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Essential Oils and Chitosan Applications to Protect Apples against Postharvest Diseases and to Extend Shelf Life

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Abstract: Apple fruits are susceptible to pathogenic fungi such as *Botrytis cinerea* and *Penicillium expansum* which are responsible for significant pre- and postharvest fruit losses. Given the strong restriction in the use of synthetic agrochemicals, especially during the postharvest phase, alternatives are currently sought for a more sustainable management of apple storage. The antifungal activity of thyme, clove, anise, camphor, and juniper essential oils (EOs) was evaluated with in vitro tests and the minimum inhibitory concentration (MIC) was determined. Thyme and anise EOs showed a MIC value of 0.5 and 1 mL L^{-1} against *B. cinerea*, respectively. A MIC of 0.5 mL L^{-1} was found for clove EO against *P. expansum*. Camphor and juniper EOs were found to be less effective. Although in those experiments the antifungal activity of EOs was proven, when EOs were applied in in vivo conditions they did not demonstrate the same effectiveness. In order to preserve EO inhibitory performances, edible coatings combining polysaccharide matrices (chitosan, arabic gum, and xanthan gum) with EOs were tested. After considering consistency, uniform coverage of the fruit surface, and antifungal properties of the matrix, chitosan was identified as the most suitable component for EO encapsulation. Treatments with chitosan in combination with thyme EO on apple fruits (cv. 'Braeburn') showed inhibitory effects on infection caused by *B. cinerea* (−48% of infected wounds compared to untreated). Similarly, clove EO combined with chitosan reduced the *Penicillium* infections in apple cv. 'Golden Delicious' by 62%. These results therefore suggest the effectiveness of the use of EOs encapsulated with chitosan for the control of postharvest diseases of apple fruits during storage.

Keywords: fruit storage; encapsulation; thyme; clove; edible coating; antifungal activity

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Citation: Soppelsa, S.; Van Hemelrijck, W.; Bylemans, D.; Andreotti, C. Essential Oils and Chitosan Applications to Protect Apples against Postharvest Diseases and to Extend Shelf Life. *Agronomy* **2023**, *13*, 822. https://doi.org/10.3390/agronomy13030822

Academic Editor: Pedro Javier Zapata

Received: 22 February 2023
Revised: 6 March 2023
Accepted: 9 March 2023
Published: 11 March 2023

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1. Introduction

Fruit quality decline and postharvest diseases represent major causes of economic losses for the horticultural sector. Considering the large number of different postharvest diseases, gray and blue mold caused by *Botrytis cinerea* Pers. and *Penicillium expansum* Link. are certainly among the most common and detrimental [1]. The postharvest application of synthetic fungicides is currently the standard technique to counter the spread of these pathogenic fungi during storage of conventionally produced fruits and vegetables [2]. In a recent study by EFSA (European Food Safety Authority), 1.4% of the food samples analyzed were found to have a residue concentration exceeding the maximum residue level (MRL), whereas 40.6% contained one or more pesticide residues below the MRL but above the limit of quantification for residues [3]. Consumers have an increasing awareness about aspects related to food quality and safety, including the health risks linked to pesticide residues in fruit and vegetables [4]. This awareness is leading toward an increased relevance of the organic agriculture sector, where organic food is perceived as healthier and safer by the consumers [5]. Scientific research regarding organic agriculture has been almost exclusively oriented towards preharvest management practices, whereas postharvest methods for organic products appear to have been neglected [6]. Physical methods

such as the modification of temperature, light, radiation, atmosphere, and pressure are consolidated practices to maintain the quality of organic fruits during the storage period [7]. A large proportion of losses and waste occurs after the storage period, during processing, packaging, and distribution (e.g., wholesalers, supermarkets) [8]. Extending the shelf life during these phases can lead to the reduction in fruit losses, while preserving fruit quality, flavor, and texture [9]. The use of edible coatings, which allows the creation of a physical barrier around the fruits by means of natural additives, can be considered as a valuable technique to extend the shelf life of organic fruits [6,10,11]. Among the natural substances able to be used against fruit postharvest decay, essential oils (EOs) have gained interest in recent years due to their antimicrobial, antibacterial, antiviral, biodegradable, and eco-friendly properties [12–15]. In more detail, the EO's action against postharvest fungi can be generally detected at the level of the fungal cell membrane (disruption of the cell membrane integrity) or at the level of the fungal inner cell metabolism (dysfunction of the fungal mitochondria, inhibition of efflux pumps) [16]. EOs often have a complex composition containing terpenes (monoterpenes, sesquiterpenes, diterpenes, norterpenes), phenylpropanoids, and sulfur and nitrogen compounds [17]. This complexity generally prevents the development of resistance by pathogens [18]. The broad range of compounds found in EOs is linked both to the plant genetic origin and to the organs (e.g., leaves, roots, flowers, fruits, seeds) used for the EO's extraction. One of the most studied EOs is that extracted from the leaves and flowers of thyme plant (*Thymus vulgaris* L., Fam. *Lamiaceae*). This EO was shown to have an inhibitory activity in in vitro tests against several fungi such as *B. cinerea*, *P. expansum*, and *P. digitatum* Pers. [19–21]. Another highly effective EO is that extracted from the leaves, stem, and buds of the clove plant, *Syzygium aromaticum* L. or *Eugenia caryophyllata* Thumb. (Fam. *Myrtaceae*) [22]. The application of clove EO, or its main component (eugenol), has been shown to have a strong antimicrobial activity as demonstrated in some in vitro assays against several fungi such as *B. cinerea*, *P. expansum*, *P. digitatum*, *P. italicum* Wehmer, and *Monilia fructigena* Pers. [23–25]. Although interesting evidence has been obtained in in vitro tests, the application of EOs to extend product shelf life is still limited due to high costs, potential toxicity, and changes in organoleptic properties of the fruits [26–28]. Moreover, antimicrobial properties of EOs appear significantly reduced under in vivo conditions because of their physical properties (e.g., some components are highly volatile) and the high concentrations needed to obtain a satisfactory antimicrobial activity [14,29,30]. In order to tackle the problems related to the in vivo postharvest application of EOs, the encapsulation technique is seen as a valid strategy to minimize the required quantities of EOs, thus reducing costs and undesired effects [28]. The encapsulation of EOs results in the reduction in volatility, immiscibility in water, oxidation, and light-induced reactions, thus promoting the permanence of the oil on the treated surface [31]. Thanks to this technique, small particles (i.e., small oil droplets) are enclosed by a coating (a matrix), forming a capsule [31]. Common matrix components for edible coatings are polysaccharides (e.g., chitosan), proteins, lipids, and resins. In addition, plasticizers and surfactants are often included to increase the flexibility and the stability of the final formulation [32].

