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Effect of hot water treatment on peach volatile emission and *Monilinia fructicola* development

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The effect of hot water treatment (HWT) to control peach brown rot was investigated. Peaches were dipped in water at 60°C for 60 s and artificially inoculated with *Monilinia fructicola* conidia. HWT failed to control brown rot if applied before inoculation and microscopic observations revealed a stimulatory effect on germ tube elongation of *M. fructicola* conidia placed immediately after HWT on the fruit surface, compared to the control. The influence of fruit volatile emission due to HWT was performed on the pathogen conidia exposed to the headspace surrounding peaches. The results showed an increase of *M. fructicola* conidial germination ranging from 33 to 64% for cultivars Lucie Tardibelle and Red Haven heat-treated peaches, respectively, compared to the control. The volatile blend emitted from heat-treated fruit was analysed by solid-phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS). Fifty compounds were detected by SPME/GC-MS in volatile blends of cv. Lucie Tardibelle peaches and significant differences in volatile emission were observed among heated and control fruit. Using PTR-ToF-MS analysis, acetaldehyde and ethanol were detected at levels 15- and 28-fold higher in heated fruit compared to unheated ones, respectively. *In vitro* assays confirmed the stimulatory effect (60 and 15%) of acetaldehyde (0·6 μL L^{-1}) and ethanol (0·2 μL L^{-1}) on *M. fructicola* conidial germination and mycelial growth, respectively. The results showed that volatile organic compounds (VOCs) emitted from heat-treated peaches could stimulate *M. fructicola* conidial germination, increasing brown rot incidence in treated peaches when the inoculation occurs immediately after HWT.

Keywords: acetaldehyde, brown rot, ethanol, heat treatment, *Prunus persica*, VOCs

Introduction

Peaches are climacteric fruits, which deteriorate quickly at ambient temperature due to their rapid ripening process and susceptibility to abiotic and biotic stresses. Among biotic stresses, brown rot caused by *Monilinia* spp. is one of the main diseases affecting stone fruit and limiting the extension of fruit storage (Sisquella *et al.*, 2013). The disease is favoured by the occurrence of weather conditions such as high humidity, warm temperatures and abundant rainfall prior to harvest. The infections, occurring in the field, can remain quiescent until fruits ripen, allowing the pathogen to overcome host defences (Mari *et al.*, 2009). Generally, the main fruit losses occur during storage, at retail and consumer sites, reaching high values (more than 50%; Larena *et al.*, 2005). The control of brown rot depends on an integrated strategy based on cultural practices and fungicide spray programmes in the field, because postharvest fungicide treatments are not allowed in European countries. During recent decades, consumer interest in 'organic' and 'safe' food products has increased, leading to the evaluation of more environmentally-friendly postharvest treatments to reduce losses and maintain high fruit quality during storage. Heat treatments are particularly promising because of their complete safety (no concern during application, 'zero residue' on fruit), ready use without registration rules and efficacy in fungal pathogen control and insect disinfestation. Heat treatments are environmentally friendly and recommended as alternative treatments to replace pesticide applications, especially with regard to fresh produce. These treatments help to eradicate pathogens or pests that are present on the fruit surface, maintaining the overall quality of the fresh produce during the supply chain.

Some studies have, in particular, proved the high efficacy of hot water treatment (HWT) in the control of brown rot, when applied alone (Spadoni *et al.*, 2013) or combined with peracetic acid (Sisquella *et al.*, 2013); however, temperatures lower than 65°C should be used with peaches to avoid physical injury. The primary mode of action of HWT is a direct effect on infection structures of fungi present on the fruit surface or in the first layers under the skin (Palma *et al.*, 2013); a collapse of mitochondrial membrane potential of *M. fructicola* spores after heat treatment was observed in inoculated peaches (Liu *et al.*, 2012). Many

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studies on fungal disease control proved the curative activity of HWT in different fruit species (Vincente *et al.*, 2005; Neri *et al.*, 2009; Karabulut *et al.*, 2010); in particular, a significant reduction of *M. fructicola* disease was observed on peaches subjected to HWT (60°C for 1 min) applied 48 h post-inoculation (Spadoni *et al.*, 2014). The use of heat as a preventive treatment in disease control has been less studied; however, a resistance induction was hypothesized because an increase of enzyme activity responsible for the host defence mechanism after HWT was reported (Liu *et al.*, 2012). In order to carry out the practical application of HWT on a large scale, the effects of treatment on fruit should be examined in detail. However, only a few studies have focused on the influence of heat on fruit volatile emission, as well as on the influence of fruit volatile organic compounds (VOCs) on fungal pathogen growth.

The present study investigated the influence of a preventive HWT of peach on *M. fructicola* pathogenicity and aimed to evaluate the following objectives: (i) the effects of the treatment on the development of *M. fructicola* artificial infections; (ii) the direct effects of VOCs emitted by heat-treated peaches on *M. fructicola* growth by *in vitro* and *in vivo* trials; (iii) the identification of VOCs produced after fruit HWT with solid-phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) and with proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS); and (iv) the effects of pure volatile compounds on *M. fructicola* growth.

Materials and methods

Fruit

Peach (*Prunus persica*) cultivars Springbelle, Royal Summer, Red Haven and Lucie Tardibelle were grown in commercial orchards located in Emilia Romagna (Italy) following standard cultural practices and chemical spray programmes, while no fungicide treatments against *Monilinia* spp. were performed. Fruits without visible wounds or rots and homogeneous in size were harvested at commercial maturity, stored at 0°C and used within a couple of days after harvest.

