agriculture

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# The Preharvest Application of Essential Oils (Carvacrol, Eugenol, and Thymol) Reduces Fungal Decay in Lemons

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**Abstract:** Lemon postharvest losses are mainly due to the presence of fungal diseases. Current postharvest decay strategies rely on synthetic chemical fungicides; however, consumers are demanding that fruit is free of any chemical residue. The use of new natural alternatives, including essential oils, is emerging due to their potential antimicrobial activity. Therefore, the aim of this work is the elucidation of the effect of carvacrol, eugenol, and thymol, individually and in combination, applied in preharvest. Three different concentrations (100, 500, and 1000 µL/mL) of carvacrol, eugenol, and thymol were individually applied and in combination in 'Fino' and 'Verna' lemon cultivars. The fungal incidence (mainly *Penicillium digitatum* and *P. italicum*) was evaluated weekly for 35 days. Moreover, the main different quality parameters (weight loss, firmness, colour, total soluble solids, titratable acidity, and total phenolic content) of lemons were evaluated at harvest and after 35 days of cold storage. The results showed that carvacrol at the lowest concentration (100 µL/L) provided the lowest fungal incidence with a non-negative effect on the lemon quality parameters during storage, while the highest concentrations and the combination of essential oils resulted in the opposite effect. Therefore, carvacrol applied at 100 µL/L in preharvest could be an eco-friendly alternative to the current fungicides to control lemon decay, while maintaining their optimal quality.

**Keywords:** *Citrus limon*; fungal incidence; postharvest losses; preharvest treatment; quality



**Citation:** Gutiérrez-Pozo, M.; Serna-Escolano, V.; Giménez-Berenguer, M.; Giménez, M.J.; Zapata, P.J. The Preharvest Application of Essential Oils (Carvacrol, Eugenol, and Thymol) Reduces Fungal Decay in Lemons. *Agriculture* **2023**, *13*, 1437. https://doi.org/10.3390/agriculture13071437

Academic Editor: Giuseppe Lima

Received: 16 June 2023
Revised: 18 July 2023
Accepted: 20 July 2023
Published: 20 July 2023



## 1. Introduction

*Citrus* spp. fruit production is mainly based in the Mediterranean area and is one of the largest fruit sources for human consumption. Lemons at harvest need to meet the quality requirements imposed by consumers, such as size, colour, firmness, acidity, and bioactive content [1,2]. Maintaining these attributes during storage is essential in providing consumers with high-quality lemons with a long shelf-life, and therefore, increasing the lemon market throughout the entire year. It is considered that postharvest losses, mainly due to the presence of postharvest diseases and physiological disorders, can range between 30 and 50% of the total production. These losses involve the generation of waste, as well as important economic consequences [3]. The main postharvest diseases that directly affect the production of lemons are green mould, blue mould, and sour rot caused by *Penicillium digitatum*, *Penicillium italicum*, and *Geotrichum citri-aurantii*, respectively. They are all considered as wound pathogens that require a lesion on the lemon fruit peel to start developing [4]. Fungal spores are massively produced during their infection and easily propagated through the air to adjacent fruit at any stage [5].

Current strategies to control lemon decay rely on synthetic chemical fungicides that allow for the maintenance of fruit quality at harvest and during cold storage [6]. The two main fungicides that are currently being applied in postharvest are imazalil and thiabendazole. However, the concern of consumers about the use of fungicides and their implication for human health and the environment is resulting in a demand for fruit free of any chemical residue and a more environmentally friendly solution [7]. Indeed, the increasing demand for organic fruit, such as lemons, is obliging farmers to adapt their strategies to the legislative requirement for organic production.

Many researchers have focused their attention on the development of new natural alternative methods to control fruit and vegetable decay. Among those alternatives, in recent decades, the use of essential oils and plant extracts has been extensively studied [8]. Essential oils (EOs) are secondary metabolites from the plant that present antimicrobial activity and a high content of antioxidant compounds. The main role of EOs is to attract and repel insects and protect against heat and cold. They possess GRAS (Generally Recognised As Safe) status, therefore their antifungal activity is receiving strong attention for its application as a food preservative. Therefore, the biological effect of EOs has been widely documented by different authors, elucidating their capability to disrupt the permeability of mycelial cell walls and inducing plant defence mechanisms [9–13].

The use of EOs such as carvacrol, thymol, and eugenol, among others, has been previously studied in vitro to control fungal growth. Numerous studies have proven the strong antifungal effect of *Thymus* spp. against *P. digitatum*, *P. italium*, and *G. citri-aurantii* [14–19]. *Thymus* spp.'s main essential oils are carvacrol and thymol [20]. Both essential oils have been tested individually and in combination in vitro against *P. digitatum* and *P. italium* in liquid and solid media, showing an inhibiting effect, where thymol displayed the highest efficacy [21]. They have also reported high efficacy encapsulated in HP-β-Cyclodextrin against the in vitro growth of *G. citri-aurantii* [22]. Indeed, carvacrol was tested in vitro against *Botrytis cinerea* and showed the inhibition of fungal growth [23]. A different EO, whose effect has also shown antifungal activity against *Penicillium* spp. in vitro, is eugenol, which can be naturally found in clove (*Syzygium aromaticum* L.) and cinnamon oil [24,25].