With this study we aimed to (i) identify those EOs with the highest in vitro antimicrobial efficacy against *B. cinerea* and *P. expansum*; (ii) test different coatings (e.g., chitosan, arabic gum, xanthan gum) for their efficiency as encapsulating matrices of selected EOs; and (iii) assess the effects of EOs alone or encapsulated to control postharvest pathogenic fungi in vivo on apple fruits.

2. Materials and Methods

2.1. Isolates of Pathogens and Apple Fruits

Two pathogen species, *Botrytis cinerea* (PCF 1104 and PCF 2144) and *Penicillium expansum* (PCF 187), were isolated from infected apples, following the methodology described in Daniel et al. [33]. Apple fruits (*Malus domestica* Borkh.) of the cultivars 'Golden Delicious' (susceptible to *P. expansum*) and 'Braeburn' (susceptible to *B. cinerea*) were used for the in vivo assays. Apples were harvested in 2017 from the apple orchards at the Pcfruit npo

research center (50°46′22.05″ N, 5° 9′37.51″ E) and afterward stored for five months in refrigerated conditions (2 ± 1 °C temperature; 90 ± 5% relative humidity) without any postharvest treatment. Apples selected for in vivo assays were characterized by their uniform maturation stage, fruit size, and absence of visible defects (Table S1, Supplementary Material).

2.2. Essential Oils (EOs) and Edible Coatings

Details about EOs and coating products used in the experiments are reported in Table 1.

Table 1. Characteristics of products used in the experiments.

Substance	Origin	Description	Commercial Name
Thyme EO	*Thymus vulgaris* (Fam. Lamiaceae)	EO obtained after distillation of thyme leaves/flowers (main component: thymol)	Essential oil, Vitalis Dr. Joseph s.r.l., Italy
Juniper EO	*Juniperus communis* (Fam. Cupressaceae)	EO obtained after distillation of juniper berries (main component: α-pinene)	Essential oil, Vitalis Dr. Joseph s.r.l., Italy
Clove EO	*Syzygium aromaticum* (Fam. Myrtaceae)	EO obtained after distillation of clove flower buds (main component: eugenol)	Essential oil, Vitalis Dr. Joseph s.r.l., Italy
Camphor EO	*Cinnamomum camphora* (Fam. Lauraceae)	EO obtained after distillation of wood/leaves (main component: camphor)	Essential oil, Vitalis Dr. Joseph s.r.l., Italy
Anise EO	*Pimpinella anisum* (Fam. Apiaceae)	EO obtained after distillation of fruits (main component: anethole)	Essential oil, Vitalis Dr. Joseph s.r.l., Italy
Chitosan	Crustaceans shell	Obtained from chitin, component of the shells of crustaceans (main component: polysaccharides)	Molekula Ltd., Shaftesbury, Dorset, UK
Arabic gum	*Acacia* tree	Obtained from the sap of the acacia trees (main component: polysaccharides)	MP Biomedicals, LLC. Solon, OH, USA
Xanthan gum	*Xanthomonas campestris*	Obtained after fermentation of sugars (i.e., sucrose) by *X. campestris* (main components: polysaccharides)	Doves Farm Foods Ltd. Company, Berkshire, UK

2.3. Experiment 1—In Vitro Tests

2.3.1. Spore Germination Assay

Different concentrations of all the EOs listed in Table 1 (0.05, 0.1, 0.5, 1, 5 mL L^{-1}) were tested in vitro against *B. cinerea* and *P. expansum.* The surfactant Tween 20 was added to each treatment and tested alone at a concentration of 0.1% (*v*/*v*). Fungal spore suspensions were prepared by transferring pathogen spores into Potato-Dextrose-Broth (PDB) medium. The concentration was quantified with a hemocytometer (Bürker-Türk, Marienfeld, Germany) and adjusted to 10^5 conidia mL^{-1}. Experiments were carried out in a 96-well plate. In each well, 100 µL of conidial suspensions was mixed with 100 µL of EO products at different concentrations. Afterwards, the plate was incubated at 24 °C for 24 h. Germination was determined by observing conidia under a light microscope (Supplementary Material, Figures S1 and S2). Each treatment was replicated 4 times and 50 conidia were counted per replicate. Results were reported as percentage of spores that did not germinate, determined as follows:

$$Spore\ not\ germinated\ (\%) = \frac{number\ of\ spores\ not\ germinated}{total\ number\ of\ spores\ evaluated} \times 100 \quad (1)$$

The minimum inhibitory concentration (MIC), corresponding to the lowest concentration of essential oil for a total inhibition of conidial germination, was determined. To allow the comparison of spore germination with a synthetic fungicide, a solution of cyprodinil and fludioxonil (Switch®, Syngenta Crop Protection, Belgium) was used at 0.0005 and 0.001 mL L^{-1} concentrations. The protocol also included a further treatment (control), without any EO or surfactant application.

2.3.2. Mycelial Growth Assay

Potato-Dextrose-Agar (PDA) was autoclaved and cooled in a water bath (until 40 °C). Afterward, different concentrations (0.1, 0.5, 1 mL L^{-1}) of essential oils were incorporated into the sterile PDA. The PDA medium was also integrated with 0.1% (v/v) Tween 20 to enhance essential oil solubility. The mixtures were poured into Petri plates (20 mL per plate), and after the solidification of the medium, two small PDA squares (4 mm × 4 mm) obtained from one-week old fungal culture (see Section 2.1) were placed in each Petri dishes as inoculum sources (four replications for each tested products and concentration). The plates were incubated at 24 °C in the dark for 3 days. The synthetic fungicide Switch® (Syngenta Crop Protection, Belgium) was used as positive control at different concentrations (0.0005, 0.001, and 0.002 mL L^{-1}).

The antifungal efficacy was expressed as percentage of mycelial growth inhibition (MGI) compared with the control, according to the following formula:

$$MGI(\%) = \frac{(C - T)}{C} \times 100 \quad (2)$$

where C and T represent the fungal colony diametral grown in control or treated Petri dishes, respectively [34]. Finally, the minimum inhibitory concentration (MIC), defined as the lowest concentration of essential oil for no visible pathogen growth, was also determined.

2.4. Experiment 2—In Vivo Tests

2.4.1. Preparation of Polysaccharide Matrices and EO Encapsulation

Three polysaccharides (chitosan, arabic gum, and xanthan gum) were tested as encapsulation matrix and prepared as described by Perdones et al. [35], Maqbool et al. [36], and Sharma and Rao [37] with some modifications. In more detail, chitosan (1% w/v) was dispersed in an aqueous solution of glacial acetic acid (0.5% v/w) for 12 h with a magnetic stirrer. The arabic gum (AG) was prepared by mixing 10 g of AG powder in 100 mL of distilled water. The solution was stirred for 3 h at 40 °C with a magnetic stirrer. The xanthan gum (XG) was prepared by dissolving 0.5 g XG powder in 100 mL of distilled water at 40 °C under magnetic stirring for 3 h. Afterwards, glycerol as plasticizer (0.5% w/v), Tween 20 as surfactant (0. 1% v/v), and $CaCl_2$ as cross-linker agent (0.2% w/v) were added to each matrix [32,38] and the mixtures stirred for 2 additional hours at room temperature in order to obtain the three different homogenous coating solutions. EO (0.2% v/v) was incorporated into the solutions using a homogenizer (Precellys® 24 Tissue Homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France) at 6800 rpm for 3 cycles of 30 s.

