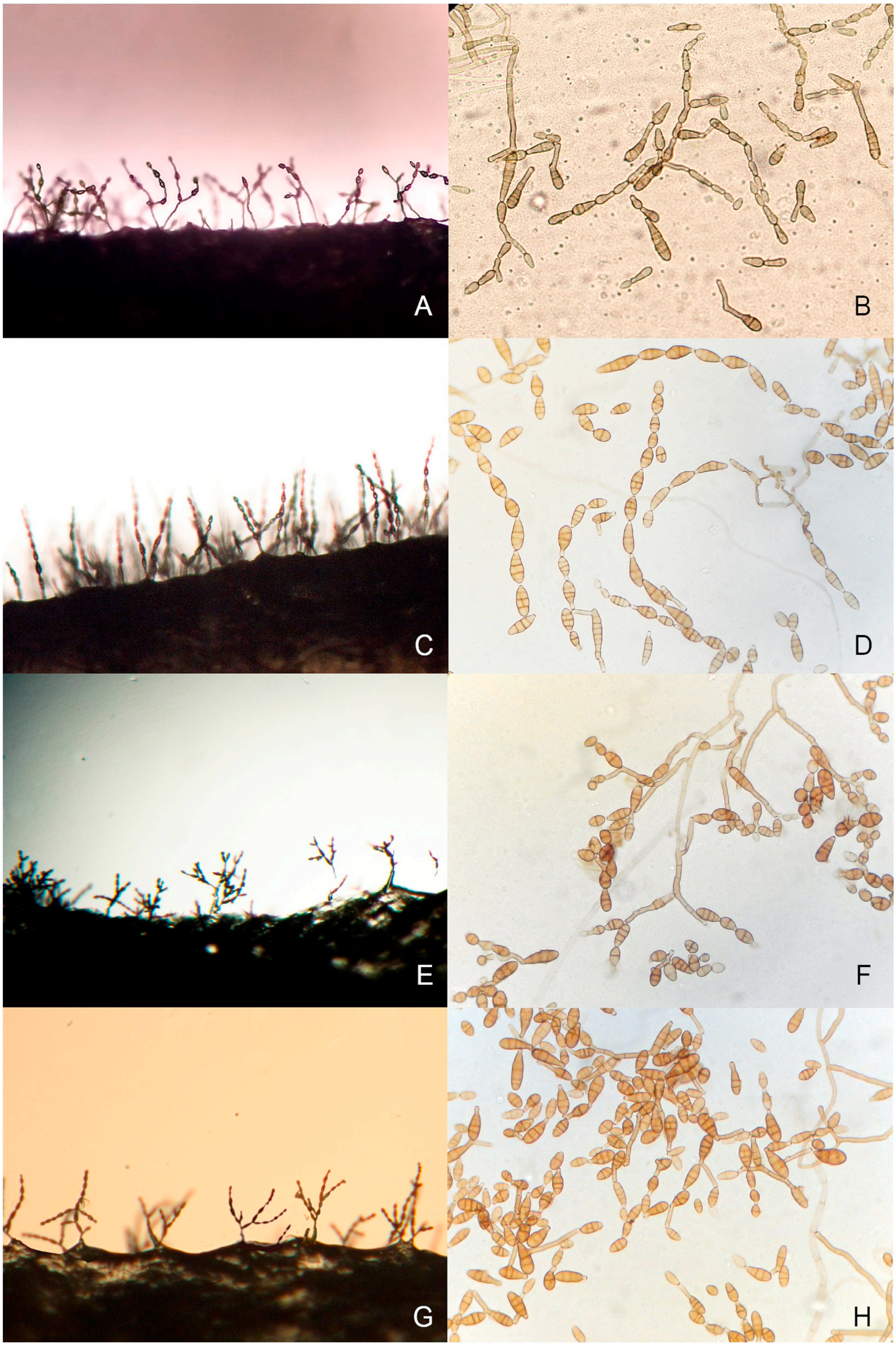

Fig. 1. Microscopic characteristics of *Alternaria* species-groups in a lateral cut at 100 × (A, C, E, G) and at 1000 × in slide preparations with lactic acid (B, D, F, H). A–B: *A. infectoria* sp.-grp.; C–D: *tenuissima* sp.-grp.; E–F: *A. arborescens* sp.-grp.; G–H: *A. alternata* sp.-grp.

consisting of long unbranched chains of conidia (from 7 up to 15), borne from primary conidiophores of varying length, generally short (Fig. 1c and d). Secondary conidiophores were infrequent, but when present, they mainly originated from the conidial body, generating short perpendicular branches. Most conidia were ellipsoidal with a greyish to light brown or tan colour, and smooth walls. Colonies on PCA were grey to greyish brown, while on DRYES these strains exhibited cottony light green to greyish green colonies (Fig. 2c and d).

