Mycobiology

Mini-review

Alternaria in Food: Ecophysiology, Mycotoxin Production and Toxicology

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Abstract *Alternaria* species are common saprophytes or pathogens of a wide range of plants pre- and post-harvest. This review considers the relative importance of *Alternaria* species, their ecology, competitiveness, production of mycotoxins and the prevalence of the predominant mycotoxins in different food products. The available toxicity data on these toxins and the potential future impacts of *Alternaria* species and their toxicity in food products pre- and post-harvest are discussed. The growth of *Alternaria* species is influenced by interacting abiotic factors, especially water activity (a_w), temperature and pH. The boundary conditions which allow growth and toxin production have been identified in relation to different matrices including cereal grain, sorghum, cottonseed, tomato, and soya beans. The competitiveness of *Alternaria* species is related to their water stress tolerance, hydrolytic enzyme production and ability to produce mycotoxins. The relationship between *A. tenuissima* and other phyllosphere fungi has been examined and the relative competitiveness determined using both an Index of Dominance (I_D) and the Niche Overlap Index (NOI) based on carbon-utilisation patterns. The toxicology of some of the *Alternaria* mycotoxins have been studied; however, some data are still lacking. The isolation of *Alternaria* toxins in different food products including processed products is reviewed. The future implications of *Alternaria* colonization/infection and the role of their mycotoxins in food production chains pre- and post-harvest are discussed.

Keywords *Alternaria* species, Ecology, Food products, Mycotoxins, Physiology

There are approximately 300 species in the genus *Alternaria* which can colonise a wide variety of plants, especially in the phyllosphere (aerial plant parts), as saprophytes of senescent plant residue, in soil and as fungal pathogens of specific crops causing distinct symptoms. The most commonly reported species include *Alternaria alternata*, *A. tenuissima*, *A. arborescens*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria*. They colonise a range of plants including cereals, oilseeds, tomatoes, cucumbers, cauliflowers, peppers, apples, melons, tangerines, oranges, lemons, and sunflower seeds [1, 2]. *Alternaria* colonisation of ripening ears can result in black pointed grain and impact directly on flour colour of bread making wheats [3]. Indeed, *Alternaria* species can contaminate other cereals such as sorghum and result in toxin contamination. Studies in the UK and in China suggest *Alternaria* causes both sooty mould of ripening ears in conjunction with *Cladosporium* species but can affect crops as a weak pathogen depending on conditions during anthesis to harvest, pre-harvest and post-harvest in wheat and barley where poor drying regimes have been employed [4, 5]. *A. tenuissima* has been shown to be the major species isolated from Argentinean wheat [6]. It has been isolated more frequently than *A. alternata* and *A. infectoria*, which have been reported as the predominant species in cereals in several studies worldwide [7-11]. Currently 13 genera including *Allewia*, *Embellisia*, *Lewia*, *Nimbya*, *Sinomyces*, *Teretispora*, *Ulocladium*, etc are recognized as synonym of *Alternaria*. *Alternaria* complex contains 24 internal sections (sect.) and 6 monotypic lineages (see Fig. 1) [12].

Approximately 30 metabolites with possible toxicity are known from various species of *Alternaria* (Fig. 2) [1]. Alternariol (AOH), altenariol monomethyl ether (AME),

Mycobiology 2015 June, **43**(2): 93-106
http://dx.doi.org/10.5941/MYCO.2015.43.2.93
pISSN 1229-8093 • eISSN 2092-9323

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Received February 26, 2015
Revised March 2, 2015
Accepted April 29, 2015

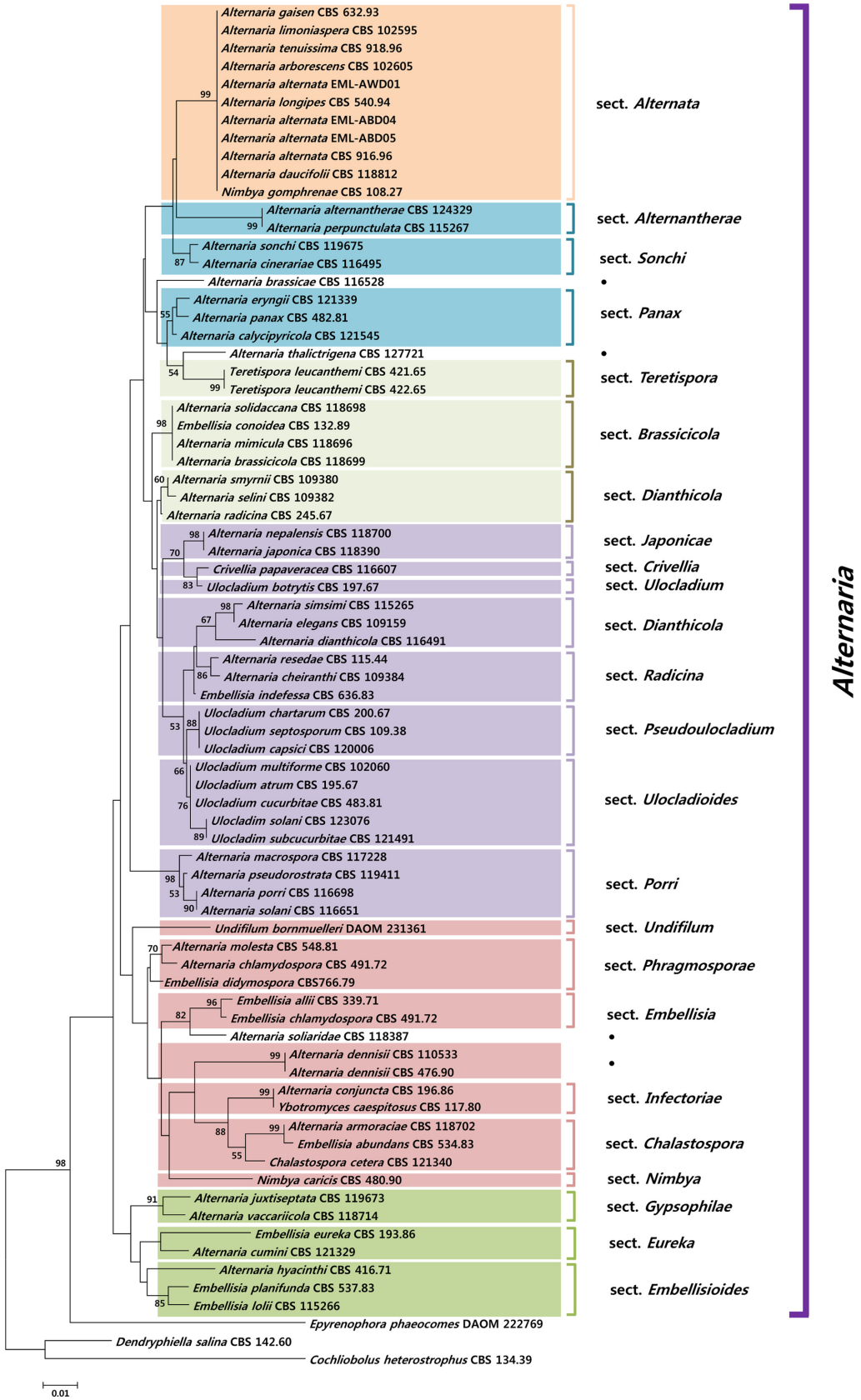

Fig. 1. Neighbor-joining phylogenetic tree showing 24 sections including *Alternata*, *Alternantherae*, and *Sonchi* in *Alternaria* complex belonging to Ploeosporaceae based on internal transcribed spacer rDNA sequences. Bootstrap percentages are presented at the nodes. The monotypic lineages are indicated by black dots based on the phylogeny constructed by Woudenberg *et al.* [12].

