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In vitro antifungal activity and *in vivo* edible coating efficacy of insect-derived chitosan against *Botrytis cinerea* in strawberry

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ABSTRACT

Strawberry is a perishable fruit, susceptible to development of rot by a range of fungi, in particular *Botrytis cinerea*. Chitosan represents an alternative to agrochemicals for improving shelf-life and fighting fungal pathogens. A chitosan-based coating derived from pupal exuviae of *Hermetia illucens* has been recently formulated for improving shelf-life of strawberry stored at 4 °C and mixed condition (4 °C and room temperature). The effects of a decolored (PEDEC) and not decolored (PEND) chitosan from the black soldier fly were evaluated and compared with commercial chitosans from crustaceans (CCs), *in vitro* and *in vivo*. An inhibition/reduction of fungal growth and a disturbance of normal fungal morphology were observed, being MIC of 0.5 mg mL^{-1} and 1 mg mL^{-1} and growth inhibition of 70 % and 4% for PEND and PEDEC, respectively. Both edible coatings distributed *via* aerograph showed equal or better potential application than CCs in controlling *B. cinerea* in strawberry post-harvest treated. Different effects for chitosans depended on their different molecular weight and deacetylation degree distributions, and the presence or absence of melanin pigments in their structure. PEND could act directly against the fungus, with effects predominantly associated with fungitoxic properties; PEDEC might principally provide viable alternatives, such as the elicitation of biochemical defense responses in fruits, for example through total phenols, in particular the flavonoids.

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.), Rosaceae family, is the most commonly consumed berry fruit worldwide because of its highly appreciated flavor and nutritional value [1]. Unfortunately, it is a particularly perishable fruit and its shelf-life can be of only 1 or 2 days at ambient conditions and 5–7 days under refrigeration, depending on the cultivar type [2,3]. During post-harvest storage strawberries are susceptible to physical and mechanical injury, drying, physiological disorders and, above all, the development of rots caused by a range of fungi [4]. This is due to high rate of metabolism, delicate external structure, lower pH, optimal water activity for fungal growth, high levels of sugars and other nutrients [5]. In order to achieve a better conservation of this fruit, refrigeration and modified atmospheres are the mainly adopted tools, and non-thermal technologies are under study in recent years [6]. Among them, exogenous post-harvest treatments, such as spraying, coating, or dipping are widely used with the aim of decelerating the respiration rate and water loss, preserving fruit consistency, reduce the growth of pathogens as far, so to extend the shelf-life and quality during storage [1].

Botrytis cinerea (Pers.) is a saprophytic fungus that occupies the second place among all fungal pathogens for economic and scientific importance [7]. It has the ability to attack leaves, stems, flowers and fruits of a wide host range, with >200 species over both temperate and

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tropical areas, determining severe pre- and post-harvest losses (annually estimated between 10 and 100 billion USD) [8].

Gray mold rot caused by *B. cinerea* is the most serious disease of strawberry post-harvest decay [9], leading to fruit losses up to 60 % [10]. Fungal inoculum comes mainly from the field, where latent infection arises at flowering stage, being symptomless underneath the sepal until fruit ripening [11]. The disease usually starts close to the pedicel and then spreads to the whole fruit. Then, the fungus produces a dense layer of grayish spores that can quickly move among fruits through air, water-mediated dispersal and by nesting [12].

Gray mold is usually managed through the use of chemical fungicides with application time from flowering to ripening, but chemical control has often low efficacy because *B. cinerea* possesses wide host diversity, short life cycle, prolific reproduction and high genetic variability, with consequent high capacity of possible mutations of genes that confer resistance to many compounds and reduce the efficacy of chemical fungicides [13]. Moreover, considering the well-known harmful effects of chemical products used in agriculture (agrochemicals), such as pesticides, on both environmental and human health, continuous efforts are being made to develop complementary, or rather alternative, control strategies to such products [14]. Among alternative products, chitosan is gaining more and more interest, in particular to improve quality and extend shelf-life of perishable fruits like strawberries [15].

Chitosan is the most important derivative of chitin, resulting from the full or partial removal of acetyl groups (CH_3-CO), which determines its deacetylation degree (DD). It is a natural polysaccharide of glucosamine and *N*-acetylglucosamine units joined by β-1,4-glycosidic links, widely distributed in living organisms, such as crustaceans, fungi and insects. It is non-toxic, biodegradable, biocompatible and soluble in slightly acidic solutions. Furthermore, it has antioxidant and antimicrobial effects, good mechanical properties and film forming capacity [10,16]. Marine crustacean exoskeletons, specifically shrimps and crabs, have been the primary source of chitosan [17], but with many difficulties in recent years because the availability of crustacean shells depends on the fishing industry and seasonal [18]. The enormous costs and harsh conditions, as well as metal contamination in seafood worldwide [19–23], make the process not environmentally sustainable [24] and dangerous for human health [19].

Insects represent an emerging source of chitin, and therefore chitosan, because they are on average made up of 10–15 % chitin, 30–45 % protein, 25–40 % fat [25], chitin being up to 35 % of the exoskeleton of larvae of some insects [26]. Because of their large numbers and because they are easy to breed, the insects provide an abundant resource for large-scale chitin production compared to crustacean, considering overall that the contents and characteristics of insect chitin and chitosan can favorably compete with those of commercials derived from crustaceans [27].

The black soldier fly (*Hermetia illucens*), Diptera, Stratiomyidae family, is a non-pest fly able to convert organic waste into nutrient-rich biomass [28,29]. *H. illucens* has gained great attention in recent years because it can be favorably used as a source of valuable molecules, such as antimicrobial peptides, lipids and chitin, through the valorization of agri-food industry waste [18,30–35]. In general, all developmental stages and related biomasses of the insect can be a good source of chitin and chitosan with a degree of purity and chemical characteristics comparable to those of the commercially available polymer. Specifically, pupal exuviae, one of the waste products of *H. illucens* farming, with high yields of chitin and chitosan, represents a valid alternative and sustainable source of the polymers [36].

