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Chitosan/mandarin essential oil-based films on citrus fruits for the control of the medfly attack and to prevent the occurrence of grey and blue mould in post-harvest

Prangthip Parichanon [a,1], Priscilla Farina [a,1], Isabel Vicente [b], Marco Cesarini [a], Eliverta Hotaj [a], Sabrina Sarrocco [a,*], Elisa Pellegrini [a], Barbara Conti [a]

[a] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56126, Pisa, Italy
[b] Department of Microbiology and Genetics, Institute for Agribiotechnology Research (CIALE), University of Salamanca, C/ Del Duero, 12, Campus Villamayor-Parque Científico, 37185, Villamayor, Salamanca, Spain

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ABSTRACT

Citrus fruits, widely consumed around the world, can be negatively affected by pests and fungal infections during their cultivation, handling, transportation, and storage, thus resulting in substantial yield losses and food waste. The use of natural preservatives like chitosan (CHT) and essential oils (EOs) is a promising approach for reducing chemical inputs to preserve food products.

This study investigated the effects of CHT, extracted from crab shells and the fungus *Pleorotus ostreatus*, alone and in combination with mandarin (*Citrus* × *reticulata* Blanco, Rutaceae) essential oil (MEO), as an oviposition deterrent towards *Ceratitis capitata* (Diptera: Tephritidae), the Mediterranean fruit fly, and growth inhibitor of *Penicillium* (*P. expansum*, *P. digitatum,* and *P. italicum*) spp. fungi, the causal agents of apple and citrus rot.

A solution of 1.0% CHT of both origins (from crab shells and *P. ostreatus*) added with MEO resulted as the best combination to significantly reduce the oviposition percentage of *C. capitata* as well as mycelial growth and spore germination of *Penicillium* isolates and their pathogenic activity on *Citrus japonica* Thunb. (kumquats) fruits.

According to results here collected, CHT added with MEO represents a valid combination to be used as an edible film and coating as part of an integrated control strategy to improve the shelf-life of fresh citrus fruits. Furthermore, fungal CHT, here used for the first time in combination with MEO, can be an excellent alternative to reply to the eating habits and necessities of the final consumers.

1. Introduction

Citrus fruits are widely consumed around the world thanks to their juicy and tasty flavours and the abundance of vitamins, minerals, and antioxidants (Lyu et al., 2020; Lu et al., 2023). Although citrus fruits have mass production, pests and pathogenic fungi can affect their quality both during cultivation and post-harvest (Zacarías et al., 2020) with significant yield losses and food waste.

Ceratitis capitata (Wiedemann, 1824) (Diptera: Tephritidae), also known as the Mediterranean fruit fly or medfly, is native to sub-Saharan Africa (De Meyer et al., 2004), but it is currently established across Africa, parts of South America, the Middle East, Europe, and Australia due to multiple introductions through the trade of infested fresh fruit (Weldon, 2022). The wide range of hosts of *C. capitata* includes wild and commercial plants, especially apples, apricots, figs, mangoes, peaches, pears, persimmons, plums, strawberries, and numerous citrus fruits (Raga et al., 2004; Araujo et al., 2005). Being *C. capitata* highly invasive, polyphagous, and easily transported as hidden eggs or larvae, it is enlisted as a priority pest in areas not yet invaded (*e.g.*, Japan and the USA).

Among plant pathogenic *Penicillium* species, *P. digitatum* (Pers.) Sacc. 1881, *P. expansum* Link 1809, and *P. italicum* Wehmer 1894 are those more frequently affecting fruits during storage. They are the causal agents of serious post-harvest diseases due to their ability to enhance virulence by locally modulating the host's environmental pH (Prusky et al., 2004). Some *Penicillium* species can produce mycotoxins, fungal

* Corresponding author. Via del Borghetto 80, 56124, Pisa, Italy.
E-mail address: sabrina.sarrocco@unipi.it (S. Sarrocco).
[1] Prangthip Parichanon and Priscilla Farina contributed equally.

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secondary specialized metabolites harmful for human health (Otero et al., 2020). Mycotoxins represent a big threat especially when contaminating baby food such as fruit juices (Sarrocco and Vannacci, 2018).

Nowadays, chemicals are widely used to protect fruits, but they are unsafe for human and environmental health (Rani et al., 2021). Several studies have been conducted to improve the quality of fresh food by using natural preservatives, in line with the European policy (Matrose et al., 2021; Chowdhury et al., 2022). Essential oils (EOs), extracted from aromatic plants, can be used as natural food flavours and preservatives (Angelini et al., 2022). Mandarin [*Citrus* × *reticulata* Blanco (Rutaceae)] essential oil (MEO) is one of the most important citrus fruits extracts widely exploited by the food industry. Due to its antimicrobial, antifungal, insecticidal, and antioxidant activities, it is used as fungicide to reduce post-harvest decay or as enhancer of storage quality of horticultural products (Mossa et al., 2021; Saengwong-Ngam et al., 2022).

Chitosan (CHT) is the second-most abundant polysaccharide found in nature after cellulose (Abenaim and Conti, 2023) and has been reported to possess good film-forming and bioactive properties (Gao et al., 2019). CHT-based films and coatings have been found to perform as an excellent matrix to be loaded with the bioactive compounds of EOs (Dinu et al., 2021).

This study aimed to use MEO, which was selected by trained panelists according to its sensory profile on fruit (Bedini et al. (2020), CHT from crab shells and the fungus *Pleurotus ostreatus*, and the corresponding MEO-enriched CHT solutions for two main purposes: i) to prevent oviposition of *C. capitata* on *Citrus japonica* Thunb., which can occur when storage conditions are optimal for the insect and asymptomatic but attacked fruits in the field allow the presence of adults during the storage period with the consequent progression of the infestation; ii) to reduce the growth of *Penicillium* spp. isolates, as well as their pathogenicity, *in vitro* and *in vivo* on the same target fruit. The evaluation of the efficacy of CHT produced by fungi represents an innovative approach to reply to the request of new eating habits representing the future for human diets.

2. Materials and methods

2.1. Mandarin essential oil selection and chemical characterization

The EO used in this study was selected based on a previous sensorial evaluation reported in Bedini et al. (2020). A panel of expert sensorial analysts, trained according to the Department of Agriculture, Food, and Environment (DAFE) of the University of Pisa internal procedure, determined that MEO was the most suitable to match the sensory profile of fresh fruit.

