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Natural occurrence of mycotoxins and toxigenic capacity of *Alternaria* strains from mouldy peppers

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ABSTRACT

Sweet pepper (*Capsicum annuum*) is an important crop cultivated worldwide, with Argentina being one of the major producers in South America. The fruit is susceptible to several fungal diseases, leading to severe economic losses for producers. In this study, *Alternaria* was found as the prevalent genus in mouldy peppers (50% fruits infected). Morphological identification revealed that all 64 *Alternaria* isolates belonged to small-spored species, most of them corresponding to *A. tenuissima, A. arborescens* and *A. alternata* species-groups. Their secondary metabolite profile was evaluated *in vitro*; alternariols were synthesized by most of the isolates (91% for alternariol and 92% for alternariol monomethyl ether). A high number of *Alternaria* spp. also produced tenuazonic acid (64%), altenuene (84%) and tentoxin (72%). In addition, damaged pepper fruits were analysed for the presence of tenuazonic acid and alternariols. A total 32 out of 48 spoiled pepper fruits were contaminated with at least one of these metabolites. Half of the samples were positive for tenuazonic acid (range 8–11,422 μg/kg), while alternariol and its monomethyl ether were less frequently detected (21 and 29%, respectively) and at lower concentrations. This is the first report on the natural occurrence of *Alternaria* mycotoxins in Argentinean sweet pepper, and highlights a consumer risk when mouldy fruits are used in industrialized products because these compounds are not destroyed by conventional heat treatments.

1. Introduction

Sweet pepper (*Capsicum annuum*) is an important crop cultivated worldwide, grown throughout tropical, sub-tropical and temperate regions (Edirisinghe et al., 2014). Argentina is one of the major producers in South America, with a yield of approximately 138,800 tons in 2013 (FAOSTAT, 2016). It is consumed as fresh raw vegetables, pickles, roasted or cooked, dry ground or as canned peppers and it has also application for cosmetic and pharmaceutical purposes (Javaid and Iqbal, 2014). It has a great nutritive value, especially in vitamins A, C and E, as well as high antioxidant content.

This vegetable is susceptible to several pre- and post-harvest diseases that cause severe economic losses for producers, including anthracnose, caused by *Colletotrichum capsici*, and infections by *Botrytis cinerea* and *Alternaria* spp. (Edirisinghe et al., 2014; Troncoso et al., 2005). *Alternaria* is a ubiquitous fungal genus frequently isolated from different plant crops, which contains plant pathogenic as well as saprophytic species. Small-spored *Alternaria* spp. have been reported as contaminant of several foods, such as cereals, fruits, and vegetables, causing both pre- and post-harvest decay (Andersen et al., 2015; Armitage et al., 2015; Logrieco et al., 2009). Some *Alternaria* species have been isolated from pepper, including *A. alternata*, *A. brassicae*, *A. solani*, *A. capsici*, *A. dauci*, *A. longipes*, *A. porri* and *A. tenuissima*; with *A. alternata* and *A. solani* being the most commonly reported species from this plant (Nasehi et al., 2014; Simmons, 2007). *Alternaria* causes a rot in pepper fruits that originates with water-soaked lesions and turns to velvety grey as spores are produced. Besides pathogenic species that colonize plants through flowers, saprophytic species can also infect fruits if their skin is injured by insects, chilling, mechanical damage, sunburn, or calcium deficiency (blossom-end rot) (Hochmuth and Hochmuth, 2009; Wall and Biles, 1993).