2.4.2. Preparatory Assay: Method of Application (Spraying or Dipping)

The apple fruit surface was first sterilized with 5% sodium hypochlorite solution (NaOCl) for 2 min, then rinsed in water for 2 min and dried at room temperature. Following the preliminary indications of experiment 1, thyme and clove EOs (0.2% v/v concentration) were tested in combination with edible coatings (chitosan, arabic gum, and xanthan gum) against gray mold (thyme EO) and blue mold (clove EO). Treatments were performed either by spraying or dipping the fruits, following the methodology described below:

- EO spray. Apple fruits were wounded at the equatorial region with a sterile tip (3 mm diameter × 3 mm deep, two wounds $fruit^{-1}$). Fruits were afterward treated (see Section 2.4.1) by spraying the products on the apple surface. After 2 h, fruits were inoculated by laying on each wound 10 µL of a conidial suspension (10^5 conidia mL^{-1}

for *B. cinerea* on 'Braeburn' apples and 10^3 conidia mL^{-1} for *P. expansum* on 'Golden Delicious' apples).

- EO dipping. Apple fruits were wounded as described above. Fruits were then dipped in product solutions (see Section 2.4.1) for 1 min (30 sec for fungicide control). After 2 h, fruits were inoculated as described above.
- Fungicide spray or dipping. Apple fruits were wounded as described above. Fruits were then sprayed or dipped in a solution of the synthetic fungicide Switch® (0.05% *w*/*v*) and after 2 h inoculated as above.
- Control. Apples were wounded, treated with water, and then inoculated.

The protocol also included untreated fruits inoculated with water to exclude any unwanted pathogen contamination. After treatments, apples were kept in a climate chamber at 20 °C and 100% RH to allow pathogen germination. The infection incidence was assessed at 3, 5, and 7 days after inoculation and data were expressed as inhibition rate, calculated as:

$$Inhibition\ rate(\%) = \frac{(C - T)}{C} \times 100 \quad (3)$$

where C and T are the percentage of infected wounds in control or treated fruits, respectively. For the preparatory assay (screening test), 3 replications, each consisting of 8 apple fruits per treatment, were used.

2.4.3. Extended Assay: Fruit Dipping with EOs and Chitosan as Coating Matrix

Based on the outcomes of the previously described preparatory assay, chitosan was identified as the most suitable edible coating. This polysaccharide (1% *w*/*v*) was combined with thyme and clove EO (0.5% *v*/*v*) against *B. cinerea* and *P. expansum*, respectively. Moreover, chitosan, thyme EO, and clove EO alone were tested in order to verify the effectiveness of individual components. Inoculation (*B. cinerea* on 'Braeburn' and *P. expansum* on 'Golden Delicious') and dipping treatments were performed as described in Section 2.4.2. After treatments, apples were kept in a climate chamber at 20 °C and 100% RH to allow pathogen germination and development. Disease incidence and development was assessed at 3, 5, and 7 days after inoculation (monitoring was extended to 11 days for *B. cinerea* only) (Figures S3 and S4). Assessments consisted of visual observations of rotting and were expressed as follows:

$$Infected\ wounds\ (\%) = \frac{number\ of\ infected\ wounds}{total\ number\ of\ wounds} \times 100 \quad (4)$$

Moreover, the disease severity was determined by measuring the diametric length of the symptomatic spot. For the extended assay, 4 replications, each consisting of 24 apple fruits per treatment, were used.

2.5. Statistical Analysis

Data normality was examined with the Shapiro–Wilk test and homogeneity of variance was evaluated using Fligner–Killeen's test. Differences among treatments were assessed through the analysis of variance (ANOVA) followed by the Least Significant Differences (LSD) post hoc test ($p < 0.05$). Data expressed in percentage were arcsine-transformed prior to the application of the ANOVA. When assumptions of ANOVA were not met, the Kruskal–Wallis test was applied. All analyses were carried out in R v. 3.3.1. (R Development Core Team 2016). Values were expressed as mean ± standard error (SE).

3. Results and Discussion

3.1. Experiment 1—In Vitro Tests

Thyme and clove EOs were the most effective in reducing the percentage of germinated spores (Figure 1A,B, Figures S1 and S2). The minimum inhibitory concentration (MIC) against *B. cinerea* was observed at 0.5, 0.5, and 1 mL L^{-1} for thyme, clove, and anise

EOs, respectively (Figure 1A). Similar concentration ranges of thyme, clove and anise EOs were effective in inhibiting *B. cinerea* under both in vitro and in vivo conditions [39,40]. Regarding *P. expansum*, clove, thyme, and anise EOs were able to inhibit spore germination at concentrations of 1, 1, and 5 mL L^{-1} respectively, with an efficacy that was not significantly different from that of the chemical control with fungicide at 0.001 mL L^{-1} (Figure 1B). Clove and thyme EOs were partially effective (60% MIC) against *P. digitatum* (causal agent of *Citrus* decay) at 0.5 mL L^{-1} [41]. Monoterpenes (carvacrol, thymol, and camphor), which are present in thyme extracts, may be responsible for EO microbial inhibition by interfering with fungal cell wall enzymatic metabolism [12,42]. Similarly, eugenol, a component of clove EO, was able to reduce cell wall functionality in fungi (*Candida albicans*), therefore inhibiting mycelial growth [43]. Spore germination was only partially inhibited by juniper and camphor EOs, even when the highest concentration was applied (5 mL L^{-1}) (Figure 1A,B). In other tests conducted on juniper EO against *Alternaria alternata* (Fr.) Keissler, *Aspergillus versicolor* Tiraboschi, and *P. funiculosum*, higher MICs (above 10 mL L^{-1}) were effective, suggesting that the concentration range we tested was still too low to be able to inhibit spore germination [44].