MEO extracted from *C.* × *reticulata* peels was purchased from Erba Vita Group S.p.A. (Chiesanuova, San Marino), and its chemical characterization was carried out at the Department of Pharmacy of the University of Pisa by Gas Chromatography/Electron Impact-Mass Spectrometry (GC/EI-MS). The predominant compound is limonene (83.6%), followed by lower amounts of linalool (6.0%), sabinene (3.0%), myrcene (2.4%), and 13 other compounds (between 0.1 and 0.8%) (Bedini et al. (2020).

2.2. Citrus japonica fruit selection

The oviposition behaviour in *C. capitata* may be, according to different studies, based on chemical and visual cues (Papaj et al., 1989; Levinson et al., 2003). Since we aimed to exclude the interference of such stimuli with the protective effect given by the treatments proposed in the present work, we chose kumquats homogeneous in shape, dimensions, colour (bright orange), and ripeness.

Before the beginning of the trials, thirty organic kumquats were weighed and measured (length and diameter) using a Vernier calliper. The mean weight ± standard error (SE) of the kumquats was 8.99 ± 0.15 g (n = 30). About the dimensions, the mean length ±SE of kumquats was 3.22 ± 0.05 cm, the mean diameter ±SE 2.37 ± 0.02 cm (n = 30), and the surface roughly 20 cm^2.

Furthermore, *C. capitata* prefers to lay eggs in pre-existent wounds on the citrus fruits to by-pass the numerous EO-glands in the flavedo, whose content might be lethal for eggs and newly hatched larvae that should burrow through the peel before reaching the pulp (Papaj et al., 1989). To avoid such influence on the insect pest as well as the alteration of the mycelial growth and spore germination evaluation, we selected kumquats free from any abiotic or biotic lesions.

2.3. Chitosan and Mandarin essential oil-enriched chitosan formulations

Chitosan from crab shells was purchased from Sigma-Aldrich (St. Louis, MO, USA), while CHT from *P. ostreatus* was provided from Chibio Biotech Co., Ltd. (Qingdao, China). All the CHT formulations were prepared following the procedure developed by Peng and Li (2014) and adapted by Farina et al. (2022). For the 1.0% (*w/v*) plain CHT solutions, the powder was dissolved in demineralized water added with 1.0% (*v/v*) lactic acid (Farmitalia Carlo Erba Reagents S.p.A., Cornaredo, Italy). Chitosan solutions were agitated at 250 r.p.m. for 1–4 h (based on the amount of product processed at a time) on a hot plate stirrer (New type: VELP Scientifica, Usmate, Italy) at 25 °C. For MEO-enriched CHT formulations, 0.05, 0.4, 0.5, 0.8 or 1.0% (*v/v*) of MEO – according to the different trials performed –, 0.5% (*v/v*) of vegetal glycerol (A.C.E.F. S.p.A., Fiorenzuola d'Arda, Italy), and 0.6% (*v/v*) of Tween® 80 (Sigma-Aldrich) were added to the plain CHT solutions. The formulations were agitated for 10 min on the hot plate stirrer at 18 °C and 500 rpm. All the solutions were stored at 4 °C and warmed to 18 °C before use.

2.4. Ceratitis capitata mass rearing

The *Ceratitis capitata* specimens used for the oviposition deterrence trials have been reared at the DAFE of the University of Pisa since 1970. Adult flies are kept in a 75 × 75 × 115 cm polyester and knitted mesh (650 μm opening) tent (BugDorm-2400 Insect Rearing Tent, MegaView Science Co., Ltd., Taichung, Taiwan), provided with water and a solid diet composed of sucrose and yeast extract (Sigma-Aldrich) (5:1) *ad libitum*. Eggs are laid through the knitted mesh and fall into water-filled polyvinyl chloride (PVC) trays (30 × 15 × 4 cm). On alternate days, eggs are collected by sedimentation and used to inseminate a medium modified from Tanaka et al. (1969) and composed of wetted wheat bran (70% total, two parts of bran and three of water), sucrose (17%), yeast extract (11%), citric acid (1%, Thermo Fisher Scientific, Waltham, MA, USA), and sodium benzoate (1%, Sigma-Aldrich). The trays containing the medium inseminated with eggs are stored in a transparent polypropylene box (73 × 50 × 16 cm) closed with a lid. Mature larvae abandon the diet to pupate on the bottom of the box. Pupae are collected and moved into a clean tent where the adults will emerge. The rearing is maintained at 25 ± 2 °C, with relative humidity of 60–65%, and illuminated by fluorescent lamps (14,000 lux) to obtain a 12 h/12 h of darkness/light photoperiod.

2.5. Ceratitis capitata oviposition deterrence trial on kumquats

To test the protection given over 96 h to organic kumquats by CHT and MEO-enriched CHT solutions against the oviposition of *C. capitata*, we adopted the methodology reported in Farina et al. (2022), with minor modifications due to the different biology of the species involved. We moved two hundred *C. capitata* unsexed adults (10–15 days old, 1:1 sex ratio) from the rearing tent to a 47.5 × 47.5 × 93 cm nylon and knitted mesh (160 μm opening) cage (BugDorm-4M4590DH, MegaView Science Co.). The cage was provided with water, the solid diet, and a beaker filled with 300 mL of water and covered with cotton gauze to maintain the humidity. The cage was kept under laboratory conditions (25 ± 2 °C, relative humidity of 60–65%, and constant illumination).

The arrangement of the kumquats in the cage is schematized in Fig. 1. At each inner corner of the cage, we secured a styrofoam platform (12 × 7 × 2.5 cm) carrying four toothpicks. On each toothpick, we inserted a kumquat, previously rinsed, dried, and pierced where the stalk leaves the small cavity after the removal (to minimise the lesion size).

In total, there were 16 kumquats per cage: four untreated as controls, four sprayed with 200 μL of a 1.0% MEO solution in ethanol (EtOH) (corresponding to 2.0 μL EO/kumquat or 0.1 μL EO cm^{-2}), four spread with 400 μL of the 1.0% plain CHT solution (from crab shells or *P. ostreatus*), and four spread with 400 μL of the 1.0% CHT + 0.5% MEO (corresponding to 2.0 μL EO/kumquat or 0.1 μL EO cm^{-2}) formulation. Before use, all the treated kumquats were placed under a vertical fume hood for 5 (to let the EtOH evaporate) to 60 (to let the CHT dry) minutes. For both CHT kinds, three independent replicates of the procedure were prepared.

