Alternaria spp. and their mycotoxins in tomatoes. A scientific approach from field to food industry

Núria Estiarte Piñol

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Alternaria spp. and their mycotoxins in tomatoes. A scientific approach from field to food industry.

DISSERTATION

to obtain the degree of Doctor by the University of Lleida in the Agricultural and Food Science and Technology Program

by

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HACEN CONSTAR

Que, bajo su dirección, la Sra. Nuria Estiarte Piñol ha realizado el trabajo de investigación titulado "*Alternaria* spp. and their mycotoxins in tomatoes. A scientific approach from field to food industry", que presenta para optar al grado de Doctora por la Universitat de Lleida. Considerando que el trabajo realizado constituye tema de Tesis Doctoral, autorizan su exposición y defensa en la Universitat de Lleida.

Y para que así conste, se expide el presente a 1 de junio de 2016.

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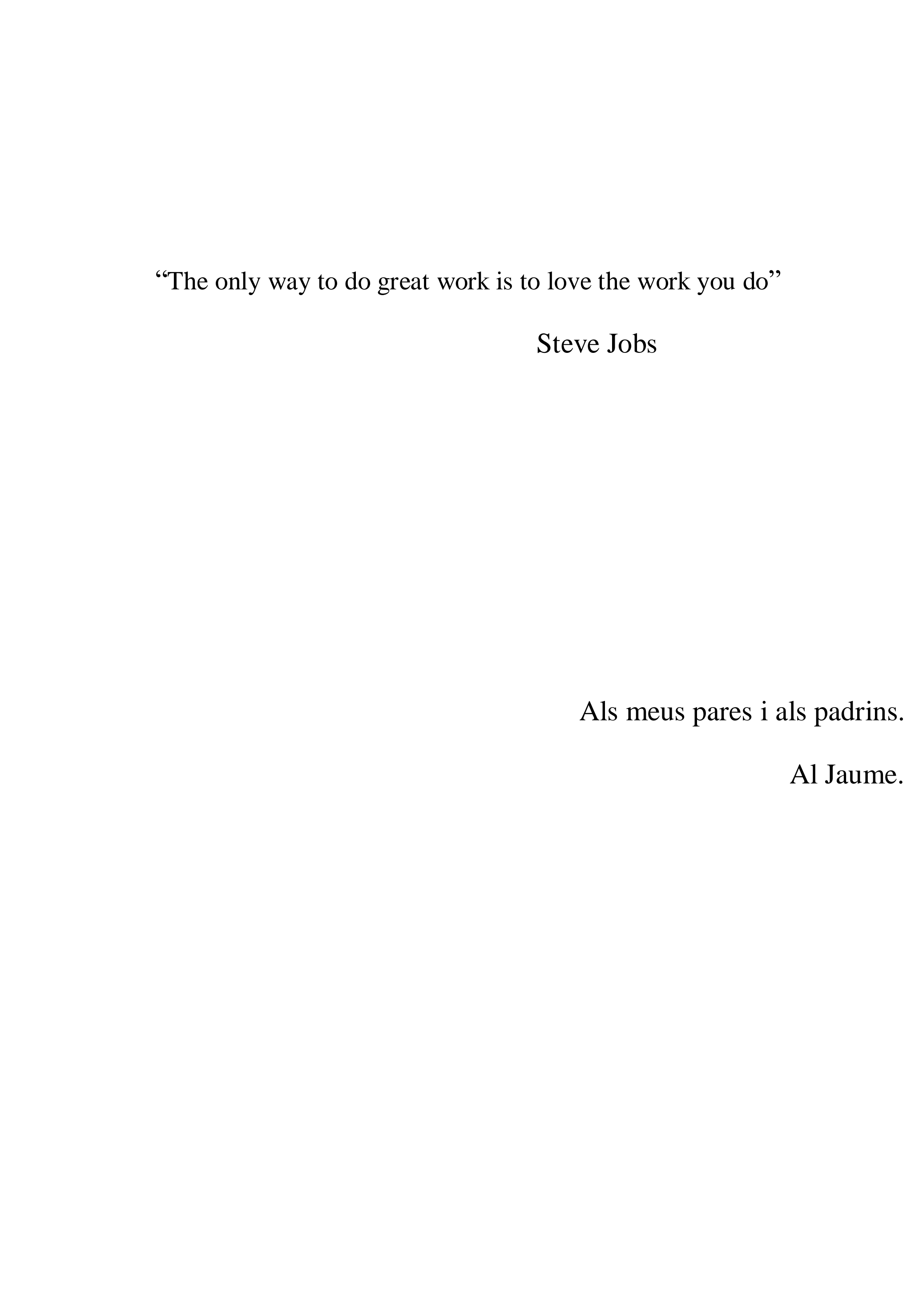

"The only way to do great work is to love the work you do"

Steve Jobs

Als meus pares i als padrins.

Al Jaume.

LIST OF ABBREVIATIONS

1-MCP	1-methylcyclopropene
AA	Amino acid adenylation domain
AAL TA1, TA2	*A. alternata* f. spp. *lycopersici* toxins
ACP	Acyl carrier protein
AF	Aflatoxin
AFM1	Aflatoxin M_1
ALT	Altenuene
AltR	Alternariol regulator
AME	Alternariol monomethyl ether
AOH	Alternariol
AT	Acyltransferase
ATX-I, -II, -III	Altertoxin I, -II, -III
BLAST	Basic local alignment search tool
CCP	Critical control point
CD	Condensation domain
CS	Chalon-and Stilben-Synthase (N)/(C)
DFMO	Difluoromethylornithine
DH	Dehydratase
DMI	Sterol-demethylation inhibitor
DON	Deoxynivalenol
EFSA	European Food Safety Authority
ER	Enoyl reductase
FB	Fumonisin B_1, B_2
HPLC	High-performance liquid chromatography
HT-2	HT-2 toxin
IARC	International agency of research on cancer
KR	Ketreductase
KS	β-ketoacyl synthase
MeT	Methyltransferase
NAD	NAD binding domain

NIV	Nivalenol
NLX	Norlichexanthone
ODC	Ornithine decarboxylase
OTA	Ochratoxin A
PAT	Patulin
PCR	Polymerase chain reaction
PKS	Polyketide synthases
PMA	Propidium monoazide
PTI	Polyamine transport inhibitor
QoI	Quinone outside inhibitor
qPCR	Quantitative polymerase chain reaction
RCP	Representative concentration pathway
STX III	Stemphyltoxin III
T-2	T-2 toxin
TeA	Tenuazonic acid
TEN	Tentoxin
UDG	Uracil DNA Glycolase Superfamily
UV	Ultraviolet
ZEN	Zearalenone

RESUM

La Comissió Europea va demanar a l'Autoritat Europea en Seguretat Alimentària (EFSA) avaluar el risc de les toxines produïdes per les espècies d'*Alternaria,* sovint presents en productes alimentaris i pinsos, amb el propòsit que la pròpia Comissió Europea i les autoritats competents dels diferents estats membres poguessin considerar la possibilitat d'establir mesures de control i, a la vegada, omplir els buits de coneixement existents. Com a resultat, al 2011, l'EFSA va emetre un comunicat científic titulat "Opinió científica sobre el risc existent per animals i la salut pública en relació a la presència de toxines d'*Alternaria* en productes alimentaris i pinsos". Va ser arran de la publicació d'aquest comunicat que va sorgir la idea de desenvolupar la present Tesi Doctoral.

Per iniciar aquesta Tesi Doctoral, en primer lloc, es va avaluar l'estat de la qüestió. Amb aquest objectiu es van escollir dues empreses alimentàries de la província de Lleida i es va iniciar la recol·lecta de mostres de tomata durant dues campanyes de collita compreses entre el 2012 i el 2014. D'aquestes mostres de tomata es va determinar la presència d'espècies viables d'*Alternaria* mitjançant la tècnica PMA-qPCR. A més, també es va analitzar la presència de dues micotoxines d'*Alternaria,* l'alternariol (AOH) i l'alternariol monometil èter (AME). Els resultats van demostrar la presència d'*Alternaria* en tomates fresques destinades per a la producció de derivats de tomata en ambdues industries alimentàries. Curiosament, es va observar que els nivells d'*Alternaria* augmentaven en les mostres preses dins de la planta de producció. Aquest fet, probablement era degut a què les tomates fresques es submergien en banys d'aigua, els quals probablement estaven altament contaminats amb *Alternaria*. En relació a la presència d'AOH i AME al llarg del processat industrial, els resultats van demostrar que els nivells d'ambdues toxines disminuïen a mesura que avançava el procés, exceptuant les mostres de concentrat de tomata, en les quals aproximadament el 50% de les mostres estaven contaminades en un interval comprés entre 22.6 i 137.4 μg/kg de tomata.

En segon lloc, es va intentar abordar el tema des d'una perspectiva més global, del camp a la taula. Primerament, es van estudiar dos components de la família de proteïnes Velvet (VeA i LaeA) i, a la vegada, es va indagar en el metabolisme de les poliamines en *Alternaria alternata.* L'objectiu d'ambdós estudis era trobar noves diana útils per al disseny de nous fungicides, ja que s'ha observat l'aparició de fongs resistents a determinats fungicides comunament emprats comercialment. Els resultats derivats d'aquests estudis van demostrar que, tant LaeA com VeA, estaven involucrats en funcions essencials d'*A. alternata*, incloent el creixement fúngic, el desenvolupament asexual i la biosíntesi d'AOH i AME. Per tant, seria convenient explorar més a fons ambdós components per tal d'utilitzar-los, en un futur, com a possibles diana antifúngics. En relació al metabolisme de les poliamines, va ser interessant observar que dos anàlegs de les poliamines (AMXT-2455 i AMXT-3016) van obtenir resultats prometedors a l'hora de controlar la infecció fúngica provocada per *A. alternata,* ja sigui en tomateres o en tomates fresques, tot i que en aquest darrer cas caldria optimitzar el tractament. El següent pas dut a terme, ja en un context de postcollita, va ser analitzar els efectes de l'1-metilciclopropè (1-MCP) en *A. alternata* infectant tomates en relació al creixement fúngic i a la producció d'AOH i AME. Els resultats van demostrar que la infecció per *Alternaria* era superior en aquelles tomates tractades amb 1-MCP quan les condiciones d'emmagatzematge eren favorables al creixement d'*Alternaria*. En canvi, el tractament amb 1-MCP de les tomates inoculades amb *A. alteranta* no va afectar la producció d'AOH i AME en dues de les tres varietats de tomata emprades. Finalment, es va estudiar l'estabilitat de l'AOH i l'AME durant el procés industrial de productes derivats de tomata. Els resultats van demostrar que ambdues micotoxines són altament estables al llarg del processat industrial. Es va observar que el rentat de les tomates, especialment si s'utilitza aigua clorada, és l'etapa industrial que aconsegueix reduir la contaminació d'AOH i AME més eficientment.

RESUMEN

La Comisión Europea pidió a la Autoridad Europea en Seguridad Alimentaria (EFSA) evaluar el riesgo de las toxinas producidas por las especies de *Alternaria*, a menudo presentes en productos alimentarios y piensos, con el propósito de que la propia Comisión Europea y las autoridades competentes de los distintos estados miembros pudieran considerar la posibilidad de instaurar medidas de control y, a su vez, llenar los vacíos de conocimiento existentes. Como resultado, en 2011, la EFSA emitió un comunicado científico titulado "Opinión científica sobre el riesgo existente para animales y la salud pública en relación a la presencia de toxinas de *Alternaria* en productos alimentarios y piensos". Fue a partir de la publicación de este comunicado que surgió la idea de desarrollar la presente Tesis Doctoral.

Para iniciar esta Tesis Doctoral, en primer lugar, se evaluó el estado de la cuestión. Con este objetivo se escogieron dos industrias alimentarias de la provincia de Lleida y se inició la toma de muestras de tomate durante dos campañas de cosecha (2012-2014). En estas muestras de tomate se determinó la presencia de especies viables de *Alternaria* mediante la técnica PMA-qPCR. Paralelamente, también se analizó la presencia de dos micotoxinas de *Alternaria*, el alternariol (AOH) y el alternariol monometil éter (AME). Los resultados demostraron la presencia de *Alternaria* en tomates frescos destinados a la producción de derivados de tomate en ambas industrias alimentarias. Curiosamente, se observó que los niveles de *Alternaria* aumentaban en las muestras recogidas dentro de la planta de producción. Este hecho quizá se debió a que los tomates frescos se sumergían en baños de agua, los cuales probablemente estaban altamente contaminados con *Alternaria*. En relación a la presencia de AOH y AME a lo largo del procesado industrial, los resultados demostraron que los niveles de ambas toxinas disminuían a medida que avanzaba el procesado, exceptuando las muestras de tomate concentrado, ya que aproximadamente el 50% de estas muestras estaban contaminadas en un intervalo entre 22,6 y 137,4 µg/kg de tomate.

En segundo lugar, se intentó abordar el tema desde una perspectiva más global, del campo a la mesa. Primeramente, se estudiaron dos componentes de la familia de proteínas Velvet (VeA y LaeA) y, a su vez, se indagó en el metabolismo de las poliaminas en *A. alternata*. El objetivo de ambos estudios fue encontrar nuevas diana útiles para el diseño de nuevos fungicidas, ya que se había observado la aparición de mohos resistentes a determinados fungicidas comúnmente empleados a nivel comercial. Los resultados derivados de este estudio demostraron que, tanto LaeA como VeA, estaban involucrados en funciones esenciales de *A. alternata*, incluyendo el crecimiento fúngico, el desarrollo asexual y la biosíntesis de AOH y AME. Por lo tanto, sería conveniente explorar más a fondo ambos componentes para poderlos utilizar, en un futuro, como posibles diana antifúngica. En relación al metabolismo de las poliaminas, fue interesante observar que dos análogos de las poliaminas (AMXT-2455 y AMXT-3016) obtuvieron resultados prometedores a la hora de controlar la infección fúngica provocada por *A. alternata*, ya fuera en plantas de tomate o en tomates frescos, aunque en este último caso sería necesario optimizar el tratamiento. El siguiente paso llevado a cabo, en un contexto de poscosecha, fue analizar los efectos del 1-metilciclopropeno (1-MCP) en el crecimiento fúngico y la producción de AOH y AME por parte de *A. alternata*, infectando para ello tomates y tratándolos con este compuesto. Los resultados demostraron que la infección por *Alternaria* era superior en aquellos tomates tratados con 1-MCP cuando las condiciones de almacenamiento eran favorables al crecimiento de *Alternaria*. En cambio, el tratamiento con 1-MCP de los tomates inoculados con *A. alteranta* no afectó a la producción de AOH y AME en dos de las tres variedades de tomate usadas. Finalmente, se estudió la estabilidad del AOH y AME durante el proceso industrial de productos derivados de tomate. Los resultados demostraron que ambas micotoxinas fueron altamente estables a lo largo del procesado industrial. Se observó que el lavado de los tomates, especialmente si se utilizaba agua clorada, fue la etapa industrial que consiguió reducir la contaminación de AOH y AME más eficientemente.

ABSTRACT

The European Commission asked the European Food Safety Authority (EFSA) to review the risk of *Alternaria* toxins in food and feed based on the available information, with the purpose of enabling the European Commission and the competent authorities in the Member States to consider the need for a possible follow up and to fill the knowledge gaps. As a result, in 2011 the EFSA emitted a scientific report entitled "Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food". It was from this publication that aroses the idea of developing this Doctoral Thesis.

The first step of this Doctoral Thesis was to assess the state of the matter. With this aim, two different food industries from the province of Lleida (Spain) were selected, and tomato samples were collected during two seasons (2012-2014). From these samples the presence of viable *Alternaria* spp. was detected, using a PMA-qPCR technique specially developed for this purpose, and, besides, the occurrence of two *Alternaria* mycotoxins, alternariol (AOH) and altenariol monomethyl ether (AME), was analyzed. Results evidenced the presence of *Alternaria* spp. in raw tomatoes used for the production of tomato derived products in both industries. Interestingly, it was observed that levels of viable *Alternaria* spp. increased once inside the production plant, probably because the immersion of raw tomatoes in washing baths with contaminated water. Dealing with the presence of AOH and AME throughout the tomato production chain, results showed that their levels decreased along the production of tomato derivatives, except for the concentrated product in which almost 50% of samples were contaminated with AOH in a range between 22.6-137.4 μg/kg of tomato.

A second approach to the problem was to address the matter considering a global perspective, from the field to the food industry. Firstly, we focus our study in analyzing two components of the Velvet family (VeA and LaeA) and the polyamine metabolism in order to find new targets useful to design new fungicides,

since it has been demonstrated that there are appearing problems of fungal resistance towards some of the existent fungicides. Results demonstrated that LaeA and VeA are involved in essential functions of *A. alternata*, including mycelial growth, asexual development, and AOH and AME production, and therefore, it could be convenient to explore more deeply their use as antifungal targets. In relation to polyamine metabolism, it was interesting to find out that two polyamine analogs (AMXT-2455 and AMXT-3016) demonstrated promising results to control *Alternaria* infection on tomato plants even though in tomatoes it needed some improvements. Secondly, in a postharvest context, we analyzed the effects of 1-methylcyclopropene (1-MCP) on *A. alternata* infecting tomatoes in relation to fungal growth and mycotoxin production. It was observed that black spot disease produced by *A. alternata* was significantly more severe on 1-MCP treated tomatoes when storage conditions were favorable for fungal growth. In contrast, 1-MCP treatment did not significantly affect AOH and AME biosynthesis in two of the three assayed varieties. And finally, we study the stability of AOH and AME along the industrial process of tomato derivate products, observing that both mycotoxins were quite stable throughout the tomato production chain. Results pointed out that the washing step, especially using chlorinated water, was the most efficient measure to decrease both AOH and AME initial levels.

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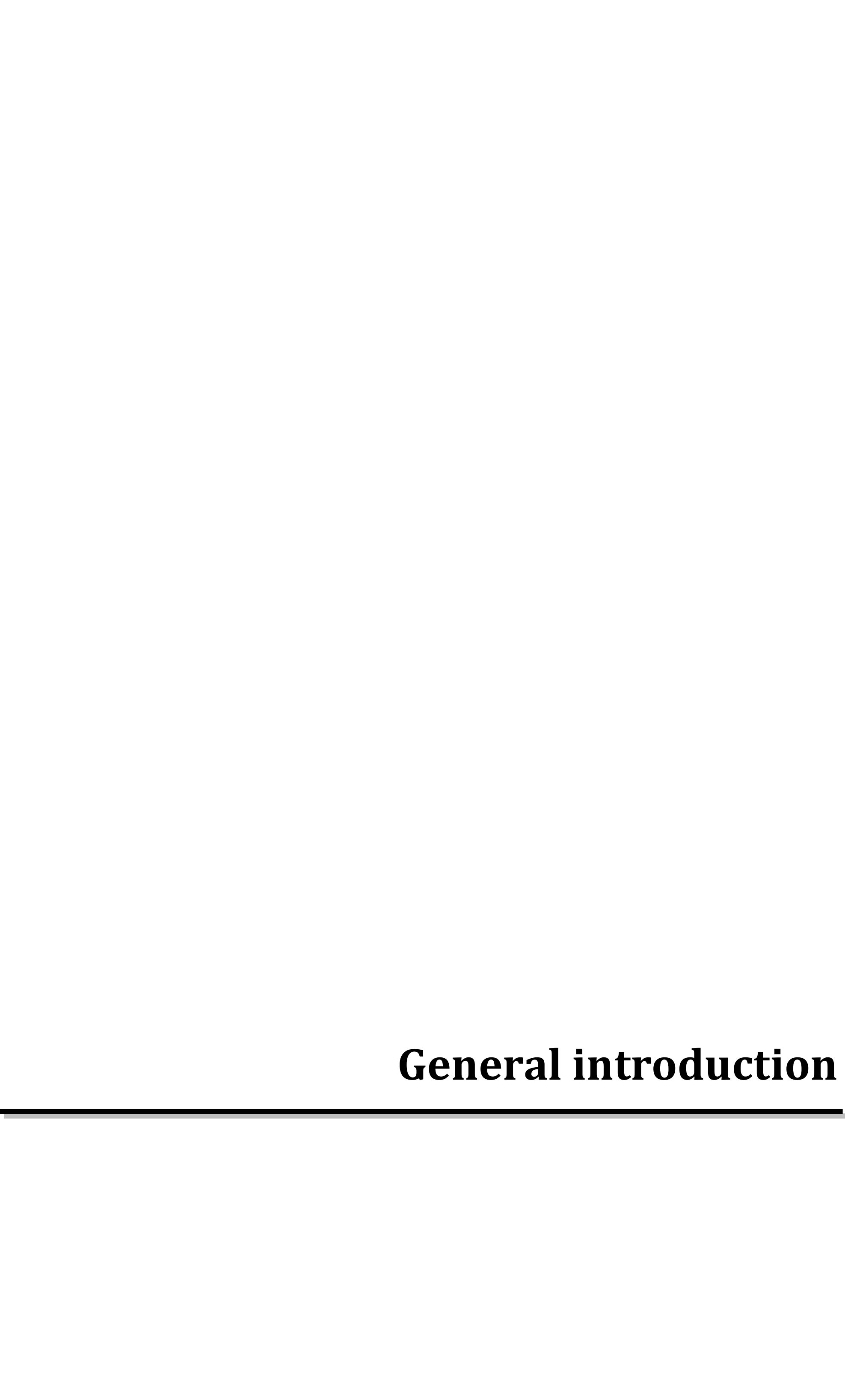


General introduction

1. Molds and mycotoxins

Fungi are microscopic organisms belonging to the group of eukaryotes, which includes microorganisms such as yeasts and molds, and multicellular fungi commonly known as mushrooms. The difference between yeasts and molds lies in their living structure and their methods of reproduction. Yeasts are microscopic organisms consisting of solitary cells that reproduce by budding. In contrast, molds occur in long pluricellular filaments known as hyphae, which grow by apical extension. The network that hyphae form is called mycelium and it is considered to be a single organism. Filamentous fungi are divided into three or two groups, depending on the reference source: Zygomycetes, Deuteromycetes and Ascomycetes, although most recent theories include Ascomycetes in the Deuteromycetes group (Pitt and Samson, 2007; Taylor, 2011). Most fungi that cause food spoilage and have the capacity to produce toxic metabolites are included in the Deuteromycetes group. In this group, the hyphae are divided by cross-walls, called septa, which delimit hyphae in different compartments. Each compartment can contain one or multiple genetically identical nuclei. Reproduction in the Deuteromycetes is via asexual spores called conidia. Traditionally, the mode of formation and the shape of conidia have been used for taxonomical purposes.

Fungi and human civilization have shared a long history together. References of filamentous fungi were found in Greek literature (Galagan et al., 2005). Several stones representing mushrooms were found in Guatemala and were associated with the Mayan culture (Lowy, 1971). In the most recent history, fungi have largely been used on account of their beneficial properties, such as in biological processes to produce several food commodities or for the production of antibiotics. Additionally, various enzymes produced by fungi are used industrially and as biological pesticides to control weeds, plant diseases and insect pests. However,

although fungi can be beneficial for our society, they can also be very detrimental, causing plant diseases and producing several secondary metabolites, some of them toxic for humans and animals.

The number of known species of fungi is estimated as at least 74 K but could be even higher. In 1990, the magnitude of fungal diversity was estimated conservatively at 1.5 M species (Hawksworth, 1991; Hawksworth, 2001).

The last decade has witnessed a revolution in genomics. In the fungal kingdom, the first fungal genome was sequenced in 2003 (Galagan et al., 2003) and, since then, the number of available fungal genome sequences has increased and will probably increase exponentially. All these data are an excellent contribution for sectors such as biology, medical research and industrial profits (Galagan et al., 2005).

1.1. Molds in the food industry

Many fungi are beneficial in the food industry, especially for several biological processes and fermented foods, since they positively contribute to developing the desired organoleptic characteristics. This is the case of the production of some cheeses (blue cheese, soft ripened cheese and rind-washed cheese), sausages and soy sauce. However, the presence of molds in crop fields or in food commodities is not always associated with beneficial properties, quite the opposite, since they are responsible for causing the spoilage of fruits, vegetables and food commodities.

The most significant fungal genera responsible for food spoilage and, at the same time, mycotoxin producers, are *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* and *Alternaria* (Rodríguez et al., 2015). Until recently, fungal spoilage of raw fruits or vegetables was generally associated with an aesthetic issue, although nowadays we know that the problem is more severe than that and is linked to a significant constraint in global food safety. This is because some of them produce toxic metabolites, called mycotoxins, which are dangerous to humans and animals, the

chemical characteristics of which will be discussed in the next section (Pitt and Hocking, 1997).

For farmers, the presence of molds in crop fields represents important economic losses. To control fungal pests, farmers usually spray land with fungicides. However, for consumers the threat comes from the toxins that these fungi can produce. If fungal contamination is not controlled in the crop fields, the raw material that will enter the food industry can be highly contaminated with fungal toxins. In this respect, many countries have adopted regulations to limit mycotoxin exposure (Ramos, 2011b). Hence, early detection of undesirable fungi in raw materials or pre-processed foods, followed by corrective actions to avoid risks associated with mycotoxin accumulation or food spoilage, are needed. However, it is important to mention that the presence of toxigenic or spoilage molds in foods does not always indicate that mycotoxin production or food spoilage occurs.

1.2. Mycotoxins

1.2.1. What are mycotoxins?

The term “mycotoxin” combines the terms *mykes*, the Greek word for molds, and *toxicum*, the Latin word for toxic or poisonous. Generally, mycotoxins are low-molecular-weight natural products produced by filamentous fungi as secondary metabolites. They are generally produced at the end of the exponential growth phase. Mycotoxins evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route such as ingestion, inhalation or skin contact (Bennett, 1987; Bennett and Klich, 2003). The physiological function of mycotoxins is not yet completely understood, although some theories point to some kind of metabolic control mechanism within the fungus, as well as a defense mechanism against other organisms, or even an evolutionary advantage for colonizing certain biological niches (Ramos, 2011a). The toxic effect of

mycotoxins on animal and human health is referred to as mycotoxicosis, the severity of which depends on the toxicity of the mycotoxin, the extent of exposure, age, sex and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed. Furthermore, the kind of living organism which is affected is also important as, while some mycotoxins can be detrimental for one organism, the same ones could be innocuous for another (Drusch and Aumann, 2005; Peraica et al., 1999).

1.2.2. A little bit of history

Mycotoxins have likely always existed, but recognition of the true chemical nature of such entities of fungal metabolism did not occur until recent times. Looking back in time, it has been speculated that a mycotoxicosis could have been the cause of the last of the Ten Plagues in Ancient Egypt. The firstborn could have passed away following the opening of the grain storage facilities, whose contents was contaminated by toxic fungi, due to the growth of mycotoxigenic molds during the harvest, which was supposed to be especially rainy, probably because of the Seventh Plague (Marr and Malloy, 1996; Ramos et al., 2011). More references to mycotoxicosis are associated with the bubonic plague that took place in Europe from the 14th to the 17th century. It has been reported that there were several years of abundant rain, which triggered the production of mycotoxins on the grain and could have caused the death of many rats. With this scenario, the fleas were forced to quest for an alternative host that turned out to be humans (Matossian, 1986). More recent intoxications are dated between the 1940s and 1950s, when episodes of lethal disease in humans arose in Russia during the early years of the Second World War. In this case, the cause of the intoxication was attributed to T-2 toxin, a metabolite biosynthesized by *Fusarium sporotrichioides* (Richard, 2003). These are just some of the outbreaks caused by different mycotoxins over history, although there are more cases. In fact, it was an episode of mycotoxicosis on turkey poults that favored the appearance of modern mycotoxicology, which began

with the discovery of aflatoxins in 1961 (Richard, 2007). From then until 1975 is considered to be the golden era for mycotoxins, as many scientists focused their research on their study. In the beginning, mycotoxin research was closely related to research on fungal antibiotics. For instance, patulin was first isolated from *Penicillium patulum* during the search for antibiotics in 1941 (Singh, 1967).

1.2.3. A risk for human health

Toxicological studies of mycotoxins have usually been led by agriculturalists, chemists, microbiologists, and veterinarians rather than toxicologists, who usually tend to focus on the study of hazardous chemicals, such as polyaromatic hydrocarbons, heavy metals, and organic pesticides. Hence, mycotoxins research is usually conducted by researchers who are often unfamiliar with the basic principles of toxicology, which may sometimes be an issue.

Table 1. IARC classification of the main mycotoxins (IARC, 1993).

IARC number	Definition	Mycotoxins
1	The mycotoxin is carcinogenic to humans	AFs
2A	The mycotoxin is probably carcinogenic to humans	–
2B	The mycotoxin is possibly carcinogenic to humans	AFM1, FBs, OTA, sterigmatocystin
3	The mycotoxin is not classifiable as to its carcinogenicity to humans	DON, NIV, PAT, T-2/HT-2, ZEN, citrinin, fusarenon-X
4	The mycotoxin is probably not carcinogenic to humans	–

AFs: aflatoxins; AFM_1: aflatoxin M_1; FBs: fumonisins B_1, B_2 and B_3; OTA: ochratoxin A; DON: deoxynivalenol; NIV: Nivalenol PAT: patulin; T-2/HT-2: T-2 toxin/ HT-2 toxin; ZEN: zearalenone.

The International Agency for Research on Cancer (IARC) has classified several mycotoxins according to human health hazards (Table 1). This classification just

includes those mycotoxins that are regularly found in food and feedstuff and are legislated by international or national organizations (aflatoxins, ochratoxin A, patulin, and different toxins produced by *Fusarium* spp.). However, in the last decade, another group of toxins produced by *Alternaria* spp. has attracted the attention of researchers and the European Community, although these latter mycotoxins will be further described in the following chapters.

The European Union and many other countries, in view of the real and harmful hazard related to mycotoxin consumption, have adopted regulations to limit exposure to mycotoxins by final consumers. At the same time, the Codex Alimentarius Commission has developed a code of practice for the whole system to control mycotoxin contamination in feed and food (CAC, 2003; EC, 2006).

1.2.4. Risk of mycotoxin contamination

Generally, there are two causes responsible for the contamination of food products with mycotoxins. The first cause comes from the contamination of agricultural commodities in the field and upon postharvest storage. The second cause is associated with residues from animal-derived food via mycotoxin-contaminated feed (Drusch and Aumann, 2005). Methods to control mycotoxins are mainly preventive and include good agricultural practices and sufficient drying of crops after harvest (Lisker and Lillehoj, 1991). There is considerable on-going research on methods to prevent preharvest contamination of crops. These approaches include developing host resistance through plant breeding and through enhancement of antifungal genes by genetic engineering, use of biocontrol agents, and targeting regulatory genes in mycotoxin development (Brown et al., 1998; Rajasekaran et al., 2006). However, until now, none of these methods has solved the mycotoxin problem, because their formation is often unavoidable. In fact, nowadays, most of the efforts aimed at solving the mycotoxin problem simply involve the removal of mycotoxin-contaminated commodities from the food supply

through government screening and regulation programs, and food safety programs in industries (Bennett and Klich, 2003).

In general, mycotoxin exposure is more likely to occur in parts of the world where poor methods of food handling and storage are common, where malnutrition is a problem, and where few regulations exist to protect exposed populations. However, even in developed countries, specific subgroups may be more vulnerable to mycotoxin exposure. In the United States, for example, Latin American populations consume more corn products than the rest of the population and, usually, corn products are more vulnerable to contamination by several mycotoxins (Barrett, 2000; Bennett and Klich, 2003). In developed countries, the main problems with mycotoxins are associated with animal health, because animal feeds are more likely to contain mycotoxins and thus animal mycotoxicosis occurs, but they are difficult to diagnose due to subtle or nonspecific effects (CAST, 2003).

1.2.5. Climate change and mycotoxins

There is strong evidence indicating that climate change may affect mycotoxins in food and feed (Magan et al., 2011; Paterson and Lima, 2010; 2011). Climate change or extreme climatic events are already part of our everyday life and can be seen more and more frequently (Sant'Ana, 2010). Concentrations of methane, carbon dioxide, nitrous oxide and chlorofluorocarbons in the atmosphere are increasing, resulting in environmental warming, greater precipitation, or drought (Paterson and Lima, 2011). Climate change will affect climatic parameters, such as temperatures or rainfall, but also food security. In the fields, climate change will affect yields and quality of food crops. Spoilage and contamination of toxigenic fungi on crops may also change, because they are highly susceptible to environmental factors. Consequently, mycotoxin biosynthesis could also be affected, although this change will likely be region-dependent, positive for some regions and negative for others. In those regions in which there may be an increase in the occurrence of mycotoxins, this can have significant effects on human and

animal health, because mycotoxins can be carcinogenic and, at the same time, they are difficult to destroy during processing (Magan et al., 2011). This concern will have an especially negative impact in those countries where the environmental conditions are characterized by high humidity and temperatures that favor fungal proliferation, resulting in contamination of food and feed. It will be even worse in developing countries, where the poor socio-economic status of many of the inhabitants predisposes them to the consumption of mycotoxin contaminated products, either directly or at various points in the food chain, this being a significant risk with serious impacts on their health (Wagacha and Muthomi, 2008).

2. The genus *Alternaria*

Alternaria is a ubiquitous fungal genus that includes saprophytic and pathogenic species. It is abundant in the atmosphere and in the soil and is also known as a serious plant pathogen associated with a wide variety of substrates, including seeds, plants and agricultural commodities. Some of the substrates susceptible to its colonization are plants including cereals, tomatoes, cucumbers, cauliflowers, peppers, apples, melons, tangerines, oranges, lemons, and oilseeds, among others. The most commonly reported species include *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria* (Barkai-Golan, 2008; EFSA, 2011; Robiglio and Lopez, 1995). *Alternaria* spores are one of the most common and potent airborne allergens and sensitization to *Alternaria* allergens has been determined to be one of the most important factors in the onset of childhood asthma in arid regions (Halonen et al., 1997).

2.1. Morphology, taxonomy and molecular analysis

Alternaria was originally described by Nees von Esenbeck (1816), based on *A. tenuis* as the type specimen. Nowadays, the defining characteristics of *Alternaria* include dark multicelled conidia with transverse and longitudinal septa that occur in chains or borne singly (Fig. 1). The conidial shape is usually ovate or obclavate with a final beak (Ellis, 1971). Although *Alternaria* morphological traits are often variable and easily degenerate in laboratory conditions, they are still considered the main keys for taxonomic *Alternaria* identification, based on the presence or absence of a beak, conidial and beak shape, and conidial arrangement.

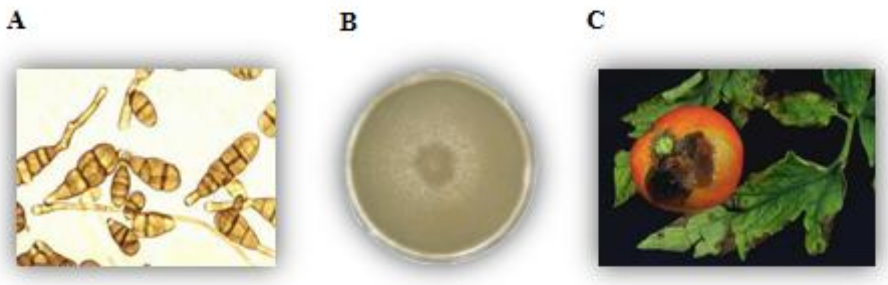


Figure 1. A. *Alternaria* spp. conidia. **B.** *Alternaria alternata* grown on PDA. **C.** *Alternaria* spp. infecting a tomato and a tomato plant.

Several years after its discovery, as a result of a lifetime study on *Alternaria*, Simmons (2007) summarized *Alternaria* taxonomy based on morphological characteristics and 275 *Alternaria* species were recognized (Woudenberg et al., 2013). However, probably due to the lack of molecular techniques, *Alternaria* genus has suffered from considerable taxonomic uncertainty and flux since its inception. In 2000, the first molecular phylogenetic analysis of *Alternaria* was presented and revealed that the *Stemphylium* spp. were phylogenetically distinct from *Alternaria* and *Ulocladium* spp., while most *Alternaria* spp. and *Ulocladium* spp. were placed together in a large clade with internally nested phylogenetically distinct clades referred to as the *alternata*, *radicina*, *porri*, *brassicicola* and

ulocladium species-groups (Pryor and Gilbertson, 2000). Later studies, supported by the analysis of the five-gene combined dataset (*gpd*, *Alt a1*, Actin, Plasma membrane ATPase, Calmodulin), have reported that *Alternaria* and *Ulocladium* are two distinct and strongly phylogenetic lineages (Lawrence et al., 2013).

Traditional methods to identify and classify microorganisms, including fungi, are based on microscopy, culture and biochemical tests. These techniques remain the core basis and standard practice in most laboratories but new techniques, such as nucleic acid-based assays, have emerged and are being rapidly introduced. These new methodologies offer several advantages over traditional ones, such as effectiveness, rapidness, reliability and sensibility. These techniques have been useful for sequencing, taxonomy studies, gene function and expression studies, among others. Lately, polyphasic approaches, combining traditional morphology, molecular sequence analysis and secondary metabolite profiling, have been successfully used for the identification of plant pathogenic *Alternaria* spp. (Andersen et al., 2008). However, other polyphasic studies of *Alternaria* showed an overabundance of morphological, chemical and molecular variations that made species identification, chemotaxonomy and even molecular phylogeny unachievable (Andersen et al., 2015; 2009).

Approximately 300 species are included in the genus *Alternaria* and a large morphological diversity has been observed among them. Therefore, considerable and great efforts have been made to organize taxa into subgeneric species-groups (Simmons, 2007). Nevertheless, the large variability is not just at a morphological level but also at a genetic level, even when strains are of the same species (Aradhya et al., 2001; Guo et al., 2004). In this respect, it was recently demonstrated at the genomic level that individual strains of *Alternaria* spp. may possess a thousand or more predicted unique genes (Dang et al., 2015). This characteristic complicates even more the task of identifying and classifying *Alternaria*.

2.2. Ecophysiology

As mentioned in previous sections, the taxonomy of *Alternaria* has been regularly revised in the past decade and it has been reported to be particularly controversial. When isolates were identified using traditional methods, based on morphological characteristics of conidia, most of them were classified as *A. alternata*, thus leading to a general belief that this was the most common species in food products. Consequently, the few ecophysiology studies available in the literature on *Alternaria* have been developed for *A. alternata* (Vaquera et al., 2016). In this respect, it has to be taken into consideration that, although different *Alternaria* spp. might be closely related, their ecophysiology might differ. Despite that, there is the general notion that temperature and water activity (a_w) are among those factors that may influence germination and growth of *Alternaria* spp. The optimum temperature for sporulating, in general, is close to 25 ºC, although the optimum range for growing can be from 22 to 28 ºC. However, the maximum temperature for growing is near 36 ºC, while the minimum is frequently described to be -3 ºC, which enables it to grow under cold storage and continue to grow on cabbage, celery or other vegetables stored at 0 °C, or on certain apple cultivars stored at or below 0 °C (Sommer, 1985). In relation to a_w, *Alternaria* spp. grow at high values (0.99), although the minimum a_w needed for their development is 0.84 (Magan and Lacey, 1984; Oviedo et al., 2011).

2.3. Plant diseases associated with *Alternaria* spp.

Alternaria spp. are plant pathogens that cause damage in the field and are one of the most common species recorded in agricultural commodities. *Alternaria* is also known as a saprophyte in food and feed products, and as a common pathogen of stored fruits and vegetables that causes postharvest economic losses. Taking into account that *Alternaria* can also grow at low temperatures, even at -3 ºC, the fungus can continue to grow during storage or transport. In addition to spoiling

fruits and vegetables through disease development, *Alternaria* spp. are also capable of producing a wide range of toxic metabolites, of which a high proportion are phytotoxins that play a role in fungal pathogenicity, and several of them are mycotoxins that turn out to be harmful to humans and animals (Barkai-Golan and Paster, 2008a). In the context of fresh fruits and vegetables for human consumption, *A. alternata* is especially important, as new taxonomic tools have shown that, among all *Alternaria* spp., this species is predominant in several crops, including fruits and vegetables.

Tomatoes are very prone to contamination by *Alternaria* spp. because of their soft skin. In fact, *Alternaria* spp. have been reported to be the main pathogen of harvested tomatoes (Morris et al., 2000). The disease produced by *Alternaria* is generally associated with black spot disease, which is of worldwide economic importance and appears as dark brown to black, smooth, only slightly sunken lesions, which are of firm texture and can become several centimeters in diameter. Infection can occur at the stem end of the fruit, or through mechanical injury, cracking from excessive moisture during growth, or chilling (Agrios, 2005; Pitt and Hocking, 1997). Black spot disease does not just affect tomatoes, since it can also be a devastating foliar and seed-borne disease resulting in severe yield reductions in crops such as cabbage, broccoli, potatoes, canola and rapeseed, among others. *Alternaria* black spot disease on tomatoes occurs on green and ripe fruit affected by physiological alterations, such as nutritional deficiency or skin sunburn. The disease is favored by warm, rainy weather or dew formation on the fruit surface, and is more severe if infection occurs when the fruit is ripe rather than green. The disease may cause substantial losses, especially on tomatoes for canning (Logrieco et al., 2003).

3. *Alternaria* mycotoxins

Alternaria spp., besides being deleterious on causing food spoilage and being a potent airborne allergen, are of particular interest to mycotoxicologists because they can produce approximately 30 metabolites with possible toxicity (Robiglio and Lopez, 1995). Alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), tentoxin (TEN), and altertoxins I, II, and III (ATX-I, ATX-II and ATX-III) are considered to be some of the most important toxic metabolites produced (Fig. 2). *A. alternata* is the most common *Alternaria* species in harvested fruits and vegetables, and the most important mycotoxin-producing species.

3.1. Physicochemical characterization

The first *Alternaria* toxins were isolated and characterized in the years 1953-1986 (EFSA, 2011). Since then, physicochemical characteristics have only been reported in the literature for some of the *Alternaria* toxins, which are divided into 5 different classes based on their chemical structure:

1. The dibenzopyrone derivatives: AOH, AME, and ALT
2. The perylene derivatives: ATX-I, ATX-II, ATX-III and stemphyltoxin III
3. The tetramic acid derivatives: TeA and iso-TeA
4. AAL-toxins, abbreviation for *A. alternata* f. sp. *lycopersici* toxins
5. Miscellaneous lactones: TEN

AOH and AME crystallize from ethanol as colorless needles, and melting points with decomposition are 350 °C and 267 °C, respectively. They are soluble in most organic solvents and give a purple color reaction with ethanolic ferric chloride. ALT crystallizes as colorless prisms melting at 190-191°C. ATX-I is an

amorphous solid melting at 180 °C, which shows a characteristic bright yellow fluorescence under UV light. Yellow-orange fluorescence is characteristic for ATX and violet-blue for AOH, AME and ALT. TeA is colorless, and it is soluble in methanol and chloroform (EFSA, 2011).

Figure 2. Structure of the different toxins produced by *Alternaria* spp. (Lee et al., 2015).

Alternaria toxins can be partly metabolized in plants and form conjugated metabolites. These modified or "masked" mycotoxins, in which the toxin is usually bound to a more polar substance such as glucose, amino acids and sulfates, are of concern as they can probably release their native precursors after enzymatic hydrolysis in the digestive tract of organisms. They are referred to as masked

mycotoxins because they have the ability to escape from established analytical methods due to differences in polarity between the native precursors and their metabolites. Research has mainly focused on the parent mycotoxins, while very limited data are available on the occurrence of masked mycotoxin metabolites in food and feed (Berthiller et al., 2005).

3.2. *Alternaria* mycotoxins production

Since the discovery of mycotoxins, many studies have focused on understanding molecular mechanisms leading to their biosynthesis and, in this respect, there have been significant advances. To date, the most studied and well characterized mycotoxin biosynthesis pathways are for aflatoxin and sterigmatocystin (Yu et al., 2004; Yu and Leonard, 1995). However, there is still scarce information on the biosynthesis of *Alternaria* mycotoxins, despite the importance of *Alternaria* mycotoxins as a contaminant of food and feed commodities.

Biosynthetic routes for AOH were first studied by Thomas (1961), who suggested that this metabolite might be synthesized by head-to-tail condensations of acetate units. Later, the enzyme alternariol-O-methytransferase was isolated from *A. alternata* that had O-methyltransferase activity and converted AOH to AME (Gatenbeck and Hermodsson, 1965; Hiltunen and Söderhäll, 1992).

In general, it is considered that the predominant classes of fungal secondary metabolites include polyketides, non-ribosomal peptides, terpenes, and alkaloids. AOH is thought to be formed by the polyketide route of biosynthesis, which is a common pathway for the formation of many fungal secondary metabolites (Crawford and Townsend, 2010; Gulder et al., 2011). Fungal polyketide synthases (PKSs) are crucial for the first steps of the biosynthesis of several mycotoxins and other secondary metabolites. Recently, applying BLAST searches with amino acid sequences of 50 known PKSs from different fungi, ten putative PKSs were identified in an *A. alternata* genome (Fig. 3 and 4), claiming that one of them,

PksJ, is supposedly responsible for catalyzing the first steps of the biosynthesis of AOH and AME (Saha et al., 2012). It has been suggested that AOH biosynthesis could take place in peroxisomes. In addition, *altR* encodes a protein that has homology to other fungal specific transcription factors and has also been described as being involved in AOH production. Another interesting fact was to find a second gene cluster, the *pksH*, which appears to influence the biosynthesis of AOH, influencing the expression of *pksJ*. Thus, two PKS enzymes would contribute to one product and one transcriptional regulator, AltR, would be involved in *pksJ* induction or repression (Saha et al., 2012).

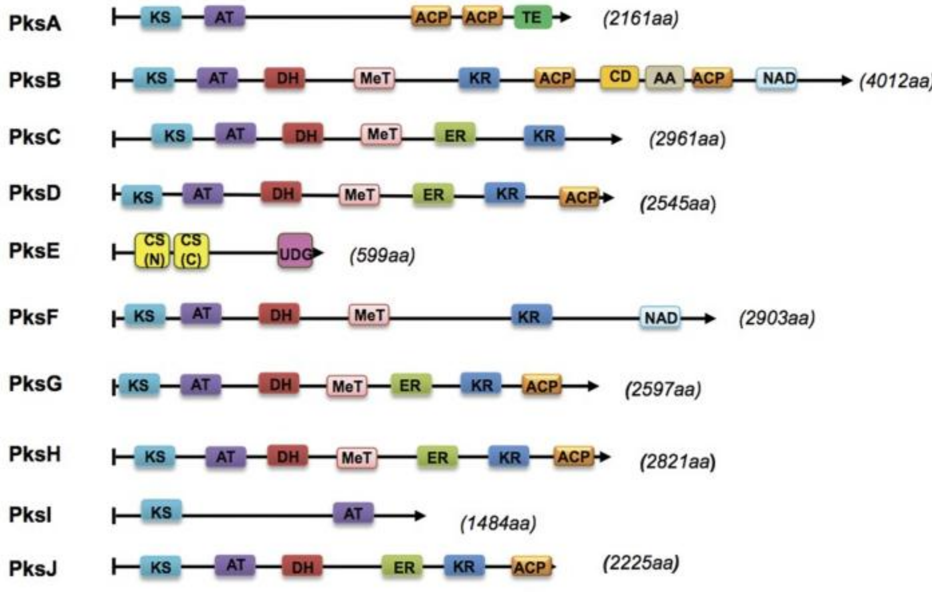


Figure 3. Proposed architecture of the ten PKSs found on an *A. alternata* genome. KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; MeT, methyltransferase; ER, enoyl reductase; KR, ketreductase; ACP, acyl carrier protein, CD, condensation domain; AA, Amino acid adenylation domain; CS, Chalon- and Stilben-Synthase (N)/(C); UDG: Uracil DNA Glycolase Superfamily; NAD, NAD binding domain. Figure from Saha et al. (2012).

On the other hand, another report was published recently dealing with AOH and AME biosynthesis in the wheat pathogen *Parastagonospora nodorum*, an important wheat pathogen in Australia and worldwide, which was recently described as also producing AOH (Tan et al., 2009). Chooi et al. (2015) performed a BLASTP search with the PKS gene sequences that they had compiled in *P. nodorum* and they did not identify any close homolog of *A. alternata* PksH and PksJ in the *P. nodorum* genome. Interestingly, they identified another PKS from *A. alternata* (PksI) sharing significant homology (86% identity) to SnPKS19 in *P. nodorum*. On performing a BLASTP in the GenBank protein database, they found that the closest characterized homolog for SnPKS19 and PksI was a norlichexanthone (NLX) synthase (GsfA) in the griseofulvin pathway in *Penicillium aethiopicum*. From the structure similarity between the two proteins, it was hypothesized that it was highly likely that the PKS gene *SnPKS19* may encode the biosynthesis of AOH. Additionally, it was observed that Δ*SnPKS19* mutants did not produce AOH, which definitely may confirm that *SnPKS19* is required for AOH biosynthesis in *P. nodorum*.

Hence, it seems that there are controversial results between the ones reported by Saha et al. (2012) and the findings described by Chooi et al. (2015). In contrast to PksH and PksJ, which are PKSs commonly involved in biosynthesis of aliphatic compounds, both the SnPKS19 and pksI genes encode a PKS that is typically involved in biosynthesis of aromatic polyketides (Chooi and Tang, 2012). Interestingly, Saha et al. (2012) observed that pksI was transcribed and that its expression correlated to the timing of AOH production, suggesting that pksI is unlikely to be a pseudogene. Nevertheless, pksI was not investigated further in their study. Chooi et al. (2015) explained that it is quite common for the RNA silencing technique, the methodology used by Saha et al. (2012), to result in off-target effects. For example, highly conserved KS domains could also have been targeted in the study by Saha et al. (2012) and, hence, it is possible that the

transcription of other PKS genes was also knocked down due to the potential complementary nature of the nucleotide sequences.

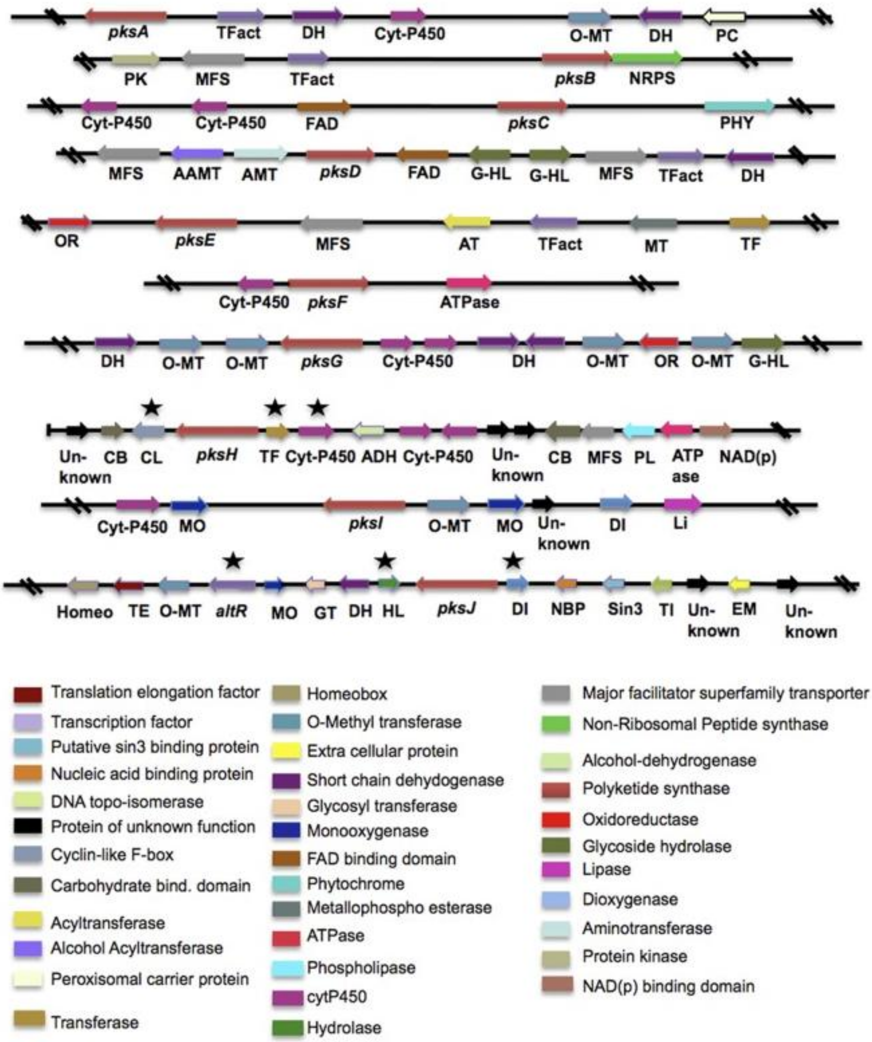


Figure 4. Organization of polyketide biosynthesis gene clusters in *A. alternata* proposed by Saha et al. (2012). Each arrow indicates the direction of transcription deduced from the analysis of the nucleotide sequences. The asterisks indicate the genes of the *pksJ* and *pksH* clusters, which were silenced in addition to *pksJ* and *pksH* in the study by Saha et al. (2012).

Taking all into consideration, it seems possible that *pksI* might be the gene responsible for AOH production in *A. alternata*. Nevertheless, further investigation is required to verify the genes involved in the biosynthesis of AOH in *A. alternata*.

All in all, the identification of the true PKSs responsible for the biosynthesis of AOH may open up the possibility of developing PCR-based detection methods for gene expression analyses before AOH levels are actually detectable. However, there is still a long way to go before achieving complete understanding of the mechanisms of mycotoxin biosynthesis and their regulation in any *Alternaria* spp.

3.3. Toxicity and biological effects of *Alternaria* mycotoxins

Exposure to *Alternaria* toxins has been linked to a variety of adverse health effects in humans and animals. *In vitro*, it has been observed that *Alternaria* isolates grown in laboratory culture are toxic to chickens and rats, human cell cultures, and were also teratogenic and fetotoxic in mice (Griffin and Chu, 1983; Harvan and Pero, 1976; Ostry, 2008; Pero et al., 1973; Sauer et al., 1978). Culture extracts of *A. alternata* have been described as being mutagenic *in vitro* and carcinogenic in rats fed with contaminated feed (Combina et al., 1999). Additionally, AOH and AME are suggested as playing an important role in the etiology of human esophageal cancer, as it was observed that a high consumption of foodstuff contaminated with *A. alternata* was linked to a higher incidence of this cancer (Liu et al., 1992). Nevertheless, there is a high degree of heterogeneity in the susceptibility of mammalian cell lines to the toxic effects of *Alternaria* toxins, as each toxin may have its own effects.

Due to their possible harmful effects, *Alternaria* toxins are of concern for public health. However, currently there are no regulations on *Alternaria* toxins in food and feed in Europe or in other regions of the world.

Table 2. Main toxic effects caused by *Alternaria* toxins (Pavón-Moreno et al., 2012)

Mycotoxin	Toxic effects
AOH	Mutagenic activity in bacterial cultures Genotoxic activity in animal cultures Cytotoxic activity in prawn cultures (*Artemia salina*)
AME	Mutagenic activity in bacterial cultures Genotoxic activity in animal cultures Precarcinogenic alterations in oesophageal mucosa of mice Cytotoxic activity in prawn cultures (*Artemia salina*)
ALT	Cytotoxic activity in prawn cultures (*Artemia salina*)
TeA	Internal bleeding in dogs and birds Precarcinogenic alterations in oesophageal mucosa of mice Hematologic disorder in people (Onyalai)
ATX	Mutagenic activity in Ames test Genotoxic activity in cell cultures of mice (ATX-I and ATX-III)

3.4. Occurrence of *Alternaria* mycotoxins in food commodities

There are currently no statutory or guideline limits set for *Alternaria* mycotoxins, although their relevance in food and feed is currently under discussion. Responding to this concern, the European Commission asked the European Food Safety Authority (EFSA) to assess the risk for animal and public health due to *Alternaria* mycotoxin exposure in order to enable the European Commission and the competent authorities in the Member States to consider the need for a possible follow-up and to fill the knowledge gaps. In 2011, the EFSA published a report on the risks of *Alternaria* toxins for animal and public health (EFSA, 2011). The scientific report stated that there are not enough relevant data on toxicity of *Alternaria* mycotoxins, and that more information is needed on their toxicokinetics and on the occurrence of *Alternaria* toxins in food and feed across the European countries in order to know the real exposure of consumers. The European Commission also asked for more studies related to the influence of food and feed processing to enable a risk assessment to be performed.

Several studies have reported the presence of *Alternaria* mycotoxins in different food commodities such as olives (Visconti et al., 1986), mandarins (Logrieco et al., 1990), peppers (Monbaliu et al., 2009), apples and their derived products (Delgado and Gómez-Cordovés, 1998; Stinson et al., 1981), wheat and other grains (Monbaliu et al., 2010), among others (Ackermann et al., 2011; da Motta and Valente Soares, 2001; Noser et al., 2011; Terminiello et al., 2006; Van de Perre et al., 2014; Visconti et al., 1987).

It has been mentioned in previous sections that many soft-skinned vegetables and fruits, including tomatoes, are especially susceptible to fungal invasion. In this regard, *Alternaria* spp. have been reported to be the most frequent fungal species invading tomatoes (Barkai-Golan and Paster, 2008b). While the direct consumption of a moldy tomato by any consumer is improbable, there exists the possibility that moldy tomatoes are used for the production of tomato products such as ketchups, purées, juices, sauces, scalded peeled tomatoes, among others.

In 2006, a study was carried out on the occurrence of *Alternaria* mycotoxins in 80 tomato purées in Argentina. They found that 39 of these purées were contaminated with *Alternaria* mycotoxins. TeA was found in 23 samples (up to 4,021 µg/kg), AOH in 5 (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 (Terminiello et al., 2006). In another study with several commercial tomato pastes, small amounts of TeA were detected at levels of 0.01-0.1 µg/g (Scott and Kanhere, 1980). In a different study, a search was performed for AOH, AME and TeA in 80 samples of tomato products processed and sold in Brazil, endeavoring to cover a wide range of products and several commercial brands. While no AME or AOH was detected in any of the samples, TEA was found in 7 samples of tomato pulp (39-111 ng/g) and 4 samples of tomato purée (29-76 ng/g) (da Motta and Valente Soares, 2001). In another study undertaken by Pavón et al. (2012), they found that sun-dried tomatoes showed a high incidence of AOH and AME contamination, as 66.7% of samples were contaminated. The drying process, particularly during the early

stages when the environmental conditions are appropriate for *Alternaria* growth and mycotoxin production, could probably be the explanation for the high incidence of *Alternaria* mycotoxins in sun-dried tomato samples. On the contrary, only a few samples were positive for AOH, AME or ALT in canned tomato products, ketchup and tomato sauce. 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 (Lopez et al., 2014). This type of data might help EFSA to evaluate whether legislation will be required, based on the risk assessment.

4. Production chain of tomato products

Tomatoes and their by-products are widely consumed worldwide. As mentioned in previous sections, tomatoes are very susceptible to *Alternaria* decay. For consumers, the health concern does not lie in moldy tomatoes, but rather in the final processed product. It should be taken into consideration that, in the food industry, raw fruits used for processing do not usually have the best quality, and the entrance of moldy tomatoes into the food production chain is quite possible.

In this section, the fate of *Alternaria* spp. and its mycotoxins during the production chain will be discussed.

4.1. Preharvest

Generally, fruits may be contaminated by *Alternaria* spp. in crop fields and, once in the food industry, during storage. Green and ripe fruits can be affected by black mold disease, which is influenced by physiological alterations, such as nutritional deficiency or skin sunburn. Warm rainy weather or dew formation on the fruit

surface also favors the disease, which is more severe if infection occurs when fruit is ripe rather than green (Logrieco et al., 2003). To avoid fungal contamination, farmers usually spray crop fields with fungicides. In fact, fungicides have been used in agriculture for well over a century. Nevertheless, over time it was discovered that plant pathogenic fungi can adapt to fungicide treatments by mutation, leading to resistance and loss of efficacy. Over the last few years, some reports have described the resistance of certain *Alternaria* spp. towards some fungicides (Avenot and Michailides, 2007; Lucas et al., 2015; Miles et al., 2014). For the purpose of finding efficient new fungicides, it is necessary to explore new targets and new strategies for resistance management. With this aim, it is essential to continue to investigate the molecular basis of *Alternaria*, in order to find those targets that could inhibit its growth or that may inhibit mycotoxin biosynthesis.

When contamination has occurred, *Alternaria* spp. may begin the biosynthesis of mycotoxins, although not all species belonging to *Alternaria* genus produce toxins. Additionally, conditions to which the fungus is exposed may not be ideal for mycotoxin biosynthesis.

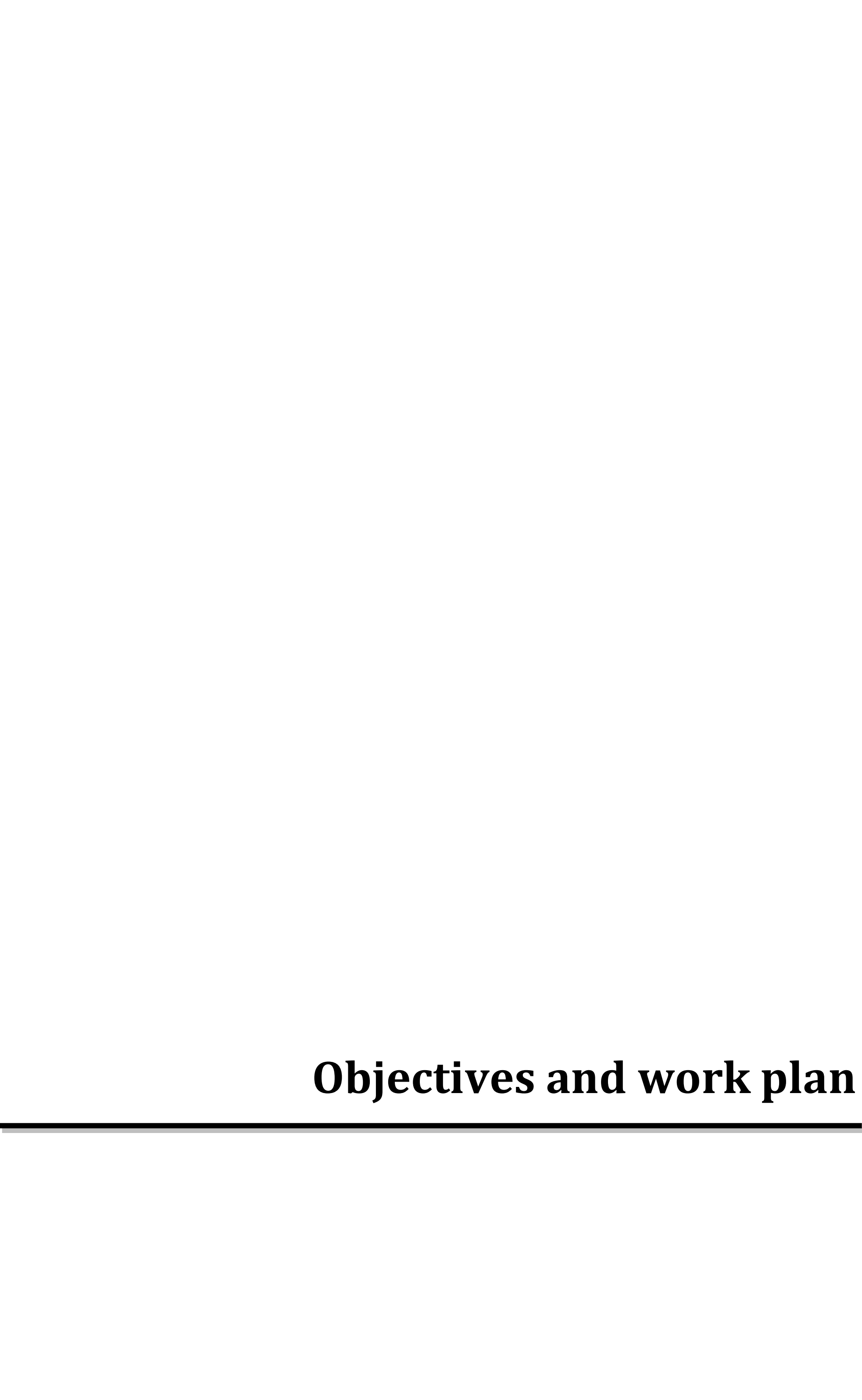
4.2. Postharvest

Contamination of raw fruits with *Alternaria* spp. may occur in the field or during the storage process, in which, if conditions are favorable for fungal growth, fungi will keep growing and mycotoxin levels may also increase, resulting in a bioaccumulation of toxins that can be dangerous for consumer health. It should be taken into account that *Alternaria* has the ability to grow even at low temperatures and, therefore, it can also spoil food commodities during refrigerated transport and storage (Barkai-Golan, 2008). The duration of the storage period will depend on the industry, either because the food production plant is overfull, or because fruits are not yet at the appropriate ripening stage.

The control of tomato ripening is one of the main goals of producers and traders in tomato marketing, since accelerated ripening after harvesting leads to decay development. In this regard, the use of technologies that minimize or inhibit ethylene action enables the postharvest life of tomatoes to be extended, this being a concern during transportation and commercialization. The use of 1-methylcyclopropene (1-MCP) is a commercial strategy already used to control ethylene production. 1-MCP interacts with ethylene receptors and thereby prevents ethylene-dependent responses (Blankenship and Dole, 2003; Sisler and Blankenship, 1996; Sisler and Serek, 1997). How ethylene and 1-MCP affects physiology and quality of fruits has been studied broadly. Nevertheless, scarce literature considers the effects of ethylene and 1-MCP on fruit pathogens, and it has been described that 1-MCP is not innocuous to fungi, although it may have different effects depending of the pathogen affecting the fruits (Akagi and Stotz, 2007; Janisiewicz et al., 2003; Jiang et al., 2001; Ku et al., 1999).