The most effective EO in reducing *B. cinerea* mycelial growth was thyme. In more detail, incorporation of thyme EO at concentrations of 0.5 and 1 mL L^{-1} into the PDA medium resulted in full inhibition against *B. cinerea* (Figures 2A and S3). Similarly, thyme EO, encapsulated in a chitosan-based film, was found to be effective in preventing avocado soft rot caused by *Clonostachys rosea*, while maintaining fruit firmness and the overall nutritional quality [45]. EO from anise showed an efficacy comparable to that observed with synthetic fungicide, but only at 1 mL L^{-1}. Even at high dosage (1 mL L^{-1}), clove EO was not able to provide a complete inhibition against gray mold (MGI between 70–90%). The most effective EO in reducing *P. expansum* mycelial growth was clove. In detail, concentrations of 0.5 and 1 mL L^{-1} of clove EO were effective in inhibiting mycelial growth in a way that was not significantly different from that of the tested fungicide (Figures 2B and S4). In contrast, the inhibitory activity of EOs from thyme and anise was rather low when low concentrations (0.5 and 0.1 mL L^{-1}) were used. Juniper and camphor EOs showed the lowest MGI rate as compared to the other treatments, therefore confirming the low inhibitory effect shown in the spore germination test. As a general consideration, for some EOs (thyme, clove, anise) concentrations of at least 0.5 mL L^{-1} appeared to be required in order to determine inhibitory effects on fungal growth, whereas for other EOs (juniper and camphor) higher concentrations are probably needed to provide a certain control over fungal development.

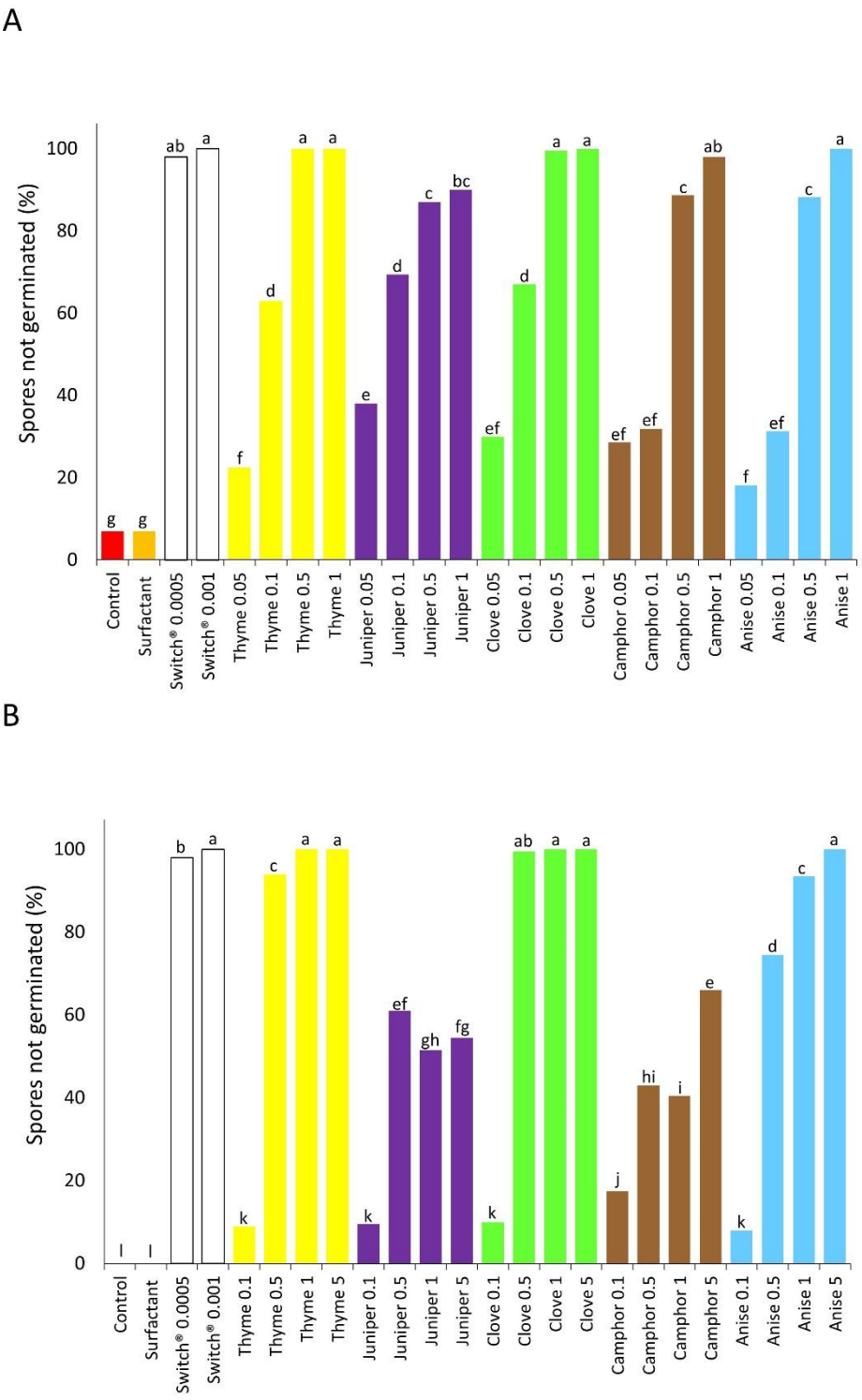


Figure 1. Spore germination assay in vitro. Percentage of spores not germinated (*Botrytis cinerea*—panel (**A**) and *Penicillium expansum*—panel (**B**)). Spores were evaluated after 24 h at 24 °C. Different letters in each picture mean significant differences by LSD test ($p < 0.05$). n = 4; The concentrations are expressed in mL L^{-1}.