Forty out of 360 isolates were identified as *A. arborescens* sp.-grp. Their distinctive characteristic was the presence of long primary

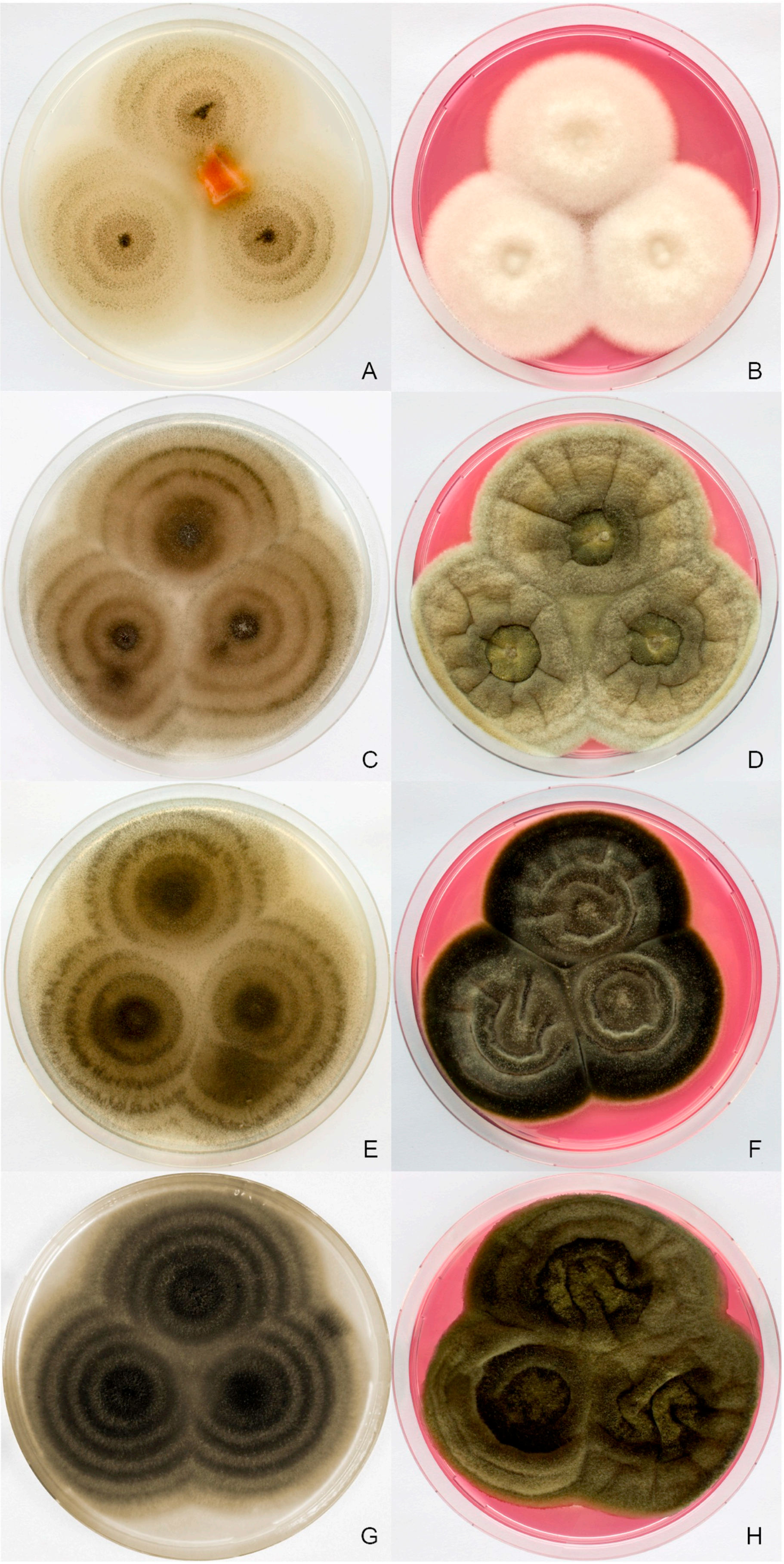

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Fig. 2. Macroscopic characteristics of *Alternaria* species-groups in PCA (A, C, E, G) and DRYES (B, D, F, H) plates. A–B: *A. infectoria* sp.-grp.; C–D: *A. tenuissima* sp.-grp.; E–F: *A. arborescens* sp.-grp.; G–H: *A. alternata* sp.-grp. Please note that the orange spot in this figure. A corresponds to a piece of carrot from the medium.

conidiophores with a terminal cluster of branching conidial chains (Fig. 1e and f). Secondary conidiophores originating mostly from conidial apex were regularly observed. Conidia were darker than the formerly described isolates, most commonly ovoid and with rough walls. Colonies on PCA were dark brown to black, with 4 to 6 well-defined concentric rings of growth and sporulation. DRYES colonies were dark green and sulcate, with velutinous texture (Fig. 2e and f).

Seven isolates were identified as *A. alternata* sp.-grp. They presented short primary conidiophores with multi-branched chains of 4–10 conidia, frequently with lateral secondary conidiophores (Fig. 1g and h). Conidia shapes showed a wide diversity, ranging from ellipsoidal to ovoid; the former were grey to tan and presented smooth wall, while the later were dark brown to black with rough wall. Colonies in PCA were dark, usually with well-defined areas of growth and sporulation, and of granulated aspect, while colonies in DRYES showed characteristics comparable both to *A. tenuissima* and *A. arborescens* sp.-grp. (Fig. 2g and h).

The remaining 32 isolates exhibited intermediate characteristics between *A. tenuissima*, *A. arborescens* and *A. alternata* sp.-grp. and were referred to as *Alternaria* sp.

3.2. Secondary metabolite profiles

The *Alternaria* spp. isolates from Argentinean crops showed high chemical diversity. A total of 78 different metabolites were detected in the *Alternaria* spp. extracts. The statistical mode for metabolite production was 19 metabolites, and the maximum number of compounds produced by a strain was 33. The chemical structure of 31 out of 78 compounds was unequivocally confirmed by MS/MS and UV–Vis data, and identical