Edible coating based on chitosan is used as a tool to improve food appearance and maintain its quality [5]. It acts as a barrier against moisture and oxygen during processing, handling and storage of fruits, including strawberries, and possessess an intrinsic antimicrobial effect because it also act as an antifungal barrier, inhibiting the germination of fungal spores and reducing the decay caused by fungi present in fruits [5,9,16]. Chitosan-based coatings have been successfully used to preserve quality and reduce food losses during storage by inhibiting the growth of microorganism [37]. It was obtained a high antifungal activity against *B. cinerea* under both *in vitro* and *in vivo* conditions, as well as a significantly reduction of incidence and disease severity of gray mold during storage of strawberries coated by dipping with chitosan nanoparticles containing essential oil extracted from *Pistacia atlantica* [38]. Recently, chitosan film-forming dispersions were applied to strawberry fruits as coatings by immersion, demonstrating antioxidant and potential antimicrobial activities [39]. A chitosan-based coating derived from pupal exuviae has been recently formulated and positively evaluated for retarding the decay and improving shelf-life of tomatoes, peaches, nectarines, apricots, strawberries by post-harvest treatment [40–42]. In particular, chitosan from *H. illucens* was effective in preserving and enhancing some crucial post-harvest physicochemical parameters and nutraceutical properties of strawberries stored at room temperature (RT), at 4 °C and at mixed storage conditions (4 °C + RT), thus confirming black fly soldiers as an innovative source of chitin and chitosan to be used in a sustainable agricultural system [41]. On the other hand, an insect-derived chitosan has never been used as an edible coating to control fungal diseases in strawberry fruits. On these bases, this study was thus motivated to investigate, for the first time, the antifungal activity against the main strawberry decay responsible, the fungus *Botrytis cinerea*, by two different chitosans, both derived from *H. illucens* pupal exuviae, but characterized by different physicochemical properties *i.e.* their molecular weights and the presence or not of melanin pigments in the molecule. Specifically, the main objectives of this study were to evaluate the insect-chitosans effects on conidial germination and mycelial fungal growth and morphology, with the aim to gain knowledge on different abilities by the two chitosans and identify the best formulation as a post-harvest edible coating treatment to control gray mold disease on the new Lucanian strawberry (*Fragaria x ananassa* Duch.), cultivar *Melissa*, at different fruit storage conditions.

2. Material and methods

2.1. Preparation of chitosan

Chitosan was produced from pupal exuviae (PE) of *H. illucens* according to Triunfo et al. [36]. PE were provided by Xflies s.r.l (Potenza, Italy). Decolorized (PEDEC) and not decolorized (PEND) chitosan with deacetylation degree (DD) of 87 ± 2 % and the measured viscosity-average molecular weight (M_v) of 75 ± 4 and 145 ± 5 kDa respectively, were obtained [41,42]. Two commercial chitosans with high and low molecular weight (MW), HMW and LMW respectively, were used as controls: HMW with M_v of about 364± 4 kDa and with a DD of 90 ± 1 %; LMW with M_v of about 205 ± 10 kDa and DD of 80 ± 5 % (Sigma-Aldrich Co., St. Louis, MO, USA). For the evaluation of antifungal activity, stock solutions of chitosan were prepared. Chitosan samples were dissolved in 1 % acetic acid solution (*v/v*) at 5 mg mL^{-1} (*w/v*), stirred, filtered and stored at 4 °C until the use.

2.2. Fungal isolate

Botrytis cinerea isolate was provided from the SAFE (School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata) culture collection. The mycelium was grown on Potato Dextrose Agar (PDA) for 14 days in the dark at 22 °C and the conidia were harvested in 10 mL of distilled water by gently scraping with a sterile glass rod. The suspension was filtered through a double layered sterile gauze to remove mycelium and adjusted to concentration of 2×10^7 conidia mL^{-1} for the experiments with a hemocytometer (Thoma counting chamber, BLAUBRAND$^{(R)}$, Wertheim, Germany).

2.3. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The experiment was carried to establish the preliminary *in vitro* ability of insect-chitosans to exert a fungistatic and/or fungicidal effect against *B. cinerea* and to define, at the same time, which concentration could be the suitable to induce them. Indeed, MIC is defined as the lowest concentration of an antimicrobial product that fully inhibits fungal conidial germination as a possible fungistatic outcome, while MFC is the concentration that does not allow any mycelial growth because a fungicidal effect is induced. It was determined by micro-dilution method in 96-well microtiter plates with 40 μL of chitosan stock solution or sterile water (dH_2O) to 150 μL of Luria Bertani (LB) broth and 10 μL of spore suspension or dH_2O, in a final volume of 200 μL (chitosan stock solution diluted 1:5), according to Attjioui et al. [43] with the following modifications: 2×10^7 conidia mL^{-1} of *B. cinerea* were used as initial spore concentration; LB broth was used as culture media; plates were incubated at 22 °C.

The experiment included 6 serial dilutions for each tested chitosan with concentrations of 1, 0.5, 0.25, 0.12, 0.06 mg mL^{-1} with or without fungal spore suspensions. Considering that acetic acid itself possesses a known antimicrobial activity, depending on the concentration, also acetic acid alone was tested as control [44]. Therefore, corresponding serial dilutions were also made for acetic acid to obtain solutions with 0.20, 0.10, 0.05, 0.025 and 0.012 % (*v/v*) of acetic acid. The highest concentrations (5 and 2.5 mg mL^{-1}) were excluded as it was not possible to discriminate the antifungal activity of chitosan from its solvent, acetic acid (data not shown). LB broth with only 2×10^7 conidia mL^{-1} of *B. cinerea* suspension represented the positive control, while LB constituted the blank.

Fungal growth was spectrophotometrically assessed (Microplate reader mod. MULTISKAN FC, Thermo Scientific, Fisher Scientific, Segrate, Italy) measuring the optical density (OD) at 600 nm, daily, for 5 days. The OD reading of each concentration of tested chitosan treatment and LB medium alone, with no inoculum, was subtracted from OD of the corresponding inoculated well in order to eliminate the background OD level deriving from chitosan, acetic acid and LB [45].

To determine the chitosan fungistatic or fungicidal effect, 40 μL of each tested sample were spread on a PDA Petri dish. The concentration that did not allow any mycelial growth, after 72 h of plate incubation at 22 °C, was considered as MFC [46].

2.4. Effects of chitosan on in vitro fungal growth and morphology

In order to investigate the effects on the mycelium growth and hyphal structure of *B. cinerea*, chitosan at the concentration deemed suitable according to the obtained results from MIC experiment, *i.e.* 0.5 mg mL^{-1}, was added in PDA medium and then an agar plug from 7 days old fungal PDA culture was placed in the centre of the plate and incubated for 6 days in the dark at 25 °C.

The inhibition percentage of mycelial growth (GI) was assessed by the following formula:

$$GI\% = [(Rc\text{-}Rt)/Rc] \times 100$$

where *Rc* is the radius of the colonies grown on PDA substrate without chitosan and *Rt* is the radius of the colonies grown on PDA substrate amended with chitosan, measured according to Muñoz and Moret [47].

