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Effect of red thyme oil (*Thymus vulgaris* L.) vapours on fungal decay, quality parameters and shelf-life of oranges during cold storage

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Abstract

This work has been aimed at studying the effect of red thyme oil (RTO, *Thymus vulgaris* L.) on the shelf-life and *Penicillium* decay of oranges during cold storage. RTO vapours significantly reduced ($P \leq 0.05$) the percentage of infected wounds, the external growth area and the production of spores in inoculated orange fruit stored for 12 days at 7°C in a polypropylene film selected for its appropriate permeability. Among the RTO compounds, p-cymene and thymol were the most abundant in packed boxes at the end of cold storage. The RTO vapours did not affect the main quality parameters of the oranges, or the taste and odour of the juice. The results have shown that an active packaging, using RTO vapours, could be employed, by the citrus industry, to extend the shelf-life of oranges for fresh market use and juice processing.

Keywords: essential oils, active packaging, *Penicillium* decay, GC-MS, citrus fruit, shelf-life

Running title: Control of orange fungal decay by essential oil volatile compounds

The chemical compounds studied in this article

Thymol (PubChem CID: 6989); p-cymene (PubChem CID: 7463); ɣ-terpinene (PubChem CID: 7461); polypropylene (PubChem CID: 76958)

1. Introduction

Orange (*Citrus sinensis* L. Osbeck) is one of the most important types of commercialised fruit in Mediterranean countries, and its production has increased significantly over the last decade (Strano, Altieri, Admane, Genovese & Di Renzo, 2017). Green mould, caused by *Penicillium digitatum* Sacc., accounts for 90% of the postharvest losses of citrus fruit (Kellerman, Erasmus, Cronj & Fourie, 2014), whereas *P. italicum* Wehmer, a nesting-type fungus which is responsible for blue mould, can directly attack healthy fruit regardless of injury (Ladaniya, 2008). The current methods used to limit *Penicillium* growth on orange fruit are the use of sodium hypochlorite solutions or peracetic acid as disinfectants, and the application of waxes containing chemical fungicides (Danyluck, Friedrich, Dunn, Zhang & Ritenour, 2019; Strano et al., 2017). However, the application of synthetic fungicides creates concern about environmental pollution, human health and the development of fungicide-resistant strains. Therefore, natural antimicrobial substances should be considered as alternatives (Baruzzi, Pinto, Quintieri, Carito, Calabrese & Caputo, 2015). Essential oils (EOs) are natural substances endowed with antimicrobial action against the various spoilage microorganisms that contaminate fruit and vegetables (Reyes-Jurado, Navarro-Cruz, Ochoa-Velasco, Palou, López-Malo & Ávila-Sosa, 2019). Different EOs, such as cinnamon, cloves, lemon grass, oregano, thyme, nutmeg and basil, are generally recognised as safe (GRAS) compounds in the USA (U.S. Code of Federal Regulations, 2013). Moreover, EO compounds, such as carvacrol, carvone, cinnamaldehyde, citral, p-cymene, eugenol, limonene, menthol, linalool, vanillin, citral and thymol, have been registered by the European Commission (EU Reg. 1334/2008) for use as food flavourings (Prakash, Kedia, Mishra & Dubey, 2015). The application of EOs to citrus fruit to control fungal decay has often been reported (Sivakumar & Bautista-Baños, 2014). Microemulsified clove oil applied in a vapour phase (He, Ren, Lung, Zhang, Wang & Sun, 2016) or the addition of EO compounds to waxes (Castillo, Pérez-Alfonso, Martínez-Romero, Guillén, Serrano & Valero, 2014) have been found to effectively control *Penicillium* spp. contamination on citrus fruit. As regards thyme essential oil (*Thymus vulgaris* L.), one of the most frequently used

EOs during postharvest storage, Yahyazadeh, Zare, Omidbaigi, Faghih-Nasiri and Abbasi (2009) reported the control of *P. digitatum* and *P. italicum* growth on oranges after using thyme oil inside polyethylene bags, while Plaza, Torres, Usall, Lamarca and Vinas (2004) reduced *P. digitatum* incidence on orange fruit by applying *Thymus vulgaris* oil in wax and using high barrier (OPP/PE/PA/EVOH/PA/PE-20/50) or low barrier (PA/PE 20/70) multilayer plastic bags as packaging. Conversely, the application of thyme essential oil to soaking pads failed to reduce *Penicillium* incidence on fruit (Plaza et al., 2004). However, several sensory defects have been detected on oranges treated with EOs (Plaza et al., 2004; Yahyazadehet al., 2009), when packaging not specifically selected for essential oil application was used. Moreover, the previous studies on the effect of EOs on orange quality were carried out at 20°C or 25°C, and did not consider the cold storage condition or its effect on the volatility of EO compounds. The effect of EOs on the quality parameters of fruit has been largely investigated using edible coatings (Sivakumar & Bautista-Baños, 2014), but limited data are available on their application under vapour phase (López-Gómez, Ros-Chumillas, Buendía-Moreno, Navarro-Segura, & Martínez-Hernández, 2020; Santoro et al., 2018; Servili, Feliziani & Romanazzi, 2017). Weight loss and the ascorbic acid concentration are the main quality parameters affected by EO vapour treatments on fruit; significant reductions in the loss of vitamin C and weight during storage were found in peaches and nectarines treated with thyme (*Thymus vulgaris* L.) and savory essential oils (Santoro et al., 2018), and the combination of modified atmosphere packaging and thyme oil (*Thymus vulgaris L.*) vapours was found to reduce the weight loss in avocado fruit (Sellamuthu, Mafunu, Sivakumar, Soundy & Korsten, 2013).

Given the above background, the objective of this study has been to assess the hypothesis that antifungal active red thyme essential oil (RTO, *Thymus vulgaris* L.) vapour concentrations, defined by *in vitro* assays, were compatible with the preservation of the main quality and sensory parameters of oranges under *in vivo* cold storage conditions.

In this study, we used RTO, applied in the vapour phase, to control *Penicillium* spp. decay on orange fruit during cold storage. After the selection of an appropriate packaging material for RTO

application in the vapour phase, the growth of fungal strains over the surfaces of the orange fruit and the concentration of the main EO compounds within the package atmosphere were monitored during cold storage. In addition, a comparison of the main quality parameters and sensory characteristics was carried out on un-spoiled oranges cold-stored in air and under RTO vapours.

2. Materials and Methods

2.1 Red thyme essential oil and fungal strains

Commercially available red thyme essential oil (RTO, *Thymus vulgaris* L.) was purchased from Bristol Botanicals Ltd (Bristol, UK). Other details are reported in Supplementary Methods (SM1).The RTO is obtained through distillation of dried thyme leaves. The RTO was diluted in n-hexane (HiPerSolv Chromanorm® for HPLC, VWR International, Darmstadt, Germany) for the antimicrobial assays as already reported (Cosentino et al., 1999).

P. digitatum ITEM 9569 and *P. italicum* ITEM 9571 were obtained from the ITEM (Agri-Food Toxigenic Fungi Culture Collection, Bari, Italy) microbial collection of the Institute of Sciences of Food Production (Bari, Italy; http://server.ispa.cnr.it/ITEM/Collection/) and maintained on Potato Dextrose Agar (PDA, Biolife Italiana Srl, Milan, Italy) at 4 °C. Fungal strains were incubated for one week on PDA at 25°C. Spore suspensions were prepared by flooding and suspending 1-week-old cultures of the strains in 10 mL of sterile distilled water. Spore suspensions were filtered through sterile gauze and the concentration adjusted to approximately 1.0 x 10^7spores/mL using a Thoma counting chamber.

2.2 *In vitro* antifungal effect of RTO

2.2.1 Vapour contact assay

Antimicrobial screening of RTO was performed in vapour phase to evaluate the antimicrobial activity on the mycelium growth. Vapour contact antifungal activity was assessed by means of the disc volatilization assay (Supplementary Methods, SM2).

125

126 2.2.2 Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal
127 Concentration (MFC) of red thyme essential oil (RTO, *Thymus vulgaris* L.)
128 The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of
129 RTO were ~~also~~ determined measuring the antifungal effect on the mycelium biomass at estimated
130 RTO concentrations of 33.4, 13.3 and 6.7 µL/L. PDA plates exposed to hexane were used as
131 controls. The PDA plates were covered with PT-500 cellophane membranes (Pacifici Corrado
132 S.n.c., Rome, Italy), inoculated with 100 µL of *P. digitatum* and *P. italicum* spore suspensions,
133 placed into the HDPE plastic boxes, and incubated at 25°C for 72 h. The dry weight of the mycelia
134 biomass on the plates exposed to RTO or to air was determined after drying the membranes with
135 mycelium for 24 h at 60°C in an oven. The lowest RTO concentration that significantly inhibited
136 mycelium biomass development ($P \leq 0.05$) was defined as the MIC.
137

138 2.3 Selection of the packaging material for RTO applications
139 2.3.1 Selection based on permeability
140 In order to select the most effective packaging material for RTO application in the vapour phase,
141 the gas composition that was able to sustain the growth of both *Penicillium* strains on oranges was
142 determined considering three different plastic materials: polyethylene PE-25, polyethylene PE-40
143 (Corapack, Brenna, Italy) and polypropylene PP 30 µm (Carton Pack, Rutigliano, Italy).
144 The maximum concentration of CO_2 that did not inhibit the radial growth of either strain was
145 preliminary defined as the limit that had to be respected, during the shelf-life experiments. The CO_2
146 produced by oranges was assayed considering the three types of plastic film. Six oranges (weight of
147 1.03 ± 0.05 kg and producing a volume of about 1 L) were packed into cardboard boxes (29x19.5x9
148 cm, Antonio Sada & Figli Spa, Pontecagnano Faiano, Salerno, Italy), which were then closed with
149 one of the 3 plastic films and incubated at 7 °C for 14 days. Four replicates were prepared for each