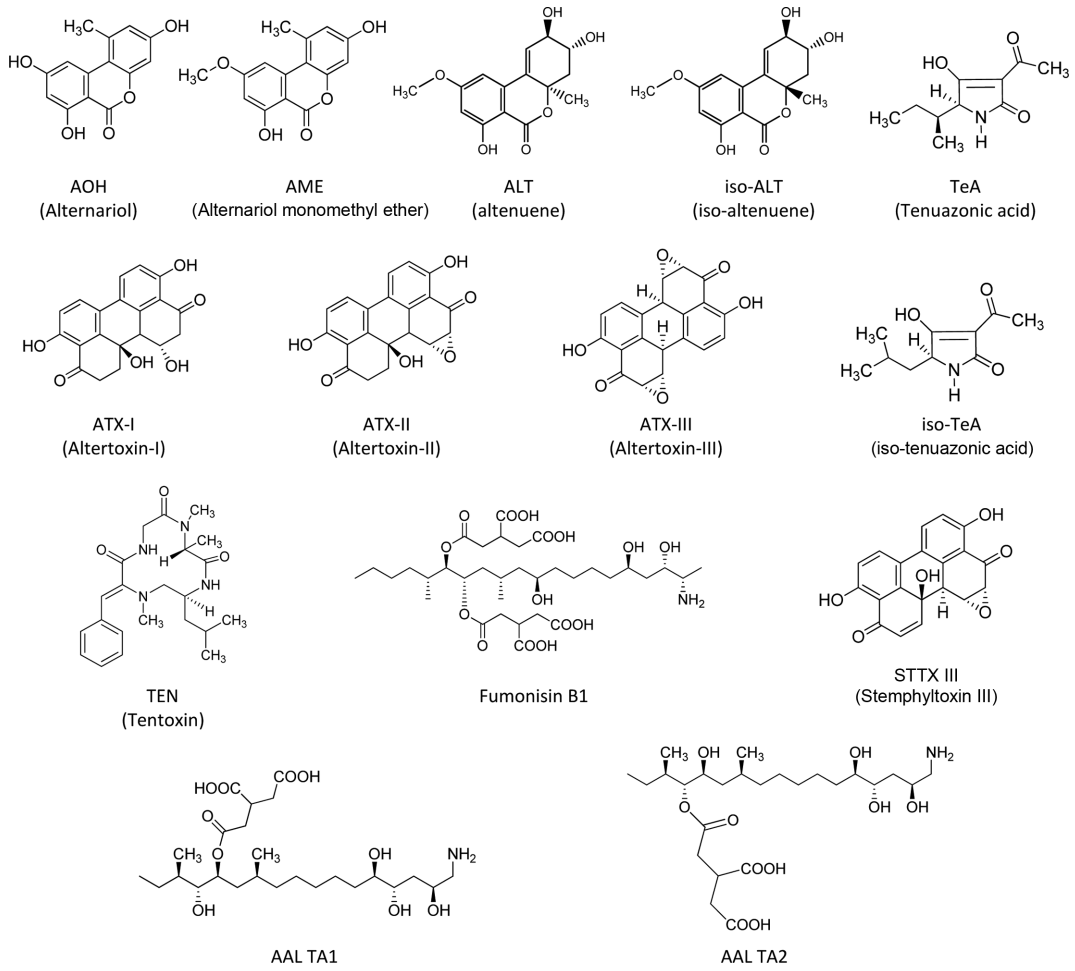

Fig. 2. Structure of the different toxins produced by *Alternaria* species. Key to toxins: AOH, alternariol; AME, alternariol monomethyl ether; iso-ALT, iso-altenuene; TeA, tenuazonic acid; iso-TeA, iso-tenuazonic acid; TEN, tentoxin; ATX-I, altertoxin I; ATX-II, altertoxin II; ATX-III, altertoxin III; STTX III, Stemphyltoxin III; AAL TA1 and TA2 toxin, *Alternaria alternata* f. sp. *lycopersici* TA1 and TA2 toxin; fumonisin B1.

altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), and altertoxins I, II, and III are considered to be some of the important toxic metabolites [1]. Previous studies have demonstrated that TeA, AOH, and AME are the most common *Alternaria* toxins present in Argentinean wheat [13].

Of particular health concern is the association found between *A. alternata* contamination in cereal grains and the high levels of human oesophageal cancer in China [14, 15]. The European Food Safety Authority (EFSA) [16] suggested in 2012 that *Alternaria* toxins are of high concern for public health. The European Standing Committee has recommended that EU member states should collect data on the occurrence of *Alternaria* toxins in food commodities. The Netherlands has recently completed a survey and found that AOH, AME, TeA, and TEN were detected in one or more food commodities whilst ALT was not found in any samples. TeA was found in 27% of samples at high concentrations in cereals, tomato sauces, figs, wine, and sunflower seeds. Only incidental occurrence was found in apples, citrus fruit, tomatoes, and olives [17]. This type of data will inform EFSA on whether legislation will be required based on the hazard analysis assessment.

This review will consider the information available on (1) the ecological factors, growth and hydrolytic enzyme production by *Alternaria* species, especially of *A. alternata* and *A. tenuissima*, (2) the relative competitiveness of *Alternaria* against other fungi, (3) the relationship between environmental conditions and mycotoxin production, (4) the toxicology of the key *Alternaria* toxins, (5) contamination of different products, and (6) future drivers which will influence *Alternaria* research in relation to mycotoxin contamination of food commodities.

ENVIRONMENTAL FACTORS, GERMINATION AND GROWTH OF *ALTERNARIA* SPECIES

Some studies reported a relationship between temperature and water activity on the germination and growth of *Alternaria* species. Magan and Lacey [4] carried out studies

Table 1. Summary of the range of environmental conditions which allow germination, growth and toxin production (based on data from several studies) by *Alternaria alternata* and *A. tenuissima* (psychrotolerant)

Factors	Germination	Growth	Toxin production
Temperature (°C)	1~35	< 1 and > 35	< 10 and > 35
Water activity (a_w)	0.84~0.995	< 0.85	< 0.90
pH	2.5~10	< 2.5 and > 10	< 2.5 and > 9

on *A. alternata* strains responsible for black point of wheat grain. The impact of different temperatures and water activity (a_w) conditions on temporal germination and growth were examined in detail. They showed that germination occurred over the range 5~35°C, with minima a_w of 0.84~0.85 at 25°C at pH 6.5. The temperature range was 5~30°C with minima of 0.86 a_w at 20~25°C at pH 4.0 (Table 1). For a strain of *A. alternata* isolated from wheat, growth was optimum at about 25°C and growth occurred over the a_w range 0.88~0.89 to 0.995 a_w (Fig. 3A) [18]. Thus growth occurred over a narrower range of a_w × temperature conditions than that for germination [4]. These data were used over the whole a_w × temperature range to construct a contour map of optimum and marginal conditions for growth using isopleth lines joining conditions of similar growth rates (Fig. 3B). Subsequently other studies with *A. alternata* strains from sorghum, and *A. tenuissima* from wheat (Argentina) have been similarly examined. Thus, Patriarca *et al.* [19] found that two strains of *A. tenuissima* had a broad temperature range for growth with optimum at 25~30°C at 0.98 a_w. At 0.95 a_w growth was reduced by > 50~60% regardless of temperature. The optimum temperature at 0.95 a_w was 30°C. These strains grew similarly at 0.95 a_w and 34°C and 30°C. Statistically, all factors, a_w, temperature, strain and their interactions significantly affected growth rate ($p < 0.0001$). Oviedo *et al.* [20] found *A. alternata* strains from soya beans grew well at 25~35°C on soya-based media. This is unusual as the strains from temperate cereals are usually unable to grow or grow very slowly at ≥ 35°C.