At time zero (T0), we prepared a double set of kumquats (32 samples). One set (*i.e.*, 16 samples) was placed inside the cage with the flies right after the drying of the treatments, and then we counted the number of eggs laid after 48 h (T1). At T1, we replaced the set of fruits with the other one prepared at T0 and stored, in the meantime, under the same environmental conditions. After 48 more h (T2, 96 h after the beginning of the trial), we counted the eggs laid on the new set of fruits to evaluate the duration of the protection given by the treatments.

Since, according to Levinson et al. (2003), females force the ovipositor through the flavedo to create an egg cavity within the albedo or, otherwise, exploit pre-existing lesions (Papaj et al., 1989), to expose the eggs laid, we peeled kumquats under a stereomicroscope (Leica EZ4, Leica Biosystems Italia, Buccinasco, Italy). Indeed, we found and counted the eggs laid under the flavedo (epicarp), inside the albedo (mesocarp), and around the toothpicks inserted in the fruit. The protection offered by the different treatments against *C. capitata* was assessed as oviposition percentage, according to the following formula:

$$Oviposition\ percentage = NT \div NCG \times 100$$

with *NT* as the number of eggs laid on a specific treatment (4 samples per cage) and *NCG* as the total number of eggs laid in a cage (16 samples per cage).

To assess the differences in the oviposition of *C. capitata* among treatments, data were submitted to one-way analysis of variance (ANOVA), with the oviposition percentages (after angular transformation) as the dependent variables and each treatment as the main factor. For multiple comparisons of means in the oviposition deterrence trial, Tukey's HSD *post-hoc* test ($P \leq 0.05$) was used. Data were processed by the free software PSPP (GNU Project, Boston, MA, USA).

2.6. Toxicity trial on Ceratitis capitata eggs

To test the toxic effect of the proposed treatments on the eggs of *C. capitata*, we adopted the methodology reported in Farina et al. (2021) with minor modifications. In detail, filter paper squares (4.47 × 4.47 cm, surface 20 cm^2) were poured with 250 μL of EtOH (control) or 250 μL of the 0.8% EtOH solution of MEO (corresponding to 0.1 μL EO cm^{-2}). For the treatments involving CHT, the liquid amount was doubled due to its viscosity, even if the MEO concentration was maintained. Thus, 500 μL of the 1.0% plain CHT solution (from crab shells or *P. ostreatus*) or 500 μL of the 1.0% CHT + 0.4% of MEO (corresponding to 0.1 μL EO cm^{-2}) formulation were applied on the filter paper squares. Before use, EtOH was made to evaporate from paper under the vertical fume hood for 5 min. The treated paper squares were placed in glass Petri dishes (diameter 10 cm) and wetted with 350 μL of water; then, 50 *C. capitata* eggs (0–12 h old) collected from the water-filled PVC trays described in paragraph 2.4 were arranged on their surface using a wet, thin brush. Petri dishes containing the eggs were kept in a climatic chamber (KW Srl, Siena, Italy) at 25 °C in darkness. The egg's hatchability was checked every 24 h for 3 days (*i.e.*, after 24, 48, and 72 h), counting the empty chorions with the help of the stereo microscope. At each daily check, the paper squares were moistened again with 250 μL of water.

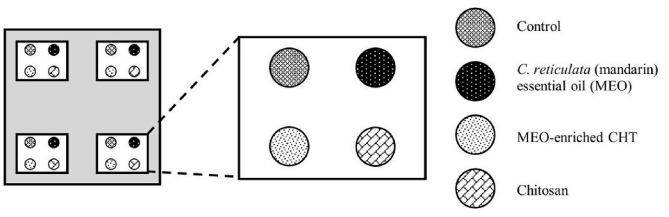

Fig. 1. Schematic representation of the kumquats arrangement in the oviposition deterrence trial. The grey square represents the cage seen from above; the patterned circles are the fruit samples (one control and three different treatments); the white rectangles are the styrofoam platforms into which the kumquats are inserted using a toothpick.

Egg mortality data were corrected using Abbott's (1925) formula. For each treatment, five independent replicates were prepared. To assess the toxic effect on *C. capitata* eggs, data were submitted to one-way ANOVA, with the egg mortality percentages (after angular transformation) as the dependent variables and each treatment as the main factor. For multiple comparisons, Tukey's HSD *post-hoc* test ($P \leq 0.05$) was used. Data were processed by the free software PSPP (GNU Project, Boston, MA, USA).

2.7. Fungal isolates and growth condition

Penicillium expansum, *P. digitatum*, and *P. italicum,* isolated from several affected fruits (apple, lemon, orange, tangerine, and grape) and collected from different Italian geographic origins, were deposited in the fungal collection at DAFE of the University of Pisa and stored on Potato Dextrose Agar (PDA, 39.0 gL^{-1}, Sigma-Aldrich) under mineral oil at 4 ± 1 °C. When actively growing colonies were needed, they were grown on PDA at 24 °C under a 12 h/12 h of darkness/light photoperiod. A preliminary pathogenicity test on citrus fruits (orange, lemon, and kumquats) was performed on the 30 isolates here collected (*data not shown*) to select, for each of the three fungal species, those most aggressive to be further used in the present work, as described in paragraphs 2.8, 2.9, and 2.10.

2.8. Antifungal activity of chitosan

To assess the effect of CHT on fungal growth and find its most appropriate concentration to be used for the following tests, CHT extracted from crab shells was tested by using a quick protocol based on multi-well microplate spectrophotometric readings (Sarrocco et al., 2019). A spore suspension of each isolate (made from colonies grown on PDA for 1 week at 20 °C) was inoculated in each well – at the final concentration of 10^6 spore mL^{-1} – in 150 μL of Potato Dextrose Broth (PDB, 24.0 gL^{-1}, Sigma-Aldrich) added with three concentrations of CHT (0.1%, 0.5%, and 1.0%). PDB without CHT was used as a control. For each isolate and medium, three independent replicates, each consisting of six wells, were prepared. Optic Density (OD) values were obtained by spectrophotometric measurements at 595 nm (Bio-Rad 680 Microplate reader, Milan, Italy) every 4 h during the day (12 h at night). The OD values were used to create growth curves that, in their linear phase, were submitted to analysis of variance regression (by GraphPad Prism 8) in order to compare the slopes when fungi were grown in PDB *vs*. those in the presence of the three concentrations of CHT, assuming *P* slope ≤ 0.05 as a significant level.