Moreover, the effect of these EOs in vivo, when they are applied in postharvest, has also been studied. In oranges, the use of thymol coating at 2% was tested against *P. digitatum* development, reducing the decay incidence [26]. A different study using the dipping method looked at the effect of *Thymus vulgaris*, whose main components are carvacrol and thymol, on "Navel" oranges, resulting in a great reduction in fruit decay at 400 µL/L [27]. Further, a recent study has applied carvacrol and eugenol nano-emulsions to "Newhall" navel oranges to confer resistance against *P. digitatum* [28]. In "Valencia" oranges, the optimal concentration of *Thymus vulgaris* applied in postharvest that achieved the highest fungal decay reduction was 1000 µL/L [18]. Moreover, the combined application of eugenol, thymol, and carvacrol (250 µL/L) in sweet cherries significantly reduced the ripening process and increased the antioxidant content, parameters that improve the defence system of the fruit [29].

In lemons, the antifungal efficacy of thymol, carvacrol, and a combination of both was tested against *P. digitatum* and *P. italicum*. Both EOs were applied with wax in the packing lines at different concentrations (10–500 µL/L), resulting in a higher reduction in the infected fruit surface in those lemons treated with 500 µL/L [21,30]. Serna-Escolano et al. [18] carried out a preventive and curative study with thymol encapsulated in HP-β-CDs against *G. citri-aurantii*, where a reduction in the incidence and severity of damage was observed compared to the non-treated lemons.

The use of EOs as a fungicide alternative has been widely studied in postharvest in citrus fruit. However, it could be interesting to study the effect that these EOs could have on the fruit when they are applied as a preharvest treatment. Moreover, evaluating the effect of pure EOs, instead of the whole plant extract, has also been demanded by many researchers due to the differences detected in antifungal efficacy when the whole plant extract is applied [31,32]. Only a few studies have been carried out in preharvest, such as those on tomatoes [33,34], lettuce [35–37], and strawberry [38,39]. However, there is no literature available on citrus fruit in preharvest. Therefore, the aim of this work was the elucidation of the effect of carvacrol, eugenol, and thymol, individually and in combination, in preharvest on lemon fungal incidence, and the impact on their quality parameters at harvest and after 35 days of cold storage.

## 2. Materials and Methods

### *2.1. Plant Material and Experimental Design*

The experiments were carried out in two consecutive growing seasons (2021–2022 and 2022–2023) in an outdoor commercial plot of lemon trees located in Fortuna (Murcia, Spain) under organic agronomic practices. Two different lemon cultivars were used for these experiments, 12-year-old 'Fino 49' lemon trees grafted on *Citrus macrophylla* and 15-year-old 'Verna' lemon trees grafted on *Citrus aurantium* planted at 7 m × 5 m. 'Fino 49' lemon trees were treated and harvested in January and 'Verna' lemon trees were treated and harvested in June. For the experiment, three blocks of five lemon trees were selected for each of the treatments. In the growing season 2021–2022, lemon trees were treated with carvacrol (CV), eugenol (EG), and thymol (TH) at three different concentrations (100, 500, and 1000 µL/L) according to previous work [18,29,40]. Based on the results obtained in the first season, the concentrations that provided the best results were selected for the second growing season (2022–2023): (1) CV at 100 µL/L; (2) a combination of CV and EG, both at 100 µL/L (CV + EG); (3) a combination of CV and TH, at 100 and 500 µL/L, respectively (CV + TH); and (4) a combination of the three EOs, CV and EG at 100 µL/L and TH at 500 µL/L (CV + EG + TH). All EO treatments were prepared by diluting the pure EOs (Sigma-Aldrich, Madrid, Spain) in tap water with 0.1% Tween 20 as a surfactant. In both seasons, control trees were treated with an aqueous solution containing 0.1% of wetting solution. All treatments were applied by spraying 5 L per tree on leaves and lemons. Two treatments of EOs were applied, 1 month and 5 days before harvest. 'Fino' and 'Verna' lemons were harvested at a yellow commercial ripening stage when they presented the optimal diameter (around 55 mm) requested by the market. Then, lemons were transferred to the lab within a 2 h period. Afterward, lots of 15 fruits (3 replicates of 5 lemons each) uniform in size, colour, and without any physical damage were selected for each of the treatments and sampling days and stored for 35 days at 10 °C and 85% of relative humidity (RH). Three lots of lemons from each treatment were selected at different sampling days (0, 7, 14, 21, 28, and 35) during the cold storage period for the analytical determinations.