After slides preparation, the mycelium characteristics were revealed under the optical microscope (Axioskop, Zeiss, Jena, Germany) at 100× resolution.

2.5. In vivo experiment

2.5.1. Plant material

Strawberry (*Fragaria x ananassa* cv "Melissa") fruits (Copyright © 2022 Nova Siri Genetics) were provided by APOFRUIT Italia soc. coop. agricola (Scanzano Jonico, Matera, Italy) in June 2023. The fruits were produced in an organic farm without the addition of chemical pesticides in accordance with organic farming regulations and transported to the laboratory in refrigerated conditions. Strawberries were chosen considering the following characteristics: uniformity of size, surface color and ripening degree; absence of any defects or damages [11]. The experiments were carried out on the same day.

2.5.2. Effect of chitosan coating treatment on B.cinerea disease control

A decay study was conducted to verify the effect of PEND, PEDEC, HWM chitosan treatments on the growth of *B. cinerea* on strawberries during storage at 4 °C for 14 days or at 4 °C for 7 days and further 3 days at room temperature (totally 10 days).

After surface sterilisation with 1 % Sodium-hypochlorite solution for 2 min, fruits were rinsed 3 times with sterile dH_2O and then the excess water allowed to evaporate under laminar flow hood.

Coating solutions were prepared according to Tafi et al. [40], dissolving the required amount of each chitosan in solvent solution (1 % acetic acid *v/v*, 0.2 % Tween-80 *v/v*, 2 % glycerol *v/v*) until complete dissolution of chitosan by stirring for 16 h. Thereafter, pH was adjusted to 3.2. Coating solutions with 0.5 % and 1 % of chitosan were used and distributed by an aerograph.

The infection was carried out by dipping each fruit for 5 s in a solution of *B. cinerea* 1×10^4 conidia mL^{-1}.

The strawberries were separated into 10 groups: 1) healthy and untreated (NEGATIVE CONTROL); 2) only infected with *B. cinerea* (Bc CONTROL); 3) healthy and treated with solvent solution (SOLV); 4) infected with *B. cinerea* and treated with solvent solution (Bc SOLV) 5–7) infected with *B. cinerea* and treated with 0.5 % of PEND, PEDEC, and HWM coating solution; 8–10) infected with *B. cinerea* and treated with 1 % of PEND, PEDEC, and HWM coating solution.

At the observation time, disease incidence (DI) was calculated as the percentage of infected fruits out of the total number of examined fruits for each treatment. The disease severity (DS) was calculated according to an empirical scale with six degrees: 0, healthy fruit; 1, 1–20 % fruit surface infected; 2, 21–40 % fruit surface infected; 3, 41–60 % fruit surface infected; 4, 61–80 % fruit surface infected; 5, >81 % of surface infected and showing sporulation. DS for each treatment was the average class (from 0 to 5) value of examined fruits. Finally, the McKinney's index (MI%), that includes information on both DI and DS, was calculated according to McKinney [48] and Romanazzi et al. [11] by the following formula:

$$MI\% = \sum [(c \times F)/(N \times C)] \times 100$$

where *c* is the category of the disease class recorded for the fruits, *F* is the disease frequency, *N* is the total number of examined fruits and *C* is the highest class of disease intensity that occurred on the empirical scale, that is 5 in our case.

The experiment was set up with 3 replications of 9 fruits.

2.5.3. Extraction and quantification of total phenolic and total flavonoid concentration

Methanolic extracts of strawberry fruits were obtained and used for the determination of total phenolic (TPC) and flavonoid (TFC) contents, according to the methods described in Triunfo et al. [41] and Tafi et al. [40]. TPC was determined by the Folin–Ciocalteau reagent method, measuring the absorbance at 723 nm after 1 h and was expressed as mg of gallic acid equivalent (GAE) g^{-1} of fresh weight (FW). TFC, expressed as mg quercetin equivalent (QE) g^{-1} FW, was determined by the $AlCl_3$ method, measuring absorbance at 510 nm.

2.6. Statistical analysis

All experiments were carried out in triplicate. Normal distribution of data was tested by the Shapiro-Wilk test at $p < 0.05$ and homoscedasticity was tested performing the Bartlett's test ($p < 0.05$). Not normally distributed data (*in vitro* experiments) were analyzed using Kruskall-Wallis H test; normally distributed data (*in vivo* experiment), expressed as mean ($n = 3$) ± SD, were analyzed according to one- or two-way ANOVA followed by Tukey's HSD test ($p < 0.05$) or by Bonferroni test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). R (version 4.2.3, R Foundation for Statistical Computing, Vienna, Austria) using the software RStudio IDE (release 2023.06.0 + 421) to write and run R code was used.

3. Results

3.1. MIC and MFC determination induced by chitosans against B. cinerea

To exclude an eventual interference of acetic acid on chitosan activity, the effect induced on fungal growth by acetic acid alone, at the same amounts used to dissolve chitosan, was considered. Acetic acid was not able to fully inhibit the mycelial growth (Table 1).

The effect induced by chitosans at the six tested concentrations on *B. cinerea* is reported in Fig. 1.

Commercial HMW chitosan induced no mycelial growth at the highest tested concentration (1 mg mL^{-1}), to which the minimum inhibitory concentration (MIC) was assigned. At the same time, a significant reduction of the fungus was obtained also at 0.12 and 0.06 mg mL^{-1} (Fig. 1a). Similarly, 1 mg mL^{-1} was the MIC for LMW chitosan, which also induced a significant reduction of *B. cinerea* growth at the lowest concentrations. An unexpected and significantly higher than the positive control OD value was detected at 0.25 mg mL^{-1} (Fig. 1b).

The fungal growth was fully inhibited by PEND at 1 and 0.5 mg mL^{-1}, so determining this latter as MIC. Noteworthy, also at lower concentrations PEND determined a significant reduction of *B. cinerea* (Fig. 1c).

MIC attributed to PEDEC was the maximum tested concentration (1 mg mL^{-1}) where no fungal growth was observed. Moreover, in the other cases, a significant reduction of *B. cinerea* was determined by PEDEC, in particular at 0.5 and 0.25 mg mL^{-1} (Fig. 1d).

When samples derived from each well tested in microdilution assay were spread on PDA plate, after 72 h incubation at 22 °C, the fungicidal effect was induced only by PEND at 1 mg mL^{-1}, so determining only for that chitosan at that concentration the minimum fungicidal concentration (MFC).