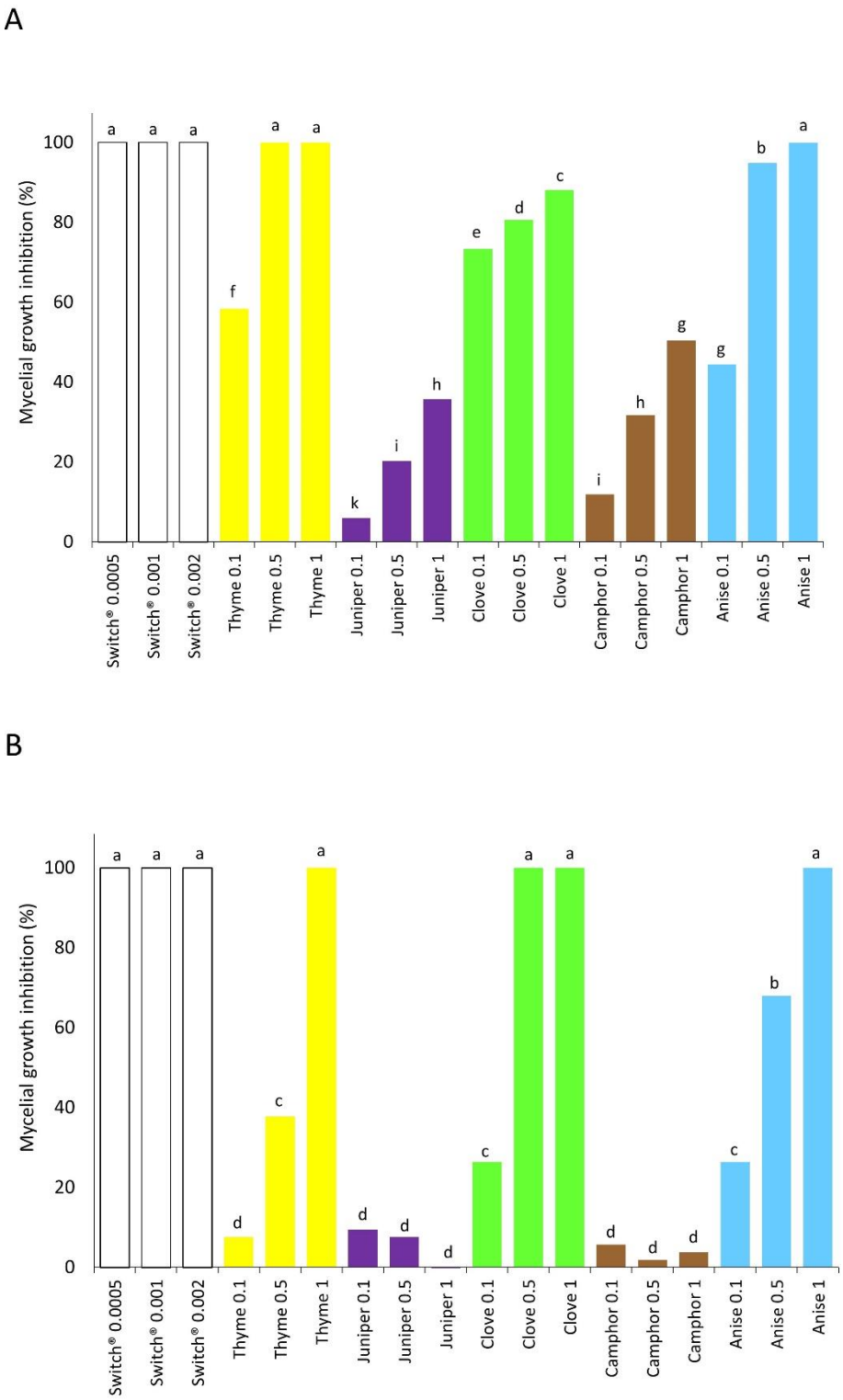


Figure 2. Mycelial growth assay in vitro. Percentage of inhibition of mycelial growth (MGI) of *Botrytis cinerea* (panel (**A**)) and *Penicillium expansum* (panel (**B**)). Colony radius was measured after 3 days at 24 °C. Different letters in each picture mean significant differences by LSD test ($p < 0.05$). n = 4; The concentrations are expressed in mL L^{-1}.

3.2. Experiment 2—In Vivo Tests

The in vitro tests enabled identification of the most effective EO against *B. cinerea* (thyme EO) and *P. expansum* (clove EO). The in vivo screening test enabled identification of the EO dipping application method as being more effective in controlling the growth

of *B. cinerea* and *P. expansum* compared to the spray application (Figures 3 and 4). In more detail, the application of thyme coated with chitosan resulted in a *B. cinerea* inhibition rate of around 80%, which was not statistically different from that obtained with the fungicide, 5 and 7 days after inoculation (Figure 3A). The arabic and the xanthan gum used as coating agents reduced the thyme EO efficacy in inhibiting fungal infection by 20–40% as compared to the combination of chitosan plus thyme when applied by dipping, whereas differences were not significant when spray applications were compared (Figure 3B). Dipping applications of chitosan and arabic gum with clove EO were able to provide a high fungal (*P. expansum*) inhibition after 7 days from inoculum (80–90% inhibition rate, which was not significantly different from that of the fungicide) (Figure 4A). Spray application of chitosan + clove EO reduced the inhibition rate to approximately 40%, whereas spray application with the arabic gum as the coating maintained a high percentage of fungal inhibition (around 70%), even after 7 days from inoculum (Figure 4B). The use of xanthan gum, independently from the method of application, was generally combined with a lower inhibition rate after both *B. cinerea* (Figure 3) and *P. expansum* (Figure 4) inoculation. The antifungal property of chitosan, used alone or in combination with essential oils, has been well studied during the storage of different fruit species [35,42,46–49]. Moreover, chitosan-based edible coatings containing essential oils from grape and sea buckthorn seeds prolonged shelf life of fresh-cut organic apple and strawberry slices [50]. Chitosan can interfere with the phospholipid components of the cell wall membrane, causing inhibitory effects on fungal RNA and protein cellular metabolism [51,52]. Arabic and xanthan gum were also tested as coating agents with essential oils to prevent postharvest fruit decay. Anthracnose disease in banana and papaya fruits during storage was significantly reduced after the application of cinnamon oil (4%) combined with arabic gum at 10% *w*/*v* [36]. Moreover, the use of cinnamon EO (when applied through either fumigation or spray), reduced gray mold disease in table grape, probably as a consequence of the antimicrobial activity given by antioxidant enzymes (i.e., peroxidase and polyphenoloxidase) obtained after the EO treatment [53,54]. Similarly, peppermint essential oil in combination with xanthan gum was effective in extending strawberry shelf life by preserving fruit quality and limiting postharvest decay [55].

Given the indications provided by the screening test, chitosan was chosen as the coating agent of EOs to be tested with the dipping method of application on apples 'Braeburn' and 'Golden Delicious', against *B. cinerea* and *P. expansum* infection, respectively. After 11 days from inoculum with *B. cinerea*, around 33% of wounds of 'Braeburn' apples showed infection symptoms (Figures 5A and S5). Dipping treatment with thyme EO alone was not able to significantly reduce disease incidence (percentage of infected wounds) during the monitoring period. Chitosan alone reduced the disease incidence only until 5 days from inoculum, whereas differences with inoculated control were not significant from 7 to 11 days after infection. The combination of the thyme EO and chitosan significantly reduced the percentage of infected wounds (around 20% after 11 days from inoculum), even though apples treated with the fungicide (Switch®) showed the lowest disease incidence, with values close to zero (no infected wounds) for the whole experiment duration (Figure 5A). Lesion average dimension progressively increased with time, reaching higher values in the control-inoculated apples (4 cm lesion diameter after 11 days, Figure 5B). Application of chitosan alone or in combination with thyme EO significantly reduced lesion severity as compared to control, whereas the thyme EO showed no severity reduction.