The development of *Alternaria* in numerous vegetables has been associated with the production of several toxic metabolites in the infected fruits (Andersen et al., 2006; Graf et al., 2012). Most *Alternaria* species are capable of synthesizing a great variety of secondary metabolites, including some compounds that are considered mycotoxins while others

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are still debated. The most common *Alternaria* toxins found in foodstuff are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), altertoxins I, II, III (ATX-I, -II, -III) and tentoxin (TEN) (Alexander et al., 2011; Ostry, 2008). These compounds are classified in different groups according to their structure: dibenzopyrone derivatives (AOH, AME, ALT), perylene derivatives (ATX-I, -II, -III), tetramic acid derivatives (TeA) and miscellaneous structures, such as the cyclic tetrapeptide tentoxin (TEN). TeA has been reported to be toxic to a wide spectrum of viruses, bacteria, fungi and plants, as well as being acutely toxic for several animal species such as mice, chicken and dogs (Andersen et al., 2015; Asam and Rychlik, 2013; Logrieco et al., 2003, 2009). It has been associated with human haematological disorders like Onyalai, a form of thrombocytopenia, which only seems to occur in central southern Africa (Steyn and Rabie, 1976). There are several reports on the mutagenicity and genotoxicity of AOH and AME in bacteria and mammalian cells *in vitro* but data on whole animal studies with a natural exposure route are insufficient in the literature. High doses of AME caused dysplastic changes on mice oesophagus (Yekeler et al., 2001), while both AOH and AME presence has also been associated with high levels of human oesophageal cancer in China (Andersen et al., 2015; Logrieco et al., 2009; Ostry, 2008). Altertoxins (ATXs) have been reported to be more potent mutagens and acutely toxic to mice than AOH and AME (Scott, 2004), and ALT showed cytotoxic activity on *Artemia salina* (Pavón et al., 2012). Other *Alternaria* metabolites are reported to be phytotoxins; TEN is a non-host-specific toxin, causing chlorosis in the seedlings of many plants (Lou et al., 2013).

There are currently no statutory or guideline limits set for *Alternaria* metabolites, although their relevance in food and feeds is still under discussion. The European Food Safety Authority report concluded that more information is needed on their toxicokinetics, natural occurrence, and influence of food and feed processing on the incidence of the toxins in these products to enable their risk assessment (Alexander et al., 2011).

No data are available on the literature about the frequency of *Alternaria* spp. or other pathogenic fungi in peppers cultivated in Argentina. Moreover, little is known about the toxigenic potential of *Alternaria* strains causing pepper fruit rot worldwide. Considering this, the objectives of this work were to evaluate *Alternaria* contamination of peppers, the toxigenic potential of the isolates and the natural occurrence of the main mycotoxins from this genus in the fruits.

2. Materials and methods

2.1. Samples

Sixty six mouldy pepper fruits (*Capsicum annum*) were obtained from two organic producers from Buenos Aires, Argentina. Fruits at two ripening stages (11 green and 37 red) with visible spoilage were selected in the field during harvest. Each fruit was collected in an individual plastic bag and kept at 5 °C for a maximum of 24 h until processed for isolation of fungi.

2.2. Isolation and identification of fungi

Isolation of fungal material from the lesions was done by transferring a representative portion of contaminated tissue to the centre of a Dichloran Chloramphenicol Malt Agar (DCMA) plate (Pitt and Hocking, 2009). The internal zone of each fruit was also sampled and plated in the same way as for injuries. DCMA plates were incubated for 5–7 days at 25 °C in the dark. Fungal growth was isolated onto Potato Dextrose Agar (PDA) plates (Pitt and Hocking, 2009). Fungal identification of the isolates at genus level was carried out by macro and microscopic examination of the colonies according to Pitt and Hocking (2009) and Samson et al. (2004). Isolates belonging to *Alternaria* genus were further morphologically identified to species-group level according to Simmons (2007). Briefly, isolates were inoculated in Potato Carrot Agar (PCA) and incubated for 7 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 patterns of the cultures were examined directly on the plates using a stereomicroscope. Further examination (length of primary and secondary conidiophores, secondary conidiophores shape, conidial shapes, sizes, colours and ornamentation) was done at ×400 magnification using slide preparations made with adhesive tape mounted in lactic acid. Macroscopic characteristics of isolates were observed on Dichloran Rose Bengal Yeast Extract Sucrose Agar (DRYES) (Samson et al., 2010) plates inoculated in three points and incubated 7 days at 25 °C in darkness. Malt extract and yeast extract were purchased from Oxoid Ltd. (Basingstoke, Hants; England).