4.3. Tomato processing

There is scarce information on the stability of *Alternaria* mycotoxins during the distinct food processing operations. In fact, this was one of the points that the EFSA claimed in its scientific report, in which it was specifically detailed that there was a need to increase the studies on the influence of food and feed processing on *Alternaria* toxins. In the production of heat-treated tomato by-products, all live structures of *Alternaria* spp. will be destroyed during the food processing, mainly due to the effect of heat, but it is uncertain what happens to mycotoxins. Different studies reveal that *Alternaria* mycotoxins could remain quite stable during the industrial process, which consequently may end up with relatively high contents of *Alternaria* mycotoxins in the end products, such as juices, sauces and purées. The high stability of *Alternaria* mycotoxins during food processing could be an important hazard for human and animal health.



Objectives and work plan

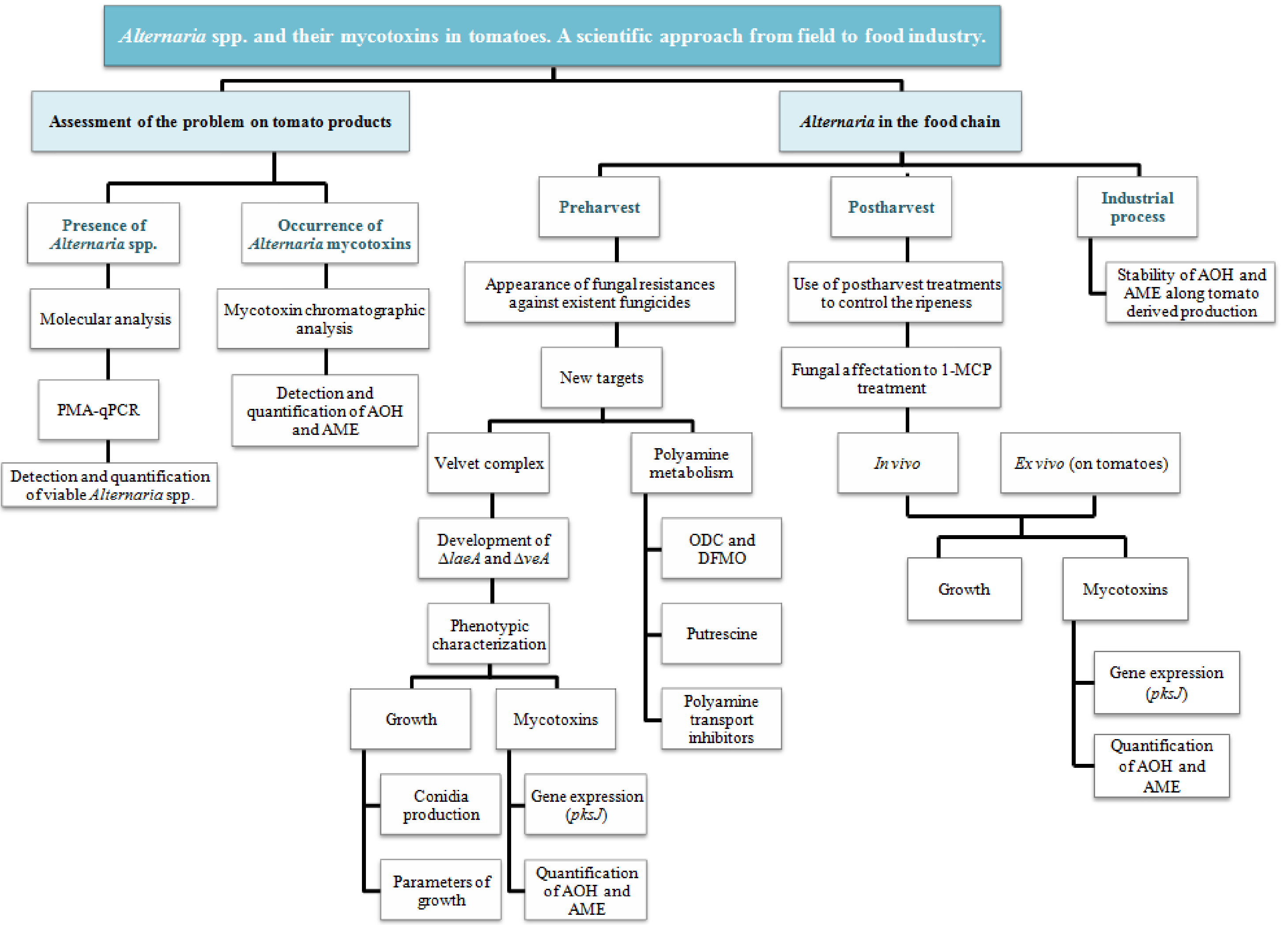
In 2011, the European Commission asked the European Food Safety Authority (EFSA) to assess the risk for public health related to the presence of *Alternaria*, based on the information available. The purpose of the report requested, which was entitled "Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food" (EFSA, 2011), was to enable the European Commission and the competent authorities in the Member States to consider the need for a possible follow-up to fill the knowledge gaps.

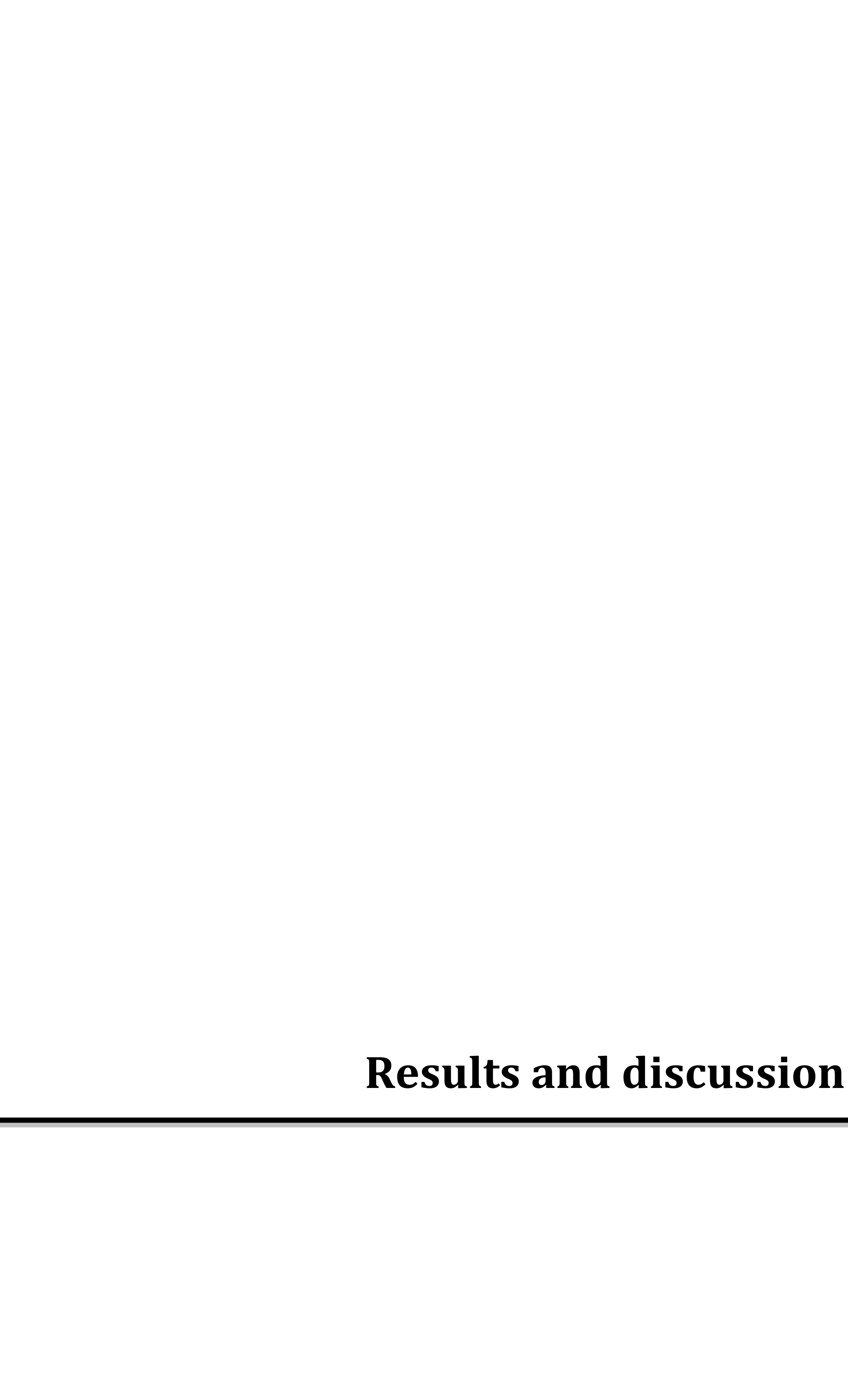
This Doctoral Thesis arose from the publication of the EFSA's scientific opinion, as this report concluded that there are still limited data about several aspects of *Alternaria* toxins, such as, for example, the data on occurrence of *Alternaria* toxins in food or the influence of food processing on these toxins. Therefore, taking into account the EFSA's conclusions and recommendations, it was decided to develop this PhD **for the purpose of increasing the information about certain aspects of *Alternaria* spp. and their mycotoxins during the food chain in Spain**. The food matrix chosen to develop all the experimental assays included in this document was the tomato. This matrix was chosen because of its high consumption in Spain and worldwide. However, not only the fresh fruit was considered, but also its by-products, such as sauces, juices, ketchups, among others. Additionally, the EFSA reported that several *Alternaria* mycotoxins were generally found in tomatoes and tomato products. It is important to mention that the *Alternaria* strain used for most of the assays was *Alternaria alternata*, since it is considered to be the most important mycotoxin-producing species and one of the most abundant in harvested fruits and vegetables, especially tomatoes (Bottalico and Logrieco, 1992). The two *Alternaria* mycotoxins assessed in all the studies were AOH and AME, because they are the most analyzed and characterized mycotoxins in food commodities worldwide in relation to *Alternaria* spp.

The general objectives of this Doctoral Thesis are described below. To achieve each goal, it was necessary to set specific objectives.

I. Assess the presence of *Alternaria* spp. and the occurrence of their mycotoxins in the food industry.
   - Develop an easy and effective technique to assess the quantitative presence of viable *Alternaria* spp. in tomato by-products.
   - Collect tomato samples during different harvest seasons and from two different food industries (one conventional and the other a manufacturer of organic products) and analyze the presence of viable *Alternaria* spp. and the occurrence of AOH and AME during different processing stages.

II. Investigate some important concerns in relation to *Alternaria* and its mycotoxins from field to fork.
   - At preharvest: Identify new targets suitable for designing new fungicides to overcome the problem of the appearance of fungal resistance against existing fungicides.
   - At postharvest: Examine the effect that postharvest treatments applied to raw fruits have on the fungi, focusing on growth parameters and mycotoxin production.
   - In the food industry: Analyze the effect that the industrial process for producing tomato by-products has on the stability of AOH and AME. Also, assess the effects of UV radiation on the levels of AOH and AME.


Alternaria spp. and their mycotoxins in tomatoes. A scientific approach from field to food industry.
Assessment of the problem on tomato products
Presence of *Alternaria* spp.
Molecular analysis
PMA-qPCR
Detection and quantification of viable *Alternaria* spp.
Occurrence of *Alternaria* mycotoxins
Mycotoxin chromatographic analysis
Detection and quantification of AOH and AME
Alternaria in the food chain
Preharvest
Appearance of fungal resistances against existent fungicides
New targets
Velvet complex
Development of ΔlaeA and ΔveA
Phenotypic characterization
Growth
Conidia production
Parameters of growth
Mycotoxins
Gene expression (*pksJ*)
Quantification of AOH and AME
Polyamine metabolism
ODC and DFMO
Putrescine
Polyamine transport inhibitors
Postharvest
Use of postharvest treatments to control the ripeness
Fungal affectation to 1-MCP treatment
In vivo
Ex vivo (on tomatoes)
Growth
Mycotoxins
Gene expression (*pksJ*)
Quantification of AOH and AME
Industrial process
Stability of AOH and AME along tomato derived production



Results and discussion

Chapter I

Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. contamination in tomato products

Ana Crespo-Sempere, Núria Estiarte, Sònia Marín, Vicente Sanchis, and Antonio J. Ramos

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Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. contamination in tomato products

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Abstract

Alternaria is a common contaminating genus of fungi in fruits, grains, and vegetables that causes severe economic losses to farmers and the food industry. Furthermore, it is claimed that *Alternaria* spp. are able to produce phytotoxic metabolites, toxic to plants, and mycotoxins, unsafe for human and animal health. DNA amplification techniques are being increasingly applied to detect, identify, and quantify mycotoxigenic fungi in foodstuffs, but the inability of these methods to distinguish between viable and nonviable cells might lead to an overestimation of mycotoxin-producing living cells. A promising technique to overcome this problem is the pre-treatment of samples with nucleic acid intercalating dyes, such as propidium monoazide (PMA), prior quantitative PCR (qPCR). PMA selectively penetrates cells with a damaged membrane inhibiting DNA amplification during qPCRs. In our study, a primer pair (Alt4-Alt5) to specifically amplify and quantify *Alternaria* spp. by qPCR was designed. Quantification data of qPCR achieved a

detection limit of 102 conidia/g of tomato. Here, we have optimized for the first time a DNA amplification-based PMA sample pre-treatment protocol for detecting viable *Alternaria* spp. cells. Artificially inoculated tomato samples treated with 65 µM of PMA, showed a reduction in the signal by almost 7 cycles in qPCR between live and heat-killed *Alternaria* spp conidia. The tomato matrix had a protective effect on the cells against PMA toxicity, reducing the efficiency to distinguish between viable and nonviable cells. The results reported here indicate that the PMA-qPCR method is a suitable tool for quantifying viable *Alternaria* cells, which could be useful for estimating potential risks of mycotoxin contamination.

Keywords: *Alternaria* spp.; Mycotoxins; Propidium monoazide; Quantitative polymerase chain reaction; Tomato.

1. Introduction

Alternaria is a genus of fungi which includes saprophytic and pathogenic species that affect field crops, reducing the yield and causing post-harvest decay of various fruits, grains, and vegetables, which consequently leads to economic losses to farmers and the food industry (Logrieco et al., 2003). Tomatoes are highly susceptible to fungal invasion due to their thin skin and *Alternaria* is the most common fungus found on mouldy tomatoes (Andersen and Frisvad, 2004; Barkai-Golan and Paster, 2008a; Pitt and Hocking, 1997). More specifically, *Alternaria alternata, A. arborescens, A. tenuissima, A. tomaticola, A. tomato,* and *A. tomatophila* (former *A. solani*) are the primary *Alternaria* species found in raw tomatoes and tomato products (Andersen et al., 2008; Somma et al., 2011; Weir et al., 1998).

Alternaria spp., besides being commonly associated with several plant diseases, play an important role in the production of mycotoxins. The most relevant mycotoxins produced by *Alternaria* spp. are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA), altenuene

(ALT), altertoxins (ATXs), stemphyltoxin III, and *Alternaria alternata* f. sp *lycopersici* toxins (AAL-toxins), which have the potential to cause several health problems in humans and animals (EFSA, 2011; Logrieco et al., 2009; Scott, 2001). Among all *Alternaria* spp., *A. alternata* has been regarded as the most important mycotoxin-producing species (Barkai-Golan, 2008). However, *A. arborescens*, *A. tenuissima*, *A. tomato*, *A. solani*, and *A. tomatophila* are also known to produce AOH, AME, ATX-I, -II, -III, and TeA (Andersen et al., 2008; Andersen and Frisvad, 2004; Andersen et al., 2002; Pollock et al., 1982).

When dealing with food safety and plant pathology, rapid determination of the presence of fungi is essential to take the appropriate corrective actions to help the industry lower the contamination levels in the final products, particularly when a post-harvest storage is required. Nucleic acid-based methods are being increasingly applied to detect, identify, and quantify mycotoxigenic fungi in foodstuffs (Edwards et al., 2002; Mackay, 2004; Niessen, 2007; Zur et al., 1999). Polymerase chain reaction (PCR) has replaced complex and time-consuming microbiological tests, based on the growth of the studied microorganism in different types of culture media, for the amplification of specific genomic markers. Particularly, quantitative real-time PCR (qPCR) is a technique that allows the detection, identification, and quantification of DNA and RNA present in a food sample (Hayat et al., 2012; Postollec et al., 2011; Rodríguez, Isabel Luque et al., 2011). However, there are still limitations in the use of nucleic acid-based techniques. One of these obstacles is the inability to discriminate between the nucleic acids of viable and dead microorganisms, as the DNA from dead cells can remain intact for several days or even weeks (Josephson et al., 1993). Hence, the DNA from dead cells can serve as a template in PCR amplification, overestimating viable *Alternaria* spp. cells, which are the potential mycotoxin producers. Therefore, these techniques are not suitable for assessing the potential risk of fungal contamination in foodstuffs, particularly when the raw material is stored before being processed. To overcome this problem, propidium monoazide (PMA)

combined with qPCR has been proposed to differentiate dead and viable forms, or to detect and quantify only viable cells. PMA is a nucleic acid-intercalating fluorophore that can penetrate through the damaged membranes of dead cells. Once inside the cell, and after exposure to strong visible light, PMA binds to the DNA of dead cells, leaving the DNA from viable cells unlabeled. The unlabelled DNA from the viable cells is amplified, while the PMA bound to the DNA of dead cells inhibits the activity of the polymerase and no amplification of this latter DNA occurs. Although there are several studies in which PMA-qPCR is used for detecting and quantifying viable bacterial cells (Cawthorn and Witthuhn, 2008; Elizaquível, Sánchez, Selma et al., 2012; Josefsen et al., 2010; Nocker et al., 2006; Nocker et al., 2009; Pan and Breidt Jr., 2007; Zhu et al., 2012), few studies have focused this technique on fungi (Vesper et al., 2008), and none on *Alternaria* spp. Here we developed a specific and sensitive PMA treatment combined with qPCR in order to detect, identify, and quantify *Alternaria* spp. viable cells in tomato samples.

2. Material and Methods

2.1 Fungal isolates and culture conditions

All the isolates used in this study are listed in Table 1. Fungal reference strains were provided by the *Centralbureau voor Schimmelcultures* (CBS, The Netherlands) and the Spanish Type Culture Collection (*CECT*, Spain). Fungal strains were stored as conidial suspensions in 40% glycerol at −20 ºC. Ten *Alternaria* spp. isolates obtained in our laboratory from tomatoes were also included. The identification of these ten isolates was previously confirmed by sequencing a beta-tubulin gene region with the Beta3-Beta4 primers (Peever et al., 2004).

To prepare the conidial suspensions strains were grown on Petri dishes containing Potato Dextrose Agar (Biokar Diagnostics, France), at 26 °C for 6 days, in the dark. Conidia were collected with a sterile solution of Tween 80 (0.005% v/v) and filtered through Miracloth (Calbiochem, USA). Conidial concentration was determined using a Thoma counting chamber.

Table 1. Fungal strains used in this study indicating species and origin.

Species designation	Strain	Origin
Alternaria alternata	CECT 20560	Pepper-Spain
Alternaria alternata	CBS 116329	Apple-Germany
Alternaria alternata	CECT 2662	Tomato-UK
Alternaria alternata	CBS 119115	Cherry leaf-Greece
Alternaria arborescens	CBS 109730	Tomato-USA
Alternaria tenuissima	CBS 124278	Cherry-Denmark
Alternaria tomaticola	CBS 118814	Tomato-USA
Alternaria tomato	CBS 114.35	Tomato
Alternaria tomatophila	CBS 109156	Tomato leaf-USA
Alternaria solani	CBS 105.51	Tomato
Botrytis cinerea	CECT 2100	*Vicia faba*-UK
Colletotrichum cocades	CECT 21008	-
Colletotrichum dematium	CBS 12525	Leaf *Eryngium campestre*-France
Colletotrichum gloesporoides	CECT 21015	-
Fusarium oxysporum	CECT 2866	Tomato
Geotrichum candidum	CBS 117139	Tomato-Brazil
Rhizopus microsporus var. *rhizopideformis*	CBS 607.73	Cereals-Yugoslavia
Rhizopus oryzae	CECT 2339	-
Rhizopus stolonifer	CECT 2344	-
Stemphylium eturmiunum	CBS 124279	Flower, apple-Denmark
Stemphylium lycopersici	CBS 122639	Diseased leaves of tomato-China
Ulocladium botrytis	CECT 20564	Valencia, Spain
Alternaria spp.	Alt 3.1; Alt 1.4; Alt 30 Alt 35; Alt tp13; Alt tp15; Alt tp18; Alt 05 Alt 06; Alt 09	Tomato-Spain

CECT: Spanish Type Culture Collection; CBS: Centralbureau voor Schimmelcultures; Alt: strains isolated and conserved at the Applied Mycology Unit Culture Collection of the University of Lleida (Spain).

2.2 DNA extraction

Cultures were grown in 500 µL of Malt Extract broth (2% w/v malt extract, 0.1% w/v peptone, 2% w/v glucose) for 2 days at 26 ºC. The mycelial extract was recovered after 10 min of centrifugation at 17500 x g and 300 µL of DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) was added. The mycelial suspension was lysed by vortexing with five 2.8 mm Precellys metal beads (Bertin Technologies, France) for 10 min. After a centrifugation at 17500 x g for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) was added to the supernatant. The supernatant was stored at -20 ºC for 10 min and then centrifuged (17500 x g, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by addition of one volume of isopropanol. After a 5-minute incubation time at room temperature, the DNA suspension was centrifuged (17500 x g, 10 min). The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA resuspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3 Primer design

A primer pair, Alt4 (5'-CTTTTGCGTACTTCTTGTTTCC-3') and Alt5 (5'-CAGGCATGCCCTTTGGATAC-3'), was designed for specific amplification of *Alternaria* spp. based on sequence alignments of the internal spacer regions (ITSs) from several *Alternaria* spp. strains and other related fungal co-contaminants of tomato products. Additionally, another primer pair, Alt6 (5'-AACTTTCAACAACGGATCTCT-3') and Alt7 (5'-ATGCTTAAGTTCAGCGGGTA-3'), was designed in a conserved ITS region, to obtain amplifications of all DNA samples from *Alternaria* spp. and co-contaminants strains. Thus, DNA amplification using Alt6-Alt7 primers could serve as a control of DNA integrity to prevent false negative amplifications. Both pair of primers, Alt4-Alt5 and Alt6-Alt7, were designed using OLIGO V5 software (http://www.oligo.net).

2.4 Primer-specific PCR detection

Alt4-Alt5-specific PCR assays were performed in a GeneAmp® PCR System 2700 (Applied Biosystems, USA). Amplification reactions were carried out in volumes of 10 µL containing 10 ng of DNA, 16 mM $(NH_4)_2SO_4$, 67 mM TrisHCl pH 8.8, 0.01% (v/v) Tween 20, 1.5 mM $MgCl_2$, 250 µM (each) dNTP, 0.5 µM of each primer, and 0.5 U of DFS-Taq DNA Polymerase (BIORON, Germany). PCR reactions were performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 66 ºC for 45 s, and extension at 72 °C for 60 s with a final extension of 10 min. PCR products were detected in 1.5% (w/v) agarose ethidium bromide gels in TAE 1X buffer (40 mM Tris-acetate, 1 mM EDTA, and 20 mM acetic acid). A TrackIT 100 bp DNA ladder (Invitrogen, USA) was used as the molecular size marker.

2.5 Fungal detection in artificially contaminated samples

Tomatoes were surface-sterilized by dipping them into a NaClO solution (0.1% w/v Cl) for 5 min and then immersed in 70% ethanol for 1 min. Excess water was removed by placing the tomatoes in a laminar flow bench. For tomato inoculation, fungal conidia suspensions of *A. alternata* CECT 20560 were prepared in distilled water containing Tween 80 (0.005% v/v). Two hundred grams of tomatoes were dipped into 200 ml of phosphate buffered saline (PBS), 138 mM NaCl, 2.7 mM KCl, 10 mM $NaH_2PO_4 \cdot 2\ H_2O$, and 2 mM KH_2PO_4, containing the conidia suspensions (10^5, 10^4, 10^3, 10^2, and 10 conidia/g tomato) and homogenized in a Masticator stomacher for 60 s (IUL instruments, Spain). Another two hundred grams of non-inoculated tomatoes were used as the negative control. A triplicate of 50 ml aliquots were taken from each stomacher bag, filtered through a Miracloth (Calbiochem, USA) and centrifuged at 15000 x g for 10 min. Pellets were resuspended in 2 ml of PBS, which were then treated with PMA to finally perform the DNA extraction as previously detailed (2.2). Additionally, in order to see if the detection limit could be improved, the artificially inoculated tomato samples were incubated at 26 ºC for 20 h prior DNA extraction.

Presence and quantitative detection of *Alternaria* spp. was performed by qPCR. PCR reactions were performed in a final volume of 10 μL containing 1X SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA), 250 nM of each primer, and 4 μL of template DNA. All amplifications were performed on a CFX96 TouchTM Real-Time PCR Detection System (BIO-RAD, USA). The standard protocol included one cycle at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s, and 66 °C for 30 s. Reactions were done in triplicate. In all cases, a negative amplification control was included using 4 μL of water instead of DNA. For the preparation of standard curves five different concentrations of conidia (10^2, 10^3, 10^4, 10^5, and 10^6 conidia/g tomato) were used to artificially contaminate the tomatoes as previously described. The quantification cycle (Cq) value that determines the cycle number at which fluorescence increases above background, was plotted against the logarithm of starting quantity of template for each dilution. Amplification efficiency was calculated from the slope of the standard curve ($E=10^{-1/slope}$; % Efficiency =(E−1)×100 (Kubista *et al.*, 2006)). In addition, melting curves were programmed in order to check the specificity of the Alt4-Alt5 primers.

2.6 Treatment of samples with PMA

In order to optimize PMA treatment for quantifying viable *Alternaria* conidia, PMA toxicity was tested. Aliquots of 500 μL of PBS with 10^6 conidia/ml were treated with different PMA concentrations (20, 30, 40, and 50 μM). The treatment of conidia with PMA, stocks of 20 mM in water (BIOTIUM, USA), consisted in the addition of the reagent to the samples, an incubation period of 20 min in the dark at room temperature (with occasional mixing to allow reagent penetration), and 10 min of exposure to light using a photo-activation system. A closed box with refractory walls and blue wavelength light-emitting diodes (LED, 6000 mcd) was constructed and placed 7 cm from a 24-well microplate containing the samples. After the PMA treatment, 1/100 and 1/1000 dilutions from the 10^6 conidia/ml aliquots were plated on PDA Petri dishes. After a 24-hour incubation period at 26 ºC, colony forming units (CFU) were counted. To determine the suitability of the

PMA-qPCR technique for distinguishing viable and non-viable conidia, PMA treatments were performed in three different samples of 500 µL of PBS containing 10^6 conidia/ml: live conidia, dead conidia (treated in a hot bath at 85 ºC for 1 h), and live:dead conidia where both samples were mixed in equal proportions. Loss of cell viability for dead conidia was confirmed by plating on PDA media and incubating for 24 h at 26 ºC. Each sample was treated in triplicate to ensure reproducibility of the results. After PMA treatment, samples were centrifuged (15000 x g, 10 min), conidia resuspended in 300 µL of DNA extraction buffer, and DNA extractions were performed as previously described.

Additionally, to evaluate the toxicity and the efficiency of PMA in a tomato matrix, 200 grams of tomatoes were homogenized with PBS (1:1 dilution) using a Masticator stomacher (IUL instruments, Spain) for 60 s. Aliquots of 50 ml were taken from the stomacher bag, filtered through a Miracloth (Calbiochem, USA), and centrifuged at 15000 x g for 10 min as described in fungal detection for tomato samples in section 2.5. For assessing efficiency, tomato pellets were resuspended with 2 ml of PBS containing 10^6 conidia/ml of live conidia, dead conidia, or live:dead conidia in equal proportions. For evaluating PMA toxicity in the tomato matrix, 50, 60, and 65 µM of PMA concentrations were tested.

2.7 Statistical analysis

All statistical analyses were performed using Statgraphics Plus 5.1 (Statpoint Technologies Inc., USA). One-way analysis of variance (ANOVA), with a significance level of p=0.05, was carried out to determine significant differences between the means.

3. Results and Discussion

3.1. Primer set specificity

The Alt4-Alt5 primer set was designed to detect, identify, and quantify *Alternaria* spp. The specificity of these primers was tested by PCR amplification of the most common *Alternaria* spp., responsible of the decay of fruits and vegetables and the main co-contaminant fungi present in tomatoes (Table 1). As shown in Figure 1A, amplification of all DNA samples from *Alternaria* spp. (*A. alternata*, *A. arborescens*, *A. tenuissima*, *A. tomato*, *A. tomatophila*, *A. tomaticola,* and *A. solani*) were obtained. *Ulocladium botrytis* DNA was also amplified. Conversely, DNA samples from fungal co-contaminants (*Geotrichum candidum*, *Colletotrichum dematium*, *Colletotrichum cocades*, *Colletotrichum gloesporoides*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizopus oryzae*, *Rhizopus microsporus* var. *rhizopodiformis, Rhizopus stolonifer, Stemphylium eturmiunum*, and *Stemphylium lycopersici*) did not amplify any product. This negative amplification was not due to low DNA integrity, since Figure 1B shows the amplification of the same DNA samples but using the Alt6-Alt7 primer pair instead of the Alt4-Alt5. Additionally, DNA samples from ten *Alternaria* spp. isolates from our own collection (Table 1) were amplified with the Alt4-Alt5 primers (Figure 1C).

A close phylogenetic relationship has been established between *Alternaria* spp. and *Ulocladium* spp. in some studies (Andersen and Hollensted, 2008; Chou and Wu, 2002). Although it is possible to distinguish *Alternaria* spp. from *U. botrytis*, detailed morphological and chemical analyzes are required. Nonetheless, it has been observed that the genus *Ulocladium* contains species that are responsible for the decay of nuts, fruits, and cereals, plant pathogens, and mycotoxin producers. ATX-I and TeA are two of the mycotoxin produced by *Ulocladium* spp., which are also produced by *Alternaria* spp. (Andersen and Hollensted, 2008; EFSA, 2011; Scott, 2001; Schlegel et al., 2001). Hence, it has not been possible to distinguish between *Alternaria* spp. and *U. botrytis* using Alt4-Alt5 primers, but the joint

detection of both genera could be an advantage for the food industry because of their related mycotoxigenic profile.

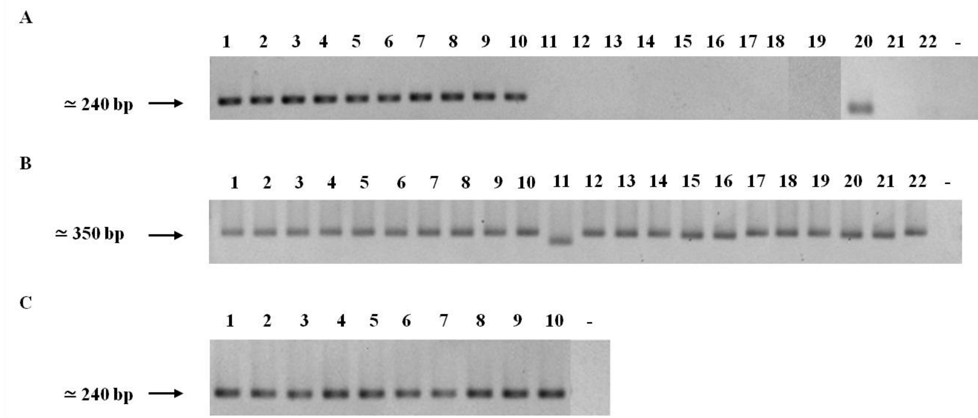

Figure 1. Specificity of *Alternaria* PCR primer set. **A.** Amplification of the most common *Alternaria* spp. present in fruits and vegetables and the main co-contaminant fungi present in tomatoes using Alt4-Alt5 primers. **B.** Control of the false negative amplifications between the most common *Alternaria* spp. present in fruits and vegetables and the main co-contaminant fungi present in tomatoes using Alt6-Alt7 primers. 1: *Alternaria alternata* CECT 20560; 2: *Alternaria alternata* CBS 116.329; 3: *Alternaria alternata* CECT 2662; 4: *Alternaria alternata* CBS 119.115; 5: *Alternaria arborescens* CBS 109.730; 6: *Alternaria tenuissima* CBS 124.278; 7: *Alternaria tomaticola* CBS 118.814; 8: *Alternaria tomato* CBS 114.35; 9: *Alternaria tomatophila* CBS 109.156; 10: *Alternaria solani* CBS 105.51; 11: *Geotrichum candidum* CBS 117.139; 12: *Colletotrichum dematium* CBS 125.25; 13: *Colletotrichum cocades* CECT 21008; 14: *Colletotrichum gloesporoides* CECT 21015; 15: *Botrytis cinerea* CECT 2100; 16: *Fusarium oxysporum* CECT 2866; 17: *Rhizopus oryzae* CECT 2339; 18: *Rhizopus microsporus var. rhizopodiformis* CBS 607.73; 19: *Rhizopus stolonifer* CECT 2344; 20: *Ulocladium botrytis* CECT 20564; 21: *Stemphylium eturmiunum* CBS 124279; 22: *Stemphylium lycopersici* CBS 122639; (-): negative control and M: molecular ladder weight of 100 bp (Invitrogen, USA). **C.** Amplification by PCR of *Alternaria* isolates from tomato. 1: Alt tp15; 2: Alt 09, 3: Alt 30; 4: Alt 35; 5: Alt 05; 6: Alt tp13; 7: Alt tp18; 8: Alt 06; 9: Alt 3.1; 10: Alt 1.4.

3.2. Amplification efficiency and standard curves using qPCR

Alt4 and Alt5 primers were also used for qPCR amplification. To test the efficiency and specificity of the primers, standard curves were generated using *A. alternata* (CECT 20560) DNA in a tomato matrix.

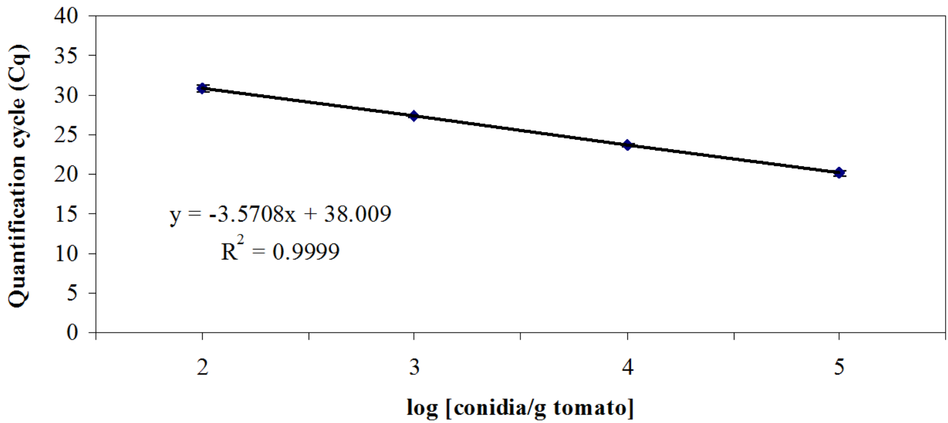

Figure 2. Standard curves obtained with SYBR Green I using five tomato food matrix samples inoculated with *A. alternata* (CECT 20560) conidia with different concentration each sample. The concentrations assayed were: 10^6, 10^5, 10^4, 10^3 and 10^2 conidia/g of tomato. After inoculation the tomato sample was treated as a current sample. At the end it was carried out a DNA extraction. The figure shows a strong linearity between Cq values and the concentration of *A. alternata* conidia assayed (R^2=0.9999) and an efficiency of 90.57%.

As shown in Figure 2, a strong linear relationship (R^2=0.9999) was found between the DNA extracted from the tomato food matrix inoculated with different concentration of conidia (10^5, 10^4, 10^3, and 10^2 conidia/g) and Cq values. When Cq values were plotted against conidia concentrations, a slope of -3.5708 was obtained, indicating an efficiency of 1.9057 (90.57%). This efficiency is considered acceptable according to Postollec et al. (2011). Furthermore, it was found that the Alt4-Alt5 primer pair, amplified all *Alternaria* spp. and *U. botrytis* DNA by qPCR, confirming the results obtained by conventional PCR.

Additionally, for *A. solani*, *A. tomato*, and *A. tomatophila* a single melting peak (83 ºC) was observed, while for the rest of positive samples the melting temperature was 82 or 82.5 ºC. Thus, primer pair Alt4-Alt5 is specific and efficient and can be used for accurate quantification of *Alternaria* spp. and *U. botrytis* contamination in tomato products.

3.3. Evaluation of the detection limit using qPCR in artificially inoculated tomato samples

The limit of detection (LOD) was assayed in artificially inoculated tomatoes with different concentrations of *A. alternata* (CECT 20560) conidia (10^5, 10^4, 10^3, 10^2, and 10 conidia/g of tomato). Additionally, the effect of pre-enrichment on contaminated tomato samples prior DNA extraction was studied. Enrichment of raw inoculated tomatoes was done in PBS at 26 ºC for 20 h. The aim of the additional enrichment was to improve the detection and quantification limits; however, a LOD of 10^2 conidia/g of tomato was obtained for both assays, with or without the enrichment step (Table 2). When tomato samples were inoculated with 10^2 conidia/g of tomato with no enrichment step, 6.6 x 10^2 conidia/g of tomato were detected. In contrast, 1.5×10^4 conidia/g of tomato was determined with a 20 h enrichment step. Thus, an inoculation of 10 conidia/g of tomato was not enough to produce a signal in the qPCR. No detectable signal was observed in the negative controls. Our results are similar to those in previous studies focused on the quantification of DNA by qPCR methods. Rodríguez et al. (2011) evaluated the sensitivity of detection for different ochratoxin A producing moulds in artificially inoculated food matrices, obtaining a LOD that ranged from 10 to 1 conidia/g. Similarly, Diguta et al. (2010) developed a qPCR system to quantify and identify *B. cinerea*, one of the major pathogens present in grapes. A LOD of 6.3 pg of DNA was obtained, corresponding to 540 conidia without the enrichment step. Selma et al. (2008) established a useful qPCR protocol to detect and quantify conidia in wine grapes inoculated with *Aspergillus carbonarius*, achieving a LOD of 5 conidia/reaction without incubation.

Table 2. Limit of detection of *A. alternata* in artificially contaminated tomato samples using the primers Alt4-Alt5 for the qPCR.

Inoculated conidia/g tomato	Detected conidia /g tomato (without incubation)	Detected conidia /g tomato (after 20 h incubation at 26 ºC)
10^5	$4.4x10^5 \pm 3.1x10^5$	$3.7x10^6 \pm 3.2x10^6$
10^4	$6.4x10^4 \pm 1.6x10^4$	$2.4x10^6 \pm 1.8x10^6$
10^3	$7.1x10^3 \pm 1.1x10^3$	$5.6x10^4 \pm 6.5x10^4$
10^2	$6.6x10^2 \pm 3.4x10^2$	$1.5x10^4 \pm 1.6x10^3$
10	-	-
0	-	-

Although the LOD achieved in our study (10^2 CFU/g of food matrix) is similar to that obtained with conventional culture methods, the aim of using PMA-qPCR instead of standard plate counts was to reduce the analysis time, since results can be obtained in 24 hours instead of the 5-7 days needed otherwise. It should be considered that *Alternaria* spp. grow slowly, so a longer enrichment step might be required in order to detect lower concentrations of conidia. There are no legislations regarding the presence of fungi in food products, only the level of mycotoxin contamination, which must be below the value established by the law (CEC, 2006). Therefore, mycotoxin analysis is undoubtedly an essential requirement. However, the detection of viable cells, which have the possibility to grow and produce mycotoxins, might be useful in order to apply corrective measures in an industrial context, particularly when the vegetables are stored before processing.

3.4. PMA sample pre-treatment combined with qPCR to detect and quantify viable *Alternaria* conidia

One of the limitations of PCR and qPCR is the inability to discriminate between live and dead cells. To bypass this problem, the use of PMA has been tested in *Alternaria* spp. cells, being PMA concentration a key factor to effectively discriminate between viable and non-viable cells. An adequate concentration must be added to detect exclusively viable cells, but no cytotoxicity should be observed.

Hence, live cells from an *A. altermata* (CECT 20560) culture were exposed to four different concentrations of PMA (20, 30, 40, and 50 µM), and toxicity was measured as detailed above (2.6). Additionally, to study the influence of the food matrix on PMA toxicity, PMA was added to artificially inoculated samples reaching final dye concentrations of 50, 60, and 65 µM. As shown in Figure 3A no differences in CFU counts were observed between samples in PBS treated with PMA concentrations of 20, 30, and 40 µM and the untreated live cells. However, a statistically significant toxic effect (p=0.05) was found when conidial suspensions were treated with 50 µM of PMA. Interestingly, no cytotoxic effect was observed for live cells in tomato matrices exposed to 50, 60, and 65 µM of PMA, based on plate count data (Figure 3B). This finding suggests that the tomato matrix might hinder PMA entry into the cell and higher PMA concentrations would be required for an efficient discrimination between viable and non-viable cells. The maximum PMA concentrations with no cytotoxic effects were 40 and 65 µM in PBS and food matrix respectively, thus, these were used in subsequent experiments.

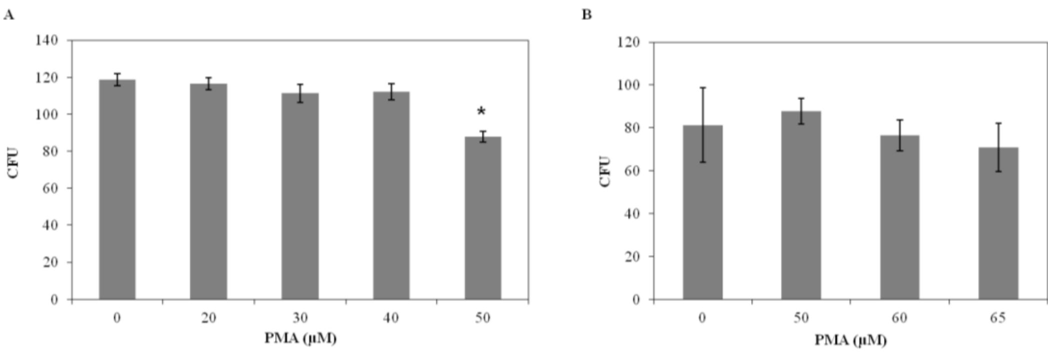

Figure 3. PMA toxicity to conidia suspensions of an *A. alternata* culture (CECT 20560) in PBS (**A**) or in a tomato matrix (**B**). Colony forming units (CFU) were counted in PDA plates after an incubation of 24 h at 26 ºC. (*) indicates statistically significant differences (ANOVA, $p < 0.05$). Error bars represent standard deviations obtained from three independent replicates.

PMA specificity was obtained by comparing qPCR quantification cycle values for treated live and dead cells. A signal reduction of 8.86 cycles was observed between live and heat-killed conidia in PBS treated with 40 µM of PMA (Figures 4 and 5). Additionally, the subtraction of Cq values of PMA-treated live cells from samples with PMA-treated live-dead (50:50) cells in PBS was 0.98 cycles, which correlates with the fact that in one PCR cycle almost all the DNA is duplicated (E=1.9057).

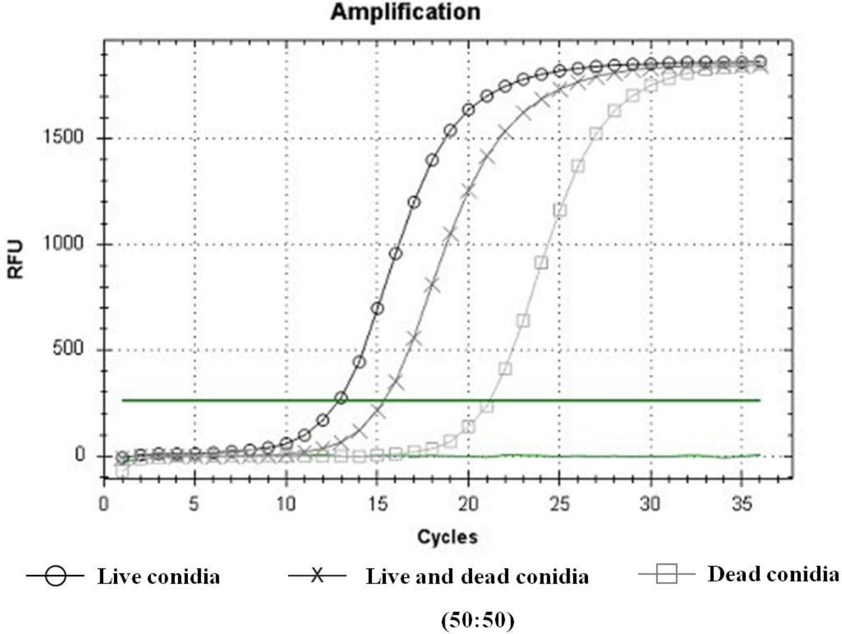

Figure 4. Amplification curves obtained by qPCR using a 40 µM PMA treatment. Curves correspond to live *A. alternata* (CECT 20560) conidia, a mix of live and heat killed conidia (50:50) and heat killed conidia in PBS. Analyses were performed in triplicate. RFU: relative fluorescence units.

Therefore, the DNA quantified in PMA-treated live-dead (50:50) cells belonged to live cells, whereas the amplification of the DNA from dead cells may be inhibited due to PMA activity. Conversely, when exposing fungal cells in the tomato matrix to 40 µM of PMA, a difference of 3.23 Cq between live and heat-killed conidia

was found (Figure 5), confirming the tomato matrix effect observed in the PMA toxicity assay described above. PMA efficiency was shown to increase with higher PMA concentrations, achieving a difference of 6.85 Cq between live and heat-killed conidia in the tomato matrix treated with 65 µM of PMA. Furthermore, the subtraction of Cq values of PMA-treated live cells from samples with PMA-treated live-dead (50:50) cells in tomato matrix was 1.04 cycles.

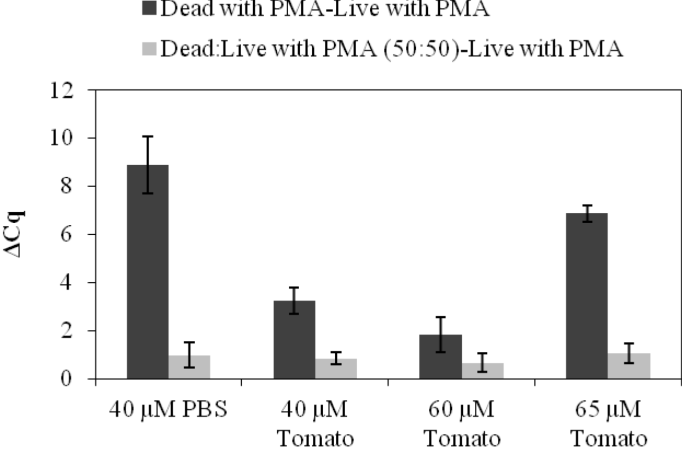

Figure 5. Effect of different PMA concentrations on Cq values. Results are presented as the difference of Cq values between dead and live cells treated with PMA and the difference of Cq values between dead:live (50:50) and live cells treated with PMA. Error bars represent standard deviations obtained from three independent replicates.

Since the first description by Nocker et al. (2006), PMA has been applied to a wide variety of microorganisms including bacteria (Elizaquível, Sánchez, Selma et al., 2012; Kralik et al., 2010; Nocker et al., 2009), yeast (Andorrà et al., 2010), virus (Fittipaldi et al., 2010; Sánchez et al., 2012), and fungi (Vesper et al., 2008). PMA concentration, incubation conditions, and light source proposed as optimal are highly variable depending on both the microorganism and buffers or the food matrices containing the microorganisms. For instance, a 6 µM of PMA has been proposed for the detection of viable yeast in wine (Andorrà et al., (2010), while

100 µM of PMA was used to identify *Escherichia coli* O157:H7 in vegetables (Elizaquível, Sánchez, Selma et al., 2012). Consequently, the efficiency of the treatment varies among samples, with a 7 to 13.6 Cq reduction between live and dead cells for pure bacterial cultures (Kralik et al., 2010; Nocker, Sossa et al., 2007), a 6 to 11 Cq reduction for yeast in wine (Andorrà et al., 2010), a reduction that ranges from 1 to 3 Cq for detecting *Vibrio parahaemolyticus* in seafood (Zhu et al., 2012), or a 1.3 to 3 reduction in log_{10} CFU for bacteria in vegetables (Elizaquível, Sánchez and Aznar, 2012; Yáñez et al., 2011). Vesper et al. (2008) have published the only study in which PMA has been used to identify viable cells in fungi. These authors studied the effect of PMA in several fungal strains (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Mucor racemosus*, *Rhizopus stolonifer*, and *Paecilomyces variotii*) in air and water samples, achieving a Cq reduction of 6 to 9 cycles between live and dead cells. In our study, a Cq reduction of 8.86 (a 302.9 fold reduction) between live and heat-killed cells in PBS was observed for samples treated with 40 µM of PMA, while a difference of 6.85 Cq (82.86 fold reduction) was found in the case of live and dead cells in the tomato matrix exposed to 65 µM of PMA. Furthermore, it seems dead cells are somehow *protected* by the tomato matrix, reducing PMA toxicity and the capacity to inhibit qPCR DNA amplification in these cells.

Other useful techniques have been proposed to reveal viable cells, such as RNA detection by reverse-transcriptase PCR (RT-PCR) (Kidon Sung, 2005; Sheridan et al., 1998). This technique has also been assayed in food matrices, often combined with qPCR (Bleve et al., 2003; Hierro et al., 2006; M. Vaitilingom, 1998; M.B. Mayoral, 2006; Pavón, González, Martín and García, 2012). The detection of highly unstable RNA, specifically mRNA, which is only produced by metabolically active cells, would allow identifying viable cells only. In contrast to highly persistent DNA that can remain stable from days to three weeks (Josephson et al., 1993), RNA degrades more rapidly after cell death. Nonetheless, the instability that makes RNA a suitable target for detecting viable cells, would turn it

to a difficult target to work with because it is prone to contamination due to inadequate sample processing, unsatisfactory storage, or due to contamination with RNA-degrading enzymes. All these drawbacks lead to reproducibility problems. Furthermore, it should be considered that mRNA depends on the metabolic activity of the cell, so the quantification could be overestimated when there is an active physiological state or underestimated if the cells are alive but latent (Nocker and Camper, 2006).

The activation of PMA using blue LED photoactivation systems instead of a halogen light source is another advantage initially proposed by Vesper et al. (2008), which was tested in our work for *Alternaria* spp. The problem of using high-wattage halogen light sources (≥ 600 W as recommended by the PMA manufacturer, BIOTIUM) is that cell membranes could be damaged by the heat emitted by the lamp, making them susceptible to PMA entry. Therefore, activation periods are usually < 2 minutes in PMA treatments using high-wattage halogen light sources (Andorrà et al., 2010; Josefsen et al., 2010; Nocker, Sossa et al., 2007). To avoid excessive heating, some authors have suggested to place the sample on ice, before or after the activation. Conversely, longer activation periods, from 10 to 15 minutes, which could help improve PMA efficiency, have been carried out using LEDs (Elizaquível, Sánchez and Aznar, 2012; Fittipaldi et al., 2010; Vesper et al., 2008).

In this work, we have developed a fast, sensitive, and efficient technique based on the pre-treatment of the sample with PMA combined with qPCR, which allows detecting DNA from viable *Alternaria* spp. cells. In tomato samples, detection of DNA from dead cells is around 82.86-fold lower when PMA is used in comparison with live cells. This methodology can be useful to the food industry as it could be employed as a preventive tool to detect the risk of contamination in foodstuffs with potentially mycotoxigenic fungi as is the case of *Alternaria* spp., which are responsible of the production of mycotoxins such as AOH, AME, TEN, TeA, ALT, ATXs, stemphyltoxin III, and AAL-toxins.

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Chapter II

Quantification of viable conidia of *Alternaria* spp. and occurrence of alternariol and alternariol monomethyl ether during the food processing of tomato products

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Quantification of viable conidia of *Alternaria* spp. and occurrence of alternariol and alternariol monomethyl ether during the food processing of tomato products

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Abstract

During two harvest seasons, the presence of conidia from *Alternaria* spp. and the occurrence of two *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME), was investigated throughout the food production chain of two industries, one used organic fruits and the other did not. For this purpose a propidium monoazide (PMA) treatment followed by a quantitative Real Time PCR (qPCR) was used to detect and quantify the viable conidia exclusively. Results demonstrated that 68.4% of the total raw fruits analyzed were contaminated with viable *Alternaria* spp. Fungal contamination was also found in mashed tomatoes analyzed before the heat treatment with medians around 350 and 90 conidia/g of tomato, for the organic and the not organic industry, respectively. High levels of *Alternaria* conidia were also found in waste by-products such as rejected tomatoes

in sorting tables. Regarding the occurrence of AOH and AME, only few samples were contaminated with AME (5 out of 127). In contrast, 35% of raw tomatoes were positive for AOH in the organic industry and 21% in the other one. AOH was also present in samples analyzed before the heat treatment, with a median of 96.5 μg/kg for mashed tomatoes and 257.4 μg/kg for peeled tomatoes (in the organic industry). While almost no mycotoxins were found in the final products (tomato purée and scalded peeled tomatoes) of the organic industry, in the conventional one 47% of the tomato concentrates were contaminated with levels that ranged between 22.6 and 137.4 μg/kg of tomato. Latest samples had been concentrated previously, reason that may explain these high levels of contamination.

Keywords: *Alternaria* spp.; AOH; AME; tomatoes

1. Introduction

The genus *Alternaria* includes several species that cause plant diseases in many crops and can spoil various fruits, grains, and vegetables during post-harvest storage and transport. *Alternaria* spp. are known to produce several secondary metabolites, some of which are mycotoxins. The most common mycotoxins associated with *Alternaria* in food commodities include alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA), altenuene (ALT) and altertoxins (Barkai-Golan, 2008; EFSA, 2011; Logrieco et al., 2009; Ostry, 2008). There is still scarce information about the toxicological effects of *Alternaria* mycotoxins though some studies suggest they are harmful to human and animals (Brugger et al., 2006b; Liu et al., 1992; Pero et al., 1973; Pfeiffer et al., 2007; Pollock et al., 1982).

The European Commission asked the European Food Safety Authority (EFSA) to assess the risk for animal and public health regarding *Alternaria* mycotoxin exposure. This way, the European Commission and the competent authorities in

the Member States could consider the need for a possible follow up, including filling the knowledge gaps. In 2011 the EFSA emitted a scientific opinion in relation to the presence of *Alternaria* toxins in feed and food (EFSA, 2011). A total of 11730 analytical data were considered for this assessment, which included reports of the occurrence in food and feed and data published in the scientific literature. Results were characterized by a high proportion of values below the limit of detection (LOD) and the limit of quantification (LOQ). The EFSA concluded that there was a need for more representative occurrence data on *Alternaria* toxins in food and feed across the European countries in order to establish the real exposure by consumers. Additionally, the EFSA asked for more studies related to the influence of food and feed processing on *Alternaria* toxins.

Tomatoes and their derived products are widely consumed over the entire world but, unluckily, due to their thin skin they are very susceptible to fungal decay. In this sense, it has been reported that *Alternaria* spp. are the primary cause of black mold disease on raw tomatoes and are considered to be the major postharvest spoilers of fresh tomatoes (Andersen and Frisvad, 2004; Morris et al., 2000). Green and ripe fruits can be affected by the black mold disease which is influenced by physiological alterations, such as nutritional deficiency or skin sunburn. Warm rainy weather or dew formation on the fruit surface favor the disease, which is more severe if infection occurs when fruit is ripe rather than green (Logrieco et al., 2003). While the direct consumption of a moldy tomato by a consumer is unlikely, there exists the possibility that moldy tomatoes are used for the production of tomato products such as ketchups, purées, juices, sauces, among others. There are several reports that describe the presence of *Alternaria* mycotoxins in different tomato commodities (Ackermann et al., 2011; da Motta and Valente Soares, 2001; Noser et al., 2011; Terminiello et al., 2006; Van de Perre et al., 2014; Visconti et al., 1987). Not only tomatoes are affected by *Alternaria* spp. since other studies have detailed the presence of *Alternaria* toxins in olives (Visconti et al., 1986), mandarins (Logrieco et al., 1990), peppers (Monbaliu et al., 2009), apples and their

derived products (Delgado and Gómez-Cordovés, 1998; Stinson et al., 1981), wheat and other grains (Monbaliu et al., 2010), among others.

Tomato fruit can be contaminated in crop fields and can enter the food industry with a high rate of contamination, which can become higher after a period of storage if conditions are favorable for fungal growth. Once inside the production plant, limited information is available regarding the stability and fate of *Alternaria* toxins during storage and processing. There are some studies that indicate mycotoxins are highly stable and are not completely destroyed throughout the food processing. Dealing with *Alternaria* toxins, Siegel et al. (2010) investigated the stability of AOH, AME and ALT upon bread baking using flour spiked with mycotoxins. They found that mycotoxins were barely degraded during wet baking, while significant degradation was observed upon dry baking. Similarly, Combina et al. (1999) studied the effect of thermal processing on the stability of AOH, AME and TeA using a sunflower flour matrix. The authors found that the most effective treatment to reduce AOH and AME was heating samples at 121 ºC for 60 minutes. Hence, if food industries use raw fruits contaminated with *Alternaria* spp., capable to produce mycotoxins, these toxic compounds could be present in final products. The Food and Agriculture Organization of the United Nations (FAO) specifies that all those materials susceptible to be contaminated with natural toxins, have to fulfill the levels indicated by the national or international regulations (Dauthy, 1995). However, *Alternaria* mycotoxins are not regulated yet and so, no controls are required for these toxins.

The aim of this work was to investigate the fate of conidia from *Alternaria* spp. throughout the industrial food chain. For this purpose the propidium monoazide (PMA) treatment, which is a DNA binding dye that allows detecting exclusively the viable conidia, has been attached to a quantitative Real Time PCR (qPCR) to quantify the viable conidia present in each one of the samples analyzed. Additionally, the occurrence of AOH and AME has been assessed on several

production stages of two different industries, one of them with organic tomatoes as raw material and the other one with conventional tomatoes. Results might help to evaluate if any type of regulation is needed for these toxic contaminants.

2. Material and Methods

2.1 Analytical standards and chemicals

Standards of AOH (~96%) and AME (~96%) were supplied by Sigma–Aldrich (Alcobendas, Spain). A stock solution was prepared for each standard by dissolving 5 mg of the purified mycotoxins in ethanol reaching a final concentration of 1000 µg/mL. From the stock standard solutions, working standard solutions at a concentration of 15 µg/mL were prepared. AOH and AME concentration was checked by UV spectroscopy. All standards were stored at -20 ºC in sealed vials until use.

Acetonitrile (99.9%) and methanol (99.9%) were both HPLC (high-performance liquid chromatography) grade and were supplied by J.T. Baker (Deventer, The Netherlands). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA).

2.2 Tomato sample collection in the food industry

Tomato samples were collected from two different food industries along two harvest seasons, 2012/2013 and 2013/2014. Both food industries were located in the province of Lleida (Spain). One of the industries worked with organic fresh tomatoes while the other used non organic tomatoes. Samples were collected randomly in all the production stages where it was physically possible, since in some of the stages the process was completely automated and samples could not be collected (Fig. 1). In the organic industry, raw tomatoes were collected from

wooden pallets before getting in the production plant. Once inside, samples were picked before the heat treatment (peeled tomatoes and mashed tomatoes).

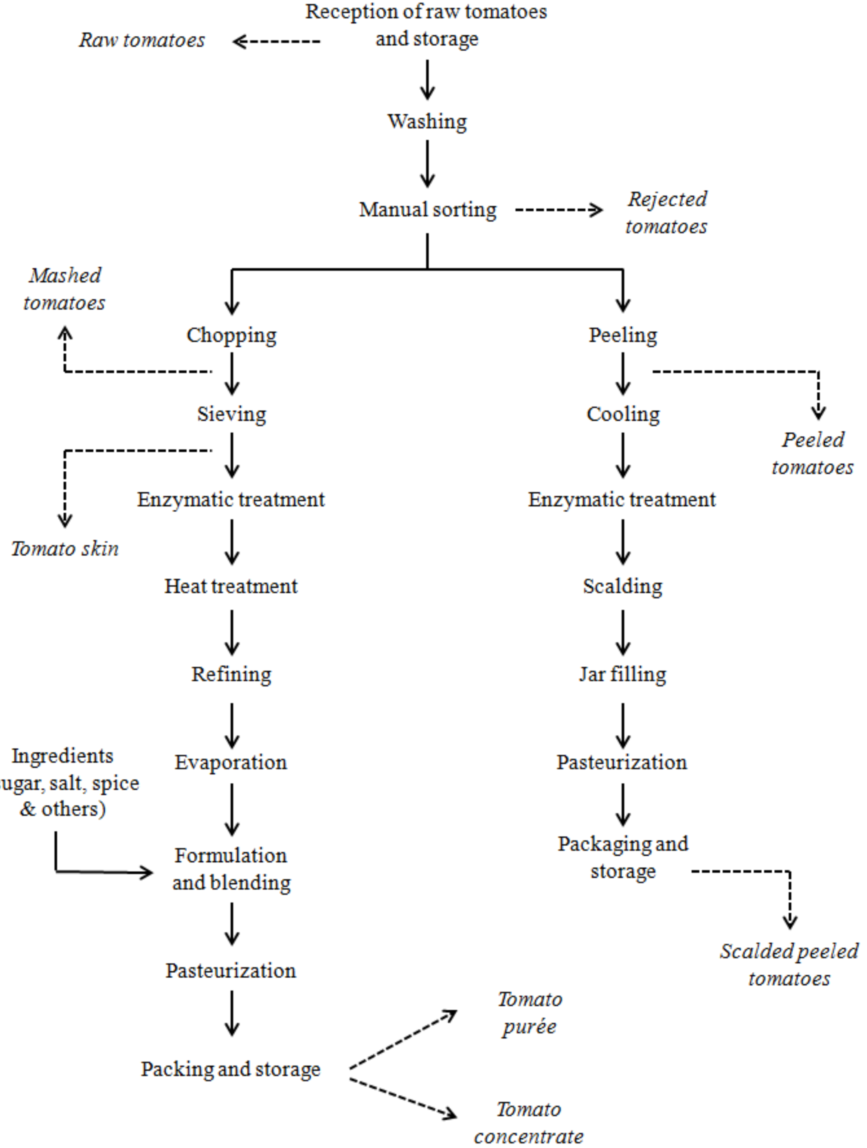

Figure 1. Production flow chart of derived tomato products. Italic letters specify the type of tomato samples that were collected in the organic and conventional industry for the assessment of *Alternaria* spp. presence and occurrence of AOH and AME.

Waste by-products such as rotten tomatoes, rejected during the manual selection or tomato skin peels, obtained from the production of scalded peeled tomatoes, were also collected. The final products picked from the organic industry were tomato purée and peeled tomatoes. From the conventional industry, raw tomatoes were collected before getting in the production plant as well and before the heat treatment (mashed tomatoes). In this case, the final product collected was tomato concentrate.

2.3 Quantification of viable *Alternaria* conidia

2.3.1 Sample preparation

Collected samples were put in plastic bags and once in the laboratory were weighed (around 1 kg). All these data was later used to process the results. Tomato samples were mixed in a beaker with one volume of phosphate buffer saline (PBS, 138 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4•2 H_2O, and 2 mM KH_2PO_4) and were homogenized with a blender (Turbo Habana, Palson, Spain). From the mixture, two aliquots of 50 mL each one were collected for the analysis of AOH and AME (see section 2.4). These samples were frozen at -20 ºC until performing the mycotoxin extraction and the HPLC analysis. Similarly, for assessing the presence of viable conidia of *Alternaria* spp., two aliquots of 50 mL each were taken from the sample and DNA extraction was carried out immediately.

2.3.2 DNA quantification from viable conidia using the PMA-qPCR technique

Tomato samples were filtered through a Miracloth paper (Calbiochem, USA) and centrifuged at 15000 *x g* for 10 min. Pellets were resuspended in 2 mL of PBS. Next, the PMA treatment was carried out combined with the qPCR (PMA-qPCR) to quantify viable conidia from *Alternaria* spp. The PMA treatment, DNA extraction and qPCR detection and quantification were performed as detailed in Crespo-Sempere et al. (2013). In the current study, the LOD was considered the lowest DNA concentration that can be detected with 95 percent confidence that it

is a true detection. For its calculation, a six-point calibration curve was developed using six different conidia concentrations of *A. alternata* (10, 10^2, 10^3, 10^4 and 10^5 conidia/g of tomato), which were used to artificially contaminate different samples of tomato purée. Not contaminated tomato purée was considered a negative control. Next, the PMA-qPCR method was applied to these contaminated tomato purée samples. A five-point calibration curve was developed using the *A. alternata* DNA extracted from each one of the tomato samples (Fig. 2).