3.2. Influence by chitosans added in the PDA medium on mycelial growth and hyphal morphology of B. cinerea

Fig. 2 shows the inhibition expressed as percentage relative to the control (%). All chitosans were used at the concentration of 0.5 mg mL^{-1} and were able to differently decrease radial growth of *B. cinerea* with respect to the control (assigned inhibition of 0 %). Specifically, the mycelial growth inhibition percentages (GI%) were 70 %, 56 %, 4 %, and 3 % for PEND, HMW, PEDEC, and LMW, respectively. Because of the low efficacy of LMW we decided not to further consider this commercial chitosan in the subsequent experiments.

The interaction between chitosans and the hypha led to changes in their morphology. As shown in Fig. 3, hypha appeared to be shriveled, coiled and containing vacuolar structures (Fig. 3b). Moreover, some damaged hyphae (Fig. 3c) and formless conidiophores (Fig. 3d) were observed.

Table 1
Growth of *Botrytis cinerea* in presence of solutions with different percentage of acetic acid. Different letters indicate significant differences between the different values, according to Kruskall-Wallis H test at $p < 0.05$.

	OD600 nm				
Acid acetic	0.20 %	0.10 %	0.05 %	0.025 %	0.012 %
B. cinerea	0.095 ± 0.003 ^{c}	0.443 ± 0.147 ^{bc}	0.676 ± 0.174 ^{b}	0.909 ± 0.301 ^{a}	0.815 ± 0.256 ^{ab}

3.3. Effect of chitosan coating treatment on B. cinerea disease control on strawberry

The effect of coating with chitosans on strawberry preservation at 4 °C is shown in Table 2. After 14 days, gray mold symptoms were clearly observed in the positive control (Bc CONTROL), which exhibited a 100 ± 0 % incidence, which determined the significantly highest MI% (26.7 ± 6.7 %). Similar behavior was shown by fruits inoculated and treated with solvent (Bc SOLV). On the contrary, HMW and PEDEC at 1 %, and PEND at 0.5 % were able to fully protect the fruits as no symptoms were observed. Anyway, also the other treatments allowed a significant disease reduction with respect to the Bc CONTROL in terms of both incidence (reduction of 66.7 %) and McKinney Index, that was better for Bc PEDEC 0.5 and Bc PEND 1 than Bc HMW 0.5. Notably, PEND treatments showed higher efficiency at 0.5 %.

In the condition of 7 days at 4 °C and further 3 days of exposure at room temperature, altogether the disease increased in all treatments, as expected (Table 3). PEND at 0.5 % resulted in the best treatment able to control *B. cinerea* with significantly lower values with respect to Bc CONTROL and Bc SOLV, apart from the severity from this latter. In particular, Bc PEND 0.5 decreased the incidence of 25.9 %, the disease severity from 3.6 ± 0.2 to 1.9 ± 0.6 and the McKinney index from 71.1 ± 3.8 (Bc CONTROL) and 55.6 ± 3.8 (Bc SOLV) to 28.9 ± 3.8. On the other hand, also the other coating treatments determined reduced values of McKinney Index significantly different from Bc CONTROL and also from Bc SOLV in the case of Bc PEDEC 1 and Bc PEND 1. Remarkably, PEDEC at the highest, and PEND at both doses used for preparing coating reduced gray mold more effectively than commercial chitosan samples.

3.4. Effect of chitosan coating on phenolic compounds of strawberry fruits

The impact of chitosan coating on preserving the phenolic compound content in the local strawberry cultivar *"Melissa"* in the control of *B. cinerea* disease was evaluated by quantifying the content of total phenols (TPC) and total flavonoids (TFC), as reported in Table 4.

In strawberry fruits, application of the chitosan treatments preserved and induced an increase in TPC compared to negative or infected control during storage under the two conditions used.

Strawberries not inoculated with *B. cinerea*, stored at both 4 °C and 4 °C + RT, showed higher TPC when treated with chitosan from *H. illucens*. At 4 °C + RT, in particular, PEND 0.5 and PEDEC chitosan showed the best coating solutions, while PEND 1 chitosan showed a TPC statistically similar to that of HMW 1. The negative and solvent control exhibited the lowest TPC for all storage conditions. In presence of *B. cinerea* infection, the TPC of strawberries showed a significant decrease for all treatments, compared to uninfected fruits for all storage conditions tested (Table 4). Bc-infected strawberries showed the highest TPC only when treated with chitosan from *H. illucens*. In particular, PEDEC chitosan proved to be the best coating solutions during *B. cinerea* disease control in all storage conditions. As for uninfected strawberries, the control and solvent coating exhibited the lowest TPC at all storage conditions (Table 4).

As observed for TPC, chitosan from *H. illucens* showed to preserve and increase the TFC in strawberries under both storage conditions, with the unique exception for PEDEC 0.5 at 4 °C, that was not significantly different from both negative control and solvent (Table 4). At 4 °C, HMW 1 also demonstrated a similar coating effect to insect chitosan. The