150 plastic film material, and nine gas measurements were carried out, using Check-Point Dansensor
151 PBI (Ringsted, Denmark), during cold storage.
152
153 2.3.2 Selection based on the challenge-test
154 The selection of the packaging material was also carried out considering the antifungal effect of
155 RTO vapours on inoculated oranges using the three plastic films, as detailed in Supplementary
156 Methods (SM3), where the fruit material and inoculum preparation are presented. The plastic
157 material that maximized the antifungal action of red thyme oil vapours on oranges was selected for
158 further assays.
159
160 2.4 *In vivo* antifungal effect of RTO
161 Oranges (6 for each replicate, N = 3) were packed in a cardboard box (Antonio Sada & Figli Spa,
162 Pontecagnano Faiano, Salerno, Italy) and each fruit was wounded (24 wounds per replicate, total
163 wounds = 72) and inoculated separately with *P.digitatum* ITEM 9569 and *P. italicum* ITEM 9571
164 strains, as described in Supplementary Methods (SM3.1) . Other wounded oranges, supplemented
165 with a sterile saline solution (NaCl 9 g/L), were used as controls (6 per replicate, N = 3). Inoculated
166 and non-inoculated orange fruit was stored in a climate controlled storage chamber at 15°C for 48 h
167 in order to simulate a pre-harvest orange contamination before cold storage, as already carried out
168 for the study of the microbial decay of other fruit (Pinto, Caputo, Quintieri, de Candia & Baruzzi,
169 2017). After this incubation period, the samples (3 replicates composed of 6 oranges for each
170 *Penicillium* strain and sampling time) were packed in air (Passive atmosphere samples, PA) or in
171 the presence of RTO (PA + RTO). Oranges under RTO vapours were packed using the
172 polypropylene film (PP, 30 µm), selected on the basis of its permeability to O_2 and CO_2, and for the
173 control of the fungal decay, as described in the Supplementary Results. The sealed (Impulse Auto
174 Sealer, Mercier Corporation, Taipei, Taiwan) bags were stored in the same climate-controlled
175 storage chamber at 7 °C, 70% RH, ventilation rate of 0.1 L min^{-1}, for 16 days (sampled after 8, 12

and 16 days) for *P. italicum* ITEM 9571 and for 12 days (sampled after 4, 8 and 12 days) for *P. digitatum* ITEM 9569. Unwounded and non-inoculated orange fruit was stored in air or under RTO vapours in the cardboard boxes closed with PP 30µm for 16 days at 7 °C as negative control samples. The percentage (%) of wounds with visible mycelium growth and with fungal spores was recorded for each strain and each sample at each sampling time. In addition, the diameter of mycelium growth of each wound was measured in two perpendicular directions by means of a vernier caliper and expressed as area (cm^2).

2.5 Gas-chromatographic analyses

2.5.1 GC-MS analyses of RTO

Thymol, p-cymene, and γ-terpinene, the three main compounds of RTO, were quantified by means of Gas Chromatography-Mass Spectrometry (GC–MS), as previously described by Cutillas, Carrasco, Martinez-Gutierrez, Tomas and Tudela (2018), albeit with some modifications.

The GC-MS analyses were performed in a gas chromatograph 680 coupled with a Clarus SQ 8T mass spectrometer (Perkin Elmer). The gas chromatograph was equipped with a split/split-less inlet, . an ELITE 5-MS column (Perkin Elmer) (0.30 m length × 0.25 mm inner diameter × 0.25 µm full thickness), and helium (48kPa ~7 psi) as the carrier gas. The essential oil (0.1%) was injected (1 µL) into the column at a split ratio of 2; the injector temperature was 250 °C. The mass spectra were taken under positive electron impact ionization mode, with a mass range from 60 to 210 amu, a solvent delay of 5 min and a transfer line of 250 °C. The chromatographic programme was: 50°C for 2 min, a gradient of 25 °C/min until 240°C and then 240°C for 2 min. The concentration of each compound was calculated, by means of area interpolation on the calibration curve built using external analytical standards (Sigma-Aldrich Srl, Milan, Italy). The selective ion monitoring mode was exploited, considering 119 m/z for p-cymene, 93 m/z for γ-terpinene and 135 m/z for thymol.

2.5.2. Respiration rate, headspace gas composition

The respiration rate was measured during storage using a closed system, as reported by Fratianni et al. (2017) by means of GC-TCD analysis. About 1 kg of oranges, for each treatment and replicate (n = 3), was put into 6 L sealed high-density polyethylene (HDPE) plastic jars (one jar per replicate), and CO_2 was allowed to accumulate to 0.1% (standard concentration of CO_2). The respiration rate was expressed as mL CO_2 kg^{-1} h^{-1}.

In addition, the headspace gas composition (O_2 and CO_2) in each PP 30 µm package was monitored daily using a gas analyser (CheckPoint, PBI Dansensor, Ringsted, Denmark).

2.6 Effect of packaging with RTO vapours on the quality parameters of orange fruit under cold storage

Oranges (6 per replicate, N = 3) were stored in air (Air) or in the cardboard boxes closed with PP 30 µm with RTO (Passive atmosphere plus RTO, PA+ RTO) or without RTO (Passive atmosphere, PA) at 7 °C for 12 days in order to assess the effect of RTO on the main quality parameters during postharvest cold storage. Samples were analysed at harvesting and after 4, 8 and 12 days, to determine the quality parameters and sensory characteristics. The main RTO compounds were identified and quantified, at the same sampling time, by means of headspace-gas-chromatography-mass-spectrometry (HS-GC-MS) analysis.

2.6.1 Colour, weight loss, titratable acidity and pH of the juice

The CIELAB colour parameters (L^*, a^* and b^*) were measured on 5 random points of the orange peel using a colorimeter (CR-400, Konica Minolta, Osaka, Japan), as already reported by Cebadera-Miranda et al. (2019) on blood oranges. Then, ΔL^* and ΔE^* were calculated (Cáceres, Díaz, Shinya, & Infante, 2016).

The weight loss of each replicate was calculated as a percentage of the weight at day 0.

Orange juice was obtained by squeezing 6 half oranges from each replicate in a squeezer (Juicer SZP 25 B2 - Silver Crest, Hamburg, Deutschland) for 1 min at 14,000 rev. min^{-1}. Titratable acidity

228 was determined by means of an automatic titrator (PH-Burette Crison Instrument, Barcelona, Spain)
229 with 0.1 mol L^{-1} NaOH to final pH value of 8.1, using 10 mL of juice. The results were expressed as
230 % of citric acid. The total soluble solid (TSS) content of the juice, expressed in °Brix, was
231 measured using a refract-meter (model DBR35, XS Instruments, Italy). The pH of the juice was
232 measured using a pH meter (PH-Burette 24 Crison Instrument, Barcelona, Spain).
233
234 2.6.2 Antioxidant activity, total phenol content and ascorbic acid
235 The same extraction was carried out to analyse the antioxidant activity and total phenols: for each
236 replicate, 5 g of juice was homogenized in a methanol: water solution (80:20 v/v) for 1 min, using
237 an Ultraturrax (IKA T18, Staufen, Germany), and then centrifuged (Prism C2500-R, Labnet,
238 Edison, NJ, USA) at 13540 *g* for 5 min at 5 °C. The supernatant (methanolic extract) was used for
239 the DPPH assay as previously reported (Cefola et al., 2014). The results were expressed as
240 milligrams of Trolox per 100 grams of fresh weight (fw) using a Trolox calibration curve (82–625
241 µM; $R^2 = 0.999$).
242 Total phenolic content was determined according to the method of Singleton and Rossi (1965). The
243 results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight. The
244 calibration curve of the gallic acid was prepared with five points, from 50 to 500 µg mL^{-1}, with R^2 =
245 0.999.
246 Other parameters were evaluated in the orange juice. Orange juice was removed from the extractor
247 and passed through cotton gauze to remove the seeds and pulp, and it was then centrifuged at 5000
248 x g for 5 min to remove smaller debris. The orange juice, obtained from different samples, was
249 immediately evaluated to establish the sensory characteristics and was stored at -20°C for further
250 analyses.
251 The HPLC method was used to determine the ascorbic acid (AA) in the orange juice with a Rezex
252 ROA H^+ 8% column (300mm×7.8mm, Phenomenex, Torrance, CA), as described by Mikulic-
253 Petkovsek, Slatnar, Schmitzer, Stampar, Veberic and Koron (2013). The column temperature was

set at 55°C. The standard calibration curve fell within the 0.0025–0.04 % w/v range. The AA concentration was expressed as mg 100 g^{-1}fresh weight (FW). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated on the basis of signal-to-noise ratios (S/N) of 3 and 6, respectively. The LOD value was 2.4 mg $100g^{-1}$ FW. The LOQ value corresponded to 4.8 mg $100g^{-1}$ FW.

2.6.3 Headspace-gas-chromatography-mass-spectrometry (HS-GC-MS) analysis

As far as the quantification performed in the vapour phase trials is concerned, the concentration (expressed as µL/L) of each compound was determined at the MIC level (section 2.2.2). An estimated RTO concentration of 33.4 µL/L was obtained in a plastic box (600 mL), which was closed quickly and sealed tightly with a parafilm; the boxes were incubated at 25°C for 96 h. The headspace gas was extracted at each sampling time using a gas-tight SGE microsyringe (Supelco) and analysed by means of HS-GC-MS. The head-space volatiles were analysed using the previously described GC–MS apparatus with an integrated headspace autosampler, as reported in Arancibia, López-Caballero, Gómez-Guillén, and Montero (2014).