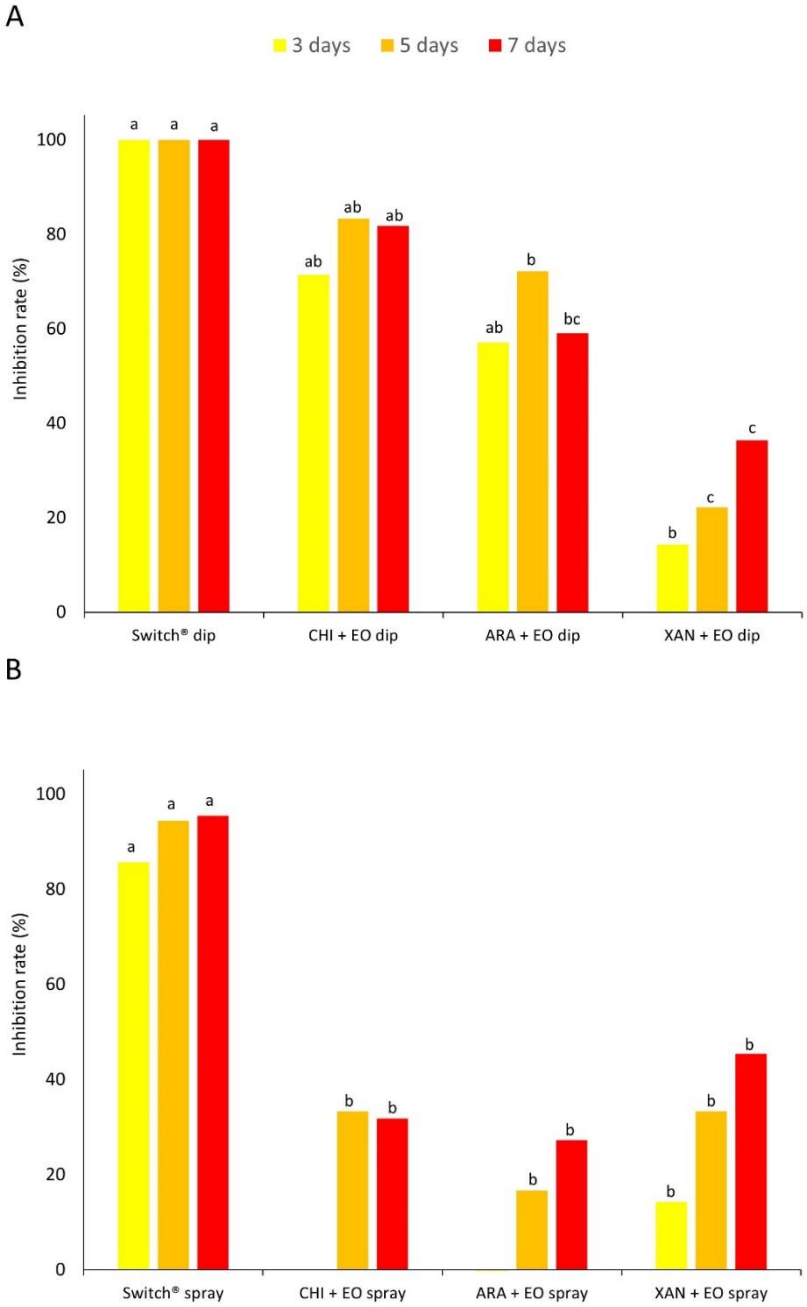


Figure 3. Preparatory assay in vivo: comparison between methods of application. Antifungal activity of thyme essential oil (EO) in combination with chitosan (CHI), arabic gum (ARA), xanthan gum (XAN) on apples cv. Braeburn. Data are expressed as percentage of inhibition rate in comparison to untreated control, after dipping treatment (panel (**A**)) or spraying treatment (panel (**B**)) at 3, 5, and 7 days from inoculation with *B. cinerea*. Different letters for each evaluation period mean significant differences by LSD test ($p < 0.05$). n = 3.

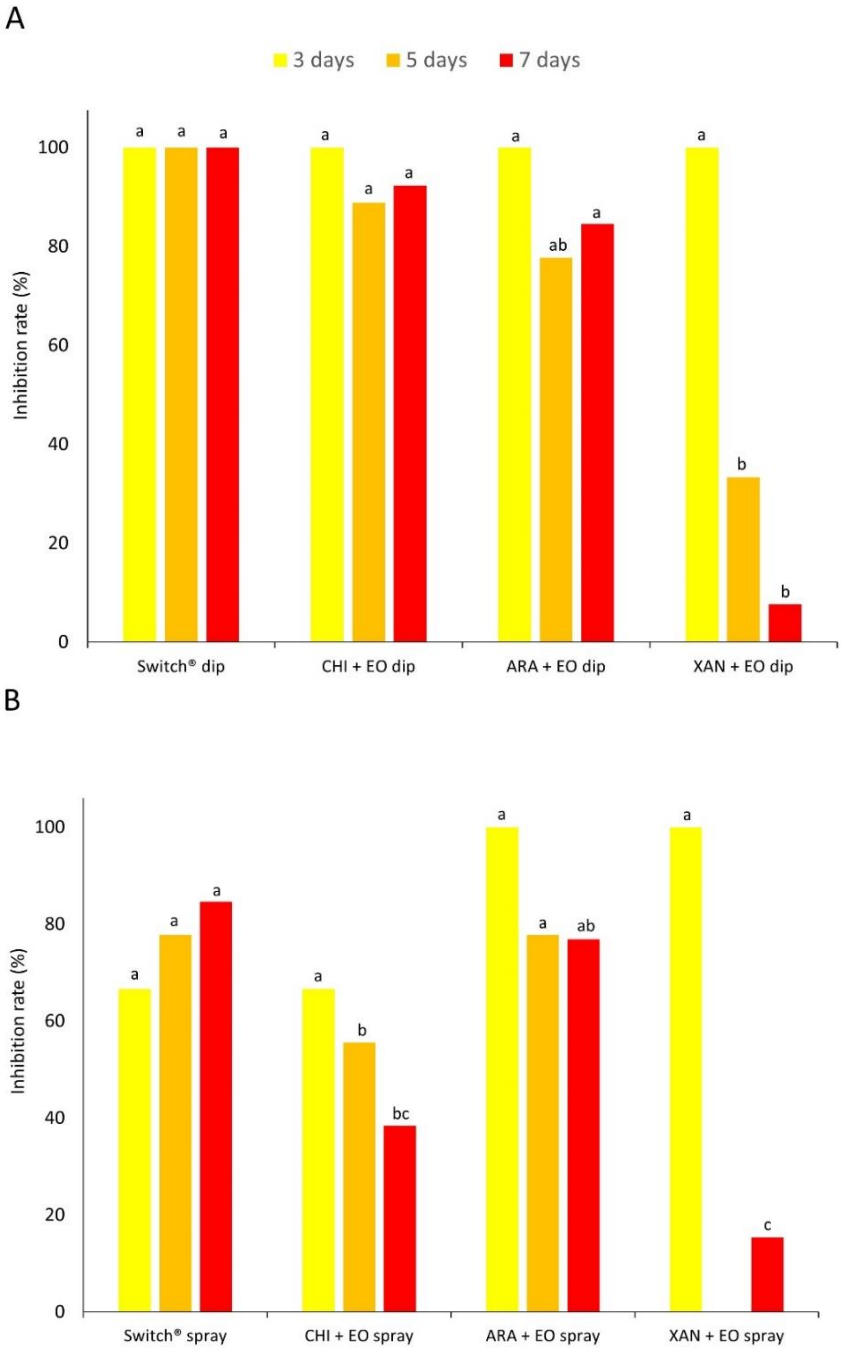


Figure 4. Preparatory assay in vivo: comparison between methods of application. Antifungal activity of clove essential oil (EO) in combination with chitosan (CHI), arabic gum (ARA), xanthan gum (XAN) on apples cv. Golden Delicious. Data are expressed as percentage of inhibition rate in comparison to untreated control, after dipping treatment (panel (**A**)) or spraying treatment (panel (**B**)) at 3, 5, and 7 days from inoculation with *P. expansum*. Different letters for each evaluation period mean significant differences by LSD test ($p < 0.05$). n = 3.