retention index to standards when available. The absence of important compounds like alternariols, altertoxins, and tenuazonic acid were manually verified in the MassHunter Quant Software. Limit of detection of the strongly ionizing tenuazonic acid and the related tetramic was 5–10 ng/cm^2, while the less strongly ionizing alternariols and altertoxins were in the 30–60 ng/cm^2 range.

The 31 identified metabolites were selected for multiple correspondence analysis (MCA), and the result of the analysis is given in Fig. 3. The percentage of variance explained by the two dimensions was low (39.2%). All isolates belonging to *A. infectoria* sp.-grp. (= section *Infectoriae*) were clearly segregated from the rest of the strains. On the other hand, strains belonging to *A. tenuissima*, *A. arborescens*, and *A. alternata* sp.-grps. (= section *Alternaria*), and the isolates classified as *Alternaria* sp. were grouped in a cloud without evident distinction in relation to the species-groups or the food of origin.

Isolates belonging to *A. infectoria* sp.-grp. produced 9 identified metabolites that were not synthesized by any of the isolates belonging to the other species-groups. These were infectopyrones, novae-zelandins and phomapyrones (syn. phomenins). Table 2 shows their frequency of production by the isolates belonging to this sp.-grp.

The metabolites produced by isolates belonging to *A. tenuissima*, *A. arborescens*, and *A. alternata* sp.-grps., as well as the strains classified as *Alternaria* sp. are shown in Table 3. The metabolite profiles of these morphological sp.-grps. were similar, and no consistent differences could be established among them, neither specific species-groups metabolites could be identified. Most of the compounds that are considered *Alternaria* mycotoxins were produced by isolates from these groups. Both alternariol and its monomethyl ether were the most frequently produced by strains from these groups, 83% of the strains were able to synthesise these metabolites. Altenuene (72%), tentoxin (66%), tenuazonic acid (62%) and isopropyl tetramic acid (a TeA derivative, 55%) were the following mycotoxins in frequency in terms of number of producers. Other metabolites produced in high frequencies were pyrenochaetic acid A (78%) and altechromone A (72%).

