Alterations in volatile metabolites profile of fresh tomatoes in response to *Alternaria alternata* (Fr.) Keissl. 1912 infection

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ABSTRACT

Alternaria alternata (Fr.) Keissl. 1912 is one of the main fungal pathogens that infect tomato (*Solanum lycopersicum* L.) during cold storage affecting postharvest quality and marketing. During fungal infections, fruits and fungi release specific volatile metabolites (VM) that could alter the fruit aroma, or could mediate resistance response in the fruit, or they also could suggest the possible status of fungal attack. The detection of the VM released during the tomato-*Alternaria* interaction could contribute to the development of ecofriendly and harmless strategies for its control. In this study, the profile of VM of fresh tomatoes inoculated with *A. alternata*, were analyzed by solid phase microextraction and gas chromatography-mass spectrometry (SPME-GC-MS) during storage at 15 and 20 °C for 48 h, respectively. Changes in the profile of VM were observed between control and inoculated fruit since the first few hour post-inoculation. Some VM (3-methyl-2-butenal, dimethyl disulfide, 1-butenol, hexanol, 2-methyl-1-butanol acetate, among others) were only detected in inoculated fruit, so they appear to be synthesized by the presence of the pathogen. Also, a marked increase of 3-methyl-1-butanol and 6-methyl-5-hepten-1-one were observed in inoculated fruit, and they were progressive over time particularly at 20 °C. In conclusion, *A. alternata* induced changes in the profile of volatile metabolites released by tomato fruit. Some of the VM released during tomato-*A. alternata* interaction, were synthesized or stimulated by the fungal attack. These results contribute to the current knowledge about the profile of VM released during the fruit-pathogen interaction.

Key words: Volatile metabolites, fresh tomatoes, *Alternaria alternata*, cold storage temperatures, SPME-GC-MS.

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Received: 26 April 2017.
Accepted: 2 August 2017.
doi:10.4067/S0718-58392017000300194

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a commercially important vegetable crop worldwide, with a production about 200 million tons on 4.8 million hectares (FAOSTAT, 2015). Consumers highly accept it because of the quality, sensory characteristics and important source of nutrients. However, due to its physiology and high water content, it is highly perishable and susceptible to fungal attack, affecting the quality and leading to significant post-harvest losses. After harvest and under cold storage conditions, tomato is susceptible to infection caused by necrotrophic fungus *Alternaria alternata* (Fr.) Keissl. 1912 (Zitter and Wien, 1984; Ruelas et al., 2006), which is one of the most common pathogen causing spoiled tomatoes and it is responsible for black mold disease (Troncoso-Rojas and Tiznado-Hernández, 2014). The Alternaria rot symptoms are concentric dark brown spots on the fruit surface with abundant conidia, frequently developed near the peduncle scar or at the blossom-end of the fruit (Snowdon, 2010). *Alternaria alternata* can attack the fruit from the first ripening stages and remain quiescent until conditions of temperature (around 20 °C), relative humidity (90-95%), and the maturity of the fruit are optimal for the development of infection (Prusky et al., 2013).

The fungal infection starts when the spore arrives on the plant surface. At this moment a communication system between plant and pathogen is activated, which triggers the induction of defense mechanisms of the plant. Some of these events considered as part of the defense mechanisms include the production of reactive oxygen species, synthesis of phytoalexins such as phenolic compounds (Ruelas et al., 2006). Besides, modifications in structural defenses (random creation of links between cell wall polymers, lignification) increase the activity of pathogenesis-related proteins (Cota et al., 2007), among others (Thatcher et al., 2005). Also, volatile metabolites (VM) released during the infection can alter the fruit flavor. Some volatiles act as signals of plant diseases (Jansen et al., 2011), or as an antimicrobial agent (Neri et al., 2015), keeping the inactive state of fungi, and contribute to plant resistance against pathogens (Quintana-Rodriguez et al., 2015).

During the fungal attack, the fruit metabolism is altered resulting in the synthesis of new volatile compounds or changes in the levels of the existing ones. Vikram et al. (2004) reported an increase in the abundance of 1-butanol, 1-hexanol, ethyl acetate and hexanoic acid ethyl ester in apple infected with *Botrytis cinerea*, *Penicillium expansum*, *Mucor piriformis*, and *Monilinia* sp. In