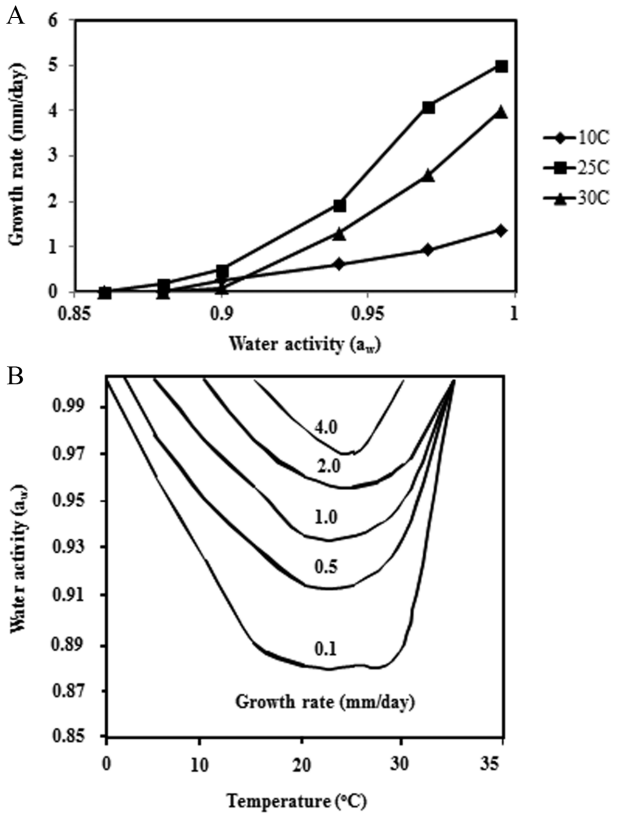

Fig. 3. A, Effect of water activity (a_w) × temperature effects on growth of a strain of *Alternaria alternata* on a wheat-based medium; B, The growth profile of *A. alternata* on wheat-based medium showing the optimum and boundary conditions for growth. Numbers on the isopleths join conditions with a similar growth rate (mm/day). Adopted from Magan N and Aldred D [18].

Tomatoes are highly susceptible to fungal decay because of their thin skin and soft tissue, and *Alternaria* is the main fungus responsible for spoilage. Pose *et al.* [21] found that *A. alternata* strains from tomato fruits grew at 6~35°C and 0.982~0.922 a_w on synthetic tomato-based medium. Optimum growth was at 21°C and 0.982 a_w. At 0.982 a_w growth was reduced by > 80% when temperature was decreased from 21°C to 6°C. Vaquera *et al.* [22] showed that germination and growth occurred over the range 6~30°C and 0.995~0.95 a_w for *A. arborescence*, the cause of stem canker. The minimum germination times were at 25~30°C and freely available water (0.995 a_w), and optimum at 30°C/0.995 a_w. At cooler temperature (6°C) growth was reduced to < 20% of the optimum. Thus refrigeration of tomato fruits might extend its shelf-life but this may not prevent deterioration by *Alternaria*. Overall, studies suggested that growth of different strains from the same plant niche is similar for those isolated from cereals, tomatoes, sorghum, sunflower and soya beans. However, this may not be true for mycotoxin production, which can vary significantly between strains (e.g., *Aspergillus flavus* and aflatoxin production).

INTERACTIONS AND COMPETITIVENESS OF *ALTERNARIA* SPECIES

Since *Alternaria* species are common components of the airspora and components of the phyllosphere of many plants, they inevitably come in contact with a diverse fungal and bacterial community. Heavy pigmentation of their spores may provide good UV resistance and contribute to their effective colonization of the phyllosphere/phylloplane of many plant surfaces. However, they also must have effective tolerance to abiotic factors and produce the necessary hydrolytic enzymes to facilitate effective competitiveness in these ecological niches. For example, increased populations of *Alternaria* on ripening ears of wheat coincided with reduced populations of *Fusarium* species [4, 23]. Azcarate

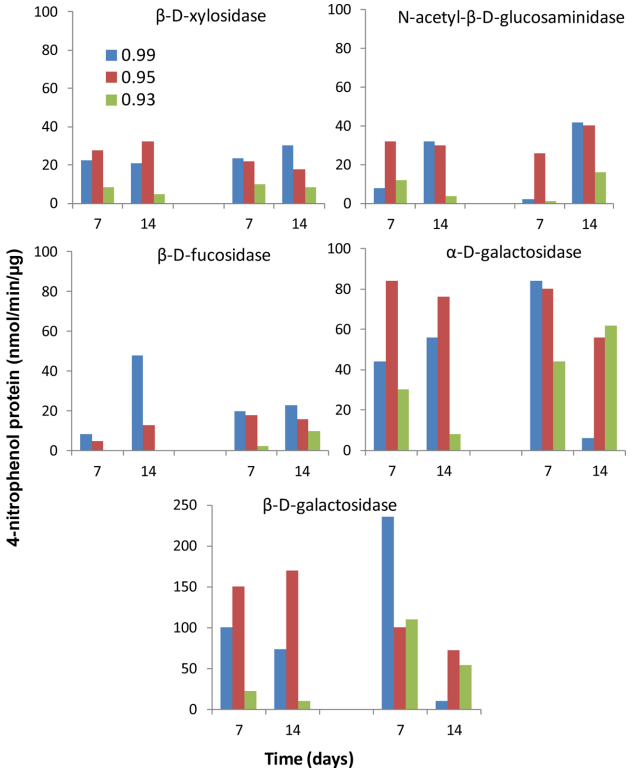

Fig. 4. The effect of water activity (a_w) and time on the specific activity of 5 different hydrolytic enzymes by a strain of *Alternaria tenuissima* on wheat grain. Please note the difference in scale for one of the enzymes. Adopted from Hope R [26].

et al. [13] found that wheat from the 2004~2005 Argentinean harvest was highly contaminated with *Alternaria* species (100% samples, 4,107 isolates) but the incidence of *Fusarium* sp. was considerably lower (46% samples, 54 isolates). Previously, in Canada, Wallace and Sinha [24] using multivariate statistical approaches also showed that higher populations of *Alternaria* reduced *Fusarium* (*F. culmorum*; *F. graminearum*) colonization. Because *Fusarium* species produce type B trichothecenes in cereals pre-harvest this has important health implications.

Expression of hydrolytic enzymes can be affected by environmental factors [25]. β-D-galactosidase was highly expressed at the earlier time of infection and only at high a_w conditions (Fig. 4) [26]. Other important enzymes were α-D-galactosidase, N-acetyl-β-D-glucosaminidase, β-D-fucosidase, and β-D-xylosidase. Interestingly, temporal enzyme production was often higher at 0.95 a_w than at 0.995 or 0.93 a_w. However, even at 0.93 a_w there was significant enzyme production, especially of the two galactosidases. This suggests that *A. tenuissima* can rapidly colonise food matrices and these enzymes facilitate colonization over a range of interacting abiotic conditions.