2.3. Secondary metabolite profiles of Alternaria isolates

A total of 64 *Alternaria* isolates from pepper were subjected to metabolite profiling. DRYES plates were inoculated at three points and incubated 14 days at 25 °C in darkness. Extraction was carried out at micro-scale using a method described by Andersen et al. (2005) for *Alternaria* metabolites. 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 stream of N_2 and dissolved in 400 μL methanol. The methanol extract was filtered through a 0.45 μm 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 diode array detector (DAD) and high-resolution (HR) maXis 3G QTOF mass spectrometer (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 4.0 and TargetAnalysis 1.2 (Bruker Daltonics) by the aggressive dereplication approach (Klitgaard et al., 2014), using accurate masses, isotopic patterns, as well as retention time (when known). For this study, a database of 678 known and putative compounds from *Alternaria, Lewia,* and other related genera was used, tentatively identifying them based on accurate mass (deviation <1.5 ppm) and isotopic pattern (isotope fit <50).

2.4. Natural occurrence of Alternaria mycotoxins in target pepper samples

To evaluate the presence of the main *Alternaria* mycotoxins in the peppers, 48 of 66 damaged fruits were selected after fungal isolation and stored at −18 °C for a maximum of one week until analysis. Mycotoxin extraction was carried out following the method described by Terminiello et al. (2006) for tomato products. Briefly, each fruit was cut into small pieces and homogenized with 150 mL of methanol in a blender at 3000 rpm for 3 min. Then, 60 mL of 20% ammonium sulphate solution was added and the mixture was mechanically agitated at 300 rpm for 10 min. The mixture was filtered by gravity, and defatted with 40 mL of hexane. Then 50 mL of cold distilled water were added to the remaining aqueous phase, and extracted with 20 mL of chloroform twice to collect the fraction with neutral metabolites (AME and AOH). The aqueous phase was acidified to pH 2 with HCl 6 N and two extractions with 20 mL chloroform were made. The organic fractions were collected in a separating funnel, washed with 30 mL water, filtered

through anhydrous sodium sulphate and collected to analyse acid metabolites (TeA). All chloroform extracts were evaporated in a rotary evaporator at 40 °C. The residues were dissolved in 2 mL HPLC grade methanol and stored at −18 °C until analysis. Quantification was performed by an HPLC-UV system consisting of a Shimadzu LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 μL loop and a Shimadzu UV detector Model SPD-6A. The analytical column was a Phenomenex Jupiter 4.6 × 250 mm 5 μ C_{18}. The mobile phase was methanol:water (80:20) containing 300 mg/L $ZnSO_4 \cdot H_2O$ for AOH and AME, and methanol:water (90:10) containing 300 mg/L $ZnSO_4 \cdot H_2O$ for TeA. A flow rate of 0.4 mL/min was used. The wavelength for recording chromatograms was 258 nm for AOH and AME, and 280 nm for TeA. Standards of TeA (as a copper salt), AME and AOH were purchased from Sigma-Aldrich (St. Louis, MO, USA). From all solid standards, individual stock solutions of 0.5 mg/mL were prepared gravimetrically in methanol and stored at −18 °C. The copper salt was reconverted to tenuazonic acid as described by Scott and Kanhere (1980). Working standard solutions of 5 μg/mL of each toxin were then prepared. The precise concentration of working solutions was confirmed by UV-spectroscopy. A calibration curve was constructed for quantification purposes using toxin standards and correlating peak area *versus* concentration. Detection limits of the method, established as the minimum concentration of the toxins detected in the samples that allowed confirmation by the diode array detector, were 2.8 μg/kg, 1.1 μg/kg and 2.9 μg/kg for TeA, AOH and AME, respectively. Recovery experiments were performed by spiking non-contaminated red peppers, provided by the organic producers, at four levels of addition (10, 100, 250 and 500 μg/kg) with three replicates by level. Mean recoveries of TeA, AOH, and AME from triplicate samples were 77.7, 83.3, and 84.0% at 10 μg/kg with coefficients of variation (RSDs) of 8.6, 8.0, and 7.4%, respectively; 89.7, 89.3, and 90.0% at 100 μg/kg with RSDs of 4.5, 4.7, and 6.2%, respectively; 92.7, 95.3, and 97.0% at 250 μg/kg with RSDs of 4.9, 3.7, and 4.7%, respectively; and 85.3, 93.0, and 96.7% at 500 μg/kg with RSDs of 2.9, 4.7, and 2.6, respectively. Average recoveries at the four levels of addition were 86%, 90% and 92% for TeA, AOH and AME, respectively. Quantities reported were corrected by recovery.