In addition, two compounds, altertoxin-I and alterperylenol, were detected in both the *A. infectoria* sp.-grp. (50% and 25%, respectively) and in the other small-spored *Alternaria* sp.-grps. (47% and 39%, respectively). However, no other metabolite (known or of unknown structure) was found in common between the two groups.

4. Discussion

The *Alternaria* strains isolated from different Argentinean crops, including fruits and cereals, were classified in only four morphological species-groups according to Simmons (2007): *A. tenuissima*, *A. arborescens*, *A. alternata*, and *A. infectoria*. The isolates from *A. infectoria* sp.-grp. were clearly distinguished morphologically from the rest; particularly, the colour of the colonies in DRYES (greyish white to white) was a diagnostic characteristic (Andersen and Thrane, 1996; Andersen et al., 2002; Dugan and Peever, 2002; Serdani et al., 2002), together with the presence of secondary conidiophores longer than those from the other sp.-grps. These features have also been reported as distinctive of *A. infectoria* sp.-grp. in recent studies (Gannibal and Lawrence, 2016; Lawrence et al., 2014). The morphological traits of the other three sp.-grps. were very similar, making their identification difficult, due to overlapping of characteristics, and morphological plasticity. Among this group of isolates, the *A. tenuissima* sp.-grp. was by far the most abundant in all food crops analysed, followed by *A. arborescens* in tomato fruits. *A. alternata* was the least abundant in all type of crops. As previously stated by Andersen et al. (2015), *A. alternata sensu stricto* is a less predominant species and several strains that have been labelled as *A. alternata* in the literature are in fact members of *A. tenuissima* or *A. arborescens* sp.-grp.

The isolates belonging to the *A. infectoria* sp.-grp., *i.e. Alternaria* section *Infectoriae*, presented a specific secondary metabolite profile, clearly different from the rest of the groups belonging to section *Alternaria*. None of the well-known *Alternaria* toxins, that are considered relevant in foods, namely AOH, AME, TeA, TEN or ALT (EFSA, 2011, 2016), were produced by pure culture isolates of this section (white colonies without any sectors of green on DRYES), which is in agreement with several reports and papers (Andersen and Thrane, 1996; Webley et al., 1997; Andersen et al., 2002; Serdani et al., 2002; Andersen et al., 2009). The main metabolites of *Alternaria* section *Infectoriae*, based on the frequency of producing strains, would be the infectopyrones, phomapyrones and novae-zelandins, as well as perylene quinone derivatives, such as altertoxin-I and alterperylenol. Our findings corroborate the results by Zwickel et al. (2018), except that none of our *A. infectoria* sp.-grp. (= section *Infectoriae*) strains produced alternariols, tenuazonic acid, or any of the above mentioned toxins.

On the other hand, the metabolite profiles of isolates from *A. tenuissima*, *A. arborescens*, *A. alternata* and morphologically related strains, belonging to *Alternaria* section *Alternaria*, were chemically indistinguishable from each other. The importance of this section lies in the wide range of toxic compounds, mycotoxins and phytotoxins, that can be synthesized by most of its members. The alternariols (AOH and AME) were the most frequent metabolites produced *in vitro* by the strains of this section (83% of producers). Other metabolites from the same biosynthetic pathway, such as altenuene, or 3-hydroxyalternariol 5-*O*-methyl ether, were also detected in high frequency. TeA, another major *Alternaria* toxin, and the only *Alternaria* metabolite for which a limit in food has been established at regional level in Bavaria, Germany (Rychlik et al., 2016), could be produced *in vitro* by more than 60% of the members of the section, together with some derivative compounds, like isopropyl tetramic acid. The high number of strains capable of synthesising these mycotoxins, as well as tentoxin, altertoxins, and