According to the results here obtained, the 1.0% CHT solution was chosen for further experiments. Based on this result, and to also check the antifungal efficacy of CHT extracted from *P. ostreatus*, the same spectrophotometric experiment was repeated on three *Penicillium* isolates, one for each species, by inoculating fungal spores in PDB

containing 1.0% of fungal CHT.

2.9. Antifungal activity of Mandarin essential oil

The effect of MEO on the growth of the three *Penicillium* species was tested following two approaches, *i.e.*, by evaluating the effect of its volatile compounds and directly adding the EO in the growth medium as a poisoning compound.

For the first experiment, 20 mL of Yeast Extract Sucrose Broth (YESB, Sigma-Aldrich), added with MEO at the final concentrations of 1.0, 0.5, and 0.05% (*v/v*) starting from a stock solution of MEO prepared in sterile distilled water + 0.01% Tween® 80 (Chidi et al., 2020), was inoculated in 50 mL Falcon tubes with an aqueous spore suspension, made from colonies grown on PDA at 20 °C in darkness, at a final concentration of 10^3 spore mL^{-1}. YESB not added with MEO was used as a control. For each isolate and medium, three independent replicates were prepared. All Falcon tubes, closed with Parafilm® to keep the volatiles within the growth environment, were incubated at 20 °C in darkness on a rotary shaker (75 rpm) for 6 days, and the mycelium was collected by filtering it through a sterile Miracloth® layer. Mycelium was maintained at 40 °C and weighed twice a day until the weights reached a constant value between two following measures.

The effect of MEO's volatiles on *Penicillium* spp. spore germination was tested *in vitro* on Water Agar (WA, 20 g L^{-1}) added with three concentrations of MEO (0.05, 0.5, and 1.0%), while only WA was used as a control. One hundred μL of a 10^3 spore mL^{-1} aqueous spore suspension, made from colonies grown on PDA for 1 week at 20 °C, was spread on plates containing WA. A filter paper disk of 1 cm diameter, soaked in a water +0.01% Tween® 80 solution containing MEO at 0.05, 0.5, and 1.0%, was placed on the lid of each WA plate. Inoculated plates, closed with Parafilm® to keep the volatiles within the growth environment, were incubated upside down at 20 °C in darkness. For each isolate and EO concentration, three independent replicates were prepared. After 72 h, the number of germinated spores in presence of each concentration of MEO was counted. Mycelial weight and number of germinated spores were submitted to analysis of variance ANOVA by the free software PSPP (GNU Project, Boston, MA, USA) to compare with that obtained in control, assuming $P \leq 0.05$ as a significant level.

2.10. Combined effect of chitosan and Mandarin essential oil on fungal growth and fruit infection

As a final validation of the selected CHT + MEO formulation, an *in vivo* test was performed on kumquats to evaluate its on the pathogenicity of the three fungal species. Organic kumquats were inoculated with 20.0 μL of an aqueous fungal spore suspension (10^6 spore mL^{-1}) and the formulation of 1.0% CHT (from scrab shells) + 1.0% MEO on a wound (1 cm × 1 mm × 2 mm) made with a sterile scalpel on the fruit surface, previously sterilized in 100% EtOH for 30 s. Inoculation through the wound was made to resemble the entry size of the pathogen that can infect only through the wounds on the surfaces of fruits (Yang et al., 2019).

Fruits inoculated with the fungal spore suspension (of each of all the three isolates on kumquats) without CHT and MEO were used as controls. Kumquats were closed within a plastic bag to maintain humidity in order to put the pathogen into the best conditions for fruit infection and incubated for 5 days at 20 °C in darkness. At the end of the incubation, the number of fruits (expressed as a percentage) showing signs of infection (such as rot and/or fungal sporulation) were registered and, after angular transformation, submitted to ANOVA by the free software PSPP (GNU Project, Boston, MA, USA) to compare the pathogenicity of the isolates in presence of CHT + MEO with that on control fruit, assuming $P \leq 0.05$ as a significant level. The experiment was repeated three times.

3. Results

3.1. Protection of the kumquats against the oviposition by Ceratitis capitata

Regarding the oviposition by *C. capitata* on organic kumquats, all the proposed treatments effectively discouraged it, compared to the untreated control samples, at both the considered times (T1 = 48 h and T2 = 96 h).

When using CHT from crab shells, the results of one-way ANOVA analysis showed significant differences among the samples at both sampling times ($F_{3,11} = 20.55$, $P < 0.001$ and $F_{3,11} = 127.78$, $P < 0.001$, respectively). At T1, Tukey's HSD post-hoc test ($P < 0.05$) indicated that there were no statistical differences among the treatments, while, at T2, the MEO-enriched CHT solution was the most statistically effective (Fig. 2).

In detail, at T1, 40.70 ± 1.92% of eggs were laid in the control kumquats, 25.29 ± 3.42% in the fruits treated with CHT from crab shells, 15.22 ± 2.47% in the MEO-treated ones, and 18.78 ± 1.77% in those treated with the MEO-enriched CHT solution. At T2, the oviposition percentages were, respectively, 48.72 ± 1.03, 20.14 ± 0.89, 19.15 ± 2.09, and 11.97 ± 1.41% (Fig. 2).

Even when using CHT from *P. ostreatus*, the results of one-way ANOVA analysis showed significant differences among the samples at both sampling times ($F_{3,11} = 69.89$, $P < 0.001$ and $F_{3,11} = 103.40$, $P < 0.001$, respectively). According to Tukey's HSD *post-hoc* test ($P < 0.05$), at T1, it is the MEO EO-enriched CH solution that gave the most significant protection to kumquats; at T2, they were the MEO-enriched CHT solution again and plain CH from *P. ostreatus*.