mangoes, some volatiles such as ethyl boronate, thujol, 1-butanol, ethyl propanoate, and styrene were synthesized in response to infection caused by *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. Also, an increase in the abundance of ethyl butanoate, 1-methyl-4-(1-methylethylidene) cyclohexene, ethanol, ethyl acetate, ethyl hexanoate, limonene, and ethyl octanoate were observed (Moalemiyan et al., 2006). In fresh tomato, few studies had been carried out to detect the emission of volatile compounds during fungal infection. One of them was published by Ibrahim et al. (2011), who studied the emission of volatile compounds released from tomatoes inoculated with three different fungi: *Fusarium oxysporum*, *Aspergillus flavus*, and *Aspergillus niger*. 1,2-Dimethyl benzene, methyl *cis*-11-octadecenoate, isopropylbenzene, and adogen73 (oleic acid amide) were the most predominant volatiles in fruit inoculated with *F. oxysporum*. The most dominant volatile in fruit inoculated with *A. flavus* were 9-octadecenoic acid (Z), octane, nonane and butylated hydroxytoluene; and in tomato fruit inoculated with *A. niger*, the most predominant were 9-octadecenoic acid (Z), decane, octane, and 1-methylene-1*H*-indene. These results indicate that the type and abundance of the volatile emission are affected by the host, pathogen, and environmental factors. To the best of our knowledge, no scientific information is available in the literature about the volatile metabolites released from fresh tomato infected with *A. alternata*. Therefore, the profile of volatile metabolites released during tomato fruit and *A. alternata* interaction at 15 and 20 °C, were determined in the present study. The detection of specific VM released during tomatoes-*A. alternata* interaction and under different temperatures could provide valuable information about their metabolism, or reflect the fruit damage caused by the pathogen, which may allow us to understand better the infection process of *A. alternata* in tomatoes, which in turn could contribute to the development of ecofriendly and harmless strategies for its control.

MATERIALS AND METHODS

Plant material

Tomato plants (*Solanum lycopersicum* L.) 'Rutgers' were grown in a shade house, in a semiarid climate, at the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, A.C., Sonora, México). There were three rows of 10 plants in each row. Fruits were harvested and sorted by size, color (light red; USDA color classification), and lack of visible defects to get a homogeneous sample. In addition, the fruits had a similar respiration rate (20.28 ± 1.8 mL CO_2 kg^{-1} h^{-1}) and ethylene production (4.5 ± 1.2 µL kg^{-1} h^{-1}). The respiration rate and ethylene production analyses were performed with gas chromatography according to the methods reported by Troncoso-Rojas et al. (2005).

Inoculation process of tomato fruits with *A. alternata*

An *A. alternata* strain was previously isolated from infected tomatoes and maintained under refrigerated conditions. The strain was cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan, USA); the colony, conidium morphology and sporulation characteristics of this specie were confirmed according to those described by Simmons (2007), Troncoso-Rojas et al. (2005), and Lawrence et al. (2013). The colony of *A. alternata* had a dark olive-green color, felty to wooly texture, with a prominent (2 to 5 mm) white margin and a diameter of over 80 mm after 8 to 10 days of growth. Conidial were chains of yellowish-brown color, beaked, ovate in shape with the presence of transverse and longitudinal septa, short conidiophores in bushy clumps, 10-15(-20) μm, with rare to no branching. These characteristic agree to those reported by Pryor and Michailides (2001) for *A. alternata* strain (EGS 34-016, GenBank accession number AF347031.1). A conidia suspension was prepared using colonies from 7-10 d of growth. Conidia was obtained by gently scraping the surface of the mycelium using sterile distilled water, and the spore concentration was adjusted to 10^4 conidia mL^{-1}.

Freshly harvested fruits were randomly divided into two groups of eight fruits each, which were dipped into a 200 mg L^{-1} NaHOCl for 1 min, rinsed with sterile water and air dried in a laminar flow cabinet at room temperature before treatments. Subsequently, one group was inoculated by immersion in the conidia suspension of *A. alternata* for 3 min. The control group was immersed in sterile distilled water and allowed to dry at room temperature. Control and inoculated fruits were stored during 48 h at 15 °C, 90-92% RH (storage condition), and 20 °C, 85-90% RH (simulated shelf-life period).

Determination of volatile metabolites of the *A. alternata* growing in Petri plates

The fungus was planted on potato dextrose agar (PDA) and incubated at 26 °C. After 10 d of fungal growth, the Petri plates were maintained at 20 °C. On one side of the Petri plate, a small hole was made and covered with tape. A sampling of the headspace was done by the solid phase microextraction (SPME) technique following the method reported by Bianchi et al. (2009), with some modifications. An SPME device with a carboxen/polydimethylsiloxane (CAR/PDMS, 85/75 μm film thickness) coating (Supelco-Sigma-Aldrich, Palo Alto, California, USA) was used. The fiber was inserted through the small hole in Petri plates and exposed for 60 min to volatile compounds released by fungus into headspace; then, it was removed from the plates and desorbed in the injector port of the gas chromatograph (GC Varian 3400x, Palo Alto, California, USA) for 5 min at 250 °C. Three plates were used in this experiment.