### *2.2. Decay Incidence of Lemons*

Decay incidence was determined weekly in a parallel experiment where lemons were stored in a commercial facility. For this experiment, ten boxes of 100 lemons from each of the different treatments were stored in the commercial storage at 10 °C and 85% RH. Decay incidence was evaluated after 7, 14, 21, 28, and 35 days by identifying and discarding lemons with disease symptoms [41]. The fungal decay was expressed as decay accumulated; the number of lemons decayed on every previous sampling day were summed up. The formula used was:

$$\text{Decay (\%)} = (\text{total decayed fruit}/\text{total evaluated fruit}) \times 100$$

### *2.3. Physiological and Quality Parameters*

Individual fruit were weighed at day 0 and after 35 days of storage, and weight loss (WL) was expressed in percentage (%). Firmness was also measured individually in each fruit using a TX-xT2i texture analyser (Stable Microsystems, Godalming, UK) coupled to a steel plate that caused a deformation of 5% of the fruit diameter and was expressed as N $mm^{-1}$. The respiration rate was measured on three replicates of five lemons placed in a 0.5 L glass jar for 60 min at room temperature, and 1 mL of the atmosphere was analysed and quantified in a gas chromatograph (Shimadzu 14B-GC) coupled to a thermal conductivity detector. The respiration rate was expressed as mg of $CO_2$ $kg^{-1}$ $h^{-1}$ [42]. The colour was measured individually on each sampling day using a Minolta colourimeter (CRC200; Minolta, Osaka, Japan) and it was expressed as Hue Angle. All parameters were measured in each of the treatments, and results were expressed as mean ± SE. The juice from four lemons was mixed and used to measure total soluble solids (TSS) and titratable acidity (TA) in duplicate in each sample, and the peel was mixed and frozen in liquid nitrogen for further analysis. A digital refractometer (Hanna Instruments, Woonsocket, RI, USA) was used for the TSS and the TA was determined via the titration of 0.5 mL of juice diluted in 25 mL of distilled water with NaOH 0.1 mM until pH 8.1 using an automatic titrator (OMNIS, Metrohm AG, Herisau, Switzerland). The results (mean ± SE) of TSS and TA were expressed as °Brix and g citric acid equivalent 100 $mL^{-1}$, respectively.

### 2.4. Total Phenolic Content

Total phenolic content (TPC) was measured by homogenizing 2 g of frozen flavedo with 15 mL of water: methanol (2:8, $v/v$) containing 2.0 mM NaF. After centrifugation of the extracts at 10,000× $g$ and 4 °C for 20 min, TPC was measured in duplicate in each flavedo sample using the Folin–Ciocalteau reagent, as previously reported [43]. The results (mean ± SE) were expressed as mg of gallic acid equivalent to 100 g of fresh weight (FW).

### 2.5. Statistical Analysis

Statistical analysis was performed using the SPSS software v. 20.0 for Windows. The results were expressed as the mean ± SE of three replicates. The data sets were tested for normality and homoscedasticity using the Saphiro–Wilk and Levene's tests, respectively. An analysis of variance (ANOVA) was conducted on datasets (decay incidence, weight loss, firmness, colour, TSS, TA, and TPC) to determine significant differences between treatments ($p$-value < 0.05). When statistically significant differences were detected, post hoc parametric comparisons were carried out for each pair using the Duncan test.

## 3. Results

### 3.1. Effect of EOs in the Decay Incidence of Lemons

The decay incidence was studied weekly during 35 days of storage at 10 °C in two consecutive growing seasons (2021–2022 and 2022–2023). In the first season, three concentrations of CV, EG, and TH were tested (100, 500, and 1000 μL/L) individually in 'Fino' and 'Verna' lemon trees (Figure 1). In 'Fino' lemons, all the preharvest treatments applied significantly ($p < 0.05$) reduced the fungal decay, with the exception of TH at the highest concentration, while in 'Verna', all the treatments applied significantly ($p < 0.05$) reduced the fungal incidence in lemons during the whole storage period. The decay incidence of 'Fino' lemons was higher than 'Verna'. After 35 days of storage, a decay incidence of 16.60 ± 0.79% was observed in the control 'Fino' lemons (Figure 1A). While those lemons treated with CV at 100, 500, and 1000 μL/L presented 5.70 ± 0.30, 7.70 ± 0.47, and 9.10 ± 0.55%, respectively. An increasing tendency was also observed in those lemons treated with EG (7.30 ± 0.30, 8.90 ± 0.31, and 10.80 ± 0.29%) and TH (10.10 ± 0.38, 8.50 ± 0.43, and 14.30 ±0.67%) at 100, 500, and 1000 μL/L. A decay incidence of 6.00 ± 0.26% was observed in control 'Verna' lemons (Figure 1B), observing the lowest incidence in lemons treated with CV at 100 μL/L (2.90 ± 0.18%) and increasing as a higher concentration of CV was applied. Similar results were observed for EG, while for TH, the intermediate concentration (500 μL/L) was the one providing the lowest incidence (3.40 ± 0.22%). Therefore, the application of CV and EG at 100 μL/L and EG at 500 μL/L significantly ($p < 0.05$) reduced the decay incidence in lemons of both cultivars.