In detail, at T1, 52.02 ± 5.94% of eggs were laid in the control kumquats, 21.22 ± 5.12% in the fruits treated with CHT from *P. ostreatus*, 19.64 ± 5.28% in the MEO-treated ones, and 7.10 ± 2.34% in those treated with the MEO-enriched CHT solution. At T2, the oviposition percentages were, respectively, 54.86 ± 2.50, 16.50 ± 0.71, 20.68 ± 2.11, and 7.95 ± 1.12% (Fig. 3).

3.2. Toxicity on Ceratitis capitata eggs

The results of one-way ANOVA analysis showed no significant differences in terms of ovicidal effect among different treatments ($F_{4,24} = 0.65$, $P = 0.636$), as visible in Fig. 4. In detail, the egg mortality percentage was 6.0 ± 3.1% for MEO, 8.24 ± 1.6% for CHT from crab shells, 4.64 ± 2.3% for the same CHT enriched with MEO, 7.20 ± 1.6% for CHT from *P. ostreatus*, and 4.24 ± 2.3% for the same CHT enriched with MEO (Fig. 4).

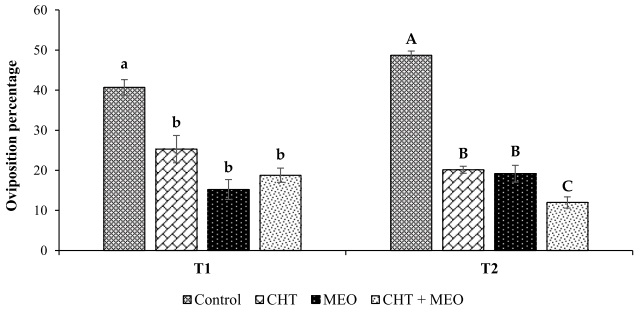

Fig. 2. Oviposition of *Ceratitis capitata* on kumquats after 48 (T1) and 96 h (T2) from the treatment with chitosan from crab shells (CHT), mandarin essential oil (MEO) and the corresponding MEO-enriched CHT solution (CHT + MEO). Data are represented as mean ± standard error (SE) with $n = 3$. For each time, different letters (small letters a-b for T1, capital letters A-C for T2) correspond to significant differences among the proposed treatments (Tukey's HSD, $P \leq 0.05$).

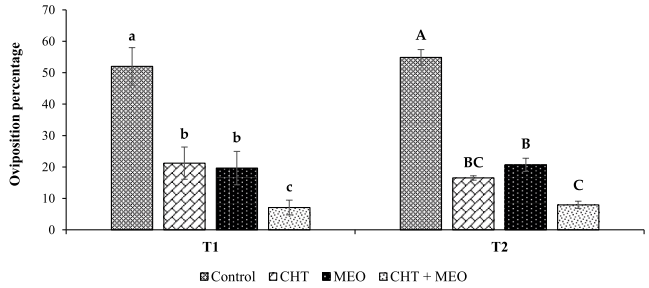

Fig. 3. Oviposition of *Ceratitis capitata* on kumquats after 48 (T1) and 96 h (T2) from the treatment with chitosan from *Pleurotus ostreatus* (CHT), mandarin essential oil (MEO) and the corresponding MEO-enriched CHT solution (CHT + MEO). Data are represented as mean ± standard error (SE) with $n = 3$. For each time, different letters (small letters a-b for T1, capital letters A-C for T2) correspond to significant differences among the proposed treatments (Tukey's HSD, $P \leq 0.05$).

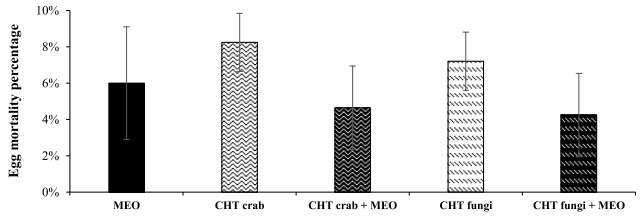

Fig. 4. Ovicidal effect of mandarin essential oil (MEO), chitosan (CHT) from crab shells and *Pleurotus ostreatus* (fungi) and the correspondent MEO-enriched CHT solutions on *Ceratitis capitata* eggs after 72 h. Data are represented as mean ± standard error (SE) with $n = 5$.

3.3. Antifungal activity of chitosan

Concerning *P. expansum* isolates (Table 1), a significant reduction of growth rate (*P* slope <0.001) was registered for all three strains already when in presence of the minimum concentration of CHT here tested (0.1%), while, at the other two concentrations, no fungal growth was measured, thus demonstrating a complete inhibition of the ability of the spores to germinate.

A similar behavior was observed for both *P. digitatum* (Table 2) and *P. italicum* (Table 3) isolates, where 0.1% of CHT resulted in a significant reduction of fungal growth rate, and the two higher concentrations did not allow the germination of the spores (slope = 0.000).

Analysis of variance of regression of the growth curves resulted in a total inhibition of spore germination for the three strains (slope = 0.000; *P* slope <0.0001), as already observed for the CHT from crab shells, thus confirming no differences between the two CHT kinds.

3.4. Determination of Mandarin essential oil concentration

When dry weight values were submitted to statistical analysis, the highest concentration of MEO (1.0%) resulted in being able to significantly reduce the growth of the three fungal isolates (Fig. 5) with a reduction of around 70%, 60%, and 50% for *P. digitatum*, *P. expansum*, and *P. italicum*, respectively. Concerning *P. digitatum* and *P. italicum*, also the 0.50% concentration of MEO resulted in a significant reduction, by around 40% and 30%, respectively, for both the fungal isolates. No significant effects were registered for all tested fungi at the lower concentration (0.05%).

Results of statistical analysis are shown in Fig. 6, where a significant reduction of developed colonies was registered for *P. digitatum* when in presence of volatile compounds released by the three different (0.05, 0.50, and 1.0%) MEO solutions, with a decrease of around 60% of colonies when MEO was added at the highest concentration. The inhibition capability increased from the lowest to the highest EO concentration. Conversely, in *P. expansum,* all three MEO concentrations significantly reduced the number of developed colonies (on an average of around 30% in presence of each of the three concentrations), but no differences were measured among the three solutions. Finally, no significant effects were observed for *P. italicum*.

According to the results here obtained, 1.0% CHT and 1.0% MEO were selected as the most effective and further used in combination for their antifungal activity against all three *Penicillium* species involved.