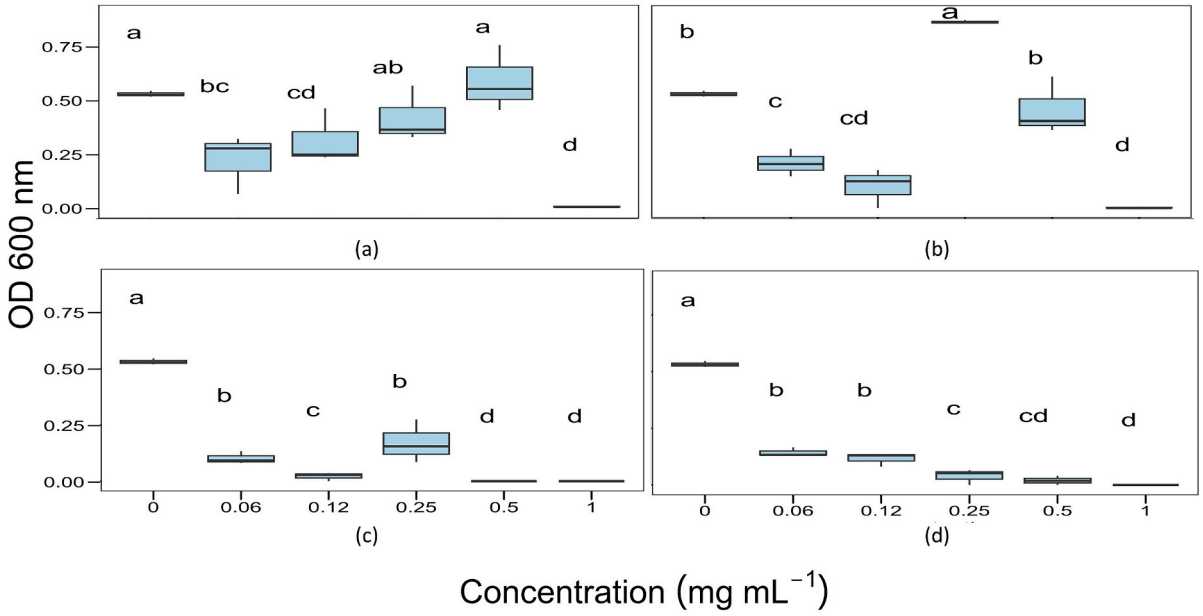

Fig. 1. Box plots absorbance (OD600 nm, $n = 3$) of *Botrytis cinerea* growth in microdilution assay for commercial chitosan with high **(a)** and low **(b)** molecular weight, unbleached (PEND) **(c)** and bleached (PEDEC) **(d)** chitosan from *Hermetia illucens* pupal exuviae, at the six different concentrations, from 0 (positive control including only conidia suspension in LB) to 1 mg mL^{-1}. Different letters indicate significant differences between observed absorbance values for each tested chitosan concentration, according to Kruskall-Wallis H test at $p < 0.05$.

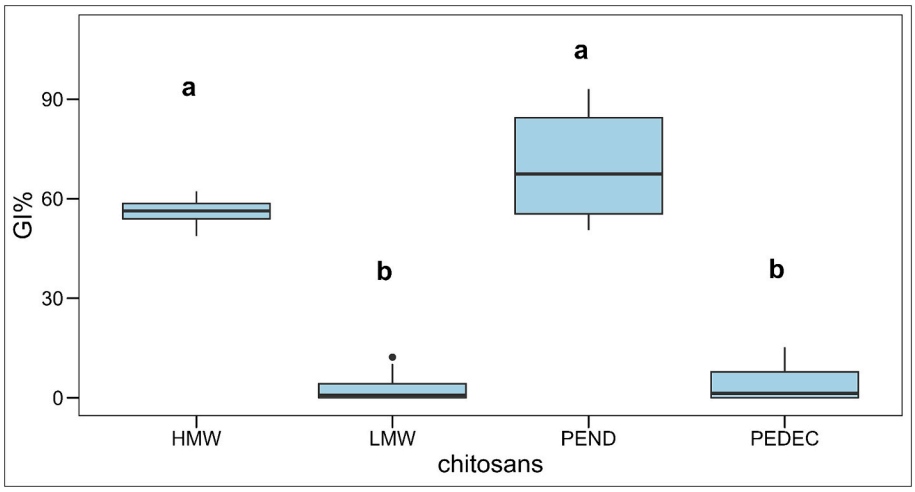

Fig. 2. Box plots representing the percentage of *Botrytis cinerea* growth inhibition (GI%) by chitosans (HMW = chitosan at high molecular weight; LMW = chitosan at low molecular weight; PEND = unbleached pupal exuviae chitosan; PEDEC = bleached pupal exuviae chitosan) in the coating solution added in the media at the concentration of 0.5 mg mL^{-1}. Different letters indicate significant differences between values for each tested chitosan, according to Kruskall-Wallis H test at $p < 0.05$.

TFC in strawberries were less concentrated in both the negative and solvent controls than those treated with chitosan.

Unlike observed for TPC, post-harvest treatment with chitosan from *H. illucens* preserved TFC in strawberries infected with *B. cinerea*, compared to uninfected fruits, particularly PEDEC chitosan at both 4 °C and 4 °C + RT. For all the other treatments, TFC showed a significant decrease with respect to uninfected fruits. As for TPC, the TFC in Bc-infected strawberries was significantly less concentrated in the control and solvent than in those treated with insect chitosan, which showed the highest values (Table 4). Indeed, chitosan from *H. illucens*, more PEDEC than PEND, proved to be the best coating in controlling strawberry fungal decay, preserving and increasing TFC, especially compared to commercial chitosan.

4. Discussions

Strawberry is characterized by a high post-harvest respiration rate, being a non-climacteric fruit, thus leading to a rapid deterioration, in particular if stored at room temperature [1,49]. To help maintain the quality of fresh fruit, a thin layer of edible material can be applied on its surface during post-harvest processing for reducing the use of non-biodegradable packaging materials [50]. The polysaccharide-based coatings are considered safe for both the environment and consumers. In particular, because of its biocompatibility, biodegradability and environmental non-toxicity, chitosan-based edible coatings are considered a suitable tool for improving the shelf-life of strawberry fruits in a safe way [51,52] by counteracting the oxidative stress [53]. Moreover, chitosan represents a safe fungicidal agent to be used as an alternative to agrochemicals for post-harvest treatments to fight several pathogens, in