Based on the results of the first season, during the second season CV at the lowest concentration was tested individually and in combination with EG and TH at 100 μL/L and 500 μL/L, respectively (Figure 2A,B). Similar results were observed in both cultivars, where the use of CV at 100 μL/L and in combination with EG and TH resulted in a significant reduction in fungal decay compared to the control lemons. After 35 days of storage, a decay incidence of 14.30 ± 0.56 and 6.30 ± 0.47% was observed in 'Fino' and 'Verna' control lemons, respectively, while the treatment with CV at 100 μL/L presented 4.90 ± 0.31% of fungal incidence in 'Fino' and 3.80 ± 0.21% in 'Verna' lemons. The use of a combination of

CV + EG and CV + TH also reduced the fungal incidence in 'Fino' (8.60 ± 0.43, 9.20 ± 0.42) and 'Verna' lemons (4.60 ± 0.25, 5.00 ± 0.37), respectively. However, the combination of the three EOs (CV + EG + TH) did not significantly ($p < 0.05$) reduce the fungal decay in both cultivars, being 13.80 ± 0.25% in 'Fino' and 7.70 ± 0.46 in 'Verna' after 35 days of storage.

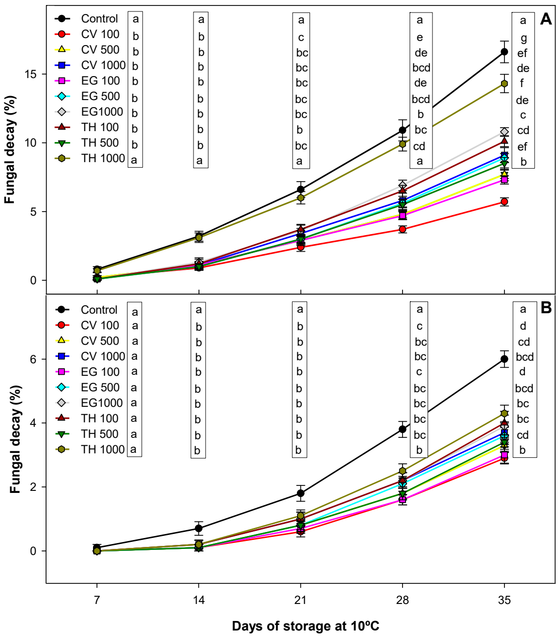


**Figure 1.** Fungal decay incidence (%) in 'Fino' (**A**) and 'Verna' (**B**) lemons from the 2021–2022 growing season; the groups were the control and treated at preharvest with carvacrol (CV), eugenol (EG), and thymol (TH) at three different concentrations (100, 500, and 1000 µL/L) after 7, 14, 21, 28, and 35 days of storage at 10 °C. Significant differences ($p < 0.05$) between treatments are presented with different lower-case letters, corresponding to the order of the treatments in the legend.

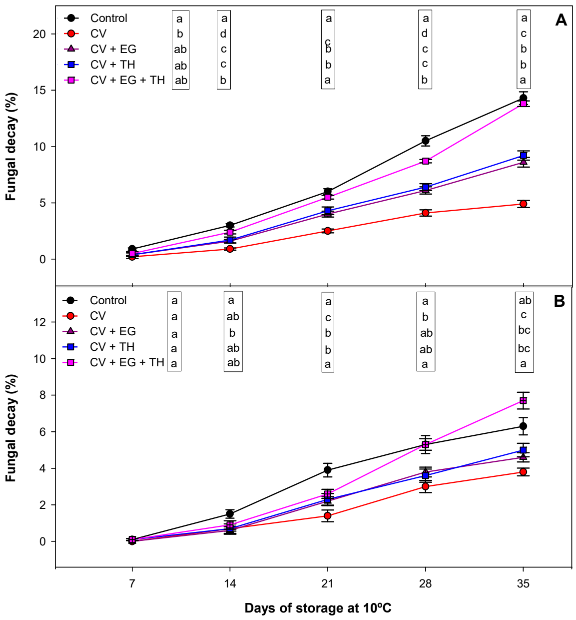


**Figure 2.** Fungal decay incidence (%) in 'Fino' (**A**) and 'Verna' (**B**) lemons from the 2022–2023 growing season; the groups were the control and treated at preharvest with carvacrol 100 µL/L (CV), eugenol 100 µL/L (EG), and thymol 500 µL/L (TH) after 7, 14, 21, 28, and 35 days of storage at 10 °C. Significant differences ($p < 0.05$) between treatments are presented with different lower-case letters, corresponding to the order of the treatments in the legend.