Identification and quantification of the RTO compounds in the headspace of the cardboard boxes containing oranges and packed with PP-30 µm film were also performed. In this case, the sampling was carried out, using a gas-tight SGE microsyringe (Supelco), with the help of a 20 mm PTFE/GC-MS Grade Ultra Pure Silicone Septa attached to the outer surface of the cardboard boxes. Three biological replicates were analysed at each sampling time (0, 4, 8 and 12 days at 7°C). Since the vapour fraction of RTO is mainly characterised by thymol, p-cymene and γ-terpinene, the total RTO amount was calculated considering their relative percentages.

2.6.4 Sensory parameters of orange fruit and juice samples

The sensory profile of the oranges was obtained by applying a descriptive sensory analysis (Cozzolino et al., 2016), with a group of 10 trained panelists (made up of 5 females and 5

males).The visual quality (VQ) was evaluated for both the fruit and juice samples, the characteristic odour and off-odour were then scored and, finally, samples were tasted to score the characteristic flavour and off-flavour. The VQ, characteristic odour and characteristic flavour were evaluated using a hedonic scale ranging from 5 to 2 (5 =excellent; 4 =good; 3= fair, limit of sensory acceptability; 2=unusable or poor). The off-odour and off- flavour of the fruit and juice samples were scored according to a rating scale of 2 to 5 (2 =strong; 3= severe; 4= moderate; 5= poor). Each panelist evaluated one set per replicate and storage treatment, working in an individual booth and drinking water for oral rinsing purposes.

2.7 Statistical analysis

One- or two- way ANOVA ($P \leq 0.05$) was applied through the SPSS software (SPSS, Inc., IBM Corp., Chicago, IL, USA). Multiple comparisons of the individual means were conducted for each sample with Fisher's least significant difference (LSD) multiple range test or the Tukey (HSD) test at 95% or 99% confidence intervals.

3. Results

3.1 *In vitro* antimicrobial activity of RTO against *Penicillium* spp.

3.1.1 Vapour contact assay~~s~~

The antimicrobial activity of RTO was determined against *Penicillium* spp. strains by means of vapour contact assay~~s~~.

The antifungal activity of RTO in the vapour phase is reported in Table 1S. The hexane vapours did not affect the mycelium growth of either *Penicillium* strain. On the basis of the GC–MS analysis, the RTO applied in these experiments showed that the three main compounds were thymol (43.4 ± 2.1%), p-cymene (37.5 ± 1.6%) and γ-terpinene (19.1 ± 1.1%) (Table 2S). The exposure of *Penicillium* strains to 66.7 µL/L of RTO reduced the mycelium growth in comparison to plate

controls (Table 1S). However, this RTO concentration did not show a fungicidal effect since the spore germination was detected in Petri dishes at the end of incubation; therefore, the MFC value was not achieved for the highest RTO concentration in the vapour phase.

3.1.2 Definition of the MIC values

MIC values, calculated by means of vapour contact assay~~s~~, were defined for the red thyme oil (RTO, *Thymus vulgaris* L.) (Section 3.1.1).

The estimated MIC values of RTO were 33.4 µL/L for *P. digitatum* ITEM 9569 and 13.3 µL /L for *P. italicum* ITEM 9571 (Table 3S). An RTO concentration of 33.4 µL/L, was able to significantly reduce the dry mycelium biomass of both *Penicillium* strains; the release of the main RTO compounds was quantified over time for this concentration by means of HS-GC-MS analysis. The total released RTO, as calculated on the basis of the amount of the three main compounds, was 48, 50 and 57 µL/L at 0, 1 and 3 days of incubation, respectively (Fig. 1). The total released RTO was essential to define the concentration to be used in shelf-life trials with oranges. The percentage concentration of each compound is reported in Fig. 1. The concentration of the three compounds in air at T0 (about six hours after loading red thyme oil on paper discs) was about 32, 13, 3 µL/L for p-cymene, γ-terpinene and thymol, respectively. These concentration values changed to 41, 14, and 1.7 µL/L after three days of storage at 25°C. On the basis of these results, p-cymene was found to be the most volatile compound of RTO, as it showed the highest concentration of the RTO compounds during incubation.

3.2 *In vivo* antimicrobial activity of RTO against *Penicillium* spp.

3.2.1 Selection of the packaging material for RTO postharvest application

The selection of the packaging film for RTO application during cold storage was based on CO_2 permeability and the effect on *Penicillium* decay on the oranges.~~-~~Preliminary results showed that,

above the CO_2 concentration of 10%, the radial growth of both *Penicillium* strain was inhibited (data not shown). Therefore, the CO_2 concentration that should not have been exceeded by the plastic film was set at 10%.

A permeability test with oranges showed that the equilibrium between CO_2 and O_2 was reached over the 10-11% range for the three plastic films. However, this equilibrium was achieved after 3 days of cold storage for the polyethylene films (PE-25 and PE-40) and after 6 days for the polypropylene film (PP) (Fig. 1S). These results were not sufficient to allow any single plastic film to be chosen for further *in vivo* experiments. For this reason, a preliminary challenge test was carried out to evaluate the spoilage pattern at the end of incubation.

The challenge tests on the three packaging films highlighted that the superficial growth of both *Penicillium* strains on oranges was affected significantly ($P \leq 0.05$) by the packaging condition. The surficial growth of both strains was lower under the polyethylene films (PE-25 and PE-40) than under the polypropylene film (PP) (Table 6S), in accordance with the faster accumulation of CO_2 in PE films (Fig. 1S).

The percentage of infected wounds of oranges packed under RTO vapours, recorded after 19 days at 7 °C, was not reduced, regardless of the packaging film. However, the surficial growth area was reduced significantly for both strains when only the PP film was used (Table 4S). This result could be due to the appropriate permeability of this plastic material that maximises the antifungal action of RTO vapours.

In addition, the growth of strains in the air did not differ when the PP film was used, in comparison to that detected on un-packed inoculated oranges at 25°C (data not shown).

The film permeability test and the reduction in the surficial mycelium area of both *Penicillium* strains on the inoculated oranges overall indicated that the PP film was the best packaging film to evaluate the effect of RTO on oranges under commercial or challenge-test conditions.

3.2.2 Antimicrobial effect of RTO on oranges during cold storage

Once the most active EO in the vapour phase and the best packaging film had been selected, it was possible to proceed with challenge-tests, using *Penicillium* spp., on healthy oranges under commercial conditions.

The wounded but non-inoculated oranges did not develop fungal growth during 12 days of cold storage, regardless of the packaging condition (Air or RTO). However, at the end of cold storage, that is, on day 16, *Penicillium* decay was observed on 5.9 ± 2.1 % of the wounds on the oranges stored in air, whereas no infected wound was detected in the samples stored with RTO.

Two-way ANOVA analysis revealed that the RTO treatment and incubation period significantly affected ($P \leq 0.05$) the percentage of wounds infected by *P. italicum* ITEM 9571, whereas the RTO treatment, the incubation period and their interaction significantly affected ($P \leq 0.05$) the external growth area. The percentage of wounds with mycelium growth and the surficial growth area on the oranges cold stored in air increased from day 8 to day 16 (Table 1-A). Both parameters were reduced (30% for the decay incidence and 60% for the external growth area) on oranges packed with RTO after only 8 days of cold storage. The external growth area did not increase significantly in RTO-treated samples up to day 16. (Table 1-A). The production of *P. italicum* spores on oranges treated with RTO vapours was only detected on day 16, and accounted for 9.4% of the infected wounds (Table 1-A). As far as the control of RTO vapours on *P. italicum* ITEM 9571 is concerned, it can be hypothesised that the treatment produced a double effect of reducing the external growth and delaying the sporulation phase.

Two-way ANOVA analysis showed that the RTO treatment significantly affected ($P \leq 0.05$) the percentage of wounds infected by *P. digitatum* ITEM 9569, whereas the incubation period significantly affected ($P \leq 0.05$) the surficial growth area (Table 1-B). The growth rate was higher for this strain than for *P. italicum* (Table 1-A). The RTO treatment did not reduce the number of wounds colonised by *P. digitatum* throughout the cold storage period, but effectively controlled surficial growth during the first 8 days of cold storage. At the end of incubation, spores were also

383 detected on oranges packed with RTO, although a reduction of 35% was observed in comparison to
384 the samples stored under passive atmosphere (Table 1-B).
385 The RTO treatment had a stronger effect on delaying the sporulation of *P. digitatum* than on
386 controlling the development of external growth. This result is consistent with MIC data calculated
387 for the vapour phase, where the values were higher for *P.digitatum* ITEM 9569 than for *P. italicum*
388 ITEM 9571. Therefore, *in vivo* trials confirmed the results obtained by *in vitro* tests.
389 Overall, RTO vapour treatment was more efficient against the more sensitive strain with a reduced
390 growth rate. An example of inoculated oranges stored under passive atmosphere or under passive
391 atmosphere with RTO is reported in Fig. 2S.
392
393 3.3 Effect of the active packaging on the main quality parameters of oranges during cold storage
394
395 3.3.1 Quantification of the main RTO compounds released during cold storage
396 The quantification of the main RTO compounds released in the vapour phase during cold storage is
397 reported in Table 2.
398 GC-MS spectra revealed additional peaks belonging to different classes of volatile compounds,
399 including terpenes and aldehydes, as previously reported for orange fruit and its juice (Centonze et
400 al., 2019; Zhang et al., 2017). The main volatile compound related to oranges was D-limonene.
401 The amount of RTO loaded on the Whatman paper discs (33 µL RTO/L air) was selected to obtain
402 an RTO concentration of 40-50 µL/L in the vapour phase, considering a mean total volume of 6.8 L
403 and a headspace volume of 5.8 L for each package. It is interesting to note that the average
404 concentration was 30.8 ± 3.0 µL/L for 12 days of cold storage, remaining stable inside the
405 packages. However, changes in the relative concentration were found for three main RTO
406 compounds. The most volatile compound was p-cymene, whose concentration decreased by about
407 25% after 12 days; the thymol concentration increased about ten-fold, in comparison to the initial
408 amount. It is possible to conclude, on the basis of the RTO composition in a liquid state (see Table

