Highlights:

- 1-MCP could increase growth of *A. alternata* if conditions favor fungal development.
- In 1-MCP treated tomatoes, mycotoxin biosynthesis depends on tomato variety.
- Although no mycotoxin was detected at 6d-10 °C, *pksJ* gene expression was observed.
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*

Estiarte, N. 1, Crespo-Sempere, A. 1,2, Marín, S. 1, Sanchis, V. 1 and Ramos, A.J. 1*

1 Applied Mycology Unit, Food Technology Department. University of Lleida. UTPV-XaRTA, Agrotecnio Center, Lleida, Spain. nestiarte@tecal.udl.cat; smarin@tecal.udl.cat; vsanchis@tecal.udl.cat; ajramos@tecal.udl.cat. 2 Present address: Valgenetics S.L. University of Valencia Science Park, Valencia, Spain. acrespo@valgenetics.com

*Corresponding author. Email: ajramos@tecal.udl.cat; Tel (+34) 973 702811; Fax (+34) 973 702596

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-methylocyclopropene (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 (10^6 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, 2008; Bottalico and Logrieco, 1992; Ostry, 2008).
In the last few 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/cm². 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 °Brix. 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 m³. The 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 tomatoes treated with MCP-1 was compared with the color of the
skin of 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 m³ 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 fruit 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 at 10 ºC (see Table 1). After the 1-MCP treatment at 10 ºC, all set 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/mm² and
0.034 ng/mm², respectively. The LOD was based on a signal-to-noise ratio of 3:1. The
limit of quantification (LOQ) was calculated as $3 \times$ LOD. All solvents were HPLC grade and all chemicals were analytical grade.

### 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 $\alpha$-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 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 °C 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.

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 °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 (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 °C 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’-ACACTAGCACAGTCGGTTCCA-3’) and pksJ_R (5’-ATGGCCGCGTACTACCCAG-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’-ACCCCTTGGCCAGTGGTTACCAG-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, 2001).

Gene expression measures were derived from biological triplicates.

\[
R = \frac{(E_{\text{target gene}})^{\Delta Cp_{\text{target gene}}} (\text{Mean untreated samples} - \text{Mean treated samples})}{(E_{\text{reference gene}})^{\Delta Cp_{\text{reference gene}}} (\text{Mean untreated samples} - \text{Mean treated samples})}
\]
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 °C. In contrast, cultures that had been incubated one more week at 20 °C 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). Regarding mycotoxin production, after 6 days incubated at 10 °C, cultures did not produce AOH or AME either with or without 1-MCP. It was not until the second week of incubation at 20 °C 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. 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 \textit{pksJ} expression neither after the first week of incubation at 10 °C nor after the second week at 20 °C.

3.2 Effect of 1-MCP on \textit{A. alternata} growth on tomato fruit

3.2.1 Fruit quality measurements

Before analyzing the effect of 1-MCP on \textit{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. \textit{Egara} cultivar was besides firmer and the one with the lowest quantity of SS and TA (Table 4).

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 (Fig. 3). 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. During the 6 days at 10 °C, 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. However, when *A. alternata* was grown for one more week at 20 °C, 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. 4-6, 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.

### 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. 4-6). 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 °C), 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.

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 physiological changes and, although no mycotoxins were detected during the first 6 days of storage at 10 °C 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 \textit{pksJ} gene was less expressed on \textit{Alternaria} inoculated on tomatoes treated with 1-MCP in comparison with the controls.

Regarding \textit{pksJ} expression of \textit{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 \textit{pksJ}. On the Egara cultivar treated with 1-MCP, \textit{A. alternata} showed AOH production during the first 6 days at 10 °C (Fig. 4, 0.194 μg/g tomato) and this data seems to be congruous with the study of the \textit{pksJ} gene expression. In that case, an upregulation of $3.00 \log_2$ of the \textit{pksJ} gene was observed, which means an 8 times increase in the expression of the \textit{pksJ} 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 \textit{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; Diaz 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 (Efiuvwevwe 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 °C) 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 et 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.