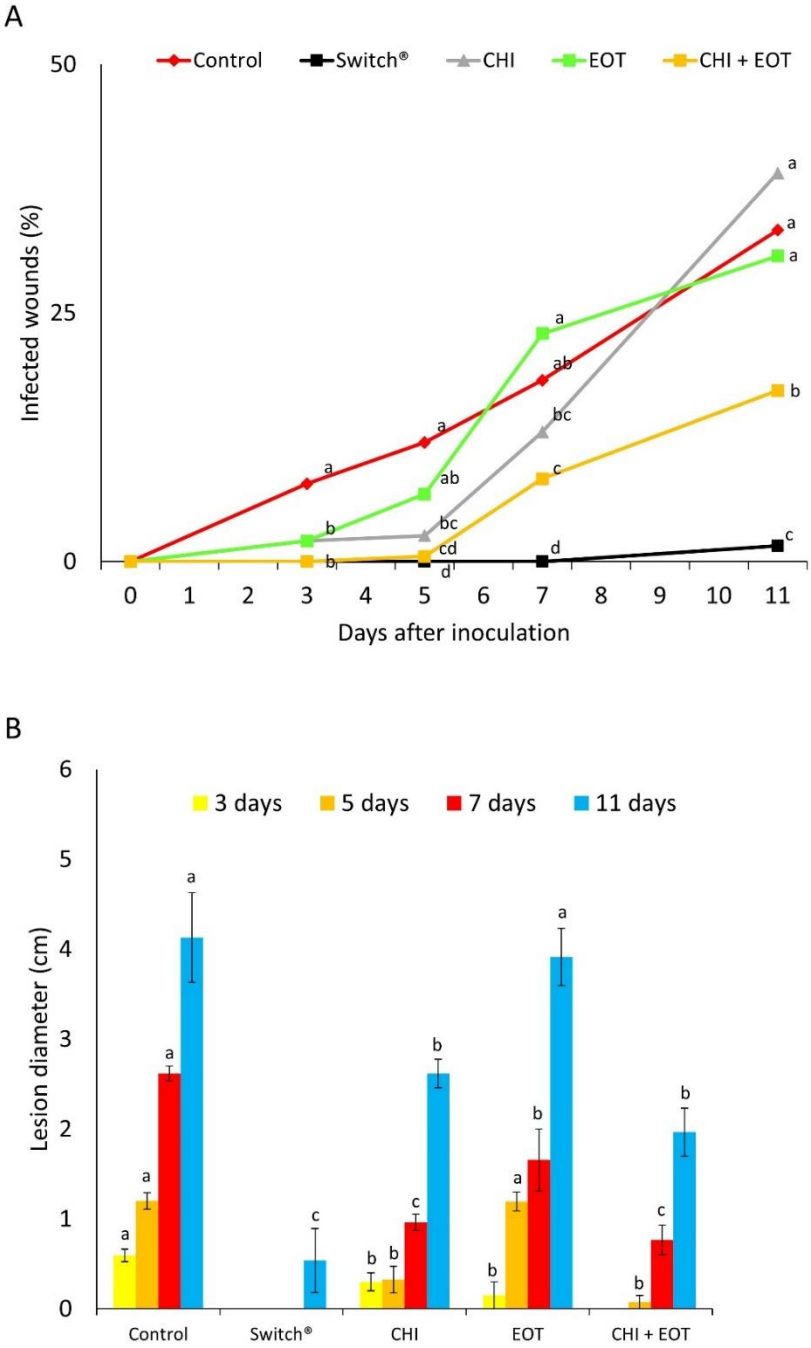


Figure 5. Extended assay in vivo: fruit dipping with EOs and chitosan as coating matrix. Antifungal activity of chitosan alone (CHI), thyme EO alone (EOT), or in combination (CHI + EOT). Data are expressed as percentage of infected wounds (panel (**A**)) and mean lesion diameter (panel (**B**)) at 3, 5, 7, 11 days from inoculation with *B. cinerea* on Braeburn apple fruits. Different letters for each evaluation period mean significant differences by LSD test ($p < 0.05$). Vertical bars indicate mean ± SE, n = 4. Water-inoculated apples showed no contaminations (data not shown).

'Golden Delicious' apples inoculated with *P. expansum* showed symptoms in around 80% of the infected wounds (Figures 6A and S6). Both chitosan and clove EO alone were partially effective in reducing disease incidence, even though their combination provided an even higher protection (around 25% of infected wounds after 7 days). As in the 'Braeburn'–*B. cinerea* combination, dipping 'Golden Delicious' apples in a solution containing the fungicide before the inoculation resulted in the most complete disease inhibition (Figure 6A), the percentage of infected wounds being below 5% after 7 days

from inoculum. None of the tested treatments reduced the disease severity measured at 5 and 7 days after the inoculum, and the final lesion length was around 1.5 cm on average (Figure 6B).