409 2S), that p-cymene and thymol showed the highest and lowest volatility, respectively. The vapour
410 phase showed stable γ-terpinene concentrations of about 23% (corresponding to *ca*. 7 µL/L).
411
412 3.3.2 Changes in the orange quality parameters under different cold storage conditions
413 The composition of the atmosphere inside the PA samples changed during storage (Fig. 3S),
414 showing the decrease of O_2 concentration and the increase of CO_2 concentration. On the contrary,
415 the PA+ RTO samples did not show any significant changes in the composition of the atmosphere
416 throughout the cold storage period (Fig. 3S).
417 The effects of the packaging condition, storage time and their interaction on the quality parameters
418 are shown in Table 3.
419 Among the colour parameters, only *b** and the *Chroma* values were significantly affected by the
420 storage time factor, and they showed an increase (about 2.5% and 2%, respectively) during cold
421 storage (Table 3).
422 The antioxidant activity was affected by the storage time and by the interaction of the two factors.
423 At the beginning, the fresh samples showed an antioxidant activity of about 70 mg Trolox 100 g^{-1}
424 fw. This value decreased in all the samples until the 8^{th} day, and then increased on day 12, albeit
425 only for the orange fruit stored in air; no significant differences were found, at any time, for the PA
426 or PA + RTO samples (Fig. 5S).
427 As regards the total phenol content, all the samples started with values of 95 mg_{GAE} 100 g^{-1} fw on
428 day 0. The results obtained from the multifactor ANOVA test showed that both the packaging
429 condition and the storage time significantly affected the total phenol content (Table 3). Samples
430 stored in Air showed higher mean values than the PA and PA+ RTO ones. A significant reduction
431 in the mean values was in particular observed during the 12 days of cold storage (about -8.5%;
432 Table 3); however, no significant differences were found, at any time, between the PA and PA +
433 RTO samples, as already found for the antioxidant activity.

All the analysed factors (packaging condition, storage time and their interaction) significantly affected the respiration rate (Table 3). It remained almost constant in the samples stored in air until the end of storage, while it increased for the oranges packed under PA, and did not show significant differences related to RTO.

The total soluble solid content was only affected by the packaging condition (Table 3); the lowest values were found in the samples stored in PA+ RTO, although the values in the samples stored in air or in the packed fruit without RTO were similar.

The weight loss and pH of the juice of the oranges were not affected by the packaging condition, storage time or their interaction.

The AA concentration in the juice samples extracted from oranges stored in air, in passive atmosphere or in passive atmosphere with RTO, is reported in Fig.2.

Two-way ANOVA analysis showed that the storage time, packaging conditions and their interaction significantly affected ($P \leq 0.05$) the AA concentration. All the samples showed a significant reduction in the AA content in the orange juice at the end of cold storage in comparison to the initial concentration. However, the samples stored in air showed lower AA concentrations than the samples packed with PP plastic material (PA or PA + RTO) during cold storage (Fig. 2). The AA concentrations in these latter samples did not differ significantly during the cold storage period.

3.3.3 Sensory evaluations

The visual quality of the fruit was affected significantly by the packaging conditions and by the storage time. The oranges stored under different conditions were scored above the marketable limit at each sampling time during the entire storage period.

As expected, off-odour, evaluated on the whole oranges, showed that the PA+ RTO treatment resulted in a lower value (about 2.3) than the values assigned to the Air and PA samples (about 4.8). This sensory characteristic of the PA+RTO samples was found to be attenuated once the orange

460 flavedo was removed. After the opening of the packages, the off-odours of the PA+ RTO packed
461 fruit were no longer detected after 12 hours of storage at 25°C. According to multifactor ANOVA,
462 the characteristic odour of the oranges was influenced by both the packaging conditions and the
463 storage time factors and by their interaction, and a general decrease in score was observed over the
464 cold storage period (data not shown). The results of the sensory evaluation of the juice obtained
465 from the two ANOVA tests showed that all the factors were significant.
466 The juice VQ decreased until the 8th day, showing lower scores in the PA + RTO samples; it then
467 continued to decline in the PA and it increased slightly for the fruit subjected to other treatments,
468 and reached the highest score in the Air samples (Fig. 6S, panel A). The characteristic odour of the
469 juice decreased over the first 8 days (Fig. 5S, panel B), and the PA+ RTO samples showed the
470 lowest values. After the 8th day, it increased more in the Air than in the packed fruit (Fig. 6SB). The
471 juice off-odour was evaluated as being poor or moderate for all the samples throughout the
472 incubation period. (Fig. 6S, panel C).
473 The storage time produced a greater effect on the reduction of the characteristic juice flavour than
474 on the appearance of the juice off-flavour. Juice off-flavours were completely absent at the
475 beginning of cold storage so the samples were scored 5. The appearance of some unusual flavours
476 was detected in all the samples at the end of cold storage.
477
478 **4. Discussion**
479 In the current work, red thyme oil (RTO, *Thymus vulgaris* L.) was used in the vapour phase to
480 control *Penicillium* spp. contamination of oranges and to extend the shelf-life of citrus fruit.
481 The RTO concentration that inhibited the mycelium growth of both strains was, on average, 51.7
482 µL/L in the vapour contact assay. A previous study (Yahyazadeh et al., 2009) showed that vapours
483 released from filter papers soaked with thyme (*Thymus vulgaris* L.) oil (from 200 to 800 µL/L)
484 effectively controlled the development of *Penicillium* strains on citrus fruit. The measurement of the
485 total RTO concentration in the vapour phase, performed by means of HS-GC-MS, showed that

mycelium development was completely inhibited at *ca.*50 µL/L in the *in vitro* assay; however, a lower concentration (30.8 µL/L on average) was sufficient to significantly reduce the surficial growth of both strains on packed oranges.

Our results indicate that, among the assayed films, the polypropylene one was the most suitable packaging film for RTO application in the vapour phase. The selection of a suitable packaging film is an important step in the control of the O_2/CO_2 ratio, which is fundamental to maintain acceptable sensory properties of fruit during cold storage. In addition, as demonstrated in this work, the selection of the packaging material plays a pivotal role to maximize the antifungal action of the essential oil vapours.

Changes in the permeability of the polypropylene packaging material were found to prevent the build-up of CO_2 in a headspace atmosphere during the cold storage of minimally processed pomegranate (*Punica granatum* L., cv. Acco) arils for 15 days (Hussein, Caleb, Jacobs, Manley & Opara, 2015). Changes in the O_2/CO_2 ratio of orange segments induced anaerobic conditions that negatively affected the sensory attributes, regardless of whether mono or multilayer polypropylene packaging material was applied during storage (Karacay and Ayhan, 2010).

Yahyazadeh et al. (2009) reported changes in the organoleptic properties of oranges (alcoholic taste or bad fruit flavour) when using polyethylene films without a proper gas permeability. These negative effects were amplified by the presence of EO vapours. On the contrary, our results show that it is possible to reduce the percentage of wounds colonised by *Penicillium* spp. on oranges during cold storage, and to preserve most of the chemical and sensory characteristics.

The main effect of RTO vapours was a reduction in the colonisation of the fruit skin by mycelium and, as a secondary effect, a delay in the production of spores, with a strain-dependent effect on decay incidence values (Table 1A and B). Oranges were inoculated with spores that started to germinate within a few days; the data related to the incidence values showed that RTO had no, or a very limited effect on spore germination. On the contrary, RTO vapour significantly reduced the external growth of mycelium, thus suggesting that its main antifungal effect was against young

512 hyphae rather than spores. Indeed, on day 8, the RTO vapours had reduced the external growth of *P.*
513 *italicum* ITEM 9571 and *P. digitatum* ITEM 9569 on oranges by approximately 60 and 42%,
514 respectively.
515 These results are in agreement with those of Plaza et al. (2004), who reported that thyme oil
516 (*Thymus vulgaris* L.) vapours affected the growth of the external mycelium more than the fungal
517 decay incidence. In our case, the antifungal effect was strain dependant, as also reported by
518 Yahyazadeh et al. (2009) for oranges packed in polyethylene bags with thyme (*Thymus vulgaris* L.)
519 and clove oil vapours.
520 The antifungal action mechanism of essential oil compounds has been well documented; it is
521 known, for example, that thymol, the main terpene compound present in essential oil from different
522 *Thymus* species, causes damages to the cell membrane by interacting with ergosterol, disrupts Ca^{2+}
523 and H^{+} homeostasis (Pavoni et al., 2019) and affects the mycelium morphology, thereby changing
524 the localisation of chitin within the hyphae (Nazzaro, Fratianni, Coppola, & De Feo, 2017).
525 However, in our trial, p-cymene was the most abundant RTO compound in the cardboard boxes
526 during the cold storage of oranges (Table 2). The antimicrobial activity of p-cymene is
527 controversial. As reviewed by Marchese et al. (2017), p-cymene did not show antifungal activity
528 against *Rizhopus oryzae* and *Aspergillus niger*. However, *Ciminum cymininunm* L. seed essential
529 oil, mainly composed by p-cymene (47.08%), showed antifungal activity against *A. flavus*.
530 Although limited data are available on the antifungal mechanism of p-cymene, recent results have
531 shown an effect on conidia production, on the cell membrane function, and on the transcription and
532 regulation of genes involved in mycotoxin production (Tian, Woo, Lee, & Sook Chun, 2018). Like
533 other monoterpene hydrocarbons, ɣ-terpinene affects membrane permeability and induces protein
534 and lipid leakage (Pavoni et al., 2019).
535 Here, the release kinetics of RTO compounds in a packaging system has been reported, for the first
536 time, with reference to the application of EOs to citrus fruit during cold storage. The concentration
537 of RTO in the cardboard boxes containing oranges was lower than that detected in the plastic boxes.