Pathogen

The isolate of *M. fructicola* (MFA55), obtained from the authors' collection and previously identified by sequencing of ribosomal DNA ITS regions (Mari *et al.*, 2012), was maintained on potato dextrose agar (PDA) at 4°C until use. In order to obtain a good sporulation of the pathogen, it was inoculated on V8 agar (V8A; 250 mL V8 juice, 40 g agar, 1 L distilled water) and incubated at 25°C with a 12 h dark/12 h light cycle for 10 days. Conidial suspensions were prepared by washing the colonies with sterile distilled water containing 0·05% (v/v) Tween 80, quantified with a haemocytometer and diluted to the required concentration for each assay.

Influence of preventive HWT of fruits artificially infected with *M. fructicola*

The effect of preventive HWT to control *M. fructicola* infections was assayed on unwounded and wounded cv. Red Haven peaches. Fruits were previously washed with sodium hypochlorite (1% w/v) and rinsed in distilled water for 1 min to remove pathogen natural inoculum from the fruit surface and left to dry at room temperature for 1 h. Subsequently, a batch of unwounded fruits was heated using the methodology proposed by Spadoni *et al.* (2013) with slight modifications. Peaches were inserted in metallic grid baskets and submerged for 60 s in a stainless steel tank fitted with an electric resistance heater and thermostat containing water at 60°C. Control fruits were dipped in water at room temperature (20°C) for 60 s. After 0, 0·25, 1, 3 and 6 h from the treatment, all fruits were inoculated by dipping in 5 L of a *M. fructicola* conidial suspension (5×10^3 conidia mL^{-1}) and kept for 5 days at 20°C. Subsequently, the percentage of infected fruits was recorded. The sample unit was represented by four replicates of six fruits each and the experiment was performed twice. Another batch of fruits, previously disinfected, was heat-treated by immersion at 60°C for 60 s and after 0, 0·25, 1, 3 and 6 h all fruits were wounded with a sterile nail ($2 \times 2 \times 2$ mm) and inoculated, introducing 20 μL of a *M. fructicola* conidial suspension (10^3 conidia mL^{-1}) into the wound. The control was represented by peaches dipped in water at 20°C, wounded and inoculated as cited above. All fruits were kept for 5 days at 20°C and the lesion diameters (mm) were recorded. The sample unit was represented by four replicates of six fruits each and the experiment was performed twice.

Effect of HWT on *M. fructicola* germ tube elongation on fruit surface

To study the stimulation of *M. fructicola* on heat-treated fruit, pathogen conidial germination was examined on the fruit surface by microscopic observations. For this purpose, cv. Red Haven peaches were sterilized and treated by heat as described above. Immediately (T0) and 24 h after treatment (T24), peaches were sprayed with a conidial suspension of *M. fructicola* (10^6 conidia mL^{-1}), distributing an aliquot of 5 mL suspension per fruit. Fruits immersed in water at room temperature and inoculated as described above represented the control. After inoculation, the fruits were placed in separate glass boxes ($32 \times 24 \times 20$ cm) covered on the bottom with a paper leaf soaked with 10 mL distilled water in order to maintain a high relative humidity (>95%). The boxes were hermetically sealed by Parafilm and incubated at 20°C. To observe the germ tube elongation of germinated conidia on the fruit surface, a fruit epidermal layer (4×4 mm) was removed with a sterile scalpel to a depth of about 0·5 mm in three different positions per fruit at 18 h post-inoculation (hpi), in both conditions (T0 and T24). The tissue pieces were placed on a glass microscope slide; the conidia were stained with 1–2 drops of lactophenol blue solution (Sigma-Aldrich) and visualized with a Nikon Eclipse TE2000-E as described by Guidarelli *et al.* (2011). The sample unit was represented by three microscope observations (replicates) of 10 germ tubes each for both inoculation times (T0 and T24).

Influence of volatile blend emitted from heat-treated peaches on *M. fructicola* conidial germination

A pathogen conidial suspension (10^3 conidia mL^{-1}) was prepared as described above and an aliquot of 100 μL was spread on malt extract agar (MEA). Peach cultivars Springbelle, Red Haven and Lucie Tardibelle were heat-treated by dipping in water at 60°C for 60 s. Immediately after the treatment, fruits were placed inside the glass boxes described above. The plates inoculated with *M. fructicola*, opened and overturned to directly

expose the plate surface to VOCs produced by the fruit, were inserted in the same boxes. A mesh was used to avoid any physical contact between pathogen and fruit surface. Boxes containing fruits immersed in water at room temperature represented the control. Ten to 12 peaches per cultivar, depending on fruit size, were placed in each box with eight overturned inoculated plates. Boxes were sealed with a Parafilm double layer and incubated at 20°C for 4 days. The conidial viability was measured as colony-forming units (CFUs).

Analysis of VOCs emitted by heat-treated peaches

PTR-ToF-MS analysis

VOCs emitted by Lucie Tardibelle peaches were analysed following the procedure described in previous studies on apple (Soukoulis *et al.*, 2013) using a commercial PTR-ToF-MS 8000 apparatus (Ionicon Analytik GmbH) in its standard configuration (V mode). Before measurement, a batch of four peaches was immersed in water at 60°C for 60 s, while control peaches were immersed in water at room temperature for the same time. Immediately after treatment, each individual fruit was placed in a glass jar (1 L) provided with two Teflon/silicone septa on opposite sides. To standardize the measurements, all samples were then equilibrated at room temperature for 30 min prior to analysis. Peaches were consequently closed hermetically for 10 min inside the glass jars to accumulate the volatile compounds emitted. VOCs were then measured by direct injection of the headspace mixture into the PTR-ToF-MS drift tube via a heated (110°C) peek inlet for 10 min. The sampling time per channel of ToF acquisition is 0·1 ns, amounting to 350 000 channels for a mass spectrum ranging up to m/z = 400, with the following conditions in the drift tube: drift voltage 600 V, temperature 110°C and pressure 2·25 mbar. Every single spectrum is the sum of 28 600 acquisitions lasting for 35 μs each.