3.5. Combined effect of chitosan and Mandarin essential oil on fungal growth and fruit infection

The result of the combined effect of CHT from crab shells and MEO on *Penicillium* spp. growth is shown in Table 4. For all three species, no

Table 1
Growth of *Penicillium expansum* isolates in presence of different concentrations of chitosan. Abbreviations: CHT, chitosan, PDB, Potato Dextrose Broth.

Medium	slope	R^2	*P*	*P* slope
	***P. expansum* RC**			
PDB	0.018	0.75	<0.0001	
PDB + CHT 0.1%	0.001	0.69	<0.0001	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. expansum* Ba-4**			
PDB	0.010	0.89	<0.0001	
PDB + CHT 0.1%	0.004	0.75	<0.0001	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. expansum* 05**			
PDB	0.013	0.97	<0.0001	
PDB + CHT 0.1%	0.007	0.88	<0.0001	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			

Analysis of variance of regression: slope = growth rate (optic density h^{-1}); R^2 and *P* = significance of the growth curve; *P* slope = significance of the difference between the slope in presence of CHT and that in PDB (control). $P \leq 0.05$ was assumed as a significant level.

Table 2
Growth of *Penicillium digitatum* isolates in presence of different concentrations of chitosan. Abbreviations: CHT, chitosan, PDB, Potato Dextrose Broth.

Medium	Slope	R^2	*P*	*P* slope
	***P. digitatum* PD-010**			
PDB	0.013	0.86	<0.0001	
PDB + CHT 0.1%	0.008	0.83	<0.0001	**< 0.0001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. digitatum* An-4**			
PDB	0.005	0.70	<0.0001	
PDB + CHT 0.1%	0.003	0.70	<0.0001	**0.03**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. digitatum* RC**			
PDB	0.006	0.88	<0.0001	
PDB + CHT 0.1%	0.001	0.50	<0.0001	**< 0.0001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			

Analysis of variance of regression: slope = growth rate (optic density h^{-1}); R^2 and *P* = significance of the growth curve; *P* slope = significance of the difference between the slope in presence of CHT and that in PDB (control). $P \leq 0.05$ was assumed as a significant level.

Table 3
Growth of *Penicillium italicum* isolates in presence of different concentrations of chitosan. Abbreviations: CHT, chitosan, PDB, Potato Dextrose Broth.

Medium	slope	R^2	*P*	*P* slope
	***P. italicum* To-010**			
PDB	0.005	0.90	<0.0001	
PDB + CHT 0.1%	0.001	0.50	0.022	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. italicum* L70**			
PDB	0.007	0.88	<0.0001	
PDB + CHT 0.1%	0.001	0.56	0.0023	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			
	***P. italicum* RC**			
PDB	0.013	0.96	<0.0001	
PDB + CHT 0.1%	0.003	0.71	<0.0001	**< 0.001**
PDB + CHT 0.5%	0.000			
PDB + CHT 1.0%	0.000			

Analysis of variance of regression: slope = growth rate (optic density h^{-1}); R^2 and *P* = significance of the growth curve; *P* slope = significance of the difference between the slope in presence of CHT and that in PDB (control). $P \leq 0.05$ was assumed as a significant level.

spore germination (slope = 0.000) occurred when in presence of the combination of CHT and MEO, thus resulting in a significant inhibition of fungal growth (*P* slope <0.001).

Similar results were obtained for the combination of CHT from *P. ostreatus* (1.0%) added with MEO, resulting in nofungal spore germination of the three fungal isolates (slope = 0; *P* slope <0.001) compared to PDB control.

The presence of CHT (from crab shells) + MEO reduced percentages of infected fruit inoculated with all three isolates, mainly for *P. digitatum*, where a significant reduction ($P \leq 0.05$) of the number of affected kumquats (80% less) was registered when in presence of CHT + MEO.

Table 4
Growth of *Penicillium digitatum, P. expansum*, and *P. italicum* in presence of a combination of chitosan (CHT, 1.0%) and mandarin essential oil (MEO, 1.0%).

Medium	Slope	R^2	*P*	*P* slope
	P. digitatum			
PDB	0.005	0.82	<0.0001	
PDB + CHT + MEO	0.000	0.04	0.0748	**< 0.001**
	P. expansum			
PDB	0.007	0.86	<0.0001	
PDB + CHT + MEO	0.000	0.03	0.2147	**< 0.001**
	P. italicum			
PDB	0.011	0.95	<0.0001	
PDB + CHT + MEO	0.000	0.16	0.0026	**< 0.001**

Analysis of variance of regression: slope = growth rate (optic density h^{-1}); R^2 and *P* = significance of the growth curve; *P* slope = significance of the difference between the slope in presence of CHT and that in Potato Dextrose Broth (PDB; control). $P \leq 0.05$ was assumed as a significant level.

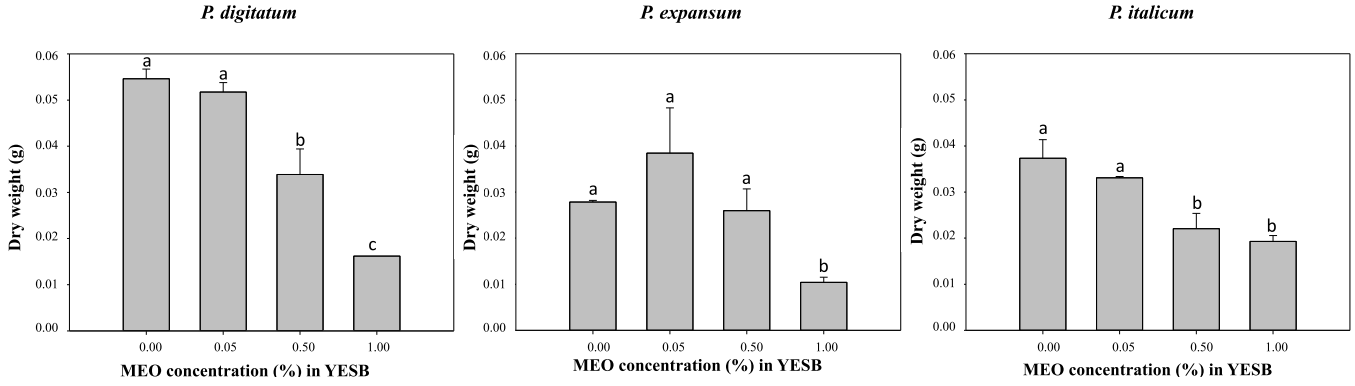

Fig. 5. Growth (expressed as mycelial dry weight) of *Penicillium digitatum*, *P. expansum*, and *P. italicum* in liquid medium added with mandarin essential oil (MEO) at different concentrations. Within each graph, different letters (a–c) correspond to values significantly different from the control (YESB medium without MEO), assuming $P \leq 0.05$ as a significant level.