Previous studies [4, 25, 27] have examined the macroscopic interactions between *A. alternata* and different phyllosphere fungi (Table 2). When paired against *A. alternata* on

Table 2. Interactions between *Alternaria alternata* and other phyllosphere fungi based on the macroscopic interactions

Fungal species in specific a_w	Interacting fungal species						I_D
	A.a.	C.c.	C.h.	E.n.	F.c.	V.l.	
0.98 a_w							
A. alternata	-	1	1	1	1	1	5
C. cladosporioides	1	-	1	0	1	1	4
C. herbarum	1	1	-	0	1	1	4
E. nigrum	1	5	4	-	2	1	13
F. culmorum	1	1	1	2	-	1	6
V. lecanii	1	1	1	1	1	-	5
0.95 a_w							
A. alternata	-	1	1	0	1	1	4
C. cladosporioides	1	-	1	0	0	1	3
C. herbarum	5	5	-	0	1	1	4
E. nigrum	5	5	5	-	3	4	22
F. culmorum	1	4	4	3	-	1	13
V. lecanii	1	1	1	0	1	-	4

Modified from Magan N and Lacey J [4].
Interaction scores: 1:1, intermingling; 2:2, mutual antagonism on contact; 3:3, mutual antagonism at a distance; 4:0, dominance on contact; 5:0, dominance at a distance. I_D: Index of Dominance.
A.a., *A. alternata*; C.c., *C. cladosporioides*; C.h., *C. herbarum*; E.n., *E. nigrum*; F.c., *F. culmorum*; V.l., *V. lecanii*.

Table 3. Interactions between grain fungi on wheat-based media at 25°C

Fungal species in specific a_w	Interacting fungal species								I_D
	A.c.	A.n.	A.v.	P.bc.	P.c.	P.h.	P.p.	P.r.	
0.98 a_w									
A. alternata	1	2	1	0	0	1	4	1	10
C. clodosporioides	2	0	0	0	1	1	0	1	5
C. hebarum	2	0	0	0	1	1	0	1	5
E. nigrum	1	2	4	2	3	0	1	1	14
F. culmorum	4	4	1	4	1	1	4	1	20
0.95 a_w									
A. alternata	1	1	1	2	1	0	1	1	8
C. cladosporioides	2	1	1	0	1	1	1	1	8
C. herbarum	2	1	1	1	0	1	1	1	8
E. nigrum	4	3	1	3	3	3	1	5	23
F. culmorum	1	4	1	1	4	0	1	2	14

Modified from Magan N and Lacey J [4].
Key to fungi: A.c., *Aspergillus candidus*; A.n., *A. nidulans*; A.v., *A. versicolor*; P.bc., *Penicillium brevicompactum*; P.c., *P. cyclopium*; P.h., *P. hordei*; P.p., *P. piceum*; P.r., *P. roqueforti*.

wheat-based medium, *E. nigrum* and *F. culmorum* are more competitive. In contrast, *A. alternata* is a superior competitor to several species of *Aspergillus* and *Penicillium* at a range of a_w values (Table 3).

Another aspect of interactions between different fungi is the ability to utilize different carbon sources. Previously, Marin *et al.* [28] demonstrated that $a_w \times$ temperature modified the total C-source utilization and that the Niche Overlap Index (NOI) for an individual species. Later Lee

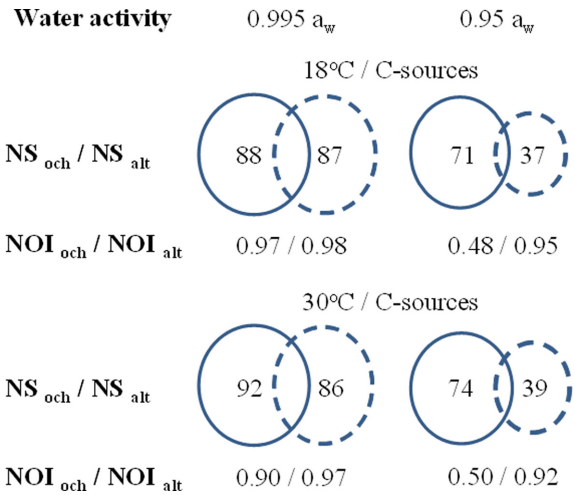

Fig. 5. Diagramatic example of the impact of environmental factors on Niche sizes (dotted line circle: *Alternaria alternata* [NS alt]; solid line circle: *A. ochraceus* [NS och]) and Niche Overlap Index (NOI) between *A. alternata* and *Aspergillus ochraceus*. Modified from Magan N and Aldred D [18] and Lee HB and Magan N [29].

and Magan [29] demonstrated the importance of using a group of C-sources relevant to the commodity of interest to avoid miss-interpretation of the results. Recently, Mohale *et al.* [30] showed that rates of C-source utilization as well as total number of C-sources used were important in understanding the interaction between different species such as between toxigenic and atoxigenic strains of *Aspergillus flavus* (Fig. 5) [27, 29]. This illustrates the relative niche sizes of *Aspergillus ochraceus* (= *A. westerdijkiae*) and *A. alternata* [27, 29] in relation to temperature and a_w. Niche size changed with temperature and a_w. The NOI for each species is based on the difference between the numbers of C-sources utilized individually over those utilized in common. NOI values > 0.90 indicate occupation of the same niche, and those < 0.90 indicate occupation of separate niches.

Comparison of the total C-sources utilized by *F. culmorum*, *A. tenuissima* and other phyllosphere fungi commonly found in wheat grain [26] shows that both the total number utilized and the NOI of *F. culmorum* and that of the other species varies with temperature and a_w (Table 4). Between-species variation in C-source utilization may be further modified by fungicide/preservative applications [18, 25]. These interactions may also affect mycotoxin production although few if any studies have examined impact on *Alternaria* toxins.

A recent study by Müller *et al.* [31] examined the role of *Alternaria* mycotoxins in interactions between *A. tenuissima* and *F. culmorum* and *F. graminearum*. They added specific mycotoxins such as AOH, TeA, or the trichothecene deoxynivalenol (DON) or zearalenone (ZEA) to wheat kernels and observed interactions between strains of these species. Grain spiked with DON or ZEA resulted in growth of *A. tenuissima* strains and increased AOH and TeA production, with degradation of the *Fusarium* toxins. In contrast, the *Fusarium* species behaved differently. For example, TeA decreased growth of *F. graminearum* and *F. culmorum* while DON production was decreased and ZEA significantly increased. Strains of both *Fusaria* could degrade AOH. Although neither a_w nor temperature were varied in this study, the results suggest complex interactions between phytopathogenic fungi and the role of mycotoxins [18, 25].

Table 4. Niche size (NS) and Niche Overlap Index (NOI) determined on carbon source relevant to wheat [18]

Fungal species in specific temperature	0.93 a_w			0.995 a_w		
	NS	NOI (F.c.)	NOI (spp.)	NS	NOI (F.c.)	NOI (spp.)
15°C						
F. culmorum	18			16		
A. tenuissima	3	0.17	1	17	1	0.94
C. herbarum	14	0.78	1	17	1	0.94
F. graminearum	0	0	0	17	0.94	0.88
F. poae	0	1	1	18	1	0.89
M. majus	2	0.11	1	18	1	0.89
P. verrucosum	17	0.94	1	18	1	0.89
25°C						
F. culmorum	18			17		
A. tenuissima	7	0.39	1	17	0.94	0.94
C. herbarum	11	0.61	1	16	0.88	0.94
F. graminearum	7	0.39	1	18	1	0.94
F. poae	18	1	1	18	1	0.94
M. majus	0	0	0	16	0.88	0.94
P. verrucosum	15	0.83	1	18	1	0.94

Adopted from Hope R [26].
Assays carried out at two a_w levels at 15°C and 25°C.
NOI (F.c.), NOI (spp.): NOI of *F. culmorum* (F.c.) and that of the other species.