### *3.2. Effect of EOs in the Quality Parameters of 'Fino' and 'Verna' Lemons*

Quality parameters were analysed weekly, although data has only been presented at harvest and after 35 days of storage. Weight losses (WL) were studied and presented after 35 days of storage at 10 °C in 'Fino' (Figure 3A) and 'Verna' lemons (Figure 4A). No significant ($p < 0.05$) differences were observed in WL between treated and control lemons. 'Fino' and 'Verna' control lemons presented a 4.60 ± 0.22 and 3.80 ± 0.26% of WL, respectively, presenting 'Verna' lemons with slightly lower WL than 'Fino' ones. Firmness is another important attribute related to fruit quality, no significant ($p < 0.05$) differences were detected between treatments at harvest and after 35 days of storage in both cultivars (Figures 3B and 4B). Significant differences (Table 1) were detected between sampling days (0 and 35), where a reduction in firmness of 55 and 15% were observed in control 'Fino' and 'Verna' lemons, respectively. Therefore, 'Fino' lemons showed a higher loss of firmness during storage compared to 'Verna'. The colour of lemons was also studied based on the CIELAB parameters, where the Hue Angle of 'Fino' and 'Verna' lemon was represented (Figures 3C and 4C). No significant differences were observed between treatments in both cultivars, neither between sampling dates in 'Fino' lemons, being 91.37 ± 0.39 and 91.41 ± 0.32 h° in control lemons at harvest and after 35 days of storage, respectively. However, the Hue Angle of 'Verna' lemons significantly (Table 1) decreased during storage in all treatments, with the control ones showing a Hue Angle of 95.05 ± 0.43 h° at harvest and 92.15 ± 0.43 h° after 35 days of storage.

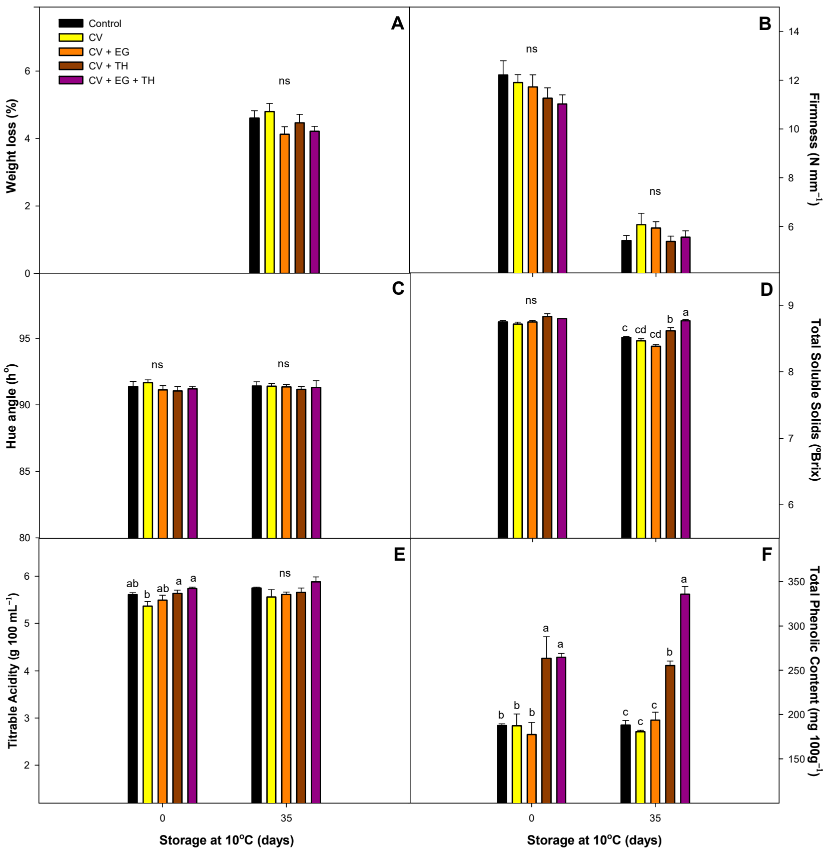


**Figure 3.** Effect of preharvest treatment with carvacrol at 100 µL/L (CV), carvacrol and eugenol at 100 µL/L (CV + EG), carvacrol and thymol at 100 and 500 µL/L (CV + TH), and carvacrol, eugenol, and thymol at 100, 100, and 500 µL/L (CV + EG + TH) in weight loss (**A**), firmness (**B**), Hue Angle (**C**), total soluble solids (**D**), titratable acidity (**E**) and total phenolic content (**F**) in 'Fino' lemons at harvest and 35 days of storage at 10 °C. Significant differences ($p < 0.05$) between treatments are presented with different lower-case letters. No significant differences are presented with 'ns'.

**Table 1.** F values resulted from the analysis of variance (ANOVA) in weight loss, firmness, Hue Angle, total soluble solids, titratable acidity, and total phenolic content datasets. Significant differences were presented with asterisks (*: *p*-value < 0.1, **: *p*-value < 0.05, ***: *p*-value < 0.01). When no significant differences were detected, 'ns' was included.

| Cultivar | Weight Loss | Firmness | Hue Angle | Total Soluble Solids | Titratable Acidity | Total Phenolic Content |
|---|---|---|---|---|---|---|
| 'Fino' cultivar | | | | | | |
| Time | - | 36.82 *** | ns | 155.06 *** | 4.00 ** | 3.94 * |
| Treatment | ns | ns | ns | 17.48 *** | 4.58 *** | 41.84 *** |
| Interaction Time × Treatment | - | ns | ns | 8.78 *** | ns | 4.00 *** |
| 'Verna' cultivar | | | | | | |
| Time | - | 22.66 *** | 65.56 *** | 90.82 *** | ns | 8.25 *** |
| Treatment | ns | ns | 2.03 * | 8.87 *** | ns | 3.64 ** |
| Interaction Time × Treatment | - | ns | ns | 5.91 ** | ns | ns |