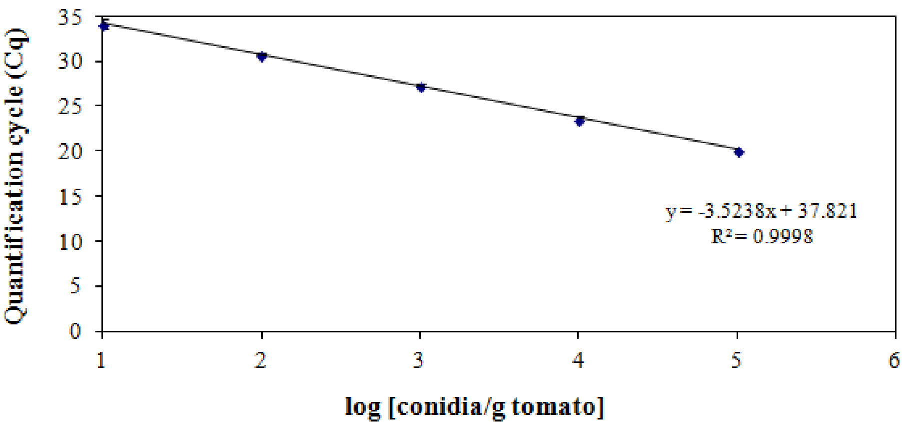

Figure 2. Standard curve obtained with SYBR Green I using five tomato food matrix samples artificially inoculated with *A. alternata* conidia with different concentration each sample (10^6, 10^5, 10^4, 10^3, 10^2 and 10 conidia/g of tomato).

The mean of the quantification cycles (Cq) of all the replicates with the lowest DNA concentration (10 conidia/g of tomato) was used to calculate the LOD by using the following equation:

$$log_{10}\, Concentration = \frac{Cq - b}{m}$$

Eq. 1

Where m is the slope of the regression line and b is the interception point with the y-axis (Fig. 2). According to Eq. 1, the LOD was considered 11 conidia/g of tomato. The LOQ was considered the lowest concentration that could be quantified reliably. The Cq of the LOQ was determined using the following equation:

$$Cq_{LOQ} = Cq_{LOD} - 2(\sigma_{LOD})$$

Eq. 2

Where the Cq_{LOD} was the average of all the replicates with the lowest DNA concentration detectable and the σ, their standard deviation. According to Eq. 1 and Eq. 2, the LOQ established for this work was 28 conidia/g of tomato. The LOD and LOQ have been improved compared to our previous work (Crespo-Sempere, Estiarte et al., 2013). Once the LOD and the LOQ were determined, all values below the LOD were considered 0, while values between the LOD and the LOQ were substituted by 28 conidia/g of tomato.

2.4 Assessment of natural occurrence of AOH and AME

2.4.1 AOH and AME extraction

For the AOH and AME extraction procedure, 20 g of each tomato sample were mixed with 60 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric) in a small glass beaker and homogenized for 15 minutes using a uniform magnetic mixer. The solution was left for approximately 10 minutes, to favor precipitation by gravity. Then, 6 mL of the supernatant was transferred to a centrifuge tube and diluted with 15 mL of a 0.05 M sodium dihydrogen phosphate solution (pH 3 adjusted with *o*-phosphoric) and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract was passed by gravity filtration through a previously conditioned Bond Elut Plexa SPE cartridge (200 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the cartridge was done with 5 mL of methanol followed by 5 mL of miliQ water. The SPE

column was washed with 5 mL of water followed by air drying on the manifold. Finally, elution was carried out with 5 mL of methanol and 5 mL of acetonitrile. Sample extracts were dried in a speed vacuum concentrator at room temperature and stored at -20 ºC until the HPLC analysis. Prior to HPLC injection, samples were resuspended in 500 µL of a water-methanol solution (50:50 v/v) and sonicated for 1 minute.

2.4.2 HPLC analysis

Separation, detection and quantification of AOH and AME were performed on a HPLC system consisting of a Waters 2695 Alliance Separations Module connected to a UV/Visible dual λ absorbance Detector Waters 2487, using a reversed phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a Spherisorb guard column (5 µm ODS2, 4.6 x 10 mm, Waters, Millford, MA, USA). Columns were set at a temperature of 35 ºC. For chromatographic separation of AOH and AME the flow rate was 0.5 mL/min and the injection volume was 100 µL. Absorption wavelength was 258 nm. The mobile phase consisted of a double distilled miliQ water and methanol-water (70:30 v/v) according to the gradient described in Table 1.

Table 1. HPLC gradient used for the chromatographic analysis of AOH and AME.

Time (min)	Water	Methanol-water (70:30 v/v)
0	100	-
7	100	-
9	20	80
10	20	80
12	-	100
41	-	100
43	10	90
45	30	70
46	50	50
47	70	30
50	100	-

The retention times were 24 minutes for AOH and 32 minutes for AME. For mycotoxin quantification, working standard solutions were used to perform a ten-point calibration curve (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). The LOD of the analysis was 10 µg/kg of tomato for AOH and 12 µg/kg of tomato for AME, based on a signal-to-noise ratio of 3:1. All solvents were HPLC grade and all chemicals were analytical grade. The LOQ was calculated as 3 × LOD. Method performance characteristics for AOH and AME are summarized in Table 2.

Table 2. Recovery and repeatability of AOH and AME analysis on tomatoes.

	AOH			AME		
Spiking level (µg/kg tomato)	**n**	**Recovery [a] (%)**	**RSDr [b] (%)**	**n**	**Recovery (%)**	**RSDr (%)**
500	3	115.5 ± 2.1	1.8	3	106.0 ± 8.2	7.7
1000	5	99.0 ± 2.4	2.4	5	93.8 ± 3.1	3.4
2000	3	79.8 ± 3.2	4.0	3	78.0 ± 3.6	4.6

[a] Mean value ± standard deviation.
[b] RSDr = relative standard deviation.

2.5 Statistical analysis

All statistical data were analyzed assuming a non-parametric distribution. Multiple comparisons were made with the Wilcoxon test, which compares the medians between pairs. The p-value was established as 0.05. All statistical analyses were performed with the JMP program.

3. Results and Discussion

3.1 Presence of viable *Alternaria* spp.

The primer set Alt4-Alt5 used in this study was designed previously (Crespo-Sempere, Estiarte et al., 2013) to detect and quantify with the qPCR several

Alternaria spp. such as *A. alternata, A. arborescens, A. tenuissima, A. tomato, A. tomatophila, A. tomaticola* and *A. solani*. Primer set Alt4-Alt5 was not able to distinguish between *Alternaria* spp. and *Ulocladium botrytis*, but the joint detection of both genera could be an advantage for the food industry since *U. botrytis* is also considered a plant pathogen and a mycotoxin producer (Andersen and Hollensted, 2008). All these species are commonly associated with the decay of fruits and vegetables, especially in tomatoes. For this study a total of 175 samples were analyzed between 2012 and 2014. Some of these samples were collected from an industry with organic tomatoes as raw fruits, specifically 115 samples (Fig. 3).

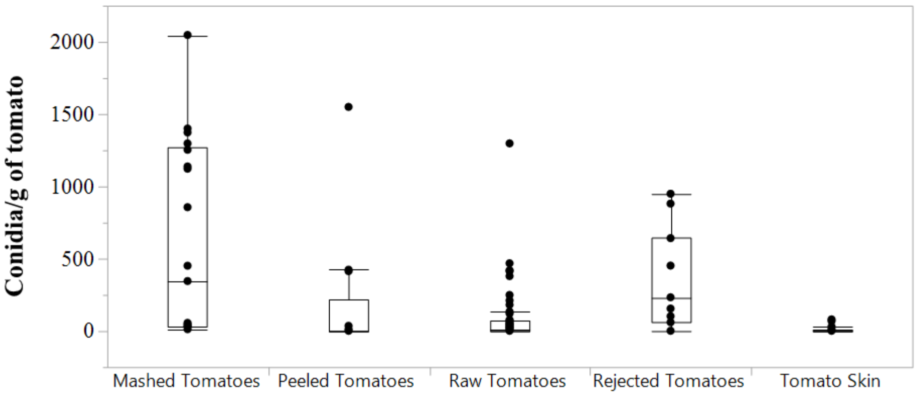

Figure 3. Distribution of the concentration of viable *Alternaria* conidia during the tomato processing in an industry with an organic production. The horizontal line within the box represents the median sample value. The ends of the box represent the 3rd and 1st quartile. The whiskers represent the lowest datum still within 1.5xIQR of the lower quartile, and the highest datum still within 1.5xIQR of the upper quartile.

The other 60 tomato samples were taken from a food industry that used non organic raw tomatoes (Fig. 4). The PMA-qPCR technique allows detecting exclusively the viable conidia. Results demonstrated the presence of viable

Alternaria in 127 out of 175 samples, which means that 72.6% of the samples were contaminated. Among these samples, 31 out of 127 had an amount of contamination below the LOQ (28 conidia/g of tomato), which represents 24.4% of the whole positive samples. Within the positive samples, 57.8% corresponded to the organic industry, while the 42.5% had been collected from the conventional company. The box plots illustrated in Figs. 3 and 4 show the distribution of fungal concentration for all the production stages analyzed.

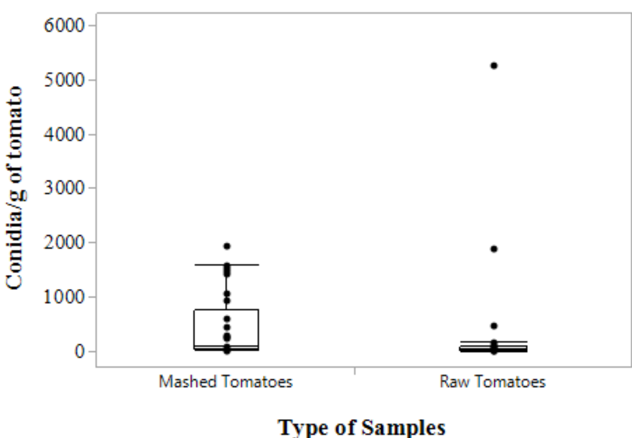

Figure 4. Distribution of the concentration of viable *Alternaria* conidia during the tomato processing in an industry with a conventional production. The horizontal line within the box represents the median sample value. The ends of the box represent the 3rd and 1st quartile. The whiskers represent the lowest datum still within 1.5xIQR of the lower quartile, and the highest datum still within 1.5xIQR of the upper quartile.

Several production stages were analyzed for the presence of viable *Alternaria* spp. in the organic industry. From the whole raw tomato fruits collected, 29 out of 48 were positive for viable conidia, which represent the 60.4% of the raw material. Regarding the conventional company, 80.6% of the raw tomatoes were contaminated with viable conidia. Comparing both industries, the conventional company had the highest percentage value of raw samples contaminated with viable conidia though the median for both cases was very similar and the statistic

analysis did not show significant differences between both industries (p-value > 0.05). Importantly, in both industries it was observed that the contamination with *Alternaria* was very heterogeneous. In this sense, it has to be taken into account that samples were collected in different days along the whole two seasons and depending on the day, the ripening stage of fruits and the percentage of rotten tomatoes were different, which may affect the total contamination of *Alternaria* spp. In fact, warm rainy weathers or dew formation on the fruit surface favor *Alternaria* disease. Additionally, depending on the ripening stage of the fruit, infection could be more sever. As riper is the fruit, more susceptibility to fruit decay (Logrieco et al., 2003). All these variables may influence fungal concentration levels. Despite the variability of *Alternaria* concentration, the black mold disease was observed in many tomatoes when collecting samples. Actually, it has been reported that *Alternaria* spp. are the primary cause of black mold disease in raw tomatoes (Morris et al., 2000). Reports on the occurrence of *Alternaria* spp. in fruits are numerous, especially in tomatoes. Harwig et al. (1979) studied which molds were present in rotten tomatoes and observed that 15 out of 41 were *Alternaria* spp., which represents the 37%. These results were supported some years later by Andersen and Frisvad (2004), who isolated fungal colonies from several moldy tomatoes and found that the most predominant genus was *Alternaria* (40%), followed by *Penicillium* (25%), *Stemphylium* (15%), and *Cladosporium* (10%). Among *Alternaria* spp., *A. tenuissima* was the most frequent species. More recently, Pavón et al. (2012) detected and quantified by RT-qPCR the presence of *Alternaria* spp. in raw and processed fruits. Their analysis demonstrated the presence of *Alternaria* in 46 out of the 80 raw samples analyzed, which represents the 58%.

Before entering in the production plant, tomatoes are washed. Afterwards, rotten tomatoes are manually sorted in sorting tables. In the organic industry, these rejected tomatoes were assessed for the presence of *Alternaria* and it was observed that 81.8% of the samples were contaminated with *Alternaria* conidia. However,

even though tomatoes had been washed and selected manually, the concentration of viable *Alternaria* did not decrease at all on the following production stages. In the non organic industry the same tendency was observed. The Wilcoxon test showed that in both industries, mashed and rejected tomatoes were significantly different compared to the rest (p-value < 0.05). Mashed tomatoes had higher concentration of viable *Alternaria* compared to raw tomatoes. A possible explanation could be due to the immersion of raw fruits into water baths. Commonly, in the food industry there are two different immersions in water. The first one occurs before entering in the production plant and it is used to wash fruits and transport them to the hand sorting tables. The second immersion in water takes place after the manual selection because that way, tomatoes are transported easily. The frequency of water replacement in these baths will depend on the food industry but it may be low. Hence, this water becomes highly contaminated and fruits may become contaminated through it. Following the water bath, all tomatoes used for the production of purées or sauces go to a chopping tank where they are chopped and mixed all together. The mashed tomatoes analyzed in this study were collected from this tank. It may be important to notice that, when producing scalded peeled tomatoes the contamination was lower. In this case, tomatoes are not blended all together and the skin is removed. Additionally, for the production of scalded peeled tomatoes the quality of raw fruits is usually better and, probably, less contaminated with fungi. Actually, assessing fungal contamination of tomato skin samples, results showed that these samples were not contaminated at all as the median was below our LOD. All these factors together may help to decrease the total fungal contamination when producing scalded peeled tomatoes.

The presence of *Alternaria* conidia was not analyzed on the final products as it was assumed that fungi should be killed during the heat treatment and so, no viable conidia would be found.

3.2 Occurrence AOH and AME along the food production chain

A total of 277 tomato samples were analyzed for the presence of both AOH and AME during two different harvest seasons. From these samples, 184 were collected from the organic industry, while the other 93 samples were collected from the conventional one. As previously done for the assessment of the presence of *Alternaria*, samples were collected from different stages of the industrial production chain. In general, the occurrence of AOH was by far higher than AME, as just 5 out of 277 tomatoes were contaminated with AME.

Table 3. Natural occurrence of AOH and AME in several tomato products obtained from an industry with an organic production.

		AOH			
	n	**Positive Samples (%)**	**Range***	**Mean***	**Median***
Raw tomatoes	48	17 (35%)	95.4-1318.6	386.8	261.4
Peeled tomatoes	18	4 (22%)	117.4-445.5	269.4	257.4
Mashed tomatoes	20	3 (15%)	95.4-100.3	97.4	96.5
Tomato skin	14	5 (36%)	153.1-209.8	169.5	162.6
Rejected tomatoes	10	4 (40%)	111.2-265.3	165.2	142.1
Scalded peeled tomatoes	51	0	ND	ND	ND
Tomato purée	23	1 (4%)	0.0-26.4	-	-

		AME			
	n	**Positive Samples (%)**	**Range***	**Mean***	**Median***
Raw tomatoes	48	1 (2%)	0.0-136.8	-	-
Peeled tomatoes	18	0	ND	ND	ND
Mashed tomatoes	20	2 (10%)	100.9-105.6	103.2	103.2
Tomato skin	14	1 (7%)	0.0-502.2	-	-
Rejected tomatoes	10	1 (2%)	0.0-197.8	-	-
Scalded peeled tomatoes	51	0	ND	ND	ND
Tomato purée	23	0	ND	ND	ND

*The range, the mean and the median were calculated just for positive samples. Units for range, mean and median were µg/kg of tomato.

All samples contaminated with AME had been collected in the organic industry (Tables 3 and 4). As described in Table 3, the group of samples that reached the highest percentage of contamination for AOH in the organic industry were the set of samples of rejected tomatoes (40%), followed by tomato skin (36%) and raw fruits (35%). The two final products analyzed, scalded peeled tomatoes and tomato purée, were not contaminated at all. Just one sample of purée was found to be positive. In fact, it is reasonable that the highest percentage of contamination for AOH is found among rejected samples because these fruits were rotten and they were discarded from the production flow due to their high fungal contamination. In contrast, the contamination of AOH or AME in mashed tomatoes, which were highly contaminated with *Alternaria* conidia, was not high at all. This could be explained by the fact that the contamination of mashed and peeled tomatoes possibly takes place when tomatoes are dipped into water during the washing step. Hence, these *Alternaria* spp. did not have time to biosynthesize mycotoxins since samples were collected immediately after the washing step.

Table 4. Natural occurrence of AOH and AME in several tomato products obtained from an industry with a conventional production.

		AOH			
	n	**Positive Samples (%)**	**Range***	**Mean***	**Median***
Raw tomatoes	34	7 (21%)	336.5-1436.9	1000	1165.2
Mashed tomatoes	29	4 (17%)	92.7-107.9	101	101.2
Tomato concentrate	30	14 (47%)	22.6-137.4	45	39.0

		AME			
	n	**Positive Samples (%)**	**Range***	**Mean***	**Median***
Raw tomatoes	34	0	ND	ND	ND
Mashed tomatoes	29	0	ND	ND	ND
Tomato concentrate	30	0	ND	ND	ND

*The range, the mean and the median were calculated just for positive samples. Units for range, mean and median were µg/kg of tomato.

In the conventional industry (Table 4), results showed that considering only positive samples, the median of contamination for AOH in raw fruits (1165.2 µg/kg of tomato) was higher than the one in the organic industry (261.4 µg/kg of tomato). However, when AOH concentration from contaminated and not contaminated tomatoes was considered, there were no significant differences between raw fruits from the organic and from the conventional industry (p-value > 0.05). Similarly, no significant differences were found between mashed tomatoes from the organic and the conventional industry (p-value > 0.05). In both industries, the assessment of the presence of AOH and AME in different production stages demonstrated that toxin levels decreased throughout the production chain. However, in the conventional industry 47% of the final products assessed were significantly more contaminated with AOH compared to the final products from the organic industry (p-value < 0.05), where almost no contaminated samples were found. It is important to mention that in the conventional industry the final product was tomato concentrate, so, the reason for this difference could be the concentration step.

Currently there are no regulations of *Alternaria* toxins in food and feed in Europe or in other regions. Terminiello et al. (2006) investigated the presence of AOH, AME and TeA in 80 samples of tomato purée. Thirty-nine out of 80 samples were contaminated with *Alternaria* mycotoxins. Levels of AOH ranged between 187 to 8756 µg/kg, for AME it was 84 to 1734 µg/kg and for TeA, 39 to 4021 µg/kg. More recently, cereal, fruit and vegetable products have been analyzed for AOH and AME contamination by Asam et al. (2010). Both toxins were frequently detected in vegetable products. These authors found that AOH levels ranged between 2.6 and 25 µg/kg, while for AME it was 0.1 to 5 µg/kg. Indeed, they reported that tomato products were especially affected. Ackermann et al. (2011) also found the presence of AOH in 93% of samples of tomato products.

In conclusion, in the present work there is enough evidence of the presence of *Alternaria* spp. in tomatoes used for the production of tomato derived products since 68.4% of the analyzed raw samples were contaminated with viable *Alternaria* spp. From all these contaminated samples, 75.6% had levels of contamination above 28 conidia/g of tomato. Maximum levels almost reached 2000 conidia/g of tomato in both industries. In this sense, controlling the water used for the washing may be a good preventive measure to reduce the contamination of tomatoes. Additionally, it has been shown the presence of, at least, two *Alternaria* mycotoxins. The levels of both toxins decreased throughout the production process of tomato products. Nevertheless, 47% of the tomato concentrate samples analyzed in one of the industries were contaminated with AOH, which may represent an important hazard for human health. It is important to mention that no significant differences were found between data of raw and mashed tomatoes from the organic and the not organic industry in relation to the presence of viable *Alternaria* spp. Dealing with AOH occurrence, no significant differences were observed among raw fruits and mashed tomatoes from the organic and the conventional industry though tomato concentrate samples were significantly more contaminated with AOH than scalded peeled tomatoes and tomato purée. Considering that *Alternaria* is the most frequent fungus responsible of tomato fruit decay, the assessment of the presence of *Alternaria* DNA or their mycotoxins could be considered a good parameter to determine the quality of the raw material that enters a food company. However, the lack of standardized protocols for this kind of analysis could be an important limiting factor.

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Chapter III

LaeA and VeA are involved in growth morphology, asexual development, and mycotoxin production in *Alternaria alternata*

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LaeA and VeA are involved in growth morphology, asexual development, and mycotoxin production in *Alternaria alternata*

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Abstract

Alternaria alternata is a common filamentous fungus that contaminates several fruits, grains and vegetables causing important economic losses to farmers and the food industry. *A. alternata* is a mycotoxigenic mould, which jeopardizes human and animal health. Two of the most common *A. alternata* mycotoxins found in food and feed are alternariol and alternariol monomethyl ether. There is little information about the genetics and the molecular regulation within the *Alternaria* genus. Hence, in this study we examined the role of LaeA and VeA, two regulatory proteins belonging to the velvet family, which have been described to be involved in several functions in many fungi including secondary metabolism. We found that deletion of *laeA* and *veA* genes, respectively, drastically reduced sporulation and strongly comprised mycotoxin production, both *in vitro* or during pathogenesis of

tomato fruits. We have also studied how the loss of *laeA* and *veA* may affect expression of genes related to alternariol and alternariol monomethyl ether biosynthesis (*pksJ* and *altR*), and to melanin biosynthesis (*cmrA*, *pksA*).

Keywords: *Alternaria* spp.; AOH; AME; tomatoes

1. Introduction

The *Alternaria* genus of ascomycete fungi is comprised of saprophytic and pathogenic species causing plant diseases in many field crops and post-harvest decay of various fruits, grains and vegetables. Besides their importance due to economic losses worldwide for farmers and the food industry, *Alternaria* is also a matter of concern due to its ability to produce a wide variety of different toxic secondary metabolites, named mycotoxins. Among all *Alternaria* spp., *A. alternata* has been regarded as the most important mycotoxin-producing species (Barkai-Golan and Paster, 2008b; EFSA, 2011). Despite it being well documented and described that *Alternaria* mycotoxins are harmful for human and animals (Brugger et al., 2006a; Liu et al., 1992; Pero et al., 1973; Pfeiffer et al., 2007; Pollock et al., 1982), currently there are no regulations on *Alternaria* toxins in food and feed in Europe or other regions of the world.

Since the discovery of aflatoxins produced by *Aspergillus* spp., many studies have been focused toward the understanding of molecular mechanisms leading to mycotoxin biosynthesis. Although there has been significant progress on the molecular knowledge of some mycotoxins, still there is scarce information on the biosynthesis of *Alternaria* mycotoxins. Recently, Saha et al. (2012) have identified ten putative polyketide synthases (PKSs) in *A. alternata*, suggesting that one of them, PksJ, was supposedly responsible to catalyze the first steps of the biosynthesis of alternariol (AOH) and alternariol monomethyl ether (AME), two of the most common *Alternaria* mycotoxins. Interestingly, they also found another

gene, *altR*, that had homology to other fungal transcription factors, which was found to be involved in *pksJ* induction (Saha et al., 2012). On the other hand, another report has been published lately dealing with AOH and AME biosynthesis in the wheat pathogen *Parastagonospora nodorum* (Chooi et al., 2015), which has been described to also produce AOH (Tan et al., 2009). In this latest study, it was not found a gene with close homology to *pksJ* in the *P. nodorum* genome but, interestingly, it was described another protein, SnPKS19, that was required for AOH biosynthesis and, additionally, SnPKS19 shared significant homology to PksI, a PKS that was also described by Saha et al. (2012) in *A. alternata* genome. Hence, further investigation is required to verify the gene responsible of AOH synthesis in *A. alternata*.

The fungus, *A. nidulans* is one of the best genetically characterized eukaryotic systems and has been quite useful for studying secondary metabolite biosynthesis mechanisms. For example, the heterotrimeric velvet complex was first characterized in this species (Bayram, Krappmann, Ni et al., 2008). The velvet family proteins, LaeA, VeA and VelB, are fungal specific and have a marked functional plasticity in different species, but they are structurally highly conserved among ascomycetes and basidiomycetes (Calvo, 2008; Ni and Yu, 2007). It has been found in several fungal species that the velvet complex is involved in the regulation of diverse cellular processes, including control of asexual and sexual development, growth morphology, secondary metabolism and virulence (Bayram, Krappmann, Ni et al., 2008).

Although there are just few studies regarding the function of VelB (Bayram, Krappmann, Ni et al., 2008; Chang et al., 2013; Lan et al., 2014; López-Berges et al., 2013; Yang et al., 2013), several researchers have described the function of LaeA and VeA in different fungal species. The nuclear localized LaeA protein was first described in *Aspergillus* spp. as a global regulator of secondary metabolism (Bok and Keller, 2004a). VeA was originally discovered in *A. nidulans* as an

inhibitor of light-dependent conidiation (Käfer, 1965). It was later reported that the velvet complex and aspects of sexual and asexual development are strongly linked with secondary metabolite biosynthesis (Calvo et al., 2004; Calvo et al., 2002; Chang et al., 2001; Kato et al., 2003). In an earlier study, Crespo-Sempere et al. (2013) reported that darkness stimulated *A. carbonarius* secondary metabolite production, whereas light propitiated sexual or asexual development. This effect seemed to be regulated, in part, by the velvet complex and the environmental conditions triggered by light and darkness (Bayram, Krappmann, Ni et al., 2008). The molecular mechanism proposed by Bayram et al. (2008) elucidated that, in *A. nidulans*, VeA transport to the nucleus is inhibited by light while, in the dark, most VeA protein is found in the nuclei. During darkness, VeA migrates to the nucleus through interactions with other elements that involve an importin α, KapA, and the velvet-like protein B (VelB). In the nucleus, VeA interacts with LaeA and their union triggers secondary metabolism pathways in this species.

Although there is some information about the role of LaeA and VeA in different fungi, nothing has been reported about their function(s) in any *Alternaria* spp. Hence, this is the first work highlighting some processes involving LaeA and VeA in *Alternaria*. For this purpose we have deleted *laeA* and *veA* genes in *A. alternata* and analyzed how these deletions affect growth morphology, asexual development (sporulation and germination), mycotoxin production, virulence when infecting tomato fruit, and expression of genes related to the velvet complex in *A. alternata* (the melanin and the mycotoxin biosynthesis pathways).

2. Material and Methods

2.1 Fungal strains, fruit material and growth conditions

The *A. alternata* strains used in this study were CBS 116.329 (isolated from apple), provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht, The

Netherlands), and ATCC 66981 (isolated from peanut), kindly provided by C. Lawrence (Virginia Bioinformatics Institute, Virginia, USA). *A. tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain).

To prepare conidial suspensions, strains were grown on Potato Dextrose Agar plates (PDA; Biokar Diagnostics, France) in the dark at 26 °C for 14 days. Conidia were collected with a scalpel within a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, The Netherlands) and filtered through Miracloth (Calbiochem, USA). Conidial concentration was determined using a Thoma chamber. Fungal strains were stored as conidial suspensions at -20 °C with 40% glycerol.

For the study of growth infection, wild-types, Δ*veA*, and Δ*laeA* mutants were inoculated in tomato fruits (*Solanum lycopersicum* var. *palladium*). The tomato variety was selected taking into account its susceptibility to *Alternaria* spp. infection.

2.2. Genomic DNA extraction

Cultures were grown for 2 days at 26 ºC on 500 µL of Malt Extract broth (2% w/v malt extract, 0.1% w/v peptone, 2% w/v glucose). Mycelium was recovered after 10 min of centrifugation (17500 *x g*) and 300 µL of DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added. The mycelium suspension was vortexed with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies, France) during 10 min. After centrifugation at 17500 *x g* for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) were added to the supernatant. Then, the supernatant was stored at -20 ºC for 10 min and centrifuged (17500 *x g*, 10 min). The DNA-containing supernatant was transferred to a new tube, and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 min of incubation at room temperature the DNA suspension was

centrifuged (17500 *x g*, 10 min). The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3. Construction of *veA* and *laeA* deletion strains

A Blastx algorithm with LaeA nucleotide sequence from *A. flavus*, *A. nidulans* and *C. heterostrophus* (Accession numbers AY883016, AY394722 and JF826792) and VeA from *A. flavus*, *A. nidulans* and *C. heterostrophus* (Accession numbers DQ296645, AF109316 and JF826791) as queries was performed in the *A. alternata* genome, recently sequenced by Dang et al. (2015) using a 454 Titanium deep sequencing technology (Roche, Indianapolis, USA). To construct the *laeA* and *veA* gene replacement plasmids (Fig. 1A), 1.7 kb upstream and downstream fragments from the promoter and terminator regions of *laeA* and *veA* genes were cloned into the plasmid vector pRF-HU2 (Frandsen et al., 2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs, USA), as described previously by Crespo-Sempere et al. (2011). The specific primers used for amplifying the promoter and terminator regions were A-VA, A-VB, A-VE and A-VF for *veA* and A-LA, A-LB, A-LE and A-LF for *laeA* (Table 1, Fig. 1B) including vector-specific 9 bp long overhangs containing a single 2-deoxyuridine nucleoside in the 5' end, which ensured directionality in the cloning reaction. Upstream and downstream fragments were amplified by PCR from genomic DNA of *A. alternata* (CBS 116.329 and ATCC 66981) with DFS-Taq DNA Polymerase (Bioron, Germany). Cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58 °C for 1.5 min and 72 °C for 3 min, and a final elongation step at 72 °C for 10 min. Both DNA inserts and the digested vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs, USA) to obtain plasmids pRFHU2-VEA and pRFHU2-LAEA (Fig. 1A).

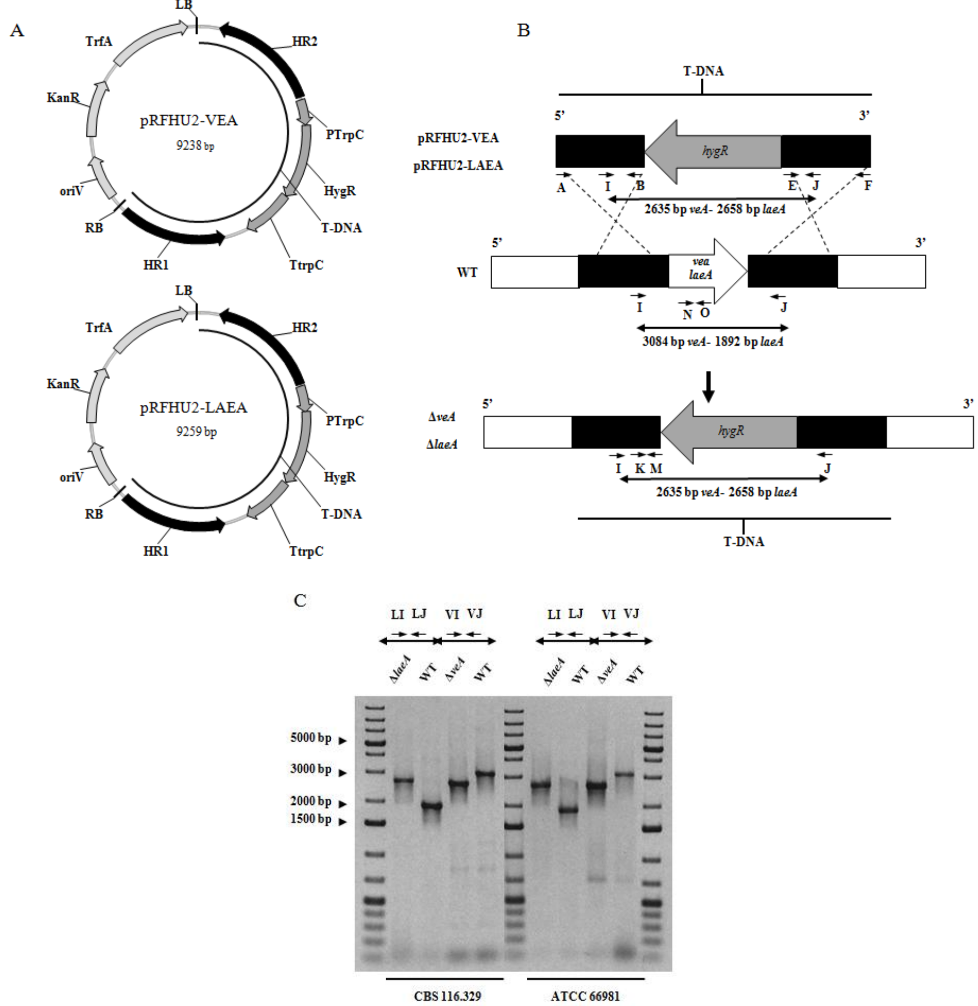


Figure 1. Deletion of the *veA* and *laeA* genes in *A. alternata*. **A.** Physical map of pRFHU2-VEA and pRFHU2-LAEA plasmids. LB = Left Border, HR2 = Homologous flanking region 2, PTrpC = Tryptophan promoter from *A. nidulans*, HygR = hygromycin phosphotransferase, TtrpC = Tryptophan terminator from *A. nidulans*, HR1 = Homologous flanking region 1, RB = Right Border, oriV = origin of replication in *E. coli*, KanR = kanamycin resistance, TrfA = replication initiation gene (broad-host-range). **B.** Diagram of the strategy of *veA* and *laeA* replacement with the hygR selectable marker from pRFHU2-VEA and pRFHU2-LAEA by homologous recombination to generate the Δ*veA* and Δ*laeA* null mutants. Primers used in the construction of plasmids and those used for the analysis

of the transformants are shown. **C.** PCR analysis of the expected amplification band patterns with A-LI/A-LJ set or primers for *laeA* detection on wild-type (WT) and Δ*laeA* strains, and A-VI/A-VJ set or primers for *veA* detection on the wild-type and Δ*veA* mutants.

Table 1. Primers sequences used in this study.

Primer name	Primer sequence (5′→3′)
A-LA	GGTCTTAAUCGCTCGCTACCAGGTCACCG
A-LB	GGCATTAAUGCCTGTCCATGCCACTCACG
A-LE	GGACTTAAUCGCCAGACACCCACCTAGCC
A-LF	GGGTTTAAUCCTGTACGAGCGACGACTCCC
A-VA	GGTCTTAAUTGCAAGTCCGTGCGATATTTCCGT
A-VB	GGCATTAAUGGTCGTCGATGAACAGCACCA
A-VE	GGACTTAAUATGGTCTTCAGCCTGTACGTTGC
A-VF	GGGTTTAAUTGCCACACCACCCTCAAAGCG
A-LD	AAGTGCAATCGAGATGGTCAAGT
A-LG	TGCCACCTGTGCAACGTCA
A-VD	CCTCCGCCTTCCGCAAACTCT
A-VG	GACAGGTATTCCGATGCCCTT
RF1	AAATTTTGTGCTCACCGCCTGGAC
RF2	TCTCCTTGCATGCACCATTCCTTG
A-LI	TCCCCTTCCGACAGAGTGTTACCG
A-LJ	CGTCGCGTTGGTTGCTGAT
A-VI	CCAGGGCTCCCAGAAAGATGTGA
A-VJ	AGCACACACATAACGCCGTACTCCA
A-LK	CTTCCGACAGAGTGTTACCGCCAT
A-LM	ACGCTCGGAGTTTGACCCAACC
A-VK	CCATACACCAGCGGACCTACCAC
A-VM	CACTGCTGGTCGCCTTCACG
A-BTF	ACAACTTCGTCTTCGGCCAGT
A-BTR	ACCCTTTGCCCAGTTGTTACCAG
A-LN	CCGCCCTCGCTCCAATGGTCA
A-LO	GCGGTCCTTTTCAGCCTCGTC
A-VN	GCTACTTCATCTTCCCCGACCTGT
A-VO	TGACCTCTGCCAGATTCTCGAAC
pksJ_F	ACACTAGCACAGTCGGTTCCCA
pksJ_R	ATTGGCCGCGTACTACCCAG
AltR_F	AAACACCGCTTGAGGAACGCCAGA
AltR_R	AAAGCGTGCCATTGCCGATACCAG
pksA_RT_fwd	GATTGCCATCGTCGGTATG
pksA_RT_rev	GGCTCATCGATGAAGCAAC
cmr1_RT_fwd	GAAATGTCACCTGCGCAAAC
cmr1_RT_rev	GTCTTGGGCTGCGATAATG

An aliquot of the mixture was used directly in chemical transformation of *E. coli* DH5α cells without prior ligation. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing using primers A-VD and A-VG for *veA*, A-LD and A-LG for *laeA*, and RF1 and RF2 for both (Table 1). Then, plasmids pRFHU2-VEA and pRFHU2-LAEA were introduced into chemically competent *A. tumefaciens* AGL-1 cells.

Transformation of *A. alternata* was done as described previously by Crespo-Sempere et al. (2011) using *A. tumefaciens* AGL-1 cells carrying the plasmids pRFHU2-VEA and pRFHU2-LAEA. Equal volumes of IMAS-induced bacterial culture (De Groot *et al.*, 1998) and conidial suspension of *A. alternata* (10^6 conidia/mL) were mixed and spread onto nitrocellulose membrane filters (Sartorius Stedim Biotech, Germany), which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the membranes were transferred to PDA plates containing 100 µg/mL of hygromcycin B (Calbiochem, USA), as the selection agent for fungal transformants, and 200 µg/mL of cefotaxime (Calbiochem, USA) to inhibit growth of *A. tumefaciens* cells. Hygromcycin resistant colonies appeared after 6 to 7 days of incubation at 26 °C.

To ensure a correct deletion of *veA* and *laeA* and the absence of ectopic insertions the conventional PCR and the quantitative PCR (qPCR) were used to determine gene copy number (GC) of the T-DNA inserted in *A. alternata*. Firstly, disruption of *veA* and *laeA* was confirmed by PCR analyses of the transformants (Fig. 1C).

The insertion of the selection marker at the correct homologous site was checked with the primer pair A-VI and A-VJ for *veA* and A-LI and A-LI for *laeA* (Table 1). To exclude the possibility that T-DNA was integrated elsewhere in the genome, the qPCR was used instead of a Southern blot analysis. Hence, to determine the number of T-DNA molecules that had been integrated in the genome of each selected transformant, a qPCR analysis was carried out following an already

demonstrated methodology described by several authors (Crespo-Sempere, Selma-Lázaro et al., 2013; López-Pérez et al., 2015; Solomon et al., 2008) and firstly described in a filamentous fungus by De Preter et al. (2002). Two primer pairs, (Fig. 1B, Table 1), were designed within the T-DNA in the promoter region of the target genes, close to the selection marker, A-VK and A-VM for *veA* and A-LK and A-LM for *laeA*. qPCR reactions were performed in a final volume of 10 µL, containing 1X of SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA), 250 nM of each primer and 1 µL of template DNA. All amplifications were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA). The standard protocol included one cycle at 98 °C for 2 min, followed by 35 cycles at 98 °C for 5 s and 58 °C for 30 s. Reactions were done in triplicate for each knockout mutant candidate. qPCR efficiency (E) for each pair of primers was calculated from the slopes of the standard curve (Lee et al., 2006). The number of T-DNA copies that have been integrated in the genome of the transformant was calculated according to the Eq. 1, based on Pfaffl (2001) and Rasmussen (2001b), which depends on E and the Crossing point (Cp) value of the transformant versus the wild-type strain, and normalized in comparison to a reference gene that is present with the same copy number in both wild-type strain and transformant.

$$Copy\ number = \frac{\left(E_{target\ gene}\right)^{\Delta Cp\ _{target\ gene}\ (wild\ type - transformant)}}{\left(E_{reference\ gene}\right)^{\Delta Cp\ _{reference\ gene}\ (wild\ type - transformant)}}$$

Eq. 1

The gene of β-tubulin (Protein identification n° 417073) was chosen as reference gene, using A-BTF and A-BTR primers (Table 1). All primers were designed using the OLIGO Primer Analysis Software V.7.

2.4 Phenotypic studies of Δ*veA* and Δ*laeA* disrupted mutants

2.4.1 Mycelial growth, mycotoxin production and sporulation assessment

For growth assessment, mycotoxin production and sporulation quantification, PDA plates were inoculated centrally with 5 µL of conidia suspensions (10^5 conidia/mL) of the wild-type strain of *A. alternata* and the Δ*veA* and Δ*laeA* knockout strains. Cultures were incubated at 26 ºC under two different conditions, white light (Mazda, 23 W CFT/827, 1485 lumen) or darkness.

Mycelial growth was determined by measuring daily two perpendicular diameters of the growing colonies over four days. Mycotoxin production (AOH and AME) was quantified in five day old cultures. To this aim, three 5 mm agar plugs were removed from the inner, middle and outer part of the colonies. Plugs were weighed to ensure a standardization of the method. Plugs were homogenized in 750 µL of acetonitrile-methanol-water (45:10:45 v/v/v), adjusted to pH 3 with *o*-phosphoric acid, for 60 minutes. Samples were filtered (Millex-HV 0.45 µm, 25 mm, Millipore Corporation, USA) into another vial and mycotoxin extracts were dried in a speed vacuum concentrator at room temperature. Samples were stored at -20 °C until HPLC analysis. Prior to analysis, the extracts were resuspended in 500 µL of a water-methanol solution (50:50 v/v). Separation, detection and quantification of AOH and AME was performed on a HPLC system consisting of a Waters 2695 Alliance Separations Module connected to a UV/Visible dual λ absorbance Detector Waters 2487, using a reversed phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a Spherisorb guard column (5 µm ODS2, 4.6 x 10 mm, Waters, Millford, MA, USA). Columns were set at a temperature of 35 ºC. For chromatographic separation of AOH and AME the flow rate was 0.5 mL/min and the injection volume was 100 µL. Absorption wavelength was 258 nm. The mobile phase consisted of a gradient of double distilled miliQ water (MiliQ Academic Millipore, USA) and methanol-water (70:30 v/v) according to the gradient described in Table 2. The retention times

were 24 minutes for AOH and 32 minutes for AME. For mycotoxin quantification, working standard solutions were used to perform a ten-point calibration curve for the mycotoxins (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). The detection limit of the analysis was 10 ppb for AOH and 12 ppb for AME, based on a signal-to-noise ratio of 3:1. All solvents were HPLC grade and all chemicals were analytical grade.

Sporulation assessment was carried out by scraping the surface of 14 day old cultures with the help of a scalpel. Conidia and mycelium were homogenized in a sterile solution of 0.005% (v/v) Tween 80. Conidia concentration was measured by using a Thoma counting chamber and results were expressed as conidia/mm^2.

Assays were performed with independent biological triplicates and technical triplicates. All comparisons were analyzed by One way ANOVA followed by the Tukey's honestly significant different test (HSD), using Statgraphics Centurion Version XVI. Significance was defined as $P < 0.05$.

2.4.2 Germination and hyphal growth

Conidia were grown on a sterilized microscope slide in which a PDA drop had solidified. Once the medium was solid, it was inoculated with 25 µL of a conidial suspension (10^4 conidia/mL). Slides were placed in Petri dishes that contained a sterilized filter paper previously moistened with 400 µL of water. Cultures were grown at the dark at 20 ºC. Conidia germination and hyphal growth was monitored after 16 and 36 hours with a light microscope Leica DM2000 coupled to a DFC290 HD digital camera (Leica Microsystems, Germany).

2.5 Gene expression analysis

PDA Petri dishes were inoculated with 100 µL of a conidial suspension (10^5 conidia/mL), homogeneously spread and incubated at 26 °C under two different conditions, light or darkness. After 5 days, mycelium was collected, frozen in liquid nitrogen and stored at -80 °C before nucleic acid extraction. RNA was

extracted from 1 g of mycelium previously grounded to a fine powder with a mortar and a pestle with liquid nitrogen. Pulverized mycelium was added to a pre-heated (65 ºC) mixture of 10 mL of extraction buffer: 100 mM Tris–HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% (v/v) β-mercaptoethanol and 5 mL of Tris-equilibrated phenol. The extract was incubated at 65 °C for 15 min and cooled before adding 5 mL of chloroform-isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 *x g* for 20 min at 4 °C, and the aqueous phase was re-extracted with 10 mL of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated during 3 hours at -20 ºC by adding 3.3 mL of 12 M LiCl. After centrifugation at 27200 *x g* for 60 min, the pellet was washed with 500 µL of 70% ethanol. The resultant pellet was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides. Then, RNA was washed again with 500 µL of 70% ethanol, and, finally, dissolved in 100 µL of water. RNA concentration was spectrophotometrically measured and verified by ethidium-bromide staining of an agarose gel. Total RNA was treated with DNase (TURBO DNase, Ambion, USA) to remove contaminating genomic DNA. Single-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instructions (Invitrogen, USA).

Gene-specific primer sets, A-LN/A-LO and A-VN/A-VO, were designed for *laeA* and *veA* gene expression analysis, respectively (Table 1). To assess the involvement of deleted genes on mycotoxin biosynthesis, we studied gene expression of PksJ (Protein identification nº AAPT02903) and a putative transcriptional factor AltR (protein identificacion nº AAPT02898), both claimed to have an essential role in AOH and AME biosynthesis in *A. alternata* (Saha et al., 2012), using the primer pairs pksJ_F/pksJ_R and altR_F/altR_F. Additionally, two primer pairs, pksA_RT_fwd/pksA_RT_rev and crm1_RT_fwd/cmr1_RT_rev (Fetzner et al., 2014), were used to analyze the involvement of LaeA and VeA in

melanin metabolic pathways by studying gene expression of a PKS (PksA), required to melanin biosynthesis, and a putative transcription factor (CmrA) that controls the expression of at least three structural genes for melanin biosynthesis (Fetzner et al., 2014).

Real-time qPCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) to monitor cDNA amplification. The primer pair A-BTF/A-BTR (Table 1) was designed within the β-tubulin gene to be used as a reference gene. Gene expression measures were derived from biological duplicates and technical triplicates. Gene expression ratios (R) were calculated using the formula described by Pfaffl et al. (2002) and detailed in Eq. 2.

$$R = \frac{(E_{target\ gene})^{\Delta Cp\ _{target\ gene}\ (MEAN\ wild\ type - MEAN\ transformant)}}{(E_{reference\ gene})^{\Delta Cp\ _{reference\ gene}\ (MEAN\ wild\ type - MEAN\ transformant)}}$$

Eq. 2

2.6 Fungal growth infection on tomato fruits

Growth infection and mycotoxin production of Δ*laeA* and Δ*veA* mutants were assayed on tomato fruits. Previously to perform mycotoxin extraction, AOH and AME free tomatoes were artificially spiked with *Alternaria* mycotoxins to assess recovery and repeatability data. Recovery and repeatability results are listed in Table 3.

To analyze *Alternaria* spp. artificial infection on tomatoes, fruits were previously surface-disinfected with 10% sodium hypochlorite for 1 minute and rinsed with tap water for 10 minutes. Once dried, tomatoes were four-times injured with a sterilized awl. Inoculation was performed placing 5 µL of a conidial suspension (10^4 conidia/mL) in each wound. Control tomatoes were also injured but no conidial suspension was used. Tomatoes were stored into plastic bags at 20 ºC and

70% RH for two weeks. Five tomatoes were considerate a single replicate and the assay was performed in quadruplicate.

Diameter lesion size was measured two weeks after the inoculation. For mycotoxin production assessment, plugs of 7 mm of diameter and 0.5 mm of thickness were removed where there was the fungal infection. Three plugs were taken from each tomato. All the plugs from the same replicate were put into a stomacher bag. Mycotoxin extraction was done by adding 30 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric) and homogenizing for 2 minutes with the stomacher. The mixture was homogenized for 15 extra minutes under a uniform magnetic field. The solution was left for 10 minutes approximately to favor precipitation by gravity. Then, 6 mL of the supernatant were transferred to a centrifuge tube and diluted with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3) and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract were passed by gravity through a previously conditioned Bond Elut Plexa SPE cartridge (200 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the cartridge was done with 5 mL of methanol following 5 mL of miliQ water. The SPE column was washed with 5 mL of water followed by air drying on the manifold. Finally, elution was carried out with 5 mL of methanol and 5 mL of acetonitrile. Sample extracts were dried under nitrogen flow and stored at -20 ºC until HPLC analysis. Previous to HPLC injection, samples were resuspended in 500 µL of the mobile phase solution (water-methanol, 50:50 v/v). HPLC conditions were the same as previously described.

3. Results

3.1 Identification of the LaeA and VeA orthologs in *A. alternata*

In order to identify LaeA and VeA homologs in *A. alternata* we interrogated the *A. alternata* genome sequences available at Alternaria Genomes Database using Blast

alignment approaches (Dang et al., 2015). Blastx searches were performed using LaeA and VeA nucleotide sequences (coding and genomic) from various species including, *A. flavus* (GenBank accession numbers AY883016 and DQ296645, respectively), *A. nidulans* (GenBank accession numbers AY394722 and AF109316, respectively) and *Cochliobolus heterostrophus* (GenBank accession numbers JF826792 and JF826791, respectively). Once LaeA and VeA homologs were identified from the *A. alternata* genome, their amino acid sequences were used to analyze the similarity with LaeA and VeA from other fungal species. Several candidate homologs were found using a Blastx search against the NCBI database (Table 4).

Table 4. Amino acid similarities between LaeA and VeA proteins from *A. alternata* and amino acid sequences deposited on the NCBI using the Blastx algorithm.

Prot ID AAT_PP02962 LaeA *A. alternata*			
Organism	**Accession Nº**	**E value**	**Identity**
S. lycopersici	KNG51967.1	3e-141	94%
P. tritici-repentis	XP_001934837.1	2e-143	93%
B. maydis	AEP40318.1	4e-134	90%
A. nidulans	AAQ95166.1	3e-62	43%
A. flavus	XP_002374839.1	3e-60	43%

Prot ID AAT_PP09942.1 VeA *A. alternata*			
Organism	**Accession Nº**	**E value**	**Identity**
B. maydis	XP_014080488.1	1e-151	90%
P. tritici-repentis	XP_001933979.1	0.0	82%
S. lycopersici	KNG51195.1	0.0	76%
A. flavus	ADF30049.1	3e-68	60%
A. niger	GAQ41759.1	2e-73	55%

The hits with the highest identity percentage belonged to *Stemphylium lycopersici, Pyrenophora tritici-repentis* and *Bipolaris maydis*, also known as *C. heterostrophus*. All of them belong to the Dothideomycetes class and even share subclass (Pleosporomycetidae), order (Pleosporales) and family (Pleosporaceae). For LaeA, all the values for percentage identity were equal to or higher than 90% (E-values close to 0.0), which indicate that LaeA is a highly conserved protein in fungi including *Alternaria*. For VeA, the percentage identity was lower than 90% for *C. heterostrophus* and *S. lycopersici*, which may indicate that LaeA is more conserved than VeA. Alignment of LaeA and VeA with *A. flavus, A. nidulans* or *A. niger* showed lower percentage identity values. These results are not surprising as all *Aspergillus* spp. belong to the Eurotiomycetes class.

3.2 Knockout of *laeA* and *veA* genes in *A. alternata*

Targeted gene disruption of *laeA* and *veA* was performed to investigate the role of both transcriptional factors in *A. alternata*. The first step of the gene deletion strategy was to construct the pRFHU2-LAEA and pRFHU2-VEA plasmids (Fig. 1A) using the USER friendly cloning technique. The resultant mixture was used to transform chemically competent *E. coli* DH5α cells. Positive transformants were selected as kanamycin-resistant colonies and screened by PCR. Afterwards, plasmids were introduced into chemically competent *Agrobacterium tumefaciens* cells (AGL-1). The following step was transformation of *A. alternata* by co-cultivation with *A. tumefaciens*. Positive transformant colonies were those able to grow on a hygromicin B medium. The gene replacement strategy followed in order to disrupt *veA* and *laeA* is shown in more detail in Fig. 1B.

The correct disruption of *laeA* and *veA* genes was verified by PCR using the primers A-LI and A-LJ for *laeA* and the primer pair A-VI and A-VJ for *veA*. Fig. 1C shows the expected amplification band patterns for wild-type and disrupted Δ*laeA* and Δ*veA* strains.

Table S1. Determination of the number of T-DNA copies integrated in the genome of *A. alternata*. Gene copy number (GC) quantification was conducted by qPCR according to Eq 1. The genes *laeA* and *veA* were used as the target genes for Δ*laeA* and Δ*veA* transformants, respectively. The *β-tubulin* gene was used as a reference gene. Wild-type strains, ATCC 66981 and CBS 116.329 were used as controls. Efficiencies for *laeA*, *veA* and *β-tubulin* genes were 2.134, 2.34 and 2.115, respectively. All reactions were conducted in triplicate.

Strain		Cp_{laeA}	Cp_{veA}	$Cp_{\beta\text{-tub}}$	ΔCp_{target}	ΔCp_{ref}	GC
ATCC 66981							
Wild-type	Wild type	23.76±0.16	23.06±0.03	24.22±0.13	0.00	0.00	1.00
Δ*laeA* -27	Knockout	23.38±0.02		23.53±0.06	0.38	0.69	0.80
Δv*eA* -29	Knockout		22.86±0.19	23.74±0.28	0.20	0.48	0.83
CBS 116.329							
Wild-type	Wild type	24.34±0.20	23.53±0.12	24.63±0.13	0	0	1.00
Δ*laeA* -31	Knockout	23.59±0.06		23.74±0.16	0.75	0.89	0.91
Δv*eA* -37	Knockout		22.91±0.15	23.92±0.11	0.62	0.71	1.00
Δv*eA* -6	Ectopic		21.25±0.03	24.13±0.27	2.28	0.5	4.78
Δv*eA* -18	Ectopic		23.01±0.05	25.05±0.13	0.52	-0.42	2.13

When we used the primers for amplifying *laeA* and *veA* in the wild-type strain, the band fragments obtained were 1892 and 3084 bp, respectively, while for Δ*laeA* strain the band was about 2658 bp and for Δ*veA* about 2635 bp. Thus, the hygromycin resistance marker had been integrated properly by homologous recombination disrupting *veA* and *laeA*, though this did not exclude the possibility that the T-DNA had been integrated elsewhere in the genome (ectopic transformation). Hence, to assess the number of T-DNA copies integrated in the genome a qPCR analysis was carried out, confirming that most of the mutants contained a single T-DNA integration (see supporting information; Table S1). Two

ectopic transformants have been also included as an example of an ectopic transformation. The wild-type strains were used as controls and the β-tubulin gene was used as the reference.

3.3 Involvement of LaeA and VeA in mycelial and hyphal growth, and conidiation

To determine in which fungal functions LaeA and VeA were involved, several experiments were performed. For this purpose, two different strains of *A. alternata* were used and the wild-type of each strain was used as the control. For each wild-type strain one knockout of LaeA and one knockout of VeA were used to carry out the assays. When results were inconclusive, assays were repeated but using different knockouts to verify results.

Wild-type and disrupted *laeA* and *veA* strains were grown on PDA for 14 days, under light or dark conditions. As shown in Fig. 2, the CBS 116.329 wild-type colonies grew in a brown-grey uniform layer while Δ*laeA* and Δ*veA* mutants were less-pigmented and grew as a white-grey velvet cover with a radial ring, which was clearly observed in Δ*veA* colonies, especially in those grown on the dark. Nevertheless, in the ATCC 66981 colonies, instead of being brown-grey pigmented (as observed in CBS 116.329), the green color was more predominant. Furthermore, while in CBS 116.329 a clear loss of pigmentation was observed compared to wild-type colonies, in ATCC 66981 there was a remarkable difference in color between wild-type and Δ*veA*, but not when it came to Δ*laeA* colonies. No remarkable differences were found between colonies grown in the dark and colonies grown under light conditions and neither in the reverse plate appearance (data not shown).

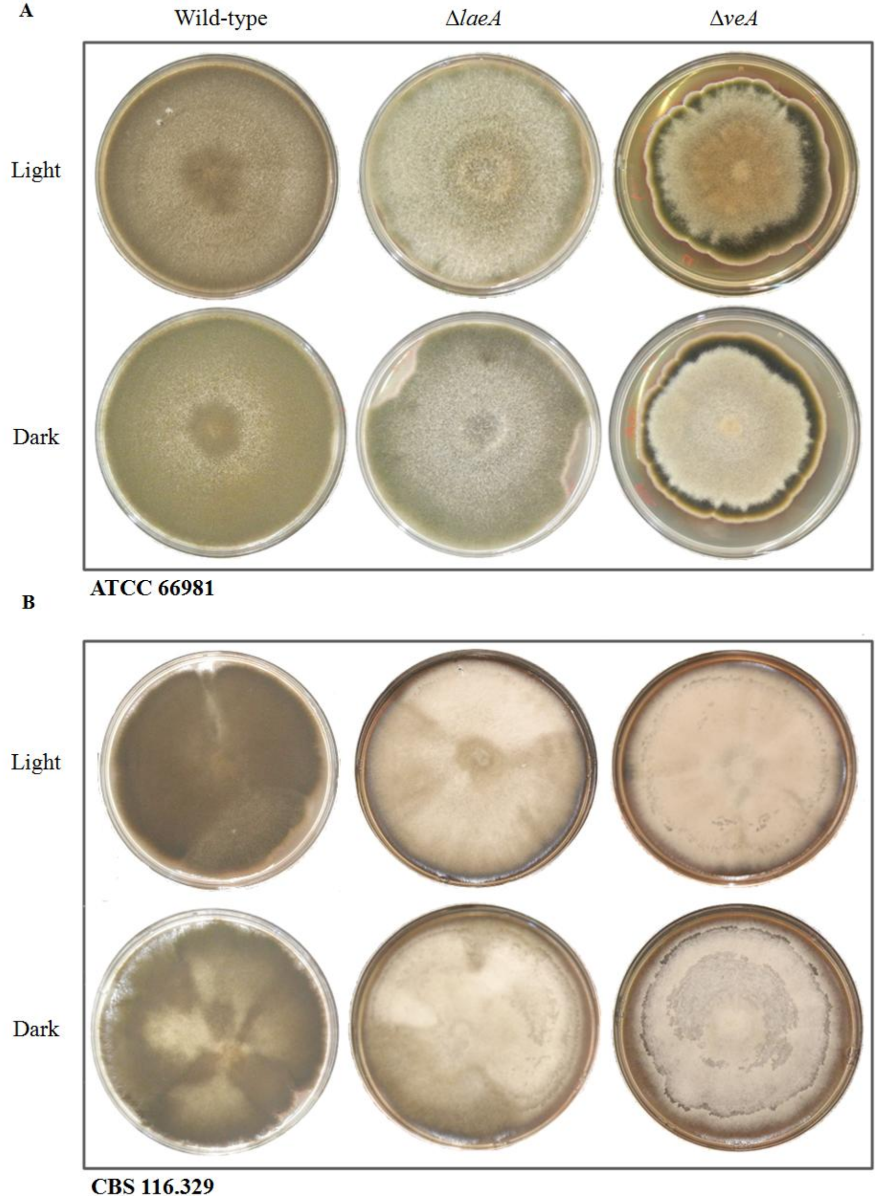


Figure 2. Front (top) colony view of the wild-type, Δ*veA* and Δ*laeA A. alternata* strains inoculated on PDA plates incubated for 14 days at 26 ºC under dark or light conditions.

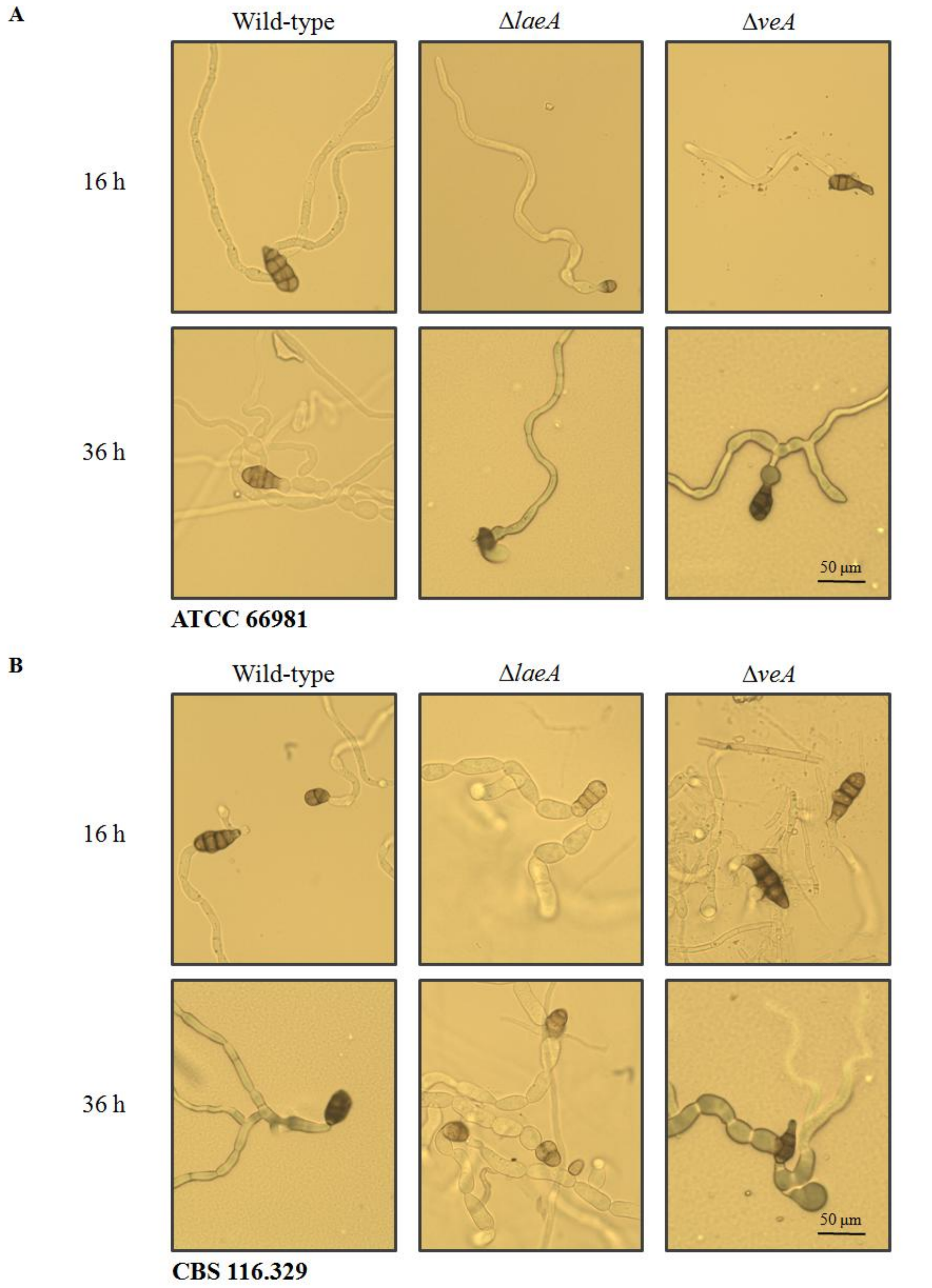


Figure 3. Conidia and hyphal growth of wild-type, Δ*laeA* and Δ*veA* strains (CBS 116.329 and ATCC 66981) photographed after 16 and 36 h of inoculation.

Conidia and hyphal growth was assessed after 16 and 36 h of inoculation (Fig 3). No significant differences were found between wild-type and Δ*veA* strains. In both strains there were large and small dark colored conidia. Approximately half of the conidia were obclavate to obpyriform and four-celled, while the other half were short and one-celled. Nevertheless, we did observe a difference in Δ*laeA* spores, especially those of ATCC 66981. In general, Δ*laeA* conidia reduced the conidial length and instead of having three or four cells, they were one or two-celled with a short beak. We did not observe differences on the hyphal growth on any of the analyzed strains.

To assess fungal growth, two perpendicular diameters of the colonies were measured over four days (Fig. 4). For ATCC 66981, results showed that wild-type was the strain that achieved the biggest diameter on the fourth day while Δ*veA* was the smallest. For CBS 116.329, (Fig. 4) Δ*laeA* was the strain with the fastest growth on the fourth day, while no significant differences were observed between the wild-type and the Δ*veA* transformant. There were no significant differences due to light conditions, regardless of the strain.

To investigate the involvement of LaeA and VeA in conidiation, conidial suspensions were prepared from 14 day old cultures. Results (Fig. 5) demonstrated that both proteins are linked in some way with conidia production as *laeA* and *veA* deletion resulted in a drastic reduction of conidia production for both strains. Both for ATCC 66981 or CBS 116.329, the wild-type colonies produced the highest amount of conidia, but while the wild-type from the ATCC 66981 grown on the dark produced more than $3.8 \cdot 10^4$ conidia/mm^2, the wild-type from the CBS 116.329 produced about $2.9 \cdot 10^4$ conidia/mm^2. In the case of ATCC 66981 cultivated on the light, the loss of LaeA and VeA resulted in a conidia reduction of 84% for Δ*laeA* and 92% for Δ*veA*. When colonies were cultivated in the darkness, the reduction was about 65% and 91% for Δ*laeA* and Δ*veA*, respectively. The involvement was more remarkable when it came to CBS 116.329 transformants. In

this case, the conidia concentration of Δ*laeA* and Δ*veA* mutants was on or below the limit of quantification of the counting method. It is to be noted that for both strains and their corresponding transformants, the colonies produced more conidia under dark conditions though this difference was only statistically significant for Δ*laeA* mutants from ATCC 66981 and for the wild-type colonies from CBS 116.329.