Spectra analysis

The external calibration automatically done by the acquisition programme provided a poor mass accuracy, thus internal calibration of ToF spectra was performed off-line (Cappellin *et al.*, 2010). Signal losses caused by the detector dead time and duty cycle were corrected as reported by Cappellin *et al.* (2011). Data preprocessing on ToF spectra was carried out to remove the baseline and noise reduction was achieved by averaging over the 30 consequent ToF spectra corresponding to the same sample, thereby allowing the improvement of the signal-to-noise ratio by about five times. For peak identification and area extraction, the procedure followed was according to Cappellin *et al.* (2011). The experimental m/z values reported were up to the third decimal. VOC concentrations were expressed in ppbv (part per billion by volume) and were calculated from peak areas according to Lindinger *et al.* (1998), using tabulated values for the reaction rate coefficient (Cappellin *et al.*, 2012). When the reaction rate coefficient was not available within the literature, a constant value ($k = 2 \times 10^{-9}$ cm^3 s^{-1}) was employed.

SPME/GC-MS analysis

For the SPME/GC-MS analysis, a similar procedure to the one employed for the PTR-ToF-MS analysis was used. Before measurement, control fruits were immersed in water at room temperature for 60 s while hot water-treated peaches were immersed in water at 60°C for 60 s. To standardize the measurements, all samples were equilibrated at room temperature for 30 min prior to analysis. Each individual fruit was placed in a glass jar (1 L) provided with two Teflon/silicone septa on opposite sides and kept at room temperature for 30 min for volatile compound collection. The sample unit was represented by one fruit (replicate) repeated three times per treatment.

Headspace SPME/GC-MS

Headspace volatile compounds were collected by a 2 cm Solid Phase Microextraction fibre coated with divinylbenzene/carboxen/polydimethylsiloxane 50/30 lm (DBV/CAR/PDMS; Supelco), inserted through a Teflon/silicone septum using a manual holder (Supelco). The fibre was exposed to the peach headspace for 30 min. Volatile compounds adsorbed on the SPME fibre were desorbed at 250°C in the injector port of a GC interfaced with a mass detector operating in electron ionization mode (EI, internal ionization source; 70 eV) with a scan range from m/z 35 to 300 (GC Clarus 500; PerkinElmer). Separation was achieved on an HP-Innowax fused silica capillary column (30 m, 0·32 mm ID, 0·5 μm film thickness; Agilent Technologies). The GC oven temperature programme consisted of 40°C for 3 min, then 40–220°C at 4°C min^{-1}, stable at 220°C for 1 min, and then 220–250 at 10°C min^{-1}, and finally 250°C for 1 min. Helium was used as the carrier gas with a constant column flow rate of 2 mL min^{-1}. Compound identification was based on mass spectra matching with the standard NIST05/Wiley98 libraries and retention indices (RI) of authentic reference standards.

Effect of pure VOCs on *M. fructicola* growth

Acetaldehyde and ethanol, the main VOCs emitted from heated peaches, were purchased as pure compounds from Sigma-Aldrich and tested for their effects on *M. fructicola* conidial germination and mycelial growth in *in vitro* assays. Aliquots of 100 μL of a conidial suspension of *M. fructicola* or a plug (6 mm diameter) from an actively growing pathogen culture were respectively spread or placed in the centre of MEA plates. In each case, aliquots of 0·2 μL L^{-1} of pure ethanol or 0·6 μL L^{-1} of pure acetaldehyde were placed using a microsyringe on a paper filter (Whatman no. 1, 90 mm diameter) positioned inside the cover, as described by Neri *et al.* (2009). VOC concentrations were selected in order to roughly match the corresponding concentrations derived from VOC emission by heat-treated fruits determined with PTR-ToF-MS (see Results). The dishes were quickly closed, sealed with Parafilm, and incubated at 25°C. Control samples were represented by Petri dishes inoculated with pathogen but treated with distilled water instead of a chemical compound. The CFUs were recorded after 48 h of incubation, while the mycelium growth was recorded after 5 days as colony diameter (mm). For both compounds tested and for each biological fungal parameter, five Petri dishes (replicates) were used. The assay was performed three times.

Statistical analysis

All data regarding infected fruits, culturable conidia test and germ tube elongation were subjected to one-way analysis of variance (ANOVA) using STATISTICA FOR WINDOWS (Statsoft Inc.). Separation of means was performed using the least significance difference (LSD) test, $P < 0.05$. For the statistical analysis of VOC data, specific scripts were developed using R (R Foundation for Statistical Computing). Pairwise multiple comparisons were made using the Tukey's post hoc test at the same significance level. All experiments were carried out in a complete randomized block design.

Results

Influence of a preventive HWT of fruits artificially infected with *M. fructicola*

HWT applied before inoculation with *M. fructicola* failed to control the development of brown rot, both in intact and wounded peaches. The incidence of infection in heated intact fruits was in fact 100% for all times of inoculation tested (0–6 h), significantly higher than the incidence of disease observed in untreated fruits, which ranged from 15 to 50%, for fruits dipped in water at 20°C immediately or 6 h after inoculation with *M. fructicola*, respectively (Fig. 1). Similarly, heated fruits wounded and inoculated with the pathogen showed lesion diameters significantly larger than those observed in untreated fruits, except for the inoculations performed immediately (0 h) and 6 h after HWT (Fig. 2). However, all wounds of heated and control fruits were infected over 80% in all assayed times (data not shown).