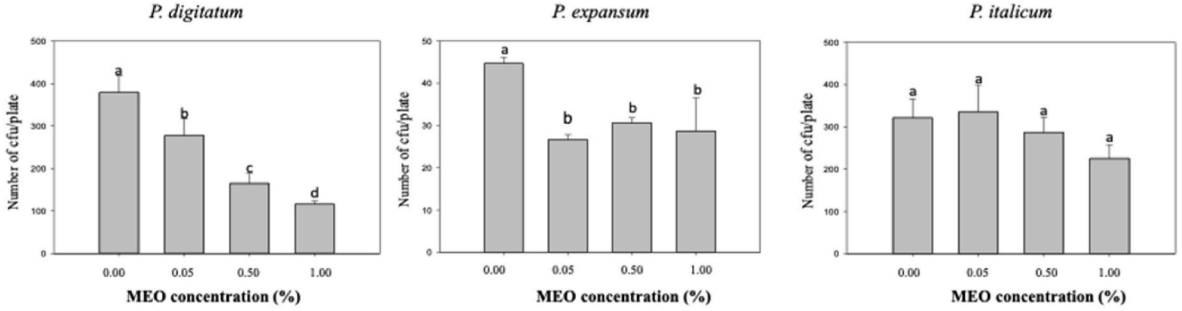

Fig. 6. Growth (expressed as the number of developed colonies, cfu) of *Penicillium digitatum*, *P. expansum*, and *P. italicum* on Water Agar in presence of volatile compounds released by mandarin essential oil (MEO) added at different concentrations. Within each graph, different letters (a–d) correspond to values significantly different from the control (YESB medium without MEO), assuming $P \leq 0.05$ as a significant level.

4. Discussion

On kumquats, Prokopy and Duan (1998) observed that the act of ovipositor boring of a naive female is significantly enhanced by the presence of another egg-laying female on the fruit. For the validity of our finding, having in our trials about one hundred females in each cage (two hundred specimens per trial with a 1:1 sex ratio), we can consider the socially facilitated oviposition behaviour respected. According to Robertson et al. (1995), this practice would communicate to the second ovipositing female the acceptability of the fruit previously selected by the already experienced female.

The complete composition of the MEO from peels employed in this study is reported in Bedini et al. (2020). Limonene resulted as the main compound (83.6%) and linalool among the minor ones (6.0%). Regarding the use of EOs as repellents towards *C. capitata*, restricting the research only to those extracted from *Citrus* fruits and their monoterpenes, there are some previous attempts. Levinson et al. (2003) observed that oviposition by *C. capitata* was not affected when treating mock wax fruits (both in reddish-yellow and whitish-coloured samples) with *Citrus* × *sinensis* (L.) Osbeck EO (D-limonene 90.4%, linalool 1.0%, myrcene 0.9%) in concentrations from 0.004 to 0.039 μL cm^{-2}. Conversely, they observed 76% deterrence with 3.9 μL cm^{-2} and complete oviposition suppression starting from 9.8 μL cm^{-2} in the whitish samples only, hence demonstrating how also colour can influence oviposition. In our trials, the concentration of MEO was roughly 0.1 μL cm^{-2}, but, however, we obtained a statistically significant reduction of oviposition (compared to the control) in real, orange-coloured kumquats. Ioannou et al. (2012) reported that 1.0 μL of D-limonene stimulates oviposition in *C. capitata*, while it is linalool the monoterpene able to suppress egg deposition. More recently, the deterrent effect of linalool on *C. capitata* females was further verified under laboratory conditions and in the field (where, conversely, males were attracted by the compound) (Papanastasiou et al., 2020). Additionally, D-limonene and linalool were shown to be toxic by topical application on *C. capitata* adults of both sexes, particularly on males (LD_{50} for limonene on males 8.34, on females 31.72 nL/fly; LC_{50} for linalool on males 10.37, on females 49.39 nL/fly) (Papanastasiou et al., 2017).

On *C. capitata* eggs, the contact toxicity was previously evaluated by Ruiz et al. (2014) using the pure compounds citral and D-limonene (LC_{50} of, respectively, 22.44 and 77.06 μL mL^{-1}) and ether extracts of *Citrus* × *paradisi* Macfad. var. Foster Seedless and *Citrus limon* var. Eureka peels (LC_{50} of, respectively, 72.94 and 113.46 μL mL^{-1}). Although such extracts are not EOs, they similarly contain D-limonene as the main component (82.7 and 72.5% for grapefruit and lemon, respectively) and smaller amounts of linalool, sabinene, and myrcene. The MEO we used for our trials only exerted 6.0% of egg mortality. The MEO concentration we used (0.1 μL EO cm^{-2}, corresponding to 8 μL mL^{-1}) was, indeed, lower than the one used by Ruiz et al. (2014) and enough to act as a repellent towards the adults but not as a toxic agent to eggs.

The same accession of the *C.* × *reticulata* EO here employed proved to be attractive at low concentrations (0.6 and 1.19 μL mL^{-1}), but repellent at higher concentrations (23.87 μL mL^{-1}) to the fruit fly *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae). Furthermore, it caused a 75% oviposition deterrence at the concentration of 4.2 μL mL^{-1} (Bedini et al., 2020).

Our results also demonstrated that MEO has significant effects on the growth of *P. expansum, P. digitatum,* and *P. italicum*. The increasing concentration of MEO improved the inhibition of mycelial growth and spore germination in all the tested strains, probably due to the destruction of the endomembrane system of fungal cells, as described by Hu et al. (2019). Viuda-Martos et al. (2008) revealed that the antifungal activity of MEO was mainly due to the high content of D-limonene, linalool, and citral (the first two active compounds are present in our MEO as well). These monoterpenes may cause membrane rupture, which leads to cell wall disruption and lysis (Parichanon et al., 2022). Furthermore, their accumulation in the cell damages cytoplasmic organelles and the cell membrane of mold, resulting in conidia rupture, content leakage, mycelium shriveling, and hyphae collapse (Tariq et al., 2019). All this information could support the effect that MEO showed both on mycelial growth and spore germination in all the *Penicillium* isolates here tested. In addition to the direct effect that MEO exerted against fungi, even its volatiles were able to affect spore germination, although in a concentration-dependent way. We demonstrated that the amount of EO employed is relevant for reducing spore germination, in accordance with Droby et al. (2008).