Acknowledgements

We thank the Postharvest Programme of IRTA, especially to J. Usall, E. Costa and R. Altisent, for kindly offering us their postharvest chambers and laboratories and for helping us with the fruit quality measurements. We are also grateful to the Catalonian Government (XaRTA-Reference Network on Food Technology) for their financial
support. N. Estiarte thanks the Secretaria de Universitats i Recerca del Departament d'Economia i Coneixement of the Generalitat de Catalunya for the pre-doctoral grant.

5. References


Ku, V.V.V., Wills, R.B.H., Ben-Yehoshua, S., 1999. 1-Methylcyclopropene can differentially affect the postharvest life of strawberries exposed to ethylene. HortSience 34, 119-120.


Figure captions

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.

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 log2 ratio between 1-MCP treated and untreated samples. 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. Error bars indicate standard errors.

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

Figure 4: 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.

Figure 5: 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.

Figure 6: 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.
Table legends

Table 1: Description of the treatments assayed. The 1-MCP treatment (0.6 μL/L) was assessed during 24 h at 10 °C. The positive sign (+) implies that samples were inoculated with *Alternaria*, treated with 1-MCP and incubated at 10 °C (6 days) or 20 °C (7 days). The negative sign (+) implies that samples were not inoculated with *Alternaria*, not treated with 1-MCP and not incubated at 10 °C (6 days) or 20 °C (7 days).

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

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

Table 4: Fruit quality parameters of tomato varieties tested (Egara, Caniles and Palladium).
## Table 1

<table>
<thead>
<tr>
<th></th>
<th>Inoculated</th>
<th>1-MCP</th>
<th>10 °C (6 days)</th>
<th>20 °C (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Treatment 5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Treatment 6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Treatment 7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Treatment 8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
### Table 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water</th>
<th>Methanol-water (70:30 v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>41</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>43</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>45</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>46</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>47</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3

<table>
<thead>
<tr>
<th>Spiking level (µg/g tomato)</th>
<th>n</th>
<th>Recovery (^a) (%)</th>
<th>RSDr (^b) (%)</th>
<th>n</th>
<th>Recovery (%)</th>
<th>RSDr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>3</td>
<td>115.5 ± 2.1</td>
<td>1.8</td>
<td>3</td>
<td>106.0 ± 8.2</td>
<td>7.7</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>99.0 ± 2.4</td>
<td>2.4</td>
<td>5</td>
<td>93.8 ± 3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>2.0</td>
<td>3</td>
<td>79.8 ± 3.2</td>
<td>4.0</td>
<td>3</td>
<td>78.0 ± 3.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\(^a\) Mean value ± standard deviation.

\(^b\) RSDr = relative standard deviation.
Table 4

<table>
<thead>
<tr>
<th></th>
<th>Weight (mg)</th>
<th>Caliber (mm)</th>
<th>Firmness (kg/cm²)</th>
<th>SS (*Brix)</th>
<th>TA</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egara</td>
<td>209.78 ± 42.81</td>
<td>81.45 ± 6.29</td>
<td>5.71 ± 2.03</td>
<td>4.93 ± 0.12</td>
<td>4.89 ± 0.47</td>
<td>L 44.56 ± 2.62</td>
</tr>
<tr>
<td>Caniles</td>
<td>74.85 ± 6.81</td>
<td>50.34 ± 1.87</td>
<td>6.78 ± 1.54</td>
<td>6.23 ± 0.38</td>
<td>5.76 ± 0.42</td>
<td>L 47.54 ± 1.79</td>
</tr>
<tr>
<td>Palladium</td>
<td>93.85 ± 11.34</td>
<td>57.15 ± 3.13</td>
<td>6.08 ± 2.15</td>
<td>5.13 ± 0.15</td>
<td>5.40 ± 0.14</td>
<td>L 48.63 ± 1.70</td>
</tr>
</tbody>
</table>
Figure

Egara

Mycotoxin production (µg/g tomato)

AOH  AME  Diameter of growth

Diameter of growth (mm)

(+) 1-MCP  (-) 1-MCP

10 °C (6 days)  10 °C (6 days) & 20 °C (7 days)

AB  a  A  a  AB  a

Click here to download high resolution image