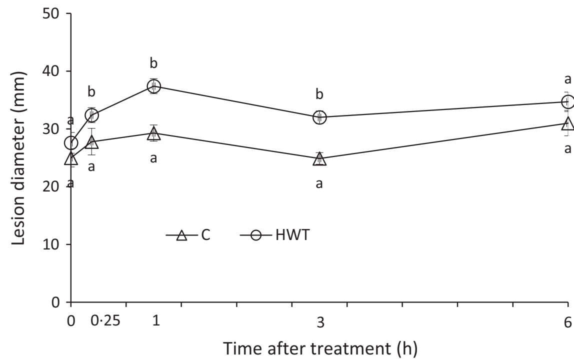

Figure 2 Effect of preventive hot water treatment (HWT) on wounded cv. Red Haven peaches inoculated with *Monilinia fructicola*. Fruits were dipped in water at 60°C for 60 s and wounded and artificially inoculated with 20 μL of a pathogen conidial suspension (10^3 conidia mL^{-1}) per wound at 0, 0·25, 1, 3 and 6 h after treatment. Control fruits (C) were treated by dipping in water at 20°C for 60 s. The lesion diameters were recorded after 5 days at 20°C. Each point represents the mean of 24 fruits ± SE. Within the same time, the same letters represent no significant differences according to LSD test ($P \leq 0{\cdot}05$).

Effect of HWT on *M. fructicola* germ tube elongation on fruit surface

The development of *M. fructicola* conidia on the surface of cv. Red Haven peaches treated with hot water and subsequently inoculated (immediately or after 24 h) was microscopically observed (Fig. 3). After 18 hpi, most conidia (>95%) had germinated both in HWT and control fruits (data not shown). In addition, the conidia inoculated immediately (0 h) after HWT showed elongation tubes significantly longer (135 ± 7·8 μm) than those observed in conidia inoculated on control fruits (58 ± 4·5 μm). The stimulatory effect was absent when the inoculation was performed 24 h after treatment. In this case no significant differences were found between germ tube length of conidia inoculated on control (73 ± 6·3 μm) and heat-treated fruits (72 ± 4·8 μm; Table 1).

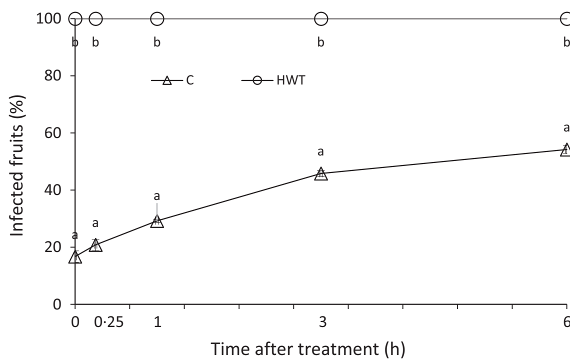

Figure 1 Effect of preventive hot water treatment (HWT) on unwounded cv. Red Haven peaches inoculated with *Monilinia fructicola*. Fruits were dipped in water at 60°C for 60 s and inoculated by immersion in a pathogen conidial suspension (4×10^3 conidia mL^{-1}) at 0, 0·25, 1, 3 and 6 h after treatment. Control fruits (C) were dipped in water at 20°C for 60 s. The percentage of infected fruits was recorded after 5 days at 20°C. Each point represents the mean of the four replicates of six fruits each ± SE. Within the same time, the same letters represent no significant differences according to LSD test ($P \leq 0{\cdot}05$).

Influence of volatile blend emitted from heated peaches on *M. fructicola* conidial germination

The influence of the volatile blend emitted from heat-treated fruits on conidial germination was assayed. A higher germination rate was observed in the conidia exposed to the headspace of heated peaches than conidia exposed to the headspace of control fruit (Table 2). The best stimulation of conidial germination was observed in cv. Springbelle fruits (64·1%), while the lowest was obtained by volatiles emitted by cv. Lucie Tardibelle heat-treated fruits (33·3%).

Detection and identification of VOCs emitted by heat-treated fruits using PTR-ToF-MS and SPME/GC-MS

The results on VOC emission showed a significant difference between HWT and control fruits. On the whole, 50 compounds were detected by SPME/GC-MS in volatile blends of cv. Lucie Tardibelle peaches (Table 3). The emission of the following compounds was significantly higher in HWT than untreated fruits: the aldehydes acetaldehyde, hexanal and nonanal, the alcohols 3-methyl-1-butanol, 3-hexen-1-ol (Z) and 1-octanol, the esters ethyl acetate, isoamyl acetate, hexyl acetate, butanoic acid 3-methyl ethyl ester and the alkane octane. In comparison, the hydrocarbons alpha-pinene, toluene and pentadecane, and an unknown compound, diminished or were not detected after the treatment. Ethanol was only detected