Chitosan from coarse crabs has low toxicity by ingestion on *C. capitata* adults, with an LC_{50} of 11,319 and 10,705 mg L^{-1} after 24 and 48 h, respectively (Rabea et al., 2015). Anyhow, all the CHT coatings in our trials were let dry before use, so we can exclude that the flies could have been exposed to this potentially toxic agent by ingestion. Likewise, on eggs, the two CHT kinds we tested exerted low toxicity (8.24 and 7.20% for, respectively, CHT from crab shells and *P. ostreatus*). A CHT coating from crustacean shells (100 and 200 g L^{-1}) was recently proposed by da Costa et al. (2021) to protect *Vitis vinifera* L. cv. Itália, but, conversely to our results on kumquats, they observed an increased number of punctures (without and with eggs) and eggs of *C. capitata* on grapes compared to their control.

In this study, the use of CHT resulted in being effective also in inhibiting spore germination and growth of all three species of *Penicillium* involved. This outcome is consistent with that reported by Meng et al. (2010), who also studied CHT and its derivatives to control phytopathogenic fungi on pear fruit by inhibiting spore germination and mycelial growth on *Alternaria kikuchiana* Tanaka and *Physalospora piricola* Nose also by stimulating the production of defense-related enzymes and enhancing antioxidative activity in the host. Furthermore, Al-Hetar et al. (2011) also studied the effect of different CHT concentrations on *Fusarium oxysporum* and showed that the inhibitory effect was found to increase as the CHT concentration increased. For this reason, even if in the tests here reported both the 0.5 and 1.0% CHT solutions could completely inhibit *Penicillium* spp., the 1.0% solution was selected for further experiments in view of the application of CHT on fruit by profiting from the induction of antioxidative activity, as explained by Al-Hetar et al. (2011).

To the best of our knowledge, our work is the first attempt to use an EO-enriched CHT solution, especially from *P. ostreatus*, to protect citrus fruits against the oviposition by *C. capitata* and evaluate its effect over 96 h. Similar approaches were attempted before, with good results, but on different substrates. For example, Farina et al. (2022) protected beef meat from the blowfly *Calliphora vomitoria* (L., 1758) (Diptera: Calliphoridae) using *Laurus nobilis* L. and *Piper nigrum* L. EOs added to CHT from crab shells, and Parichanon et al. (2023) applied the same treatments against the cheese and ham skipper *Piophila casei* (L., 1758) (Diptera: Piophilidae). Furthermore, Ascrizzi et al. (2021) used the same kind of CHT enriched with *Ferulago campestris* (Besser) Grecescu EO to disrupt the reproductive activity of *Acanthoscelides obtectus* (Say, 1831) (Coleoptera: Bruchidae) females on the common bean.

Similarly, also for *Penicillium* spp., this work reports for the first time the possibility of successfully combining CHT from *P. ostreatus* with MEO to inhibit mycelial growth and spore germination, as well as to protect the fruit from decay even.

Production of CHT from fungal mycelium is receiving increased attention for several reasons. As alternative to the limited crustacean waste supplies due to seasons sites of fishing, fungal fermentative processes are easy, cheap, eco-friendly, and geographic or seasonal limitations free (Yu et al., 2012). Additionally, physical properties of biofilms made with fungal CHT are not significantly different from those made with high and low molecular weight commercial CHT such as that derived from shrimp. Not less important, fungal-sourced CHT is gaining prominence because of its vegan appeal (Alimi et al., 2023).

Grande-Tovar et al. (2018) reviewed how CHT coatings enriched with EOs can affect fungi involved in fruit decay. However, the antifungal activity of CHT-EOs is particularly dependent on the ability to

release the antimicrobial compounds from the polymer matrix, type of EOs, fungal species, and incubation temperature. In our case, we discovered that using plain CHT compared to CHT-MEO did not allow us to obtain different results, as reported by Ali et al. (2015), who reported that using 1.0% CHT and 1.0% CHT enriched with 1.0% lemongrass EO showed no significant differences in the percentage of disease incidence of anthracnose of bell pepper. On the other hand, in the antifungal activity *in vitro*, they found that 1.0% CHT enriched with lemongrass EO can reduce the length of mycelial growth at a significant level compared to 1.0% CHT alone, suggesting CHT as a suitable carrier for EOs. Indeed, it can stabilize their volatile components and prolong their bioactivity over time, as well as create a barrier on fruit thanks to its film-forming property (Cazón and Vázquez, 2020).

5. Conclusions

This study showed that 1.0% MEO and 1.0% CHT concentrations are effective against *Penicillium* spp. by inhibiting mycelium and spore germination. Furthermore, CHT extracted from crab shells and the fungus *P. ostreatus* showed the same behavior, as they strongly inhibited *Penicillium* spp. Noteworthy, MEO-enriched CHT solutions exhibited antifungal effectiveness in controlling mold growth both *in vitro* and in kumquats. Even as oviposition deterrents, both types of MEO-enriched CHT (from crab shells or *P. ostreatus*) showed the lowest percentage of oviposition of *C. capitata* and a mildly toxic effect on its eggs.

In future studies, the quality and shelf-life stability of kumquats after the treatments, as well as consumer acceptance, should provide more evidence on the effects of MEO-enriched CHT if compared to plain CHT. As the two CHT kinds (of animal and fungal origin) proved to be effective against this insect pest and pathogenic fungi, it would be an excellent opportunity to exploit both according to the eating habits and necessities of the final consumers.

CRediT authorship contribution statement

Prangthip Parichanon: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Priscilla Farina:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Isabel Vicente:** Investigation. **Marco Cesarini:** Investigation. **Eliverta Hotaj:** Investigation. **Sabrina Sarrocco:** Writing – review & editing, Supervision, Conceptualization. **Elisa Pellegrini:** Writing – review & editing, Supervision. **Barbara Conti:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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