TSS and TA were also studied and represented at harvest and after 35 days of storage (Figures 3D and 4D,E). The TSS of 'Fino' and 'Verna' control lemons at harvest were 8.75 ± 0.02 and 6.62 ± 0.04 °Brix, respectively (Figures 3D and 4D). Significant differences (Table 1) at harvest were detected between treatments in 'Fino' lemons, where CV + EG + TH-treated lemons presented a higher TSS content compared to the control ones. Their TSS for CV-, CV + EG-, CV + TH-, and CV + EG + TH-treated lemons were 8.47 ± 0.03, 8.38 ± 0.03, 8.62 ± 0.05, and 8.77 ± 0.02 °Brix, respectively. Moreover, TSS content was maintained during storage at 10 °C in 'Fino' lemons. However, in 'Verna' lemons, TSS significantly increased during cold storage. Control lemons showed at harvest 6.53 ± 0.02 °Brix and after 35 days of storage 6.82 ± 0.06 °Brix. Further, significant differences (Table 1) were detected for 'Verna' lemons between treatments in both sampling dates, showing CV + EG + TH-treated lemons to have a lower TSS content (6.53 ± 0.02 °Brix) than the control ones after 35 days of storage. Titratable acidity was maintained in both cultivars during the storage period (Figures 3E and 4E), except for the control 'Fino' lemons, which showed a slightly higher TA after 35 days of storage. Moreover, the presence of EOs did not affect the TA of the lemons.

The total phenolic content (TPC) was evaluated in the peel of 'Fino' and 'Verna' lemons (Figures 3F and 4F). In 'Fino' lemons, significant differences (Table 1) were detected in the TPC between treatments, with CV + TH and CV + EG + TH being higher than the rest of the treatments and the control lemons at harvest and after 35 days of storage. After 35 days of storage, 'Fino' lemons treated with CV + TH and CV + EG + TH presented values of TPC 255.27 ± 5.09 and 335.79 ± 8.65 mg 100 $g^{-1}$, respectively, compared to 188.20 ± 5.05 mg 100 $g^{-1}$ that were detected in the control ones. However, in 'Verna' lemons, these significant differences between treatments were only detected after 35 days of storage, and the TPC in lemons treated with CV + EG, CV + TH, and CV + EG + TH were significantly higher than in control ones (Table 1). Values of 272.17 ± 11.27 and 250.14 ± 10.67 mg 100 $g^{-1}$ were observed in the CV + TH- and CV + EG + TH-treated lemons after 35 days of storage, compared to the 227.97 ± 12.42 mg 100 $g^{-1}$ that was detected in the control lemons.

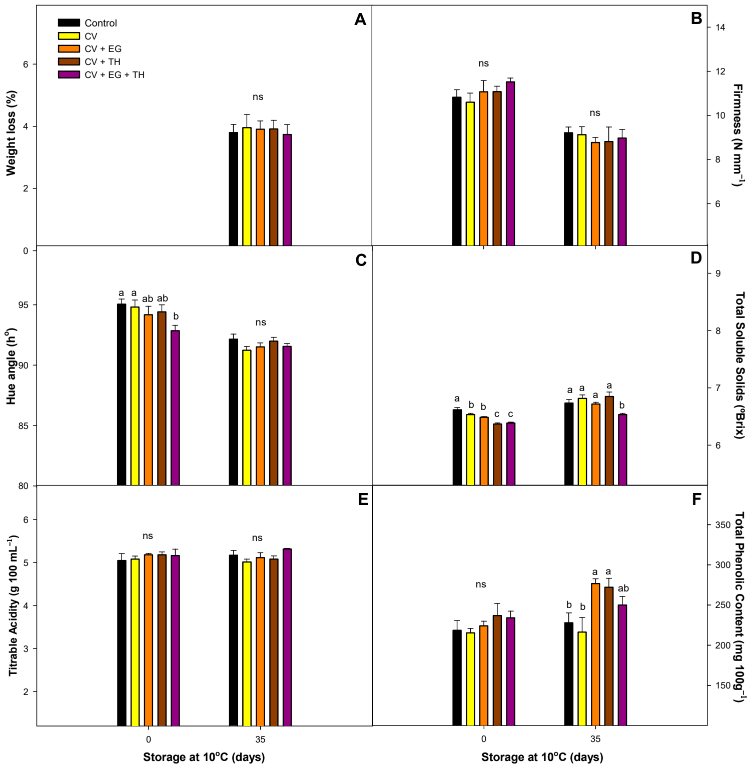


**Figure 4.** Effect of preharvest treatment with carvacrol at 100 μL/L (CV), carvacrol and eugenol at 100 μL/L (CV + EG), carvacrol and thymol at 100 and 500 μL/L (CV + TH), and carvacrol, eugenol, and thymol at 100, 100, and 500 μL/L (CV + EG + TH). Weight loss (**A**), firmness (**B**), Hue Angle (**C**), total soluble solids (**D**), titratable acidity (**E**), and total phenolic content (**F**) in 'Verna' lemons at harvest and after 35 days of storage at 10 °C. Significant differences ($p < 0.05$) between treatments are presented with different lower-case letters. No significant differences are presented with 'ns'.