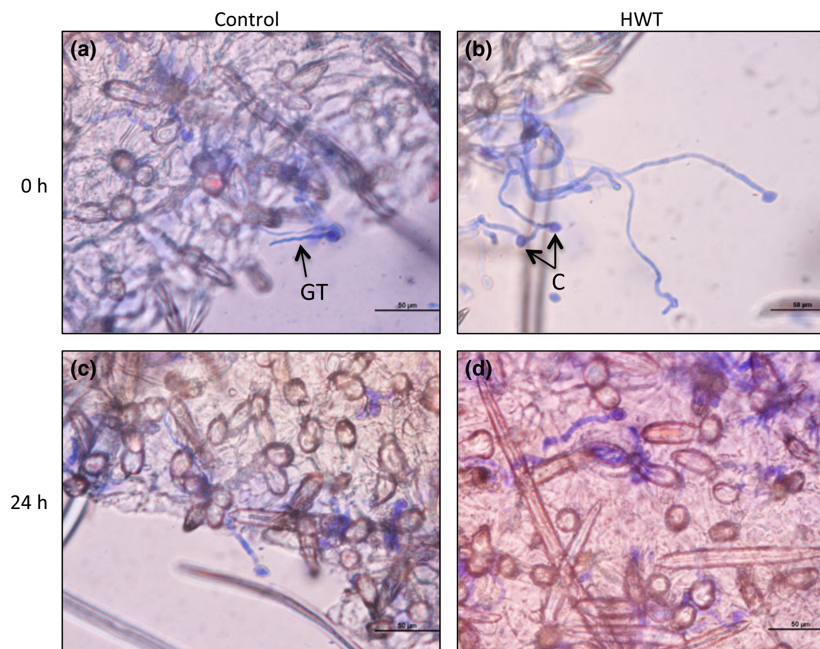

Figure 3 Influence of hot water treatment (HWT) on germ tube elongation of *Monilinia fructicola* conidia on peach surfaces. Fruits were treated by dipping in hot water (60°C for 60 s) and inoculated with a suspension of 10^6 conidia mL^{-1} immediately (0 h) and 24 h after treatment. Conidia (C) and germ tubes (GT) were stained with lactophenol blue and analysed under an optical microscope. (a, c) Control (fruits dipped in water at room temperature for 60 s) and (b, d) HWT after 18 h post-inoculation. Bar = 50 μm.

Table 1 Influence of hot water treatment (HWT) on germ tube length (μm) of *Monilinia fructicola* conidia inoculated on peach surface 0 or 24 h after treatment by spraying

	Treatment[a]	
Inoculation after treatment (h)	Control	HWT
0	57·5 ± 4·5 a	134·8 ± 7·8 b
24	72·8 ± 6·3 a	71·6 ± 4·8 a

[a]Control fruits were dipped in water at room temperature. Heat-treated fruits were dipped in water at 60°C for 60 s. Heat-treated and control fruits were sprayed with a 5 mL volume of a pathogen conidia suspension (10^6 conidia mL^{-1}). The length of conidia germ tubes was recorded 18 h post-inoculation at 20°C. Each value is the mean of 30 conidia (replicates) ± SE. Within the same row, the same letter represents no significant difference according to LSD test ($P \leq 0·05$).

Table 2 Effect of volatile blend emitted from hot water-treated (HWT) peaches on *Monilinia fructicola* conidia germination[a]

	Treatment[a]		
Cultivar	Control	HWT	Stimulation (%)
Springbelle	14·1[b] ± 1·3 b	39·0 ± 2·8 a	64·1
Red Haven	12·4 ± 1·1 b	20·8 ± 1·8 a	40·0
Lucie Tardibelle	8·3 ± 0·8 b	12·0 ± 1·3 a	33·3

[a]Heat-treated and control fruits were dipped in water at 60°C and room temperature, respectively, for 60 s, prior to being placed inside a glass box. Aliquots of 0·1 mL of conidia suspension (10^3 conidia mL^{-1}) were spread on malt extract agar dishes, incubated at 25°C for 4 days. Each plate was overturned and placed on a mesh dividing it from treated peaches inside the box.

[b]Each value is the mean number of colony-forming units of eight replicates ± SE. Within the same row, the same letter represents no significant difference according to LSD test ($P \leq 0·05$).

in heat-treated fruits, but its increase after treatment was not statistically significant due to high variance. More insights into the behaviour of ethanol emission upon heat treatment were provided by the PTR-ToF-MS measurements, and a clear and significant increase of both ethanol and acetaldehyde was found (Table 4). In fact, acetaldehyde and ethanol emission was 15- and 28-fold higher in heated fruit compared to unheated ones, respectively. Moreover, PTR-ToF-MS measured VOCs not detected with SPME/GC-ME, such as methanol, which did not show any significant variation between heated and control fruits.

Effect of pure VOCs on *M. fructicola* growth

After the PTR-ToF-MS measurements, the effect of acetaldehyde and ethanol, the main components of the volatile blend emitted from heated peaches, was assayed on *M. fructicola* growth at the concentrations observed in the headspace surrounding treated fruit (Table 5). The compounds affected the pathogen development in different ways. Acetaldehyde (0·6 μL L^{-1}) stimulated the conidial germination (+60%), while ethanol (0·2 μL L^{-1}) significantly increased the colony diameter of the patho-

Table 3 Volatile organic compounds (VOCs) emitted from cv. Lucie Tardibelle peaches treated by dipping in hot water (60°C) for 60 s (HWT) and fruit dipped in water at 20°C (Control) for the same time