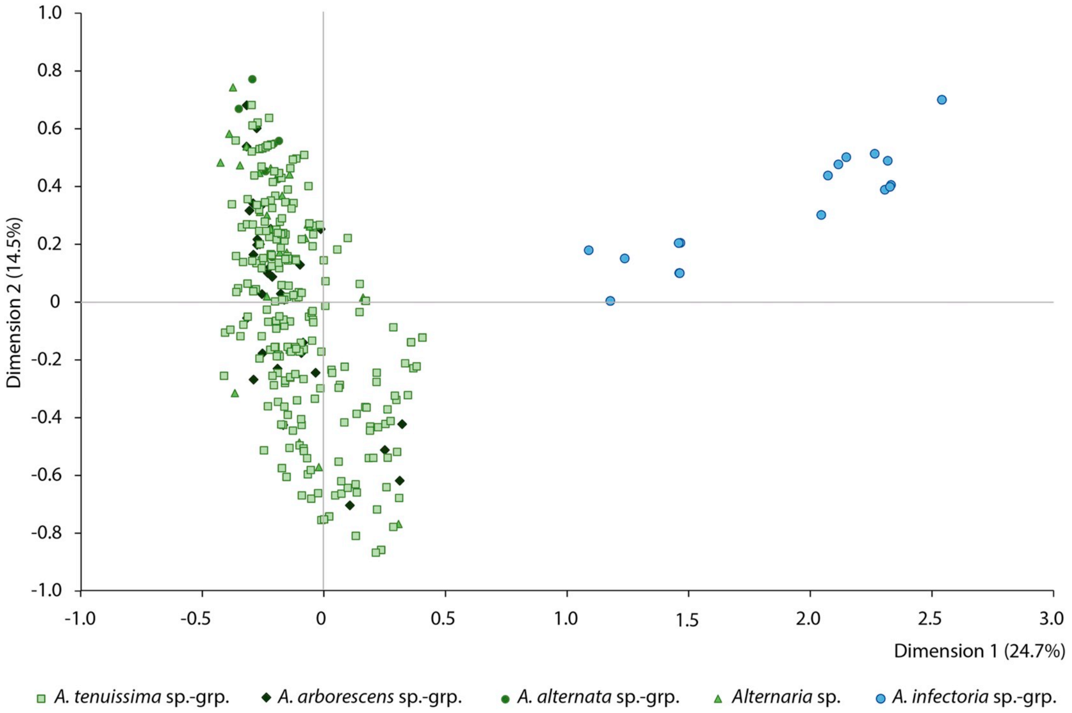

Fig. 3. Loading plot in 2 dimensions based on a multiple correspondence analysis (MCA) of a matrix consisting of 31 metabolites produced by 360 food-borne *Alternaria* strains and 4 representative strains corresponding to each species-group. The percentage of variance explained by each dimension is in brackets.

Table 2

Secondary metabolites produced by 19 food-borne Argentinean isolates from *A. infectoria* sp.-grp. and one reference strain, *A. infectoria* EGS 43.162. Metabolites in bold were produced exclusively by *Alternaria* section *Infectoriae*.

Metabolite	Number of producers (n = 20)
Infectopyrone[a]	**17**
Novae-zelandin B	**17**
Novae-zelandin A	**16**
Phomapyrone A (=phomenin A)	**14**
Phomapyrone B (=phomenin B)	**13**
Phomapyrone D (=phomenin D)	**11**
4Z-Infectopyrone	**10**
Phomapyrone E or G	**6**
Phomapyrone F	**3**
Altertoxin-I[a]	10
Alterperylenol	5

[a] *Alternaria* metabolites whose natural occurrence has been detected in food.

other biologically active compounds, makes this section the riskiest in food. Moreover, several secondary metabolites of this section have been detected as natural contaminants of different crops and food products (marked with * in Table 3) (Hickert et al., 2016; López et al., 2016; Walravens et al., 2014; Zwickel et al., 2016).

Some of the most frequently produced metabolites from members of sect. *Alternaria* are not commonly associated with the genus *Alternaria*. Pyrenochaetic acid, a secondary metabolite first isolated from *Pyrenochaeta terrestris* with phytotoxic effects (Sato et al., 1981), was the third most produced metabolite in section *Alternaria*, followed by altechromone A, a metabolite produced by *A. brassicicola*, which showed antibacterial activity, and was described as a plant growth promotor (Gu, 2009; Königs et al., 2010). Altenuisol, another compound not mentioned among the main *Alternaria* metabolites, and whose toxicity in mammalian cells has been reported *in vitro* (Nemecek et al., 2012; Pero et al., 1973) was frequently produced. Due to the high number of food-borne isolates with the capability of synthesising these compounds, more data on their toxicological effects are required to decide if their presence in food should be evaluated.