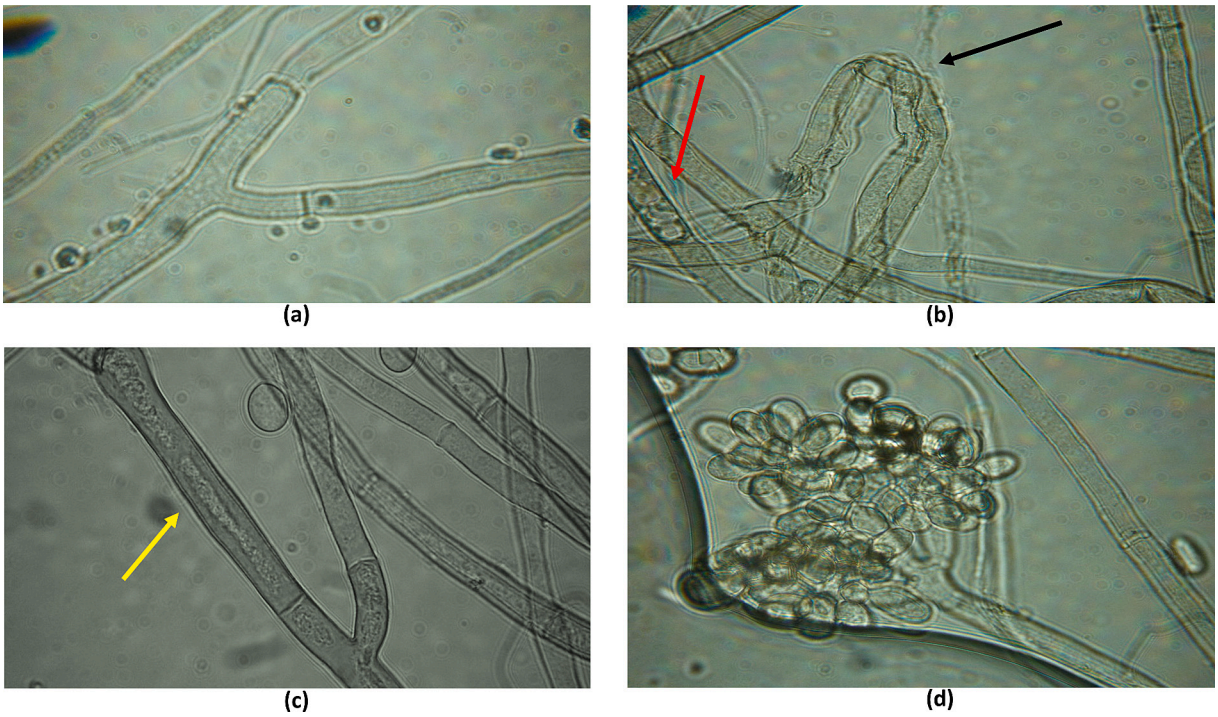

Fig. 3. Representative images showing microscopic structural changes in *Botrytis cinerea* mycelium in response to chitosans treatments at 0.5 mg mL^{-1}: **(a)** negative control mycelium; **(b)** vacuolar structures (red arrow) and hyphae shriveled and coiled (black arrow) of fungus exposed to PEND (unbleached chitosan); **(c)** damages on hyphal surface (yellow arrow) induced by PEDEC (bleached chitosan); **(d)** not well-developed conidiophore, with branches close to the main axis due to HMW (chitosan at high molecular weight) treatment. Resolution 100×.

Table 2
Disease incidence (DI), disease severity (DS), and McKinney's index (MI%) of post-harvest *B. cinerea* induced decay of strawberry fruit stored for 14 days at 4 °C.

Treatment	Incidence (%)	Severity (0–5)	McKinney Index (%)
NEGATIVE CONTROL	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}
SOLV	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}
Bc CONTROL	100.0 ± 0.0 ^{a}	1.3 ± 0.3 ^{a}	26.7 ± 6.7 ^{a}
Bc SOLV	88.9 ± 19.2 ^{a}	1.4 ± 0.5 ^{a}	24.4 ± 3.8 ^{a}
Bc HMW 0.5	33.3 ± 0.0 ^{b}	1.7 ± 0.6 ^{a}	11.1 ± 3.8 ^{b}
Bc HMW 1	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}
Bc PEDEC 0.5	33.3 ± 0.0 ^{b}	1.0 ± 0.0 ^{a}	6.7 ± 0.0 ^{bc}
Bc PEDEC 1	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}
Bc PEND 0.5	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}
Bc PEND 1	33.3 ± 0.0 ^{b}	1.0 ± 0.0 ^{a}	6.7 ± 0.0 ^{bc}

SOLV = solvent used for preparing chitosan coating; Bc = artificially inoculated fruits with *Botrytis cinerea*; HMW = chitosan at high molecular weight; PEDEC = bleached pupal exuviae chitosan; PEND = unbleached pupal exuviae chitosan; 0.5 and 1 = coating solution with 0.5 and 1 % of chitosan. Data are expressed as mean (n = 3) ± SD. Different letters in the same column indicate significant differences between values, according to one-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Table 3
Disease incidence (DI), disease severity (DS), and McKinney's index (MI%) of post-harvest *B. cinerea* induced decay of strawberry fruit stored for 7 days at 4 ° C and then exposed for 3 days at room temperature.

Treatment	Incidence (%)	Severity (0–5)	McKinney Index (%)
NEGATIVE CONTROL	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{d}	0.0 ± 0.0 ^{f}
SOLV	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{d}	0.0 ± 0.0 ^{f}
Bc CONTROL	100.0 ± 0.0 ^{a}	3.6 ± 0.2 ^{ab}	71.1 ± 3.8 ^{a}
Bc SOLV	100.0 ± 0.0 ^{a}	2.8 ± 0.2 ^{abc}	55.6 ± 3.8 ^{b}
Bc HMW 0.5	88.9 ± 11.1 ^{ab}	2.8 ± 0.6 ^{abc}	48.9 ± 3.8 ^{bc}
Bc HMW 1	88.9 ± 0.0 ^{ab}	3.1 ± 0.8 ^{abc}	53.3 ± 0.0 ^{bc}
Bc PEDEC 0.5	81.5 ± 6.4 ^{b}	3.7 ± 0.9 ^{a}	55.6 ± 3.8 ^{b}
Bc PEDEC 1	88.9 ± 0.0 ^{ab}	2.2 ± 0.3 ^{bc}	37.8 ± 3.8 ^{de}
Bc PEND 0.5	74.1 ± 6.4 ^{b}	1.9 ± 0.6 ^{c}	28.9 ± 3.8 ^{e}
Bc PEND 1	88.9 ± 11.1 ^{ab}	2.6 ± 0.4 ^{abc}	44.4 ± 3.8 ^{cd}

SOLV = solvent used for preparing chitosan coating; Bc = artificially inoculated fruits with *Botrytis cinerea*; HMW = chitosan at high molecular weight; PEDEC = bleached pupal exuviae chitosan; PEND = unbleached pupal exuviae chitosan; 0.5 and 1 = coating solution with 0.5 and 1 % of chitosan. Data are expressed as mean (n = 3) ± SD. Different letters in the same column indicate significant differences between values, according to one-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

particular *Botrytis cinerea*, that is the most dangerous for the strawberry decay [54,55]. On the other hand, the seasonality and high costs for producing chitosan from crustaceans [18,24] have led to research alternative chitosan sources, including insects [36]. In literature, chitosan from the bioconverting insect *H. illucens* has already proved to be a good alternative to crustacean chitosan both for its tested chemical and biological characteristics [36,44] and for its application potential, such as for the formulation of coatings to improve the shelf-life of foods [40–42]. Based on these needs, the current work assessed, for the first time, the ability of two different chitosans derived from *H. illucens* pupal exuviae to control *B. cinerea*. In particular, the effects of PEDEC and PEND chitosan were evaluated and compared with those of commercial chitosans, both *in vitro* and *in vivo*. In the first case, an inhibition/reduction of the fungal growth and a disturbance of normal fungal morphology were observed. At the same time, a formulation of an edible coating distributed *via* aerograph was effective to *in vivo* control the gray mold induced by *B. cinerea* in the Lucanian strawberry cultivar *Melissa* post-harvest treated.