3. Results

3.1. Fungal identification

No relationship between ripening stage of the fruit and contamination was observed. The most prevalent genus was *Alternaria*; 33 out of 66 fruits (50%) were infected with at least one *Alternaria* strain. A total of 64 isolates from this genus were recovered. *Fusarium* and *Cladosporium* were also isolated but in much lower frequency (8 and 6 isolates, respectively). The incidence of total mycota from contaminated pepper fruits is shown in Table 1. Given that *Alternaria* was by far the main contaminant of pepper; a further analysis was carried out involving their morphological identification to species-group level and evaluation of their toxigenic capacity.

All 64 *Alternaria* isolates belonged to small-spored species-groups. Following Simmons (2007) identification manual, 49 isolates out of 64 (77%) were identified as belonging to *A. tenuissima* species-group (group "H" from the manual). This group was characterized by three-dimensional sporulation patterns with conidia formed in relatively long chains of conidia (up to 15), borne from primary conidiophores of varying length. The observation of secondary conidiophores was uncommon, but when present, they originated from the conidial body. On DRYES, these strains exhibited light green to greyish colonies. Another four isolates (6%) exhibited a sporulation pattern similar to that of *A. arborescens* sp.-grp. (group "L"). The main characteristics were long primary conidiophores with a terminal cluster of branching conidial chains. Secondary conidiophores originating mainly from the conidial apex and less frequently from conidial body were regularly observed. Colonies on DRYES were dark green and sulcate. Four more isolates (6%) presented short primary conidiophores with multi-branched chains of 4–10 conidia, frequently with lateral secondary conidiophores and were identified as *A. alternata* sp.-grp. (group "J"). Colonies on DRYES were sulcate and dark green. One isolate (2%) exhibited characteristics similar to *A. armoraciae* sp.-grp. (group "M"), showing long primary conidiophores with several and short lateral branches of conidia. The six remaining isolates (9%) exhibited intermediate characteristics among groups H, J and L and were referred to as *Alternaria* sp.

Table 1
Frequency of fungal genera isolated from peppers cultivated in Argentina.

Fungal genus	Number of isolates	%[a]
Alternaria	64	79.1
Fusarium	8	9.9
Cladosporium	6	7.4
Geotrichum	1	1.2
Epicoccum	1	1.2
Harzia	1	1.2
Total	81	100

[a] Percentage of each genus over total strains isolated.

3.2. Secondary metabolite profiling of Alternaria isolates

In order to assess the toxicogenic potential of *Alternaria* isolates, a secondary metabolite profile was constructed for each strain focused on the main mycotoxins and phytotoxins reported as naturally occurring in food products. The alternariols were produced by a higher number of isolates, AOH by 58/64 (91%) and AME by 59/64 (92%). Tenuazonic acid, altenuene and tentoxin were also detected in high frequencies, 41 (64%), 54 (84%) and 46 (72%) producer isolates, respectively. Altertoxins ATX-I and ATX-II were detected in a lower number of isolates, while ATX-III was not produced by any of them. These results are summarized in Table 2.