Determination of volatile metabolites released by inoculated tomatoes

Fruits inoculated or not with the pathogen were individually placed in glass containers (400 mL capacity) and maintained at 15 or 20 °C, 85-90% RH. The lids of the containers were adapted with silicone septa PTFE (20 mm) for sampling. The SPME fiber (CAR/PDMS) was exposed to the

headspace of the container for 60 min. Samples were taken from the headspace at different times: 0, 3, 6, 9, 12, 24, and 48 h after inoculation. The experiment was repeated twice.

Gas chromatography-mass spectrometry analysis

The SPME fiber was inserted into a gas chromatograph (GC) splitless injector and maintained for 5 min at 250 °C. An Agilent 5975C GC (Agilent Technologies, Santa Clara, California, USA) equipped with a Supelcowax 10 column (30 m length, 0.25 mm id) with a 0.25 mm film thickness (100% polyethylene glycol) (Supelco-Sigma-Aldrich) was used. The chromatographic conditions were as follow. The injection port and detector temperatures were maintained at 250 °C. The oven temperature was initially maintained at 40 °C for 5 min and then increased up to 70 °C, with a rate of 5 °C min^{-1}. Afterward, it was kept at 70 °C for 1 min and then increased from 70 to 150 °C at 10 °C min^{-1}; a temperature of 150 °C was maintained for 8 min. The GC was directly interfaced with a 5975C quadruple Triple-Axis HED-MS detector (Agilent Technologies). Electron impact mass spectra were registered at 70 eV of ionization energy, and the mass spectrometer scanned from *m/z* 35 to 400. The carrier gas was helium. Three repeated measurements were determined for each sample.

The volatile metabolites were identified by comparing the retention times with those of the commercial standard and comparing their mass spectra with the database stored in the National Institute of Standards and Technology Library (NIST). The commercial standards were: hexanal, 3-methyl-1-butanol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, ethyl acetate, hexyl acetate, 1-butanol, 2-methyl acetate, nonanal, 6-methyl-5-hepten-2-one, and 2-isobutylthiazole were purchased from Sigma-Aldrich.

Measurement of Alternaria rot development on the fruit surface

On four artificially inoculated tomatoes from each treatment, the disease caused by *A. alternata* was measured after different times: 0, 3, 6, 9, 12, 24, 48, 96 and 144 h post-inoculation. The lesion size on the fruit surface was measured with a vernier, and the results were reported as the lesion size in square millimeters (mm^2).

Statistical analysis

ANOVA was conducted to determine significant differences in the abundance of volatile metabolites at both temperatures (15 and 20 °C). The ANOVA was performed based on an entirely randomized design with factorial arrangement of two factors. One of the factors was the temperature (15 and 20 °C) and the second factor was the sampling times (0, 3, 6, 9, 12, 24, and 48 h). In addition, ANOVA was performance based on an entirely randomized design with factorial arrangement of two factors, to determinate significant differences in the development of *Alternaria* rot on the fruit surface, with respect the sampling time. When significant differences ($p < 0.05$) were found, the Tukey-Kramer multiple range test ($p < 0.05$) was performed. All data were reported as means ± standard deviations. All statistical analyses were performed using the NCSS software 2010 (NCSS, Kaysville, Utah, USA).

RESULTS

VMs profile released by *A. alternata* growing on Petri plates

The profile of volatile metabolites emitted by *A. alternata* growing on PDA Petri plates and analyzed by SPME/GC/mass spectrometry is shown in Figure 1a. More than 30 peaks in the headspace of Petri plates were detected, but only 17 volatile metabolites were identified according to their retention times and to NIST library of the mass detector. Among the VM identified are toluene, methylbenzene, 2,2,4,6,6-pentamethyl-3-heptene, xylene, limonene, styrene, heptamethyl-1-nonene, azulene, among others (Table 1). After 10 d of fungal growth, the most abundant compound was styrene with 91.69% of

Figure 1. Gas chromatographic profiles of volatile metabolites detected in the headspace of *Alternaria alternata* growing on PDA plates (a), control tomato (b), and inoculated fruit with *A. alternata* (c) using solid phase microextraction and gas chromatography. Arrows point to the peaks of identified compounds.