ECOLOGY OF MYCOTOXIN PRODUCTION

The environmental conditions over which specific *Alternaria* mycotoxins may be produced have been identified. Extensive studies on *A. alternata* growth and mycotoxin production were carried out on wheat matrices [32, 33]. Comparison of growth profiles and production of three different mycotoxins by *A. alternata* on wheat grain (Fig. 6) [34] shows the optimum and marginal conditions for growth, AME, AOH, and TeA production in relation to a_w × temperature conditions. The conditions for mycotoxin production are slightly narrower in terms of temperature and a_w conditions relative to those for growth. Studies of *A. alternata* strains and production of AOH and AME on irradiated soya beans were done by Oviedo *et al.* [35, 36]. They found that optima for AOH varied with strains but were generally at around 25°C and 0.98 a_w with minima around 0.92 a_w over the temperature range tested (15~30°C). For AME the production by two strains of *A. alternata* was more consistent with optima around 25°C and 0.98 a_w, with a_w minima across the whole temperature range (15~30°C) of around 0.96 a_w. Interestingly, AME was not produced at > 30°C, while AOH was. Profiles in relation to the production of TeA have also been developed for *A. alternata* strains from soya bean-based media [20]. The profiles for TeA suggest that optimum concentrations are produced at 0.98 a_w and 25~30°C. This could be due to ecological differences or regional differences between strains as these originated from Argentina. Recent studies of *A. tenuissima* strains from Argentinian wheat samples also have a similar pattern of growth and mycotoxin production, especially at elevated temperature conditions (≥ 30°C) [19]. Nutritional status of the commodity may also be important. For example, Magan and Baxter [37] found that strains of *A. alternata* isolated from sorghum had different production patterns for TeA. TeA was produced over the range 0.99~0.93 a_w over 28 days growth periods. Even at 0.93 a_w TeA was produced with most biosynthesis occurring after 28 days, reaching levels similar to that at other more conducive a_w levels (< 0.95 a_w).

Pose *et al.* [38] studied the profiles of production for TeA, AOH, and AME by *A. alternata* strains on tomato-based media. The optimum conditions for TeA accumulation were 0.982 a_w and 21°C after 28 days of incubation. This mycotoxin was produced at 0.982 and 0.954 a_w over the range 15~35°C. After 28 days incubation at 0.982 a_w and 6°C small amounts of TeA were also detected. At lower a_w, 0.922, high concentrations of TeA were only detected at 21°C after 21 days incubation, although lower levels were also produced at 35°C after 28 days. The optimum AOH production occurred at 21°C and 0.954 a_w after 28 days. It was suggested that 21°C was the most favourable conditions for AOH synthesis over the whole a_w range (0.922~0.982) examined. The maximum concentration of AME was determined at 0.954 a_w and 35°C, although high amounts were also produced at 21°C.

Recently, data have been obtained for the first time on the impact of a_w × temperature conditions on altertoxin (ATX)-II by strains of *A. tenuissima* isolated from Argentinean cereals [19]. This showed that growth occurred over the whole temperature range tested and was optimum at 25~30°C and 0.98 a_w, and 30°C at 0.95 a_w (Fig. 7). The incubation time did not show a significant effect on ATX-II accumulation. The optimum conditions for ATX-II production were 0.98 a_w and 30°C for both strains. The

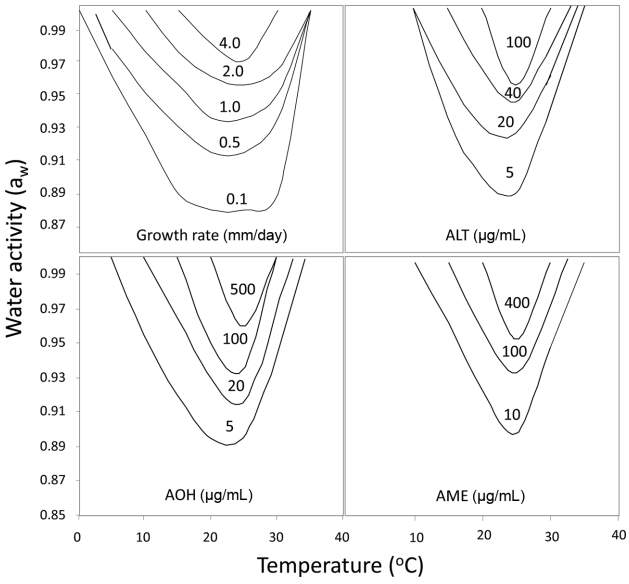

Fig. 6. Comparison of water activity (a_w) × temperature conditions over which growth and three different *Alternaria* mycotoxins can be produced on wheat. Adopted from Sanchis V and Magan N [34].

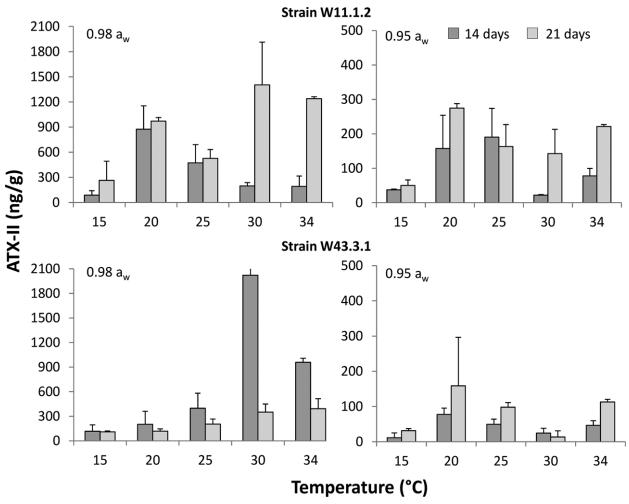

Fig. 7. Kinetics of altertoxin-II (ATX-II) production by two strains of *Alternaria tennuissima* over periods of 14 and 21 days on wheat-based medium. Bars indicate standard errors. Please note different Y-axis range for 0.98 and 0.95 water activities. Adopted from Patriarca A *et al.* [19].

strains also accumulated significant amounts of this toxin at 34°C.

Overall, studies suggest that mycotoxin production by strains is more variable than that for growth [19, 20, 37]. In addition, strains can often lose the ability to produce AME, ATE, ALT, TeA when sub-culturing continuously on rich artificial media [18].

ANALYTICAL AND MOLECULAR DETECTION OF *ALTERNARIA* TOXINS

Analytical approaches. *Alternaria* mycotoxins have been determined after separation by thin-layer chromatography, high performance thin-layer chromatography, gas chromatography, and more usually liquid chromatography (LC), mainly with ultraviolet (UV) detection, although fluorescence and electrochemical detection have also been used for other *Alternaria* mycotoxins than TeA [39].