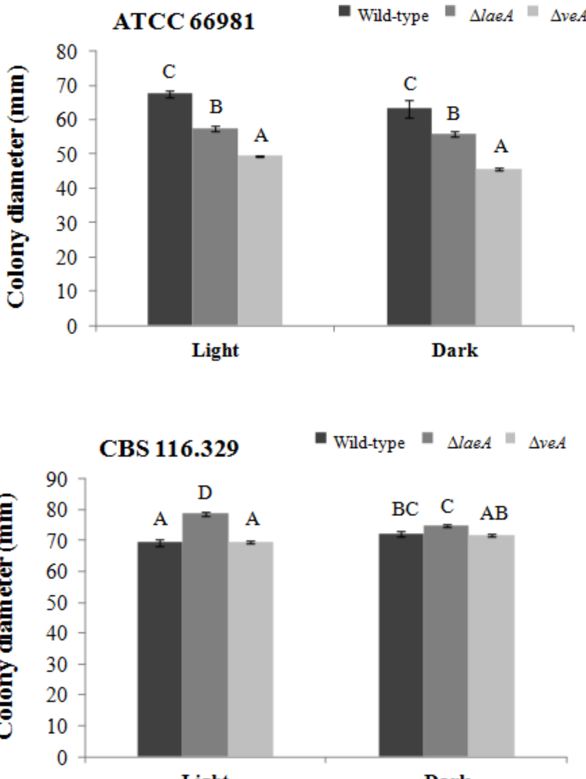


Figure 4. Effect of *veA* and *laeA* deletion on the colony growth of *A. alternata* strains (CBS 116.329 and ATCC 66981). Colonies were grown on PDA plates on the darkness or under light conditions at 26 ºC for 14 days. The colony diameter was measured on the fourth day. Error bars indicate standard errors. Different letters indicate significant differences among sample groups (ANOVA, $P < 0.05$).

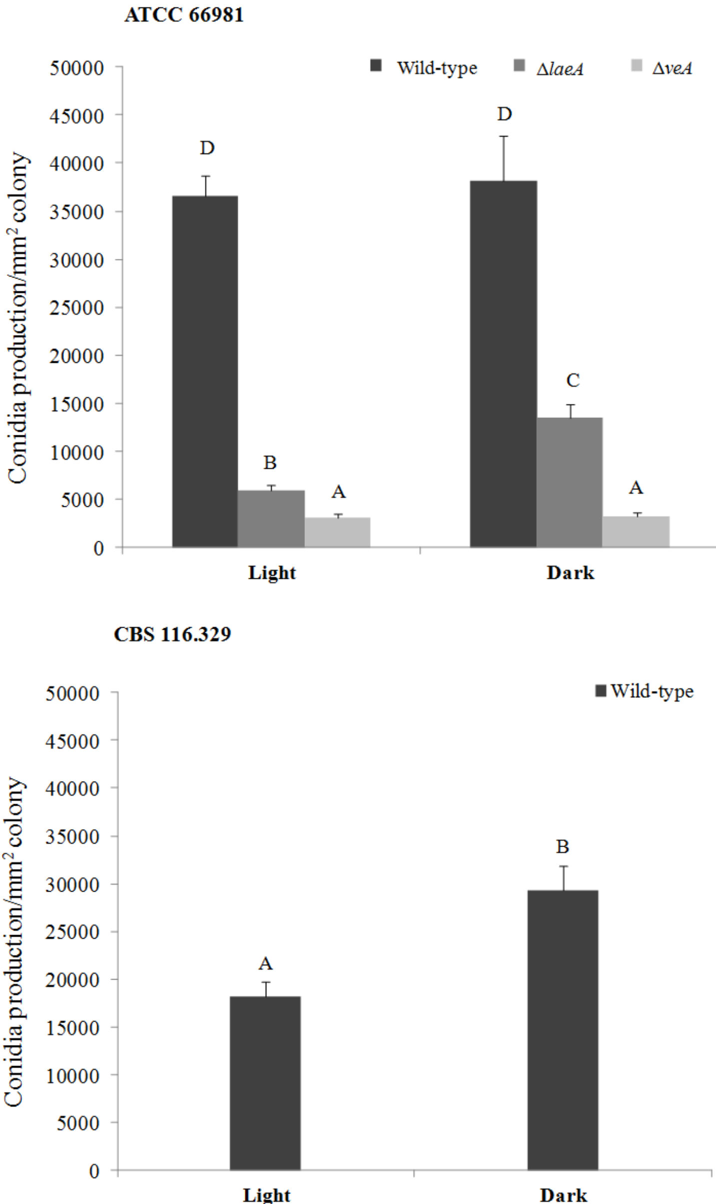


Figure 5. Conidia production per mm^2 of colony in the wild-type, Δ*laeA* and Δ*veA* strains of ATCC 66981 and CBS 116.329. Error bars indicate standard errors. Letters indicate homogeneous groups within the same day (ANOVA, $P < 0.05$).

3.4 Requirement of LaeA and VeA in mycotoxin biosynthesis

In order to investigate whether LaeA or VeA are linked to secondary metabolism related to mycotoxin biosynthesis, mycotoxin extraction was performed in 5 day old cultures. Two different *Alternaria* mycotoxins were analyzed, AOH and AME (Fig. 6). Even though results were quite different for ATCC 66981 and CBS 116.329, in both cases and for all the transformants AOH was the mycotoxin produced in a higher amount.

In the ATCC 66981 background, the colonies that produced the highest amount of mycotoxins were Δ*laeA* mutants, followed by wild-type and then Δ*veA* mutants. In relation to AOH production, Δ*laeA* mutants cultivated in light produced more than twice the amount compared to wild-type. This increase was smaller in colonies cultivated in the dark (~1.5 fold). In addition, Δ*veA* colonies produced less mycotoxin (Fig. 6). Results reveal that Δ*veA* mutant colonies compared to wild-type reduced their AOH production by 79% and 76%, in the light or the dark, respectively. Similar patterns were observed for Δ*laeA* mutants since AME production increment was about 2.8 and 3.3 fold for colonies grown on light and dark, respectively, compared to wild-type. Conversely, AME production was completely inhibited for Δ*veA* mutants incubated on light and inhibited about 82% when ATCC 66981 was grown in the darkness.

Interestingly, results from experiments with CBS 116.329-derived transformants clearly indicated that LaeA and VeA are associated with mycotoxin production in this isolate. All Δ*laeA* and Δ*veA* mutants exhibited reduced mycotoxin biosynthesis in both *in vitro* and *in vivo* experiments. Under light conditions wild-type produced the highest quantity of mycotoxins, in particular AOH compared to mutant strains. Comparing the wild-type with Δ*laeA* in light and dark, the reduction of mycotoxin production was about 94% and 91% for AOH and, 59% and 56% for AME. For Δ*veA* mutants, the decrease was drastic, as mycotoxin production was almost completely inhibited for both mycotoxins. It is noteworthy

to mention that when comparing mycotoxin production in ATCC 66981 to CBS 116.329 (all wild-type and mutant strains), CBS 116.329 wild-type grown in light is by far the highest AOH producer, with 50 ng/mm^2 being the highest amount observed, while in the case of ATCC 66981, the highest production (AOH) was observed in the Δ*laeA* mutant in light, (~11.5 ng/mm^2).

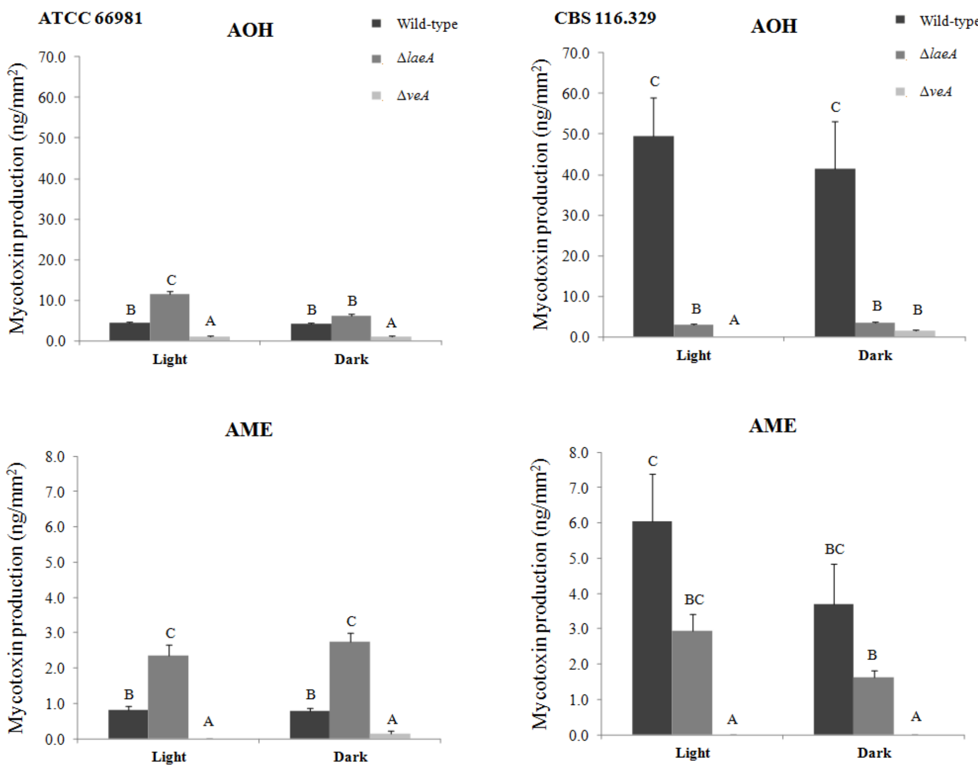


Figure 6. AOH and AME production in wild-type, Δ*laeA* and Δ*veA* strains of ATCC 66981 and CBS 116.329. Total AOH and AME production was analyzed two weeks after the inoculation. Error bars indicate standard errors. Different letters indicate significant differences among sample groups (ANOVA, $P < 0.05$).

Surprisingly, results from ATCC 66981 were not coincident with CBS 116.329 results. Thus, the same assay was repeated again with different Δ*laeA* and Δ*veA* knockouts from both strains. Results from this second assay showed that both

AOH and AME production pattern was repeated again in all transformants tested (data not shown). In addition, the morphology and the color of the ATCC 66981 mutants were different compared to the mutant colonies of the CBS 116.329 (data not shown). This may mean that there are different modes of regulation for these genes that could be strain or isolate specific.

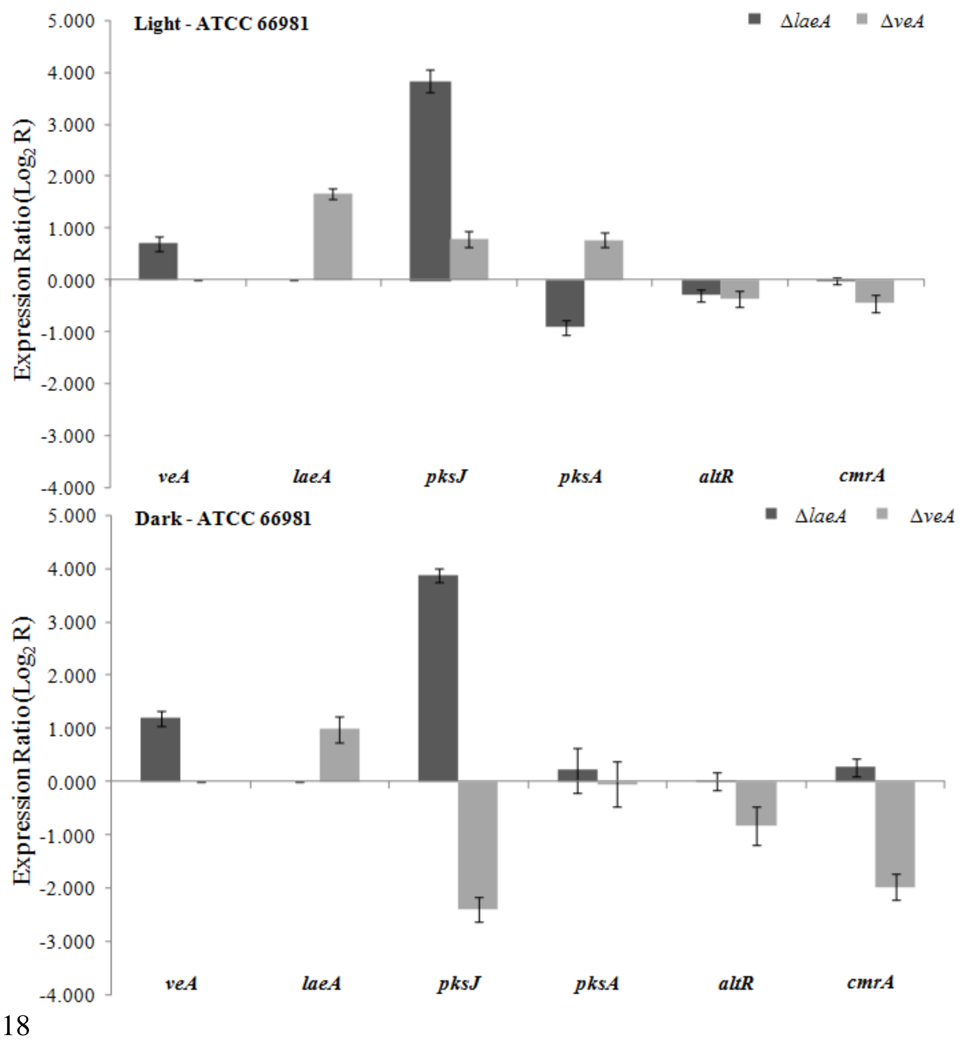
3.5 Effects of *veA* and *laeA* disruption on gene regulation

To investigate whether disruption of *laeA* and *veA* modified expression patterns of genes associated with secondary metabolism, mRNA transcript abundance was assessed using qPCR.

To demonstrate that gene disruption strategy was successful, *laeA* and *veA* gene expression was assayed in their respective deleted mutant strains and results confirmed the absence of their transcripts. *laeA* gene was also studied in Δ*veA* and *veA* in Δ*laeA* as well. Fig. 7 shows the Log_2 of the gene expression ratio compared to wild-type strains, thus, bars above the baseline indicate upregulation. Even for ATCC 66981 or CBS 116.329, *veA* transcripts seemed to be slightly upregulated in Δ*laeA* strains, (light or dark conditions). The same tendency towards up-regulation was observed for LaeA transcripts in Δ*veA* incubated either on dark or light conditions. up-regulation, while bars under the baseline indicate down-regulation.

Next, to elucidate if LaeA and VeA were involved in regulation of mycotoxin production at the transcriptional level, we carried out a gene expression assessment for *pksJ* and *altR*, both identified in *A. alternata* for being essential in AOH and AME production (Saha et al., 2012). Additionally, as we observed that deletion of *veA* and *laeA* affected the pigmentation of the colony, we studied expression of c*mra* and *pksA* (Fetzner *et al.*, 2014), genes involved in melanin biosynthesis. When studying genes related to mycotoxin production for Δ*laeA* mutants, results suggested that though mycotoxin production pattern was different for ATCC 66981 and CBS 116.329 mutants, in all cases and conditions *pksJ* was upregulated, even though this upregulation was more noteworthy for ATCC 66981 Δ*laeA* knock

outs, in which *pksJ* transcript levels were almost 4 fold higher than in wild-type. In contrast, *pksJ* expression was downregulated in a similar way in all Δ*veA* mutants, except in Δ*veA* (light) from ATCC 66981, in which there was a slight upregulation. Regarding *altR*, Δ*laeA* and Δ*veA* from both ATCC 66981 and CBS 116.329 suggested similar expression compared to the wild-type. Additionally, Fig. 7 illustrates that *cmra* was not affected by the deletion of *laeA*, as gene expression was similar to the wild-type. However, the deletion of *veA* seems to down-regulate the expression of *cmra* in all the mutants studied. The gene *pksA* appears to be less tightly regulated in some way by *laeA* or *veA*.



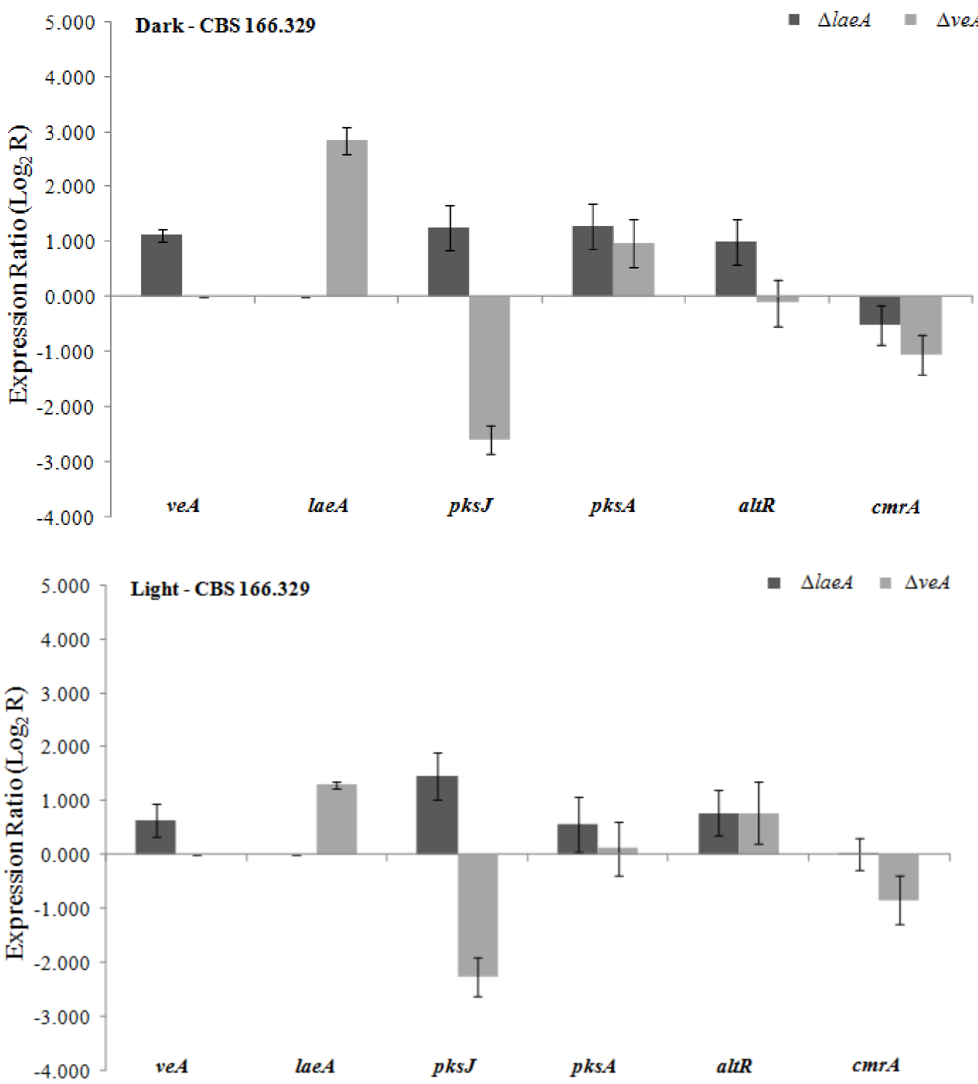


Figure 7. Gene expression analysis of *veA*, *laeA*, *pksJ*, *altR*, *pksA* and *cmrA*. RNA from mycelium was collected on the fifth day of growing on PDA medium. Bars represent *laeA* and *veA* mutant gene expression ratio compared to the wild-type on a Log_2 scale. Error bars indicate standard errors.

3.6 LaeA and VeA role in pathogenicity

Next we tested the influence of LaeA and VeA on the capacity of *A. alternata's* ability to infect tomato fruit. On tomatoes, we measured the diameter of growth and mycotoxin production expressed as µg toxin/g of tomato. Infection by both strains (wild-type and mutants) was highly variable leading to inconclusive results in regards to virulence. ATCC 66981 results for colony diameters were similar to those obtained *in vitro* (Fig. 8), being wild-types the strains with the highest growth. Conversely, CBS 116.329 strains did not show significant differences between the wild-type and the transformants.

Regarding mycotoxin production *in vivo* (Fig. 8), in the case of ATCC 66981, no major differences were observed between the wild-type and mutants. Nonetheless, as observed *in vitro* (Fig. 6), Δ*laeA* tended to produce more than the wild-type and Δ*veA*. For AOH, the production in Δ*laeA* increased 50% compared to the wild-type, while in the Δ*veA* the production was completely inhibited. For AME, the difference was larger since the Δ*laeA* mutant increased an 1800% compared to the wild-type. No AME production was observed on the Δ*veA* mutant as found for AOH.

Regarding the CBS 116.329 knock out mutants, the wild-type produced the highest level of mycotoxins. The AOH and AME level produced by Δ*laeA* transformants decreased in 96% and 90%, respectively, though no significant differences were observed as the infection was very variable. In the case of Δ*veA*, there were significant differences in diameter of infection compared to the wild-type as the mycotoxin production (AOH and AME) was completely inhibited. Collectively, our results may suggest that AOH and AME production is not a virulence factor for tomato fruit infection/colonization.

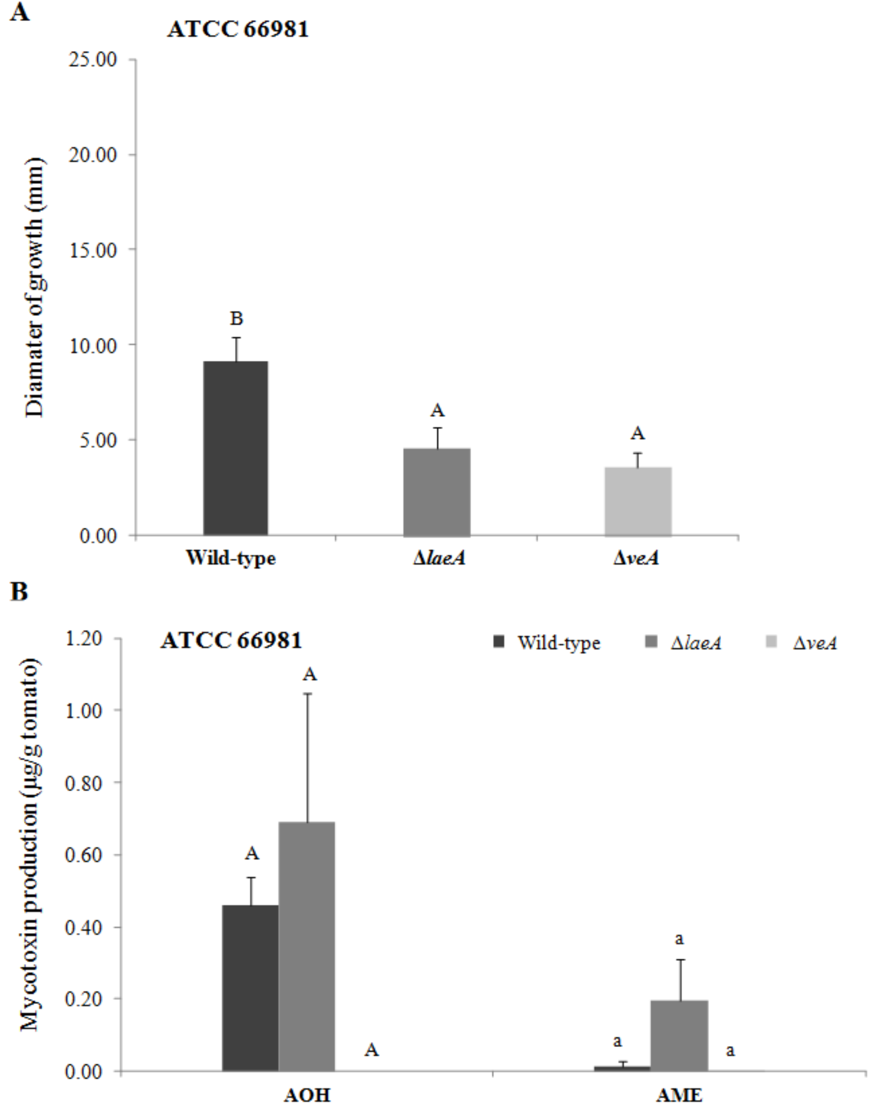


Figure 8(I). Infection capacity and mycotoxin production (AOH and AME) of Δ*laeA* and Δ*veA* strains compared to the wild-type on tomatoes. **A.** Diameter of infection of wild-type strains and Δ*laeA* and Δ*veA* null mutants of *A. alternata* on tomatoes. **B.** AOH and AME production on tomatoes. Error bars indicate standard errors. Different letters indicate significant differences among sample groups (ANOVA, $P < 0.05$).

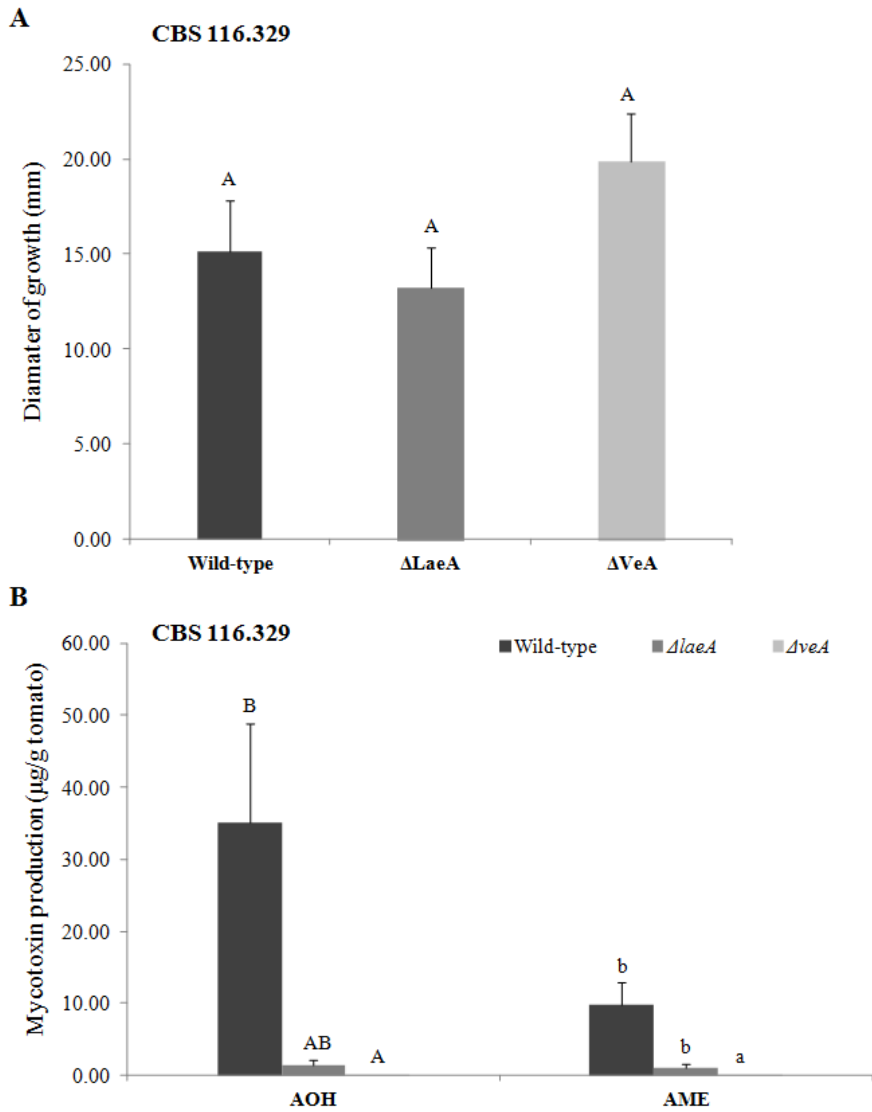


Figure 8(II). Infection capacity and mycotoxin production (AOH and AME) of Δ*laeA* and Δ*veA* strains compared to the wild-type on tomatoes. **A.** Diameter of infection of wild-type strains and Δ*laeA* and Δ*veA* null mutants of *A. alternata* on tomatoes. **B.** AOH and AME production on tomatoes. Error bars indicate standard errors. Different letters indicate significant differences among sample groups (ANOVA, $P < 0.05$).

4. Discussion

Although the genus *Alternaria* leads to important economic losses to farmers and the food industry every year, little is known about the genetic regulation of the mycotoxin biosynthesis process. Thus, detailed inspection of the target genes involved in metabolic pathways could help us to better understand the mechanisms of regulation of several routes still unclear. Once targets are identified, we could investigate strategies to control and reduce *Alternaria* infection in the field and consequently prevent the mycotoxin contamination.

Previous research on other fungi has demonstrated that the velvet or *Ve* complex has an important role in fungal growth and morphology, sexual and asexual development, germination, secondary metabolism and virulence capacity (Bayram and Braus, 2012; Bok et al., 2005; Calvo, 2008). This study is the first report that describes the role of LaeA and VeA in two different strains of *A. alternata*. Results suggest that both components are involved in essential functions of *A. alternata*, as occur in other fungi. Despite LaeA and VeA are present in several fungal genera and have a conserved protein structure, which consist of a conserved N-terminal and variable C-terminal regions, their role can be quite different depending on the genus of the fungi, and even between species of the same genus (Calvo, 2008). In fact, we have found that the loss of these proteins not always leads to the same phenotype in both *A. alternata* strains studied in this work. This could be due to the large genetic diversity among *Alternaria* strains which is also reflected in the difficulty to speciate *Alternaria* isolates by only using phenotype characteristics as pointed out by several authors (Aradhya et al., 2001; Guo et al., 2004; Pruss et al., 2014). Accordingly, Pruss et al. (2014) in their study focused on clarifying the role of the white-collar 1 (WC-1) gene in *A. alternata* did also obtain contradictory results compared to other studies published before in *A. alternata* strains (Häggblom and Unestam, 1979; Söderhäll et al., 1978).

In the case of the ATCC 66981 strain, LaeA and VeA seemed to be relevant for colony growth under either light or darkness, especially VeA, as it has been observed in *A. parasiticus* and *A. carbonarius* (Calvo et al., 2004; Crespo-Sempere, Marín et al., 2013). By contrast, for the CBS 116.329 strain, LaeA seemed to act as a negative regulator of the colony growth, while VeA did not have any significant affectation compared to the wild-type. This LaeA pattern had also been observed by Crespo-Sempere et al. (2013) in *A. carbonarius*, though in that case this increase was only observed when colonies where grown in light conditions.

Several studies postulate that the velvet complex is associated with sexual and asexual development (Bayram, Krappmann, Seiler et al., 2008; Calvo, 2008; Hee-Seo et al., 2002; Hoff et al., 2010; Jiang et al., 2011; Li et al., 2006; Yang et al., 2013). Results described in this work are consistent with these earlier studies, thus confirming that LaeA and VeA are involved in some aspects of asexual development in *Alternaria*. In *A. nidulans*, VeA acts positively regulating sexual structures, such as Hülle cells and cleistothecia, and negatively regulating asexual development (Hee-Seo et al., 2002). In *Neurospora crassa*, *C. heterostrophus*, *Fusarium graminearum*, *Fusarium verticillioides* and *Botrytis cinerea,* deletion of *ve-1*, *Chvel1*, *FgVEA*, *FvVE1* and *BcVEA* genes, respectively, also led to a significant increase in conidial production (Bayram, Krappmann, Seiler et al., 2008; Jiang et al., 2011; Li et al., 2006; Wu et al., 2012; Yang et al., 2013). By contrast, in other fungi such as in *A. carbonarius*, *A. fumigatus*, *A. parasiticus*, *Fusarium fujikuroi*, *Fusarium oxysporum* and *Penicillium chrysogenum* it has been described that VeA regulates sexual and asexual development in the opposite way, (Calvo et al., 2004; Crespo-Sempere, Marín et al., 2013; Hoff et al., 2010; Krappmann et al., 2006; López-Berges et al., 2013; Wiemann et al., 2010). Our study provides results that suggest that VeA positively regulates asexual development in *A. alternata* since in both strains tested in this study, when *veA* was disrupted conidia production drastically decreased. LaeA may also develop a

noteworthy role in sexual and asexual development. Our results indicate that LaeA positively regulates the asexual development in *A. alternata* as observed for VeA. However, this positive regulation could be less important if compared with VeA, as one of the strains (ATCC 66981) indicates that conidia reduction is more severe when deleting *veA*. This positive asexual regulation has also been described in other fungi such as *A. carbonarius*, *A. flavus*, *F. oxysporum*, F. *fujikuroi* and *P. chrysogenum* (Crespo-Sempere, Marín et al., 2013; Hoff et al., 2010; Kale et al., 2008; López-Berges et al., 2013; Wiemann et al., 2010; Wu et al., 2012), while in *C. heterostrophus* it has been described a negative regulation (Wu et al., 2012). Nevertheless, other studies performed with *A. nidulans* and *A. fumigatus* have described that *laeA* deletion hardly impaired conidial production, showing knock out mutants showed almost the same conidiation level as the wild-type strains (Bok et al., 2005; Bok and Keller, 2004a). In *A. flavus* it has been observed that in *laeA* knock out mutants conidial production was dependent of the media as in some media Δ*laeA* produced higher conidiation level than the wild-type while on others production was lower (Chang et al., 2012).

Earlier studies suggest that in *A. alternata* there is an association between developmental structures and pigment biosynthesis pathways (Fetzner et al., 2014; Kawamura et al., 1999). Among the natural pigments, the most common is melanin which biosynthesis contributes to the survival of the fungal spore by protecting against damaging UV light and it is an important virulence factor as well (Heinekamp et al., 2013; Liu and Nizet, 2009; Yin and Keller, 2011). In *A. alternata*, biosynthesis of melanin is produced primarily by the 1,8-dihydroxinapthalene (DHN) pathway (Kimura and Tsuge, 1993). Its biosynthesis requires PksA, a 1,3,6,8-trihydroxynaphtalene (THN) reductase, a scytalone dehydratase (Bmr1) and a 1,3,8-THN reductase (Bmr2) (Eliahu et al., 2007; Fetzner et al., 2014). Some of these genes encoding enzymes required for melanin biosynthesis are regulated by CmrA, a transcriptional regulator that controls spore development as well (Eliahu et al., 2007; Tsuji et al., 2000). Thus, as we observed

pigmentation differences in Δ*veA* and Δ*laeA* mutants, we studied how the loss of two proteins of the velvet complex could affect *cmrA* and *pksA*. While no important differences were observed in *pksA* gene expression in Δ*veA* and Δ*laeA* mutants, *cmrA* was down-regulated in Δ*veA* mutants. Interestingly, higher down-regulation levels of *cmrA* correlates with less pigmented colonies and with less sporulation. This may suggest that VeA could be also linked to melanin biosynthesis acting as a positive regulator of the pathway.

In *A. alternata*, LaeA and VeA are required not only for normal morphology and asexual development but also for the production of secondary metabolites. However, deletion of the transcription factors LaeA and VeA caused a different strain-specific response. It was interesting that while both Δ*laeA* and Δ*veA* transformants of the CBS 116.329 strain decreased their mycotoxin production, the ATCC 66981 Δ*laeA* strain showed increased AOH and AME production. Indeed, the mycotoxin production pattern of the strains was consistent under *in vitro* and *ex vivo* (tomato fruits) conditions. In the case of Δ*veA* null mutants, mycotoxin levels decreased in all conditions.

We also studied the expression of two of the genes putatively involved in the biosynthesis pathway and regulation of AOH, *pksJ* and *altR* (Saha et al., 2012), which encode two proteins that supposedly are sufficient for AOH production. Saha et al. (2012) observed that down-regulation of *pksJ* and *altR* caused a large decrease of AOH formation (Saha et al., 2012). In our study, while no remarkable findings were observed for *altR*, *pksJ* was overexpressed in all Δ*laeA* transformants. This overexpression was remarkably higher when it came to ATCC 66981 Δ*laeA*. For Δ*veA*, *pksJ* expression was down-regulated almost in all the strains, which correlates with mycotoxin production results. In previous studies, Crespo-Sempere et al. (2013) described a reduction of OTA production in *A. carbonarius*, both in Δ*laeA* and Δ*veA*, linked with a down-regulation of the nonribosomal peptide synthetase (*nrps*) involved in OTA biosynthesis. In *A.*

alternata this correlation has only been observed in Δ*veA* transformants. We are aware that there exists another recent theory dealing with the biosynthesis of AOH, in which is suggested that another PKS could be the responsible of the AOH production. This idea emerged from the recent study developed by Chooi et al. (2015) in which it was described that the key enzyme for the biosynthesis of AOH in *P. nodorum* was SnPKS19 and, at the same time, it was suggested that in *A. alternata* the responsible PKS could be PksI instead of PksJ, a PKS that was also described by Saha et al. (2012). Although it would have been interesting to assess the gene expression of *pksI* in the present work, the experimental assays of this study were previous to the publication of Chooi et al. (2015), so we just studied the expression of those genes already identified in *A. alternata*.

It may seem surprising that different results could be achieved in two strains of the same *Alternaria* species but, as it has been mentioned before, there is a large genetic variability among *Alternaria* strains even of the same species (Aradhya et al., 2001; Fetzner et al., 2014; Guo et al., 2004). It has been recently shown at the genomic level that individual strains of an *Alternaria* spp. may each possess a thousand or more predicted unique genes (Dang et al., 2015). Thus, other regulation mechanisms may control mycotoxin biosynthesis in a different way which could be related to overall gene content, small epigenetic modifications or instabilities of the genome, previously suggested by Pruss et al. (2014). Further research needs to be done in order to achieve an explanation of the regulation mechanism at a molecular level.

In conclusion, the research findings of this study have provided some evidence that both LaeA and VeA play an important role in morphology development, asexual differentiation and secondary metabolism in *Alternaria*. The loss of *laeA* and *veA* genes led to a drastic reduction of conidia production, suggesting that both velvet components could act as a positive regulator of asexual development. AOH and AME production is also altered in *laeA* and *veA* knockouts. However, this

appeared to be isolate/strain specific. While deletion of *veA* gene seems to strongly inhibit mycotoxin production in both strains, deletion of *laeA* increase the mycotoxin production levels in one strain while in decreased in the other. The genetic variability within *Alternaria* genus could be an explanation that may justify these differences. Hence, we believe that it is important and useful to perform these studies of target gene characterization using more than one strain of the same fungi genus in order to take into consideration the variations that could be derived from the living microorganisms. We have also pointed out that VeA could be linked to CmrA, a regulator belonging to the melanin biosynthesis pathway and the conidiation formation, as the deletion of *veA* lead to a down-regulation of *crmA*. All in all, the molecular mechanism of the velvet complex in *Alternaria* is still unclear and further studies are clearly needed to finally understand the velvet system function in *A. alternata*.

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Chapter IV

Exploring polyamine metabolism of *Alternaria alternata* to target new substances to control the fungal infection

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Exploring polyamine metabolism of *Alternaria alternata* to target new substances to control the fungal infection

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Abstract

Polyamines are essential for all living organisms as they are involved in several vital cell functions. The biosynthetic pathway of polyamines and its regulation is well established. One of the enzymes that particularly attract the attention of researchers is the ornithine decarboxylase as it is the dominant controlling factor of the entire pathway. In this work we have assessed inhibition of this enzyme with D, L-α-difluoromethylornithine (DFMO) on *Alternaria alternata* and it has drastically reduced fungal growth and mycotoxin production. This inhibition was not completely restored by addition of exogenous putrescine. Actually, increasing concentrations of putrescine on the media negatively affected mycotoxin production, which was corroborated by downregulation of *pksJ* and *altR*, both genes involved in mycotoxin biosynthesis. We have looked at the polyamine metabolism of *A. alternata* with the goal of finding targets that compromises its

growth and its capacity of mycotoxin production. In this sense, we have tested two polyamine analogs, AMXT-2455 and AMXT-3016, and we have observed that they partially control *A. alternata* viability *in vitro* and *in vivo* using tomato plants. Finding strategies to design new fungicide substances is becoming a matter of interest as resistance problems are emerging.

Keywords: *Alternaria alternata*; alternariol; alternariol monomethyl ether; polyamines; DFMO, polyamine transport inhibitor

1. Introduction

Alternaria is a common genus of ascomycete fungi that contains numerous species that are both saprophytic on organic materials and pathogenic on many plants. *Alternaria* spp. can contaminate a wide variety of crops in the fields and can cause the spoilage of various fruits, grains, and vegetables during post-harvest and transport, which causes important economic and material losses to the food industry and growers (Bottalico and Logrieco, 1992; Pitt and Hocking, 1997). During pathogenesis, several *Alternaria* species are capable of producing toxic secondary metabolites, some of which are phytotoxins that are involved in fungal pathogenicity, and some others are mycotoxins that elicit adverse effects in humans and animals. The most common group of mycotoxins associated with *Alternaria* contamination includes alternariol (AOH), alternariol monomethyl ether (AME), tentoxin, tenuazonic acid, altenuene and altertoxins. *Alternaria alternata* is one of the most common species and it has been described as the major mycotoxin-producing species of this genus (EFSA, 2011; Logrieco et al., 2009; Ostry, 2008).

Polyamines are small polycationic compounds present in all living organisms. They are essential for growth and development as they regulate several biological processes (Tabor and Tabor, 1983). In higher eukaryotic organisms, including fungi, the most common polyamines are putrescine, spermidine, and spermine.

However, a large number of fungal species do not contain spermine (Pegg and McCann, 1982; Valdés-Santiago et al., 2012; Walters, 1995). Polyamines have been frequently associated with plant stress and defense responses as it has been observed that under these situations, plants significantly accumulate free and conjugated levels of putrescine, spermidine and spermine (Alcázar et al., 2006; Richards and Coleman, 1952). This increase has been seen to go along with an upregulation of two polyamine biosynthetic enzymes, the ornithine decarboxylase (ODC) and the polyamine oxidase (Haggag and Abd-El-Kareem, 2009; Walters et al., 2002). Gardiner et al. (2009) proposed that products of the arginine-polyamine biosynthetic pathway in plants play a role in the induction of trichothecene biosynthesis during fungal infection. Thus, the pathogen would exploit the generic host stress response of polyamine synthesis as a cue for production of trichothecene mycotoxins (Gardiner et al., 2010).

During the last decades, the use of specific inhibitors and the development of mutants has been used to better understand the polyamine metabolism pathway and its regulation. In plants at least two different polyamine pathways involved in polyamine biosynthesis have been described, whereas in fungi there is a unique pathway. In animals and many fungi, putrescine is only synthesized from ornithine by ODC, which is a key enzyme of the entire pathway. This characteristic, makes this metabolic route an ideal target for controlling the growth of pathogenic fungi without altering the plant host as they can use an alternative pathway in which ODC is not involved. In this sense, some researchers have tried to design new strategies to develop new fungicides targeted on the polyamine metabolism (Crespo-Sempere et al., 2015a; Gárriz et al., 2003; Mackintosh et al., 2001; Mellon and Moreau, 2004).

Fungicides have been used in agriculture for well over a century, and initially there were no reports of losses of efficacy in the field. Nevertheless, over time it was discovered that plant pathogenic fungi can adapt to fungicide treatments by

mutations leading to resistance and loss of efficacy. This is the case of *A. alternata* contaminating nuts, or *Alternata solani* contaminating potatoes, which both achieved resistance against succinate dehydrogenase inhibitors (Avenot and Michailides, 2007; Lucas et al., 2015; Miles et al., 2014). Therefore, it is interesting to explore new targets and new strategies for resistance management. With this aim, we have explored polyamine metabolism of *A. alternata* and we have tried to find out some target, via polyamine synthesis inhibition and polyamine analogs, to achieve the control of *A. alternata* regarding tomato plant diseases. For this purpose, we have analyzed the effect of inhibiting the ODC activity on *A. alternata*. We have also studied the impact of adding exogenous putrescine and the consequences of inhibiting polyamine transport using different polyamine analogs. All these assays have been performed *in vitro* and *in vivo* using tomatoes and tomato plants.

2. Material and methods

2.1 Fungal strain growth conditions

The *A. alternata* strain used in this study was the CBS 116.329 (isolated from apple), provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). To prepare conidial suspensions, *A. alternata* was routinely grown on Potato Dextrose Agar plates (PDA, Biokar Diagnostics, France) and incubated seven days in the dark at 26 ºC. Conidia were collected with a scalpel within a sterile solution of phosphate-buffered saline (PBS) with 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, The Netherlands) and filtered through Miracloth (Calbiochem, USA). Conidial suspension concentration was adjusted to 10^6 conidia/mL using a Thoma counting chamber. To study the effect of D, L-α-difluoromethylornithine (DFMO, Enzo Life Sciencies, USA), putrescine (Sigma-Aldrich, USA), and polyamine analogs, a medium free of polyamines was prepared and 5 µL of the conidial suspension (10^6 conidia/mL) were centrally inoculated on

the plates and incubated under dark conditions at 26 ºC for seven days. This medium contained, per liter, 30 g sucrose, 1 g NH_4NO_3, 1 g KH_2PO_4, 20 g agar, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 10 mg $FeSO_4 \cdot 7H_2O$ and 100 µL of trace element solution (per 100 mL, 5 g citric acid, 5 g $ZnSO_4 \cdot 7H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 50 mg $MnSO_4 \cdot H_2O$ and 50 mg H_3BO_3). Medium pH was adjusted to 6.5 with NaOH. Putrescine, DFMO and the polyamine analogs were dissolved in water, sterilized by filtering the solution through a 0.45 µm pore size filter and added to the aforementioned autoclaved culture medium. For DFMO experiments, the concentrations tested were 2.5 mM and 5 mM. For putrescine, concentrations were 50, 500, 1000 and 5000 µM, while for the polyamine analogs, the concentrations ranged from 100 to 1200 µM. Polyamine analogs, DFMO, and putrescine were stored at −20 °C until needed. We tested seven polyamine analogs, detailed in Table 1, kindly provided by Aminex Therapeutics (USA). The polyamine transport inhibitors (PTIs) are lipophilic polyamine analogs, synthesized as polyamine transport inhibitors or antizyme inducing agents (Burns et al., 2001; Burns et al., 2009; Petros et al., 2006). The lipophilic polyamine analogs bind to the lipid membrane of the mammalian cell where the polyamine transport apparatus is blocked and, as a result, the uptake or the excretion of polyamines, or may be both, will be inhibited. The antizyme inducing polyamine analogs induce frameshifting and expression of antizyme, which is a polyamine-feedback biomolecule shown to inhibit polyamine biosynthesis and transport.

Table 1. Polyamine analogs tested against *A. alternata*.

Name	Structure
AMXT-1483	
AMXT-1505	
AMXT-2030	
AMXT-3016	
AMXT-2444	
AMXT-3938	
AMXT-2455	

2.2 Radial growth rate and sporulation assessment

Radial growth rate was determined by measuring daily, over 4 days, two perpendicular diameters of the growing colonies. Sporulation assessment was carried out by collecting all the mycelia grown on a Petri dish with the help of a scalpel and placing it on a Falcon® tube containing a sterile solution of PBS with 0.005% (v/v) of Tween 80. Tubes were vigorously shaken on the vortex and conidia were recovered by filtration through Miracloth. Conidia concentration was measured by using a Thoma counting chamber and results were expressed as conidia/mm^2 of fungal colony.

2.3 Extraction and detection of AOH and AME from culture

Mycotoxin production (AOH and AME) was quantified in seven day old cultures. To this aim, one agar plug (5 mm in diameter) was removed from the center of the colonies and extracted with 500 µL of acetonitrile-methanol-water (45:10:45 v/v/v), adjusted to pH 3 with *o*-phosphoric acid. After 60 minutes, samples were filtered (Millex-HV 0.45 µm, 25 mm, Millipore Corporation, USA) into another vial and mycotoxin extracts were dried in a speed vacuum concentrator at room temperature. Samples were stored at -20 °C until HPLC analysis. Prior to analysis, the extracts were resuspended in 500 µL of a water-methanol solution (50:50 v/v). Separation, detection and quantification of AOH and AME was performed on an HPLC system consisting of a Waters 2695 Alliance Separations Module connected to a UV/Visible dual λ absorbance Detector Waters 2487, using a reverse phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a Spherisorb guard column (5 µm ODS2, 4.6 x 10 mm, Waters, Millford, MA, USA). Columns were set at a temperature of 35 ºC. For chromatographic separation of AOH and AME the flow rate was 0.5 mL/min and the injection volume was 100 µL. Absorption wavelength was set at 258 nm. The mobile phase consisted of a gradient of double distilled miliQ water (MiliQ

Academic Millipore, USA) and methanol-water (70:30 v/v) according to the gradient described in Table 2.

Table 2. HPLC gradient used for the chromatographic analysis of AOH and AME.

Time (min)	Water	Methanol- water (70:30 v/v)
	100	-
7	100	-
9	20	80
10	20	80
12	-	100
41	-	100
43	10	90
45	30	70
46	50	50
47	70	30
50	100	-

Retention times were 24 minutes for AOH and 32 minutes for AME. For mycotoxin quantification, working standards were used to perform a ten-point calibration curve for the mycotoxins (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). The limit of detection (LOD) for AOH was 0.02 ng/mm^2 for *in vitro* assays and 0.009 µg/g tomato, while for AME the LOD was 0.034 ng/mm^2 *in vitro* and 0.012 µg/g of tomato. The LOD vas based on a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was calculated as 3 × LOD. All solvents were HPLC grade and all chemicals were analytical grade. Method performance characteristics for AOH and AME are summarized in Table 3.

Table 3. Recovery and repeatability of AOH and AME analysis on tomatoes.

	AOH			AME		
Spiking level (µg/g tomato)	**n**	**Recovery [a] (%)**	**RSDr [b] (%)**	**n**	**Recovery (%)**	**RSDr (%)**
0.5	3	115.5 ± 2.1	1.8	3	106.0 ± 8.2	7.7
1.0	5	99.0 ± 2.4	2.4	5	93.8 ± 3.1	3.4
2.0	3	79.8 ± 3.2	4.0	3	78.0 ± 3.6	4.6

[a] Mean value ± standard deviation.
[b] RSDr = relative standard deviation.

2.4 Gene expression analysis

Mycelium grown for seven days on media supplemented with different concentrations of DFMO, putrescine, and polyamine analogs was collected, frozen in liquid nitrogen and stored at -80 °C before nucleic acid extraction. RNA was extracted from 1 g of mycelium previously grounded to a fine powder with a mortar and a pestle with liquid nitrogen. Pulverized mycelium was added to a pre-heated (65 ºC) mixture of 10 mL of extraction buffer: 100 mM Tris–HCl (pH 8.0), 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% (v/v) β-mercaptoethanol and 5 mL of Tris-equilibrated phenol. The extract was incubated at 65 °C for 15 min and cooled before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 *x g* for 20 min at 4 °C, and the aqueous phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated during three hours at -20 ºC by adding 3.3 mL of 12 M LiCl. After centrifugation at 27200 *x g* for 60 min, the pellet was washed with 500 µL of 70% ethanol. The resultant pellet was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides. Then, RNA was washed again with 500 µL of 70% ethanol, and, finally, dissolved in 100 µL of miliQ water. RNA concentration was spectrophotometrically measured and verified by ethidium-bromide staining of an agarose gel. Total RNA was treated with DNase (TURBO

DNase, Ambion, USA) to remove contaminating genomic DNA. Single-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instructions (Invitrogen, USA).

Primer pairs pksJ_F/pksJ_R and altR_F/altR_R were designed to study gene expression of a polyketide synthase, PksJ, and a putative transcriptional factor, AltR, both are involved in the AOH and AME biosynthesis pathway (Saha et al., 2012). Gene-specific primer set ODC_F/ODC_R was designed for expression analysis of the polyamine biosynthetic enzyme, ODC.

Table 4. Primer sequences used in this study. The transcript ID corresponds to the *A. alternata* (ATCC 66981) genome.

Transcript ID	Gene	Primer name	Primer sequence (5′→3′)
AAT_PG02879	*pksJ*	pksJ_F	ACACTAGCACAGTCGGTTCCCA
		pksJ_R	ATTGGCCGCGTACTACCCAG
AAT_PG02875	*altR*	altR_F	AAACACCGCTTGAGGAACGCCAGA
		altR_R	AAAGCGTGCCATTGCCGATACCAG
AAT_PG07905	*ODC*	ODC_F	AGTCGTTCAGCACCTATCCC
		ODC_R	CAGGATCAATAGCCTCGACA
AAT_PG07106	*TPO4*	TOP4_F	TGCTCCTCTTCTCGCCCAT
		TOP4_R	ATGAGACCGAATAGCACACC
AAT_PG05035	*Beta-tubulin*	A-BTF	ACAACTTCGTCTTCGGCCAGT
		A-BTR	ACCCTTTGCCCAGTTGTTACCAG

The primer pair TPO4_F/TPO4_R was used to analyze the expression of TPO4, a putative polyamine transporter that is involved in the detoxification of excess polyamines in the cytoplasm (Tomitori et al., 2001). Finally, the primer pair A-BTF/A-BTR was designed within the beta-tubulin gene, which was chosen as a housekeeping gene. All primer pairs were designed using the *Alternaria* genome database (http://alternaria.vbi.vt.edu) recently published by Dang et al. (2015). *A. alternata* (ATCC 66981) genome was established as the query. Primers were

designed with the OLIGO Primer Analysis Software V.7. All primer sequences with each corresponding transcript ID are listed in Table 4. Gene expression analyses were assessed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA). qPCR reactions were performed in a final volume of 10 µL, containing 1X of SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA), 250 nM of each primer and 1 µL of template DNA. The standard protocol included one cycle at 98 °C for 2 min, followed by 35 cycles at 98 °C for 5 s and 58 °C for 30 s. Reactions were done in duplicate for each sample, checking the PCR reaction quality by analyzing the dissociation and amplification curves. The corresponding qPCR efficiency (E) in the exponential phase was calculated according to the equation: E = 10[-1/slope]. The relative expression of the target genes was calculated based on the E and the Crossing point (Cp) value of the sample versus the control. The Cp value is the cycle at which fluorescence from amplification exceeds the background fluorescence. The relative expression of the target genes was expressed in comparison to the beta-tubulin gene (reference gene), according to the following equation (Pfaffl, 2001; Rasmussen, 2001a):

$$R = \frac{\left(E_{target\ gene}\right)^{\Delta Cp\ _{target\ gene}(MEAN\ control\ -\ MEAN\ sample)}}{\left(E_{reference\ gene}\right)^{\Delta Cp\ _{reference\ gene}(MEAN\ control\ -MEAN\ sample)}}$$

Gene expression measures were derived from biological triplicates.

2.5 Inoculation and growth of *A. alternata* on tomatoes and tomato plants treated with PTIs

We tested the effect of the polyamine analogs AMXT-3016 and AMXT-2455 on *A. alternata,* tomato plants and tomato fruits. To obtain tomato plants, we grew tomato seeds (*Solanum lycopersicum* var. *paladium*) on sterilized soil in a growth chamber with a photoperiod of 16 h of light at 25 ºC and 8 h of dark at 20 ºC, and a relative humidity (RH) of 75%. Tomato seeds were kindly provided by Semillas Fitó S.A (Barcelona, Spain). The fungal inoculation on tomato plants was

performed once plants had five or more true leaves, approximately 3-4 weeks after the seeding. Prior to infection, leaves were injured by lightly rubbing the skin with carborundum dust (Carlo Erba Reagents, Italy) with a cotton-tipped applicator. Afterwards, we immediately treated with the polyamine analogs treatment by touching the leaves with a cotton-tipped previously dipped in the polyamine analogs solution (800 µM for AMXT-3016 and 1000 µM for AMXT-2455). Once excess water was dried fungal infection was performed by inoculating the leave with 10 µL of an *A. alternata* spore suspension (10^7 conidia/mL). Negative control plants were also injured with carborundum dust, soaked with the polyamine analog solution and, once dry, wetted again with 10 µL of sterile water. Tomato plants were contained individually in non-hermetic boxes and left to grow one more week on the same light and temperature conditions aforementioned but increasing the RH to 90%, which favored the fungal growth. For this experiment, each treatment was composed by five tomato plants and each plant had 5 treated leaves. Observations were performed one week after the fungal inoculation and were based on symptom appearance.

To assess polyamine analogs effectiveness on tomato fruits, we used tomato fruits (*Solanum lycopersicum* var. *paladium*). Before performing inoculation assays, we verified that this tomato variety was susceptible to *Alternaria* spp. infection by inoculating five tomatoes with an *Alternaria* conidial suspension and let inoculated tomatoes grow for one week at 20 ºC. The effectiveness of the polyamine analogs tested was measured by analyzing the diameter of the fungal infection, and the AOH and AME production. Tomato fruit were previously surface disinfected with 10% of sodium hypochlorite for 1 minute and rinsed with tap water for 10 minutes. Once excess water was evaporated, tomatoes were dipped for 10 seconds into the PTI solution (800 µM for AMXT-3016 and 1000 µM for AMXT-2455) and they were left to dry again. Tomatoes were four-times injured with a sterilized awl. Inoculation was performed placing 5 µL of a conidial suspension (10^6 conidia/mL) in each wound. Negative control tomatoes were also injured and 5 µL of water

were placed on each hole but no conidial suspension was added. Positive controls were not dipped into the polyamine analogs solutions but were inoculated with the *Alternaria* conidial suspension. Tomatoes were packaged into plastic bags and stored at 20 ºC for two weeks in the post-harvest chambers of the Institute for Food and Agricultural Research and Technology (IRTA), who kindly offer us their chambers. Inside the plastic bags the RH reached the 100%. Temperature and RH were recorded hourly by a data logger (Escort iLog RH, Portugal). Five tomatoes were considered a single replicate and the assay was performed in quadruplicate. Two weeks after the fungal inoculation, *A. alternata* growth was observed. Diameter lesion size was measured and mycotoxins were extracted. For validation, AOH and AME free tomatoes were artificially spiked with *Alternaria* mycotoxins to assess recovery and repeatability data of the method (Table 3). For mycotoxin production assessment, plugs of 7 mm of diameter and 0.5 mm of thickness were removed from the inoculation point. Three plugs were taken from each tomato. All the plugs from the same replicate were put into a stomacher bag. Mycotoxin extraction was proceed by adding 30 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric) and homogenizing for 2 minutes with the stomacher. The mixture was blended for 15 extra minutes under a uniform magnetic field. The solution was left for 10 minutes, approximately, to favor precipitation by gravity. Then, 6 mL of the supernatant were transferred to a centrifuge tube and diluted with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3) and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract was passed by gravity through a previously conditioned Bond Elut Plexa SPE cartridge (200 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the cartridge was done with 5 mL of methanol following 5 mL of miliQ water. The SPE column was washed with 5 mL of water followed by air drying on the manifold. Finally, elution was carried out with 5 mL of methanol and 5 mL of acetonitrile. Sample extracts were dried under nitrogen flow and stored at -20 ºC until HPLC analysis. Prior to HPLC injection, samples were

resuspended in 500 µL of the mobile phase solution (water-methanol, 50:50 v/v). HPLC conditions were the same as previously described.

2.6 Statistical analysis

All statistical data was analyzed using the One Way ANOVA test ($p < 0.05$). When data did not fit the ANOVA assumptions (normality and homoscedasticity), results were transformed. Tukey-HSD test (Honest Significant Difference) was used to compare means. All statistical analyses were performed with Statgraphics Centurion Version XVI.

3. Results

3.1 Inhibition of ornithine decarboxylase by DFMO

Scarce information is available about polyamine metabolism in the *Alternaria* genus. In this study, the first strategy adopted to better understand polyamine metabolism in *A. alternata* was to examine the effect of putrescine biosynthesis inhibition by using DFMO, which irreversible inhibits the ODC enzyme, responsible for catalyzing the initial step in polyamine synthesis and a key enzyme of the entire pathway (Davis et al., 1992; Metcalf et al., 1978). Two different concentrations of DFMO were tested, 2.5 and 5 mM. Results (Fig. 1) demonstrated that DFMO inhibited *A. alternata* radial growth rate (mm/day) by 10% and 26%, respectively. Additionally, not only the fungal mycelia growth was affected but also the colony morphology. Control colonies grew in a thickly green-brown uniform layer, while the colonies that have been grown on media containing 2.5 or 5 mM of DFMO lost all the pigmentation and, as a consequence, were white with much less mycelium. Regarding mycotoxin production, control colonies produced a total amount of 1.84 ng/mm^2 of AOH while no AME was detected. However, the addition of 2.5 mM of DFMO on the media decreased the AOH production to 0.78 ng/mm^2, while 5 mM completely inhibited mycotoxin biosynthesis. Growth

inhibition mediated by DFMO was readily reversed in the presence of 1 mM of putrescine. In contrast, neither mycotoxin production nor the colony color, were reversed when 1 mM of putrescine was added to the media. Colonies were less pigmented and adopted a softer green coloration.

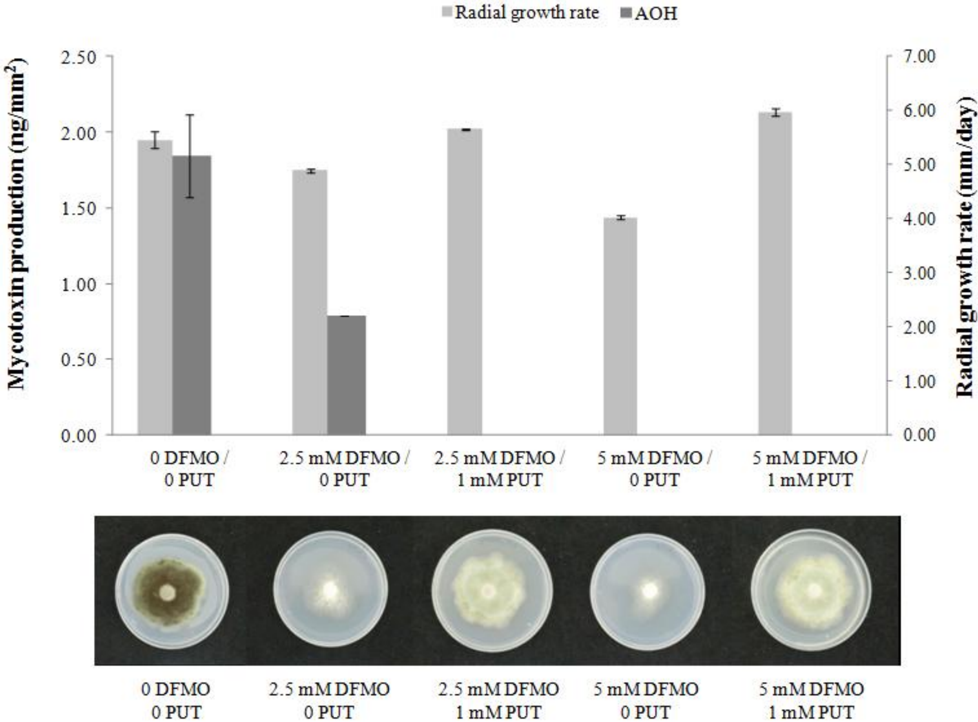

Figure 1. Effect of DFMO on AOH production (left y-axis), radial growth rate (right y-axis) and colony morphology of *A. alternata* colonies. No AME production was detected. Error bars indicate standard errors.

In order to get a deeper insight about how DFMO affected *A. alternata*, gene expression analysis was carried out. Gene expression of *pksJ* and *altR* was studied, both genes identified of being essential in the AOH and AME production pathway (Saha et al., 2012). Gene expression of *ODC* and, additionally, the expression of *TPO4*, a gene that encodes a polyamine transporter protein that recognizes putresine, spermidine, and spermine and excretes them from the cell to the

extracellular media (Igarashi and Kashiwagi, 2010; Tachihara et al., 2005; Tomitori et al., 1999; Valdés-Santiago et al., 2012).

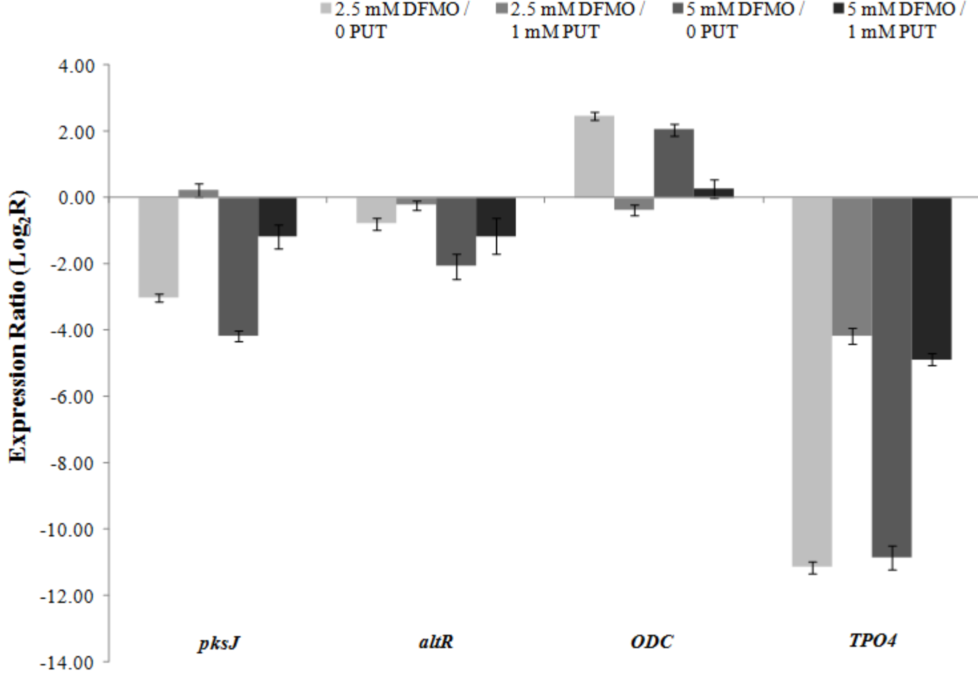

Figure 2. Relative expression of *pksJ*, *altR*, *ODC* and, *TPO4* in *A. alternata* in the presence of DFMO (2.5 and 5 mM) and presence/absence of putrescine (1 mM) with regard to expression level in the same medium without DFMO. Error bars indicate standard errors.