## 4. Discussion

This is the first work on the effect of pure EOs applied in preharvest against fungal decay in citrus fruit. The preharvest application of the selected EOs individually and in combination has resulted in a significant reduction in the fungal decay of lemons through their storage period. In the first season, a reduction of 66, 56, and 39% of the decay incidence was achieved when 'Fino' lemon trees were treated individually with CV, EG, and TH at 100 μL/L, respectively. While when TH was applied at 500 μL/L, a 49% reduction in the decay incidence was achieved. Therefore, the selection of the treatments for the second season was the lowest concentrations of CV and EG and the intermediate concentration (500 μL/L) for TH. In the second season, a 65% reduction in the decay incidence was observed in 'Fino' CV-treated lemons, compared to a 40, 35, and 3.5% reduction when the combination of EOs was applied: CV + EG, CV + TH, and CV + EG + TH, respectively. A similar reduction was achieved in 'Verna' lemons, considering that these lemons usually rot less than 'Fino' lemons. This is due to the different physiological characteristics between both cultivars, with 'Verna' lemons having a stronger and thicker peel than 'Fino' fruits, as they are more resistant in general to fungal decay [44]. Although it has been demonstrated that the use of carvacrol, thymol, and eugenol presented a synergic effect in table grape, the concentrations applied were lower compared to that applied in the present study, and a different application method was used [45]. In the present study, the application of a combination of carvacrol, thymol, and eugenol at a higher final concentration (700 μL/L) could produce significant damages in the cellular membrane of the fruit, reducing fruit quality and increasing their susceptibility to fungal decay [46]. Moreover, a previous study observed a browning effect on the skin of apricots when a high concentration of EOs was applied [47]. The use of the combination of carvacrol, eugenol, and thymol in lower concentrations could also provide a higher reduction in the fungal decay incidence, although further studies are required.

Previous in vitro studies have already evaluated the antifungal activity of *Thymus* and *Oreganum* species EOs at different concentrations (50–4000 µL/L) against *P. digitatum, P. italicum,* and *G. citri-aurantii,* achieving the best results when these cultures were subjected to the highest concentrations [14,16–19,21,23–25,43]. Further, the antifungal effect of the studied EOs when they were applied in postharvest fruits has also been widely studied in citrus against *Penicillium* spp. [21,28], *Alternaria alternata* [27], and *G. citri-aurantii* [18,22], among others. In addition, previous studies have achieved a reduction in fungal decay after dipping postharvest lemons in a solution of wax, carvacrol, and thymol at different concentrations [21,30]. However, the best results were obtained at the highest concentrations (500 µL/L), while in the present study, the concentration providing the best results was carvacrol at 100 µL/L. These differences are due to the different times of application (pre- and postharvest) and the methodology applied (dipping and vapour phase). Previous studies have already demonstrated the differential effect of EOs based on the methodology of application [32,48,49]. Moreover, the dose dependence of EOs have been previously demonstrated in postharvest, where EOs were applied at different dosages and there was not an increasing tendency [50]. This is in agreement with the results obtained in the present study, where carvacrol and eugenol application was more effective as an antifungal treatment in the lowest dose (100 µL/L), while thymol was more effective at 500 µL/L. Therefore, the present study is the first one to evaluate the effect of EOs in preharvest to control the fungal decay of lemons.

Carvacrol was more effective at the lowest dose, and the EO treatment providing the highest reduction in fungal decay overall. It is known as a phenolic compound with a fungicidal effect as it interacts with cellular membranes. The presence of a hydroxyl group destabilizes the cytoplasmic membrane and could lead to cell death, which explains its antimicrobial effect [51–53]. Thymol and eugenol are also phenolic compounds with a similar mode of action to carvacrol. Thymol has been shown to delay the germ tube development of *A. alternata*, affecting conidial cell wall, plasma membrane, and cytoplasm disorganisation, resulting in organelle destruction. Therefore, a decreased efficiency of the pathogen infection was achieved and, consequently, the disease progression was reduced [54]. However, in the present study, the use of a combination of EOs did not have an effect on the reduction of fungal decay due to the high concentration of EOs applied, as previously mentioned. The application of a higher content of EOs can become phytotoxic to the lemons, reducing fruit quality and directly affecting the integrity of the cells and, therefore, becoming more susceptible to fungal decay [55]. In the present study, the EOs showed a preventive effect, significantly reducing the fungal incidence and increasing the total phenolic content in lemons. Although the mechanism of action of EOs is still not clear, this effect could be due to an increase in phenylalanine ammonia-lyase (PAL), an enzyme implicated in the phenylpropanoid pathway leading to the synthesis of phenolic compounds, and therefore their accumulation plays an important role in plant defence mechanisms [56,57].