Recently, atmospheric pressure chemical ionisation LC-mass spectroscopy (MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been applied to the determination and confirmation of identity of AOH and AME at sub ng/mL levels [40, 41]. A multi-phase method has been developed to analyse 33 mycotoxins (including AOH and AME) in various products including peanut, pistachio, wheat, maize, cornflakes, raisins, and figs simultaneously. The mycotoxins are extracted with an acetonitrile-water mixture, diluted with water and then directly injected into a LC-MS/MS system. The mycotoxins are separated by reversed-phase LC and detected using an electrospray ionisation interface and MS/MS, using multiple reaction monitoring [42]. Rasmussen *et al.* [43] developed a method for simultaneous detection of 27 mycotoxins (including AOH, AME, TeA, and altersetin) and other secondary metabolites in maize silage. A simple pH-buffered sample extraction was developed based on the QuEChERS method. No further clean-up was performed before analysis using LC-MS/MS. More recently, Wang *et al.* [44] reported an efficient and accurate LC-MS/MS method for simultaneous determination of seventeen mycotoxins in traditional Chinese medicine. Here, the effects of four different clean-up methods, including TC-M160, TC-T220, Mycosep 227, and QuEChERS method, on the recoveries of mycotoxins were investigated. Immunochemical methods, including enzyme-linked immunosorbent assay, have not been well developed for *Alternaria* mycotoxins. Although several chromatography based techniques are suitable for *Alternaria* toxin quantification in foods and feedstuffs, and LC coupled to mass spectrometry has become the method of choice [16]. However, there are several limiting factors for the analysis of the toxins such as the efficiency of sample clean-up, the availability of sufficient amounts of standards and the lack of reference materials for foods and feedstuffs. To date, standardisation of the analytical methods or validation of their accuracy have not been completed.

Molecular approaches. There has been interest in detecting *Alternaria* spores and biomass in different food commodities, especially tomato-based products (e.g., tomato purees and tomato paste). Studies by Zur *et al.* [45] developed a PCR-based method for the detection of *Alternaria* contamination in food products by using PCR primers which are specific to the ITS1 and ITS2 of the 5.8S rRNA gene of *Alternaria* but not to microbial or tomato DNA. They tested commercial and spiked samples, and found their detection method to be a rapid and sensitive assay for *Alternaria* DNA in tomato sauces and freshly prepared products. This could be a rapid assay which could replace the Howard method which requires microscopic observation and is time consuming. Amplified fragment length polymorphism (AFLP) variability, toxin production and pathogenicity of *Alternaria* species from Argentina species isolated from tomato-based products and fruit have been determined [46]. However, this was to examine the contamination from a taxonomic viewpoint not as a rapid diagnostic tool for detection of *Alternaria* in food products.

Oviedo *et al.* [47] have used AFLP markers to identify the variability between strains of *A. alternata* and *A. infectoria* isolated from wheat in Argentina. Clear polymorphism both within and between species showed that AFLP can be used to assess the genetic variation in these two species. They suggested that this could help in the development of specific primers for detection of these species in cereal grain and other commodities.

Crespo-Sempere *et al.* [48] used propidium monoazide (PMA) combined with quantitative PCR (q-PCR) for detection of *Alternaria* in tomato-based products. This was useful for detection of viable as opposed to non-viable fungal cells in food products. They focused on Alt4, Alt5 and Alt6 and Alt7 primer pairs designed for the conserved internal transcribed spacer region. They showed that by pre-treating with PMA it was possible to detect viable *Alternaria* cells in spiked tomato-based products. However, the tomato-based matrix had a protective effect on the cells against PMA toxicity reducing the efficiency to differentiate viable and non-viable cells. They suggested this method could be a valuable tool for estimating relative risk of contamination with *Alternaria* mycotoxins. Thus an opportunity now exists to carry out molecular ecophysiological studies of *Alternaria* species in different food production chains using q-PCR approaches and perhaps integrating this with mycotoxin and colonization data to develop relative risk models as recently done with *A. flavus* and *F. verticillioides* [49, 50].

TOXICOLOGY OF *ALTERNARIA* MYCOTOXINS

Acute toxicity. A case study was reported of a single 26 year old male occasional smoker, without previous respiratory history, who experienced acute dyspnoea and bilateral inspiratory fine crackles without wheezing or fever, following exposure to natural cotton powder in the

work place. Bronchoalveolar lavage was performed and the only microorganism identified in the lavage fluid was *A. alternata*. The symptoms experienced were thought to be from *Alternaria* contamination of the cotton or from the air conditioning system within the work place. All symptoms disappeared spontaneously within 48 hr of the event, with no recurrence reported [51].

Experimentally, when sodium tenuazonate (TeA salt) was administered to mice and rats, oral LD_{50} values were reported to be 81 and 186 mg/kg in male and female mice, respectively and 168 and 180 mg/kg in male and female rats, respectively [52]. The authors do not provide any explanation for the large difference between male and female mice.

Repeat dose toxicity. Kashin-Beck disease (KBD) is an endemic osteoarthritic condition in China and other parts of Asia. The aetiology of KBD is unknown although an association with ingestion of barley grains containing an unknown metabolite of *Alternaria* species was identified in 2001 [53].

Airborne *Alternaria* allergens were detected in six mechanically ventilated air-conditioned non-industrial buildings in Montreal, Canada. *Alternaria* allergens were identified in the offices and in ventilation systems supplying the offices and of 214 workers, half reported frequent work-related respiratory symptoms. All workers underwent skin prick allergy testing for *Alternaria* allergens and those that had positive reactions reported significantly more respiratory symptoms than those that had negative skin reactions [54]. *Alternaria* spores at natural exposure ranges have also been implicated in the pathogenesis of asthma. Seven mild asthma patients were bronchial challenged with whole spores or spore extracts, which resulted in immediate-type asthma during challenge with spore extract, while whole spores induced delayed type asthma only [55].

Carcinogenicity and mutagenicity. *Alternaria* species have been suggested to have a role in the development of oesophageal cancer. AOH and AME have been isolated from *A. alternata* species which was the main contaminating fungi from grain ingested in by the population of Linxian County in China, which additionally exhibits a high incidence of oesophageal cancer [14, 15].

Extracts of *A. alternata* and also food mildewed by the extracts are tumorigenic in nude mice and are able to induce stomach tumours in rats. Using *in vitro* studies, extracts of *A. alternata* induced reverse mutation in *Escherichia coli*, unscheduled DNA synthesis in cultured human amnion FL cells, chromosomal aberrations and sister chromatid exchange in human peripheral blood lymphocytes, mutation in V79 cells and transformation of NIH 3T3 cells in *in vitro* studies [56-59]. This experimental study data showed *A. alternata* to be tumorigenic and taken together with findings from human epidemiology studies in the Linxian County of China led the authors to conclude that *A. alternata* is one of the causes of human oesophageal cancer [14].

A more recent study showed that AOH (10 μM) was able to induce significant and concentration-dependent hypoxanthine-guanine phosphoribosyltransferase and thymidine kinase mutations in Chinese hamster V79 and mouse lymphoma L5178Y $tk^{+/-}$ (MLC) cells, respectively [56]. AOH has also been reported to induce DNA strand breaks in cell-free systems [56, 57].

A genotoxic role for AOH has been identified. AOH and AME were both able to increase the rate of DNA strand breaks in human carcinoma cells (HT49 and A431 cells). In contrast AE had no such effect at concentrations up to 100 μM. In cell-free assays, AOH inhibited DNA relaxation and stimulated DNA cleavage activity of topoisomerase I, IIα, and IIβ, with the IIα isoform, being particularly targeted. Thus, AOH was characterised as an inhibitor of topoisomerase activity, contributing to its genotoxic activity [58].

There are concerns about the association found between *A. alternata* contamination in cereal grains and the high levels of human oesophageal cancer in China [14]. TeA is toxic to several animal species, e.g., mice, chicken, dogs. In dogs, it caused haemorrhages in several organs; in chicken it reduced feed efficiency, suppressed weight gain and increased internal haemorrhaging. AOH and AME might cause cell mutagenicity, could combine with the DNA isolated from human foetal oesophageal epithelium, and AOH could induce squamous cell carcinoma of the foetal oesophagus. AOH has been reported to possess cytotoxic, genotoxic and mutagenic properties *in vitro* [39].