Table 3

Secondary metabolites produced by 344 food-borne *Alternaria* isolates; 341 from Argentinean crops (262 *A. tenuissima* sp.-grp., 40 *A. arborescens* sp.-grp., 7 *A. alternata* sp.-grp., and 32 *Alternaria* sp.), and 3 reference strains (*A. tenuissima* BA 853, *A. arborescens* EGS 39.128 and *A. alternata* EGS 34.016). Metabolites in bold are considered mycotoxins (EFSA, 2016; Solfrizzo, 2017).

Metabolite	Number of producers (n = 344)
Alternariol monomethyl ether[a]	**286**
Alternariol[a]	**284**
Pyrenochaetic acid A	268
Altechromone A	249
Altenuene[a]	**248**
Tentoxin[a]	**226**
Tenuazonic acid[a]	**213**
Isopropyl tetramic acid	190
Altertoxin-I[a] **(=dihydroalterperylenol)**	**161**
Alterperylenol (=alteichin)	133
3-Hydroxyalternariol 5-*O*-methyl ether	132
Altertoxin-II (=stemphyltoxin-II)	**119**
Altenuisol[a]	117
Altersetin	117
Dihydrotentoxin	115
Altenusin	114
Alternarienonic acid	83
Altertoxin-III	**51**
Altechromone B	42
cis-Dehydrocurvularin	15
Dehydroaltenusin	12
Desmethylaltenusin	6

[a] *Alternaria* metabolites whose natural occurrence has been detected in food.

The health risk of the section *Infectoriae* remains difficult to be estimated yet, due to: a) the lack of data on their natural occurrence in food; b) scarce toxicological data. Infectopyrone is the only metabolite produced exclusively by this section which has been reported as natural contaminant in wheat from Denmark (Hertz et al., 2016) and Northern Italy (Blandino et al., 2017). There is no data on its toxicity in animal systems, but due to its structural relation with other known compounds (pyrenocines, α-pyrones), it has been suggested that infectopyrones as well as novae-zelandins could have phytotoxic activity and have been postulated as possible mycotoxins, especially the former ones, which showed cytotoxicity against murine cells (Christensen et al., 2005; Geiseler and Podlech, 2012; Ivanova et al., 2010; Larsen et al., 2003). Other metabolites from this section, the phomapyrones, showed toxicity against *Artemia salina* and insecticide activity, but results on their phytotoxicity are contradictory (Geiseler and Podlech, 2012; Pedras and Chumala, 2005; Pedras and Park, 2015). Since members of this section are frequently isolated from cereals, more toxicological studies on these metabolites are needed to establish the risk of their presence in cereal grains.

5. Conclusions

Based on the new proposed phylogenetic classification of the genus *Alternaria*, isolates contaminating Argentinean crops belong to two sections: *Alternaria* and *Infectoriae*. The profile of mycotoxins and other secondary metabolites of each section could clearly be distinguished from each other, constituting thus a useful tool to segregate isolates between those sections, and valuable information to establish the potential risk associated with the contamination of crops with members of either section. The results of this study indicate that *Alternaria* section *Alternaria* represents a substantial risk, since the members of this section are distributed in all types of crops and able to produce the main mycotoxins, such as alternariols, tentoxins and tenuazonic acids. The presence of any species or sp.-grp. from this section in food represents a similar potential risk, since their whole metabolite profile could be expressed, and it does not correlate with a particular morphological or phylogenetic species within the section. On the other hand, the health risk posed by *Alternaria* section *Infectoriae* still need to be further evaluated. However, their distribution in food is more limited since their presence seems to be only relevant in cereals crops; therefore, perylene quinone compounds, such as ATX-I and alterperylenol, must be taken into consideration in cereals, together with the main *Alternaria* toxins, due to the contribution to its accumulation by sect. *Infectoriae*.

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