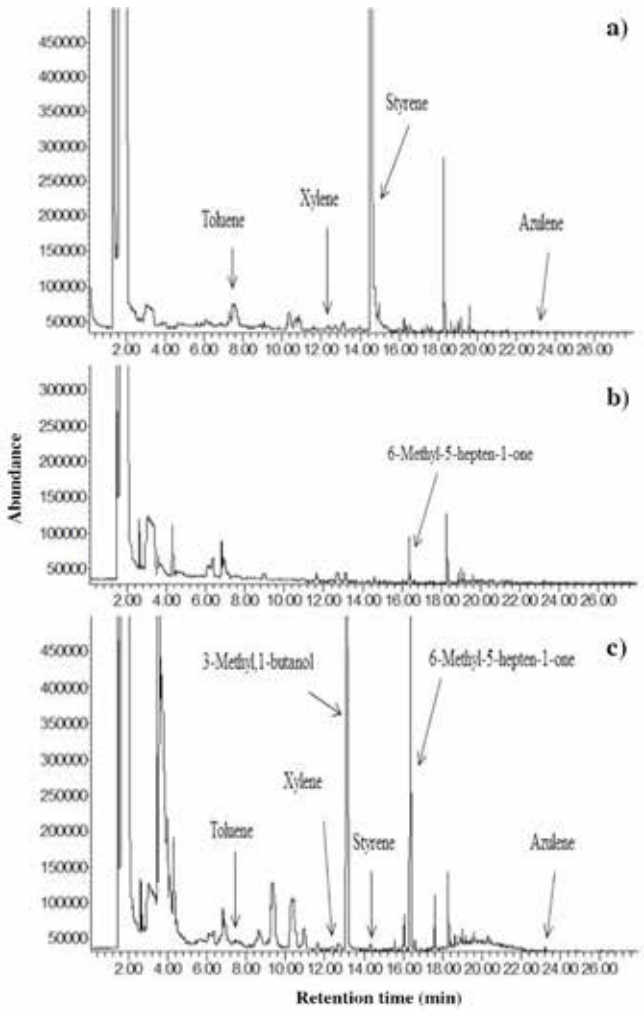


Table 1. Volatile metabolites identified in non-inoculated and inoculated 'Rutgers' tomato fruits with *Alternaria alternata* as well as in *A. alternata* growing in PDA plates. Volatiles metabolites were identified by solid phase micro-extraction and gas chromatography at 20 °C, using commercial standards (CS) and by comparison with NIST mass spectrum (MS).

Volatile metabolite	Retention time (min)	Relative abundance (%)			ID
		Control tomato fruit	Inoculated tomato fruit	*Alternaria alternata*	
Ethanol	4.03	4.02	6.24	ND	MS
Toluene	7.51	ND	1.16	2.96	MS
Dimethyl disulfide	8.66	ND	3.27	ND	MS
Hexanal	9.01	1.28	ND	ND	MS
3-Methyl,2-butenal	9.22	ND	2.11	ND	MS
2-Methyl,1butanol acetate	10.27	ND	6.54	ND	CS
Ethylbenzene	10.38	ND	ND	2.34	MS
1-3-Dimethyl benzene	10.85	ND	ND	0.86	MS
1-Butanol	10.95	ND	8.27	ND	MS
Xylene	12.40	ND	1.72	0.23	MS
Limonene	12.60	7.90	0.66	0.75	MS
3-Methyl, 1-butanol	13.13	19.27	25.40	0.26	CS
1-Ethyl-2-methyl benzene	13.66	ND	ND	0.03	MS
Styrene	14.50	ND	0.17	91.69	MS
Benzene, 1-methyl-2-2(1-methylethyl)	14.82	ND	ND	0.03	MS
1-Nitrobutane	15.56	ND	0.80	ND	MS
3-Heptene,2,2,4,6,6 pentamethyl	15.84	ND	ND	0.02	MS
2-Methyl, 1-butenol	15.99	ND	0.32	ND	MS
4-Methyl, 1-pentanol	16.08	ND	0.48	ND	MS
Methyl styrene	16.26	ND	ND	0.30	MS
6-Methyl-5-hepten-1-one	16.35	42.71	39.81	ND	CS
Butanoic acid, 2-methoxy methyl ester	16.39	ND	ND	0.02	MS
1-Hexanol	16.59	2.06	0.61	ND	MS
Nonanal	16.73	0.27	ND	ND	MS
1-Pentene,3,3,4-trimethyl-5-phenyl	16.80	ND	ND	0.03	MS
2-Isobutiltiazole	17.50	1.65	2.82	ND	MS
Heptamethyl-1-nonene	17.66	ND	ND	0.08	MS
1,2,4,5-Tetramethyl benzene (Durene)	18.60	ND	ND	0.03	MS
Benzaldehyde	19.60	0.91	ND	0.07	CS
Bicyclo (5,3,0) decapentaene (Azulene)	23.22	ND	0.106	0.08	MS

ID: Identification type; ND: non detected; MS: identification by comparison with NIST mass spectrum; CS: identification by comparison with commercial standards.

relative abundance. Pagot et al. (2007) suggest that the enzymes phenylalanine ammonia-lyase and decarboxylase of cinnamic acid are involved in the process to convert phenylalanine to styrene in the fungus *Penicillium camemberti*. On the other hand, it has been reported that phenylalanine is a product of glucose biosynthesis (Snell et al., 1996). Due to glucose is the C source in the PDA culture medium, it is possible that fungus *A. alternata* can synthesize and emit this MV as reported in *Fusarium oxysporum* (Beck et al., 2008).