Fig. 2 shows the relative expression of *pksJ*, *altR*, *ODC* and *TPO4* in the presence of DFMO with respect to control samples (0 mM DFMO and 0 mM putrescine). Thus, bars above the baseline indicate upregulation, while bars under the baseline indicate downregulation. Gene expression analysis revealed that the higher the DFMO concentration, the higher the downregulation of *pksJ* and *altR*. DFMO decreased gene expression of *pksJ* by a $\log_2$ ratio of 3.03 when treated with 2.5 mM and 4.18 with 5 mM. In contrast, with regard to *ODC* expression, it was found that both concentrations of DFMO resulted in an overexpression of the gene. *TPO4*

was the most affected gene by DFMO among the ones we studied. The colonies that had no exogenous putrescine in media indicated a $\log_2$ ratio decrease of the *TPO4* of 11.15 for 2.5 mM, and 10.86 for 5 mM, which represents a 2272 and 1859 fold change, respectively. Addition of putrescine on the media clearly reverted the effect of DFMO for *pksJ, altR* and *ODC*. However, exogenous putrescine only achieved a partial overcoming of the *TPO4* downregulation.

3. 2 Effect of exogenous putrescine addition on *A. alternata*

To assess the effect of exogenous putrescine addition on the media on which *A. alternata* was grown, different concentrations of putrescine were tested (0, 50, 500, 1000 and 5000 µM) and different parameters that could had been affected were analyzed, such as the radial growth rate, the mycotoxin production, the sporulation, and the colony morphology. Results indicated that no affectation on radial growth rate was observed on colonies containing 50 and 500 µM of putrescine though when the media contained a concentration of 1000 µM or 5000 µM, the radial growth rate decreased 11.17% and 32.00%, respectively, compared to control colonies (Fig. 3). Mycotoxin production was also affected by exogenous putrescine addition, especially AOH, as no AME was detected. The highest peak of AOH corresponded to the control colonies and, as the putrescine concentration in the media increased, AOH decreased proportionally. Colonies with 1000 µM of putrescine reduced the mycotoxin production nearly 90%, while no AOH was observed on 5000 µM plates. The effect of putrescine on the sporulation was also analyzed and it was observed that, as it has been seen with the radial growth rate and the mycotoxin production, sporulation also decreased as exogenous putrescine concentration increased in the media. This way, when the putrescine concentration was 50 µM, the sporulation decreased to 55.60% compared to the control, 35.50% when it was 500 µM and 12.73 and 7.37% when it was 1000 and 5000 µM, respectively. Fig. 3 also illustrates that putrescine also affected the color of the colonies. As putrescine concentration increased, the colonies gradually lose the

green pigmentation and tacked to almost white velvet when the concentration of putrescine reached 5000 µM.

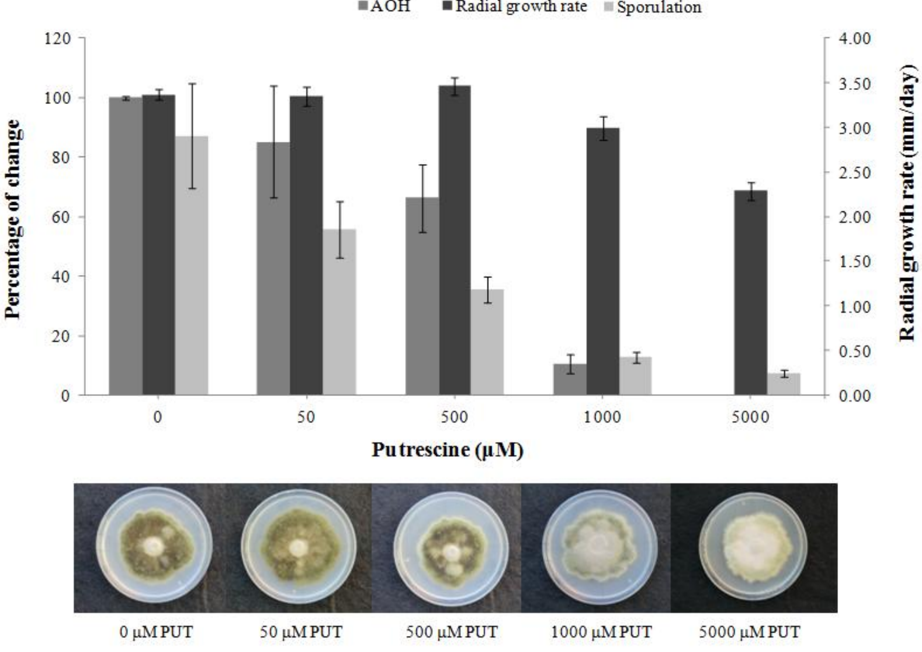

Figure 3. Effect of putrescine on mycotoxin production and sporulation is represented on the left y-axis. Results are shown as percentage of change referred to the control (without putrescine). No AME production was detected. Effect of putrescine on radial growth rate is shown on the right y-axis while colony morphology of *A. alternata* colonies with different concentrations of putrescine is represented at the bottom. Error bars indicate standard errors.

To understand the genetic impact of putrescine regarding mycotoxin production and polyamine biosynthesis pathway, we carried out gene expression of *pksJ*, *altR*, *ODC* and, *TPO4* (Fig. 4). Dealing with genes related to mycotoxin biosynthesis, when putrescine concentration was high, 5000 µM, gene expression of *pksJ* and *altR* showed a light downregulation. For *altR* this downregulation was noticeable

even at 1000 μM. Similarly, it was found that the addition of exogenous putrescine to *A. alternata* culture had little effect on the *ODC* expression.

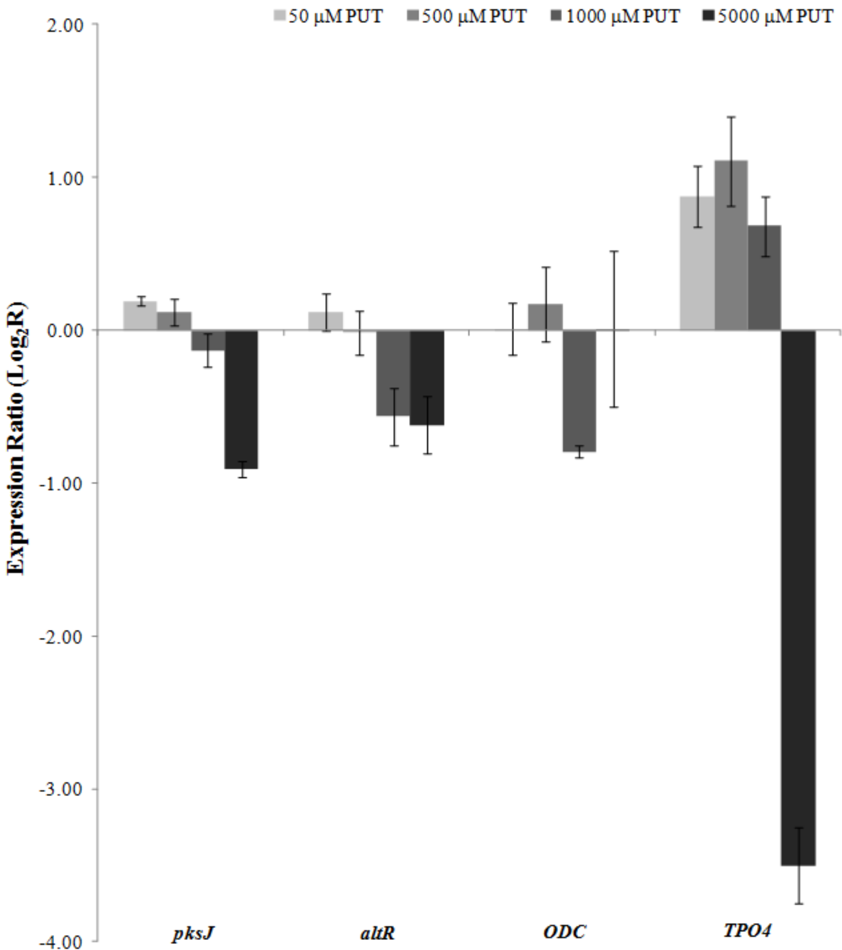

Figure 4. Relative expression of *pksJ*, *altR*, *ODC* and, *TPO4* in *A. alternata* in the presence of different concentrations of putrescine (50, 500, 1000 and 5000 μM) with regard to expression level in the same medium without putrescine. Error bars indicate standard errors.

By contrast, most significant results were found when gene expression of *TPO4* was analyzed. The slight upregulation of cultures with 50, 500 and 1000 μM of

putrescine (that correspond to 1.84, 2.16 and 1.60 fold change, respectively) greatly contrast with what happens when 5000 µM of putrescine was applied, as a downregulation of *TPO4* expression of 11.31 fold change was observed.

3.3 Polyamine analogs on *A. alternata* cultures

Fungi could get supplies of polyamines from their own production via the ODC metabolic pathway but also from importing polyamines from external sources, such as plants, which regulation is supported by polyamine transporters. Burns et al. (2001; 2009) developed a group of lipophilic polyamine analogues that potently inhibit the cellular polyamine transport system. In this context, a previous study with *Fusarium graminearum* (Crespo-Sempere et al., 2015a) supported that PTIs could affect fungal growth and mycotoxin production and even inhibit both processes. Thus, in order to evaluate the effect of polyamine analogs on *A. alternata* cultures we tested seven PTIs developed by Burns et al. (2001; 2009) and kindly provided by Aminex Therapeutics (USA) and we observed their influence on mycelial growth and mycotoxin production. For this purpose, *A. alternata* was grown on plates with different concentrations of polyamine analogs (see the list of the polyamine analogs used in Table 1). Fig. 5 shows the results obtained for all the seven polyamine analogs we tested. Data derived from this study were quite different depending on the polyamine analog used. While some of them did not have any significant effect on mycotoxin production (AMXT-2444), others increased AOH and AME synthesis (AMXT-3938). Therefore, the following strategy was to select polyamine analogs that either did not inhibit *A. alternata* growth at the highest concentration tested or did not reduce mycotoxin production. With this postulate, all polyamine analogs were eliminated excepting the AMXT-2455 and the AMXT-3016. Further on, the optimal AMXT-2455 and AMXT-3016 concentration that inhibited both fungal growth and mycotoxin production was analyzed (Fig. 6 and Fig. 7).

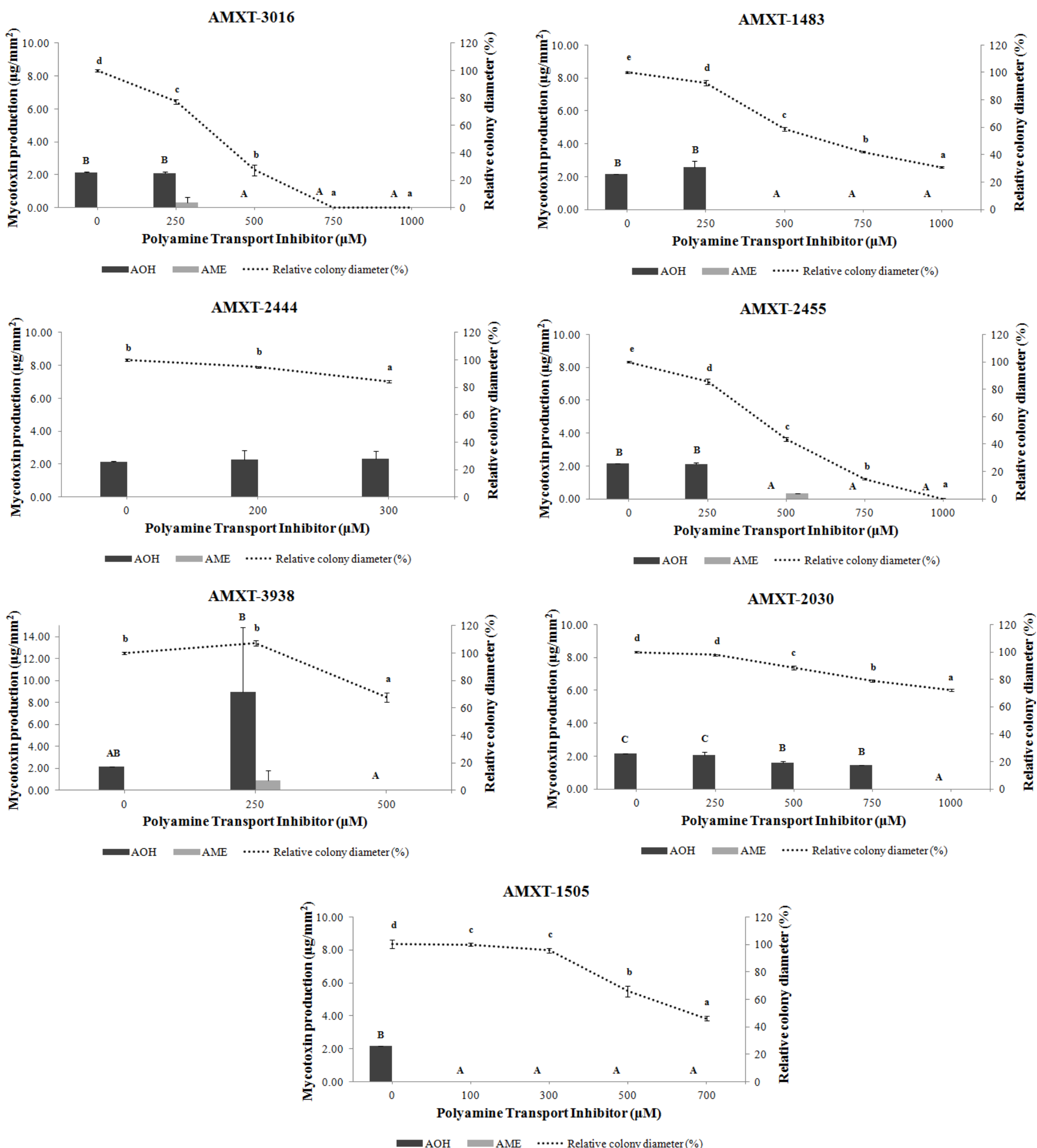

Figure 5. Effect of seven polyamine analogs on mycotoxin production (left y-axis) and colony diameter (right y-axis) referred to the control (without putrescine and without PTI) in percentage. Error bars indicate standard errors. Capital letters indicate homogeneous groups for AOH production. Lower case letters indicate homogenous groups for fungal growth. No letters indicate not significant differences among groups. All statistical data was analyzed by one-way ANOVA ($p < 0.05$). Tukey-HSD test was used to compare means.

The effect of polyamine analogs on sporulation and gene expression was also assessed. Results showed that with AMXT-2455 at 300 µM no AOH was produced and when concentration reached 900 µM both, the sporulation and the radial growth, were null (Fig. 6).

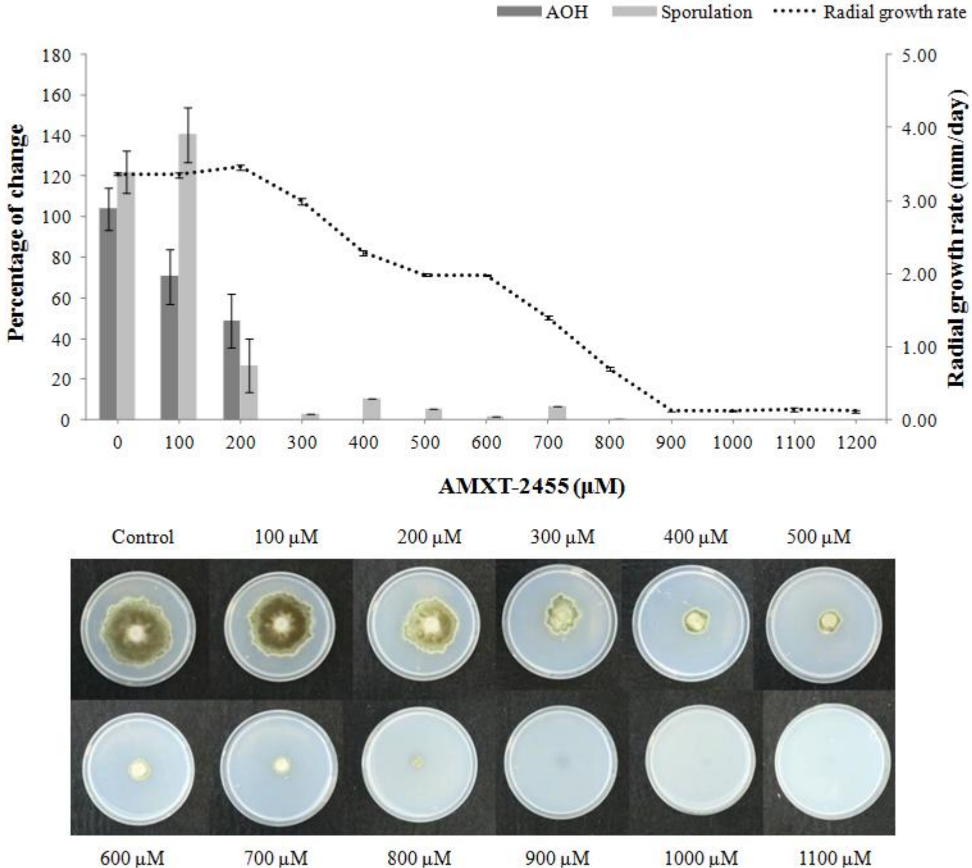

Figure 6. Effect of AMXT-2455 on mycotoxin production and sporulation (left y-axis). Results are shown as percentage of change referred to the control (without AMXT-2455). No AME production was detected. Effect of AMXT-2455 on radial growth rate (right y-axis), while colony morphology of *A. alternata* colonies with different concentrations of the polyamine analog AMXT-2455 (at the bottom). Error bars indicate standard errors.

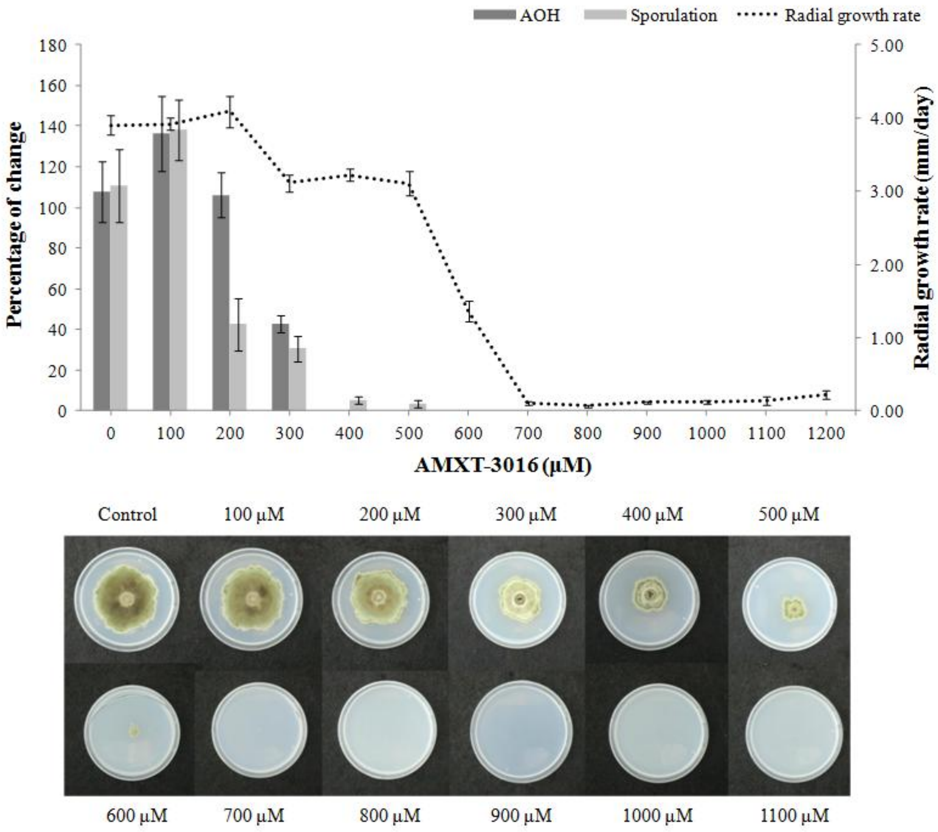

Figure 7. Effect of AMXT-3016 on mycotoxin production and sporulation is represented on the left y-axis. Results are shown as percentage of change referred to the control (without AMXT-3016). No AME production was detected. Effect of AMXT-3016 on radial growth rate is shown on the right y-axis, while colony morphology of *A. alternata* colonies with different concentrations of the polyamine analog AMXT-3016 is represented at the bottom. Error bars indicate standard errors.

Regarding the AMXT-3016, 400 µM were enough to inhibit mycotoxin production, while 600 µM were sufficient to control sporulation and, 700 µM completely inhibited radial growth. In addition to these results, we analyzed the genetic pattern of *A. alternata* when different concentrations of AMXT-2455 and AMXT-3016 were added to the media.

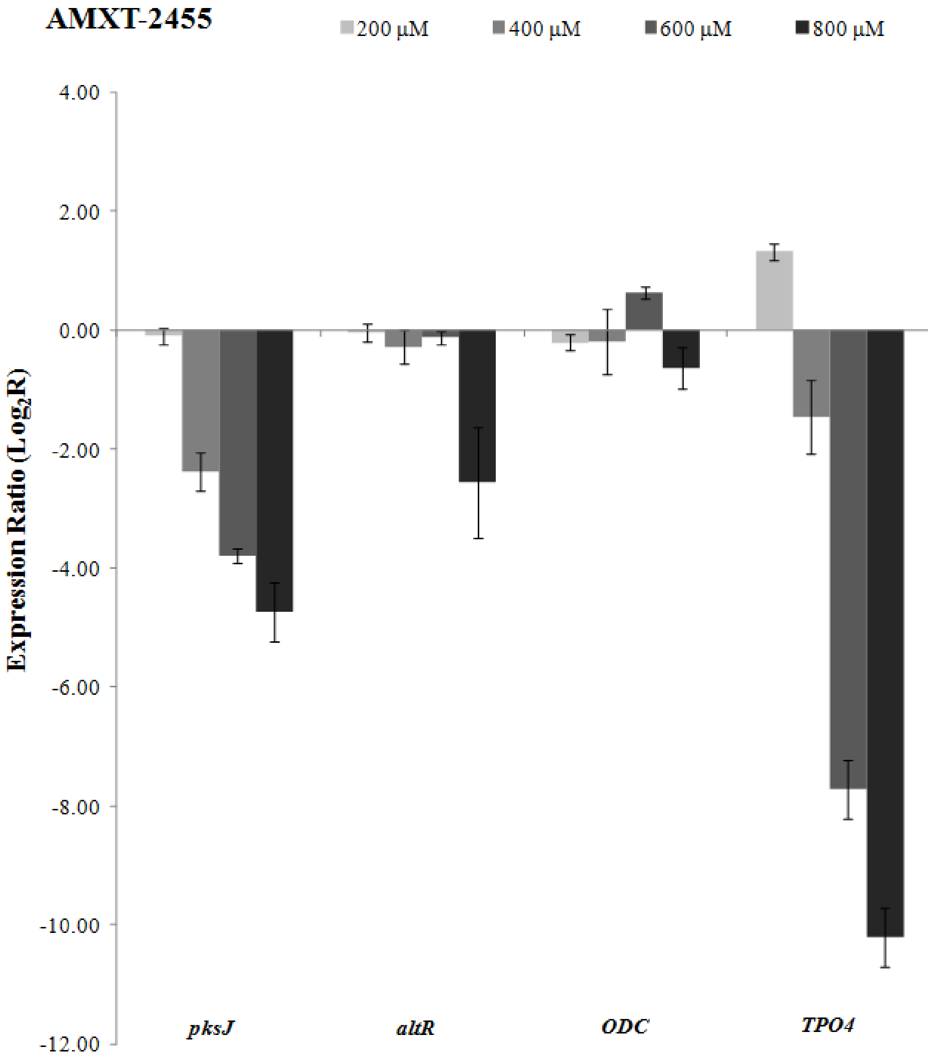

Figure 8. Relative expression of *pksJ*, *altR*, *ODC*, and *TPO4* in *A. alternata* in the presence of AMXT-2455. Bars represent gene expression ratio on a $\log_2$ scale compared to the control (grown on the same medium but without AMXT-2455). Error bars indicate standard errors.

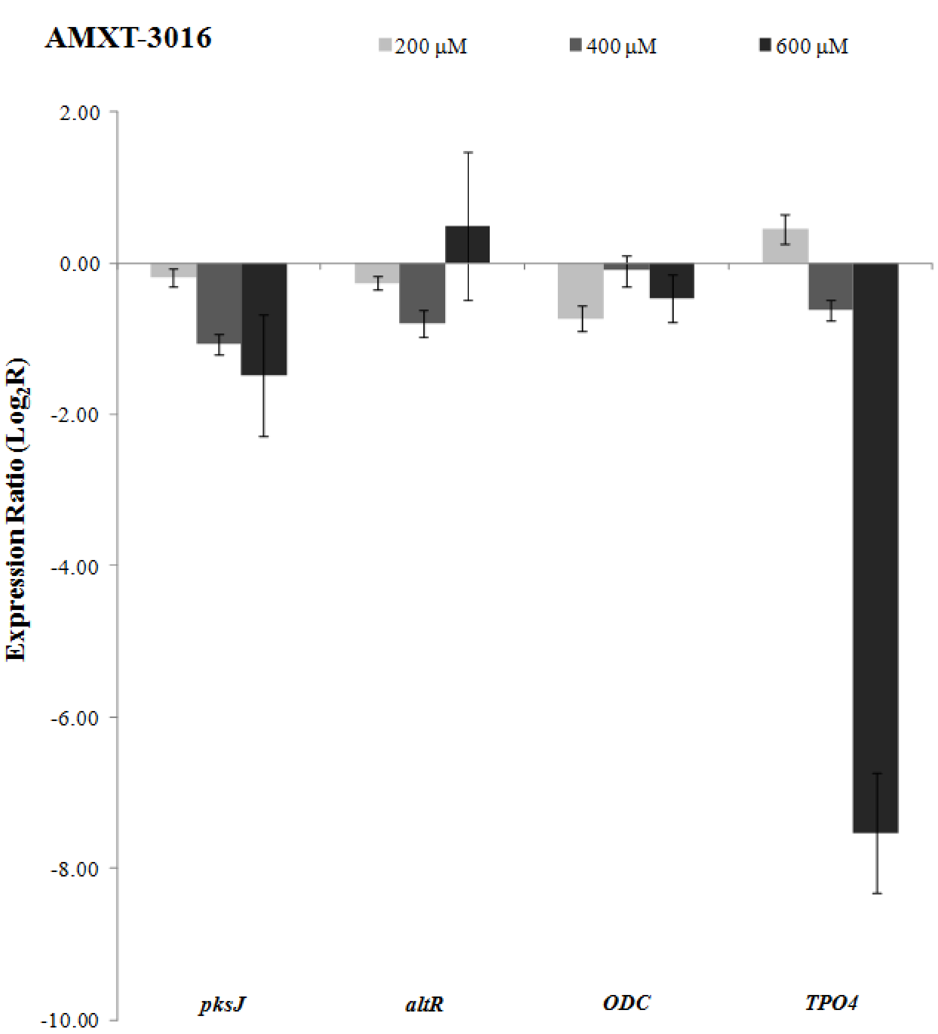

Figure 9. Relative expression of *pksJ*, *altR*, *ODC*, and *TPO4* in *A. alternata* in the presence of AMXT-3016. Bars represent gene expression ratio on a $\log_2$ scale compared to the control (grown on the same medium but without AMXT-3016). Error bars indicate standard errors.

Results shown in Fig. 8 and Fig. 9 suggest a similar pattern for both polyamine analogs. While no effect seemed to be observable when the concentration was 200 µM, when higher concentrations were applied, the *pksJ* gene expression decreased

gradually. With regard to *altR* gene expression, only AMXT-2455 at a concentration of 800 μM seemed to be enough to downregulate it. No appreciable differences compared to the control were observed regarding the *ODC* gene expression neither with AMXT-2455 nor AMXT-3016. However, both polyamine analogs seemed to have a strong effect on *TPO4*. A concentration of 200 μM seemed to lightly upregulate gene expression for 2.50 fold change in the case of AMXT-2455 and 1.38 for AMXT-3016. Nevertheless, when polyamine analogs concentrations were higher, *TPO4* downregulated its expression, reaching a fold change of 1176.27 when concentration of AMXT-2455 was 800 μM and a fold change of 184.82 when we tested 600 μM of AMXT-3016.

3.4 Ornithine decarboxylase inhibition and polyamine transport inhibition of *A. alternata* infecting tomato fruit and tomato plants

Besides *in vitro* studying how DFMO affected *A. alternata*, we also tested the effect of DFMO *ex vivo* when conidia were artificially inoculated on tomatoes. In this assay, we assessed mycotoxin production and diameter of infection and results were quite similar compared to the control tomatoes. As shown in Fig. 10, *A. alternata* had the ability to infect tomatoes even if they had been treated with 5 mM of DFMO.

We also tested AMXT-2455 and AMXT-3016 on tomato fruits and on plants in order to assess their effectiveness *ex vivo* and *in vivo* (Fig. 10). We observed that both PTIs were more efficient when we tested them on plants. While the group of control plants was severe spoiled with black spots caused by the *A. alternata* infection, the plants that had been treated with AMXT-2455 and AMXT-3016 had fewer spots though both treatments failed to completely repress *A. alternata* growth. Contrariwise, tomatoes dipped into polyamine analogs solutions did not reduce the fungal growth efficiently as no significant differences were found among infected controls and tomatoes dipped into the polyamine analogs solutions. The worst situation comes with tomatoes dipped into AMXT-2455 as, besides not

reducing the fungal growth, mycotoxin production increases after the treatment with the polyamine analog.

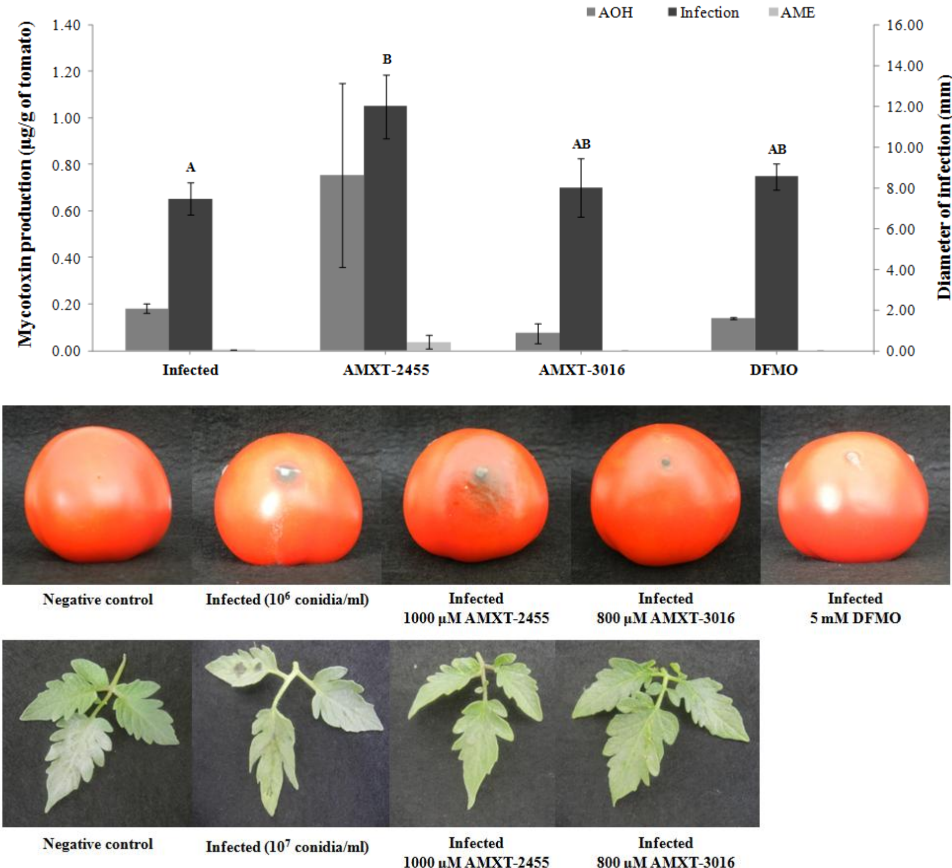

Figure 10. Effectiveness of AMXT-2455 (1000 µM), AMXT-3016 (800 µM) and DFMO (5 mM) on tomato fruit and tomato plants. Mycotoxin production on tomato fruit (left y-axis). Diameter of infection on tomato fruits (right y-axis). Error bars indicate standard errors. Capital letters indicate homogeneous groups for infection. No significant differences existed for AOH or AME groups. All statistical data was analyzed by one-way ANOVA ($p < 0.05$). Tukey-HSD test was used to compare means.

4. Discussion

It is well known that polyamines are essential metabolites present in all living organisms. In fungi, polyamines support growth and regulate several biological processes, some of which are still unknown (Tabor and Tabor, 1983). Due to its indispensable role in fungi, polyamine metabolism of phytopathogenic fungi has attracted the attention of researchers who have found in it a potential strategy to design new targets to control the problem derived from fungicide resistance.

One of the first strategies often used to manipulate and control polyamine metabolism is by inhibiting the polyamine biosynthesis pathway, specifically the activity of the ODC, the rate-limiting enzyme in this metabolic route. There are different approaches used to inhibit ODC (Bey et al., 1987). In this study we have used the DFMO, a drug that inhibits the ODC irreversibly. Although DFMO was originally studied in animals, it has also been used in fungi to better understand the polyamine metabolism. The use of DFMO is interesting as it targets a key enzyme specific for the pathogen, whereas the plant is not altered as it uses an alternative pathway of polyamine biosynthesis. The findings in this study support that ODC inhibition strongly limits fungal growth and mycotoxin production on *A. alternata*. This behavior was also observed by the use of DFMO on other pathogens such as *F. graminearum*, *Sclerotinia scletoriorum, Colletotrichum truncatum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Monilinia fructicola*, *Fusarium oxysporum*, C*ochliobolus carbonum*, *Phytophtora infestans* and, *Penicillium citrinum* (Barker et al., 1993; Crespo-Sempere et al., 2015a; Gamarnik et al., 1994; Giridhar et al., 1997; Pieckenstain et al., 2001; Rajam and Galston, 1985; Walters, 1995; West and Walters, 1989). In all these fungi, mycelial growth was overcome by addition of putrescine in the media, though in *B. cinerea* and *M. fructicola* putrescine complementation resulted in an increase in the mycelial growth above the control values. In other studies using other ODC inhibitors, similar results were found. In this context, *Aspergillus nidulans*, *Aspergillus parasiticus* or *Pyrenophora avenae*

reduced their mycelial growth when the ODC inhibitor was added to the media. Additionally, sterigmatocystin and aflatoxin biosynthesis was almost inhibited in *A. nidulans* and *A. parasiticus,* respectively (Guzmán-de-Peña et al., 1998; Guzmán-de-Peña and Ruiz-Herrera, 1997; Mackintosh and Walters, 1997). However, in this case sterigmatocystin and aflatoxin production was reverted by addition of putrescine in the media, in contrast to what we have observed on *A. alternata,* in which putrescine has reverted mycelial growth but not AOH production. Kępczyńska (1994) studied the effect of inhibiting spermidine synthesis using methyl bis-(guanylhydrazone) in *Alternaria consortiale* and they also observed reduction of mycelial growth which was not only restored with the addition of spermidine, but increased compared with the control. Thus, results show that the effect of ODC inhibitors is dependent upon the particular fungus. Birecka et al. (1986) suggested that genus-dependent differences in sensitivity to inhibitors may be due to differences in uptake of the inhibitor, ODC sensitivity and polyamine requirements. Despite these differences, it is clear that in *A. alternata* polyamines are essential for growth and for mycotoxin production and also that the ODC enzyme plays a determinant role in the polyamine biosynthesis pathway. Inhibition of this enzyme, may alter the levels of polyamines in the fungi that ultimately leads to changes in its physiology and growth (Rajam and Galston, 1985; Valdés-Santiago et al., 2012).

When DFMO was tested on *A. alternata* and gene expression patterns were analyzed, a significant downregulation of *TPO4* was observed, a gene that encodes a polyamine transport protein that excretes putrescine, spedermine, and spermine. This downregulation could be explained by the fact that inhibition of ODC by DFMO decreases global polyamine concentration in the cell. Decreased polyamines levels in the cell require less export activity by TPO4 is required. It was also interesting to find colonies grown on DFMO and supplemented with putrescine neither produced AOH nor AME, even though *pksJ* and *altR* levels were similar to the control values. In *F. graminearum*, *A. parasiticus* and, *A. nidulans* it

has been described that even ODC inhibitors repress mycotoxin production, this affectation is reverted, completely or partially, by the addition of putrescine in the media (Crespo-Sempere et al., 2015a; Guzmán-de-Peña et al., 1998; Guzmán-de-Peña and Ruiz-Herrera, 1997). However, we have not observed this reversion in *A. alternata.* Thus, two scenarios were considered. Firstly, DFMO also targets other routes of the secondary metabolism, such as mycotoxins. Secondly, putrescine itself negatively affects mycotoxin production. To investigate, different concentrations of putrescine were added to the media and we found that increasing concentrations of putrescine led to decreasing levels of AOH production and sporulation and, in a lower proportion, decreasing of the mycelium growth (Fig. 3). Conversely, in a previous study with *F. graminearum* it was observed that addition of exogenous putrescine increased mycotoxin production (Crespo-Sempere et al., 2015a). In this sense, Gardiner et al. (2009; 2010) suggested that deoxynivalenol production was strongly induced in liquid culture by various amine compounds which included putrescine or amino acids such as arginine. These contradictory results might be explained by the fact that fungi respond to changes in nitrogen availability affecting the formation of secondary metabolites (Tudzynski, 2014). Brzonkalik et al. (2011) analyzed the influence of carbon and nitrogen sources on *A. alternata* and found that arginine, which is a precursor of putrescine, was a nitrogen source that inhibited AOH and AME production. Their results were in accordance to Overhed et al. (1988), who tested the effect of sodium nitrate, glutamate and urea on AOH and AME production in *A. alternata* strains. Both studies concluded that mycotoxin production dramatically decreased when high concentrations of these nitrogen sources were added to the media. Brzonkalik et al. (2011) hypothesized that nitrate repression could be the cause of inhibition of AOH and AME by some of the nitrogen sources tested. This has been previously described for aflatoxin intermediates in *A. parasiticus* (Kashiwagi and Igarashi, 2011) or for ochratoxin in *Aspergillus ochraceus* (Abbas et al., 2009). The fungi can utilize a diverse array of compounds as nitrogen sources, although ammonium

and glutamine are preferentially used over other sources. Nevertheless, during conditions of nitrogen limitation, fungi can utilize other nitrogen supplies less easily assimilable, such as nitrate, nitrite, purines, amides, most amino acids and proteins (Marzluf, 1997). All this regulation is controlled by global regulators that control the expression of the genes for nitrogen utilization, *areA* in *A. nidulans* and *nit-2* in *Neurospora crassa* (Caddick et al., 1986; Fu and Marzluf, 1990; Kudla et al., 1990). In *A. nidulans*, when the primary nitrogen sources are not present in the media or in limiting concentration, *areA* is activated and binds to GATA specific sequences in the promoter regions. Interestingly, many of the genes that belong to mycotoxin biosynthesis clusters have GATA sequences in their promoter as well. Union of AreA to these regions carries out the blockage of mycotoxin production (Caddick et al., 1994; Fu and Marzluf, 1990; Marzluf, 1997; Tudzynski, 2014; Wilson and Arst, 1998; Woloshuk and Shim, 2013). All this may support the notion that in *A. alternata*, when putrescine is added to the media, the nitrate repression system could be activated and *areA* may participate in the nitrate-mediated negative regulation of gene transcription of AOH and AME biosynthesis. Gene expression analysis of genes involved in AOH and AME mycotoxin carried out in this study may support this hypothesis as when putrescine concentration in the media was high (1000 and 5000 μM) *pksJ* and *altR* genes showed a downregulation, which was more remarkable when the concentration was 5000 μM. However, further studies may be performed for a better understanding of nitrogen source regulation of mycotoxin production.

DFMO was originally used as a chemotherapeutic agent to interrupt cellular metabolic processes in cancer therapy. Despite its success inhibiting ODC activity, it did not achieve the goal of repressing cell growth because cell lines grown in culture could overcome the blockage of the ODC enzyme by importing polyamines from extracellular sources. This current idea was corroborated when we tested DFMO on tomatoes as we observed that *A. alternata* was able to grow similarly to the control group of samples, in which no treatment was assessed. Hence, even if

fungal cells have the polyamine biosynthesis pathway blocked, they can uptake the polyamines needed to survive from the tomato. With the aim to definitely repress cellular tumor growth, Burns *et al*. (2001; 2009) designed a group of lipophilic polyamine analogs, the polyamine analogs, which potently inhibit the cellular polyamine transport. Additionally, other polyamine analogs were characterized as antizyme inducing agents, including AMXT-3016 used in our studies. Thus, growth inhibitory effects of DFMO in combination of these polyamine analogs resulted in a tumor growth inhibition. In a previous study with *F. graminearum* (Crespo-Sempere et al., 2015a), it was observed that some of the polyamine analogs developed by Aminex Therapeutics (USA) did efficiently control mycelial growth in wheat spikes. In this context, *in vitro* tests were assessed with seven different polyamine analogs on *A. alternata* and it was observed that two of them achieved the goal of controlling *A. alternata* growth, sporulation and mycotoxin production at the same time: AMXT-2455 and AMXT-3016. Surprisingly, the polyamine analog that had been efficient for *F. graminearum* did not work for *A. alternata* (AMXT-1505). Genetic expression analysis of the mycelia corroborated that both polyamine analogs downregulated *pksJ* gene expression proportionally to the polyamine analog concentration. Both polyamine analogs were tested on tomatoes and tomato plants. Results suggested that, especially when performing treatments on tomatoes, the concentration necessary to avoid fungal growth, should had been higher. We observed that, on tomatoes, polyamine analogs failed to control *A. alternata* growth efficiently, probably, because the surface was treated with the polyamine analog but its penetration was insufficient to control the fungal development inside the fruit. So, the fungi penetrated inside the fruit and continued the infection. However, when the treatment was performed on tomato plants, the results were more successful, as both polyamine analogs achieved a reduction of the fungal infection compared to the control group of samples. Nevertheless, there were some leaves treated with polyamine analogs in which there were still some dark spots caused by *A. alternata* growth. Hence, in this case concentration

required should have been higher to achieve a complete growth repression. The possibility that polyamine analogues might be fungicidal has been the focus of interest of different researchers. In this sense, Foster & Walters (1993) showed that keto-putrescine provided substantial control of infections by six economically important plant pathogens. However, it was relatively less effective *in vitro* against *Phytophthora infestans*, *Pyricularia oryrae* and, *Pyrenophora avenae*. They also examined the fungicidal activity of N-acetylputrescine and failed to find any effect on fungal growth *in vitro* or on plant infection. Mackintosh & Walters (1997) tested six novel spermidine analogues against the oat stripe pathogen *P. avenae* and they reported that two of these analogues, N,N-dimethyl-N1-(3-aminopropyl)-1,3-diaminopropane trihydrochloride and N,N-dimethyl-N1-(3-aminopropyl)-1,4-diaminobutane trihydrochloride fairly inhibited fungal growth. Gàrriz et al. (2003) evaluated the effect of 1-aminooxy-3-aminopropane on polyamine metabolism in the phytopathogenic fungus *Sclerotinia sclerotiorum* and predicted that its ability to control plant diseases would probably be poor. However, three tri-substituted spermidines, di-p-coumaroyl-caffeoylspermidine, tri-caffeoylspermidine and tri-p-coumaroylspermidine, isolated from pollen of *Quercus alba*, were examined for antifungal activity against *P. avenae* and two of them successfully reduced mycelial growth of the oat leaf stripe pathogen (Walters et al., 2001). A mixture of diferuloylputrescine/p-coumaroylferuloylputrescine also demonstrated inhibitory activity against aflatoxin B_1 biosynthesis in *Aspergillus flavus*, although this diconjugated polyamine mixture did not display inhibitory effects on *A. flavus* growth (Mellon and Moreau, 2004).

Results with polyamine analogues may be quite promising for the control of different diseases caused by several kind of fungus on fields and crops. Nevertheless, more information is needed to pinpoint the mode of action of the polyamine analogues.

5. Conclusions

Inhibition of the polyamine biosynthesis pathway using DFMO decreases AOH production and fungal growth. Hence, polyamines might play some essential role in both biological processes. Addition of exogenous putrescine on the media reverts fungal growth but not mycotoxin production. Putrescine as a nitrogen source may affect several essential processes of the cell such as sporulation, growth rate and mycotoxin production. Some polyamine transport inhibitors seem to control fungal growth and mycotoxin production *in vitro* and promising results have been observed *in vivo.* Based on these findings, it is worthwhile to continue investigating in polyamine metabolism as a new target to control *A. alternata* diseases in plants to overcome problems derived from fungicide resistance.

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Chapter V

Effect of 1-methylcyclopropene on the development of black mold disease and its potential effect on alternariol and alternariol monomethyl ether biosynthesis on tomatoes infected with *Alternaria alternata*

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Effect of 1-methylcyclopropene on the development of black mold disease and its potential effect on alternariol and alternariol monomethyl ether biosynthesis on tomatoes infected with *Alternaria alternata*

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Abstract

Ethylene is a naturally produced plant regulator involved in several plant functions, such as regulation of fruit ripening. Inhibition of ethylene perception by using 1-methylcyclopropene (1-MCP) slows down the ripening of the fruit maintaining its quality and freshness. The use of 1-MCP is a commercial strategy commonly used in the food industry to extend the postharvest life of several fruits, including tomatoes. To assess how 1-MCP affected infection by *Alternaria alternata* on tomatoes, three different cultivars were artificially inoculated with 5 μL of an *A. alternata* conidial suspension (10^5 conidia/mL). Tomatoes were treated with 0.6 μL/L of 1-MCP for 24 hours. Spiked but untreated tomatoes were considered controls. Then, fruit were stored 6 days at 10 ºC and one more week at 20 ºC to simulate shelf-life. Fungal growth development and mycotoxin production

(alternariol, AOH and alternariol monomethyl ether, AME) were assessed both on the first and on the second week. After the first 6 days at 10 ºC, in just one variety the black mold disease was higher in the 1-MCP treated samples. However, after two weeks of storage, in all cases, tomatoes treated with 1-MCP showed more significant fungal growth disease. Regarding mycotoxin production, no large differences were observed among different treatments, which was corroborated with gene expression analysis of *pksJ*, a gene related to AOH and AME biosynthesis.

Keywords: *Alternaria*; AOH; AME; tomatoes; 1-methylcyclopropene

1. Introduction

Alternaria spp. are widely distributed in the soil and also occur ubiquitously in the air. Many species are plant pathogens that damage leaves, stems, flowers, and fruit crops in the field or cause postharvest decay of various fruits, grains and vegetables, which lead to important economic and material losses to the food industry and growers. Due to their ability for growing even at low temperatures, *Alternaria* spp. are also responsible for spoilage of commodities during refrigerated transport and storage (Barkai-Golan, 2008). *Alternaria* is also a matter of concern due to its ability to produce more than 70 secondary metabolites which are toxic to plants. A small proportion of these phytotoxins have been characterized and reported to act as mycotoxins to humans and animals. Alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid, altenuene and altertoxin-I are the main *Alternaria* mycotoxins produced in fruits and vegetables (Barkai-Golan and Paster, 2008b; Bottalico and Logrieco, 1992; Ostry, 2008). In the latest years some research has been done regarding the biosynthesis pathway of AOH and AME. Even though more research is needed, studies developed until now suggest that AOH could be formed by the polyketide route. In this sense,

some polyketide synthases (PKS) have been identified in an *Alternaria alternata* genome to be involved in the biosynthesis of AOH and AME but one of them has been claimed to be the responsible of catalyzing the first steps of the biosynthesis of AOH and AME, the PksJ (Saha et al., 2012).

Among all *Alternaria* spp., *A. alternata* has been regarded as the most important mycotoxin-producing species. Additionally, *A. alternata* is a causal agent of the black mold rot on tomato fruit, a disease frequently causing substantial damage in field and leading to postharvest losses (Logrieco et al., 2009; Reddy et al., 2000; Visconti et al., 1987). In 2011, the European Food Safety Authority (EFSA) emitted a scientific opinion on the risks for animal and public health related to *Alternaria* toxins present in feed and food. The EFSA concluded that more information was needed to fill the knowledge gaps.

Ethylene is a naturally produced plant growth regulator that has numerous effects on the growth, life development and storage of many ornamental crops, vegetables and fruits. Additionally, ethylene production plays an important role in the regulation, initiation and completion of ripening of all climacteric fruits, including tomatoes (Alexander and Grierson, 2002; Saltveit, 1999). Control of tomato ripening is one of the main goals of producers and traders in tomato marketing since accelerated ripening after harvesting leads to decay development. In this regard, the use of technologies that minimize or inhibit ethylene action enable to extend postharvest life of tomatoes, which is a concern during transportation and commercialization. The use of 1-methylcyclopropene (1-MCP) is an already commercial strategy used to inhibit ethylene production and hence to control the ripening. 1-MCP interacts with ethylene receptors and thereby prevents ethylene-dependent responses (Blankenship and Dole, 2003; Sisler and Blankenship, 1996; Sisler and Serek, 1997). Furthermore, 1-MCP has a non-toxic mode of action, negligible residue and it is active at very low concentrations (Watkins, 2006).

How ethylene and 1-MCP affects physiology and quality of fruits has been broadly studied. Nevertheless, scarce literature considers the effects of ethylene and 1-MCP on fruit pathogens. In this sense, it has been described that ethylene, besides being an essential modulator of several aspects of plant life and fruit ripening, also plays a major role in regulating plant defense responses against abiotic and biotic stresses, such as pathogen attacks (Broekaert et al., 2006; Lund et al., 1998). The 1-MCP has been described to have different effects on pathogens affecting several fruits such as natural pathogens infecting strawberries (Jiang et al., 2001; Ku et al., 1999), *Colletotrichum acutatum* and *Penicillium expansum* affecting apples (Janisiewicz et al., 2003) or *Botrytis cinerea* on pear fruit (Akagi and Stotz, 2007), among others (Biswas et al., 2014; Jing and Zi-sheng, 2011; Mullins et al., 2000; Porat et al., 1999; Su and Gubler, 2012; Zhou et al., 2006). A study on the effect of 1-MCP on the black spot disease, which is usually produced by *Alternaria* spp., found that 1-MCP stimulated the disease in Japanese pear (Itai et al., 2012). However, there is scarce information about how 1-MCP affects *A. alternata* producing black mold rot on tomatoes. Hence, in this work, growth development of *A. alternata* and AOH and AME production has been assessed both *in vitro* and on three different tomato varieties. Additionally, gene expression of a polyketide synthase involved in AOH and AME biosynthesis, *pksJ*, has been analyzed. This work aims to better understand how 1-MCP can affect infection by *A. alternata* on tomatoes.

2. Material and methods

2.1 Fungal strain growth conditions

The *A. alternata* strain used in this study was the CBS 116.329 (isolated from apple), provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). To prepare conidial suspensions, *A. alternata* was routinely grown

on Potato Dextrose Agar plates (PDA, Biokar Diagnostics, France) and incubated 7 days in the dark at 26 ºC. Conidia were collected with a scalpel within a sterile solution of phosphate-buffered saline (PBS) with 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, The Netherlands) and filtered through Miracloth (Calbiochem, USA). Conidial suspension concentration was adjusted to 10^5 conidia/mL using a Thoma counting chamber.

2.2 Fruit source

For this experiment three different tomato (*Solanum lycopersicum*) varieties, namely *palladium*, *caniles* and *egara* were used. The first two varieties were supplied by Bonnysa Agroalimentaria, a commercial orchard in Alicante (Spain) and the third one was purchased from a local shipper in Fraga (Spain). All tomato fruit were received during the turning stage, according to the USDA tomato ripeness color classification chart, and they were stored at 5 ºC until they turned to pink or light red stage. None of the tomatoes were previously treated with 1-MCP or ethylene before. Fruit were selected for uniformity in size, ripeness and absence of physical defects or apparent infections.

2.2.1 Quality parameters analysis of tomatoes

Before inoculation, quality analyses of twenty tomatoes of each variety were assessed for weight, caliber, skin color, flesh firmness, soluble solid content (SSC) and titratable acidity (TA). The caliber was determined using a digital caliper (Limit, Sweden). The surface color was measured with a CR-200 Minolta Chroma Meter (Minolta, INC, Japan). The instrument was calibrated using a standard reflector plate. Color was measured using CIE L*, a*, b* coordinates. Two readings were made in each fruit on two equatorial opposite sites. Flesh firmness was measured with a penetrometer (FT 327, Facchini srl, Italy) using a plunger of 8 mm. Before the measurement, skin was removed. Measures were made along the fruit's equatorial diameter, halfway between the steam scar and the blossom end. Results were expressed as kg/cm^2. For SSC and TA, tomatoes were squeezed. The

SSC was determined in the resulting juice with a handheld refractometer (Atago CO., LTD, Japan) and results were expressed as ºBrix. The TA of tomatoes was measured with an acid-base titration using a 0.1 N solution of sodium hydroxide as a titrant and phenolphthalein as an indicator. Ten mL of tomato juice were diluted with 10 mL of deionized water and titrated to pH 8.1. Results were expressed as g of citric acid per liter of solution.

2.3 1-MCP treatment

The 1-MCP treatment was applied with the SmartFresh™ Technology. Tomatoes that had to be treated with 1-MCP and those that had to be untreated were separated. To perform the 1-MCP treatment, samples were placed in a hermetic plastic bag of 1 m^3. 1-MCP was applied at a concentration of 0.6 µL/L, which is the commercial dose recommended. Close to the volatile product there was a fan to homogenize the atmosphere. Samples were kept inside the plastic bag for 24 hours at 10 ºC. After this period, tomatoes were stored according to the conditions indicated in section 2.5. The efficacy of 1-MCP treatment was assessed by comparison of 20 1-MCP treated tomatoes against 20 different untreated tomatoes. After 24 hours, treated and untreated tomatoes were kept for 4 days in a postharvest chamber at 20 ºC. After this period, the color of the skin of the tomatoes treated with MCP-1 was compared with the color of the skin of the untreated tomatoes with the aim of verifying that treatment with 1-MCP had worked properly.

2.4 Assessment of 1-MCP impact on *A. alternata in vitro*

For assessing how the 1-MCP treatment affected *in vitro* cultures of *Alternaria*, 5 µL of the fungal conidial suspension were placed in the centre of twelve different PDA plates. Six plates were considered control samples, so, they were not treated with 1-MCP. The other six plates were placed inside a plastic bag of 1 m^3 and the 1-MCP treatment was carried out as described in section 2.3. After the 1-MCP

treatment, all plates were stored for 6 more days at 10 ºC. After this period, three control plates and three plates treated with 1-MCP were taken to the laboratory where diameter of growth and extraction of AOH and AME from culture was assessed as described in section 2.6. Additionally, the gene expression of *pksJ*, a gene involved in the AOH and AME biosynthetic pathway (see section 2.8) was analyzed. The other plates were stored for 7 more days in a postharvest chamber at 20 ºC. After this period, all plates were taken to the laboratory, where diameter of growth, occurrence of AOH and AME and *pksJ* gene expression were assessed again.

2.5 Inoculation of fruits and storage conditions

Tomato fruit were previously surface disinfected with 10% of sodium hypochlorite for 1 minute, rinsed with tap water for 10 minutes and dried at room temperature. Prior to inoculation, tomatoes were four-times injured on the equatorial section with a sterilized awl. Inoculation was performed placing 5 µL of the conidial suspension in each wound. Negative control tomatoes were also injured but no conidial suspension was added. Tomatoes were packaged into plastic bags and stored in the postharvest chambers of the Institute of Agrifood Research and Technology (IRTA), who kindly offered us their chambers. Inside the plastic bags, the RH reached the 100%. Temperature and RH were recorded hourly by a data logger (Escort iLog RH, Portugal). There were eight different treatments, which are described in Table 1. Each treatment was contained in a wooden pallet, so one pallet was equivalent to an entire treatment. Each pallet contained 20 tomatoes. As described in Table 1, inoculation of tomatoes with the conidial suspension was done in 4 of the 8 treatments (treatment 2, 4, 6 and 8). Negative controls for the natural contamination with *Alternaria* were considered not inoculated tomatoes (treatment 1, 3, 5 and 7). From the whole treatments, just 4 treatments were treated with 1-MCP (treatment 3, 4, 7 and 8). The other treatments were not treated with 1-MCP (treatment 1, 2, 5 and 6) but they were placed in the same chamber where

treated samples were contained in the plastic bag with the 1-MCP atmosphere (see Table 1).

Table 1. Description of the treatments assayed. The 1-MCP treatment (0.6 μL/L) was assessed during 24 h at room temperature.

	Inoculated	**1-MCP**	**10 ºC (6 days)**	**20 ºC (7 days)**
Treatment 1	-	-	+	-
Treatment 2	+	-	+	-
Treatment 3	-	+	+	-
Treatment 4	+	+	+	-
Treatment 5	-	-	+	+
Treatment 6	+	-	+	+
Treatment 7	-	+	+	+
Treatment 8	+	+	+	+

After the 1-MCP treatment at 10 ºC, all sets of samples were incubated 6 more days into a postharvest chamber at 10 ºC. After these 6 days of storage, treatment 1 to 4 were taken to the laboratory where it was assessed the diameter of growth and the mycotoxin extraction from tomatoes as described in section 2.7. Additionally, gene expression of *pksJ* was also analyzed. The rest of treatments were incubated 7 more days at 20 ºC to simulate shelf-life of tomatoes. After this period, all pallets were brought to the laboratory, where the same analysis assessed for the first four treatments were carried out. For the whole assay, five tomatoes were considered a single replicate and the assay was performed in quadruplicate.

2.6 Fungal growth and extraction of AOH and AME from culture

Mycotoxin quantification and diameter of growth were assessed twice. The first assessment was done after one week of incubation in a postharvest chamber at 10 ºC and, the second assessment was done after a further week at 20 ºC to simulate

shelf-life conditions of tomatoes. Fungal growth of *Alternaria,* inoculated on PDA plates, was determined by measuring two perpendicular diameters of the growing colony. To assess mycotoxin production (AOH and AME) from fungal cultures, one agar plug (5 mm in diameter) was removed from the center of the colonies and it was placed into an eppendorf containing 500 µL of acetonitrile-methanol-water (45:10:45 v/v/v), adjusted to pH 3 with *o*-phosphoric acid. Eppendorfs containing the plugs were shaken for 10 minutes with a vortex shaker (Mo Bio Laboratories, Inc. USA). To favor mycotoxin extraction, samples were left at room temperature for 50 more minutes. Samples were filtered (Millex-HV 0.45 µm, 25 mm, Millipore Corporation, USA) into another vial and mycotoxin extracts were dried in a speed vacuum concentrator at room temperature. Samples were stored at -20 °C until the HPLC analysis. Prior to analysis, the extracts were resuspended in 500 µL of a water-methanol solution (50:50 v/v). Separation, detection and quantification of AOH and AME was performed with a HPLC system consisting of a Waters 2695 Alliance Separations Module connected to a UV/Visible dual λ absorbance Detector Waters 2487, using a reversed phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a Spherisorb guard column (5 µm ODS2, 4.6 x 10 mm, Waters, Millford, MA, USA). Columns were set at a temperature of 35 ºC. For chromatographic separation of AOH and AME the flow rate was 0.5 mL/min and the injection volume was 100 µL. Absorption wavelength was 258 nm. The mobile phase was double distilled miliQ water (MiliQ Academic Millipore, USA) and methanol-water (70:30 v/v) according to the gradient described in Table 2. The retention times were 24 minutes for AOH and 32 minutes for AME. For mycotoxin quantification, working standards were used to perform a ten-point calibration curve for both mycotoxins (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). For *in vitro* assays, the limit of detection (LOD) for AOH and AME were 0.02 ng/mm^2 and 0.034 ng/mm^2, respectively. The LOD was based on a signal-to-noise ratio of 3:1. The

limit of quantification (LOQ) was calculated as 3 × LOD. All solvents were HPLC grade and all chemicals were analytical grade.

Table 2. HPLC gradient used to detect and quantify AOH and AME.

Time (min)	Water	Methanol-water (70:30 v/v)
	100	-
7	100	-
9	20	80
10	20	80
12	-	100
41	-	100
43	10	90
45	30	70
46	50	50
47	70	30
50	100	-

2.7 Diameter of infection and AOH and AME analysis on tomatoes

The diameter of black mold rot on tomatoes was measured as the lesion size performed on the equatorial section of tomatoes. Before performing mycotoxin extraction, AOH and AME free tomatoes were artificially spiked with AOH and AME to assess recovery and repeatability data. Recovery and repeatability results are listed in Table 3. For mycotoxin production assessment, plugs of 13 mm of diameter and 5 mm of thickness were removed where there was the fungal infection. Three plugs were taken from each tomato. All the plugs from the same replicate were put into a stomacher bag. Mycotoxin extraction was proceed by adding 30 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric acid) and homogenizing for 2 minutes with the stomacher. The mixture was stirred for 15 extra minutes in a uniform magnetic field. The solution

was left for 10 minutes, approximately, to favor precipitation by gravity. Then, 6 mL of the supernatant were transferred to a centrifuge tube and diluted with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3) and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract were passed by gravity through a previously conditioned Bond Elut Plexa SPE cartridge (200 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the cartridge was done with 5 mL of methanol following 5 mL of miliQ water. The SPE column was washed with 5 mL of water followed by air drying on the manifold. Finally, elution was carried out with 5 mL of methanol and 5 mL of acetonitrile. Sample extracts were dried under nitrogen flow and stored at -20 ºC until HPLC analysis. Conditions for HPLC determination were the same as described in section 2.6. For AOH and AME analysis assessed on tomatoes, the LOD for AOH and AME were 0.009 µg/g tomato and 0.012 µg/g of tomato, respectively.

Table 3. Recovery and repeatability of AOH and AME analysis on tomatoes.

	AOH			AME		
Spiking level (µg/g tomato)	**n**	**Recovery [a] (%)**	**RSDr [b] (%)**	**n**	**Recovery (%)**	**RSDr (%)**
0.5	3	115.5 ± 2.1	1.8	3	106.0 ± 8.2	7.7
1.0	5	99.0 ± 2.4	2.4	5	93.8 ± 3.1	3.4
2.0	3	79.8 ± 3.2	4.0	3	78.0 ± 3.6	4.6

[a] Mean value ± standard deviation.
[b] RSDr = relative standard deviation.

2.8 Gene expression analysis

Gene expression analysis from *A. alternata* inoculated on tomatoes and on PDA plates was performed in order to study the effect of 1-MCP on mycotoxin biosynthesis. For RNA extraction from inoculated tomatoes, plugs of 13 mm of diameter and 5 mm of thickness were collected from each inoculation point. One

sample was considered a pull of 5 tomato plugs. Mycelium grown on PDA plates was collected with the help of a scalpel. Both type of samples were frozen in liquid nitrogen and ground to a fine powder with a mortar and a pestle with liquid nitrogen. Samples were stored at -80 °C before nucleic acid extraction. RNA was extracted from 1 g of pulverized sample, which was added to a pre-heated (65 ºC) mixture of 10 mL of extraction buffer: 100 mM Tris–HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% (v/v) β-mercaptoethanol and 5 mL of Tris-equilibrated phenol. The extract was incubated at 65 °C for 15 min and cooled before adding 5 mL of chloroform-isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 *x g* for 20 min at 4 °C, and the aqueous phase was re-extracted with 10 mL of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated during three hours at -20 ºC by adding 3.3 mL of 12 M LiCl. After centrifugation at 27200 *x g* for 60 min, the pellet was washed with 500 µL of 70% ethanol. The resultant pellet was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides. Then, RNA was washed again with 500 µL of 70% ethanol, and, finally, dissolved in 100 µL of water. RNA concentration was spectrophotometrically measured and verified by ethidium-bromide staining of an agarose gel. Total RNA was treated with DNase (TURBO DNase, Ambion, USA) to remove contaminating genomic DNA. Single-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instructions (Invitrogen, USA).

We designed a primer pair, pksJ_F (5'-ACACTAGCACAGTCGGTTCCCA-3') and pksJ_R (5'-ATTGGCCGCGTACTACCCAG-3'), to study the gene expression of a polyketide synthase, PksJ (Transcript ID AAT_PG02879), which is claimed to have an essential role in AOH and AME biosynthesis (Saha et al., 2012). The primer pair A-BTF (5'- ACAACTTCGTCTTCGGCCAGT-3') and A-BTR (5'-ACCCTTTGCCCAGTTGTTACCAG-3') was designed within the beta-tubulin gene (Transcript ID AAT_PG05035), which was chosen as reference gene. Gene

expression analyses were assessed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA). qPCR reactions were performed in a final volume of 10 µL, containing 1X of SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA), 250 nM of each primer and 1 µL of template DNA. The standard protocol included one cycle at 98 °C for 2 min, followed by 35 cycles at 98 °C for 5 s and 58 °C for 30 s. Reactions were done in duplicate for each sample, checking the PCR reaction quality by analyzing the dissociation and amplification curves. The corresponding qPCR efficiency (E) in the exponential phase was calculated according to the equation: E = 10[-1/slope]. The relative expression of the target genes was calculated based on the E and the Crossing point (Cp) value of the sample versus the control. The Cp value is the cycle at which fluorescence from amplification exceeds the background fluorescence. The relative expression of the target genes was expressed in comparison to the beta-tubulin gene (reference gene), according to the following equation (Pfaffl, 2001; Rasmussen, 2001a). Gene expression measures were derived from biological triplicates.

$$R = \frac{(E_{target\ gene})^{\Delta Cp\ target\ gene\ (Mean\ untreated\ samples - Mean\ treated\ samples)}}{(E_{reference\ gene})^{\Delta Cp\ reference\ gene\ (Mean\ untreated\ samples - Mean\ treated\ samples)}}$$

2.9 Statistical analysis

All statistical data were analyzed using the One Way ANOVA test ($p < 0.05$). Tukey-HSD test (Honest Significant Difference) was used to compare means. All statistical analyses were performed with Statgraphics Centurion Version XVI.

