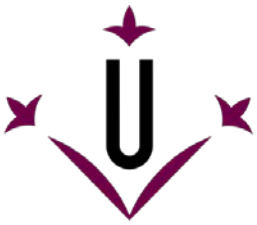

# Exploring polyamine metabolism of *Alternaria alternata* to target new substances to control the fungal infection

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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 descarboxylase 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., 2015; 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 transport inhibitors (PTIs), 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 (please change PTIs to polyamine analogs throughout manuscript) 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 PTIs, the concentrations ranged from 100 to 1200 µM. PTIs, DFMO, and putrescine were stored at −20 °C until needed. We tested seven PTIs, detailed in Table 1, kindly provided by Aminex Therapeutics (USA). The 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 PTIs bind to the lipid membrane of the mammalian cell where the 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.

### 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 the mobile phase solution (water-methanol, 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. 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.

**2.4 Gene expression analysis**

Mycelium grown for seven days on media supplemented with different concentrations of DFMO, putrescine, and PTIs 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. 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, 2001):

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

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 PTIs treatment by touching the leaves with a cotton-tipped previously dipped in the PTI 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 PTI 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 PTIs 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 PTIs tested was measured by analyzing the diameter of the fungal infection, and the AOH and AME production. Tomato fruits 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 ($10^6$

247 conidia/mL) in each wound. Negative control tomatoes were also injured and 5 µL of
248 water were placed on each hole but no conidial suspension was added. Positive controls
249 were not dipped into the PTIs solutions but were inoculated with the *Alternaria* conidial
250 suspension. Tomatoes were packaged into plastic bags and stored at 20 ºC for two
251 weeks in the post-harvest chambers of the Institute for Food and Agricultural Research
252 and Technology (IRTA), who kindly offer us their chambers. Inside the plastic bags the
253 RH reached the 100%. Temperature and RH were recorded hourly by a data logger
254 (Escort iLog RH, Portugal). Five tomatoes were considered a single replicate and the
255 assay was performed in quadruplicate. Two weeks after the fungal inoculation, *A.*
256 *alternata* growth was observed. Diameter lesion size was measured and mycotoxins
257 were extracted. For validation, AOH and AME free tomatoes were artificially spiked
258 with *Alternaria* mycotoxins to assess recovery and repeatability data of the method
259 (Table 3). For mycotoxin production assessment, plugs of 7 mm of diameter and 0.5
260 mm of thickness were removed from the inoculation point. Three plugs were taken from
261 each tomato. All the plugs from the same replicate were put into a stomacher bag.
262 Mycotoxin extraction was proceed by adding 30 mL of acetonitrile-methanol-water
263 (45:10:45 v/v/v; pH 3 adjusted with *o*-phosphoric) and homogenizing for 2 minutes
264 with the stomacher. The mixture was blended for 15 extra minutes under a uniform
265 magnetic field. The solution was left for 10 minutes, approximately, to favor
266 precipitation by gravity. Then, 6 mL of the supernatant were transferred to a centrifuge
267 tube and diluted with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3)
268 and centrifuged at 15250 *x g* for 10 minutes. Two mL of the diluted sample extract was
269 passed by gravity through a previously conditioned Bond Elut Plexa SPE cartridge (200
270 mg and 6 mL, Agilent Technologies, Santa Clara, CA, USA). Conditioning of the
271 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 ornitine descarboxylase 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