Although this is the first study where EOs are applied in preharvest in citrus fruit to evaluate their antifungal effect during postharvest storage, there are a few previous studies where these EOs have been applied in preharvest but in different crops. These previous studies have also observed a reduction in fungal incidence after applying thyme oil and clove in tomatoes [33,58]. Conversely, another preharvest study in lettuce treated with clove did not show any fungal decay incidence reduction [35]. This difference could be due to the different nature of the pathogens involved in lettuce and their morphology, as well as the fact that it was the entire EO clove that was used, instead of the pure eugenol used in this work.

During postharvest storage, fruits suffer changes in quality, such as weight loss, colour, firmness, TSS, and TA [59]. In the present study, the physiological parameters of lemons at harvest and after 35 days of storage were in general not affected by the application of

EO treatments in preharvest. Colour changes were observed during the storage of 'Verna' lemons, while no changes were observed in 'Fino'. This is due to the non-homogeneous colour that 'Verna' lemons present when they are harvested, as they have not yet reached the final characteristic yellow colour. Therefore, the application of EOs in preharvest not only reduces the fungal decay, but also maintains the physiological quality of lemons during storage. However, these results are contradictory to those achieved when the EOs were applied in postharvest to lemons, where a reduction in weight loss was observed in the treated fruits [22]. Nevertheless, the time and method of application of the EOs affect the physiological quality of the fruit, as well as the fact that, in this study, an injury and subsequent inoculation with a fungal pathogen was carried out in the fruit. Indeed, a reduction in the metabolism of the treated lemons was observed [18], while in the present study, the respiration rate (data not presented) of lemons was not affected by the EO treatments. Moreover, in previous studies, the application of thymol and carvacrol in postharvest resulted in an increase in the firmness of the treated lemons [30] and oranges [27] after cold storage due to the relation of EOs and the reduction in the softening enzyme activity in the cell wall components [23]. However, in the present study, this increase in firmness was only observed in 'Fino' lemons treated in preharvest with 100 µL/L of carvacrol and not in the lemons treated with a combination of carvacrol and thymol. This difference could be due to the time of application, as well as the need for lower concentrations of EOs when the application is carried out in preharvest. Nevertheless, contradictory results were observed in postharvest studies in oranges, where no effect on firmness was observed in oranges coated with a thymol solution [25,26]. Total soluble solids and titratable acidity results were in accordance with Pérez-Alfonso et al. [21], where thymol and carvacrol were applied in postharvest to lemons and no changes were observed between the treated and control lemons.

As part of the antioxidant compounds of lemons, the phenolic content is essential. The phenolic content of lemons at harvest and after 35 days of cold storage only increased in those cases where the fruits were treated with a combination of EOs as follows; carvacrol and thymol, carvacrol and eugenol, and carvacrol, eugenol, and thymol. This increase in the phenolic content has already been reported in lemons [22], oranges [27,28], and mandarins [60] treated with EOs. Although the combination of the three EOs did not provide the best decay incidence, it was still lower than the fungal incidence presented in the control lemons. Therefore, the increase in the polyphenol concentration could be directly related to the antifungal capacity that the EOs are providing the lemons with, since polyphenolic compounds play an important role in their accumulation in vegetable tissues as a protection mechanism against biotic and abiotic stress [56]. Indeed, previous results in preharvest have shown an increase in the TPC of strawberries [32,38,39]. This increase has been associated with the fact that applying EOs to the surface of the fruits and leaves on the tree may induce a stressful environment, resulting in an increase in the shikimic acid and phenylpropanoid metabolism pathways, enhancing the defence system of the plant [61].

## 5. Conclusions

The application of EOs in preharvest could help reduce fungal decay, one of the main problems during the postharvest storage of lemons. In the present study, it was revealed that the use of carvacrol at 100 µL/L could be a potential alternative to the current synthetic fungicides used during the postharvest storage of lemons, maintaining their quality. Indeed, the use of higher concentrations of EOs or a mix of them could have the opposite effect on the lemons, causing them to become more susceptible to fungal decay. Therefore, the use of lower concentrations than in postharvest to achieve a similar effect will be a more viable solution from a technical and economical point of view. However, future work on the optimization of EO concentration, number, and timing of applications will be required, to reduce the stress that the fruit suffers in the presence of the preharvest treatment and to effectively control fungal incidence.

**Author Contributions:** Conceptualization, M.J.G. and P.J.Z.; methodology, M.G.-P. and M.J.G.; software, M.J.G.; validation, M.J.G., P.J.Z., V.S.-E. and M.G.-P.; investigation, M.G.-P., M.G.-B. and V.S.-E.; writing—original draft preparation, M.G.-P.; writing—review and editing, M.J.G., P.J.Z. and V.S.-E.; visualization, P.J.Z.; supervision, P.J.Z.; funding acquisition, P.J.Z. and M.J.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research did not receive external funding.

**Institutional Review Board Statement:** Not applicable.

**Data Availability Statement:** The data used to support the findings of this study are included within the article.

**Acknowledgments:** The authors thank BioRender (Toronto, ON, Canada) for providing the figures used as part of the Graphical Abstract.

**Conflicts of Interest:** The authors declare no conflict of interest.

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