3. Results

3.1 Effect of 1-MCP on *A. alternata* cultures *in vitro*

To examine the effect of 1-MCP on *A. alternata*, an *in vitro* assay was assessed before testing 1-MCP on tomato fruit. Dealing with diameter of growth, no

differences were observed on cultures grown for just 6 days at 10 ºC. In contrast, cultures that had been incubated one more week at 20 ºC showed significant differences. *A. alternata* that had been treated with 1-MCP grew an average of 4.7 mm more than cultures that had not been treated (Fig. 1).

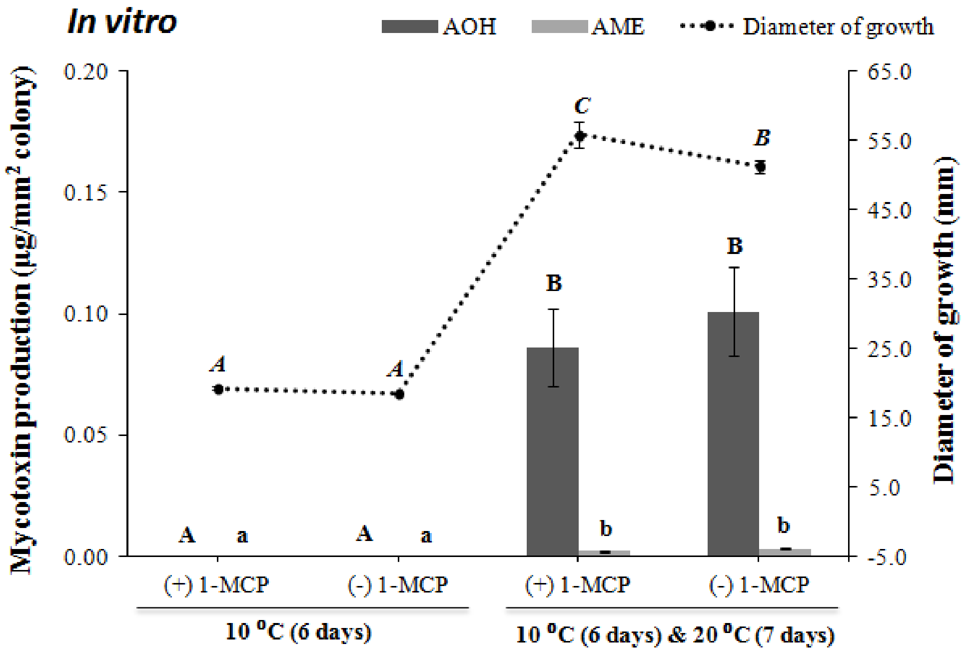

Figure 1. Effect of 1-MCP on *A. alternata* cultures grown on PDA plates. Mycotoxin production is shown on the left y-axis and diameter of infection on the right y-axis. Error bars indicate standard errors. Capital letters indicate homogeneous groups for AOH production. Lowercase letters indicate homogenous groups for AME production. Italic capital letters indicate homogenous groups for diameter of growth.

Regarding mycotoxin production, after 6 days incubated at 10 ºC, cultures did not produce AOH or AME either with or without 1-MCP. It was not until the second week of incubation at 20 ºC when fungi produced both mycotoxins. Nevertheless, quantification of AOH and AME on the second week did not show significant differences between plates that had been treated with 1-MCP and those untreated.

It is noteworthy that levels of AOH were pretty higher than AME, around 40-fold when plates were subjected to a 1-MCP treatment and more than 30-fold higher when they were not treated (Fig. 1).

To understand the genetic impact of 1-MCP regarding mycotoxin production, a study of the *pksJ* gene was carried out. The protein that encodes this gene corresponds to a polyketide synthase which is essential for the biosynthesis pathway of AOH and AME (Saha et al., 2012). Primer pair used for the amplification of *pksJ* gene (PksJ_F/PksJ_R) and primer pair used for amplification of beta-tubulin gene (A-BTF/A-BTR) were assessed for qPCR efficiency, which corresponded to 1.92 and 2.03, respectively. Results from this analysis are shown in Fig. 2.

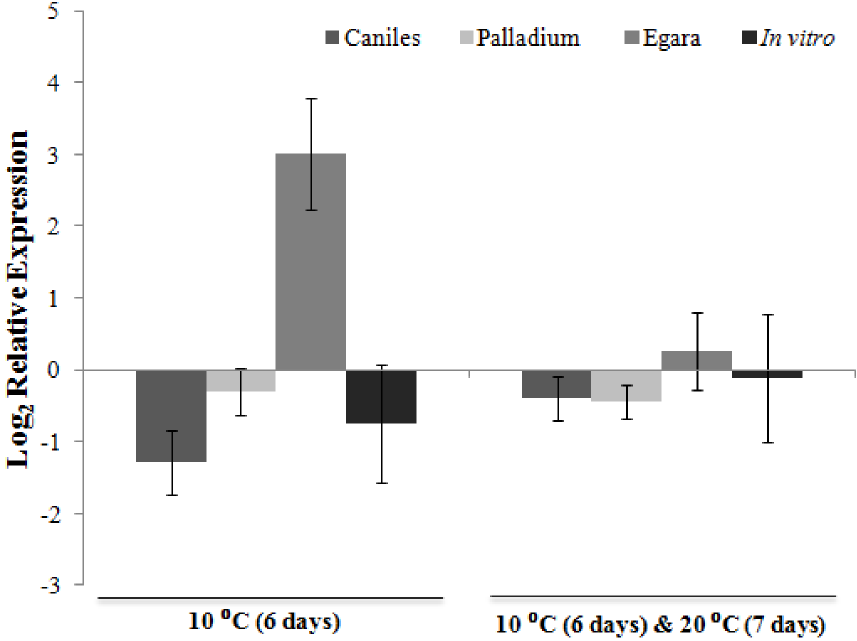

Figure 2. Relative expression of *pksJ* gene in *A. alternata* inoculated on tomatoes (Egara, Caniles and Palladium) or *in vitro* (PDA plates). Results are the $\log_2$ ratio between 1-MCP treated and untreated samples. Error bars indicate standard errors.

Values above the bar indicate an upregulation of the gene, values under the bar suggest downregulation, while values near the x-axis show a similar expression to untreated samples. 1-MCP did not affect *pksJ* expression neither after the first week of incubation at 10 ºC nor after the second week at 20 ºC.

3.2 Effect of 1-MCP on *A. alternata* growth on tomato fruits

3.2.1 Fruit quality measurements

Before analyzing the effect of 1-MCP on *A. alternata* inoculated tomatoes, several quality parameters were measured on 20 tomatoes of each variety in order to characterize tomato cultivars that had to be used for the assay. Table 4 describes all the parameters analyzed. Varieties assayed were quite different on weight and caliber. While Egara was the biggest and the heaviest tomato, Caniles was the smallest and the lightest. Egara cultivar was besides firmer and the one with the lowest quantity of SS and TA (Table 4).

Table 4. Fruit quality parameters of tomato varieties tested (Egara, Caniles and Palladium).

	Weight (mg)	**Caliber (mm)**	**Firmness (kg/cm^2)**	**SS (ºBrix)**	**TA**	**Color**
Egara	209.78 ± 42.81	81.45 ± 6.29	5.71 ± 2.03	4.93 ± 0.12	4.89 ± 0.47	L 44.56 ± 2.62 a -8.30 ± 3.85 b +19.84 ± 2.03
Caniles	74.85 ± 6.81	50.34 ± 1.87	6.78 ± 1.54	6.23 ± 0.38	5.76 ± 0.42	L 47.54 ± 1.79 a +4.79 ± 3.85 b +24.02 ± 2.66
Palladium	93.85 ± 11.34	57.15 ± 3.13	6.08 ± 2.15	5.13 ± 0.15	5.40 ± 0.14	L 48.63 ± 1.70 a +3.30 ± 4.00 b +22.21 ± 1.99

3.2.2 Verification of the efficacy of the 1-MCP treatment

Verification of the efficacy of the 1-MCP treatment was done by comparing the color of 10 tomatoes that were submitted to 1-MCP treatment with 10 tomatoes that were not. The color of fruit was checked by using a colorimeter (colorimeter

data is not shown). It was observed that tomatoes treated with 1-MCP had a different color tone compared to untreated tomatoes (Figs. 3-5). That difference was more noticeable for Egara and Palladium varieties, in which treated tomatoes had a more orange tone while the untreated were redder. For Caniles variety, this difference was not so appreciable at first sight. This may mean that 1-MCP has a different effect depending on the variety in which it is applied. After two weeks of incubation, the orange tone of the skin was still appreciable.

3.2.3 Effect of 1-MCP on the development of *A. alternata*

Once it was observed that 1-MCP seemed to affect fungal growth *in vitro*, the same assay was repeated using three different varieties of tomatoes: Caniles, Palladium and Egara. All of them had different physical (weight, caliber, firmness and color) and chemical properties (SS and TA). All tomatoes were treated as described in section 2.3 and section 2.5.

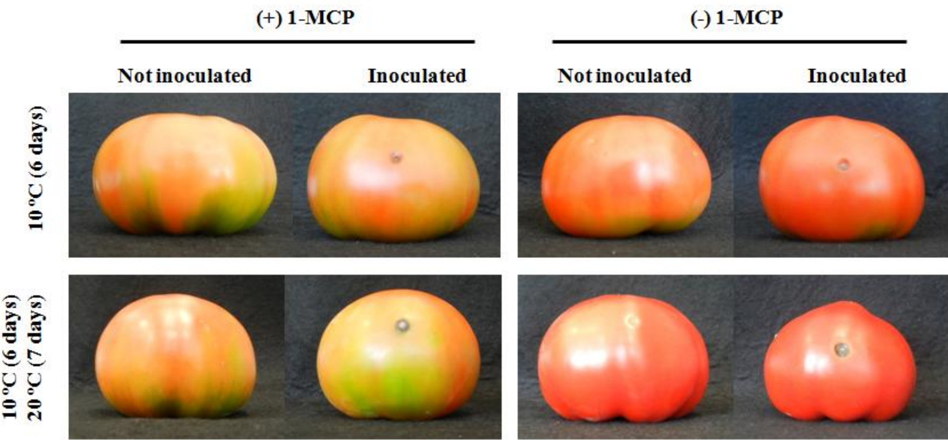

Figure 3. Color affectation and black mold rot produced by artificially inoculated *Alternaria* on 1-MCP treated and untreated Egara tomatoes. Images were taken after the first period of storage (6 days at 10 ºC) and after the shelf-life simulation (7 days at 20 ºC).

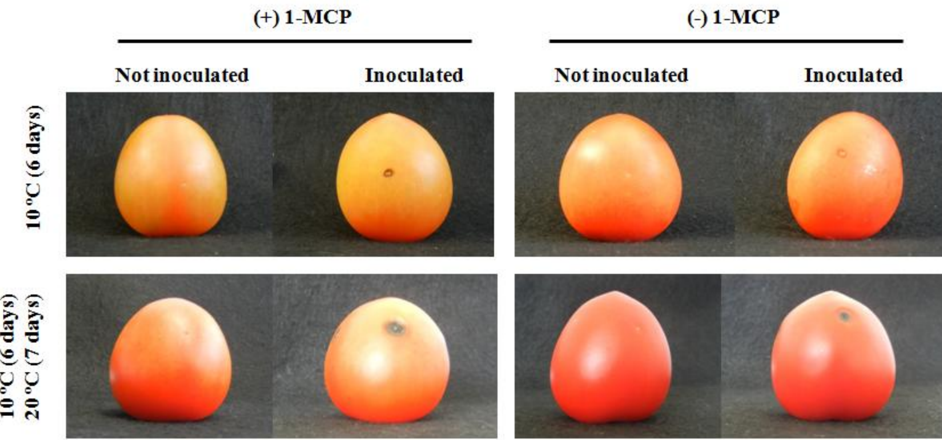

Figure 4. Color affectation and black mold rot produced by artificially inoculated *Alternaria* on 1-MCP treated and untreated Caniles tomatoes. Images were taken after the first period of storage (6 days at 10 ºC) and after the shelf-life simulation (7 days at 20 ºC).

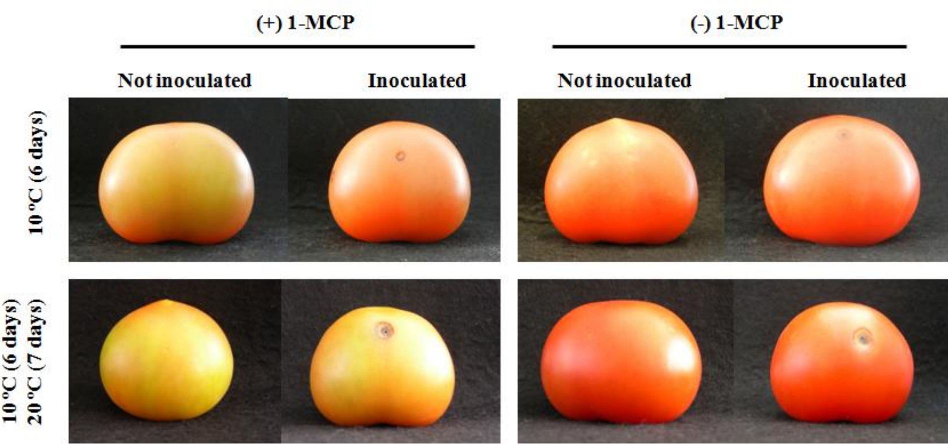

Figure 5. Color affectation and black mold rot produced by artificially inoculated *Alternaria* on 1-MCP treated and untreated Palladium tomatoes. Images were taken after the first period of storage (6 days at 10 ºC) and after the shelf-life simulation (7 days at 20 ºC).

During the 6 days at 10 ºC, as it was observed for *in vitro* cultures, untreated and treated tomatoes with 1-MCP did not show significant differences dealing with diameter of infection, except for the Egara variety (Figs. 3 and 6). In that case, the average of fungal growth of tomatoes treated with 1-MCP was 3.95 mm, while in untreated tomatoes it was 2.72 mm.

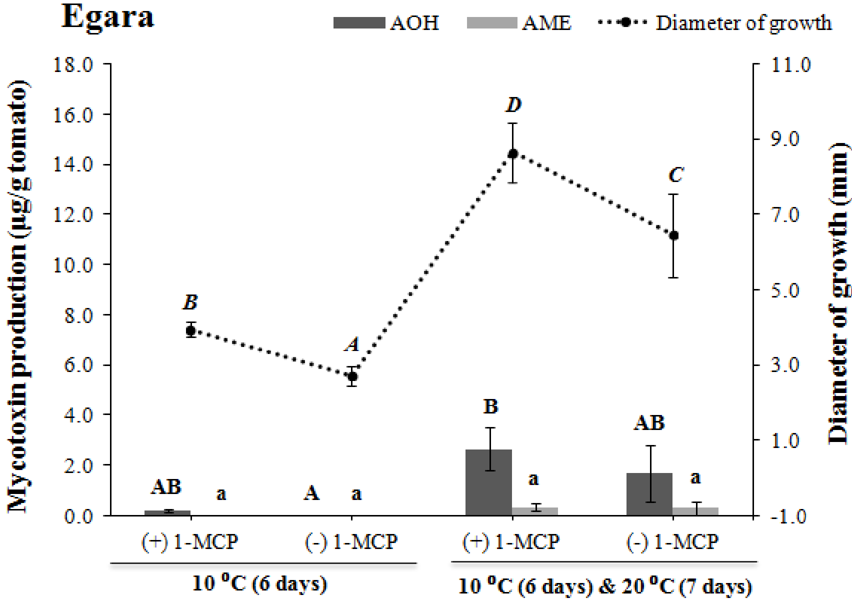

Figure 6. Effect of 1-MCP on *A. alternata* grown on Egara tomatoes with regard to AOH and AME production (left y-axis) and diameter of infection (right y-axis). Error bars indicate standard errors. Capital letters indicate homogeneous groups for AOH production. No significant differences were found for AME production. Italic capital letters indicate homogenous groups for diameter of infection.

However, when *A. alternata* was grown for one more week at 20 ºC, in two of the three tomato cultivars studied (Egara and Palladium) results yielded statistically significant differences in diameter infection between treated and untreated tomatoes. As shown in Figs. 6-8, in all cultivars, tomatoes that had been exposed to the 1-MCP gas showed a higher diameter of infection being 8.66 mm for Egara (Fig. 4), 6.84 mm for Caniles (Fig. 5) and, 7.96 mm for Palladium (Fig. 6). In

contrast, untreated tomatoes had a diameter of infection of 6.45 mm, 6.00 mm and, 5.61 mm, respectively. Hence, results reported here seem to indicate that 1-MCP may increase *A. alternata* growth when conditions are favorable for the fungus though this may be dependent on the tomato cultivar.

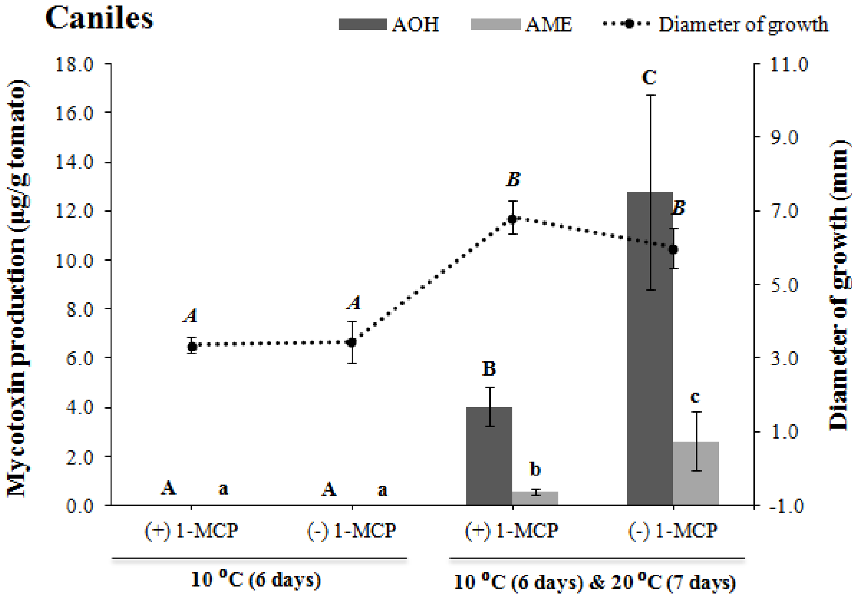

Figure 7. Effect of 1-MCP on *A. alternata* grown on Caniles tomatoes with regard to AOH and AME production (left y-axis) and diameter of infection (right y-axis). Error bars indicate standard errors. Capital letters indicate homogeneous groups for AOH production. Lowercase letters indicate homogenous groups for AME production. Italic capital letters indicate homogenous groups for diameter of infection.

3.2.4 Effect of 1-MCP on mycotoxin production of *A. alternata* and the *pksJ* gene expression

The effect of 1-MCP on AOH and AME production by *A. alternata* was tested on tomato fruit as well (Figs. 6-8). As it was observed for *in vitro* cultures, two of the three tomato cultivars did not produce AOH or AME after 6 days at 10 ° C (Palladium and Caniles). However, in one of the cultivars (Egara), after 6 days, tomatoes treated with 1-MCP contained 0.19 µg of AOH/g of tomato while

untreated tomatoes did not support any production. After one more week of storage at warmer temperature conditions (20 ºC), *A. alternata* produced both AOH and AME. In all cases, AOH production was higher than AME. Among all cultivars, Egara was the one in which *A. alternata* produced less quantity of mycotoxins. So, mycotoxin production could be a factor dependent upon the variety. No significant differences were found in relation to mycotoxin production between 1-MCP treated or untreated tomatoes, except in Caniles cultivar (Fig. 5). In that case, untreated tomatoes artificially inoculated with *A. alternata* contained more AOH and AME than treated samples. While untreated tomatoes contained an average of 12.79 µg of AOH/g of tomato and 2.62 µg of AME/g of tomato, 1-MCP treated ones contained 4.02 and 0.54, respectively.

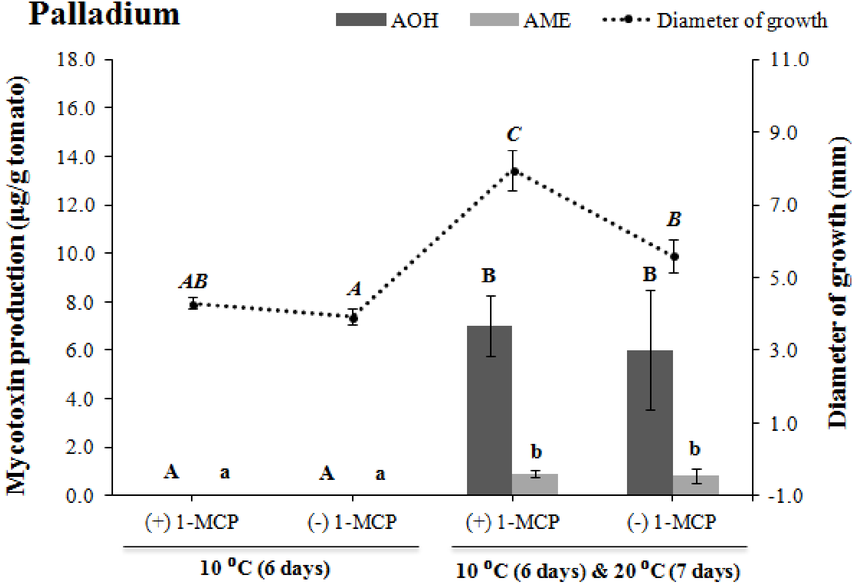

Figure 8. Effect of 1-MCP on *A. alternata* grown on Palladium tomatoes with regard to AOH and AME production (left y-axis) and diameter of infection (right y-axis). Error bars indicate standard errors. Capital letters indicate homogeneous groups for AOH production. Lowercase letters indicate homogenous groups for AME production. Italic capital letters indicate homogenous groups for diameter of infection.

As it was done for the *in vitro* test, analysis of the *pksJ* gene was assessed. Primer pair used for the amplification of the *pksJ* gene (PksJ_F/PksJ_R) on tomato samples and, primer pair used for amplification of the beta-tubulin gene (A-BTF/A-BTR) on tomato samples as well, were assessed for qPCR efficiency, which corresponded to 2.02 and 2.13, respectively. Fig. 2 illustrates the relative gene expression of samples treated with 1-MCP in relation to untreated ones. It was interesting to find out that transcript levels were not always accompanied by biochemical or phyisiological changes and, although no mycotoxins were detected during the first 6 days of storage at 10 ºC in the case of Caniles and Palladium varieties, expression of the *pksJ* gene was observed. This could be explained by the fact that during the first period of incubation, *A. alternata* may produce AOH and AME but the levels could be under our LOD. In Fig. 2, during the first 6 days, the bar corresponding to the expression of *pksJ* on Caniles, showed a downregulation of -1.28 $\log_2$, which means that the *pksJ* gene was less expressed on *Alternaria* inoculated on tomatoes treated with 1-MCP in comparison with the controls. Regarding *pksJ* expression of *Alternaria* inoculated on the Palladium cultivar, during the first week of storage under cold conditions, treated and untreated samples showed a similar gene expression of *pksJ*. On the Egara cultivar treated with 1-MCP, *A. alternata* showed AOH production during the first 6 days at 10 ºC (Fig. 4, 0.194 µg/g tomato) and this data seems to be congruous with the study of the *pksJ* gene expression. In that case, an upregulation of 3.00 $\log_2$ of the *pksJ* gene was observed, which means an 8 times increase in the expression of the p*ksJ* gene of 1-MCP treated samples over the untreated ones. When tomatoes were incubated for 14 days, AOH and AME production was observed in all cultivars (Fig. 4-6). Nevertheless, only for Caniles (Fig. 5), the results yield statistically significant differences between the samples treated with 1-MCP and the untreated. However, results from the analysis of the *pksJ* gene expression in Caniles tomatoes on the second week, showed similar expression between fungi grown on tomatoes treated with 1-MCP and without 1-MCP.

4. Discussion

1-MCP treatments markedly affect ripening of tomato fruit by inhibiting ethylene production and respiration though other quality parameters including fruit firmness, color and TA values may be also altered. It has been described that application of 1-MCP produces alterations on several parameters of tomatoes such as respiration rates, color, softening and TA values (Watkins, 2006). Only small effects on aroma volatiles were detected by Mir el at. (2004), while weight and SSC were not affected. Since 1-MCP treatments induce beneficial effects in fruit quality, such as delays in physico-chemical changes related to ripening, as well as a reduction in decay, and thereby extend the storage life of both climacteric and non-climacteric fruit, 1-MCP is a useful tool for extending the shelf-life and quality of several food products (Blankenship and Dole, 2003; Watkins, 2006). Nevertheless, although 1-MCP advantages justify its application on food products, it has to be taken into account that 1-MCP commercial use in postharvest treatments has been claimed to increase decay development due to enhanced susceptibility against external stresses such as low temperatures and pathogen infections in some fruits (Biswas et al., 2014; Díaz et al., 2002; Janisiewicz et al., 2003; Jiang et al., 2001; Ku et al., 1999). Since the discovery of 1-MCP most of studies have been focused to investigate its way of action and the effects on physiological and biochemical parameters of fruits. It is only in recent years that some studies are emerging which analyze the effect of 1-MCP on progress of pathogens that may contaminate treated fruits. In this sense, it has been observed that the effect of 1-MCP on various disorders and diseases may be variable, being species specific (Blankenship and Dole, 2003). Thus, the aim of the present work was to gain a deeper insight into the effects of 1-MCP on *A. alternata* dealing with the fungal development and mycotoxin production.

The findings of this study indicate that 1-MCP treatment, in some varieties, negatively affects the resistance of tomatoes against *A. alternata* infection and,

consequently, the disease produced by the fungus may be more severe when storage conditions are favorable for fungal growth. This hypothesis is in line with other studies that have shown that 1-MCP may increase susceptibility of fruits against pathogen infections. Díaz et al. (2002) found that treatment of tomato leaves with ethylene increased resistance to infection of *B. cinerea*, while treatment with 1-MCP increased susceptibility of tomato plants to the pathogen. On strawberries, Ku et al. (1999) observed that postharvest life of fruits at 20 and 5 °C was reduced with 1-MCP, which reduced the storage life by almost 40%. The loss of quality was due mostly to the onset of rotting. These results were later supported by Jiang et al. (2001) who found that 1-MCP treatment tended to maintain strawberry fruit firmness and color even though disease development was accelerated in fruit treated at high 1-MCP concentrations (0.5 and 1 μL/L). Working with apples, Janisiewicz et al. (2003) described that treatment of these fruit with 1-MCP increased bitter rot and blue mold decay, diseases caused by *C. acutatum* and *P. expansum*, respectively. Notwithstanding, it has been described that in some cases 1-MCP treatment alleviates diseases. In this sense, Su and Gubler (2012) evaluated 1-MCP regarding affectation of postharvest decay caused by *A. alternata*, *B. cinerea* and, *Fusarium* spp. on two different tomato cultivars. For the study, they used artificially inoculated fruit and tomato fruit with a natural level of infection. They found that after about one month of storage at 15 ºC, disease incidence and severity of individual diseases in 1-MCP treated fruit were significantly reduced compared to the untreated controls, except in one test that had been inoculated with *Alternaria*. In that case, severity of *Alternaria* rot was significantly higher on 1-MCP (1 μL/L) treated fruit compared to the untreated controls, which is in accordance to our study. However, despite Su and Gubler (2012) found that 1-MCP applied to tomatoes could decrease disorders caused by *B. cinerea*, Akagi and Stotz (2007) had previously reported that 1-MCP treatment had a relatively small effect on the rate of lesion expansion after wound inoculation

with *B. cinerea* on pear fruit, which may indicate that effects of 1-MCP could be different depending on the matrix in which it is applied.

Effect of 1-MCP could be different depending on when 1-MCP is applied and at which temperature fruits are stored. Biswas et al. (2014) determined the effect of 1-MCP application prior to cold storage with the aim of extending shelf-life of tomatoes. Results suggested that depending on the temperature in which fruits were stored, the effect regarding *Alternaria* decay was different. When tomatoes were stored at 2.5 °C, 1-MCP treatment enhanced *Alternaria* decay severity, whereas 1-MCP reduced tomato decay when fruit were ripened at 20 °C. They hypothesized that the increase of the decay rate during cold storage could be due to the fact that the ripening delay, induced by 1-MCP, may increase tomato chilling sensitivity and, consequently, it increases the decay produced by *Alternaria.* It has to bear in mind that some of the symptoms of chilling injury is the damaging of the skin, loss of firmness and pitting of the surface which may favor fruit decay and consequently facilitate fungal penetration (Efiuvwevwere and Thorne, 1988). Biswas et al. (2014) suggested to use 1-MCP to slow down tomato ripening but holding the fruit at above chilling temperature (9 to 12 °C) for long-term storage. Results from the present study are mostly in agreement with Biswas et al. (2014), as during the storage under cold conditions (10 ºC) in two out of the three varieties analyzed (Caniles and Palladium) no significant differences were observed regarding pathogen growth between treated and untreated samples. Thus, this may imply that fruits should be refrigerated but above a temperature that could cause chilling injury. However, it has to be taken into consideration that, during the shelf-life of tomatoes, in two out of three cultivars analyzed, tomatoes treated with 1-MCP evidenced more susceptibility to *Alternaria* growth. So, even though tomatoes were not affected during the cool storage, the cons of this measure might be that during the shelf-life of tomatoes treated with 1-MCP, in some varieties, fruits could be more affected by the appearance of fungal diseases.

Porat el al. (1999) studied the effects of ethylene and 1-MCP on the postharvest quality of ‘Shamouti’ oranges. They found that 1-MCP increased stem-end rots by about 7% and mold rots by about 15% compared to controls that had not been treated with 1-MCP. Additionally, treatment with 1-MCP weakened the tissue and increased the incidence of chilling injury symptoms. Thus, 1-MCP rendered fruits more susceptible to decay development regardless of the pathogen causing disease symptoms. Porat et al. (1999) suggested that a possible explanation for the negative effects of 1-MCP regarding decay susceptibility is that small amounts of endogenous ethylene produced by fruits may be required to maintain their natural resistance against various environmental and pathological stresses, so that blocking the action of endogenous ethylene by 1-MCP might have rendered the fruits more susceptible to stresses, such as low temperatures and pathogen attacks. Indeed, ethylene modulates many aspects of plant life, including various mechanisms by which plants react to pathogen attacks. This hypothesis is based on the fact that one of the earliest detectable events during plant-pathogen interaction is a rapid increase in ethylene biosynthesis and subsequent intracellular signaling which aim to control the expression of various genes involved in defense responses (Broekaert et al., 2006; Ecker and Davis, 1987; Lund et al., 1998). On *A. alternata* it has been proposed that ethylene plays a double signaling function in black spot disease, which indicates that ethylene has different effects along all the stages by which pathogen is established. Itai et al. (2012) proposed that at the time of inoculation, ethylene may induce resistance responses in the fruit by elicitation of defense proteins, whereas after successful pathogen attack, ethylene may increase *Alternaria* toxin susceptibility and promote disease symptoms by inducing necrosis in the fruit. This hypothesis is in accordance with the notion proposed by Porat et al. (1999) and may explain our results as well. Hence, when tomatoes are treated with 1-MCP the endogenous levels of ethylene of the fruits could decrease and this would enhance the susceptibility of fruits against exogenous stresses, such as low

temperatures (chilling injury) as described by Porat et al. (1999) and pathogen infections.

With regard to affectation of mycotoxin production on 1-MCP treated fruits, no large differences were observed among treatments as only for Caniles cultivar the difference between 1-MCP treated tomatoes and untreated ones was significant. At this point it is worth mentioning that *pksJ* gene expression results indicated that even when there is low or none mycotoxin detection during the first week at 10 ºC, probably because concentration is under our LOD, there was *pksJ* expression for all the varieties tested. These results confirm that AOH and AME may be biosynthesized under cool storage conditions (Barkai-Golan, 2008; Ozcelik et al., 1990). Hence, from these findings we might suggest that 1-MCP treatment does not affect AOH and AME biosynthesis significantly. However, its effect may be dependent of the tomato variety.

In conclusion, the research findings of this study have provided some evidence that 1-MCP negatively affects the resistance of three tomato varieties against *A. alternata* infection and, consequently, black spot disease produced by the fungus is significantly more severe on 1-MCP treated tomatoes when storage conditions are favorable for fungal growth. However, 1-MCP treatment has not affect significantly the biosynthesis of AOH and AME in two of the three varieties studied, which could indicate that 1-MCP may affect mycotoxin in a different way depending on the fruit variety and so, more varieties should be tested.

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Chapter VI

Stability of alternariol and alternariol monomethyl ether during food processing of tomato products

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Stability of alternariol and alternariol monomethyl ether during food processing of tomato products

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Abstract

The stability of two *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME), has been investigated during the food processing of tomato products simulating commercial processing conditions. The production stages assessed that were considered to have some effect on the stability of AOH and AME were the storage of raw fruits, fruit washing, and thermal processing. It was observed that time of storage significantly reduced the initial concentration of AOH, but only if tomatoes were stored at 35 ºC. For AME, 12 hours were sufficient to reduce the initial concentration, regardless of the temperature at which samples were stored (25, 30 and 35 ºC). The washing step achieved the highest reduction of AOH and AME. This reduction was even more efficient when using sodium hypochlorite solutions. Finally, during the heat treatment (80-110 ºC), results showed that heating tomato samples at 100 and 110 ºC, significantly

affected AOH stability, though AME seemed to not be affected by these thermal processes. The findings illustrate the importance of the different production stages on the stability and fate of *Alternaria* mycotoxins.

Keywords: *Alternaria*; AOH; AME; stability; tomatoes

1. Introduction

There are numerous published reports that detail the occurrence of well known mycotoxins such as aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, among others, in food and feed products. All these mycotoxins are regulated by the European Commission (EC, 2010), who has set maximum levels in food and feedstuff to keep these contaminants at levels which are toxicologically acceptable for human and animal consumption. However, other mycotoxins are less studied and are not regulated by any legislation. In this group, we can find the mycotoxins biosynthesized by *Alternaria* spp. The genus *Alternaria* includes several species that cause plant diseases on many crops and spoil various fruits, grains, and vegetables during postharvest storage and transport. *Alternaria alternata* is one of the most common species causing spoilage and it has been described as the major mycotoxin-producing species of this genus. The most common mycotoxins associated with *Alternaria* include alternariol (AOH), alternariol monomethyl ether (AME), tentoxin, tenuazonic acid (TeA), altenuene (ALT) and altertoxins (Barkai-Golan, 2008; EFSA, 2011; Logrieco et al., 2009; Ostry, 2008). Despite limited literature regarding the toxicity of *Alternaria* mycotoxins, it has been described that they are harmful for human and animals (Brugger et al., 2006a; Liu et al., 1992; Pero et al., 1973; Pfeiffer et al., 2007; Pollock et al., 1982).

There are several emerging studies that report the presence of *Alternaria* mycotoxins in food and feed, including tomatoes and derived tomato products

(Ackermann et al., 2011; da Motta and Valente Soares, 2001; Noser et al., 2011; Terminiello et al., 2006; Van de Perre et al., 2014; Visconti et al., 1987), olives (Visconti et al., 1986), mandarins (Logrieco et al., 1990), peppers (Monbaliu et al., 2009), apples and their derived products (Delgado and Gómez-Cordovés, 1998; Stinson et al., 1981), wheat and other grains (Monbaliu et al., 2010), among others. In most cases, fruit may be contaminated by *Alternaria* spp. in the field and, once in the food industry, during the storage. When the contamination has occurred, *Alternaria* spp. may begin the biosynthesis of mycotoxins. The spoiled products may pass through the culling step accidentally, and enter into the food production chain. When this happens, fungi can still be destroyed during the heat treatment, but it is uncertain what happens with the mycotoxins themselves. In fact, there is scarce information on the stability and fate of *Alternaria* mycotoxins throughout the food processing operations and storage. In this sense, Ozcelick et al. (1990) studied mycotoxin production by *A. alternata* in tomatoes and apples stored under various temperature conditions. They also tested if wrapping fruits had any effect on mycotoxin production. Combina et al. (1999) studied the effect of heat treatment on the stability of AOH, AME and TeA using a sunflower flour matrix. They found that the most effective treatment to reduce AOH and AME was heating samples at 121 ºC for 60 minutes. Afterwards, the heat-treated material produced was used to feed rats and toxic effects were observed on them. In the case of TeA, a heat treatment of 100 ºC for up to 90 minutes was sufficient to reduce by half its initial concentration. Scott and Kanhere (2001) also studied the stability of AOH, AME and altertoxin I (ATX-I) in apple and grape juice. Their results showed that AOH and AME were stable both in apple and grape juice at room temperatures over 27 days and after a treatment of 20 min at 80 ºC. Dealing with ATX-I, they found that it was quite stable at pH 3 in apple and grape juice. Some years later, Siegel et al. (2010) investigated the stability of AOH, AME and ALT upon bread baking and the products derived from their degradation, using whole meal wheat flour spiked with these mycotoxins. Data indicated that mycotoxins were

negligibly degraded during wet baking, while significant degradation was observed upon dry baking.

Hence, there are different studies that reveal *Alternaria* mycotoxins could remain quite stable during the industrial process, which consequently, may result in high levels of *Alternaria* mycotoxins in the finished products such as juices, sauces and purées. Still, there is little information about the stability of *Alternaria* mycotoxins throughout the production process of derived tomato products. Thus, the aim of this work is to provide information about the stability of AOH and AME through the food processing steps for tomato products. in order to identify which steps may require more attention when aiming to decrease the initial concentration of toxin contaminants.

2. Material and methods

2.1 Chemicals

Standards of AOH (~94%) and AME (~98%) were supplied by Sigma–Aldrich (St. Louis, MO, USA). A stock solution was prepared for each standard by dissolving 5 mg of the purified mycotoxins in ethanol reaching a final concentration of 1000 µg/mL. From the stock standard solutions, working standard solutions at a concentration of 15 µg/mL were prepared. AOH and AME concentration was checked by UV spectroscopy. All standards were stored at -20 ºC in a sealed vial until use.

Acetonitrile (99.8%) and methanol (99.9%) were both HPLC (high-performance liquid chromatography) grade and were supplied by Acros Organics (Morris Plains, NJ, USA). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA).

2.2 Tomato sample preparation

In our study, we chose to use small tomatoes, as lower amount of mycotoxins were needed for fruit contamination. Therefore, cherry tomatoes were used, being purchased from the local supermarket and kept at room temperature until used. All tomato samples were surface disinfected with 70% ethanol.

For heat treatment tests, instead of using raw cherry tomatoes as a matrix, a tomato juice was prepared. For this purpose, the whole cherry tomatoes were blended (Turbo Habana, Palson, Spain) until a juice homogeneous tomato matrix was achieved.

2.2.1 Spiking of samples

Before storage and washing experiments, all tomatoes were spiked with 100 µL of an AOH and AME solution containing a known concentration of both mycotoxins (0.5 µg/g of tomato) dissolved in ethanol. Once tomatoes were spiked with the mycotoxin solution, they were dried under a laminar flow hood at ambient temperature. All tomatoes were weighted individually, and all these data were used for the final analysis of results. For heat treatments tests, 20 g of tomato juice was dispensed in glass tubes and then spiked with 100 µL of ethanol containing 0.5 µg of both AOH and AME per gram of tomato. Glass tubes were sealed with cotton, covered with plastic caps and samples were mixed using a vortex.

2.3 Food production chain analyzed

To study the stability of AOH and AME along the food processing of derived tomato products, all steps were analyzed, and those susceptible of causing any alteration or instability to both *Alternaria* mycotoxins were simulated on the laboratory, where stability assays were performed (Fig. 1). Manual selection and rejection of moldy or rotten tomatoes can be an efficient measure to avoid the entrance of *Alternaria* mycotoxins in the production chain and so, no rotten tomatoes were chosen for this assay. All the experiments were performed in

triplicate. A negative control test without spiked tomatoes was prepared to ensure no AOH or AME contamination on the raw fruits used for the studies.

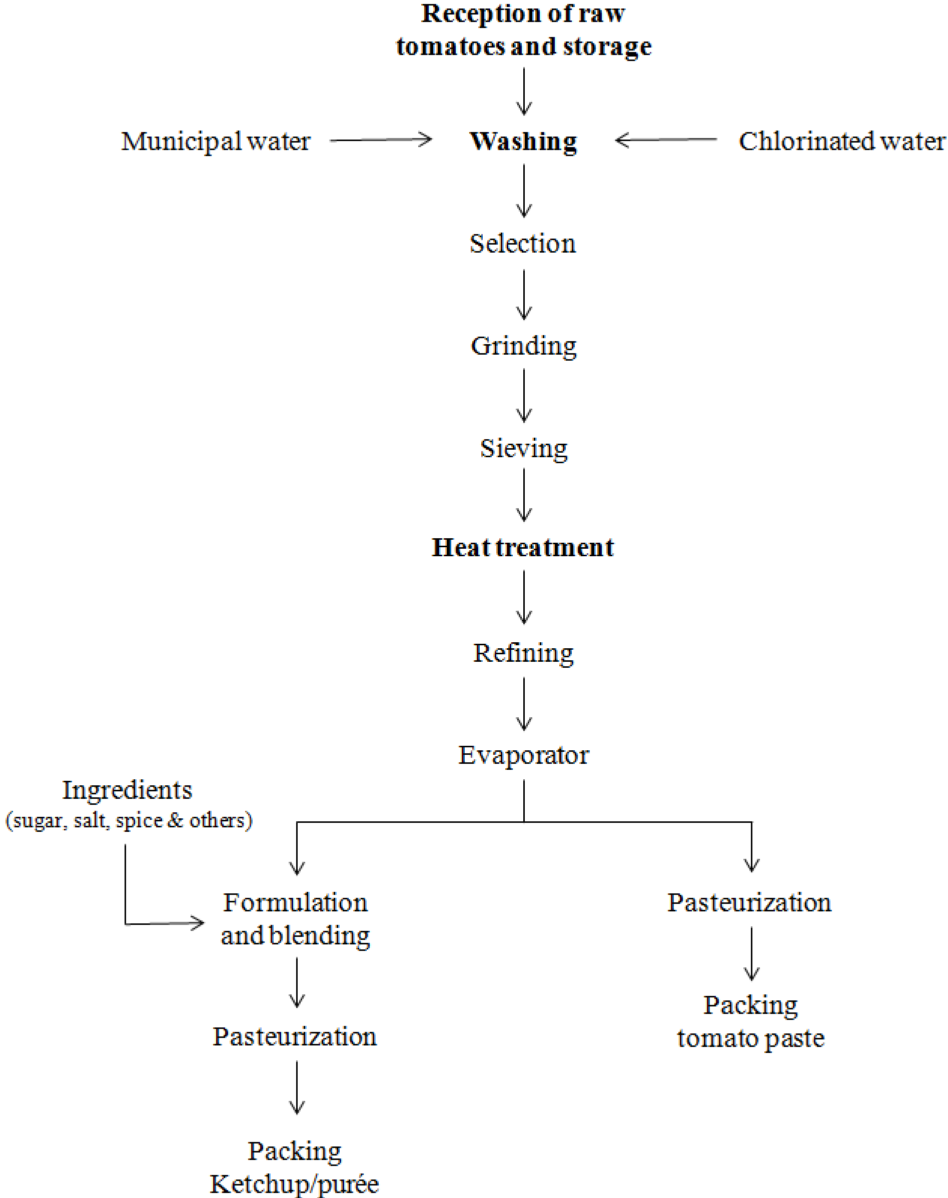

Figure 1. Production flow chart of derived tomato products. Bold letters specify the assessed steps for AOH and AME stability.

2.3.1 Storage

In this first step of the food chain, the effect of time of storage and temperature was analyzed on the stability of AOH and AME. Spiked cherry tomatoes were stored for different periods of time (12 hours, 24 hours, 2 days and 1 week) into different incubation chambers at various temperatures (25, 30 and 35 ºC) without any external light. Unstored spiked tomato samples were considered controls.

2.3.2 Washing

For the washing step, in each treatment, 5 spiked cherry tomatoes were washed with 1 liter of tap water or 1 liter of a chlorinated water solution (150 or 250 mg/L of sodium hypochlorite, NaOCl) contained in a glass beaker. To prepare the chlorine water solution, a commercial sodium hypochlorite containing 8.25% of NaOCl was used. Bleach volumes needed to prepare the desired concentration were dissolved in municipal water. To simulate the flow often used in the food industry, samples were stirred using a low homogenous magnetic field. Samples were collected from the beaker after 1, 2, 5 and 10 minutes. Fruits not dipped into water were considered controls.

2.3.3 Heat treatment

The tomato juice samples used in this step were prepared as detailed in section 2.2. Glass tubes containing the spiked samples were weighed before the assay and then placed into an oil bath for assessing the effect of heat treatment on AOH and AME stability. Temperatures tested were 80, 90, 100 and 110 ºC. Samples were taken from the oil bath after 30, 60 and 90 minutes and then they were stored into the fridge (4 ºC) until analyzed. Before AOH and AME extraction, samples were weighed again and milliQ water was added to compensate for evaporative losses. Samples were homogenized using a vortex and AOH and AME extraction was carried out (see section 2.4). Unheated spiked tomato juice samples were considered controls.

2.4 AOH and AME extraction

For the AOH and AME extraction procedure, individual raw cherry tomatoes were put in a stomacher bag containing 30 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric acid) and homogenized for 2 minutes with a stomacher (Lab-Blender 400, Ohio, USA). The resulting mixture was put in a small glass beaker and blended for 15 minutes using a uniform magnetic mixer. For tomato juice, samples were directly put in the beaker containing 30 mL of acetonitrile-methanol-water (45:10:45 v/v/v; pH 3) and mixed for 15 minutes. The solution was left for approximately 10 minutes, to favor precipitation by gravity. Then, 6 mL of the supernatant was transferred to a centrifuge tube and diluted with 15 mL of a 0.05 M sodium dihydrogen phosphate solution (pH 3 adjusted with *o*-phosphoric) and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract was passed by gravity filtration through a previously conditioned Bond Elut Plexa SPE cartridge (200 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the cartridge was done with 5 mL of methanol followed by 5 mL of miliQ water. The SPE column was washed with 5 mL of water, followed by air drying on the manifold. Finally, elution was carried out with 5 mL of methanol and 5 mL of acetonitrile. Sample extracts were dried on a speed vacuum concentrator at 35 ºC and stored at -20 ºC until HPLC analysis. Prior to HPLC injection, samples were resuspended in 500 µL of a water-methanol solution (50:50 v/v) and sonicated for 1 minute. Validation data (repeatability and recovery) of the mycotoxin extraction method are described in Table 1.

2.5 HPLC analysis

Separation, detection and quantification of AOH and AME were performed on a HPLC system model 2510 HPLC pump (Varian, Inc., Palo Alto, CA) connected to one in-line Spectroflow 757 UV/Vis absorbance detector (Applied Biosystems, Foster City, CA). A reverse phase Kinetex PFP column (5 µm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a KrudKatcher classic

HPLC in-line filter (0.5 µm depth filter, Phenomenex, CA, USA) were used. Column was set at a temperature of 35 ºC. For chromatographic separation of AOH and AME, the flow rate was 0.5 mL/min and the injection volume was 100 µL. Absorption wavelength was set at 258 nm. The mobile phase consisted of a double distilled miliQ water and methanol-water (70:30 v/v) according to the gradient described in Table 2 - HPLC gradient. Retention times were 28 minutes for AOH and 38 minutes for AME. For mycotoxin quantification, working standards were used to perform a ten-point calibration curve for mycotoxins (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). The limit of detection (LOD) for AOH was 0.009 µg/g tomato, while for AME was 0.012 µg/g of tomato. The LOD was based on a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was calculated as 3 × LOD. Method performance characteristics for AOH and AME are summarized in Table 1 (- Recoveries HPLC).

Table 1. Recovery and repeatability of AOH and AME analysis on tomatoes.

	AOH			**AME**		
Spiking level (µg/g tomato)	**n**	**Recovery [a] (%)**	**RSDr [b] (%)**	**n**	**Recovery (%)**	**RSDr (%)**
0.5	3	115.5 ± 2.1	1.8	3	106.0 ± 8.2	7.7
1.0	5	99.0 ± 2.4	2.4	5	93.8 ± 3.1	3.4
2.0	3	79.8 ± 3.2	4.0	3	78.0 ± 3.6	4.6

[a] Mean value ± standard deviation.
[b] RSDr = relative standard deviation.

2.6 Statistical analysis

All data were firstly analyzed using the multifactor ANOVA. When there was statistical significance of any of the interactions assessed (p-value > 0.05), a One Way ANOVA test vas carried out. The Tukey-HSD test (Honest Significant

Difference) was used to compare means. All statistical analyses were performed with Statgraphics Centurion Version XVI.

Table 2. HPLC gradient used for the chromatographic analysis of AOH and AME.

Time (min)	Water	Methanol- water (70:30 v/v)
	100	-
7	100	-
9	20	80
10	20	80
12	-	100
41	-	100
43	10	90
45	30	70
46	50	50
47	70	30
50	100	-

3. Results and discussion

3.1 Stability of AOH and AME during the storage

Reception of raw material is a common step in the entire food industry, which usually requires a quality inspection of the incoming fruits or vegetables. This step may also include an additional storage period, either because products are not at the appropriate ripening stage yet, or because there is no immediate need in the food production plant. Storage time will depend on the food industry. In this study, the effect of storing tomato fruits for 12 hours, 24 hours, 2 days and 1 week on the stability of AOH and AME has been analyzed. The harvest season of tomatoes usually extends from spring to summer and thus, it comes with warm weather conditions. In this work, tested temperatures were 25, 30 and 35 ºC. It was found

that the initial concentration of AME was significantly reduced after 12 h of storage, while remained constant later (Fig. 2).

For AOH, storage at 35 ºC was necessary to achieve a significant reduction of its initial concentration. Statistical analysis showed that the temperature at which tomatoes were stored did not have any significant effect. The maximum value of remaining AOH after the storage step was 75%, while the minimum was 56.33%. For AME, in general, remaining percentages were lower than AOH as the highest percentage was 66.91%, while the lowest was 48.97%. Nevertheless, as illustrated on Fig. 2, although the initial concentration of toxins decreased when tomatoes were stored for any period of time, neither of the *Alternaria* mycotoxins completely disappeared in this step, with any of the tested conditions.

Our results support the findings of Ozcelick et al. (1990), who inoculated raw tomatoes with a toxigenic strain of *A. alternata* and let the fungus grow at different temperatures (4, 15 and 25 ºC) for up to 5 weeks. They observed that when tomatoes were stored at 25 ºC, AOH and AME decreased as storage time progressed, though this decrease did not appear to be related to the temperature of storage. The authors suggested that the decrease of AOH and AME levels could be due to some degradation of mycotoxins. It is important to notice that, in their assay, after 5 weeks of storage at 25 ºC, AOH and AME were both present in tomato tissue. In another study, Dalcero et al. (1997) aimed to evaluate the presence of *Alternaria* spp. and their mycotoxins in ensiled sunflower seeds. Samples were collected when the silo was filled, and during the second and fourth months of storage. Results from this study showed that the presence of *Alternaria* spp. and the levels of AOH and TeA decreased as the time of ensiling increased. Thus, they reported that the treatment used to obtain sunflower by-products could reduce the levels of AOH and TeA mycotoxins that may have come from the raw material. In this sense, the ensiling process comprises several variables that may have an effect on the stability of *Alternaria* mycotoxins, such as changes in the pH

or modification of the dry matter. In the present study, the largest period of storage tested was 1 week.

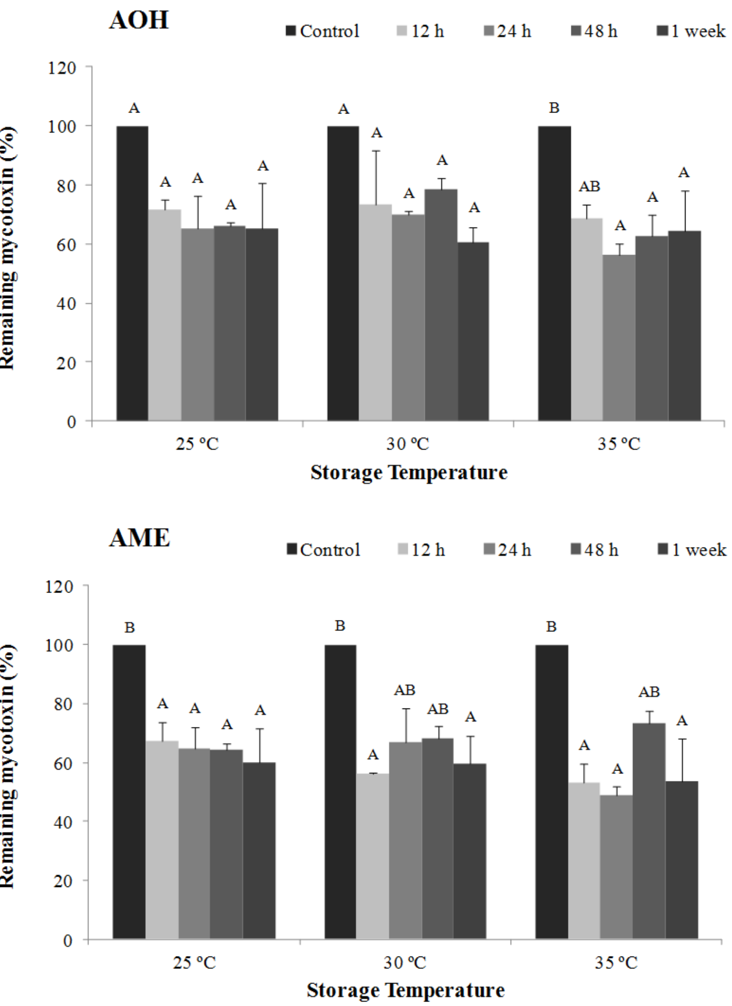

Figure 2. Stability of *Alternaria* mycotoxins during the storage of raw tomatoes. **A.** Remaining percentage of AOH. **B.** Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means between different stored temperatures tested. Capital letters indicate homogeneous groups.

There exists the possibility that longer periods of storage would have achieved larger effects on AOH and AME stability. However, it has to be taken into account that in the food industry, the storage periods for tomatoes are relatively short and it is impossible to store raw tomatoes for two or four months, unless some treatment to control the ripening of fruits is applied. Considering that AOH and AME seem to not disappear during the storage, measures applied in this step that aim to decrease the levels of toxins probably should be addressed to inhibit fungal mycotoxin biosynthesis instead of altering the chemical structure of the mycotoxins produced. With this purpose, it would be useful to find those conditions that are not favorable for fungal development and mycotoxin production. Additionally, it may be interesting to find new substances that, once applied to fruits could inhibit fungal mycotoxin biosynthesis, which may be useful both in the crop fields and during the postharvest storage.

3.2 Stability of AOH and AME during the washing

An essential step of the food production chain in most of the production plants of the food industry is the washing of raw fruits and vegetables. This step allows discarding contaminants coming from the field, such as soil, stones, insects or leaves and may help to reduce the microbial population present on the raw fruit surface. The washing can be done by spraying fruits or by dipping them into a water bath. On the other hand, common methods for sanitizing equipment, food handling tools, and raw fruits and vegetables, include the use of chlorine sanitizers. Dealing with raw fruits and vegetables, sodium hypochlorite could be used during the washing or peeling processes. In the United States of America, the regulation 21 CFR Part 173 (2015) specifies that the use of hypochlorite solutions in the washing procedures must not exceed a concentration of 2000 ppm and after the washing step, food raw products must be rinsed with potable water to ensure that all hypochlorite residue is eliminated. In this study, the washing step was assessed simulating conditions currently used in the food production plants. Thus, raw

cherry tomatoes were washed with two sodium hypochlorite levels (final concentration 150 ppm or 250 ppm). Results showed that the two factors assessed, both duration of the washing step and washing solution, and their interaction, were statistically significant in relation to the stability of the two assessed *Alternaria* mycotoxins (p-value < 0.05) (Fig. 3). According to multiple comparison tests (Tukey-HSD), for AOH, there were no significant differences between washing tomatoes with 150 or 250 ppm sodium hypochlorite solutions. However, washing tomatoes with water was significantly less efficient than washing fruits with a hypochlorite solution, at least if the washing was short (1 or 2 minutes). A One Way ANOVA test was carried out for each one of the washing solution levels, and a Tukey-HSD test was used to compare means among different times of washing (Fig. 3). Dealing with raw tomatoes washed with water, it was shown that for AOH, a water bath of just 1 minute was sufficient to significantly reduce the initial concentration of AOH. After 10 minutes of washing, the remaining percentage of AOH on tomatoes was approximately 11% (Fig. 3A). In contrast, AME was found to be more persistent on tomatoes washed with water, since 5 minutes was required to significantly reduce the initial concentration of AME. After 10 minutes of washing, the initial concentration of AME decreased to 38% (Fig. 3B). These findings may be linked to the solubility of the two mycotoxins. As far as we know, there is no experimental data regarding the solubility in water of any of these compounds. However, there are software tools that predict the solubility for a given molecule. The Toxin and Toxin Target Database (T3DB - www.t3db.ca) is a resource that was specifically designed to capture information about the toxic exposome, which is defined as the totality of all human environmental exposures from conception to death (Lim et al., 2010; Wishart et al., 2015). From this resource, it was found that the predicted solubility of AOH and AME in water, which corresponds to 0.228 mg/mL and 0.0905 mg/mL, respectively. This indicates that AOH is more soluble in water than AME, and may explain the fact that AME is more persistent on the tomato surface. When raw fruits were washed

with a sodium hypochlorite solution (150 ppm or 250 ppm), both for AOH and AME, it was observed that 1 minute was sufficient to significantly reduce the initial content of AOH about 87.52 and 88.14%, respectively, and 66.66 and 54.78% for AME, respectively. However, when using only water, the decrease was 56.746% for AOH and only 20.84% for AME. After 1 minute of washing, no significant reductions were observed for any other duration of the washing treatment. The higher reduction obtained with the sodium hypochlorite solutions may be explained by the pH of the chlorinated water, which is one of the parameters that may affect the properties of chlorine solutions (Gavin and Weddig, 2007). It was found that for the 250 ppm sodium hypochlorite solution, the solution had a pH of 9.50, while for the 150 ppm one it was 9.09. For water, the pH was 7.85. There is scarce information regarding the stability of AOH and AME at different pH levels, as most of the existent literature focuses on the effect of pH on fungal mycotoxin production. However, Siegel et al. (2010) studied the chemical stability of AOH, AME and ALT by refluxing mycotoxins in aqueous solutions with different pH values over a course of 5 hours. All three compounds were stable in a 0.15 M phosphate buffer at pH 5, but were completely degraded in 0.1 M KOH (pH 13). ALT was stable in 0.18 M phosphate/citrate buffer at pH 7, but AOH and AME were degraded. The mechanism of degradation was suggested to involve the hydrolysis of the lactone group followed by decarboxylation, both steps favored by an elevated pH. It is interesting to mention that although the optimal pH value at which mycotoxins are stable is specific for each mycotoxin, the fungal production of most of the mycotoxins is increased at acidic pH values (Gardiner, Osborne et al., 2009; Keller et al., 1997; O'Callaghan et al., 2006). Considering that, in general mycotoxins seem to be more stable at lower pH, it may be possible that when spiked tomatoes were dipped into high pH solutions, the stability of AOH and AME decreased. As the pH of the solution increased, the lower the AOH and AME stability may be. It would be of interest to further investigate the influence of the pH on the chemical structure of AOH and AME.

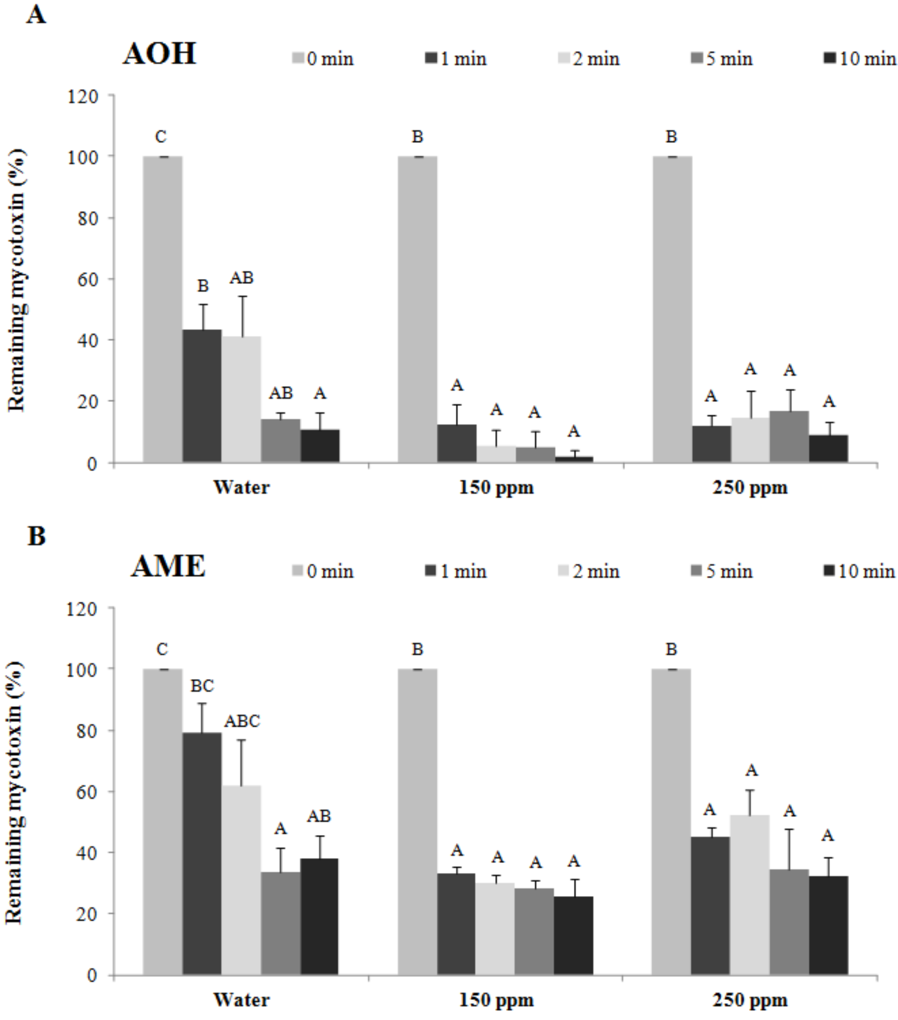

Figure 3. Stability of *Alternaria* mycotoxins during the washing of raw tomatoes. **A.** Remaining percentage of AOH. **B.** Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means between different types of washing, using just water or a concentrated sodium hypochlorite water solution (150 ppm or 250 ppm). Capital letters indicate homogeneous groups.

3.3 Stability of AOH and AME during the heat treatment

Thermal processing is essential in many food production processes. Thermal treatments currently employed include boiling, baking, frying, roasting, canning, extrusion and pasteurization. However, most mycotoxins are heat-resistant within the range of conventional food-processing temperatures (80–121 °C), so little or no reduction in overall toxin levels occur as a result of normal cooking conditions (Kabak, 2009). As far as we are aware, little information exists on the stability and fate of *Alternaria* mycotoxins during heat treatment processes. Thus, in this work we aimed to establish how heat treatments, commonly employed in the food industry to produce tomato products, affect AOH and AME. For this purpose, tomato juice samples were heated at 80, 90, 100 and 110 ºC for different periods of time (30, 60 and 90 minutes). For AOH (Fig. 4A), results showed that both factors tested (time and temperature of heating) and their interaction were statistically significant in relation to its stability (p-value < 0.05). No significant reduction in AOH was observed either at 80 ºC or 90 ºC, while significant reductions occurred at 100 and 110 ºC, with little differences between them. Indeed, Scott and Kanhere (2001) had previously studied the stability of AOH and AME in fruit juices, and observed that there were no apparent losses of *Alternaria* mycotoxins when fruit juices were heated at 80 ºC during 20 minutes. In our study, it has been observed that at 100 ºC and 110 ºC with longer time treatments (over 30 min) did not lead to higher degradation levels. After 90 minutes of heating, the remaining AOH was 67% for treatment at 100 ºC, and 56% for treatment at 110 ºC. At the heat treatment processes of up to 90 minutes tested in this study, were shown to be incapable of completely destroying AOH. Results for AME (Fig. 4B) were quite different, as no significant differences were found with the temperature or the duration of the treatment. From this result, it suggests that AME is stable when exposed to the heat treatments used in this study. As far as we know, only a few studies have assessed the thermal stability of AOH and AME. The most recent study investigating the thermal stability was conducted by Siegel et al. (2010).