297 containing 2.5 or 5 mM of DFMO lost all the pigmentation and, as a consequence, were
298 white with much less mycelium. Regarding mycotoxin production, control colonies
299 produced a total amount of 1.84 ng/mm$^2$ of AOH while no AME was detected.
300 However, the addition of 2.5 mM of DFMO on the media decreased the AOH
301 production to 0.78 ng/mm$^2$, while 5 mM completely inhibited mycotoxin biosynthesis.
302 Growth inhibition mediated by DFMO was readily reversed in the presence of 1 mM of
303 putrescine. In contrast, neither mycotoxin production nor the colony color, were
304 reversed when 1 mM of putrescine was added to the media. Colonies were less
305 pigmented and adopted a softer green coloration.
306 In order to get a deeper insight about how DFMO affected *A. alternata*, gene expression
307 analysis was carried out. Gene expression of *pksJ* and *altR* was studied, both genes
308 identified of being essential in the AOH and AME production pathway (Saha et al.,
309 2012). Gene expression of *ODC* and, additionally, the expression of *TPO4*, a gene that
310 encodes a polyamine transporter protein that recognizes putresine, spermidine, and
311 spermine and excretes them from the cell to the extracellular media (Igarashi and
312 Kashiwagi, 2010; Tachihara et al., 2005; Tomitori et al., 1999; Valdés-Santiago et al.,
313 2012). Fig. 2 shows the relative expression of *pksJ*, *altR*, *ODC* and *TPO4* in the
314 presence of DFMO with respect to control samples (0 mM DFMO and 0 mM
315 putrescine). Thus, bars above the baseline indicate upregulation, while bars under the
316 baseline indicate downregulation. Gene expression analysis revealed that the higher the
317 DFMO concentration, the higher the downregulation of *pksJ* and *altR*. DFMO decreased
318 gene expression of *pksJ* by a $\log_2$ ratio of 3.03 when treated with 2.5 mM and 4.18 with
319 5 mM. In contrast, with regard to *ODC* expression, it was found that both
320 concentrations of DFMO resulted in an overexpression of the gene. *TPO4* was the most
321 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, especifically 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.

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. 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., 2015) supported that PTIs could affect fungal growth and mycotoxin production and even inhibit both processes. Thus, in order to evaluate the effect of PTIs 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 PTIs (see the list of the PTIs used in Table 1. Fig. 5 shows the results obtained for all the seven PTIs we tested. Data derived from this study were quite different depending on the PTI 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 PTIs 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). The effect of PTIs 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). 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. Results shown in Fig. 8 and Fig. 9 suggest a similar pattern for both PTIs. 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 PTIs 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 PTIs 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 descarboxylase inhibition and polyamine transport inhibition of *A. alternata* infecting tomato fruits 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 PTIs solutions did not reduce the fungal growth efficiently as no significant differences were found among infected controls and tomatoes dipped into the PTIs 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 PTI.

## 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., 2015; 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*

447 putrescine complementation resulted in an increase in the mycelial growth above the
448 control values. In other studies using other ODC inhibitors, similar results were found.
449 In this context, *Aspergillus nidulans*, *Aspergillus parasiticus* or *Pyrenophora avenae*
450 reduced their mycelial growth when the ODC inhibitor was added to the media.
451 Additionally, sterigmatocystin and aflatoxin biosynthesis was almost inhibited in *A.*
452 *nidulans* and *A. parasiticus*, respectively (Guzmán-de-Peña et al., 1998; Guzmán-de-
453 Peña and Ruiz-Herrera, 1997; Mackintosh and Walters, 1997). However, in this case
454 sterigmatocystin and aflatoxin production was reverted by addition of putrescine in the
455 media, in contrast to what we have observed on *A. alternata*, in which putrescine has
456 reverted mycelial growth but not AOH production. Kępczyńska (1994) studied the
457 effect of inhibiting spermidine synthesis using methyl bis-(guanylhydrazone) in
458 *Alternaria consortiale* and they also observed reduction of mycelial growth which was
459 not only restored with the addition of spermidine, but increased compared with the
460 control. Thus, results show that the effect of ODC inhibitors is dependent upon the
461 particular fungus. Birecka et al. (1986) suggested that genus-dependent differences in
462 sensitivity to inhibitors may be due to differences in uptake of the inhibitor, ODC
463 sensitivity and polyamine requirements. Despite these differences, it is clear that in *A.*
464 *alternata* polyamines are essential for growth and for mycotoxin production and also
465 that the ODC enzyme plays a determinant role in the polyamine biosynthesis pathway.
466 Inhibition of this enzyme, may alter the levels of polyamines in the fungi that ultimately
467 leads to changes in its physiology and growth (Rajam and Galston, 1985; Valdés-
468 Santiago et al., 2012). When DFMO was tested on *A. alternata* and gene expression
469 patterns were analyzed, a significant downregulation of *TPO4* was observed, a gene that
470 encodes a polyamine transport protein that excretes putrescine, spedermine, and
471 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., 2015; 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., 2015). 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