538 The changes in EO concentration that were observed for the *in vitro* and *in vivo* systems could be
539 due to different packaging film permeabilities of the EO compounds, a non-optimal relative
540 humidity for EO release, and/or the absorption of the EOs by plant tissue (da Rocha Neto, Beaudry,
541 Maraschin, Di Piero, & Almenar, 2019). A high relative humidity is important to reduce the loss of
542 EO volatiles from active films during storage (Arancibia, López-Caballero, Gómez-Guillén, &
543 Montero, 2014). The release kinetics of the three RTO compounds showed a significant reduction in
544 the p-cymene concentration and an increase in the thymol concentration during cold storage, while
545 concentration of ɣ-terpinene was stable. This work demonstrates that the composition of the RTO,
546 in a real food packaging system, changes as a function of the cold storage duration. Therefore, it is
547 important to determine the total EO concentration, as well as the concentration of each EO
548 compound in the active packaging system. On the basis of the release kinetics of the RTO
549 compounds and the total RTO concentration in the packed cardboard boxes, p-cymene, ɣ-terpinene,
550 and thymol could all contribute, at different extent and in a different manner over time, to the
551 antifungal activity of the RTO. Further studies should be addressed to highlight~~ing~~ the specific
552 antimicrobial activity and possible synergic interactive effects among these essential oil
553 compounds, in order to reveal their role in exerting antifungal activity under vapour phase
554 conditions. In addition, the applications of essential oils to food in the vapour phase should also
555 consider the use of food grade solvents.
556 As far as the quality parameters are concerned, we found that most of them were first affected by
557 the extension of the storage time, and this was followed by the packaging conditions; the colour,
558 titratable acidity, and total soluble solid values were not affected by the RTO vapour treatment, as
559 previously found for strawberries (Sangsuwan, Pongsapakworawat, Bangmo, & Sutthasupa, 2016).
560 The total phenol content and antioxidant activity were affected more by the packaging in the PA
561 treatment than in the PA +RTO one; moreover, the weight losses of the orange fruit were not
562 affected by PA +RTO, unlike the results of other works that considered different fruit (Sangsuwan
563 et al., 2016; Santoro et al., 2018; Vilaplana, Pazmiño, & Valencia-Chamorro, 2018).

564 It is worth noting that the AA concentration was higher in the juice samples from oranges packed in
565 a polypropylene film than that found for fruit stored in air. Similar results were obtained by Sicari,
566 Dorato, Giuffrè, Rizzo and Albunia (2017) during the refrigeration of oranges in biaxially oriented
567 polypropylene, in comparison to non-wrapped crate storage. This result could be due to the
568 modulation of the transpiration, respiration rate and O_2 barrier properties of the packaging (Sicari et
569 al., 2017), although this hypothesis still has to be verified.
570 The packed oranges, regardless of the RTO treatment, showed a higher respiration rate than the fruit
571 stored in air, even though the effect of the essential oil vapours on the concentrations of respiration
572 gases may be limited, as reported in literature. da Rocha Neto et al. (2019) reported that the release
573 of star anise oil into apple packages reduced the CO_2 accumulated by *P. expansum* inoculated fruit
574 whereas Sangsuwan et al., (2016) found an increase in the CO_2 concentration inside packages
575 containing strawberries exposed to red thyme (*Thymus vulgaris* L.) oil vapours. In our experiments,
576 the O_2 and CO_2 concentrations were stable throughout the cold storage period in the packages
577 containing RTO vapours.
578 As regards the sensory analysis, cold storage mainly affected the characteristic odour of the whole
579 fruit and the juice flavour; the RTO odours disappeared from the whole fruit12 hours after package
580 opening. On the other hand, the RTO did not affect the visual quality of the oranges or the
581 perception of the characteristic odour of the internal part of the fruit and no RTO off-flavour or off-
582 odour was perceived in the internal parts of the fruit or in the juice. RTO is characterised by a
583 pungent odour, and the absorption of EO by flavedo was expected with a consequent negative effect
584 on the olfactory perception. Sangsuwan et al. (2016) found that a thyme oil treatment negatively
585 affected the odour, flavour and overall acceptability scores of strawberries. Conversely, a thyme oil
586 treatment did not have any negative effect on sweetness, firmness or the overall appearance of
587 organic bananas (Vilaplana et al., 2018). Moreover, avocado fruit did not show any off-flavour, and
588 showed the highest overall acceptance when a modified atmosphere packaging, combined with
589 thyme oil vapours, was considered as the storage condition (Sellamuthu et al., 2013).Therefore, the

590 impact of essential oils on the sensory characteristics of fruit could be fruit dependant and, in
591 addition, be affected by storage and packaging conditions.
592
593 **5. Conclusions**
594 The results of this study have shown the potential of applying red thyme oil (RTO, *Thymus vulgaris*
595 L.) vapours to control the *Penicillium* decay of orange fruit. In order to maximise the effect of the
596 antifungal activity of RTO, it is necessary to select a suitable packaging material with appropriate
597 gas permeability.
598 The oranges under RTO vapours showed a significant, strain dependant reduction in the percentage
599 of infected wounds, the mycelium growth area and in the occurrence of spores during cold storage.
600 Most of the quality and sensory parameters of the packed oranges, with or without RTO, did not
601 differ from those of the unpacked samples; however, the ascorbic acid loss during cold storage was
602 reduced in the polypropylene packed fruit and this resulted in a 20% higher concentration than in
603 the fruit stored in air.
604 Some differences in the sensory parameters of the fruit were found at the end of storage, although
605 they were related more to the packaging conditions (passive atmosphere) and storage period than to
606 exposure to RTO vapours. The cold storage period negatively affected the olfactory characteristics
607 of the orange fruit stored in contact with RTO vapours, albeit with a less impact on the overall
608 quality of the juice.
609 Overall, the data have highlighted a possible new strategy to reduce postharvest losses of citrus fruit
610 and to extend their shelf-life, as a result of the use of essential oil vapours combined with a suitable
611 packaging material. Novel release systems could be considered to reduce the negative sensory
612 properties of certain essential oils during food storage.
613
614
615

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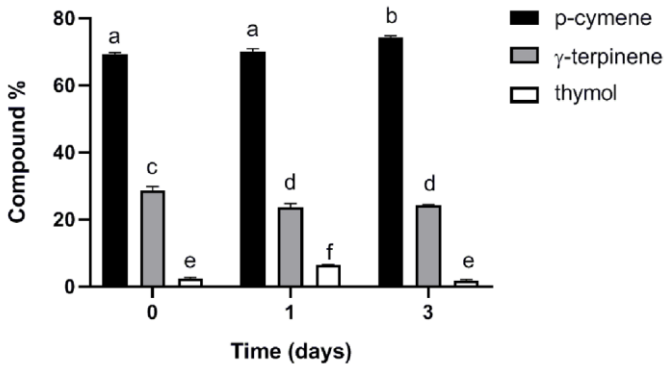

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766 Fig. 1 Thymol, p-cymene and γ-terpinene released from cellulose discs into a 600 mL plastic box
767 after 0, 1 and 3 days of incubation at 25°C. One-way ANOVA, followed by Tukey's HSD (honestly
768 significant difference) test, was performed to analyse the differences at different time-points. Bars
769 with different letters differ significantly ($P \leq 0.01$).

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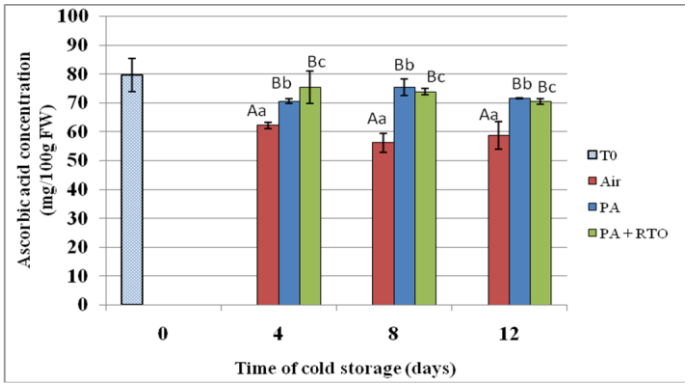

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772 Fig. 2 Ascorbic acid (AA) concentration (mg 100 g^{-1} FW) of orange juice extracted from citrus fruit
773 stored in air (Air), in passive atmosphere (PA) or in passive atmosphere with RTO vapours (PA +
774 RTO) for 12 days at 7 °C. Two way- ANOVA was applied to estimate the effects of time and of the
775 sample on the ascorbic acid concentration; the least significant difference comparison values (LSD,
776 95% confidence interval, expressed as mg 100 g^{-1} FW) were calculated for the storage time and
777 packaging condition. AA: 7.4 (storage time); 6.4 (packaging condition). Means with different
778 superscript letters differ significantly ($P \leq 0.05$). Capital letters are used to separate the mean values
779 at each sampling time on the basis of the packaging condition, whereas lowercase letters are used
780 to separate the mean values of the storage time.

781

Tables

Table 1
Mean values of the percentages (%) of the infected wounds, the infected wounds with spores and of the surficial growth area (cm^2) on the oranges inoculated with *P. italicum* ITEM 9571 (A) or *P. digitatum* ITEM 9569 (B) and stored during 16 days at 7 °C in a passive atmosphere (PA) or a passive atmosphere under RTO vapours (PA + RTO).