	Control		HWT								
Compound	Mean	SD	Mean	SD	*P* value	$P \leq 0.05$[a]	Qual (%)	LRI	LRI Bibliography (CP WAX)	RT (min)	Formula
Acetaldehyde			1·7E + 10	4·5E + 09	0·003	*	78	709	700	1·633	C_2H_4O
Octane			4·3E + 09	1·2E + 09	0·003	*	91	802		2·090	C_8H_{18}
1,3-Butadiene,2,3-dimethyl + 1,3.Pentadiene, 4-methyl	2·8E + 09	1·8E + 09			0·057		74	806		2·131	
Unknown	9·1E + 08	3·3E + 08			0·009	*		821		2·296	C_3H_6O
Ethyl acetate			1·4E + 09	2·9E + 08	0·001	*	91	900	882–890	3·147	$C_4H_8O_2$
Ethanol			1·6E + 10	1·2E + 10	0·089		86	941	925–947	3·996	C_2H_6O
3-Heptene, 2,2,4,6,6 - pentamethyl	1·7E + 10	1·3E + 10	1·9E + 10	4·7E + 09	0·780		90	1001		5·207	$C_{12}H_{24}$
α-Pinene	6·9E + 08	1·9E + 08			0·003	*	87	1028		5·901	$C_{10}H_{16}$
Toluene	7·9E + 08	4·1E + 08			0·029	*	86	1053	1028–1049	6·559	C_7H_8
Butanoic acid, 3-methyl-, ethyl ester (ethyl-3-methylbutanoate)	2·1E + 09	2·1E + 08	6·4E + 09	2·1E + 08	0·000	*	97	1084		7·367	$C_7H_{14}O_2$
2,4,4,6,6,8,8-Heptamethyl-1-nonene	1·5E + 09	1·5E + 08	2·5E + 09	8·6E + 08	0·114		59	1097		7·688	$C_{16}H_{32}$
Hexanal	1·6E + 09	4·0E + 08	3·7E + 09	7·1E + 08	0·012	*	94	1100	1089	7·781	$C_6H_{12}O$
Unknown	2·7E + 09	1·6E + 09	3·4E + 09	1·5E + 09	0·600		64	1138		9·094	$C_7H_{14}O_2$
1-Butanol-3-methyl-acetate (isoamyl acetate)			6·2E + 08	2·5E + 08	0·012	*	86	1187		10·842	$C_7H_{14}O_2$
Heptanal	2·1E + 08	1·3E + 07	4·2E + 08	2·3E + 08	0·204		83	1198	1182–1188	11·227	$C_7H_{14}O$
D-Limonene	4·4E + 08	1·8E + 08	2·6E + 08	1·9E + 07	0·170		94	1207	1187	11·540	$C_{10}H_{16}$
3-Methyl-1-butanol			1·3E + 09	5·2E + 08	0·012	*	88	1221		12·027	$C_5H_{12}O$
Furan-2-pentyl-	3·2E + 08	2·8E + 08	1·6E + 08	2·8E + 08	0·538		90	1244	1219–1225	12·823	$C_9H_{14}O$
1- Pentanol	2·4E + 08	4·2E + 08	7·E + 08	8·0E + 08	0·396		64	1263	1253–1257	13·494	$C_5H_{12}O$
Acetic acid, hexyl ester (hexyl acetate)	2·2E + 09	7·6E + 08	4·4E + 09	9·5E + 08	0·036	*	90	1284		14·249	$C_8H_{16}O_2$
2-Butanone, 3-hydroxy- (acetoin)	5·2E + 08	9·1E + 08	1·7E + 09	9·5E + 08	0·185		86	1297		14·679	$C_4H_8O_2$
Octanal	9·2E + 08	8·1E + 08	1·3E + 09	3·8E + 08	0·460		78	1300	1284–1287	14·801	$C_8H_{16}O$
3-Hexen-1-ol, acetate (Z)	1·1E + 10	4·5E + 09	2·8E + 10	1·0E + 10	0·062		90	1329		15·786	$C_8H_{14}O_2$
2-Hexen-1-ol,acetate, E-	3·3E + 08	2·9E + 08	1·2E + 09	4·9E + 08	0·062		78	1346		16·369	$C_8H_{14}O_2$
6-Methyl-5-hepten-2-one	1·4E + 09	1·4E + 09	1·8E + 09	7·2E + 08	0·670		95	1349	1315–1332	16·456	$C_8H_{14}O$
1-Hexanol			3·6E + 09	2·5E + 09	0·069		90	1364	1357–1360	16·971	$C_6H_{14}O$
3-Hexen-1-ol (Z)			1·3E + 09	6·6E + 08	0·025	*	96	1394		18·011	$C_6H_{12}O$
Nonanal	3·9E + 09	4·9E + 08	1·3E + 10	3·4E + 09	0·009	*	95	1404	1384–1390	18·316	$C_9H_{18}O$
2-Hexen-1-ol (E)			1·5E + 09	1·3E + 09	0·130			1416		18·735	$C_6H_{12}O$
Pentadecane	3·0E + 10	6·0E + 09	1·9E + 10	3·3E + 09	0·046	*	97	1500		21·468	$C_{15}H_{32}$

(continued)

Table 3 (continued)