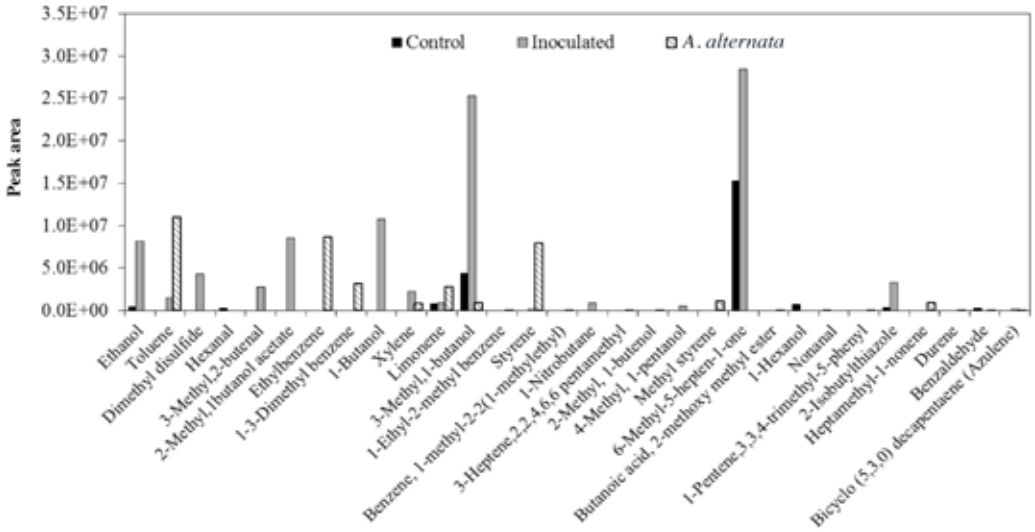
VMs profile of fresh tomato fruit

Figure 1b shows the chromatogram of the profile of VMs detected in control fruits. Approximately 20 well-defined peaks were detected, but only nine were identified (Table 1). They were classified as alcohols, aldehydes, ketones, terpenes and sulfur metabolites, such as ethanol (4.02%), 3-methyl-1-butanol (19.27%), hexanal (1.28%), nonanal (0.27%), benzaldehyde (0.91%), 6-methyl-5-hepten-2-one (42.71%), hexanol (2.06%), limonene (7.9%), and 2-isobutylthiazole (1.65%). Among these metabolites, some of the identified volatiles derive from the lipoxygenase metabolic pathway, such as hexanal and nonanal, while limonene is derived from the mevalonate pathway, and 6-methyl-5-hepten-1-one is derived from the carotenoid catabolism. Also, some of the VM identified derive from the shikimic acid pathway and amino acid metabolisms, such as ethanol, 3-methyl 1-butanol, and benzaldehyde. The most abundant volatile compound was 6-methyl-5-hepten-2-one, with 42.71% of relative abundance.

Volatile profile released during tomato-*A. alternata* interaction

The profile of volatile metabolites released by the inoculated tomatoes with *A. alternata* is shown in Figure 1c. From the figure, it is clear that more VMs were released from inoculated fruits as compared to control fruit. Among them, 17 could be identified as alcohols (ethanol, 2-methyl-1-butanol acetate, 3-methyl-1-butanol, 1-butanol, 1-pentanol-4-methyl, and 1-hexanol), aldehyde (3-methyl-2-butenal), ester (2-methyl-1-butanol acetate), ketone (6-methyl-5-hepten-1-one), aromatic hydrocarbons (toluene, styrene, xylene, and bicyclo[5.3.0]decapentaene (azulene)), sulfur compounds (dimethyl disulphide and 2-isobutylthiazole), nitrogen metabolite (1-nitrobutane), and terpene (limonene) (Table 1). Some of these VM showed a higher increase of peak area in tomatoes during the fungal attack, particularly ethanol, 3-methyl, 1-butanol, 6-methyl-5-hepten-1-one, and 2-isobutylthiazole (Figure 2). Also, VMs identified as dimethyl disulfide, 3-methyl-2-butenal, 2-methyl-1-butanol acetate, 1-butanol, 1-nitrobutane, 2-methyl-1-butenol, 4-methyl-1-pentanol, and 1-hexanol were only detected

Figure 2. Changes in the peak areas corresponding to the volatile metabolites detected in the control tomato, inoculated fruits and in the fungus *Alternaria alternata* at 20 °C.