Regarding the secondary metabolite profile of the different small-spored *Alternaria* isolates, none of the toxins could be associated with a particular species-group. AOH, AME, TeA and ALT were produced by members of all the sp.-grp. isolated from peppers; TEN was synthesized by isolates from all groups except for the isolate belonging to the *A. armoraciae* sp.-grp. On the other hand, the altertoxins were less commonly synthesized by the pepper *Alternaria* strains. ATX-I was only produced by members of the *A. tenuissima* sp.-grp. and one isolate identified as *Alternaria* sp. Similarly, ATX-II was detected in a few members of the *A. tenuissima* and *A. arborescens* sp.-grp.

3.3. Natural occurrence of Alternaria mycotoxins in target pepper samples

The presence of the main *Alternaria* mycotoxins in the pepper fruit was investigated. AOH, AME and TeA were selected for this study based on their frequency of *in vitro* production by the isolates and

Table 2
Toxicogenic capacity of *Alternaria* species-groups (n = 64) isolated from pepper.

Alternaria sp.-grp.	% isolates[a]	% producer isolates[b]						
		TeA	AOH	AME	ATX-I	ATX-II	ALT	TEN
A. tenuissima sp.-grp.	77	67	88	90	33	6	82	80
A. arborescens sp.-grp.	6	50	100	100	0	25	75	25
A. alternata sp.-grp.	6	25	100	100	0	0	100	75
A. armoraciae sp.-grp.	2	100	100	100	0	0	100	0
Alternaria sp.	9	67	100	100	17	0	100	50
Total producers[c]		64	91	92	27	6	84	72

TeA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether; ATX-I, -II, -III: altertoxins I, II, III; ALT: altenuene; TEN: tentoxin.
[a] Percentage of each species-group over total *Alternaria* isolates.
[b] Percentage of producer isolates over total isolates from the species-group.
[c] Percentage of producer isolates over total *Alternaria* spp.

their known human toxicity. A total of 32 out of 48 spoiled pepper fruits analysed were found to be contaminated with at least one *Alternaria* toxin, with TeA being the most frequent (50% of studied samples) (Table 3). This metabolite was also the one detected at the highest concentrations; although it should be pointed out that the superior extreme of the range for TeA corresponds to a single highly contaminated sample. Both AOH and AME were found in a lower number of samples (21 and 29%, respectively) and in lower concentrations.

Co-occurrence of *Alternaria* mycotoxins in mouldy pepper fruits was also evaluated (Fig. 1). TeA was found as the only contaminant in 12 out of 32 contaminated fruits (38%), but it co-occurred with alternariols in 12 more samples, four with AOH (13%), six with AME (19%) and two with both (6%). AOH was detected alone in only two fruits (6%), but in combination with the other two toxins in eight more. AME was detected alone in four of the samples (13%), and together with the other toxins in another 10 peppers.

The whole set of data from toxigenic potential of the strains and natural occurrence of the toxins in the fruits was analysed in order to establish if a correlation existed between the infection of peppers with toxin producer strains and the actual presence of the toxin in the fruit. Fig. 2 shows that TeA-producer strains were isolated from nine naturally contaminated peppers and only one pepper infected with TeA-producer *Alternaria* spp. was not contaminated with this toxin. Meanwhile, from 15 TeA contaminated pepper fruits non TeA-producing strains were isolated. For AOH and AME, the relationship between the toxicogenic capacity of the *Alternaria* spp. isolates and natural contamination of pepper fruits showed a different behaviour from that described for TeA. For both alternariols, a higher number of fruits were infected with toxigenic *Alternaria* but were not naturally contaminated with the correspondent toxin (13 and 10 peppers for AOH and AME, respectively), and a lower number of naturally contaminated peppers carried toxin-producer strains (3 and 6 peppers for AOH and AME, respectively).

4. Discussion

Alternaria was found as the most frequent post-harvest fungal contaminant of sweet peppers cultivated in Argentina. *Botrytis cinerea* and *Colletotrichum capsici* are reported in the literature as the main pathogens of peppers together with *Alternaria* spp. (Edirisinghe et al., 2014; García et al., 2015; Lakshmi Sahitya et al., 2014). However, none of these genera were isolated from the damaged fruits analysed in the present work. Greco et al. (2012) observed a similar trend in Argentinean mouldy blueberries, where *Alternaria* was the most common fungal genus, instead of the main pathogens reported in literature (such as *Rhizopus* and *Botrytis*).