522 downregulation, which was more remarkable when the concentration was 5000 μM.
523 However, further studies may be performed for a better understanding of nitrogen
524 source regulation of mycotoxin production.
525 DFMO was originally used as a chemotherapeutic agent to interrupt cellular metabolic
526 processes in cancer therapy. Despite its success inhibiting ODC activity, it did not
527 achieve the goal of repressing cell growth because cell lines grown in culture could
528 overcome the blockage of the ODC enzyme by importing polyamines from extracellular
529 sources. This current idea was corroborated when we tested DFMO on tomatoes as we
530 observed that *A. alternata* was able to grow similarly to the control group of samples, in
531 which no treatment was assessed. Hence, even if fungal cells have the polyamine
532 biosynthesis pathway blocked, they can uptake the polyamines needed to survive from
533 the tomato. With the aim to definitely repress cellular tumor growth, Burns *et al*. (2001;
534 2009) designed a group of lipophilic polyamine analogs, the PTIs, which potently
535 inhibit the cellular polyamine transport. Additionally, other polyamine analogs were
536 characterized as antizyme inducing agents, including AMXT-3016 used in our studies.
537 Thus, growth inhibitory effects of DFMO in combination of these PTIs resulted in a
538 tumor growth inhibition. In a previous study with *F. graminearum* (Crespo-Sempere et
539 al., 2015), it was observed that some of the PTIs developed by Aminex Therapeutics
540 (USA) did efficiently control mycelial growth in wheat spikes. In this context, *in vitro*
541 tests were assessed with seven different PTIs on *A. alternata* and it was observed that
542 two of them achieved the goal of controlling *A. alternata* growth, sporulation and
543 mycotoxin production at the same time: AMXT-2455 and AMXT-3016. Surprisingly,
544 the PTI that had been efficient for *F. graminearum* did not work for *A. alternata*
545 (AMXT-1505). Genetic expression analysis of the mycelia corroborated that both PTIs
546 downregulated *pksJ* gene expression proportionally to the PTI concentration. Both PTIs

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, PTIs 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. 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 PTIs achieved a reduction of the fungal infection compared to the control group of samples. Nevertheless, there were some leaves treated with PTIs 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,

572 tri-caffeoylspermidine and tri-p-coumaroylspermidine, isolated from pollen of *Quercus*
573 *alba*, were examined for antifungal activity against *P. avenae* and two of them
574 successfully reduced mycelial growth of the oat leaf stripe pathogen (Walters et al.,
575 2001). A mixture of diferuloylputrescine/p-coumaroylferuloylputrescine also
576 demonstrated inhibitory activity against aflatoxin $B_1$ biosynthesis in *Aspergillus flavus*,
577 although this diconjugated polyamine mixture did not display inhibitory effects on *A.*
578 *flavus* growth (Mellon and Moreau, 2004).
579 Results with polyamine analogues may be quite promising for the control of different
580 diseases caused by several kind of fungus on fields and crops. Nevertheless, more
581 information is needed to pinpoint the mode of action of the polyamine analogues.
582