Compound	Control Mean	Control SD	HWT Mean	HWT SD	*P* value	$P \leq 0{\cdot}05$[a]	Qual (%)	LRI	LRI Bibliography (CP WAX)	RT (min)	Formula
Dodecanal or decanal	1·5E + 09	2·5E + 08	1·9E + 09	3·7E + 08	0·227		91	1508	1493 (decanal)	21·708	
Benzaldehyde	1·6E + 09	8·0E + 08	2·1E + 09	1·1E + 09	0·515		96	1533	1516–1518	22·471	C_7H_6O
Linalool	6·1E + 10	8·3E + 09	4·5E + 10	9·0E + 09	0·081		97	1556	1530	23·199	$C_{10}H_{18}O$
1-Octanol			1·1E + 09	2·8E + 08	0·002	*	86	1567	1562–1568	23·515	$C_8H_{18}O$
Hotrienol	1·1E + 09	1·1E + 08	1·0E + 09	3·7E + 08	0·695		86	1619		25·089	$C_{10}H_{16}O$
Acetophenone	4·8E + 08	8·4E + 08	1·3E + 09	1·1E + 09	0·385		91	1660	1652	26·284	C_8H_8O
1-Nonanol	7·2E + 08	6·8E + 07	2·1E + 09	1·9E + 09	0·287		91	1669	1667	26·554	$C_9H_{20}O$
Heptadecane	1·9E + 10	9·6E + 09	2·0E + 10	3·5E + 09	0·884		97	1700		27·474	$C_{17}H_{36}$
2(3H)-Furanone,5-ethyldihydro-(γ-hexalactone)	1·8E + 09	4·5E + 08	2·5E + 09	4·5E + 08	0·159		91	1709		27·713	$C_6H_{10}O_2$
5,9-Undecadien-2-one, 6,10-dimethyl- (Z) (cis-geranylacetone)	4·9E + 08	4·6E + 08	7·0E + 08	2·7E + 08	0·538			1865		31·995	$C_{13}H_{22}O$
Octanoic acid,3-hexenyl ester (Z) (cis-3-hexenyl octanoate	7·7E + 08	4·6E + 08	6·5E + 08	4·1E + 08	0·738			1866		32·009	$C_{14}H_{26}O$
Nonadecane	8·9E + 08	5·1E + 08	1·2E + 09	3·3E + 08	0·437		96	1900		32·939	$C_{19}H_{40}$
Phenol, 2,6-bis (1,1-dimethylethyl)-4-methyl-(butylated hydroxytoluene)	1·0E + 10	5·1E + 09	1·0E + 10	4·0E + 09	0·926		98	1923		33·516	$C_{15}H_{24}O$
Benzothiazole	1·0E + 09	3·4E + 08	7·1E + 08	2·9E + 08	0·305		87	1962	1961	34·518	C_7H_5NS
Hexadecanal			1·6E + 09	1·3E + 09	0·104		87		2109	38·936	$C_{16}H_{32}O$
6-Amyl,alphapyrone	3·2E + 09	1·2E + 09	1·9E + 09	9·7E + 08	0·218		95			39·961	$C_{10}H_{14}O_2$
2H-Pyran-2-one, 6-heptyltetrahydro (δ-dodecalactone)	1·3E + 09	6·7E + 08	7·4E + 08	6·4E + 08	0·330		90			40·284	$C_{12}H_{22}O_2$

The VOCs were measured by solid-phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS).
[a]Significant differences ($P < 0{\cdot}05$) are marked with an asterisk. Reported values are expressed in chromatographic peak area units.

Table 4 Emission of selected volatile organic compounds from control and hot water-treated (HWT) cv. Lucie Tardibelle peaches as measured by proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS)

		Control		HWT		
Ion sum formula	Annotation	Mean (ppbv)	SD (ppbv)	Mean (ppbv)	SD (ppbv)	*P* value
CH_5O+	Methanol	95·7	76·5	84·4	54·6	0·755
C_2H_5O+	Acetaldehyde	27·5	22·4	445·5	294·7	0·003*
C_2H_7O+	Ethanol	5·1	3·5	143	96·3	0·003*
$C_2H_5O_2+$	Acetate fragment	6·3	1·1	16·5	9·8	0·018*
$C_{10}H_{17}+$	Monoterpenes	3·6	2·0	4·4	1·7	0·887

Significant differences ($P \leq 0{\cdot}05$) are marked with an asterisk (*).

Table 5 Effect of acetaldehyde (0·6 $\mu L\ L^{-1}$) and ethanol (0·2 $\mu L\ L^{-1}$) on *Monilinia fructicola* growth

	Treatment[a]		
	Control	Acetaldehyde	Ethanol
Colony-forming units	48[b] ± 6·2 a	77 ± 2·4 b	58 ± 6·4 a
Colony diameter (mm)	34 ± 1·1 a	32 ± 3·2 a	39 ± 0·4 b

[a]Aliquots of 0·1 mL of conidia suspension (10^3 conidia mL^{-1}) or a plug (6 mm diameter) from an actively growing pathogen culture were spread or placed in the centre of malt extract agar plates, incubated at 25°C for 2 or 5 days, respectively, prior to treatment with acetaldehyde or ethanol. Contol plates were inoculated with the pathogen but treated with distilled water.

[b]Each value is the mean of 15 replicates ± SE. Within the same row the same letters represent no significant differences according to LSD test ($P \leq 0{\cdot}05$).

gen with respect to the control (+15%). Moreover, no significant differences were found between colony-forming units of *M. fructicola* exposed to pure ethanol (58) and air (48) and between colony diameters of pathogen exposed to pure acetaldehyde (32 mm) and air (34 mm). Most of the compounds detected by SPME/GC-MS analysis in the headspace of heat-treated peaches (hexanal, isoamyl acetate, 3-methyl-1-butanol, hexyl acetate, cis-3-hexenyl acetate, trans-2-hexenyl acetate, cis-3-hexenol, trans-2-hexenol and nonanal) listed in Table 3 were also tested for their effects on *M. fructicola* as pure compounds at concentrations ranging from 0·0006 to 0·6 $\mu L\ L^{-1}$, but none of them showed any effect on pathogen conidial germination or mycelial growth (data not reported).

Discussion

Heat treatment has been reported to be an effective and safe method to control many postharvest diseases. In a previous study (Spadoni *et al*., 2014), an HWT demonstrated its high curative efficacy on peach fruits treated by dipping after artificial inoculation with *Monilinia* spp. In the present study, the effect of the hot water as a preventive treatment to control brown rot of peaches was evaluated. Data showed for the first time that heating fruit a few hours before pathogen inoculation could stimulate *M. fructicola* conidial germination. When inoculated a short time (from 15 min to 6 h) after HWT in water at 60°C for 60 s, wounded and unwounded fruits showed an incidence and a severity of disease higher than control fruit (Figs 1 & 2). These results are in contrast with the effects of HWT (dipping in water at 40°C for 5 or 10 min) applied before inoculation with *M. fructicola* observed by Liu *et al*. (2012) in a previous study, where heating was effective in reducing the lesion diameters for both times of treatment (5 and 10 min). These authors also reported an increase of chitinase, phenylalanine ammonia lyase and β-1,3-glucanase gene expression in heat-treated peaches, relating these changes to a possible role in host defence against the fungal pathogen. In the present trials, the water temperature and the duration of treatment were higher and lower, respectively, than those used in the experiments performed by Liu *et al*. (2012) and this could explain the different response of fruits to treatment.