Considering that acetic acid was used for dissolving chitosans, before using them, an eventual interference by acetic acid was excluded. The results indicated that acetic acid alone, at the same amounts used to prepare chitosans, in MIC experiments, was able to reduce more the conidial germination of *B. cinerea* as its percentage increased, but never fully inhibited it (see Table 1), as already observed in another study

Table 4
Total phenols (TP) and total flavonoids (TF) on strawberries not infected (Not Bc-infected) and infected with *Botrytis cinerea* (Bc-infected) and treated with chitosan.

	4 °C		4 °C + RT	
	TP (mg GAE g^{-1} FW)	TF (mg QUE g^{-1} FW)	TP (mg GAE g^{-1} FW)	**TF (mg QUE g^{-1} FW)**
Treatments	**Not Bc-infected**			
NEGATIVE CONTROL	0.99 ± 0.03^{e}	0.157 ± 0.04^{b}	0.72 ± 0.01^{e}	0.153 ± 0.04^{b}
SOLV	1.64 ± 0.01^{d}	0.183 ± 0.04^{ab}	1.28 ± 0.01^{d}	0.192 ± 0.03^{ab}
HMW 0.5	1.74 ± 0.02^{c}	0.204 ± 0.02^{ab}	1.36 ± 0.02^{c}	0.198 ± 0.02^{ab}
HMW 1	1.81 ± 0.01^{b}	0.221 ± 0.02^{a}	1.47 ± 0.01^{b}	0.201 ± 0.02^{ab}
PEDEC 0.5	2.05 ± 0.04^{a}	0.206 ± 0.01^{ab}	1.59 ± 0.01^{a}	0.241 ± 0.01^{a}
PEDEC 1	1.96 ± 0.01^{a}	0.219 ± 0.01^{a}	1.60 ± 0.04^{a}	0.248 ± 0.01^{a}
PEND 0.5	1.97 ± 0.01^{a}	0.242 ± 0.01^{a}	1.62 ± 0.01^{a}	0.235 ± 0.01^{a}
PEND 1	2.00 ± 0.02^{a}	0.230 ± 0.01^{a}	1.48 ± 0.02^{b}	0.214 ± 0.01^{a}
	Bc-infected			
Bc CONTROL	$0.85 \pm 0.08^{c**}$	$0.100 \pm 0.02^{c*}$	$0.51 \pm 0.01^{d***}$	$0.078 \pm 0.04^{c**}$
Bc SOLV	$0.98 \pm 0.1^{c***}$	$0.101 \pm 0.02^{c***}$	$0.57 \pm 0.01^{d***}$	$0.108 \pm 0.01^{c***}$
Bc HMW 0.5	$1.27 \pm 0.06^{b***}$	$0.132 \pm 0.01^{bc**}$	$0.83 \pm 0.04^{c***}$	$0.131 \pm 0.01^{bc**}$
Bc HMW 1	$1.26 \pm 0.06^{b***}$	$0.139 \pm 0.04^{bc***}$	$0.98 \pm 0.05^{b***}$	$0.135 \pm 0.01^{bc**}$
Bc PEDEC 0.5	$1.48 \pm 0.05^{a***}$	0.201 ± 0.04^{a}	$1.42 \pm 0.08^{a***}$	0.234 ± 0.04^{a}
Bc PEDEC 1	$1.44 \pm 0.05^{a***}$	0.208 ± 0.05^{a}	$1.30 \pm 0.05^{a***}$	0.228 ± 0.01^{a}
Bc PEND 0.5	$1.39 \pm 0.04^{ab***}$	$0.170 \pm 0.01^{ab**}$	$1.06 \pm 0.07^{b***}$	$0.155 \pm 0.02^{b***}$
Bc PEND 1	$1.32 \pm 0.03^{ab***}$	$0.159 \pm 0.01^{ab**}$	$1.11 \pm 0.05^{b***}$	0.185 ± 0.01^{ab}

SOLV = solvent used for preparing chitosan coating; Bc = artificially inoculated fruits with *Botrytis cinerea*; HMW = chitosan at high molecular weight; PEDEC = bleached pupal exuviae chitosan; PEND = unbleached pupal exuviae chitosan; 0.5 and 1 = coating solution with 0.5 and 1 % of chitosan. Data are expressed as mean ± SD ($n = 3$). Means followed by different letters in the column are significantly different ($p < 0.05$) by one-way ANOVA and Tukey post-hoc test. Asterisks indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) between not infected and infected with Bc within the same treatment (data analyzed with two-way ANOVA and Bonferroni test).

[56]. For the same reason, when chitosans were tested on strawberry fruits, a control represented by the coating with the only solvent solution used for dissolving chitosans (1 % acetic acid *v/v*, 0.2 % Tween-80 *v/v*, 2 % glycerol *v/v*), was considered. Even in this case, the solvent solution did not interfere with chitosans because values obtained from SOLV and Bc SOLV treatments were almost always not significantly different from those of NEGATIVE CONTROL and Bc CONTROL, respectively (see Tables 2, 3 and 4).

During the *in vitro* assays, it was possible to determine the minimum inhibitory concentration for all the tested chitosans. In particular, a MIC of 1 mg mL^{-1} was fixed for PEDEC, and it was the same of the both commercial HMW and LMW. Remarkably, PEND showed its MIC already at 0.5 mg mL^{-1} (see Fig. 1). Moreover, at the highest tested concentration of 1 mg mL^{-1}, PEND also induced a fungicidal effect (MFC), thus suggesting that this property could be linked to its ability better than the other chitosan samples to cause morphological modifications in the fungal cell wall [57]. Silva Júnior et al. [58] demonstrated that chitosan has the ability to induce changes in surface morphology of *B. cinerea* and cause serious damage to the cell structure. The authors observed distorted and damaged hyphae and a layer on the cell surface of the fungus, ultimately resulting in the death of hyphae and a consequent its growth inhibition. In agreement to this study, only PEND determined a fungicidal effect, together with the significant highest percentage of mycelial growth inhibition, when it was added into the PDA medium (see Fig. 2). Moreover, when observed under optical microscope, *B. cinerea* hyphae grown in this substrate enriched with PEND appeared shriveled and coiled, and contained vacuolar structures.