Based on their morphological characteristics and sporulation patterns, the *Alternaria* isolates from peppers were classified into four species-groups from Simmons (2007). All of them belonged to the small-spored *Alternaria* spp. The distribution of isolates into species-groups was similar to those observed in other vegetables and cereals cultivated in Argentina, and the majority of the strains were assigned to the *A. tenuissima* sp.-grp., as was found also in wheat, tomato, walnuts and blueberries (Andersen et al., 2015; Greco et al., 2012; Patriarca et al., 2007). The species belonging to these groups are known to be able to produce a wide variety of secondary metabolites, many of which are mycotoxins or phytotoxins.

The predominance of small-spored *Alternaria* spp. in peppers suggested an associated risk for human health. In order to evaluate the

Table 3
Natural occurrence of *Alternaria* mycotoxins in Argentinean peppers.

Toxin	N° (%) of positive fruits	Range (μg/kg)[a]	Mean (μg/kg)[a]	Median (μg/kg)[a]
TeA	24 (50)	8–11,422	1344	96
AOH	10 (21)	3–98	29	26
AME	14 (29)	7–262	56	14

TeA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether.
[a] Concentration of toxins in μg toxin per kg of fruit.

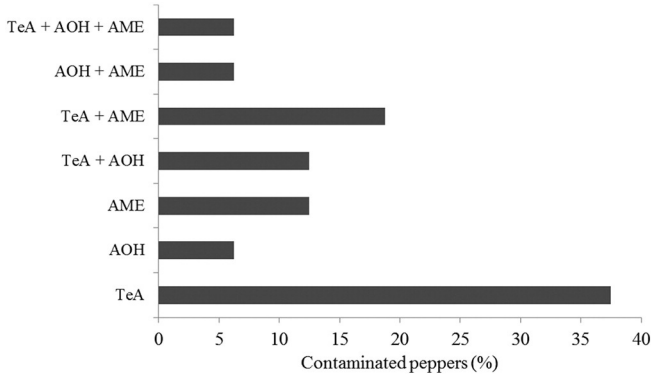

Fig. 1. Distribution and co-occurrence of *Alternaria* mycotoxins in contaminated peppers. Bars represent percentages over total toxin contaminated samples. A. TeA; B. AOH; C. AME.