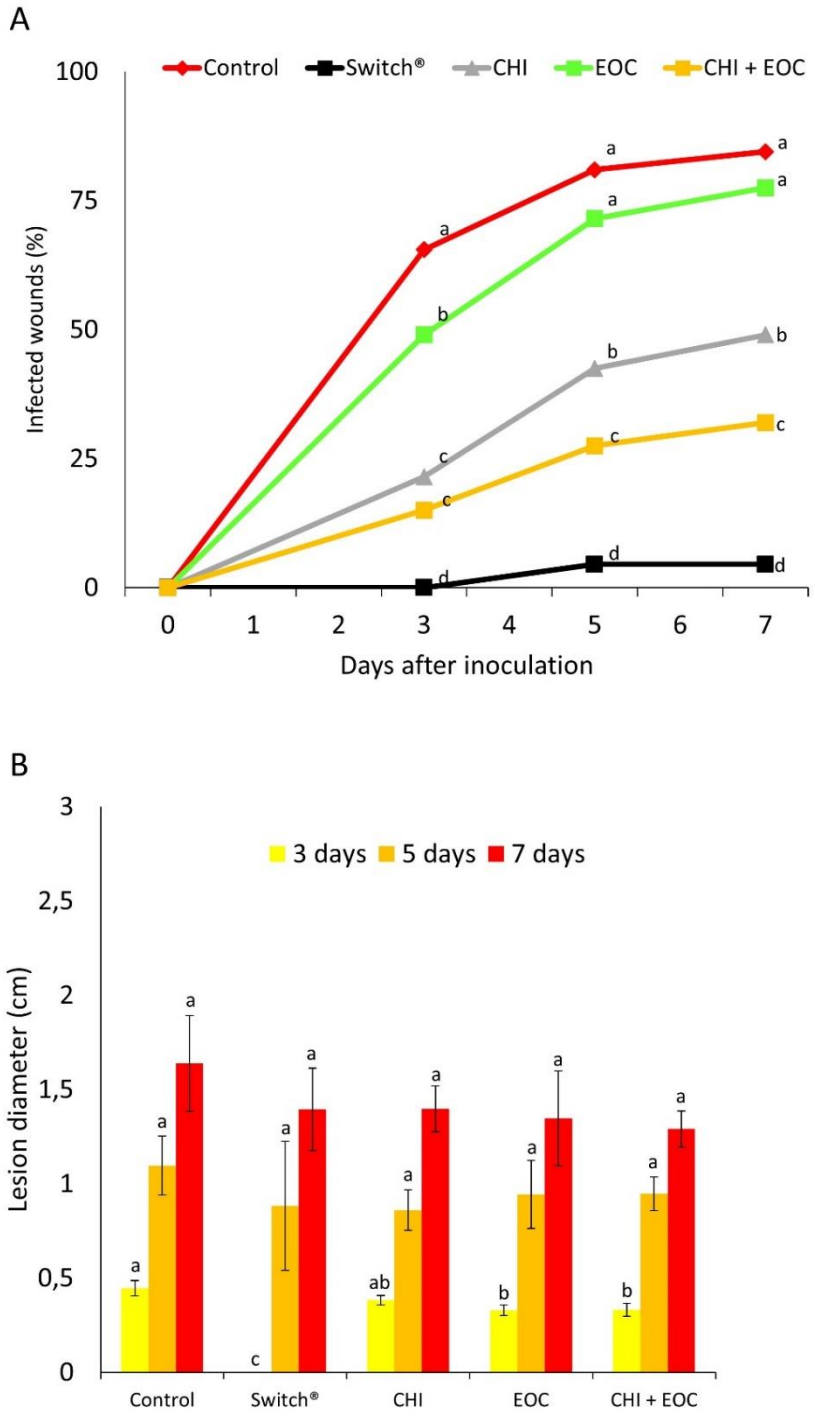


Figure 6. Extended assay in vivo: fruit dipping with EOs and chitosan as coating matrix. Antifungal activity of chitosan alone (CHI), clove EO alone (EOT), or in combination (CHI + EOT). Data are expressed as percentage of infected wounds (panel (**A**)) and mean lesion diameter (panel (**B**)) at 3, 5, 7 days from inoculation with *P. expansum* on Golden Delicious apple fruits. Different letters for each evaluation period mean significant differences by LSD test ($p < 0.05$). Vertical bars indicate mean ± SE, n = 4. Water-inoculated apples showed no contaminations (data not shown).

The combined formulation with the essential oil and chitosan generally proved to be more effective in limiting disease incidence than the single component alone (Figures 5 and 6). The application of the sole essential oil (thyme in the case of 'Braeburn' and clove for 'Golden Delicious') showed a transient effect of disease inhibition, which was detectable only until the third day after inoculation in both EO/cultivar combinations (Figures 5A and 6A). This finding contrasts with that reported by Lopez-Reyes et al. [56], who observed a gray mold control that was extended for 15 days in 'Golden Delicious' apples treated with thyme essential oil at 10% concentration. By comparison, clove essential oil at 1% concentration was ineffective in reducing the incidence of blue mold (*P. expansum*) in 'Golden Delicious' apples [33]. These contrasting findings clearly show that the efficacy of EO applications is dependent on several factors, including the genotype involved (of both pathogen and host), the means of application (alone or in combination with coating ingredients), and the components' concentrations.

4. Conclusions

The purpose of this study was to determine the in vitro and in vivo antimicrobial activity of selected essential oils and to evaluate if their encapsulation in an edible coating matrix can enhance their efficacy to extend the shelf life of apple fruits. The results of this investigation show that thyme and clove essential oils are effective against *B. cinerea* and *P. expansum* in vitro, whereas their fungal inhibitory effect is reduced under in vivo conditions. Essential oil encapsulation with polymeric matrixes (i.e., chitosan) proved to be particularly effective in reducing the postharvest disease incidence and severity of two important apple cultivars ('Golden Delicious' and 'Braeburn'). These findings suggest that encapsulated essential oils may become an interesting tool for the postharvest management of fruits, particularly when no synthetic means (pesticides) are allowed (e.g., in the organic fruit production industry). Despite these valuable outcomes, the economical sustainability of the use of these natural products, as well as their potential detrimental effect on fruit aroma and taste, must be further investigated before their use at a larger scale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030822/s1, Table S1: Fruit characterization after 5 months of cold storage, before the application of the experimental treatments; Figure S1: Spore germination of *Botrytis cinerea* after 24 h at 24 °C. Control PDB (A), surfactant Tween 20 alone (B), thyme EO 1 mL L^{-1} (C), thyme EO 0.5 mL L^{-1} (D), thyme EO 0.1 mL L^{-1} (E), thyme EO 0.05 mL L^{-1} (F); Figure S2: Spore germination of *Penicillium expansum* after 24 h at 24 °C. Control PDB (A), surfactant Tween 20 alone (B), clove EO 5 mL L^{-1} (C), clove EO 1 mL L^{-1} (D), clove EO 0.5 mL L^{-1} (E), clove EO 0.1 mL L^{-1} (F); Figure S3: Inhibition of mycelial growth of *B. cinerea* by thyme EO after 3 days at 24 °C. Control PDA, thyme EO at 0.1, 0.5 and 1 mL^{-1}; Figure S4: Inhibition of mycelial growth of *P. expansum* by clove EO after 3 days at 24 °C. Control PDA, clove EO at 0.1, 0.5 and 1 mL^{-1}; Figure S5: Apples cv. 'Braeburn' infected by *Botrytis cinerea*. Control (A) and chitosan combined with thyme EO (B) after 11 days; Figure S6: Apples cv. 'Golden Delicious' infected by *Penicillium expansum*. Control (A) and chitosan combined with clove EO (B) after 5 days.

Author Contributions: Conceptualization, S.S. and C.A.; formal analysis, S.S.; investigation, S.S. and W.V.H.; writing—original draft preparation, S.S. and C.A.; writing—review and editing, all authors; supervision, C.A.; project administration and funding acquisition, C.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. S.S. was funded by a Ph.D. grant from the Free University of Bozen-Bolzano.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding authors, upon request and agreement of the authors.

Acknowledgments: S.S. would like to thank the research center Pcfruit npo for the support and hospitality received during his research period abroad foreseen by the PhD course.

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

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