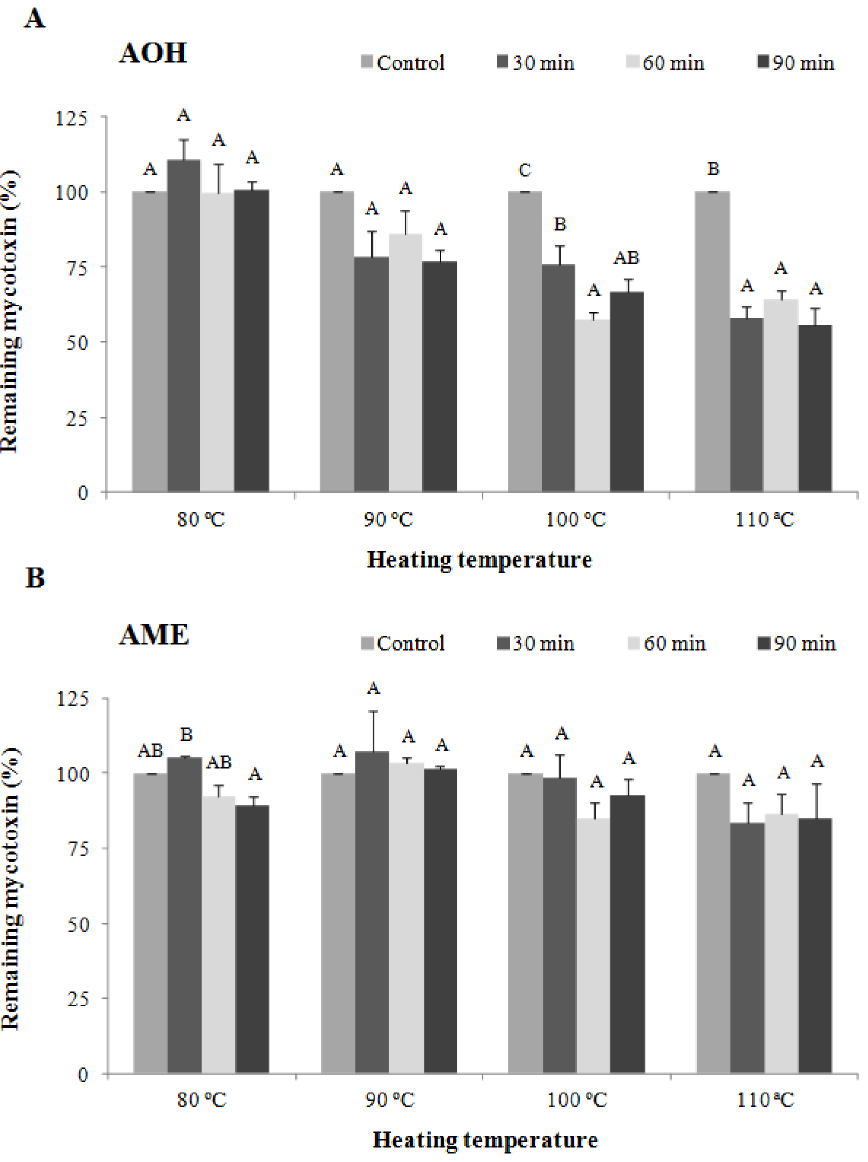

Figure 4. Stability of *Alternaria* mycotoxins during the heat treatment of tomato juice samples. **A.** Remaining percentage of AOH. **B.** Remaining percentage of AME. Error bars indicate standard errors. All statistical data was analyzed by one-way ANOVA (p-value < 0.05). Tukey-HSD test was used to compare means of the different temperatures used to assess the heat treatment effect on AOH and AME stability. Capital letters indicate homogeneous groups.

They designed a series of quantitative model experiments using spiked wheat flour, and observed that *Alternaria* mycotoxins were minimally degraded during wet baking. This study found that AOH and ALT were degraded slightly after 1 h at 230 °C, but AME was stable at all times and temperatures tested with wet baking. However, significant degradation occurred upon dry baking for all mycotoxins, though AME was observed to be the most stable, followed by AOH and ALT. Assuming that the heat treatment parameters applied to tomato samples in this work could be similar to the wet baking, their findings are partially in accordance to our results. However, in contrast to what they reported, it has been observed a significant AOH reduction when samples were heated at 100 and 110 ºC. Combina et al. (1999) also evaluated the effect of heat treatment on the stability of AOH, AME and TeA in sunflower flour. They reported that concentrations of AOH and AME remained constant when heating samples at 100 ºC (humid heat) for up to 90 minutes, while TeA concentration decreased with time to 50% after 90 minutes. In their study, the most effective treatment for reducing AOH and AME levels was heating samples at 121ºC for 60 minutes combined with a pressure of 0.1 MPa, which is not completely in accordance with what Siegel et al. (2010) reported, as they needed higher temperatures to get some effect on the mycotoxin stability. However, the combination of pressure with high temperatures may have helped to decrease the initial mycotoxin level. Kabak (2009) described that there are several factors that may play a significant role in the stability of mycotoxins, such as the initial level of contamination, the type and concentration of the mycotoxin, the heating temperature together with the time employed, the degree of heat penetration, as well as the moisture content, pH and ionic strength of food, among other factors. The differences observed among the few studies available may be explained by some of the above mentioned factors.

The future research of *Alternaria* mycotoxin stability should be a deeper analysis on the novel degradation compounds formed product of their own degradation and their toxicity. In this sense, Siegel et al. (2010) found that two chemical

compounds were formed, 6-methylbiphenyl-2,3′,4,5′-tetrol and 5′-methoxy-6-methylbiphenyl-2,3′,4-triol, from the degradation of AOH and AME under wet baking conditions, respectively. Nevertheless, the toxicological properties of the products of AOH and AME degradation are yet unknown, as they have been recently described. Thus, further studies on the toxic effects of the potential breakdown products of mycotoxins are necessary.

In this study, we have demonstrated that AOH and AME, two of the most frequent *Alternaria* mycotoxins in food and feed, are quite stable along the food production and processing chain. During the storage, neither of the two mycotoxins studied were completely destroyed. For AOH, a temperature of 35 ºC has been necessary to achieve a significant reduction of the initial concentration, although after 12 hours of storage at 35 ºC, the concentration did not decrease further, and remained constant. The minimum value of remaining AOH after the storage step was 56.33%. Regarding AME stability, statistical analyses have shown that the temperature at which tomatoes are stored does not have any significant effect on its stability. Results showed that there were significant differences between the controls and the rest of treatments though as described for AOH, prolonging the period of storage did not have a major effect on its stability. Dealing with the heat treatment, temperatures of 100 or 110 ºC significantly affect the stability of AOH. However, these heat treatment processes could not completely destroy AOH after 90 minutes of heating. Notwithstanding, AME appears to be stable when exposed to the different heat treatments employed in this study. The greatest reduction of AOH and AME occurs at the washing step. Thus, to have a good control of *Alternaria* mycotoxins, it would be recommendable to reinforce this important step in the food industry. It is important to continue the testing for the presence and levels of *Alternaria* mycotoxins in food and feed products, to increase the knowledge on the stability and the toxicity of *Alternaria* mycotoxins, and provide an estimate for public health risks for consumers.

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Chapter VII

Effects of UV radiation on alternariol and alternariol monomethyl ether levels

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Under preparation

Effects of UV radiation on alternariol and alternariol monomethyl ether levels

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Abstract

Alternaria spp. are able to produce several mycotoxins that are harmful for human and animal health. Two of the most common mycotoxins synthesized by *Alternaria* spp. are alternariol (AOH) and alternariol monomethyl ether (AME). The UV radiation is an already commercially applied technique used for reducing bacteria in unpasteurized food products and, additionally, it has also been used for the reduction and elimination of patulin. In this study it has been assessed the effects of UV radiation on AOH and AME with the purpose of using this technique for reducing both *Alternaria* mycotoxins in the food industry. However, results showed that neither AOH nor AME are affected by the UV radiation. Interestingly, it has also been observed that *A. alternata* could biosynthesize a precursor of AME in fungal cultures, which could be transformed to AME when mycotoxins extracts are exposed to the UV radiation.

Keywords: *Alternaria* spp.; AOH; AME; UV radiation

1. Introduction

The use of UV light has been largely used for water treatment, air disinfection and surface decontamination. Nowadays, UV radiation is also an already commercially used technique to reduce the levels of microbial contamination for a wide range of liquid foods and beverages. In 2000, the U.S. Food and Drug Administration (FDA) recognized UV light treatment as an alternative for thermal pasteurization of juices and beverages (FDA, 2013). Since then, the application of this non-thermal technology has increased, in part, thanks to the advantages that UV radiation offers, which include the development of more fresh products with enhanced nutritional properties as no thermal technology is applied, the low energy requirements, reduced initial investment and do not result in unacceptable organoleptic changes in comparison with thermal pasteurization (Usaga et al., 2014).

Recently, some authors have used the UV radiation to reduce the levels of specific mycotoxins, such as patulin, and at the same time reducing microbial contamination. In previous studies Dong et al. (2010) and Assatarakul et al. (2012) investigated the effects of UV treatment on patulin that had been artificially added to apple cider and to apple juice from concentrate. Results were quite promising since in both cases UV treatment was presented as a possible commercially viable alternative for the reduction and possible elimination of patulin in fresh apple cider and apple juice achieving a significant reduction of the initial level of patulin without producing quantifiable changes in the chemical composition or organoleptic properties of the cider or juice.

The CiderSure 3500 is one of the most commonly used UV juice processing units in the United States for the nonthermal processing of apple cider and fulfills the 5-log performance standard established by the federal juice HACCP regulation (Durak et al., 2012). The CiderSure 3500 UV radiation system (Fig. 1) is composed of a stainless steel outer housing surrounding an inner quartz tube

designed to carry the intended product. The juice passes through the center of eight germicidal (UV wavelength of 254 nm) low-pressure mercury lamps placed concentrically around the quartz tube. Two UV radiation sensors monitor the amount of UV radiation reaching the product every 50 ms, and the flow rate is optimized continually to ensure uniform UV radiation. The passage of the cider through the apparatus results in a temperature increase of less than 0.06 ºC (Dong et al., 2010). The UV intensity that radiates the food product is set at 254 nm.

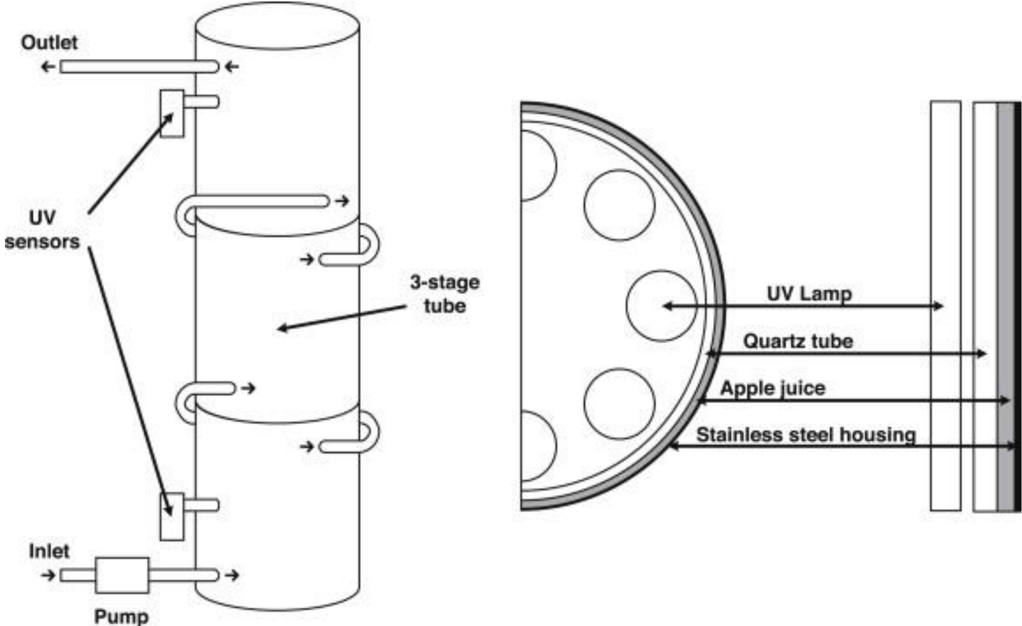

Figure 1. Schematic of the CiderSure 3500 commercial UV juice-processing unit (Usaga et al., 2015).

Ultraviolet doses were calculated by the following equation:

$$\text{UV dose (mJ/cm}^2\text{)} = [\text{irradiance}] \times [\text{exposure time}]$$

The irradiance was determined by multiplying the system's internal UV sensor readings by the radial factors, reflection factor, and absorption factor. Using these parameters, the UV dose for a single pass through the device was calculated to be

14.2 mJ/cm^2. If the juice was passed several times through the apparatus, the energy was accumulated what means that, for instance, if we pass a juice seven times through the apparatus, the total cumulative energy will be 99.4 mJ/cm^2.

With these promising results, the main goal of this study was to study the effects of UV radiation on both assessed *Alternaria* mycotoxins, AOH and AME.

2. Material and methods

Our particular aim was to know the effects of UV radiation on the stability of two *Alternaria* mycotoxins, AOH and AME. For this purpose, before assessing the effects of UV on a tomato juice artificially spiked with AOH and AME, an *in vitro* assay was carried out. From ten different PDA plates inoculated with a mycotoxigenic *A. alternata* strain (CBS 116.329, AOH and AME producer) and incubated at 26 ºC in darkness for 7 days, a mycotoxin extraction was carried out by resupending the mycelia grown on the PDA in 15 mL of an acetonitrile-methanol-water solution (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric acid). Samples were contained in falcon tubes and shacked with a vortex for 10 minutes to favor AOH and AME extraction. The extract was filtered with laboratory vacuum filters and the resultant extract was evaporated under a nitrogen flow. The dried extract was finally resuspended in miliQ water. Five mL of this solution were placed in an empty Petri dish. The Petri dish was placed opened inside a UV box unit (84x24x26 cm) that contained four UV lamps (254 nm, Enaqua, Vista, CA) positioned approximately 15 cm above the sample treatment area. Five-hundred µL of the solution were taken at different times (0, 1, 2, 5, 10, 20, 30 and, 60 minutes) and then all the volume was dried in a speedvac concentrator at 35 ºC. Samples were injected on the HPLC.

3. Results and discussion

Results obtained for AOH and AME were quite different from those obtained with patulin as it seemed that UV did not have any effect on AOH or AME, at least in an aqueous solution. Interestingly, while AOH levels were stable for all exposed times, AME seemed to increase (Fig. 2).

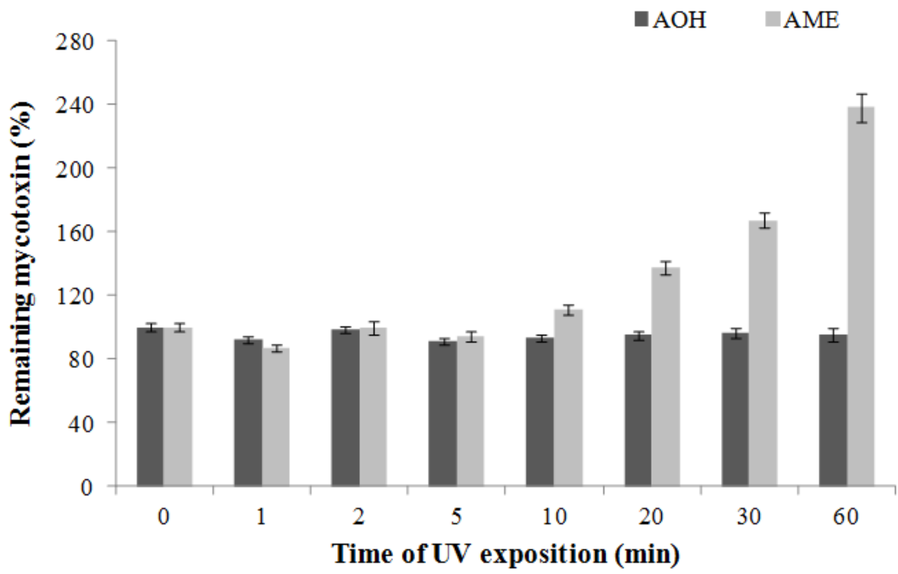

Figure 2. The remaining percentage of AOH and AME as a function of UV exposure.

Results described by Dong et al. (2010) for apple cider spiked with patulin (initial concentration of approximately 1,000 ppb) showed a toxin reduction of 43.4% after having passed through the CiderSure 3500 apparatus seven times. Each pass resulted in a significant difference of the patulin levels when compared with the previous pass. Results obtained by Assatarakul et al. (2012) were even better, in which, the UV treatment was applied to apple juice artificially spiked with patulin instead of apple cider (1,000 ppb approximately). After seven passes the remaining patulin was 5.1%, which means high inactivation efficiency. Hence, from an initial concentration of around 1000 ppb, the UV radiation treatment had achieved a

reduction of patulin to around 50 ppb, which turn to be the maximum limits in apple concentrate regulated in the United States (FDA, U.S. Food and Drug Administration. 2004). However, findings of this work seemed to indicate that UV radiation does not work for AOH and AME.

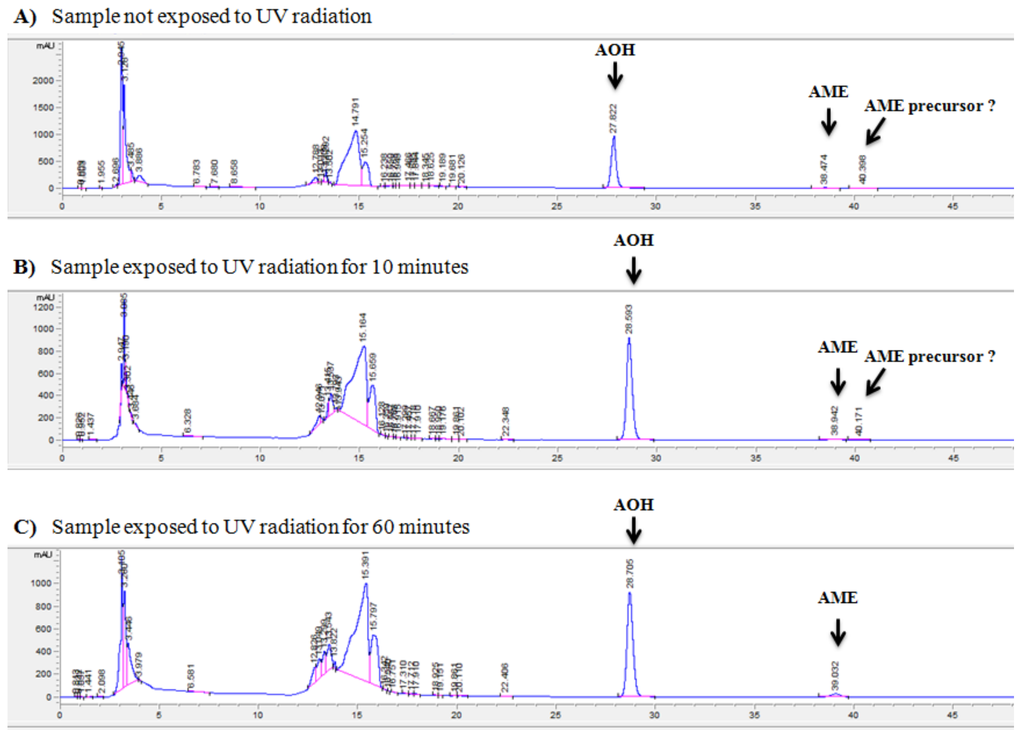

Figure 3. HPLC chromatograms of *Alternaria* mycotoxin extracts resuspended in water and exposed to UV radiation. A) HPLC chromatogram of a sample not exposed to any UV radiation. B) HPLC chromatogram of a sample exposed to a UV radiation for 10 minutes. C) HPLC chromatogram of samples exposed to a UV radiation for 60 minutes.

Dealing with the increasing peak of AME after a continuous UV radiation exposition, it was interesting to observe that in the HPLC chromatogram there was a peak very close to AME that disappeared as the time to UV exposition increased (Fig. 3). Our hypothesis was that in our mycotoxin extract, obtained from plate cultures, we had three different components of interest: AOH, AME and a

glycoside or sulfate precursor of AME. It could be possible that when the UV radiation was applied to samples, AME conjugates broke through photodegradation and that could be the reason of the increase of the AME peak.

Figure 4. Structures of AME and their possible conjugates. Adapted from Mikula et al. (2013).

There is evidence that conjugated toxins can be formed during plant metabolism. These mycotoxins are referred to as masked mycotoxins (Berthiller et al., 2013) and can pass unnoticed by conventional analytical techniques because their structure has been changed by the plant. Formation of glycosides and sulfates of several mycotoxins has been reported but, glucosylated *Alternaria* toxins have not been detected so far in relation to *Alternaria* fungi. Nevertheless, AME-3-β,D-glucoside was recently isolated from *Lysimachia clethroides* (Liang et al., 2013). Sulfated *Alternaria* mycotoxins (Fig, 4, e.g., AME-3-sulfate) were first isolated from extracts of *Alternaria* spp. found in the medicinal plant *Polygonum senegalense* and described as cytotoxic metabolites after testing *in vitro* against lymphoma cells (Aly et al., 2008; Mikula et al., 2013).

In this particular study, we cannot talk about masked mycotoxins, since no plant metabolism is involved because cultures were grown on PDA plates, but there

could exist the possibility that *A. alternata* biosynthesized a precursor of AME and this chemical compound has been modified with the UV radiation. From this chemical change, AME turned to be visible on the HPLC chromatogram. However, the identity of this supposed precursor should be verified with an UPLC-MS/MS.

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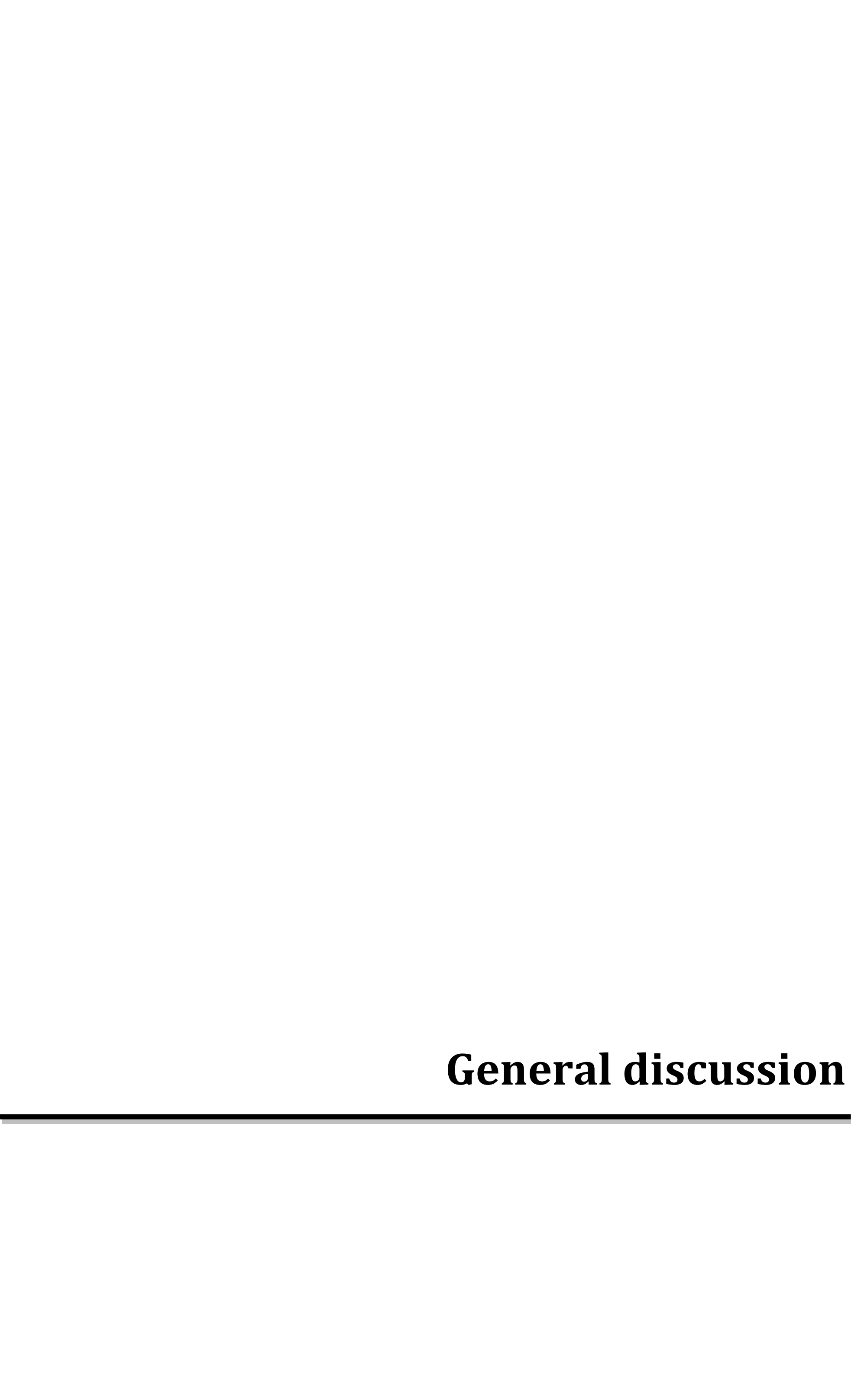


General discussion

The overall aim of this Doctoral Thesis was framed by the recommendations reported by the EFSA in the scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. This report gave rise to the idea of developing an entire project to offer a broad approach, from field to fork, to some of the issues that may worry farmers in crop fields and during the storage of raw products and, in addition, those issues that the food industry usually faces, but always bearing in mind that we were dealing with a health concern for humans and animals. An overview of the main results is presented and discussed in this section.

1. The state of the art in *Alternaria* contamination of food

The first step of this work was to assess the problem of *Alternaria* contamination in our own region. The province of Lleida, which is usually referred to as the agrarian province of Catalonia, includes strong regions of agricultural producers of several fruits and vegetables, such as apples, peaches, pears and apricots, among many others. A great part of the rural population lives from the agrarian sector and Lleida's landscape is characterized by its crop fields. The agrarian sector is reinforced by many food industries, usually devoted to the transformation and processing of raw fruits and vegetables.

Tomatoes were chosen as the food matrix to focus all the experimental assays developed in this work, since it is a fruit highly consumed worldwide, either fresh or as their by-products such as sauces, ketchups, purées or juices. In 2014, 6354 tons of tomatoes were produced in the province of Lleida, representing 12% of total tomato production in Catalonia (IDESCAT, 2014). Additionally, tomatoes are highly susceptible to fungal invasion, due to their thin skin, and *Alternaria* has

been reported to be the most common fungus found on moldy tomatoes (Andersen and Frisvad, 2004; Barkai-Golan and Paster, 2008b).

Tomato samples were collected from two different industries over two harvest seasons, from 2012 to 2014. Samples were picked from different stages of the industrial production chain. One of the industries worked only with organic raw fruits. The aim of this study was to investigate the fate of viable conidia from *Alternaria* spp. throughout the industrial processing chain and, additionally, the occurrence of AOH and AME in food samples collected, either in the intermediate stages or in the end products already prepared for human consumption.

To assess the presence of *Alternaria* spp. in food samples collected from these two food industries, a PMA pretreatment of samples, combined with a qPCR analysis (PMA-qPCR), was used to detect and quantify only viable cells (Fig. 5). Since its first description (Nocker et al., 2006), PMA has been applied to a wide variety of microorganisms, including bacteria, yeast, viruses, and fungi. Traditionally, classical culturing methods, conventional PCR or qPCR, were commonly used in food safety to detect the presence of foodborne pathogens in raw fruits or vegetables. Nowadays, classical culture techniques are usually being replaced with nucleic acid-based methods, which can detect, identify, and quantify more quickly. However, one of the limitations of PCR and qPCR is the inability to discriminate between live and dead cells, resulting in an overestimation of the target microorganism. To bypass this problem, the use of PMA has been described as effectively discriminating between viable and non-viable cells.

The safety and quality of food products cannot be assured by just testing for specific foodborne pathogens at the beginning and at the end of the industrial process. Although controls at these points are necessary, they should be reinforced with preventive measures that aim to avoid contamination during the industrial processes. In this respect, the implementation of manufacturing processes that inactivate or eliminate foodborne pathogens during the industrial chain could be

very useful. For both the food industry and the food authorities, it is important to develop effective and rapid techniques to monitor the effects of food manufacturing processes. In this respect, the use of PMA-qPCR can be used to evaluate the efficiency of decontamination food processes, as it can discriminate between live and dead cells. Thus, PMA-qPCR may be suitable to monitor the disinfection efficacy of hypochlorite, benzalkonium or ethanol, and to assess the efficiency of treatments such as pasteurization, UV treatments or ultrasonication of water (Elizaquível et al., 2014; Nocker, Sossa-Fernandez et al., 2007; Rudi, Moen et al., 2005; Rudi, Naterstad et al., 2005; Soejima et al., 2012; Zhang and Yu, 2010). However, there is still a need for improvement of some food matrices and some disinfection treatments.

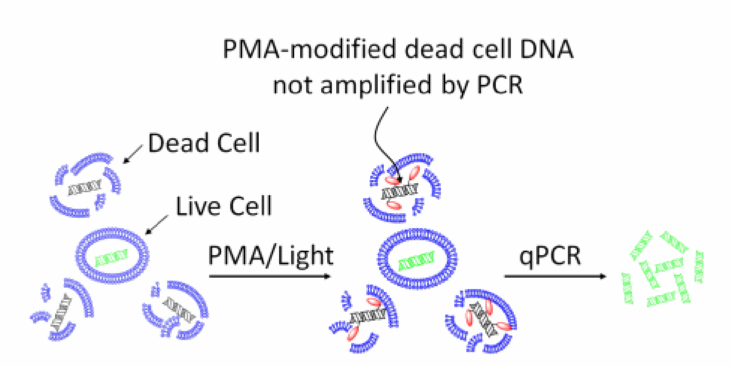


Figure 5. PMA-qPCR mechanism for selective detection of live cells. Image from www.biotium.com

For this study, a total of 175 tomato samples collected from different stages of the industrial processing of two different food industries were analyzed between 2012 and 2014. Fig. 6 describes the main results obtained for this study.

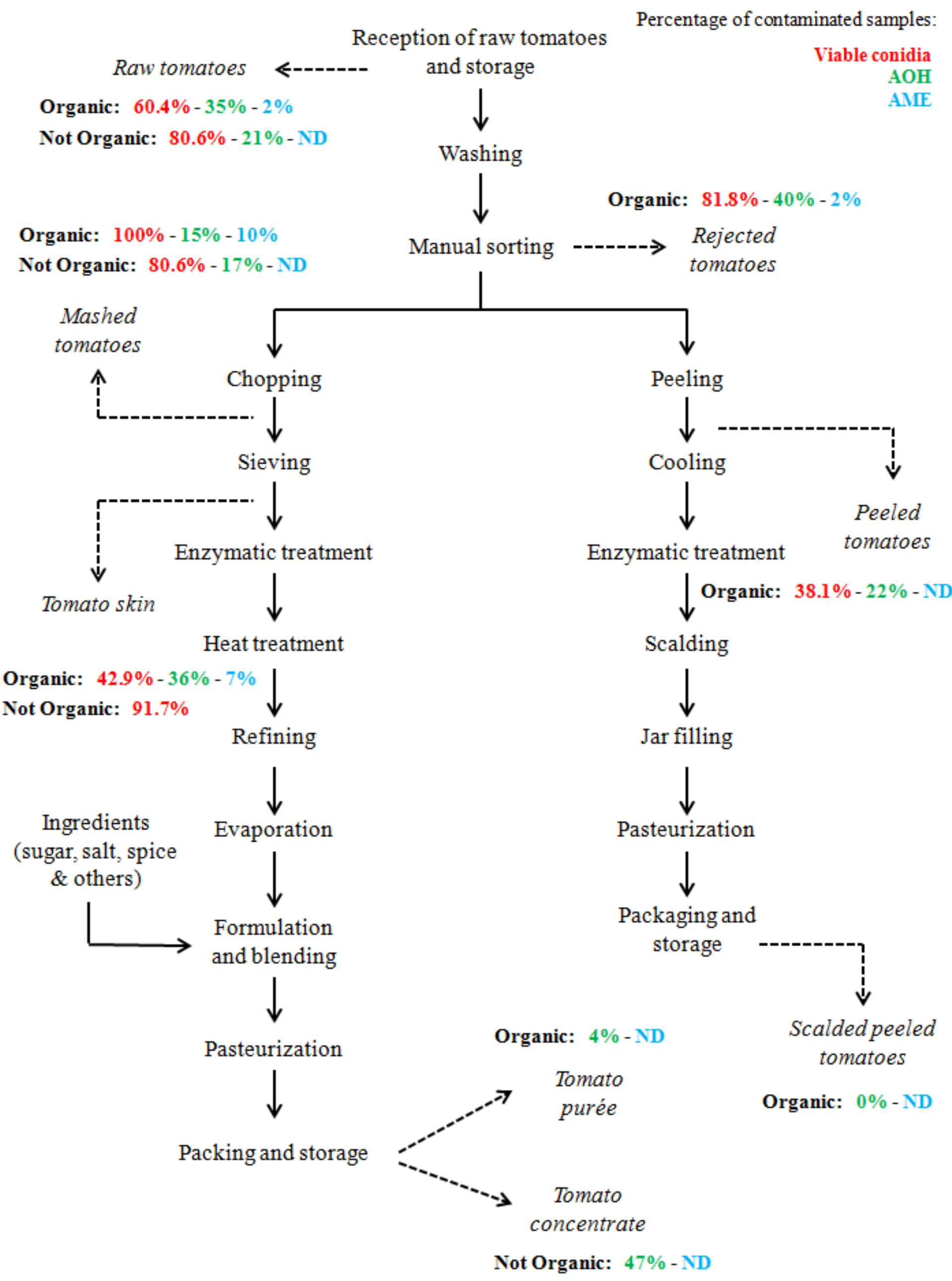


Figure 6. Summary of the results obtained from collecting tomato samples between 2012 and 2014 from two different industries. The results for samples positively contaminated with viable *Alternaria* spp. are shown in red. The percentage of positive samples contaminated with AOH and AME are shown in green and blue, respectively.

Results demonstrated the presence of viable *Alternaria* spp. in 127 out of 175 samples, meaning that 72.6% of the samples were contaminated. From among these contaminated samples, 57.5% corresponded to samples picked from the organic industry, while 42.5% were collected from the non-organic company. Tomato samples were collected from different intermediate stages of the industrial process. In fact, one of the problems that arose when collecting samples was that the industrial process was so automated, especially in the non-organic industry, that we could not collect samples from all the stages that we desired and, therefore, the groups of samples from both industries were different. Another important issue of this study was the high heterogeneity among samples from the same group. It should be taken into account that samples were picked on different days over two seasons and, depending on the day, the ripening stage of fruits and the percentage of rotten tomatoes were different, which may affect the total contamination of *Alternaria* spp. Additionally, depending on the ripening stage of the fruit, infection could be more severe. All these variables may influence fungal concentration levels. Despite the variability of *Alternaria* concentration, black mold disease was observed in many tomatoes when collecting samples.

The presence of *Alternaria* spp. in the food industry is not desirable in any industrial process, because it may mean that, for the processing, raw contaminated fruits have been used and, considering that *Alternaria* is considered a potent allergen, it may affect consumer health. However, the real problem for human and animal safety does not come from fungal cells, which will be destroyed during heat treatments, but rather from mycotoxins that viable *Alternaria* spp. may produce in the field or during storage.

In this study, the presence of AOH and AME was also assessed in two different industries responsible for the transformation of raw tomatoes into different end products (tomato concentrate, scalded peeled tomatoes and tomato purée). The presence of both mycotoxins during the industrial production chain was observed, although their levels decreased as the process moved forward. While almost no

mycotoxin contamination was found among the end products collected from the organic industry, 47% of the final tomato concentrates from the non-organic industry were contaminated with *Alternaria* spp.

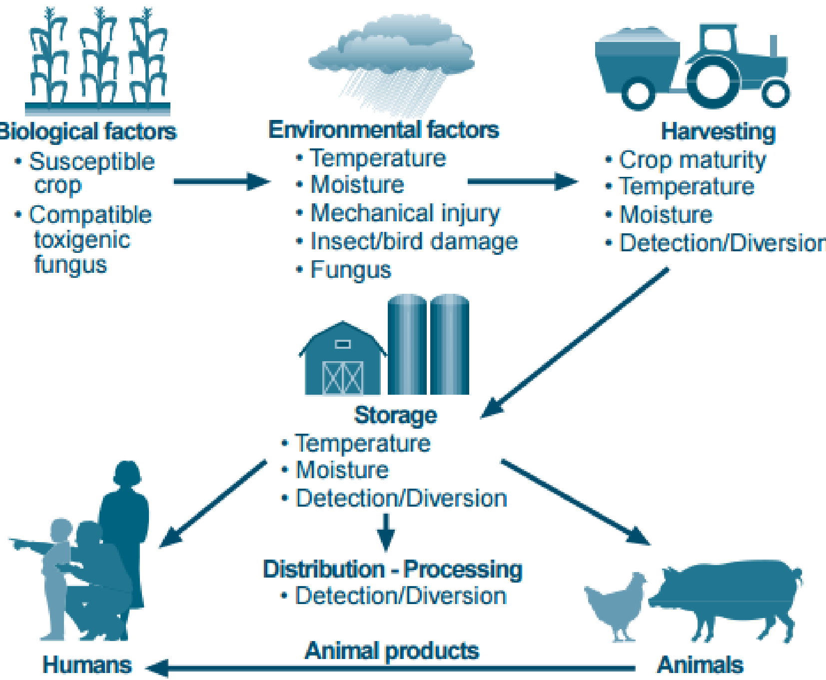


Figure 7. Factors affecting mycotoxin occurrence in the food and feed chain (CAST, 2003).

As illustrated in Fig. 7, several parameters can affect mycotoxin occurrence in the food and feed chain. In this respect, several reviews have suggested that climate change will affect the presence of mycotoxin in crop fields (Magan et al., 2011; Paterson and Lima, 2010; Paterson and Lima, 2011).

Climate change is expected to have a profound effect on the landscape worldwide, and the EU green paper on climate change in Europe has suggested that its effects could be either harmful or advantageous, depending on the region (Magan et al., 2011). In this respect, the Mediterranean zones, which include Portugal, Spain,

Southern France, Italy, Slovenia, Greece, Malta, Cyprus, Bulgaria, and Southern Romania, have been identified as a climate change hot spot, where extreme changes in temperature may occur. The annual mean temperature could increase in the order of 4–5 °C. Unfortunately, climate change does not involve just changes in temperature. It has been predicted that water availability could decrease, endangering hydropower accessibility, especially in summer. This effect, combined with the rise in temperature, could induce: (i) decreased agricultural yields (in the range of 10–30% in many regions of the South), (ii) drought, (iii) heat waves, (iv) soil and ecosystem degradation, and (v) eventually desertification. The increase in violent precipitations will augment erosion and loss of organic matter from soil. This may result in an increase in the risk of migration of pathogens, effects on plant physiology, and impacts due to invasion by pests and pathogens (Magan et al., 2011; Miraglia et al., 2009).

The consequences of climate change regarding fungal growth and mycotoxin production as an effect of changing temperatures could also vary depending on the region. In areas where the temperature will become too high for fungi to grow and to produce mycotoxins, a decrease in mycotoxin production can occur. On the other hand, in areas where it is now too cold for the fungi, the increase in the temperature due to climate change can lead to the presence of fungi on crops which did not have these problems before (Van de Perre et al., 2015). In this respect, Van de Perre et al. (2015) described that, as regards *Alternaria* spp. growth, given the more extreme scenarios of the RCP (6.0 and 8.5, Representative Concentration Pathways), in Spain there will be no significant difference in the near future (2031–2050) in relation to the growth of the mold when compared to the present. However, in the distant future (2081–2100), the mold growth could be significantly lower due to higher temperatures that may turn out to be too high for *Alternaria* to grow. A different scenario was presented for Poland, which is another important country for tomato production. In this case, all scenarios seem to indicate that, due to an increase in temperatures, growth of *Alternaria* spp. could significantly

increase compared to the present situation. It should be taken into account that the current cold temperatures of Poland do not favor *Alternaria* spp. growth and mycotoxin production, but that this could change in the near future. However, all these theories are just estimations based on mathematical models and, considering that many of the above-mentioned factors will interact among them, the effect of climate change on fungal growth and mycotoxin production can be very complex and difficult to predict. For tomatoes, several factors can alter the growth of molds on fruits and, in the same way, mycotoxin production can also vary. Colder night temperatures could lead to dew in the morning and, consequently, more cracks in tomatoes, resulting in more infestation of the fungi. More rain will also increase the contamination with fungi (Cotty and Jaime-Garcia, 2007).

If the temperature changes, it is very probable that the growth period of crops will also change. If the weather changes, it can also influence the irrigation, crop rotation and harvesting period. For example, the temperature in Spain during summer months could become too high to grow tomatoes, and thus the harvesting period could be changed to earlier months, although changing the season may also influence fungal contamination and mycotoxin production patterns. Thus, even though contamination of crops and food products with *Alternaria* mycotoxins is not a major concern at present in Spain, due to climate change it may become a great problem in the near future.

2. *Alternaria* in the food chain, from field to fork

2.1. Preharvest

Fungal contamination of crop fields is an age-old problem and an unavoidable situation representing an important issue for food safety and an economic issue for farmers. To confront this issue, additional information about fungi is needed. The

information arising from the use of novel tools such as genomics, proteomics and metabolomics provides us with the best and quickest opportunity to achieve a clear understanding of the survival of toxigenic fungi in the field, the ability of the fungus to invade crops, and the process of mycotoxin contamination under various environmental conditions.

Fungi are incredibly diverse, and recent efforts to sequence fungal genomes have demonstrated that they are rich in genes involved in the production of secondary metabolites. Since the discovery of aflatoxins produced by *Aspergillus* spp., significant progress has been made in understanding the molecular mechanisms leading to mycotoxin biosynthesis. Progress has also been made in the study of host crop resistance to fungal invasion. The information available on the production of aflatoxin is reasonably extensive, although there is still scarce information about the biosynthesis of *Alternaria* mycotoxins.

The use of new genomics seems to be the one of the keys for developing effective strategies to interrupt the fungal machinery for producing toxins, as well as to enhance host-resistance against fungal invasion. All these strategies may help to understand the genetic control and regulation of mycotoxin production by the fungus.

In this work, we used genomic techniques to i) highlight the role of two elements of the velvet complex in charge of developing essential functions of *A. alternata*, such as growth morphology, asexual development, mycotoxin production and virulence when infecting tomatoes, and to ii) investigate the polyamine metabolism of *A. alternata*. All this knowledge will be essential to explore new targets and new strategies for resistance management and, therefore, for developing new fungicides.

Although at present there are several registered fungicides for the control of the *Alternaria* spot disease, such as sterol-demethylation inhibitors (DMIs), benzimidazoles, and quinone outside inhibitors (QoI fungicides), over time it

has been discovered that plant pathogenic fungi can adapt to fungicide treatments by mutation, leading to resistance and loss of efficacy. This is the case of *A. alternata* contaminating nuts, or *A. solani* contaminating potatoes, which both achieved resistance against succinate dehydrogenase inhibitors (Avenot and Michailides, 2007).

2.1.1. Velvet complex

Genes encoding the enzymes responsible for the biosynthesis of secondary metabolites are often clustered in fungal chromosomes reminiscent of bacterial operons and, therefore, are often referred to as gene clusters. It is thought that the physical linkage of genes involved in the same biosynthetic pathway minimizes the amount of regulatory steps necessary to regulate the biosynthetic machinery and thereby contributes to physiological economization (Gacek and Strauss, 2012). In relation to the secondary metabolite gene clusters, there is a growing body of evidence suggesting that epigenetics could be the basis of their regulation (Palmer and Keller, 2010). It has been reported that gene clusters tend to be transcriptionally co-regulated by a variety of different genetic mechanisms, ranging from specific regulation by DNA binding transcription factors to global regulation via changes in chromatin structure (Gacek and Strauss, 2012; Palmer and Keller, 2010; Sarikaya-Bayram et al., 2015; Strauss and Reyes-Dominguez, 2011). Interestingly, global regulatory protein complexes involved in fungal differentiation processes, in response to environmental signals including light, nutrient deprivation or pH, have also been shown to regulate secondary metabolite gene clusters, which may link secondary metabolism to fungal development (Sarikaya-Bayram et al., 2015).

One of the protein complexes described as coordinating fungal development and secondary metabolism in several fungal species is the fungal-specific velvet domain transcription factor family, which includes the putative methyltransferase LaeA, VeA and VelB (Bok and Keller, 2004b; Calvo, 2008; Ni and Yu, 2007).

With the availability of whole genome sequences and reverse-genetics, other velvet-like proteins have been identified, such as VosA or VelC (Bayram and Braus, 2012). Fig. 8 illustrates the molecular complex formed by velvet proteins.

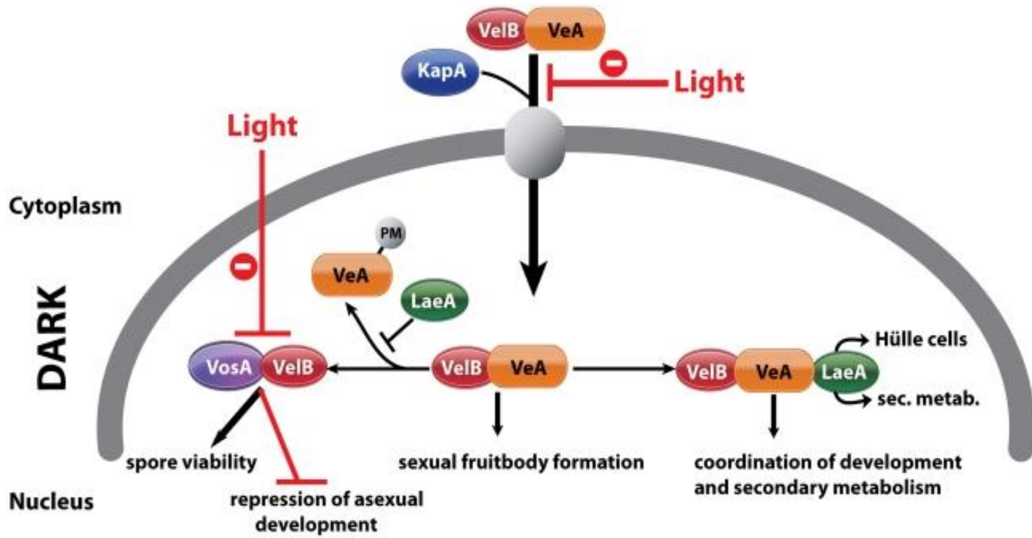


Figure 8. Molecular complexes formed by the velvet family regulatory proteins in the control of fungal development and secondary metabolite production. PM, posttranslational modification. Figure from Sarikaya-Bayram et al. (2010).

The present study is important, as it is the first report to describe the role of LaeA and VeA in two different strains of *A. alternate*, and results suggest that both components play a key role in coordinating secondary metabolism, and differentiation processes such as asexual sporulation, growth morphology and mycotoxin production.

The trimeric velvet complex, VelB–VeA–LaeA, is formed in the nucleus when the fungus is grown without light, since it has been described that VeA migration to the nucleus is light-dependent. In the dark, VeA is located mainly in the nuclei and, under light, VeA is found abundantly in the cytoplasm, reducing its entry to the nuclei by an unknown mechanism. The α-importin, KapA, is responsible for VeA translocation into the nucleus (Stinnett et al., 2007). VeA–VelB enter the nucleus

together, where they meet the methyltransferase LaeA, responsible for regulation of approximately 50% of the secondary metabolite clusters in several fungal species (Bok and Keller, 2004b). There is still a gap in knowledge about the molecular function of LaeA, although some groups have hypothesized that LaeA could regulate secondary metabolite clusters epigenetically through modification of chromatin structure, either directly or indirectly (Perrin et al., 2007; Reyes-Dominguez et al., 2010). VelB–VeA or VelB–VeA–LaeA might bind to a consensus sequence to activate sexual developmental genes as well as secondary metabolite gene clusters. However, VelB has a second function in which it can be bound to VosA, forming the heterodimer VosA-VelB. In dark conditions, this dimer can repress asexual spore formation, while in light VosA and VelB levels decrease and, consequently, it favors asexual development.

The main issue of this work has been the impossibility to describe a general model of velvet proteins for *A. alternata*, because we found that the loss of LaeA and VeA proteins, in some assays, led to a different phenotype for each *A. alternata* strain. Hence, their role could be quite different, even between strains of the same species. Table 3 and Table 4 synthesize the main results observed for each of the wild-type strains and their respective transformants for all the tests carried out in this study.

The different results observed for the two *Alternaria* strains in some of the experimental assays developed to characterize wild-types and *laeA* and *veA* knockouts could be due to the great genetic diversity among *Alternaria* strains. This diversity has also been reflected in the difficulty to speciate *Alternaria* isolates by only using morphological characteristics, as pointed out by several authors (Aradhya et al., 2001; Guo et al., 2004). It is thus quite likely that some of the strains are not classified properly in their species, or that the genetic diversity leads to a different phenotype in some of the parameters studied. In this respect, phylogenetic studies based on genetic material will be essential in helping with the classification of *Alternaria* isolates.

Table 3. Main phenotypic results observed for wild-type ATCC 66981 strain and its respective Δ*laeA* and Δ*veA* transformants.

	ATCC 66981		
	Wild-type	**Δ*laeA***	**Δ*veA***
	Light / Dark	Light / Dark	Light / Dark
Mycelial growth			
Conidia morphology	50% of the conidia were obclavate to obpyriform and four-celled and the other 50% were short and one-celled	Shorter conidia. Just one or two-celled conidia with a short beak	No differences with wild-type conidia
Colony growth	It had the biggest diameter	LaeA seems to be relevant for mycelial growth	It had the smallest diameter VeA seems to be relevant for mycelial growth
Conidia production	Produced the highest amount of conidia. No statistical differences between sporulation in the light or darkness	It drastically reduced conidia production by about 84% (light) and 65% (dark) LaeA positively regulates asexual development	It drastically reduced conidia production by about 92% (light) and 91% (dark) VeA positively regulates asexual development
Mycotoxin biosynthesis	It produced more AOH than AME	It produced the highest amount of AOH and AME	AOH and AME production was strongly inhibited
Gene expression		*pksJ* was upregulated in the light and darkness	*pksJ* and *cmrA* were downregulated in the darkness
Virulence - diameter	It showed the highest diameter of growth	It grew significantly less than the wild-type	It grew significantly less than the wild-type
Virulence - mycotoxin production	It produced more AOH than AME	It produced the highest amount of AOH and AME	AOH and AME production was completely inhibited

Table 4. Main phenotypic results observed for CBS 116.329 wild-type strain and its respective Δ*laeA* and Δ*veA* transformants.

	CBS 116.329					
	Wild-type		**Δ*laeA***		**Δ*veA***	
	Light	Dark	Light	Dark	Light	Dark
Mycelial growth						
Conidia morphology	50% of the conidia were obclavate to obpyriform and four-celled and the other 50% were short and one-celled		Shorter conidia. Just one or two-celled conidia with a short beak		No differences with wild-type conidia	
Colony growth	No differences compared to Δ*veA* KOs		It had the biggest diameter LaeA seems to act as a negative regulator of growth		No differences compared to the wild-type VeA did not have any significant impact on growth	
Conidia production	Produced the highest amount of conidia Colonies sporulated more in the darkness		Sporulation under the LOD LaeA positively regulates asexual development		Sporulation under the LOD VeA positively regulates asexual development	
Mycotoxin biosynthesis	It was a potent producer of both AOH and AME. It produced more AOH than AME		It reduced AOH and AME biosynthesis compared to the wild-type		AOH and AME production was almost completely inhibited	
Gene expression			*pksJ* was upregulated in the light and darkness		*pksJ* and *cmrA* were downregulated in the light and darkness	
Virulence - diameter	No differences compared to Δ*laeA* and Δ*veA* strains		No differences compared to the wild-type		No differences compared to the wild-type	
Virulence - mycotoxin production	It produced more AOH than AME		AOH decreased its production by 96% and AME 90%		AOH and AME production was completely inhibited	

2.1.2. Polyamine metabolism

Polyamines are essential metabolites, present in all living organisms. In fungi, polyamines support growth and regulate several biological processes, some of which are still unknown (Tabor and Tabor, 1983). Due to its indispensable role in fungi, polyamine metabolism of phytopathogenic fungi has attracted the attention of researchers, who have found in it a potential strategy to design new targets to control the problem arising from fungicide resistance.

In the present work, we have tried to investigate the fungal polyamine metabolism in three ways i) polyamine biosynthesis, ii) the role of putrescine and, iii) polyamine transport inhibitors.

One of the first strategies often used to manipulate and control polyamine metabolism is to inhibit the polyamine biosynthesis pathway, specifically the activity of the ODC, the rate-limiting enzyme on this metabolic route. For this purpose we used DFMO, a drug that inhibits the ODC irreversibly. The findings in this study support the hypothesis that ODC inhibition strongly limits fungal growth and mycotoxin production on *A. alternata*.

Someone might think that, if colonies are grown on a medium containing DFMO, supplemented with putrescine, AOH and AME production should be reverted. However, we did not observe this reversion in *A. alternata*. In our previous work, developed with *F. graminearum* (see Annex I), we found that, although DFMO reduced growth and DON production, these impacts were overcome with the addition of exogenous putrescine to the media (Crespo-Sempere et al., 2015b). Thus, in *A. alternata* two scenarios were considered regarding mycotoxin production. Firstly, we hypothesized that DFMO also targeted other regulation mechanisms or other routes of the secondary metabolism, including mycotoxins, whose impact could not be reverted by the addition of exogenous putrescine. Secondly, putrescine itself negatively affected mycotoxin production.

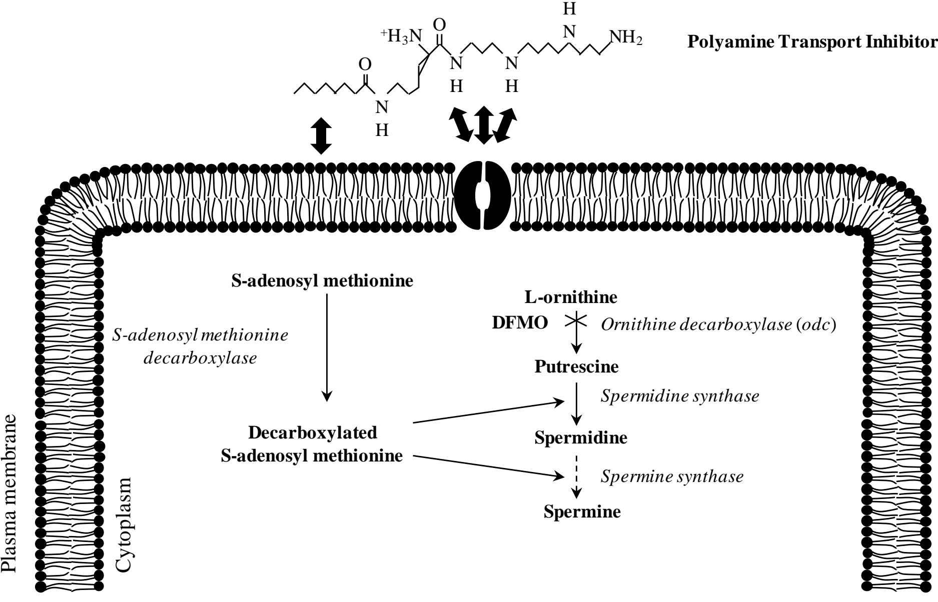


Figure 9. Polyamine biosynthetic pathway (Crespo-Sempere et al., 2015b).

To investigate the effect of putrescine on *A. alternata*, different concentrations of putrescine were added to the media and we found that increasing concentrations of putrescine led to decreasing levels of AOH production and sporulation and, in a lower proportion, a reduction in mycelium growth. Conversely, in our previous study with *F. graminearum*, it was observed that addition of exogenous putrescine increased mycotoxin production. We might have found an answer in the nitrogen source regulation of *A. alternata*. In this respect, we supported the notion that in *A. alternata*, when putrescine is added to the media, the nitrate repression system is activated and *areA* may participate in the nitrate-mediated negative regulation of gene transcription of AOH and AME biosynthesis.

It was interesting to find that DFMO strongly affected fungal growth *in vivo* (on culture plates) but, at the same time, we also observed that *A. alternata* could grow on tomatoes treated with DFMO. The reason could be that, although DFMO inhibited the activity of the ODC in the fungus, it did not achieve the goal of

repressing cell growth, because the fungus could overcome the blockage of the ODC enzyme by importing polyamines from tomatoes. Our next step was to use PTIs to block the transport of polyamines from tomatoes to fungal cells. For this purpose, we used a group of lipophilic polyamine analogs that potently inhibit the cellular polyamine transport. Interestingly, after assessing seven different PTIs *in vitro* on *A. alternata,* it was observed that two of them achieved the goal of controlling *A. alternata* growth, sporulation and mycotoxin production at the same time: AMXT-2455 and AMXT-3016. Unfortunately, neither of the PTIs was completely successful in controlling fungal tomato infection, although results were more promising when assessing both PTIs on artificially inoculated tomato plants. We concluded that polyamine analogs may be quite promising for controlling different diseases caused by several kinds of fungus in fields. Nevertheless, more information is needed to pinpoint the mode of action of polyamine analogs.

2.2. Postharvest

In this Doctoral Thesis, the effects of postharvest storage and postharvest treatments applied to fresh fruits to control their spoilage and maturity were considered of special interest. Control of tomato ripening is one of the main goals of producers and traders in tomato marketing, since accelerated ripening after harvesting leads to decay development. In this regard, the use of technologies that minimize or inhibit ethylene action enables postharvest life of tomatoes to be extended, this being a concern during transportation and commercialization.

2.2.1. Effects of 1-MCP treatments on the fungi

1-MCP is a commercial strategy already used to control ethylene production for the purpose of slowing the ripening of fruits. How ethylene and 1-MCP affect the physiology and quality of fruits has been broadly studied. Nevertheless, scarce literature considers the effects of ethylene and 1-MCP on fruit pathogens. Table 5

summarizes some of the studies carried out to date dealing with the effects of 1-MCP on different pathogens infecting fruits and vegetables.

The aim of the present study was to investigate how 1-MCP affected *A. alternata*, producing black mold rot on tomatoes. With this aim, growth development of *A. alternata*, and AOH and AME production, were assessed both *in vitro* and on three different tomato varieties.

Findings from this study indicated that 1-MCP treatment, in some varieties, negatively affects the resistance of tomatoes against *A. alternata* infection and, consequently, the disease produced by the fungus may be more severe when storage conditions are favorable for fungal growth. It was suggested that a possible explanation for the negative effects of 1-MCP regarding decay susceptibility is that small amounts of endogenous ethylene produced by fruits may be required to maintain their natural resistance against various environmental and pathological stresses, and therefore blocking the action of endogenous ethylene receptors by 1-MCP might have rendered fruits more susceptible to stresses, such as low temperatures and pathogen attacks (Porat et al., 1999). With *A. alternata*, it was proposed that ethylene displays a double signaling function with black spot disease, indicating that ethylene has different effects during the stages in which the pathogen is established. At the time of inoculation, ethylene may induce resistance responses in the fruit by elicitation of defense proteins, whereas after successful pathogen attack, ethylene may increase *Alternaria* toxin susceptibility and promote disease symptoms by inducing necrosis in the fruit (Itai et al., 2012).

With regard to the impact of mycotoxin production on 1-MCP treated fruits, no significant differences were observed among treatments in two of the three varieties studied, which could indicate that it is a fruit variety dependent factor and more varieties should be tested.

Table 5. Summary of 1-MCP treatment effects tested for several fungal pathogens on different fruits and vegetables.

Microorganism	Food matrix	Negative effects	Positive effects	No effects	Reference
A. alternata	Tomatoes	Negatively affects the resistance of fruits against fungal infection	-	No differences on AOH and AME production	Present work
B. cinerea	Tomato plants	Increased susceptibility of tomato plants to pathogens	-	-	Díaz et al. (2002)
Not specified	Strawberries	Reduced the storage life of fruits	-	-	Ku et al. (1999)
Not specified	Strawberries	Disease development was accelerated in fruit treated with high concentrations of 1-MCP	-	-	Jiang et al. (2001)
C. acutatum and *P. expansum*	Apples	Increased bitter rot and blue mold decay			Janisiewicz et al. (2003)
A. alternata, *B. cinerea* and *Fusarium* spp.	Tomatoes	-	Reduced disease incidence and severity of individual diseases	-	Su and Gubler (2012)
B. cinerea	Pears	-	-	Had a relatively small effect on the rate of lesion expansion	Akagi and Stotz (2007)
Molds	'Shamouti' oranges	Increased stem-end rots and mold rots	-	-	Porat el al. (1999)

2.3. Tomato industrial process

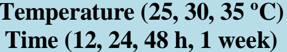
Fruits, vegetables and cereals may be contaminated by spoiling fungi in the field or during storage. When contamination has occurred, toxigenic fungi may begin the biosynthesis of mycotoxins. The spoiled products may pass through the selection stage accidentally and enter the food production chain. When this occurs, fungi can still be destroyed during heat treatment, but it is uncertain what happens to mycotoxins. In fact, there is scarce information on the stability and fate of

Alternaria mycotoxins during the food processing and storage operations. Hence, in this study we aimed to obtain information about the stability of AOH and AME during the industrial processing of tomato products in order to know which steps may require more attention so as to decrease the initial concentration of toxin contaminants.

The main drawback on conducting mycotoxin stability assays has been the use of samples contaminated artificially with the analytical standards of AOH and AME. The ideal situation would probably have been to work with naturally spoiled tomatoes with *Alternaria* spp. with a strong capacity to produce AOH and AME and to assess what happens to both mycotoxins under different conditions. However, when working with naturally spoiled products, it is difficult to control the initial concentration of contamination and it is quite complicated to obtain high levels of *Alternaria* mycotoxins under laboratory conditions.

Our findings showed that the destruction of AOH and AME during the different stages of tomato processing is limited and, therefore, none of the simulated industrial stages achieved a complete destruction of *Alternaria* mycotoxins. The greatest reduction of AOH and AME seems to occur in the washing step although, at this point, it is important to remember what we observed in one of our first studies. When we assessed the presence of viable *Alternaria* spp. and the occurrence of AOH and AME in two different food industries, we collected samples *in situ* and we observed that, after the washing step, samples were highly contaminated with *Alternaria* spp. In fact, fungal contamination increased after the washing, because the water was very dirty. Hence, even though mycotoxins are soluble in water and the washing step is a good option to remove the undesired mycotoxins, it is important that the water is clean for a good sanitization process that eliminates both mold and mycotoxins. Maybe the food industry should consider changing the water more often.

Table 6. Summary of stability results with cherry tomatoes regarding AOH and AME stability.



	AOH	**AME**
RECEPTION AND STORAGE	**Temperature (25, 30, 35 °C)** **Time (12, 24, 48 h, 1 week)**	
	Storage at 35 °C was necessary to achieve a significant reduction in AOH levels. Statistical analysis showed that the temperature at which tomatoes were stored did not have any significant effect. The maximum value of remaining AOH after the storage step was 75%, while the minimum was 56%.	AME was significantly reduced after 12 h of storage, while it remained constant later. Remaining percentages of AME were lower than AOH as the highest percentage was 67%, while the lowest was 49%.
WASHING	**Time (1, 2, 5, 10 min)** **Type of washing (tap water, 150 or 250 ppm concentrated sodium hypochlorite solution)**	
	There were no significant differences between washing tomatoes with a 150 or 250 ppm concentrated sodium hypochlorite solution. However, washing tomatoes with just water was significantly less efficient than washing fruits with a hypochlorite solution, at least if the washing was short (1 or 2 min).	AME was found to be more persistent on tomatoes washed with water, since 5 min were required to significantly reduce its initial concentration. After 10 min of washing, the initial concentration of AME decreased to 38%. AME could be more insoluble than AOH in aqueous solutions.
HEAT TREATMENT	**Temperature (80, 90, 100, 110 °C)** **Time (30, 60, 90 min)**	
	No significant reduction in AOH was observed at either 80 or 90 °C, while significant reductions occurred at 100 and 110 °C. At this temperature, treatments over 30 min did not lead to higher degradation.	No significant differences were found due to the temperature or the duration of the treatment. Therefore, AME seems to be completely stable when exposed to such heat treatments.

This last study was carried out during a PhD stay abroad in the Department of Food Science and Technology of Cornell University (NY State, USA) with

Professor Randy W. Worobo. When we were drafting the main objectives for the six-month stay proposal, one of them was to study the effect of UV radiation on tomato samples contaminated with AOH and AME with the aim of using UV treatment as a possible tool to help the food industry to prevent *Alternaria* mycotoxin contamination. Unfortunately, this study has not yet given rise to a scientific publication and, therefore, the main results are discussed in this section.

In previous studies, Dong et al. (2010) and Assatarakul et al. (2012) investigated the effects of UV treatment on patulin that had been artificially added to apple cider and to apple juice from concentrate. Results were quite promising, since in both cases UV treatment was presented as a possible commercially viable alternative for the reduction and possible elimination of patulin in fresh apple cider and apple juice. Results described by Dong et al. (2010) for apple cider spiked with patulin showed a toxin reduction of 43.4% after having passed through the CiderSure 3500 apparatus seven times (Fig. 10).