Reproductive and developmental toxicity. Experimentally, different doses of *Alternaria* species were administered to pregnant DBA/2 mice during gestation. The combined administration of AOH and AME (25 mg/kg each given together) on gestation days 9~12 led to an increased number of dead and resorbed foetuses, and runts per litter; an increase in the number of malformed foetuses was also reported. Administration of AOH at 100 mg/kg on gestation days 13~16, resulted in an increase in malformed foetuses. However, administration of AME at 50 mg/kg on gestation days 13~16 had no such effect. Feotol toxicity was apparent at 100 mg/kg of AOH and it was suggested that synergism between these may be occuring when administered together. However, as one or both *Alternaria* species occurred on different gestation days the results should be interpreted with caution [52].

Recently, it was demonstrated that ATX-II is more mutagenic than AOH in terms of DNA strand breaking in mammalian cells [59]. It induced mutations at the hypoxanthine guanine phosphoribosyltransferase gene locus at concentrations similar to that of the established mutagen 4-quinoline-N-oxide, thus proving to be at least 50 times more potent mutagen than the AOHs.

Guidelines and standards. At the present time there are no specific EU or international regulations regarding

Alternaria toxins in food [6, 40]. However, as stated earlier, EFSA [16] have recommended the collection of data throughout the EU on the relative contamination of different foodstuffs to examine the available evidence before making decisions on whether a full hazard analysis should be carried out with a view to considerations of either advisory information or eventual legislation, provided a comprehensive toxicological information is available.

CONTAMINATION OF DIFFERENT PRODUCT GROUPS BY *ALTERNARIA* SPECIES AND THEIR MYCOTOXINS

Tomatoes, vegetables and oilseeds and their products are very prone to contamination by *Alternaria* species and thus may be contaminated with *Alternaria* toxins especially at a_w levels > 0.93. There is also the potential for contamination of soft skin products including tomatoes, especially during refrigerated storage or transport, as *Alternaria* can grow slowly at low temperatures over a range of a_w levels. They sporulate easily on any crop debris and contaminate fruit surfaces. Terminiello *et al.* [60] studied the occurrence of *Alternaria* mycotoxins in 80 tomato purees processed and sold in Argentina. They found that 39 of these were contaminated with *Alternaria* mycotoxins. TeA was found in 23 samples (up to 4,021 μg/kg), 5 with AOH (up to 8,756 μg/kg), and AME in 21 samples (up to 1,734 μg/kg). In 10 samples there was co-occurrence of at least two of these toxins.

In Korea, *Alternaria* rot occurs in red pepper, tomato and paprika in both greenhouse and field crops, under moist conditions. In red pepper and paprika infection normally follows blossom end rot, which can be caused by nutritional deficiencies. Recent studies showed that several large-spored *Alternaria* species including *A. solani sensu stricto* and *A. tomatophila* cause diseases on potato and tomato. However, small-spored *Alternaria* species, especially *A. alternata*, also causes fruit rot of tomato and red pepper. *A. solani* isolates were equally aggressive on both tomato and potato, whereas *A. tomatophila* was highly aggressive to tomato [61]. In a study by Lee and Yu [62, 63], seven *Alternaria* species with large spores and long beaks, *A. cucumerina*, *A. dauci*, *A. macrospora*, *A. porri*, *A. sesami*, *A. solani*, *A. tagetica*, and *A. zinnia*, produced only AOH and AME. However, *A. brassicicola*, *A. helianthi*, *A. japonica*, *A. panax* and *A. radicina* did not produce any of the toxins [62, 63]. Thus potential exists for AOH and AME production by species with large spores and long beaks and *A. alternata* which has small conidia. Interestingly, of 45 isolates from red pepper, 23 (51.1%) produced TeA.

In the context of fresh fruits and vegetables for human consumption, *A. alternata* is especially important. New taxonomic tools have shown that this species is predominant in several crops, including fruits and vegetables. *A. tenuissima* has been reported as predominant in blueberries [64]; *A. tenuissima* followed by *A. arborescens* were the most abundant in tomatoes [65]. Polizzotto *et al.* [66] found that *A. tenuissima* and *A. arborescens* were the main *Alternaria* species contaminants of grapevine; *A. tenuissima* was the main species from this genus found in strawberries [67]. Due to lack of molecular variation, a molecular study recently pooled *A. arborescens* and *A. tenuissima* species-groups with *A. alternata* into one section [68] now comprising more than 50 species based on nucleotide sequence data [12].

There has been interest in developing methods for surveillance of tomato puree and the processed tomato-based products. Mislivec *et al.* [69] reported on the significance of TeA in fresh tomatoes for production of tomato sauce and tomato ketchup. Heavily contaminated samples of discoloured pecan nuts, sunflower seeds, sunflower seed meal and oil seed rape have also been found to contain some or all of these mycotoxins. Other sources include sorghum, wheat, rye, diseased rice and tobacco. Mycotoxigenic *Alternaria* species have also been implicated in colonizing building materials and been implicated in indoor human exposure problems. Good analytical methods for tomato products have been developed [70].

The stability of *Alternaria* toxins has not been studied in detail. However they are stable in fruit juices and wine for over 20 days or at 80°C for 20 min. When olives are pressed, only a very small percentage of the toxin is transferred to olive oil. However, heat treatment at 121°C for 60 min significantly reduced the concentrations of these toxins in *Alternaria* contaminated sunflower flour. The heat treated material caused some toxic effects when fed to rats. Because their natural occurrence is very low, risks in terms of human exposure is very limited.

Olives have occasionally been found to be contaminated with *Alternaria*. In Italy a study showed that 4 of 13 olive samples were contaminated with 2~4 *Alternaria* mycotoxins [71]. Poor quality damaged olives contained the highest contamination levels. Overall, no mycotoxins were detected in oil destined for human consumption (6 samples) or olive husks (3 samples) from olive oil mills after first pressing of the olives. In laboratory studies using the poorest olive samples, 4% of AME was transferred to the oil. Interestingly this study showed that in rice culture the strain produced significantly more TeA (3× greater).

On cottonseed the optimum environmental conditions for TeA production by *A. tenuissima* was 20°C/37.5% moisture content (= freely available water [1.00 a_w]). The absolute limiting condition of water availability was 14.9% (= 0.85 a_w). This is below the absolute minimum for germination, with growth and toxin production occurring with more available water [4, 25, 31]. It is thus possible that the a_w was not accurately controlled in these studies. At intermediate moisture levels (= 0.95 a_w) a 50% reduction in TeA was found in cottonseed, leading the authors to suggest that > 0.90 a_w and 20°C were required for TeA production. Different temperatures favour biosynthesis of these different mycotoxins by *Alternaria* spp. Visconti *et al.* [72] found similar TeA production levels for *Alternaria* from rapeseed, but no

temperature or water availability aspects were considered in their studies.

There is a need to identify the relative risk of *Alternaria* toxins in diverse food groups world-wide. Patriarca *et al.* [6] investigated the toxigenic potential of *Alternaria* strains isolated from Argentinean wheat. TeA was produced by 72% of the strains (1~14,782 mg/kg), AOH by 87% (4~622 mg/kg) and AME by 91% (7~2,625 mg/kg) when tested in autoclaved rice. All but one of 123 isolates were able to produce *in vitro* at least one of the toxins investigated. Most of the strains were able to synthesize more than one toxin: 74 isolates (60%) were positive for all three toxins, 30 (24%) for both AOH and AME, 5 (4%) for both TeA and AME, and 2 (2%) for TeA and AOH.