In order to study the stimulation of *M. fructicola* on heat-treated fruits, a microscopic analysis of fruit epidermal tissues was performed. The *M. fructicola* conidia on heat-treated and control fruits revealed a different behaviour of germination depending on peach surface condition (Fig. 3). Indeed the *M. fructicola* germ tube elongation was stimulated on the treated peach surface when the pathogen was inoculated immediately after HWT (Table 1). At 18 hpi and incubation at 20°C, the pathogen germ tube length was three times longer on the peach surface of treated fruit with respect to the control (Fig. 3a,b). Conversely, no differences were observed in germ tube elongation of conidia germinated on heat-treated and control fruit when inoculation was performed 24 h after treatment (Fig. 3c,d). This difference was presumed to be related to volatile compounds emitted by fruits after HWT and the results obtained in subsequent *in vivo* and *in vitro* trials confirmed this hypothesis. A stimulatory effect of *M. fructicola* conidial germination was observed by the volatile blend emitted by heated fruits: 64, 40 and 33% stimulation in cvs Springbelle, Red Haven and Lucie Tardibelle peaches, respectively (Table 2). The volatile emission consequent to HWT was analysed by GC/MS and PTR-MS, for the first time in the literature. Previously, most studies on peach volatiles had been focused on changes of aroma compounds dur-

ing ripening and/or their effects on fruit flavour (Lavilla *et al.*, 2002; El Hadi *et al.*, 2013). The results of PTR-ToF-MS analysis showed, in particular, a significant increase of acetaldehyde and ethanol in heat-treated fruits compared to control ones. An increase in ethylene emission in heat-treated fruit was also observed during the first hours after the treatment (data not shown). *In vitro* assays with acetaldehyde (0·6 μL L^{-1}) and ethanol (0·2 μL L^{-1}) tested as single compounds confirmed the specific stimulatory effect on *M. fructicola* conidial germination and mycelial growth, respectively (Table 5), while some VOCs emitted by heat-treated peaches detected by GC-MS (Table 3) showed no effect (inhibitory or stimulatory) on pathogen growth (data not shown). Concentrations of hexanal, isoamyl acetate, 3-methyl-1-butanol, hexyl acetate, cis-3-hexenyl acetate, trans-2-hexenyl acetate, cis-3-hexenol, trans-2-hexenol and nonanal ranging from 0·0006 μL L^{-1} to 0·6 μL L^{-1} demonstrated no activity against *M. fructicola* growth (data not reported). This is the first time that VOCs produced by heat-treated peaches have been identified and tested on pathogen growth. It has been recognized that ethanol and acetaldehyde are produced by plants under stress. The results here on peach volatile emission are in agreement with results obtained from other fruit species. For example, an increase of acetaldehyde and ethanol emission in the first hours following heat treatment was also found in mango fruits exposed to 48°C for 5 h (Mitcham & McDonald, 1993). These metabolites are also emitted by senescent fruit and/or are associated with anaerobic processes during storage (Kimmerer & Kozlowski, 1982; Loreto *et al.*, 2006). For strawberry fruits stored in a controlled atmosphere with 15% of CO_2, a primary cause of off-flavour appears to be related to the accumulation of volatile compounds such as acetaldehyde, ethyl acetate and ethanol, which are associated with the anaerobic respiration pathways (Almenar *et al.*, 2006). More recently, acetaldehyde emission was detected on wounded strawberry fruit (Neri *et al.*, 2015). From the present results, acetaldehyde, ethanol and ethylene could be considered the stress metabolites temporarily emitted by peaches as a response to heat treatment. Conversely, few studies have previously focused on fungal stimulation by VOCs (Cruickshank & Wade, 1992; Eckert & Ratnayake, 1994; Filonow, 1999; Neri *et al.*, 2015). The present study confirms the results obtained by Cruickshank & Wade (1992), who found that ethanol and acetaldehyde produced during the ripening of apricots could stimulate the mycelium of *M. fructicola* to move from latent into invasive form, because these volatiles were produced in parallel with the appearance of brown rot symptoms in fruit. In addition, Eckert & Ratnayake (1994) reported that a mixture of volatiles released by wounded oranges, including ethanol and acetaldehyde, stimulated conidial germination of *Penicillium digitatum* and *Penicillium italicum*. An inhibitory activity of many fungal pathogens by treatment with ethanol or acetaldehyde has also been reported in the literature for a number of commodities, but at concentrations notably higher than those emitted by fruit and tested in this study. A significant reduction of postharvest decay in table grapes was, for example, reported by exposure to 1500–6000 μL L^{-1} of acetaldehyde (Avissar & Pesis, 1991), by immersion in water solution with 35% (v/v) ethanol (Gabler *et al.*, 2005) or by a modified ethanol atmosphere during storage (Lurie *et al.*, 2006).

In conclusion, the results of this study showed that the VOCs emitted from heat-treated peaches could stimulate the germination of *M. fructicola* conidia and increase the incidence of brown rot in treated fruit. This factor should be considered in view of a practical application of HWTs in peach packinghouses; furthermore, fruit postharvest management should avoid new infections until 24 h after HWT. Nevertheless, due to the great number of *Monilinia* spp. infections derived from the field, fruits require a curative treatment rather than a preventive one.

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