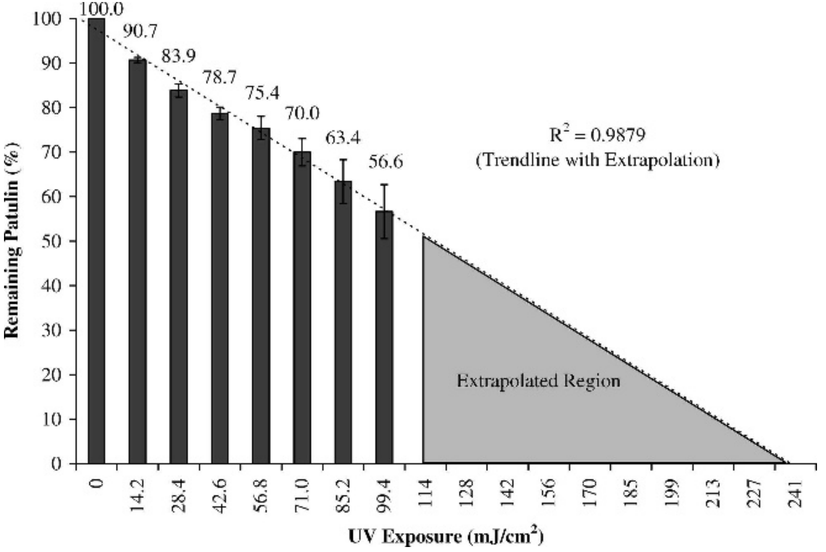


Figure 10. Relationship between percent reduction in patulin burden and UV dose with apple cider as a food matrix (Dong et al., 2010).

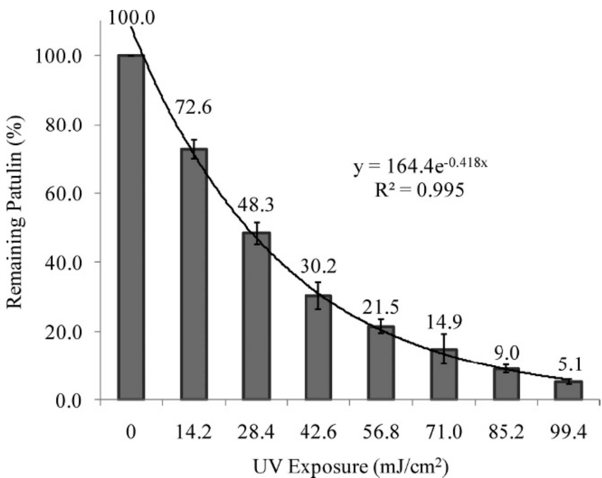
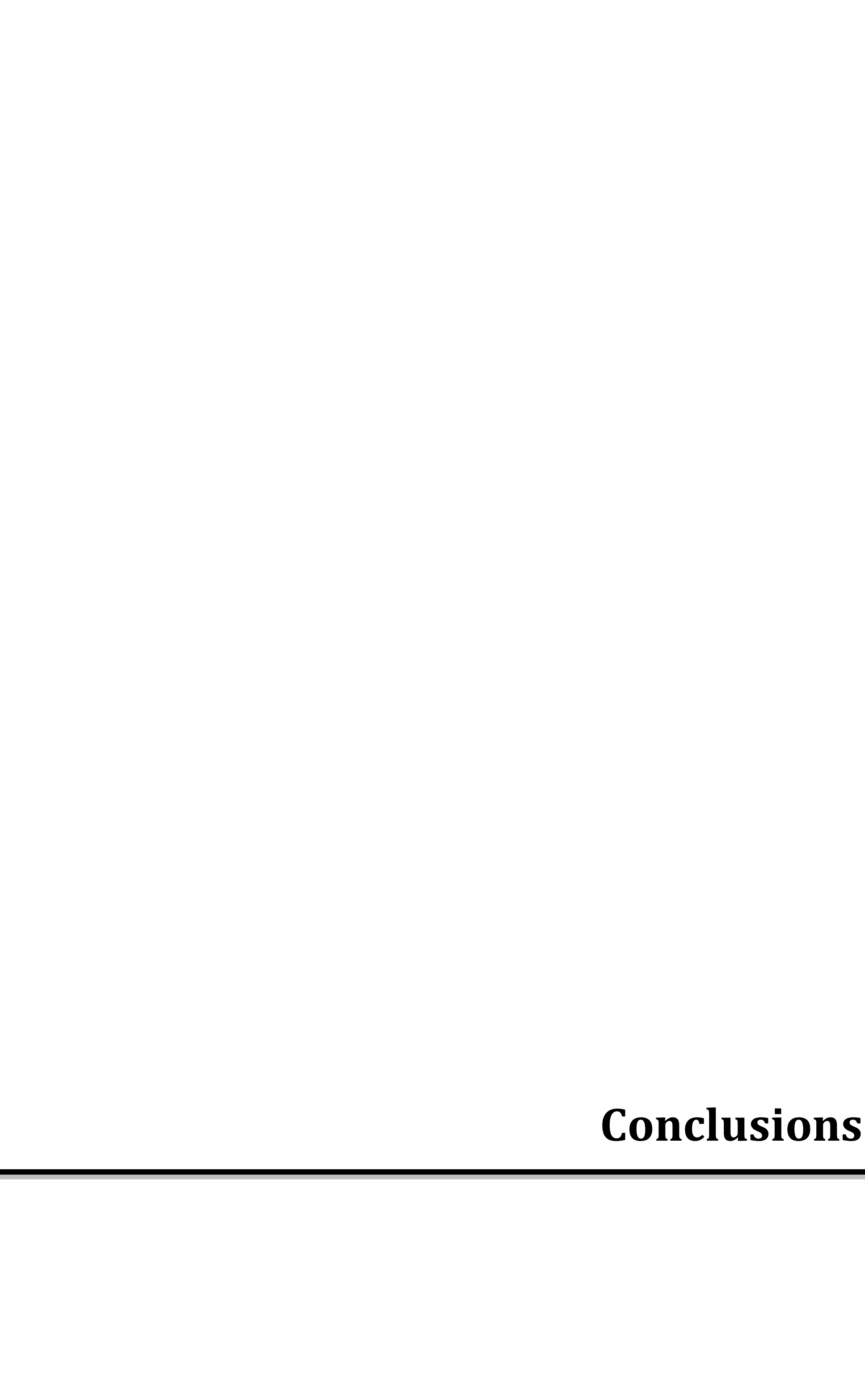


Figure 11. Percentage of patulin remaining as a function of UV exposure (Assatarakul et al., 2012).

Results obtained by Assatarakul et al. (2012) were even better. In this case, the UV treatment was applied to apple juice artificially spiked with patulin instead of apple cider. After seven passes, the remaining patulin was 5.1%, indicating high inactivation efficiency (Fig. 11).

Unfortunately, results described for AOH and AME have not been promising, as it has been observed that UV radiation does not seem to have any effect on AOH and AME. Interestingly, we observed that it could be possible that *A. alternata* biosynthesize a precursor of AME, which is modified under continuous UV exposure and, as a result, AME levels increase. However, this theory is just a hypothesis from a small test and more studies with UPLC/MSMS should be assessed to verify this idea.

This Doctoral Thesis has tried to deal with some of the aspects concerning foodstuff contamination by *Alternaria* spp. and some of the mycotoxins that these species can biosynthesize. Our main goal was to reinforce the existing information about the *Alternaria* contamination issue and its health concern. We are aware that there is still a long way to go and that more research needs to be undertaken in order to determine whether there is enough evidence indicating that the European Commission should consider the establishment of legislative measures to regulate the occurrence of *Alternaria* mycotoxins in food and feedstuff.



Conclusions

In response to the main objectives indicated at the beginning of this research, all the conclusions that we have drawn are detailed below:

Objective I: Assess the presence of *Alternaria* spp. and the occurrence of their mycotoxins in the food industry.

1. PMA-qPCR methodology has been shown to be a fast, sensitive, and efficient technique to detect DNA from viable *Alternaria* spp. cells in different types of tomato samples. This methodology can be useful to the food industry as a preventive tool to detect the risk of contamination in foodstuffs with potentially viable *Alternaria* spp. and, at the same time, as a good parameter to determine the quality of raw fruits that arrive in the food company. This study presents enough evidence of the presence of *Alternaria* spp. in tomatoes used for the production of tomato by-products. In fact, it has been observed that levels of viable *Alternaria* spp. increase once inside the production plant, probably because of the immersion of raw tomatoes in dirty water washing baths. Changing the water used for the washing step more often may be a good preventive measure to avoid an increase in the microbial load.
2. The presence of AOH and AME during the tomato production chain has also been observed, although their levels decrease during the production of tomato by-products, except with concentrated product. AOH was, by far, more predominant than AME, which was only found among the organic fruits. The non-organic food industry has shown significantly higher occurrence of AOH for raw tomatoes and for the end product compared to the organic industry.

Objective II: Investigate the main concerns in relation to *Alternaria* spp. and their mycotoxins from field to fork.

AT PREHARVEST:

Regarding Velvet complex:

1. As occurs with other fungi, LaeA and VeA are involved in essential functions of *A. alternata*.
2. Colony growth: in the case of the ATCC 66981 strain, LaeA and VeA seemed to be relevant for colony growth under both light and darkness, especially VeA. By contrast, for the CBS 116.329 strain, LaeA seemed to act as a negative regulator of colony growth, while VeA did not have any significant impact compared to the wild-type.
3. Asexual development: the loss of *laeA* and *veA* genes led to a drastic reduction of conidia production in both strains, suggesting that both velvet components could act as a positive regulator of asexual development, especially VeA.
4. AOH and AME biosynthesis: AOH and AME production is altered in *laeA* and *veA* knockouts. However, this alteration seems to be isolate/strain specific. While deletion of *veA* gene seems to strongly inhibit mycotoxin production in both strains, deletion of *laeA* increases the mycotoxin production levels in one strain (ATCC 66981), while decreasing them in the other (CBS 116.329).
5. Gene expression: *cmrA* gene was down-regulated in Δ*veA* mutants. Interestingly, higher down-regulation levels of *cmrA* correlate with less pigmented colonies and less sporulation. This may suggest that VeA could also be linked to melanin biosynthesis, acting as a positive regulator of the pathway.

Regarding polyamine metabolism:

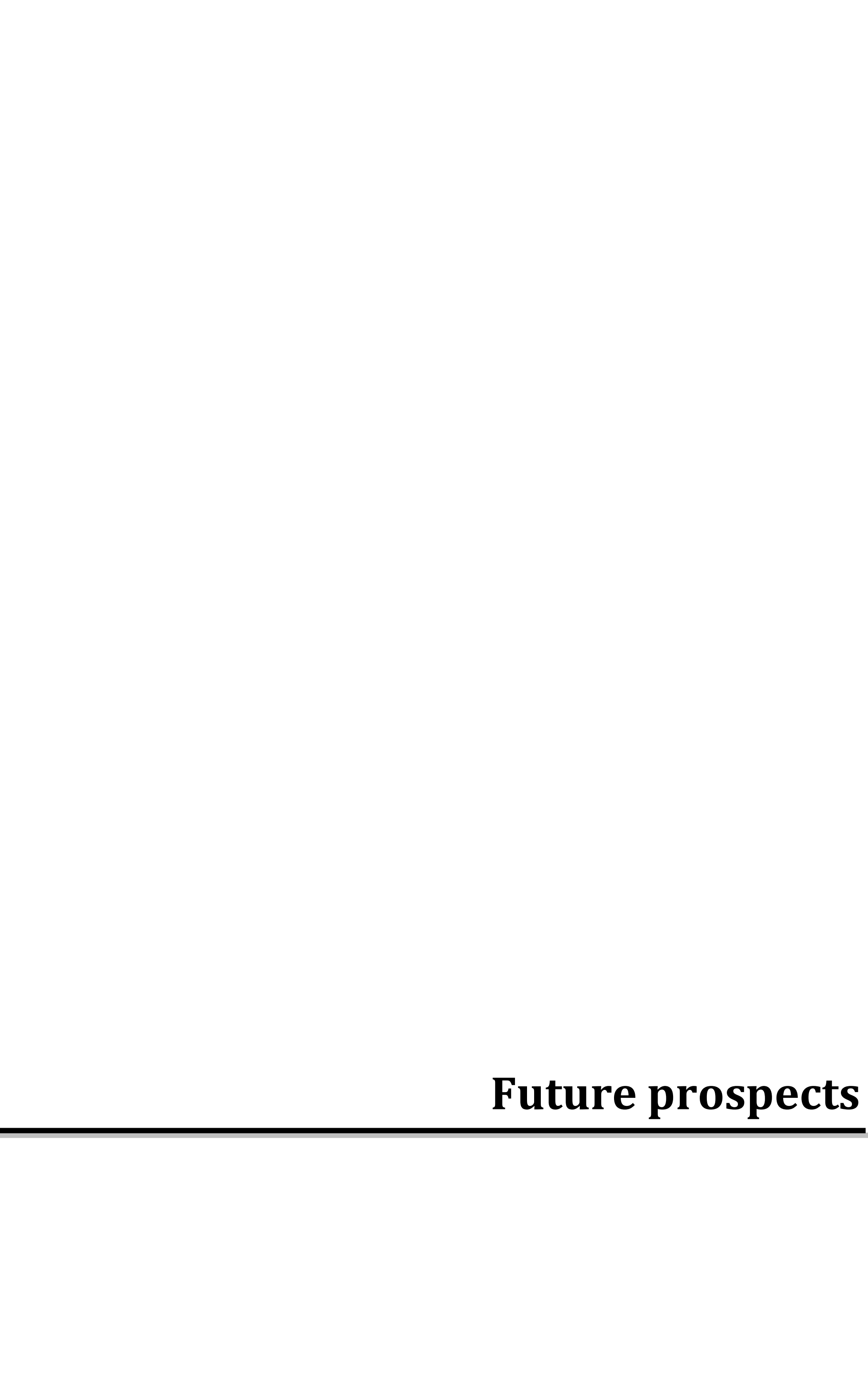
1. Inhibition of the polyamine biosynthesis pathway using DFMO decreases AOH production and fungal growth. Hence, polyamines might play an essential role in both biological processes.
2. Putrescine as a nitrogen source may affect several essential processes of the cell, such as sporulation, growth rate and mycotoxin production, probably because of the nitrate repression system that could be activated when putrescine is added to the culture media.
3. Two PTIs, AMXT-2455 and AMXT-3016, have achieved the goal of controlling *A. alternata* growth, sporulation and mycotoxin production *in vitro*.
4. On tomatoes, AMXT-2455 and AMXT-3016 failed to control *A. alternata* growth efficiently, probably because the surface was treated with the PTI but its penetration was insufficient to control the fungal development inside the fruit. The fungi thus penetrated the fruit and continued the infection.
5. On tomato plants, AMXT-2455 and AMXT-3016 achieved a reduction of the fungal infection compared to the control group of samples, although some leaves treated with PTIs still showed some dark spots caused by *A. alternata* growth. The concentration required should probably have been higher in order to achieve complete growth repression.

AT POSTHARVEST: 1-MCP negatively affects the resistance of three tomato varieties against *A. alternata* infection and, consequently, black spot disease produced by the fungus is significantly more severe on 1-MCP treated tomatoes when storage conditions are favorable for fungal growth.

1. 1-MCP treatment does not affect AOH and AME biosynthesis significantly in two of the three varieties studied, which could indicate that it is a fruit variety dependent factor, although more varieties should be tested.

IN THE FOOD INDUSTRY: AOH and AME are quite stable during the tomato production chain.

1. During storage, neither AOH nor AME were completely destroyed. For AOH, a temperature of 35 ºC was necessary to achieve a significant reduction in the initial concentration, although after 12 hours of storage at 35 ºC the concentration did not decrease anymore and remained constant. Regarding AME stability, statistical analyses have shown that the temperature at which tomatoes are stored does not have any significant effect.
2. In relation to the heat treatment, only temperatures of 100 or 110 ºC significantly affected the stability of AOH. However, the heat treatment process could not completely destroy AOH after 90 minutes of heating. On the other hand, AME seem to be completely stable when exposed to a heat treatment.
3. The greatest reduction of AOH and AME occurs at the washing step. It would thus be recommendable to reinforce this industrial step in the food industry in order to have good control of *Alternaria* mycotoxins.
4. UV radiation did not achieve a reduction in initial levels of AOH and AME in an aqueous solution.



Future prospects

The main goal of this thesis was to answer some of the requests announced by the EFSA in its scientific report "Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food" and, during these four years we have tried to reinforce the knowledge of *Alternaria* and its mycotoxins, either through a molecular way or via an analytical approach. As a product of this work, emerged this thesis that includes seven scientific publications. However, time is limited and research just the opposite and so, sometimes we have only reached the tip of the iceberg.

If I ever had the chance to continue this line of research I do believe it would be interesting to keep working on the **Velvet complex**. Some of the results achieved in this work were quite inconclusive and it would be interesting to continue the path that we started.

Another interesting line of research and, at the same time quite complex, would be to study **the role of the PksI in *Alternaria***, which has been suggested to have an essential role in the biosynthesis of AOH and AME. And, maybe little by little we would be able to decode the biosynthesis pathway of AOH and AME.

Results achieved with **polyamine analogues, AMXT-2455 and AMXT-3016,** were quite promising. Hence, it would be interesting to keep working on its mode of action and find those conditions that optimize their potential on the inhibition of *Alternaria* growth on tomatoes.

Hence, this thesis has settled down some basis that leaves the door open to several future prospects.

Annex

Annex I

Targeting *Fusarium graminearum* control via polyamine enzyme inhibitors and polyamine analogs

Ana Crespo-Sempere, Núria Estiarte, Sònia Marín, Vicente Sanchis and Antonio J. Ramos

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Targeting *Fusarium graminearum* control via polyamine enzyme inhibitors and polyamine analogs

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Abstract

Fusarium graminearum not only reduces yield and seed quality but also constitutes a risk to public or animal health owing to its ability to contaminate grains with mycotoxins. Resistance problems are emerging and control strategies based on new targets are needed. Polyamines have a key role in growth, development and differentiation. In this work, the possibility of using polyamine metabolism as a target to control *F. graminearum* has been assessed. It was found that putrescine induces mycotoxin production, correlating with an over expression of *TRI5* and *TRI6* genes. In addition, a homolog of the *Saccharomyces cerevisiae TPO4* involved in putrescine excretion was up-regulated as putrescine concentration increased while *DUR3* and *SAM3* homologues, involved in putrescine uptake, were down-regulated. When 2.5 mM D, l-α-difluoromethylornithine (DFMO) was added to the medium, DON production decreased from 3.2 to 0.06 ng/mm^2 of colony and growth was lowered by up to 70 per cent. However, exogenous putrescine could overcome DFMO effects. Five polyamine transport inhibitors were also tested against *F. graminearum*. AMXT-1505 was able to completely inhibit *in vitro* growth and DON production. Additionally, AMXT-1505 blocked *F. graminearum*

growth in inoculated wheat spikes reducing DON mycotoxin contamination from 76.87 µg/g to 0.62 µg/g.

Keywords: *Fusarium graminearum;* FHB; Deoxynivalenol; Wheat; Polyamines; DFMO

1. Introduction

Fusarium graminearum is one of the most important causal agents of the fungal disease Fusarium Head Blight (FHB) of wheat and other small-grain cereals that not only reduces yields but also reduces seed quality and contaminates grains with mycotoxins (Parry et al., 1995 and Stępień and Chełkowski, 2010). The disease occurs throughout much of the world and often with a devastating impact on wheat crops (Goswami and Kistler, 2004 and McMullen et al., 1997). The most common mycotoxins associated with *F. graminearum* are deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) which pose a health risk to humans and animals through food and feed with accumulated mycotoxins (D'Mello et al., 1999 and Desjardins and Proctor, 2007). However, DON is known to be the most commonly detected mycotoxin in cereal grains, usually at high concentrations (Canady et al., 2001). At a cellular level, DON can inhibit protein synthesis by binding to the ribosome and activating cellular protein kinases involved in signal transduction related to proliferation, differentiation, and apoptosis (Pestka and Smolinski, 2005). In mammals, DON can cause emetic effects, anorexia and immune dysregulation as well as growth, reproductive and teratogenic effects (Pestka, 2010).

To control fungal contamination, several management strategies have been employed, which include FHB-resistant wheat cultivars obtained by breeding processes or through transgenic approaches, chemical fungicides, biological control and cultural practices (Blandino et al., 2012 and Yuen and Schoneweis,

2007). However, under high inoculum conditions, chemical fungicides are the main measure to control the impact of the *F. graminearum* infection. Among the fungicides reported to be effective against FHB, triazols are the most used, which are sterol biosynthesis inhibitors (Paul et al., 2010). Nevertheless, since fungi are able to evolve and adapt, fungicide-resistant strains may arise. Indeed, a decrease of triazol fungicide efficiency has been found (Becher et al., 2010, Klix et al., 2007 and Yin et al., 2009). Additionally, several authors have reported that sub-lethal doses of some of these fungicides may increase mycotoxin production from *F. graminearum* (D'Mello et al., 1998, Müllenborn et al., 2008 and Ochiai et al., 2007). Consequently, there is a clear need to design control strategies based on new targets.

Over the past few years a number of studies have analyzed the role of polyamines (PA) in fungal metabolism, which has been recently reviewed by Valdés-Santiago et al. (2012). PA are low molecular weight aliphatic polycations with two or more primary amino groups, ubiquitously present in all living cells. PA are involved in growth, development and differentiation, being its concentration highly regulated. Main PA in fungi are putrescine, spermidine, and spermine, although some fungal species do not produce spermine. Interestingly, it has been observed that the PA biosynthetic pathway is induced in the wheat as an early response to FHB. Moreover, it has been reported that intermediates of the PA pathway promote DON biosynthesis (Gardiner et al., 2010 and Gardiner et al., 2009). In this study we have considered PA metabolism as a target to design new control strategies (Fig. 1). To this aim, we studied the effect of putrescine and a PA biosynthesis inhibitor (D, l-α-difluoromethylornithine) on growth, DON production and infection capacity. PA transport has a key role in the regulation of PA content in cell since fungi may import PA from wheat during the infection process. Thus, we also analyzed the gene expression of six proteins putatively involved in PA transport and the possibility of controlling *F. graminearum* with lipophilic PA analogs which potently can contribute to the inhibition of PA transport.

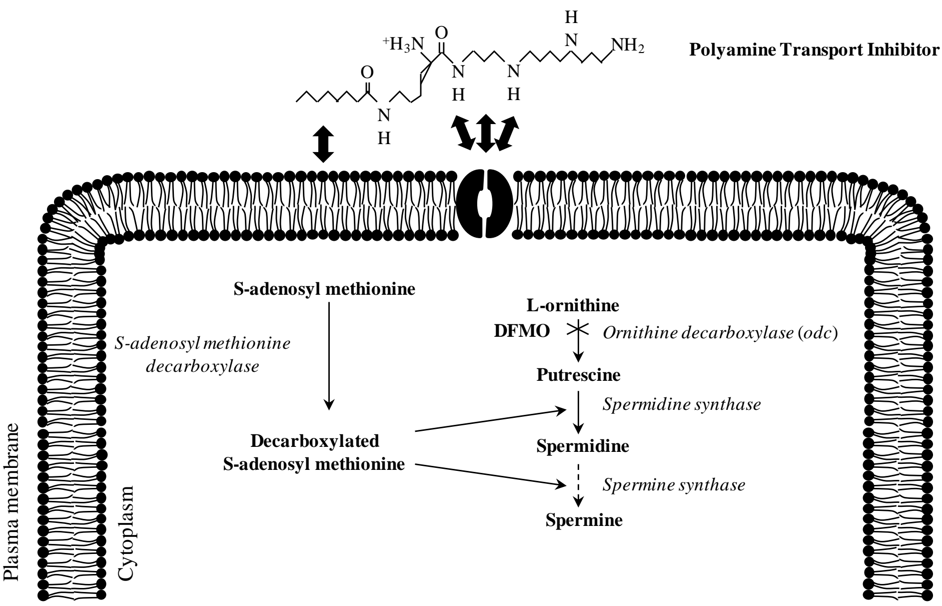


Figure 1. Polyamine biosynthetic pathway (Valdés-Santiago et al., 2012).

2. Material and methods

2.1. Strain and growth conditions

The *F. graminearum* strain used in this study was L1-2/3D, kindly provided by Dr. Belén Patiño from the Department of Microbiology III, Faculty of Biology, University Complutense of Madrid (Spain). This strain was isolated from Spanish wheat and identified by PCR using Fg16F and Fg16R primers (Nicholson et al., 1998). To enable production of macroconidia, the isolate was sub-cultured on plates containing, per liter, 30 g sucrose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl and 100 μL of trace element solution (per 100 mL, 5 g citric acid, 5 g $ZnSO_4 \cdot 7\ H_2O$, 0.25 g $CuSO_4 \cdot 5\ H_2O$, 50 mg $MnSO_4 \cdot H_2O$ and 50 mg H_3BO_3) under white light (12 h/12 h light/dark) at 26 °C for 6 days. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80, and adjusted to 10^5

conidia/mL using a Thoma counting chamber. Plates with the media mentioned above but containing 1 g of glutamine instead of 2 g $NaNO_3$, were inoculated centrally with 5 µL of conidia suspensions to study the effect of putrescine (SIGMA), D, l-α-difluoromethylornithine (DFMO) (Enzo Life Sciencies) and five Polyamine Transport Inhibitors (PTIs), detailed in Table 1, kindly provided by Aminex Therapeutics (USA).

Table 1. Polyamine transport inhibitors tested against *F. graminearum.*

Name	Structure
AMXT-1483	
AMXT-1505	
AMXT-2030	
AMXT-3016	
AMXT-3946	

The PTIs are lipophilic PA analogs, produced by N-acylation or N-alkylation of the ε-amine group of the lysine portion of the Lys-spermine conjugates (Burns et al., 2001 and Burns et al., 2009). The lipophilic PTIs would bind to the lipid membrane of the cell inhibiting the PA transport apparatus (Fig. 1). PTIs, DFMO, and putrescine were stored at −20 °C in the dark until needed. Stock solutions were prepared in water, filter-sterilized and added to autoclaved media. Putrescine concentrations assayed were 50, 500, 1000 and 5000 μM, the DFMO concentration tested was 2.5 mM based on concentrations used by Giridhar et al. (1997) and Rajam and Galston (1985), while the PTIs concentrations analyzed ranged from 50 to 1000 μM. For growth assessment, two perpendicular diameters of the growing colonies were measured after incubation for 4 days under white light (12 h/12 h light/dark) at 26 °C.

2.2. Extraction and detection of DON from culture

To extract DON, one agar plug (7 mm in diameter) was removed from the center of the colonies incubated for 7 days under white light (12 h/12 h light/dark) at 26 °C. The agar plug was shaken for 60 s in 750 μL of water and left for 60 min at room temperature. Then, vials were shaken and the extracts were filtered (Millex-HV 0.45 μm, 25 mm, Millipore Corporation) into another vial. DON extracts were stored at −20 °C until quantification. DON was measured using the RIDASCREEN® DON enzyme immunoassay test from R-BIOPHARM, according to the manufacturer's instructions.

2.3. Gene expression analysis

Mycelia were collected from 7 days old cultures grown on media amended with putrescine, DFMO or PTIs. The mycelium was frozen in liquid nitrogen and stored at −80 °C before nucleic acid extraction. RNA was extracted from the mycelium ground to a fine powder with a mortar and pestle using liquid nitrogen. Powdered mycelium was added to a pre-heated (65 °C) mixture of 10 mL of extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium

dodecyl sulfate, 1% (w/v) polyvinyl-pyrrolidone 40, 1% (v/v) β-mercaptoethanol) and 5 mL of Tris-equilibrated phenol. The extract was incubated at 65 °C for 15 min and cooled before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 × g for 20 min at 4 °C, and the aqueous phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). RNA was precipitated during 3 h at −20 °C by adding 3.3 mL of 12 M LiCl. After centrifugation at 27,200 × g for 60 min, the pellet was washed with 500 µL of 70% ethanol. The resulting pellet was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides. Then, RNA was washed again with 500 µL of 70% ethanol, and, finally, dissolved in 200 µL of water. RNA concentration was measured spectrophotometrically and verified by agarose gel electrophoresis (1.2%) and ethidium-bromide staining. Total RNA was treated with DNase (TURBO DNase, Ambion) to remove contaminating genomic DNA. Single-strand cDNA was synthesized from 5 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT) primer, according to the manufacturer's instructions (Invitrogen).

APT5newF and APT5newR were used as gene-specific primer set to amplify the TRI5 gene (Mudge et al., 2006). The rest of the primer sets were designed with OLIGO Primer Analysis Software V.7 (Table 2). Real-time RT-PCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) monitoring cDNA amplification with SsoAdvanced™ SYBR® Green Supermix (BIO-RAD). The primer pair BETA-6_F and BETA-6_R was designed within the beta tubulin gene for using it as a reference gene. The standard protocol included one cycle at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s and 56 °C for 30 s. The corresponding real-time PCR efficiency (E) in the exponential phase was calculated according to the equation: E = 10[−1/slope]. The relative expression of the target genes was calculated based on the E and the Crossing point (Cp) value of the sample versus the control. The Cp value is the cycle at which fluorescence from amplification exceeds the background fluorescence. The relative

expression of target genes was expressed in comparison to the beta tubulin gene (reference gene), according to the following equation (Pfaffl, 2001 and Rasmussen, 2001):

$$Ratio = \frac{E_{target}^{\Delta Cp\ target\ (Control-Sample)}}{E_{ref}^{\Delta Cp\ reference\ (Control-Sample)}}$$

Gene expression measures were derived from biological triplicates.

Table 2. Primers used in this study.

Gene number	Gene	Primer name	Primer sequence (5′→3′)
FGSG_03537	*TRI5*	APT5newF	CACTTTGCTCAGCCTCGCC
		APT5newR	CGATTGTTTGGAGGGAAGCC
FGSG_16251	*TRI6*	TRI6_F	CACCTTCACACGGCCAAGCAA
		TRI6_R	ATGCCGCCTAAAGTCCCGTCC
FGSG_05903	*ODC*[*]	ODC_F	ACCGCATCACTGATAGCACTCG
		ODC_R	TGGTGTATGCGCCCATATCCTC
FGSG_10284	*PAO*[*]	PAO_F	ACTCTGAGAGCGATCCCGACT
		PAO_R	GCAGACCCTCCCGCATTGTCC
FGSG_04370	*TPO1*[*]	TOP1_F	GCTTGCCGCCAACAACGGTA
		TOP1_R	GGTGTATCCAGTCCATCCGAACCA
FGSG_03725	*TPO4*[*]	TOP4_F	CCATCTTTGCTGCCGCTCCC
		TOP4_R	CCATGACCCATCGCCAACCAG
FGSG_02439	*TPO5*[*]	TOP5_F	GCCACCATCTGTCTCGCCTCC
		TOP5_R	TCCACACGACGCAGATACCGTTG
FGSG_06473	*UGA4*[*]	UGA4_F	GCCCTCCATGATCCCTGTTACCC
		UGA4_R	TGACCCCAGGCCCAGTACCAA
FGSG_03111	*DUR3*[*]	DUR3_F	AGACTTATCGCCATCTCGCACAC
		DUR3_R	ACGCCCATCCAGACATATAGCC
FGSG_00613	*SAM3*[*]	SAM3_F	ATATTCGCTTCCGCCGTGCCT
		SAM3_R	ATCCATGAGCCCACGACACCA
FGSG_09530	*BETA TUBULIN*	BETA-6_F	AGCTCACCCAGCAGATGTTCG
		BETA-6_R	GCGCATCTGGTCCTCAACCTCC

[a]Homologous genes identified by Valdés-Santiago et al. (2012) by homology with an in silico search of well-characterized Saccharomyces cerevisiae proteins.

2.4. Plant growth and inoculation

Wheat plants (*Triticum aestivum*), cultivar Califa sur, were grown in a greenhouse with temperatures that ranged from 14 to 32 °C during the day and 7–12 °C at night. Spikes of wheat were harvested at or shortly before anthesis. Spikes were surface-sterilized by dipping them into a NaClO solution (0.1% Cl) for 15 min, followed by 2 min in ethanol (70%). Excess of ethanol was removed by placing spikes in a laminar flow bench. The DFMO and AMXT-1505 PTI treatments were performed by dipping the spikes into the solutions for 2 min and placing the spikes in a laminar flow bench until they were dried (about 10 min), after which spikes were inoculated. Four different treatments were tested consisting of spikes point inoculated in the middle of the spike with 10 μL of conidial suspension (105 conidia/mL) or water, as positive and negative controls, and spikes treated with 2.5 mM DFMO or 700 μM AMXT-1505 inoculated with 10 μL of conidial suspension. Transparent plastic boxes 300 mm × 160 mm × 180 mm deep were filled with a beaker with water to give a RH of 100%, a data logger (Escort iLog RH) recording temperature and RH at 30 min intervals and a rack where spikes were placed vertically separated from one each other. Boxes were kept in an incubator at 26 °C with 12 h/12 h light/dark photoperiod for 12 days. A box with 10 spikes was used for each treatment.

3. Results and discussion

3.1. Influence of putrescine on *F. graminearum*

To examine the effect of exogenous putrescine on *F. graminearum*, the fungus was grown on plates with increasing amounts of putrescine (0, 50, 500, 1000 and 5000 μM). As Fig. 2A indicates, DON increases proportionally to putrescine concentration, resulting in almost a 23 fold induction when plates were amended with 5000 μM of putrescine. These results are similar to those reported previously by Gardiner et al. (2009) who conducted a large-scale nutrient profiling to assess

the role that different carbon and nitrogen sources play in the production of DON. These authors found that DON production was strongly induced in liquid culture by some amine compounds which included putrescine. Thus, it was confirmed that putrescine has a key role on mycotoxin production. In contrast to DON production, the growth of *F. graminearum* was not affected by the putrescine concentrations assayed. However, it would appear that the effect of putrescine on growth depends on the fungal species as Rajam and Galston (1985) found that the addition of putrescine to the culture medium resulted in a promotion of growth in *Botrytis cinerea*, *Monilia fructicola* and *Rhizoctonia solani*.

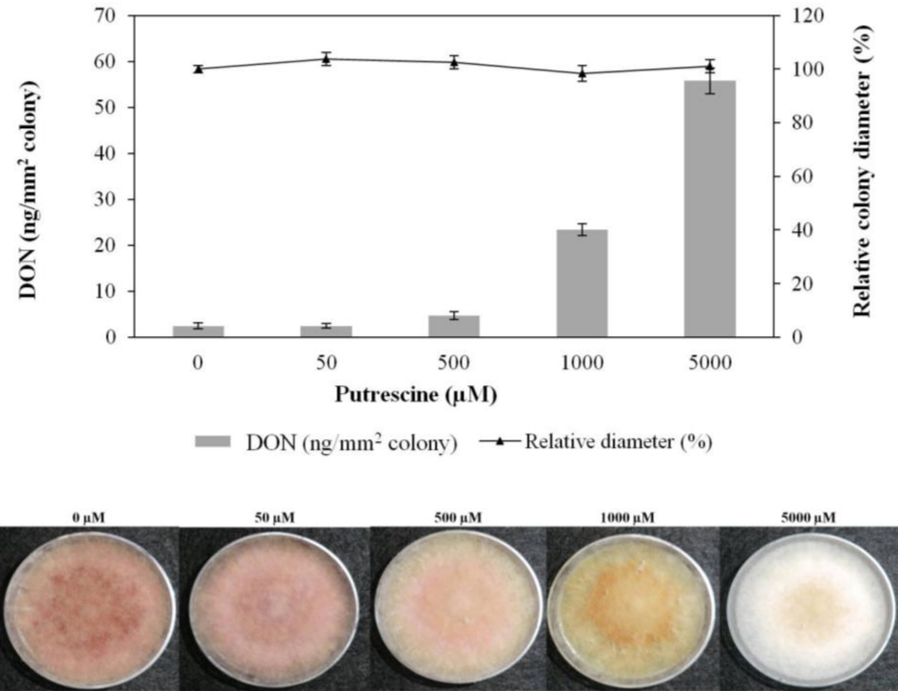


Figure 2. (Top) Effect of putrescine on DON production (left y-axis) and relative colony diameter (%) referred to the control (right y-axis) (control = 100%). (Bottom) Effect of putrescine on *F. graminearum* colonies incubated for 7 days at 26 °C (12 h/12 h light/dark).

Additionally, Fig. 2B shows the colonies of the *F. graminearum* strain incubated with increasing amounts of putrescine, which suggests that other metabolites as carotenoids may also be influenced by putrescine due to the variation of colony color among putrescine concentrations. Indeed, Rodríguez-Ortiz et al. (2009) showed regulatory connections between nitrogen metabolism and carotenogenesis and secondary metabolism in *Fusarium fujikuroi*.

To further elucidate the influence of putrescine on *F. graminearum*, a gene expression analysis was carried out (Fig. 3). We studied *TRI5* and *TRI6* genes involved in DON biosynthesis and regulation (Hohn et al., 1993 and Proctor et al., 1995), the ornithine decarboxylase (*ODC*) and polyamine oxidase (*PAO*) which are involved in PA biosynthesis and oxidation, respectively, and *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3* and *SAM3*, identified by Valdés-Santiago et al. (2012) by homology through an *in silico* search of well-characterized PA transport proteins of *Saccharomyces cerevisiae*. Fig. 3 reveals that the increment of DON production observed in Fig. 2A was correlated with an over expression of *TRI5* and *TRI6* genes, showing at 5000 µM of putrescine a log2 Ratio of 11.5 and 8.2 for *TRI5* and *TRI6*, respectively, which represent a 2896.3 and 294.1-fold change. Consequently, it appears that putrescine can modulate DON biosynthesis affecting the transcriptional regulator encoded by TRI6 and the trichodiene synthase *TRI5*, which catalyzes the first step in DON biosynthesis. It should be noted that *TRI5* was found to be positively regulated by *TRI6* (Proctor et al., 1995). These results concur with the findings of Gardiner et al. (2009) who observed that putrescine induced the fluorescence of a *F. graminearum* strain that contained a GFP gene regulated by the *TRI5* promoter.

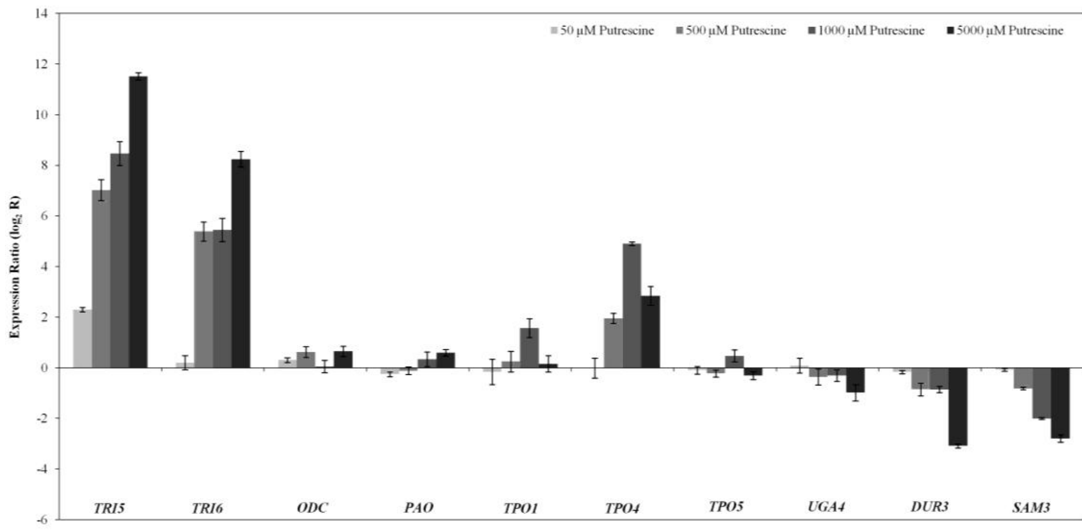


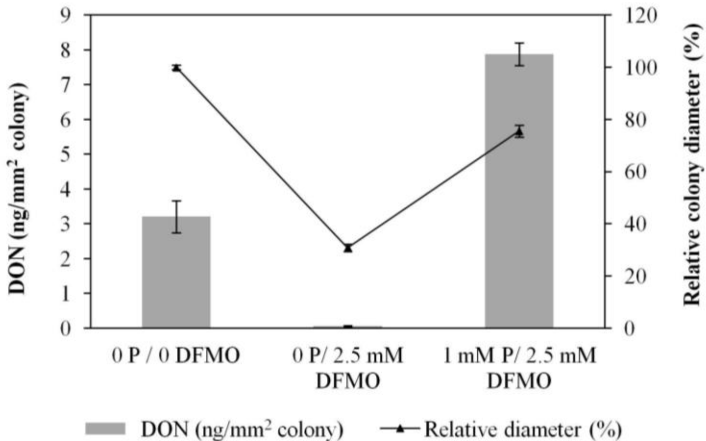
Figure 3. Relative expression of *TRI5, TRI6, ODC, PAO, TPO1, TPO4, TPO5, UGA4, DUR3* and *SAM3* in *F. graminearum* grown in the presence of different concentrations of putrescine with respect to expression level in the same medium without putrescine. Error bars indicate standard errors. Fungal colonies were incubated for 7 days at 26 °C (12 h/12 h light/dark).

By contrast, it was found that the addition of exogenous putrescine to a *F. graminearum* culture had no effect on the *ODC* and *PAO* expression. Because *ODC* catalyzes the first and committed step in the synthesis of PA, it seems that the concentration of putrescine is not self-regulatory at a transcriptional level in *F. graminearum*. Concerning to the gene expression of the putative PA transporters analyzed in this study, we found an up-regulation of *TPO4* and a down-regulation of *DUR3* and *SAM3* when cultures were amended with increasing putrescine concentrations (Fig. 3). PA transport in fungi has been poorly investigated and the existing studies are mainly focused in *S. cerevisiae* (Igarashi and Kashiwagi, 2010 and Valdés-Santiago et al., 2012). In *S. cerevisiae*, TPO1 and TPO4, located mainly in the plasma membrane, are the major PA excretion proteins (Tomitori et

al., 2001, Tomitori et al., 1999 and Uemura et al., 2005). Nevertheless, TPO5 which is located on Golgi or post-Golgi secretory vesicles and UGA4, located on the vacuolar membrane, can also catalyze the excretion of PA (Tachihara et al., 2005 and Uemura et al., 2004). Furthermore, DUR3 and SAM3 are the major PA uptake proteins in yeasts (Uemura et al., 2007). Assuming that these proteins may have similar functions in *F. graminearum*, the increase of putrescine in the culture media would lead to an over expression of *TPO4* to raise the excretion of putrescine, and a downregulation of *DUR3* and *SAM3* to reduce the putrescine uptake.

3.2. Inhibition of putrescine biosynthesis in *F. graminearum* by DFMO

To reduce PA biosynthesis, we tested the DFMO capacity to inhibit ODC, which catalyze the first and committed step in the synthesis of PA (Metcalf et al., 1978) (Fig. 1). When 2.5 mM of DFMO was added to the culture media, growth and DON production were greatly reduced as shown in Fig. 4. DON production decreased from 3.2 to 0.06 ng/mm2 of colony while the growth was lowered by up to 70 per cent. However, when the culture media was supplemented with 1 mM of putrescine, the DON production and the growth were recovered. Similar behavior was observed when *Penicillium citrinum*, *Aspergillus parasiticus* and *Aspergillus nidulans* where treated with PA inhibitors, as mycelial growth and citrinin, aflatoxin and sterigmatocystin production, respectively, were reduced by PA inhibitors (Giridhar et al., 1997, Guzmán-de-Peña et al., 1998 and Guzmán-de-Peña and Ruiz-Herrera, 1997). Additionally, PA inhibitors were able to in vitro inhibit the growth of other phytopathogenic fungi such as *B. cinerea*, *R. solani*, *Cochliobolus carbonum*, *Colletotrichum truncatum*, *Fusarium oxysporium* and *Phytophtora infestans* (Gamarnik et al., 1994, Rajam and Galston, 1985 and Walters, 1995).



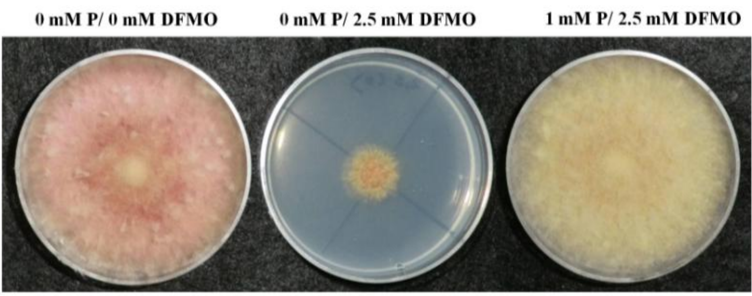


Figure 4. (Top) Effect of DFMO on DON production (left y-axis) and relative colony diameter (%) referred to the control (right y-axis) (control = 100%). (Bottom) Effect of DFMO on *F. graminearum* colonies incubated for 7 days at 26 °C (12 h/12 h light/dark).

As putrescine increased *TRI5* and *TRI6* expression and DON production (Fig. 3), it might be expected that DFMO, which binds to ODC decreasing its activity and consequently the putrescine biosynthesis, will reduce *TRI5* and *TRI6* expression. However, *TRI5* and *TRI6* gene expression was up-regulated when DFMO was added to the media (Fig. 5). Supplementation of 1 mM of exogenous putrescine together with 2.5 mM of DFMO resulted in an increase of *TRI5* and *TRI6* gene expression at similar levels to those found when *F. graminearum* cultures were amended with just 1 mM of putrescine (Fig. 3 and Fig. 5). Therefore, there is also a DON regulation system which is PA independent transcriptionally. Another

interesting finding was the up-regulation of *ODC* when *F. graminearum* cultures were treated with DFMO to inhibit the ODC activity, showing a log2 Ratio of 4.8 which represents a 27.9-fold change. With respect to the putative PA transport homologues, *F. graminearum* showed an up-regulation of *TPO1*, *TPO4* and *UGA4* as well as a down-regulation of *DUR3* in response to DFMO. Based on these results, it would seem that DFMO induces PA excretion proteins and transport to the vacuolar membrane and represses PA uptake proteins but further analysis needs to be performed.

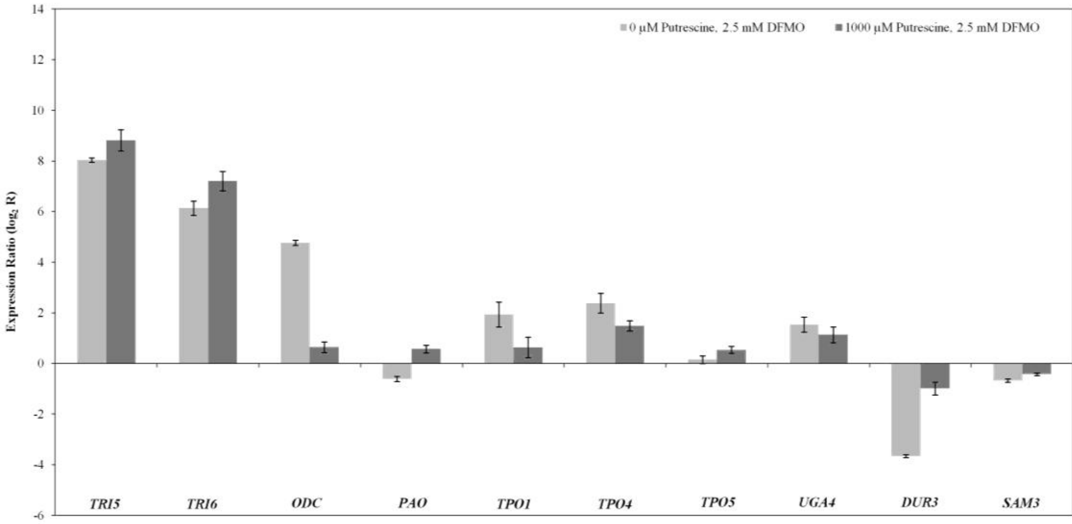


Figure 5. Relative expression of *TRI5*, *TRI6*, *ODC*, *PAO*, *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3* and *SAM3* in *F. graminearum* grown in the presence of DFMO and putrescine with respect to expression level in the same medium without DFMO or putrescine. Error bars indicate standard errors. Fungal colonies were incubated for 7 days at 26 °C (12 h/12 h light/dark).

3.3. Inhibition of PA transport in *F. graminearum*

Although DFMO inhibits largely DON production, the fungi may import PA from the wheat during the infection process, overcoming the inhibition of putrescine biosynthesis. Intracellular PA concentrations are regulated by not only biosynthesis

and degradation but also transport. Therefore, PA transport may be a useful target to control *F. graminearum* infection and DON contamination. For some years now, the inhibition of PA transport has been a goal for medical research as elevated levels of PA have been associated with different types of cancer (Nowotarski et al., 2013). In this context, Burns et al., 2001 and Burns et al., 2009 developed a group of lipophilic polyamine analogs that potently inhibit the cellular PA uptake. To investigate whether PA transport inhibitors could affect *F. graminearum*, five different PA analogs, developed by Burns et al. and kindly provided by Aminex Therapeutics (USA), were tested. To this aim, *F. graminearum* was inoculated in plates amended with 1 mM of putrescine as this could be a common PA concentration during plant infection. Results are presented as relative with respect to the control (1 mM putrescine) (Fig. 6). The observed effect on growth and DON production largely depends on the PTIs used. While AMXT-1483 reduced growth and increased DON production by as much as 180%, AMXT-1505 reduced completely both DON production and mycelial growth when 600 µM of this PTI was added to the medium. AMXT-2030 had little effect on DON production and mycelial growth and AMXT-3016 increased DON production by 270% but did not affect to the growth. In addition, AMXT-3946 reduced DON production up to 70% and increased the mycelial growth up to 210%. Of them all, we should highlight the AMXT-1505 PTI, as it was able to efficiently control *F. graminearum*. Additionally, when AMXT-1505 was applied at sub-lethal doses (<600 µM), not only it does not increase DON production as observed for other fungicides but reduces it, minimizing the risk of mycotoxin contamination (D'Mello et al., 1998, Müllenborn et al., 2008 and Ochiai et al., 2007).

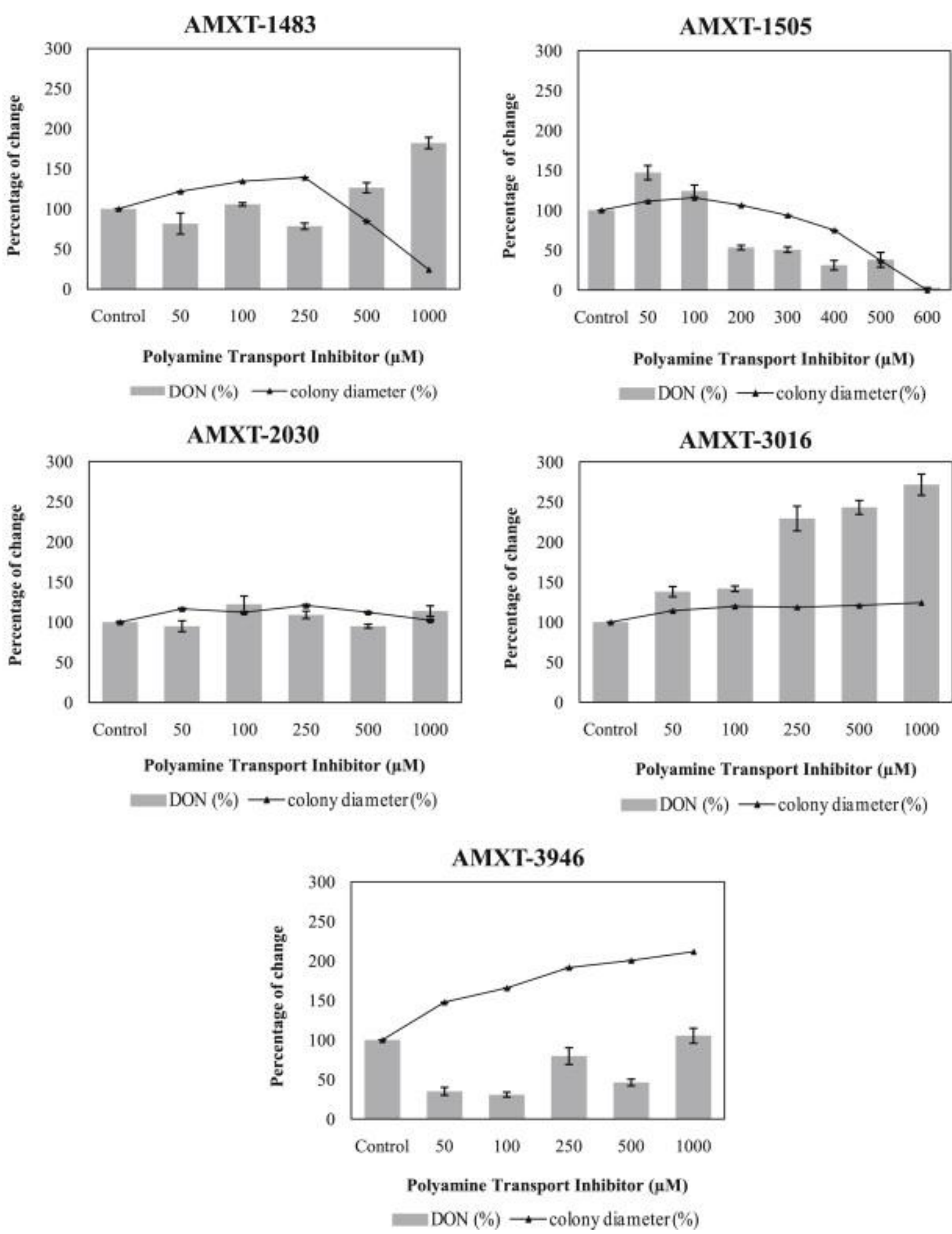


Figure 6. Effect of PTIs on DON production and colony diameter referred to the control (1000 µM putrescine) in percentage (control = 100%). Fungal colonies were incubated for 7 days at 26 °C (12 h/12 h light/dark).

To further investigate the possible mode of action of AMXT-1505, gene expression analysis of genes involved in DON biosynthesis and regulation, PA biosynthesis and oxidation and PA transport was carried out in cultures amended with 100, 300 and 500 µM of this PTI (Fig. 7). However, little changes were observed with respect to the control, showing a concentration dependent down-regulation of TPO1 and to a lesser extent TPO5. In the case that AMXT-1505 was really able to inhibit PA transport, it may lead to PA accumulation inside the cell which in turn may cause cytotoxicity. In fact, excessive level of PA may be coupled to the induction of apoptosis (Schipper et al., 2000). Nonetheless, it is difficult to speculate a mode of action for AMXT-1505 and more research is needed.

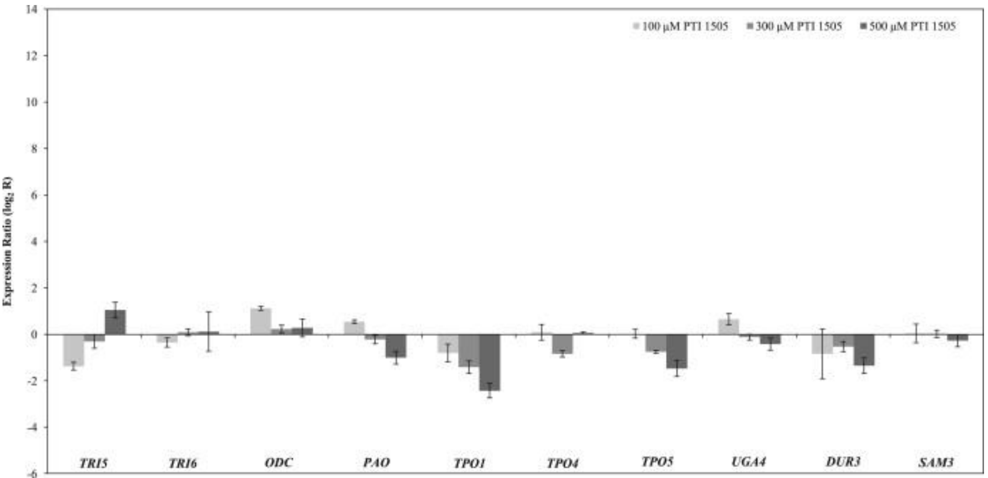


Figure 7. Relative expression of *TRI5*, *TRI6*, *ODC*, *PAO*, *TPO1*, *TPO4*, *TPO5*, *UGA4*, *DUR3* and *SAM3* in *F. graminearum* grown in the presence of 1000 µM putrescine and different concentrations of AMTX-1505 with respect to expression level in the same medium without AMTX-1505. Error bars indicate standard errors.

3.4. Antifungal activity of DFMO and AMXT-1505 against *F. graminearum* in wheat

To verify whether DFMO and AMXT-1505 are able to control *F. graminearum* growth and mycotoxin production in wheat, spikes of wheat were treated with these compounds to be later point inoculated by placing a droplet of conidia suspension in the middle of the spikes. The conditions upon which inoculated spikes were incubated were highly favorable for the *F. graminearum* growth as the inoculation was performed in spikes harvested at anthesis and incubated at 26 °C and 100% RH for 12 days. Firstly, it should be pointed out that spikes were free from external contamination as shown by the negative control in the Fig. 8. Additionally, it should be noted that *F. graminearum* grew in all the spikes from the positive control, forming a white mycelium that covered almost the entire length of the spike. Interestingly, although DFMO reduced the fungal growth in vitro by 70%, when spikes were pre-treated with DFMO, *F. graminearum* was able to grow even more than in the positive control. Conversely, AMXT-1505 was capable of controlling *F. graminearum* with only light growth in two of the spikes. As for the DON production, a concentration of 76.87 µg/g was found in the positive control while pre-treated spikes with DFMO and AMXT-1505 contained 212 and 0.62 µg/g, respectively. From the results obtained, noteworthy is the need of validating the results obtained in vitro as DFMO switched from a 98% reduction to a 276% increase of DON production. These findings may be explained considering the possibility that the fungus overcomes the inhibition of putrescine biosynthesis importing the putrescine from the wheat. Bharti and Rajam (1995) found that some DFMO treatments caused an increase of PA levels in wheat that may be responsible for the DON increase observed in Fig. 8 since higher putrescine concentrations produce higher amounts of DON as shown in Fig. 2.

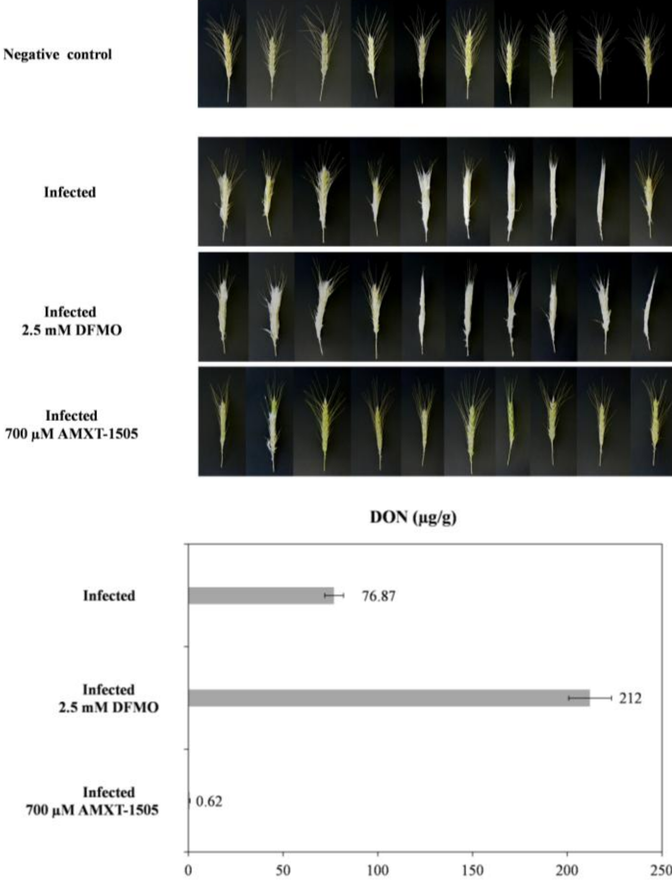


Figure 8. Activity of DFMO and AMXT-1505 against *F. graminearum* in wheat. Spikes of wheat were harvested at or shortly before anthesis, point inoculated by placing a droplet (10 µL) of conidia suspension (10^5 conidia/mL) and incubated at 26°C-100% RH with 12 h/12 h light/dark photoperiod for 12 days.

Although this kind of PTIs have not been tested before as fungicides against *F. graminearum*, some researchers have reported other PA analogs and conjugates used to control phytopathogenic fungi. The putrescine analog 1-aminooxy-3-aminopropane did not reduce mycelial growth of the phytopathogenic fungus *Sclerotinia sclerotiorum*, but perturbed polyamine metabolism inhibiting the

enzyme ornithine decarboxylase, spermidine synthase, and S -adenosyl-methionine decarboxylase in mycelial extracts (Gárriz et al., 2003). In addition, it was found that two spermidine analogs, N,N-dimethyl-N1-(3-aminopropyl)-1,3-diaminopropane trihydrochloride and N,N-dimethyl-N1-(3-aminopropyl)-1,4-diaminobutane trihydrochloride, reduced growth of the fungal plant pathogen *Pyrenophora avenae* (Mackintosh et al., 2001). Walters et al. (2001) found PA conjugates, isolated from pollen of *Quercus alba*, which have antifungal activity against *P. avenae* and Mellon and Moreau (2004) showed a combination of two polyamine conjugates that reduced aflatoxin production but had no effect on growth of *Aspergillus flavus*.

The results reported in this study indicate that the use of PA analogs is a promising approach for the control of the FHD and DON contamination. Since there is a need to find new targets to overcome resistance problems, AMXT-1055 may be useful as a target specific fungicide against *F. graminearum*, contributing to limit crop losses and to reduce the risk of contamination by mycotoxins in food and feed.

4. Conclusions

PA induces mycotoxin production in *F. graminearum*, regulating its biosynthesis at a transcriptional level since putrescine activates *TRI5* and *TRI6* gene expression. PA biosynthesis inhibitors are able to reduce growth and DON production but exogenous putrescine overcomes its effects. Some polyamine analogs inhibit growth and DON contamination *in vitro* and *in planta* and thus they are suitable as target specific fungicides against *F. graminearum*.

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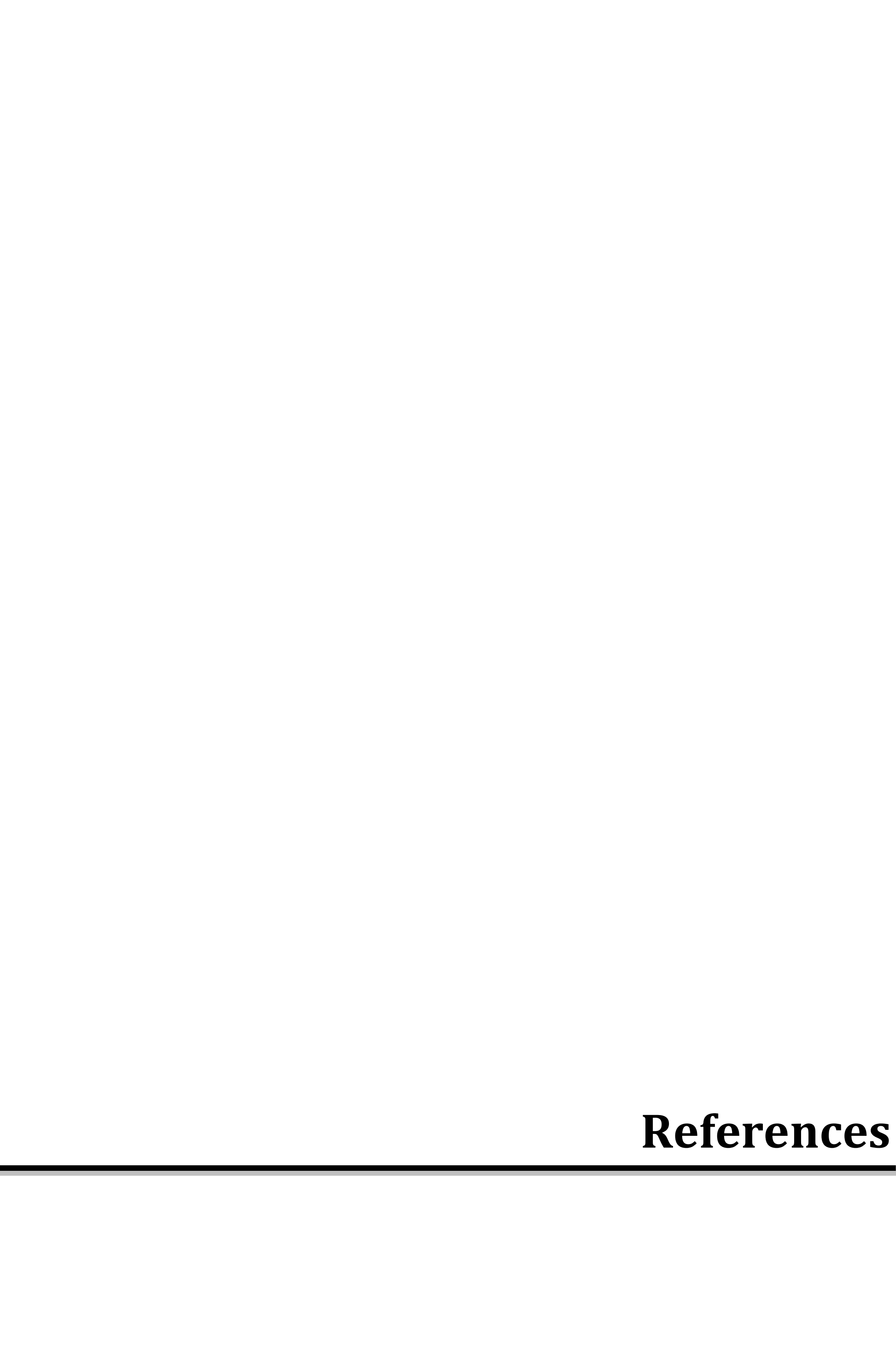

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