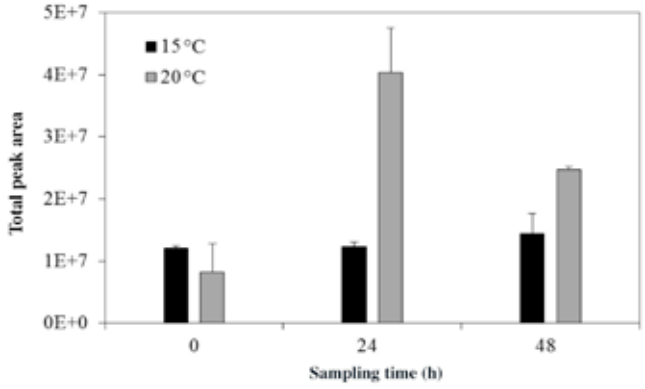
in inoculated fruit, suggesting that they are related to the phenomena of the tomato-*Alternaria* interaction.

By another side, some peaks registered in inoculated fruit were also detected in the fungus, such as toluene, xylene, styrene, and azulene, but not in control fruit (Table 1). Therefore, these volatile could consider as markers of Alternaria rot, but further studies are needed to verify this hypothesis.

Effect of post-inoculation time and temperature on the VMs profile

Temperature and time course of the infection affected the profile of VMs released by inoculated tomatoes. Figure 3 shows the VMs profile released from inoculated fruit or control, at 15 °C and 20 °C for 48 h post-inoculation (hpi). From the figure we can observe that, in most cases, VMs detected at 20 °C showed higher peak area at a different post-inoculation time than those detected at 15 °C. In inoculated fruit, toluene was detected within 1 hpi at 15 °C;

Figure 3. Total peak area of the volatile metabolites detected in tomatoes at three different times, using the solid phase microextraction fiber carboxen/polydimethylsiloxane and gas chromatography. Vertical lines represent standard deviation (n = 3).



whereas at 20 °C, it was detected after 6 hpi showing the highest peak area after 9 hpi (Figure 4a).

The volatile 2-methyl-1-butanol acetate, 4-methyl-1-pentanol and 1-butanol were mainly detected in inoculated fruit (Figures 4b, 4d, and 4f, respectively). Peak area of the ester 2-methyl-1-butanol acetate was higher at 15 °C that at 20 °C; while 4-methyl-1-pentanol showed a higher peak area after 6 hpi at 20 °C as compared to 15 °C. Similar pattern showed the emission of 1-butanol. Two of the VMs detected in control, and inoculated fruits were 3-methyl-1-butanol and 6-methyl-5-hepten-1-one. These volatiles were detected within the 1 hpi at both temperatures (Figure 4c and 4e, respectively) showing the highest peak area in inoculated fruit. Before 24 hpi, all of these VMs reached their maximum peak area at both temperatures, while control fruit had a low and constant emission of those compounds at both temperatures and throughout the evaluation period.

Alternaria rot development on the fruit surface

Figure 5 shows the lesion size caused by *A. alternata* on the fruit surface. Visible symptoms of disease were evident at both temperatures, albeit at different times. After 72 and 96 hpi, slight rot lesions appeared on the surface of tomatoes stored at 20 and 15 °C, respectively. A clear development of *Alternaria* rot on the fruit surface was observed after 144 hpi at 20 °C. The control fruit lacked visible symptoms of *Alternaria* disease.

DISCUSSION

Using the SPME-GC-MS technique, changes in the volatile metabolite profile in tomatoes inoculated with *A. alternata* were detected within the first hour post-inoculation. From the results presented here, we can mention that specific VMs are released during early stages of infection caused by the fungus in tomatoes, even at low conidia concentration

Figure 4. Effect of temperature and sampling time on the evolution of volatile metabolites released by control fruit and inoculated with *Alternaria alternata* (a: toluene; b: styrene; c: 2-methyl-1-butanol acetate; d: 3-methyl-1-butenal; e: 3-methyl-1-butanol; f: 6-methyl-5-hepten-1-one; -●- Control 15 °C; -○- Inoculated 15 °C; -▼- Control 20 °C; and -Δ- Inoculated 20 °C).