Time (days)	Percentage of infected wounds (%)		External growth area (cm^2)		Percentage of infected wounds with the spores (%)	
	PA	PA + RTO	PA	PA + RTO	PA	PA + RTO
8	62.5 ± 16.5^a	42.7 ± 1.5^b	3.2 ± 0.5^a	1.3 ± 0.5^b	47.9 ± 9.5	nd
12	80.7 ± 21.8^a	60.9 ± 13.9^a	6.7 ± 0.8^a	1.7 ± 0.2^b	79.2 ± 19.1	nd
16	91.6 ± 7.6^a	87.7 ± 10.7^a	9.9 ± 1.5^a	3.0 ± 2.1^b	91.6 ± 11.8^a	9.4 ± 10.0^b
Time (days)	Percentage of infected wounds (%)		External growth area (cm^2)		Percentage of infected wounds with the spores (%)	
	PA	PA + RTO	PA	PA+ RTO	PA	PA + RTO
4	94.4 ± 9.6^a	79.2 ± 4.2^a	3.6 ± 0.4^a	2.1 ± 0.3^b	nd	nd
8	97.2 ± 2.4^a	97.2 ± 4.8^a	10.3 ± 2.2^a	3.5 ± 1.0^b	67.3 ± 3.2	nd
12	100.0 ± 0.0^a	79.2 ± 14.7^a	18.2 ± 9.6^a	20.8 ± 5.5^a	75.0 ± 5.9^a	48.6 ± 8.6^b

nd: infected wounds with the spores not detected. One way- ANOVA was applied to estimate the effect of the time on mean values of the different parameters; the least significant difference comparison values (LSD, 95% confidence interval, expressed as % or cm^2) were calculated among the samples. Percentage of infected wounds: 26.9%; surficial growth area: 2.4 cm^2. Tukey test was applied to separate the mean values on the basis of the packaging condition (PA vs PA + RTO) at each sampling time. The means with different lowercase letters differ significantly ($P \leq 0.05$).
nd: infected wounds with the spores not detected. One way- ANOVA was applied to estimate the effect of the time on mean values of the different parameters; the least significant difference comparison values (LSD, 95% confidence interval, expressed as % or cm^2) were calculated among the samples. Percentage of infected wounds: 29.4%; surficial growth area: 11.3 cm^2. Tukey test was applied to separate the mean values on the basis of the packaging condition (PA vs PA + RTO) at each sampling time. The means with different lowercase letters differ significantly ($P \leq 0.05$).

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Table 2
Mean values of the percentages (%) of the main RTO compounds released into cardboard boxes containing oranges and sealed with PP film, during 12 days at 4 °C.

Time (days)	RTO (µL/L)	p-cymene (%)	γ-terpinene (%)	thymol (%)
0	33.0 ± 2.0^a	74.7 ± 1.4^a	23.2 ± 1.1^a	2.0 ± 0.3^a
4	36.6 ± 1.8^a	68.0 ± 3.0^a	23.7 ± 1.8^a	9.0 ± 3.0^b
8	29.1 ± 1.3^b	61.0 ± 4.0^a	22.0 ± 3.0^a	15.0 ± 3.0^c
12	27.4 ± 1.5^b	56.0 ± 4.0^a	26.0 ± 3.0^a	18.3 ± 0.7^c

One way- ANOVA was applied to estimate the effect of the time on mean values of different volatile compounds. Tukey's HSD test was performed to underline differences of compound concentration over time. The means with different lowercase letters differ significantly ($P \leq 0.05$).

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SUPPLEMENTARY CONTENT

Effect of red thyme oil (*Thymus vulgaris* L.) vapours on fungal decay, quality parameters and shelf-life of oranges during cold storage

Pinto L.[1], Cefola M.[2], Bonifacio M.A.[3], Cometa S.[4], Bocchino C.[5], Pace B.[2], De Giglio E.[3], Palumbo M.[2], Sada A.[6], Logrieco A.F.[1], Baruzzi F.[1*]

Supplementary methods (SM)

SM 1 Red thyme essential oil

Commercially available red thyme essential oil (RTO, *Thymus vulgaris* L.) was purchased from Bristol Botanicals Ltd (Bristol, UK), stored at 4 °C in dark bottles prior to use. According to the information given by the suppliers, RTO was produced by the steam distillation method. The RTO was diluted in n-hexane (HiPerSolv Chromanorm® for HPLC, VWR International, Darmstadt, Germany) for the antimicrobial assays as already reported (Cosentino et al., 1999).

SM 2 Antimicrobial activity of the RTO in-vapour phase

The antifungal activity of RTO was evaluated in vapour phase. PDA plates were inoculated with 100 µL of *P. digitatum* and *P. italicum* suspensions (1 x 10^7 spores/mL), and placed into an HDPE plastic box with an internal volume of 600 mL (Ref. 11673, Albero Forte Composite s.l., Banyeres de Mariola, Spain). Sterile discs (2 per box) were loaded with RTO at a concentration of 66.7 µL/L and placed on the inner surface of the lid. The plastic box was closed quickly and tightly, sealed with a parafilm and incubated at 25°C for 72 h. In order to define the antifungal activity in the vapour phase, the mycelium growth was classified, at the end of incubation, using the following empirical scale: 0, absence of mycelium; 1-3, stunted mycelial growth; 4-5, widespread mycelial growth. A single essential oil, showing partial inhibition of the mycelium growth (1-3 values in the empirical scale) or the absence of the mycelium growth (0 value) was selected for further experiments. The vapour contact assay was repeated twice (two independent biological replicates evaluated by two different authors) with three technical replicates.

SM 3 Challenge-test with different packaging materials

3.1 Fruit material

Oranges (*Citrus sinensis* L. cv. Navel) were purchased from a local market within 48 h of harvesting; the fruit was not coated with wax containing fungicides. Each orange was washed with sterile distilled water at 20% v/v of ethanol in order to remove dust and impurities and dried at room temperature. Orange fruit without any visible wounds, or other defects, with a weight in the 170-250 g range, was selected for both the challenge-test and the shelf-life trials.

3.2 Challenge-test

The oranges were wounded (3 mm in depth, 5 mm in diameter) using a sterile scalpel in four, evenly spaced points on the upper hemisphere of the orange (24 wounds per replicate, total wounds = 96). The wounded oranges were inoculated with *P.digitatum* ITEM 9569 and *P. italicum* ITEM 9571 spore suspensions according to the inoculum level (approximately 1.0×10^3 spores/wound) adopted by He et al. (2016), and the oranges (6 for each replicate, N = 3) were then packed in a cardboard box (29 x 19.5 x 9 cm, Antonio Sada & Figli Spa, Pontecagnano Faiano, Salerno, Italy). The samples were split into two groups: the first group of orange fruit (3 replicates composed of 6 oranges for each *Penicillium* strain and sampling time) was packed in air using the three plastic materials (PE-25, PE-40 and PP, 30 µm), sealed (Impulse Auto Sealer, Mercier Corporation, Taipei, Taiwan) and stored in a climate controlled storage chamber (Everlasting cabinet model 700 Glass, Everlasting S.r.l., Suzzara, Italy) at 7 °C, 70% RH and at a ventilation rate of 0.1 L min^{-1} for 19 days; the second group was packed in the presence of RTO vapours using the same packaging material. The EO was diluted in n-hexane (50% v/v) and applied to Grade 3 Whatman filter paper discs (90 mm circles, Whatman International Ltd, Madstone, England). The filter paper discs were divided into four pieces and attached, upside down, to the internal surface of the film. According to the previously calculated inhibiting concentration in the vapour phase of RTO, the estimated concentration was 51.7 µL/L. At the end of the incubation period, the percentage (%) of wounds with visible mycelium growth and the diameter of the mycelium growth of each wound, measured in two perpendicular directions by means of a vernier caliper and expressed as area (cm^2), were recorded.

Supplementary Results (SR)

Table 1S. Values assigned, on the basis of an empirical scale, to the mycelium growth of *P. digitatum* ITEM 9569 and *P. italicum* ITEM 9571 after exposure (72 h at 25°C) to 66.7 µL/L of RTO in vapour phase. The assay was repeated on two biological replicates (R1 and R2).

	RTO		**Hexane**	
	R1	R2	R1	R2
P. digitatum	0 ± 0	0 ± 0	5 ± 0	5 ± 0
P. italicum	0 ± 0	0 ± 0	5 ± 0	5 ± 0

Abbreviation: RTO, red thyme essential oil. Empirical scale: 0, absence of mycelium; 1-3, stunted mycelial growth; 4-5, widespread mycelial growth. The assigned values are the means of three technical replicates.

Table 2S. Chemical composition of RTO, obtained by means of GC-MS analysis.

Compound	Relative abundance (%)	Retention time (min)
terpinolene	0.04 ± 0.01	5.14
p-cymene	37.5 ± 1.6	5.20
γ-terpinene	19.1 ± 1.1	5.45
linalol	0.002 ± 0.001	5.74
terpinen-4-ol	0.02 ± 0.01	6.33
thymol	43.4 ± 2.1	6.94
carvacrol	0.09 ± 0.04	7.03
caryophillene	0.05 ± 0.02	7.81

Table 3S. Dry mycelium weight (mg) of *P. digitatum* ITEM 9569 and *P. italicum* ITEM 9571 exposed, in vapour phase, to different initial concentrations of RTO diluted in hexane (40 µL loaded on two discs) in a plastic box of 600 mL.

Strain	RTO initial concentration			
	0 µL/L	6.7 µL/L	13.3 µL/L	33.4 µL/L
P. digitatum ITEM 9569	30 ± 5[a]	36 ± 5[a]	37 ± 6[a]	nd
P. italicum ITEM 9571	35 ± 3[a]	38 ± 2[a]	19 ± 2[b]	nd

nd: dry mycelium weight < 5 mg. One way- ANOVA was applied to estimate the effect of RTO concentration on mean values of dry weight; the least significant difference comparison values (LSD, 95% confidence interval, expressed as mg) were calculated among samples for each strain. ITEM 9569: 18; ITEM 9571: 6. Means with different lowercase letters differ significantly ($P \leq 0.05$).