Greco *et al.* [64] characterized *Alternaria* species isolated from blueberries grown in Argentina. The genus *Alternaria* was the main component of the blueberry mycobiota (95%). *A. tenuissima* was the predominant species (95%), the *A. alternata* and *A. arborescens* species were also isolated in low proportion. The isolates showed a high toxigenic potential when tested in autoclaved rice; 97% of these produced AOH in a range from 0.14 to 119.18 mg/kg, 95% AME in a range from 1.23 to 901.74 mg/kg and 65% TeA in a range from 0.13 to 2,778 mg/kg. Fifty-two isolates co-produced the three mycotoxins.

With the advances in methodology for simultaneous detection of several fungal metabolites, the profiling of secondary metabolite production by *Alternaria* strains has replaced studies for the main mycotoxins. Although these investigations were mainly carried out for taxonomic purposes, knowing the full chemical potential and the distribution of *Alternaria* species on crops, is a useful tool to establish a toxicological risk assessment for food products for human consumption. Benavidez Rozo *et al.* [65] studied the profiles of secondary metabolites characteristic of *Alternaria* strains isolated from tomato affected by black mould. The isolates belonged to three species group, *A. tenuissima, A. alternata*, and *A. arborescens*. The most characteristic profile by *A. tenuissima* and *A. arborescens* strains was the simultaneous production of AOH, AME, TEN, and TeA, with the exception of some *A. arborescens* isolates that were also able to synthezise *Alternaria alternata* f. sp. *lycopersici* (AAL) toxins, while *A. alternata* isolates were able to co-produce AOH, AME, and TEN. In lower frequency strains from the three species were able to synthesize ALT, curvularin and dehydrocurvularin.

Polizzotto *et al.* [66] characterized 20 fungal endophytes belonging to the genus *Alternaria*, recovered from grapevine in different Italian regions. The isolates belonged to the *A. arborescens* and *A. tenuissima* species-group. Strains from both species consistently produced TeA (100% and 78%, respectively). Other metabolites produced by most strains were AOH, AME, ALT, and altersetin.

Andersen *et al.* [73] characterized the metabolite production of 87 *Alternaria* strains from different foods from Argentina, tomato, wheat, blueberries, and walnuts. The metabolites more frequently produced by the strains were AOH, AME, TEN, ATX (I and II), TeA, ALT, altersetin, and altenuic acid II. Several known secondary metabolites previously isolated from large-spored *Alternaria* species or other fungal genera were detected in this study, such as dehydrocurvularin, pyrenochaetic acid, alternarienonic acid and altechromone A. Eighteen major undescribed metabolites were also detected, some clearly derivatives of AOH, TEN, and TeA, while others presumable belong to new compound classes, based on their elemental composition UV/Vis and MS/MS spectra. Comparisons were made on the chemical potential of strains isolated from different sources, and no specific metabolite profile was found based on substrate, although some variations were observed in the amount of each metabolite produced. For example, the production of altenusin and pyrenochaetic acid was higher from strains isolated from blueberries than strains from the other products. Similarly, wheat might be more contaminated with TeA and alternarienonic acid than tomatoes.

Although the natural incidence of *Alternaria* and their mycotoxins in some major agricultural products have been examined in Korea [62, 63], information is overall, limited. Lee and Yu [62, 63] showed that potential mycotoxin problems might exist in some agricultural products due to the presence of species which produce large amounts of TeA, AOH, and AME. Species such as *A. dauci*, *A. porri*, and *A. radicina,* which did not produce TeA, were also not toxic to rats in feeding tests. They reported the production of major *Alternaria* mycotoxins, AOH, AME, ALT, and ATX-I by species of *A. sesami* and *A. sesamicola*, and of AME by *A. solani*. Of *Alternaria* species isolated from different agricultural sources in Korea, only a group of *A. alternata* strains was toxic to rats while those from *A. macrospora*, *A. japonica*, *A. sesami*, *A. sesamicola*, and *A. solani* were not toxic, although some species names of the isolates have been changed according to the current classification system by Woudenberg *et al.* [12]. Similar studies have isolated *Alternaria* from barley grains destined for malting purposes [10].

According to the review of safety of *Alternaria* toxins in food and feed by EFSA [16] which deals with AOH, AME, TeA, iso-TeA, ATXs, TEN, ALT, and AAL-toxins (*Alternaria alternata* f. sp. *lycopersici* toxins), feed occurrence data (1,150 results) were collected from the literature only. The highest concentrations for AOH, AME, TeA, and TEN were found in the food group 'Legumes, nuts and oilseeds' and in particular in sunflower seeds. Mean AOH levels in this food group were in the range of 22 μg/kg (lower bound [LB] mean) to 26 μg/kg (upper bound [UB] mean) with a max. value of 1,200 μg/kg. For AME, the average values were in the range 11 (LB) to 12 μg/kg (UB), with a max. value of 440 μg/kg. TeA was present in higher concentrations (LB mean, 333 μg/kg; UB mean, 349 μg/kg; max., 5,400 μg/kg). Mean values of TEN ranged from 47 (LB mean) to 50 μg/kg (UB mean) with a max. value of 880 μg/kg. Overall based on published occurrence data

about 300 feed and agricultural commodities in Europe, AOH was found in 31% of the feed and agricultural commodity samples at concentrations from 6.3 to 1,840 μg/kg (max. found in sunflower seeds). AME was found in 6% of the samples with levels ranging from 3 to 184 μg/kg (max. found in cereals). ALT was found in 73% of the samples with concentrations of between 6.3 and 41 μg/kg (max. found in wheat grains). TeA was present in 15% of the samples with levels varying between 500 and 4,310 μg/kg (max. found in oats). Azcarate *et al.* [13] investigated the natural occurrence of *Alternaria* mycotoxins in Argentinean wheat. *Alternaria* was the main component of the mycota, with an infection percentage of 100% in the samples. AOH was detected in 4 (6%) of 64 samples, with a range of 645 to 1,388 μg/kg; AME, with a range of 566 to 7,451 μg/kg in 15 (23%) of 64 samples; and TeA in 12 (19%) of 64 samples, with a range of 1,001 to 8,814 μg/kg. Co-occurrence of the toxins was also detected. Four samples contained TeA and AME, and TeA and AOH. AME was the predominant toxin, but TeA was detected in higher concentrations. The mycotoxin levels found in Argentinean wheat were higher than those reported by EFSA [16] and found in grains from Korea [62, 63].

This review has tried to integrate the available knowledge on the diversity, ecophysiology, competitiveness and the mycotoxin production capacity and toxicology of important *Alternaria* species. They are important because of the damage that they cause to economically important food crops and the contamination of a wide range of commodities with mycotoxins. The key questions which will need to be answered in the near future is whether climate change scenarios may result in a change in the dynamics of the fungal community structure in food crops and result in more economic impact and whether this could lead to more associated problems with mycotoxin contamination of products for both human and animal food and feed chains.

ACKNOWLEDGEMENTS

This study was in part supported by a grant (15162-FOD-044) from National Institute of Food and Drug Safety Evaluation under the Ministry of Food and Drug Safety, and in part by a fund (PJ01091404) from National Institute of Animal Science under Rural Development Administration, Republic of Korea.

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This article has been corrected. See Mycobiology. 2015 September; 43(3); 371.
10.5941/MYCO.2015.43.3.371

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2016-06-30

Lee H et al. Mycobiology, 2015 Jun, Vol.43, Iss 2, pp 93-106