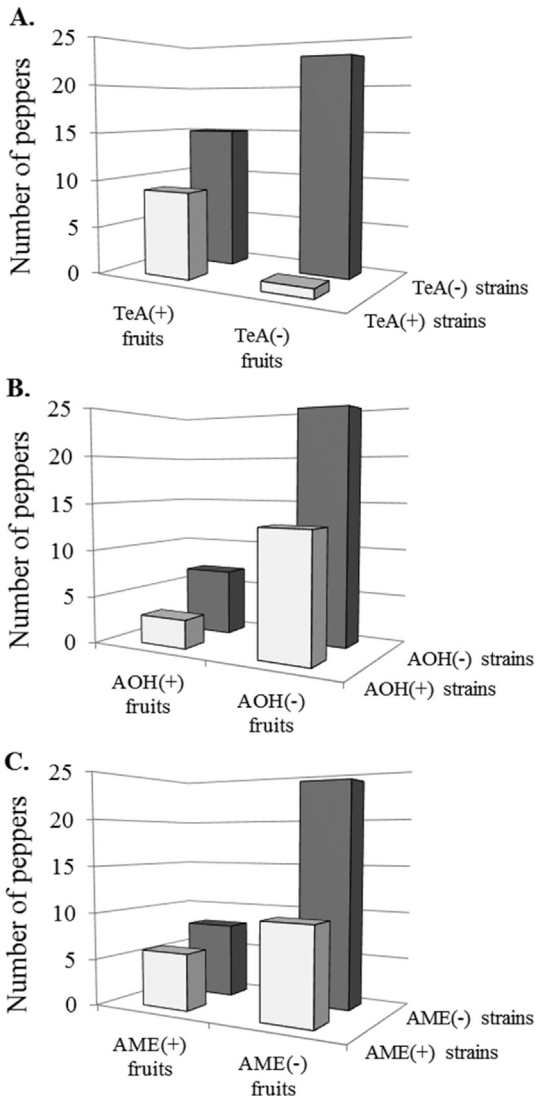

Fig. 2. Relationship between the toxicogenic capacity of the *Alternaria* spp. isolates and natural contamination of pepper fruits. □ number of peppers from which toxin producer strains were isolated. ■ number of peppers from which non-toxin producer strains were isolated.

toxigenic potential of these strains, their secondary metabolite profile was studied *in vitro*. The method used for this purpose in the present work has been applied elsewhere with chemotaxonomic aims, since it allows the detection of a high number of metabolites simultaneously (Andersen et al., 2015; Frisvad et al., 2008). In this case, it was applied to determine the potential toxigenicity of the *Alternaria* strains, and the results obtained were a useful tool to decide which mycotoxins should be further surveyed for natural occurrence in the fruits. DRYES was the selected culture medium because it has been demonstrated that *Alternaria* can express a wide variety of compounds in it (Andersen et al., 2009, 2015).

The toxigenic potential of the *Alternaria* isolates was high, with most of the strains showing the capacity to synthesize the major mycotoxins associated with this genus. Both alternariols were the toxins most frequently detected, followed by TeA, ALT and TEN. These results are in agreement with available literature. Greco et al. (2012) reported that AOH and AME were the most prevalent toxins produced in autoclaved rice by *Alternaria* strains isolated from Argentinean blueberries (97 and 95% of the isolates, respectively), followed by TeA (65% of the isolates). Similarly, Andersen et al. (2015) stated that most of the Argentinean small spored *Alternaria* strains studied from blueberry, tomato, walnut and wheat; belonging to H, L and J species-groups, were able to synthesize the main mycotoxins, detecting AOH and AME production in 83% of the isolates, TEN in 82% and TeA in 76%. On the other hand, Siciliano et al. (2015) found that TeA was the main toxin produced in Czapek-Dox medium by *Alternaria* spp. isolates obtained from cabbage, cauliflower and rocket, with 75% producer isolates; AOH and AME were produced by 57% and ALT by 50% of the analysed strains, and TEN was detected in only 4 out of 28 strains.

The altertoxins were produced in a much lower frequency than the rest of the toxins by the Argentinean pepper isolates, and were not equally distributed between the different *Alternaria* species-groups. ATX-I is the altertoxin more deeply surveyed, while ATX-II and ATX-III are less frequently reported in literature. However, the few available studies reported higher production rates for *Alternaria* spp. from different foods worldwide. Andersen et al. (2002) found that 61% of *A. tenuissima* sp.-grp. and *A. arborescens* sp.-grp. isolates from cereals and fruits were able to synthesize ATX-I; while Serdani et al. (2002) detected similar levels (58%) with isolates obtained from apple belonging to the same species-groups. In addition, all 11 *A. alternata* strains from oilseed rape studied by Visconti et al. (1992), were capable of producing ATX-I and II. On the other hand, Andersen et al. (2015), analysing *Alternaria* spp. belonging to H, J, L and M groups isolated from blueberries, tomato, walnut and wheat; found that ATX-II was the altertoxin most frequently produced (77% of the strains). ATX-I and ATX-III were detected at lower levels.