Table 4S. Mean values of the percentages (%) of infected wounds and surficial growth area (cm^2) on the oranges inoculated with *P. italicum* ITEM 9571 or *P. digitatum* ITEM 9569 and stored for 19 days at 7°C in a passive atmosphere in air (PA) or under RTO vapours (30.8 µL/L, PA + RTO) using polypropylene (PP) or polyethylene (PE-25 or PE-40) as the packaging material.

Strain	**Packaging condition**	**PP** Percentage of infected wounds	External growth area	**PE-25** Percentage of infected wounds)	External growth area	**PE-40** Percentage of infected wounds	External growth area
ITEM 9569	PA	100.0 ± 0.0^a	28.3 ± 0.0^a	100.0 ± 0.0^a	4.8 ± 2.2^a	100.0 ± 0.0^a	12.6 ± 0.0^a
	PA + RTO	100.0 ± 0.0^a	10.7 ± 1.9^b	75.0 ± 25.0^a	6.3 ± 0.8^a	75.0 ± 25.0^a	7.7 ± 0.6^b
ITEM 9571	PA	100.0 ± 0.0^a	3.5 ± 0.4^a	100.0 ± 0.0^a	2.4 ± 0.7^a	100.0 ± 0.0^a	2.3 ± 0.3^a
	PA + RTO	100.0 ± 0.0^a	1.3 ± 0.5^b	100.0 ± 0.0^a	0.9 ± 0.3^b	100.0 ± 0.0^a	2.5 ± 0.1^a

PA: Passive atmosphere; PA + RTO: passive atmosphere with RTO vapours. One way- ANOVA was applied to estimate the effect of the packaging film on the mean values of the different parameters for each strain; the least significant difference comparison values (LSD, 95% confidence interval, expressed as % or cm^2) were calculated for the samples. Percentage of infected wounds: ITEM 9569, 25.7%; area of superficial growth: ITEM 9569, 3.1 cm^2; ITEM 9571, 1.1 cm^2. The Tukey test was applied to separate the mean values according to the packaging condition (PA vs PA + RTO) for each strain and packaging film. Means with different lowercase letters differ significantly ($P \leq 0.05$).

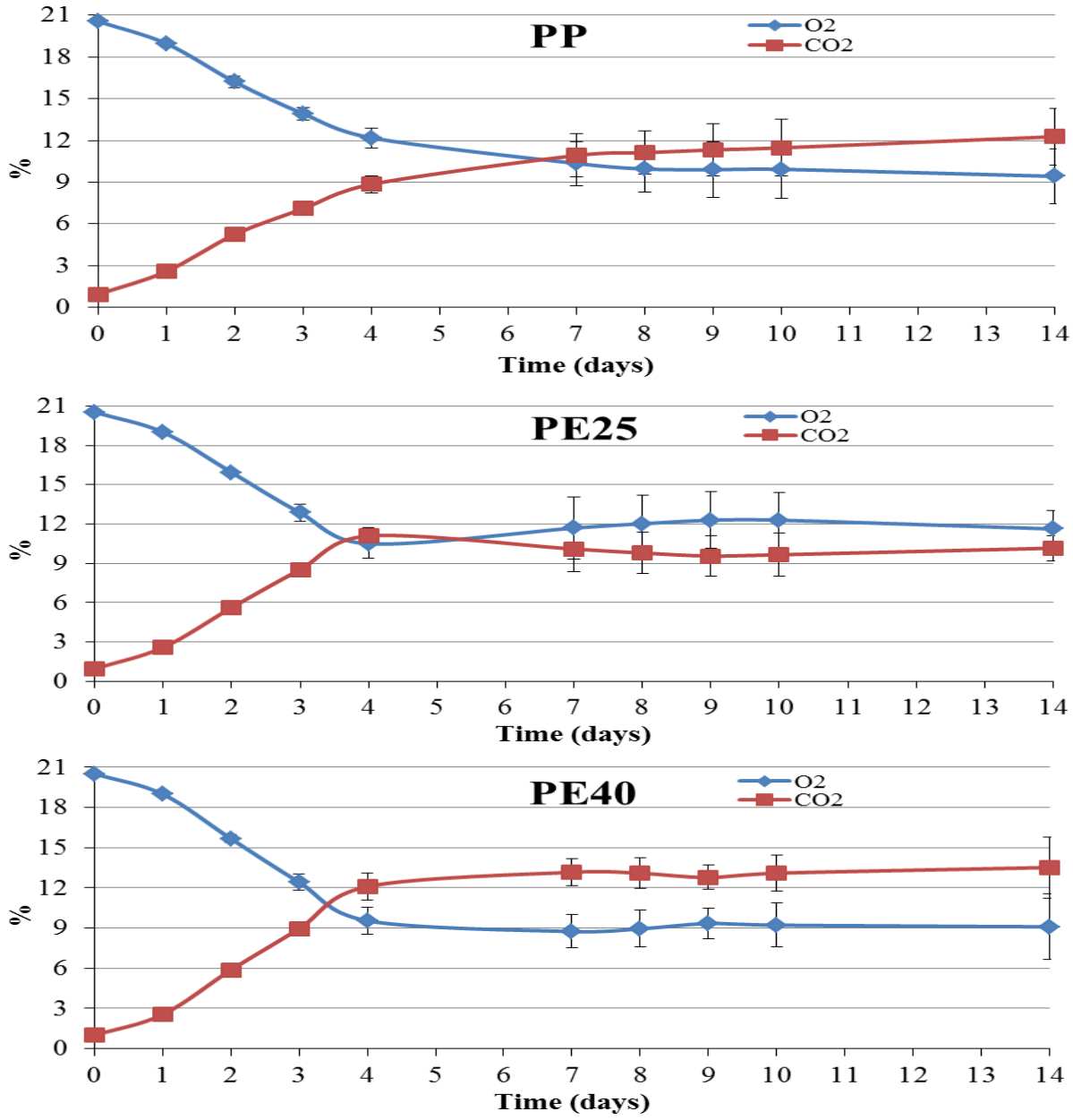

Figure 1S. Mean values of O_2 (blue lines) and CO_2 (red lines) concentrations (%) in cardboard boxes with oranges packed with PP, PE-25 or PE-40 plastic films and stored in air for 14 days at 7°C.

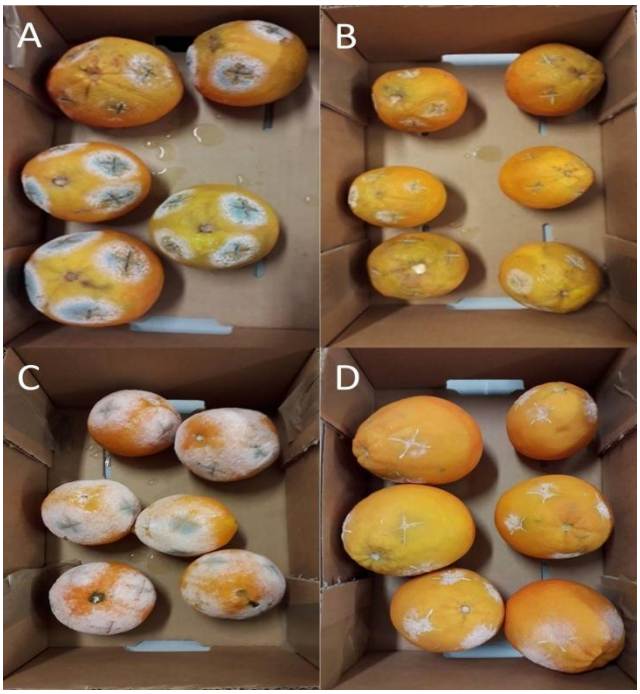

Figure 2S. Oranges inoculated with *P. italicum* ITEM 9571 and stored in a passive atmosphere in air (A) or with RTO (B) after 12 days at 7°C, or inoculated with *P. digitatum* ITEM 9569 and stored in a passive atmosphere in air (C) or with RTO (D) after 8 days of cold storage using PP as packaging material.

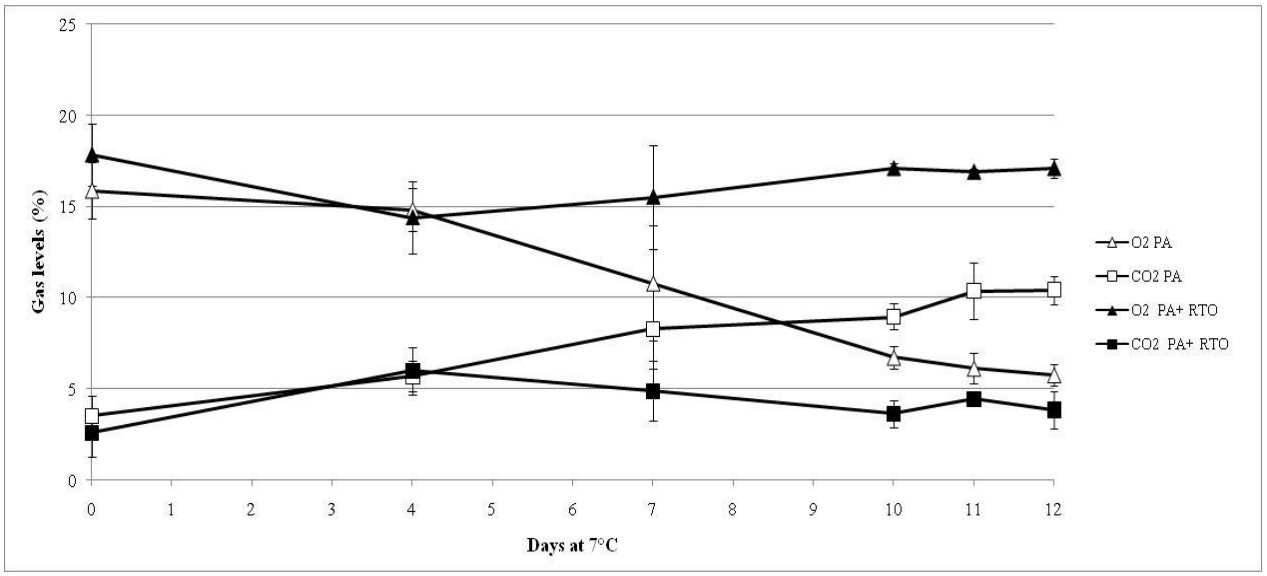

Figure 3S. Changes in O_2 and CO_2 concentration (%) of inner atmosphere of orange packages during 12 days of cold storage. Data are means ± standard deviation.