583 **5. Conclusions**

584 Inhibition of the polyamine biosynthesis pathway using DFMO decreases AOH
585 production and fungal growth. Hence, polyamines might play some essential role in
586 both biological processes. Addition of exogenous putrescine on the media reverts fungal
587 growth but not mycotoxin production. Putrescine as a nitrogen source may affect
588 several essential processes of the cell such as sporulation, growth rate and mycotoxin
589 production. Some polyamine transport inhibitors seem to control fungal growth and
590 mycotoxin production *in vitro* and promising results have been observed *in vivo.* Based
591 on these findings, it is worthwhile to continue investigating in polyamine metabolism as
592 a new target to control *A. alternata* diseases in plants to overcome problems derived
593 from fungicide resistance.
594

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**Figure captions**

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.

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.

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.

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.

Figure 5: Effect of seven PTIs 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.

Figure 6: Effect of AMXT-2455 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-2455). 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 the PTI AMXT-2455 is represented 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 putrescine on radial growth rate is shown on the right y-axis, while colony morphology of *A. alternata* colonies with different concentrations of the PTI AMXT-3016 is represented at the bottom. Error bars indicate standard errors.

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.

Figure 10: Effectiveness of AMXT-2455 (1000 μM), AMXT-3016 (800 μM) and DFMO (5 mM) on tomato fruits and tomato plants. Mycotoxin production on tomato fruits is represented on the left y-axis, while diameter of infection on tomato fruits is represented on the 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.

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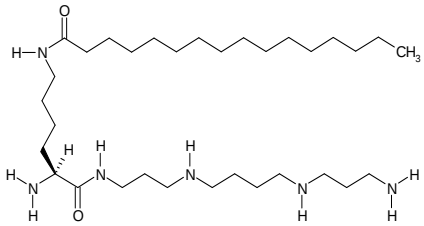
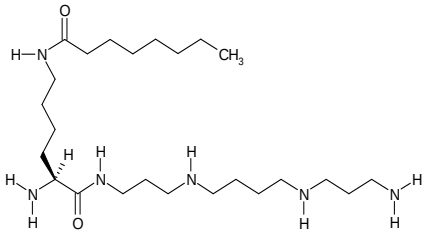
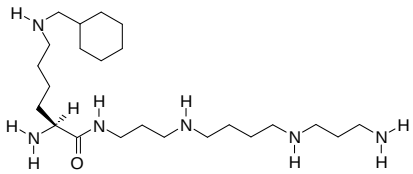
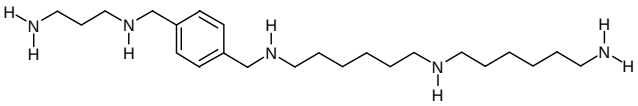
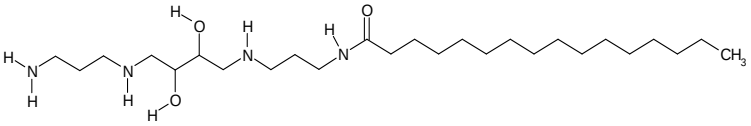
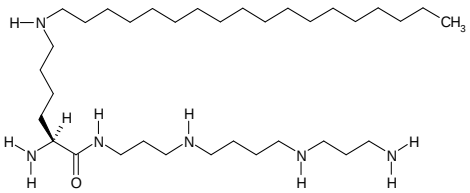
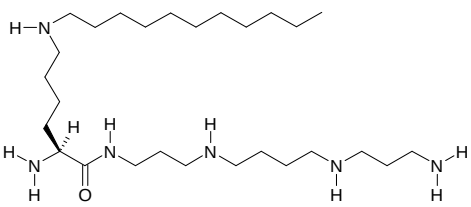
838 Table 1

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

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841 Table 2

| 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 | - |

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844 Table 3

| Spiking level (µg/g tomato) | AOH | | | AME | | |
|---|---|---|---|---|---|---|
| | 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 |

845 [a] Mean value ± standard deviation.
846 [b] RSDr = relative standard deviation.
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848 Table 4

| 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 |

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