Given the potential for mycotoxin production detected in *Alternaria* isolates from pepper, the evaluation of natural occurrence of the major toxins was carried out. Since ATXs were produced in lower frequency by *Alternaria* isolates, and human toxicity of TEN and ALT is still poorly understood, this study was focused on alternariols and TeA. The 67% of the visibly spoiled fruits analysed were contaminated with at least one of the *Alternaria* toxins investigated. TeA was found more frequently than the alternariols, and in higher levels. There are few reports concerning *Alternaria* mycotoxins incidence in this vegetable. Logrieco et al. (2009) reported concentrations of up to 54 μg/kg for TeA, 49 μg/kg of AME, and 640 μg/kg for AOH in peppers in Southern Italy. Our results showed lower levels for AOH, although TeA and AME were detected in higher amounts. Chung et al. (1998) reported much higher concentration of these toxins in pepper from Korea, ranging from small amounts to 440 mg/kg for AOH, 249–342 mg/kg for TeA and 206–294 mg/kg for AME. Yogendrarajah et al. (2014) found AME in two dry chilli samples from Belgium (70 and 222 μg/kg).

The co-occurrence of the toxins is also of relevance due to the possibility of synergistic toxicological effects. In 44% of contaminated samples, more than one mycotoxin was detected. TeA was found simultaneously with other mycotoxin in half of the fruits contaminated with this metabolite; alternariols were detected co-produced with another compound more frequently than as the only contaminant. In consequence, more studies are necessary to evaluate the interaction among mycotoxins concerning human toxicity.

Studying natural contamination with mycotoxins is of great importance since food components and storage conditions may interfere in metabolite biosynthesis of contaminant fungi. As it has been previously demonstrated in the literature, our results supported that no strict correlation can be established between the capability of production of the strain *in vitro* and its actual occurrence in the food (Pitt et al., 2000). In the present work, even though AOH and AME were the toxins most frequently produced by the isolates in DRYES medium, they were much less prevalent in pepper fruits. On the contrary, TeA was produced *in vitro* by a lower number of isolates, but more fruits were contaminated with this toxin. When studying the relationship between the natural contamination of peppers and the *Alternaria* strains isolated from the respective fruits, similar discrepancies were observed. From 15 TeA-contaminated pepper, non-TeA-producing *Alternaria* spp. were isolated. A possible explanation for this is that the producer fungus was no longer viable or cultivable, but the toxin remained in the fruit. On the other hand, several fruits were infected with strains that could produce alternariol in DRYES, but the toxin was not found in them. It appears that some components of the food may interfere in the biosynthetic pathway of alternariols, reducing their incidence. Interestingly, Siciliano et al. (2015) observed that 80% of *Alternaria* isolates from rocket and cabbage were able to produce at least one mycotoxin *in vitro,* but the same isolates seemed to lose the ability to synthesize TeA when tested *in vivo*, effect not observed with alternariols and other toxins. However, it should be noticed that the analytical technique used for TeA in the food matrix in the mentioned work presented a much lower recovery than that applied for *in vitro* detection. These results lead to the conclusion that visible deterioration caused by *Alternaria* cannot necessarily be associated with toxin accumulation in the food matrix, nor be used as a quality control for toxin contamination. Furthermore, what implies a higher risk for consumers is that the absence of fungal isolates cannot guarantee the absence of the toxin in the food.

5. Conclusions

To our knowledge, this is the first report of natural occurrence of *Alternaria* mycotoxins in mouldy peppers from Argentina. Since there are currently no international regulations about *Alternaria* toxins, safe levels for their consumption are not yet established. However, the incorporation of these compounds in food legislation is being discussed nowadays in developed countries (Alexander et al., 2011). It is generally agreed among the international scientific community that more data on natural occurrence of these toxins in different food matrices, in addition to exhaustive studies of their toxicity, including bioavailability tests and long term clinical studies, are necessary. If toxicity studies confirm the health risk associated with the presence of ALT and TEN in food products, it would be worth investigating their natural occurrence in Argentinean peppers, given the high biosynthetic potential of the *Alternaria* strains.

This work provides data about levels of major *Alternaria* mycotoxins in sweet pepper, a matrix understudied in Argentina as well as worldwide. This hazard represents a high risk when mouldy fruits are used in industrialized products because these metabolites are not destroyed by conventional heat treatments.

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