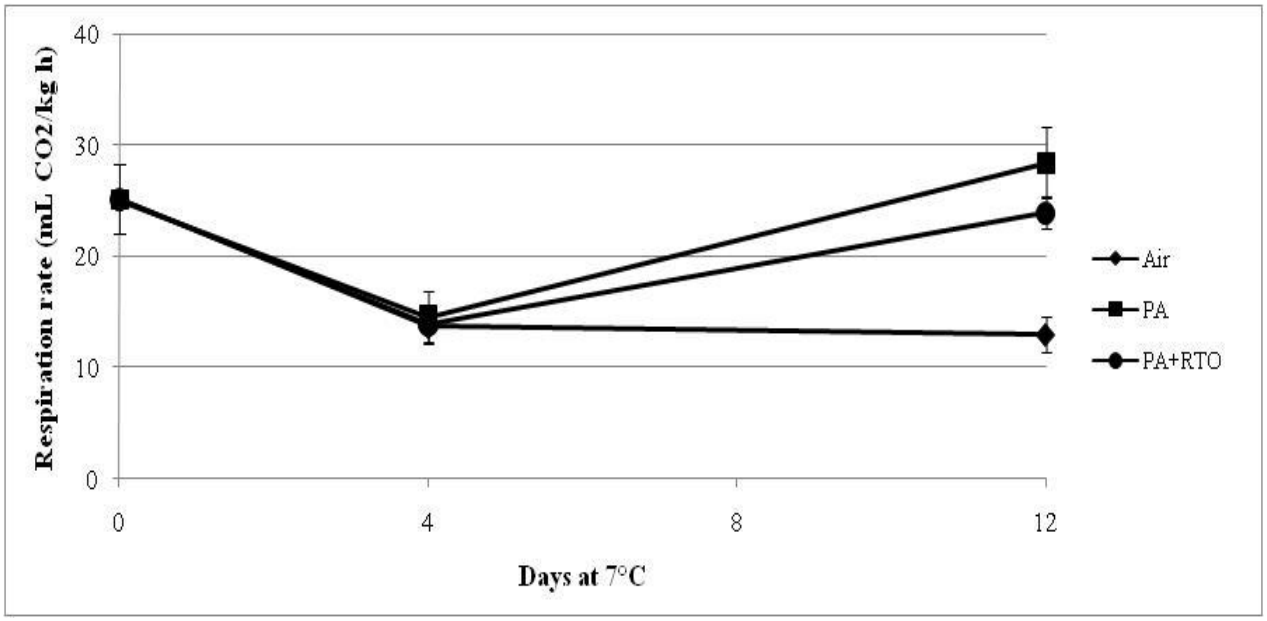

Figure 4S. Changes in the respiration rate of oranges during 12 days of cold storage. Data are means ± standard deviation.

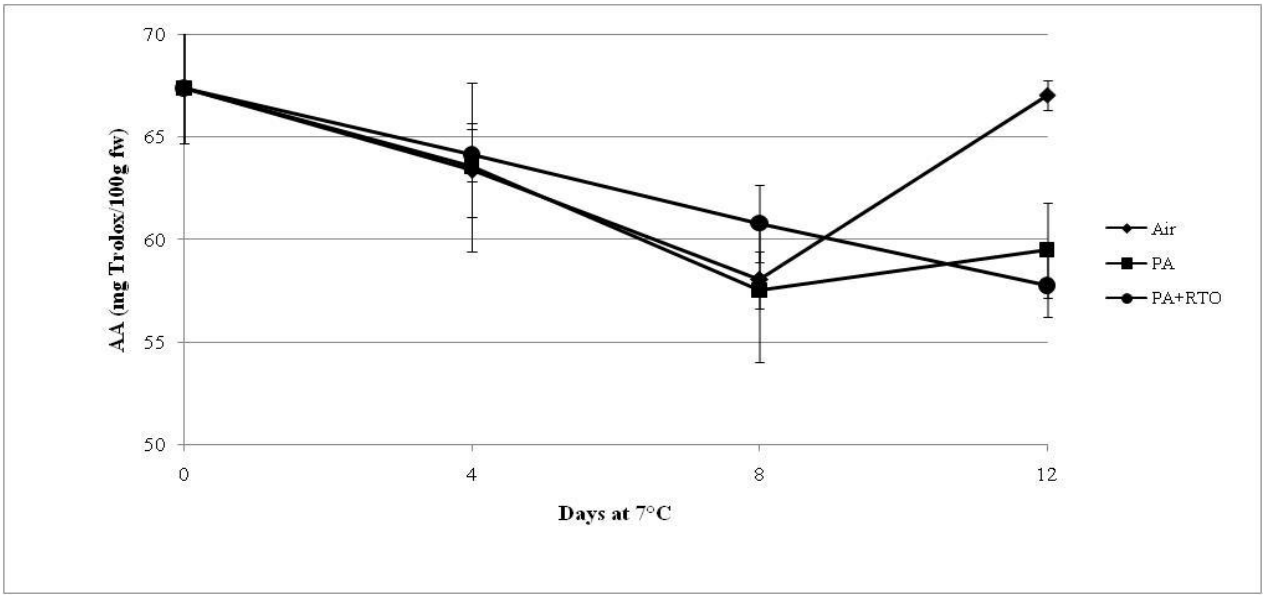

Figure 5S. Changes in the antioxidant activity of oranges during 12 days of cold storage. Data are means ± standard deviation.

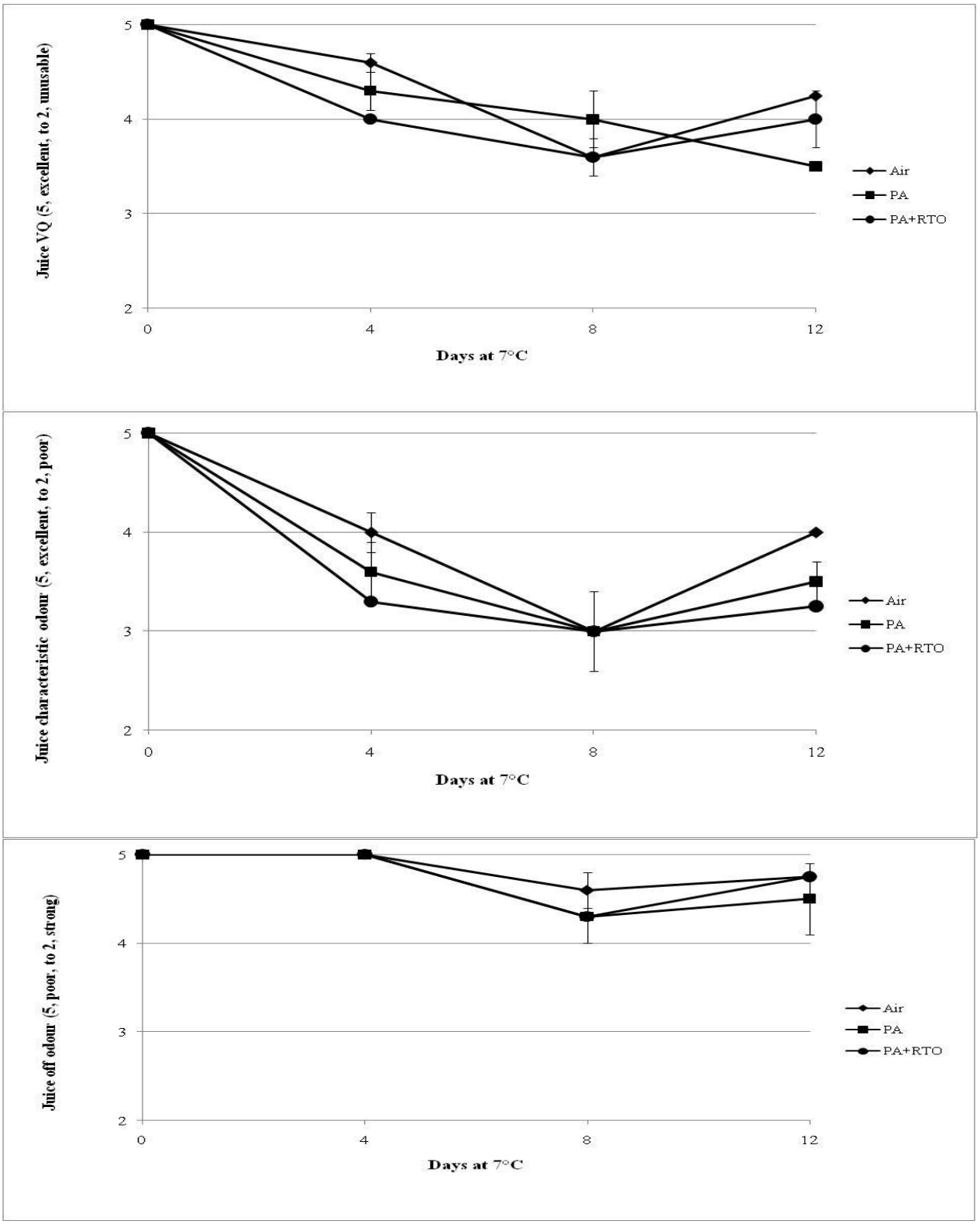

Figure 6S. Changes in juice visual quality (A), characteristic odour (B) and off-odour (C) score during the storage of orange in Air, PA or PA + RTO. Data are means ± standard deviation.

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