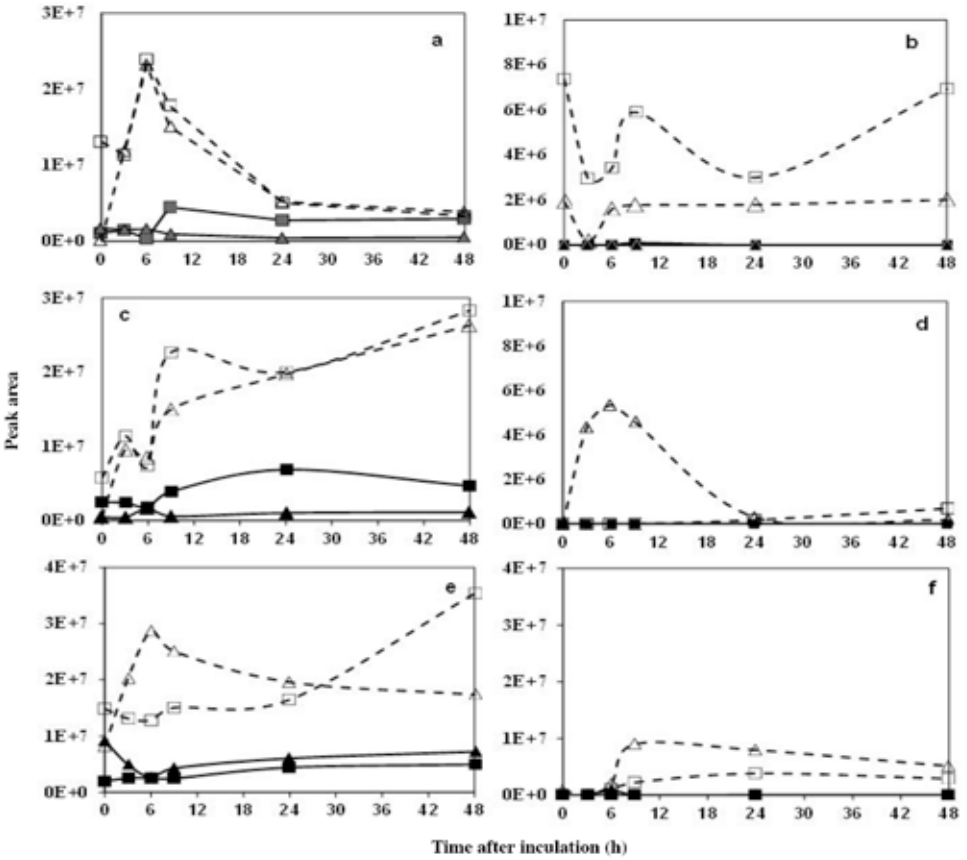
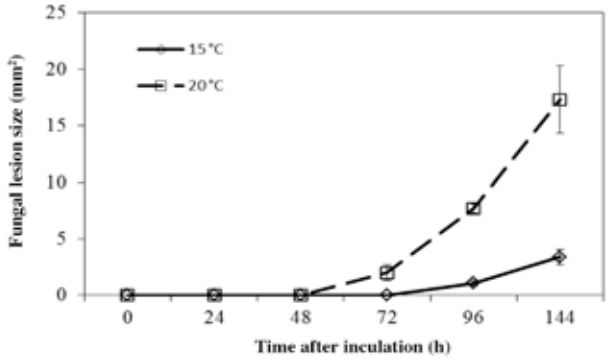


Figure 5. Alternaria rot development on the surface of tomato fruit after different hours post-inoculation at 15 and 20 °C. Vertical lines represent standard deviation (n = 4).



(10^4 conidia mL^{-1}). Fungi, plants, and fruits produce a variety of volatile metabolites that could act as signals of plant disease (Jansen et al., 2011), or as signaling molecules that play a vital role in the activation of disease resistance mechanisms. Also, could act as antimicrobial agent (Neri et al., 2015), or could serve as markers for the detection of the spoilage pathogens (Vikram et al., 2004; 2006; Moalemiyan et al., 2006). In this study, were identified some VMs released from strains of *A. alternata* growing on PDA plates. The most abundant compound was styrene, followed by toluene, 1,3-dimethyl benzene and ethylbenzene. Other VMs detected in the fungus were xylene and azulene. These results agree to those reported by Schuchardt and Kruse (2009), who conducted a study on the volatile organic compounds emitted by 14 different fungi, including *A. alternata*. The authors detected compounds like 2-methyl, 1-butanol, toluene and limonene in *A. alternata*. Styrene was found previously in other fungi, including *Fusarium oxysporum* (Beck et al., 2008), *Cladosporium* sp., *Aspergillus niger*, *Emericella* sp., *Mucor plumbeus*, *Trichoderma* spp. (Nieminen et al., 2008), and *Penicillium expansum* (Sagi-Kiss and Fodor, 2011). By another side, some VMs detected in *A. alternata* growing on PDA media, such as 3-heptene, 2,2,4,6,6-pentamethyl, butanoic acid, 2-methyl ester, heptamethyl-1-nonene, 1,2,4,5-tetramethyl benzene, and bicyclo[5.3.0] decapentaene, have not been previously reported in the literature.

Classical black mold lesions in inoculated tomatoes were observed, which indicate the active infection on the fruit 72 hpi. This result coincides with previous experiments carried out in our lab in which tomatoes was infected with the same species of *Alternaria* (Troncoso-Rojas et al., 2005; Ruelas et al., 2006; Cota et al., 2007). Also, this result agrees with other studies in which tomatoes were inoculated with *A. alternata* (Reddy et al., 2000).

The data obtained in this study show that the inoculation with *A. alternata* modified the VMs profile of control (no

inoculated) tomatoes. The profile of volatile metabolites during tomatoes-*A. alternata* interaction was characterized by the dominance of alcohols and hydrocarbons (4) and a limited presence of aldehydes, esters, terpenes, sulfur, and ketones. Of these, seven VM were only detected in inoculated fruit (dimethyl disulfide, 3-methyl-2-butenal, 2-methyl-1-butanol acetate, 1-butanol, 1-nitrobutane, 2-methyl-1-butenol, and 4-methyl-1-pentanol), so they appear to be synthesized by the presence of the pathogen. Also, some volatile compounds were detected in both inoculated and control fruit, but they had more abundance in the inoculated fruit, such as ethanol, 3-methyl-1-butanol, 6-methyl-5-hepten-1-one, and 2-isobutylthiazole. A marked increase of ethanol was observed in inoculated fruit (20 times) as compared with the control. Similar results were reported in carrot root inoculated with *Aspergillus niger*, *Botrytis cinerea* and *Fusarium avenaceum* (Vikram et al., 2004), and in mango fruit inoculated with *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* (Moalemiyan et al., 2006). The volatiles 3-methyl-1-butanol and 2-isobutylthiazole showed 5.8 and ten times more the abundance with respect to control fruit, suggesting that they were stimulated or induced in response to fungal infection.

Our results clearly showed that after the fungal attack, tomatoes released volatile metabolites such as hexanal, nonanal, 2-methylbutyl acetate, 6-methyl-5-hepten-1-one, among others, which have demonstrated in previous studies to stimulate fungal growth. In strawberry, these volatile showed a stimulatory effect on conidial germination of *Botrytis cinerea* (Neri et al., 2015). In the present study, a marked increase of 6-methyl-5-hepten-1-one was observed in inoculated fruit, even during the first few hours after inoculation, and it was progressive over time particularly at 20 °C, following a similar pattern of the *Alternaria* rot development. Therefore, it is possible that this volatile could also stimulate the growth of *A. alternata* as it occurs in *B. cinerea*; nevertheless, future studies are required to verify this hypothesis.

The detection of alcohols and aldehydes with low molecular weight in inoculated fruit is probably due to the release of VM when cuticle of tomatoes is damaged by *A. alternata*, or to the synthesis of new volatiles due to the interaction of enzymes and substrates that occur due to cellular damage. *Alternaria* is a necrotrophic phytopathogen fungus that lives quiescently in their host in the cuticular wax or intercellular space until the fruits ripen (Prusky et al., 2013; Alkan et al., 2015). It has the ability to produce cutinases (Trail and Köller, 1993) and penetrate the cuticle, the first structural barrier of fruit defense, and subsequently damages the cell membrane activating lipid peroxidation through the action of enzymes such as phospholipases and lipoxygenase (LOX) (Alkan et al., 2015). Free fatty acids of cuticle or the membrane, are rapidly catabolized by means of β-oxidation, α-oxidation, or the lipoxygenase pathway (Zhao et al., 2014), releasing C_6 volatile compounds such as hexanol, (*Z*)-3-hexenal, (*E*)-2-hexenal, or hexanal, among others. This observation is in agreement with the study reported by Jansen et al. (2009) and Rambla et al. (2014).

Some VMs were detected in tomatoes within first hours post-inoculation at both temperatures, whereas the first characteristic disease symptoms were observed on the fruit surface after 72 hpi at 20 °C. These changes in the profile of the volatile metabolites released by inoculated tomatoes with the fungus were detected before the disease symptoms were visible on the fruit surface. These results suggest that some of the volatile compounds released during the early phases of the tomato-*A. alternata* interaction could participate in the transition phenomena from the quiescent state to an aggressive colonization of the fungus. Nevertheless, more studies are needed to prove this statement.

CONCLUSIONS

Alternaria alternata induced changes in the profile of volatile metabolites released by tomato fruit. The tomatoes -*A. alternata* interaction was characterized by the dominance of alcohols and hydrocarbons with low molecular weight. The volatile 3-methyl, 2-butenal, dimethyl disulfide, 1-butenol, hexanol, 2-methyl, 1-butanol acetate, were only detected in inoculated fruit, so they appear to be synthesized by the presence of the pathogen. Also, a marked increase of 3-methyl-1-butanol and 6-methyl-5-hepten-1-one were observed in inoculated fruit, even during the first few hours after inoculation, and it was progressive over time particularly at 20 °C, following a similar pattern of the *Alternaria* rot development. These results contribute to the current knowledge about the profile of VM released during the fruit-pathogen interaction.

ACKNOWLEDGEMENTS

The authors are grateful to the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, A.C.) Project nr 6433, and Produces Foundation (Fundación Produce Sonora) from Sonora, Mexico for their financial support.

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