It is well known and documented that the antimicrobial activity of chitosan against plant pathogens depends on the physicochemical properties of chitosan, mainly MW and DD [59], as well as other factors such as pH, temperature, solubility, derivatization, type of organism [60]. In particular, MW and DD significantly influence the biological features of the polymer [61]. The results of the MIC test demonstrated that a decrease in the MW led to an increase of the *in vitro* activity to counteract spore germination and fungal growth. Indeed, PEDEC ($M_v = 75 \pm 4$; DD = 87 ± 2 %) and PEND ($M_v = 145 \pm 5$; DD = 87 ± 2 %), that possess the lowest MW, showed OD values at 600 nm always definitely lower than LMW ($M_v = 205 \pm 10$; DD = 80 ± 5 %) and HMW ($M_v = 364 \pm 4$; DD = 90 ± 1 %) at the same tested concentration (see Fig. 1). This is because the mobility, attraction and ionic interaction of the smaller chains of PEDEC and PEND chitosans are probably easier than the large ones of LMW and HMW, and therefore they can adopt an extended conformation with better binding ability, and therefore provoke greater damages to the membrane surfaces of the pathogen [59,62,63]. Our finding agrees with a study of Badawy and Rabea [64], describing that the *in vitro* antifungal activity against *B. cinerea* of depolymerized chitosan with different MWs (0.5×10^4, 3.7×10^4, 5.7×10^4, and 2.9×10^5 Da) increased as the chitosan MW decreased. On the contrary, the inhibition of mycelial growth resulted in the highest for PEND (GI = 70 %), followed by HMW (GI = 56 %) and the lowest for PEDEC (GI = 4 %) and LMW (GI = 3 %) (see Fig. 2). The discrepancies between the different responses in the two experiments may result from the different MWs and DD distributions of chitosans [60], in particular to explain the low efficacy of LMW in the inhibition assay. The DD of chitosan, that is the ratio of *N*-acetyl-d-glucosamine to d-glucosamine structural units, has an impact on the moisture absorption, intrinsic viscosity, chitosan solubility in aqueous solutions and the extent of charge distribution [65]. The microbial activity of chitosan results from the electrostatic interaction between its positively charged amino groups and negatively charged phospholipids in the pathogen's cell membrane [39]. On these bases, our result suggests that DD is probably an important factor implicated in the activity of the commercial chitosans derived from crustaceans, with the antimicrobial activity directly proportional to the DD, thus excluding LMW from having efficacy to inhibit *B. cinerea* mycelial growth [66]. In the case of insect-derived chitosans, which have a different MW and the same DD, it is likely that the poor activity of PEDEC in inhibiting mycelial growth *in vitro* may be due to the absence of pigments in its molecule, with respect to PEND [31]. Indeed, chitin in the insects is found in the form of chitin-protein fibers or chitin-melanin complex, while in the crustaceans it is linked only with proteins where calcium carbonate is deposited [67–69]. Melanin pigments present in insects are bound to chitosan through partially deacetylated amino groups [31], and it is identified as a unique feature of chitin from black soldier fly pupal exuviae [70]. Analyzing the composition of *H. illucens* prepupae, Caligiani et al. [71] individuated melanin as a possible nitrogen-containing compound, confirming the statement by Ushakova et al. [72] that the melanization process, going from the larval to adult stage, can led to an increasing content of melanin in the black soldier fly, with the maximum melanin amount being in the pupa [73]. An exclusive combination of biologically active polymers, that are chitin and melanin, is formed during the *H. illucens* life cycle, and then a stable chitosan–melanin complex is obtained [31,74,75]. Noteworthy, melanin has been shown to possess broad-range antimicrobial activity and contribute to protect the insect against bacterial and fungal infections [73]. A mechanism of membrane damage has been revealed when intracellular homogeneous melanin of the saprophytic fungus *Lachnum*

YM30 was used against the two bacteria *Vibrio parahaemolyticus* and *Staphylococcus aureus* by damaging the integrity of cell membrane, increasing the leakage of cell contents, and reducing the membrane potential [76]. Similarly, chitosan is able to disrupt and penetrate the cell membranes of fungi due to electrostatic interactions, determining a cell leakage and leading to intracellular responses [77]. Actually, melanin is vital for virulence in some plant fungal pathogens such as *Colletotrichum gloeosporioides* [78], but is not required for virulence of *B. cinerea* [79]. Due to its enhanced biological activity induced by the mutual complement between chitosan and melanin properties [31] and similar mechanisms inducing cell membrane damages [76,77], we may suppose that PEND could directly act on the *B. cinerea* cell walls with a stronger efficacy than PEDEC, despite the lower MW of the latter, and that it could reach the best antifungal activity when used at 0.5 mg mL^{-1} *in vitro* and at 0.5 % *in vivo*. In fact, strawberry fruits treated with the coating solution PEND 0.5 against *B. cinerea* showed a McKinney Index of 0 %, like the other two chitosans, but used at the highest percentage in the coating solution (1 %), in the refrigerated storage condition (see Table 3). Meanwhile, in mixed condition of storage (7 days at 4 °C and 3 days at room temperature), PEND used at 0.5 % was the only one to induce the best significant 42 % reduction of the McKinney Index (28.9 ± 3.8 %) compared to the Bc control (71.1 ± 3.8 %) due to the significant reduction of both disease incidence (26 % decrease) and severity (from 3.6 ± 0.2 of Bc control to 1.9 ± 0.6) (see Table 4). However, despite the small differences between them, our findings are in line with the statement that the biological activity of chitosan is largely dependent on several factors such as MW, DD and that the mode of action is also strongly dependent on the fungal type that in turn influences the effect of MW and DD on the activity of chitosan itself [77].

When applied to control post-harvest gray mold in wounded tomato fruit, it was demonstrated that chitosan from crustaceans with MW of 57 kDa was efficient, in term of disease incidence and total phenolic compounds enhancement in tomato fruit stored at 2 °C for 21 days and 25 °C for 3 days; moreover, among all chitosan tested with different MWs of 5, 37, 57, and 290 kDa, the lowest MW induced the smallest increase in total phenolics [57]. In the current work, where strawberry was treated by coating with chitosans derived from *H. illucens*, we observed results partially comparable with this study. Indeed, chitosan with the lowest MW PEDEC did not show the best efficiency in term of disease incidence with respect to PEND when strawberries were refrigerated and then exposed at room temperature (see Table 3), as above mentioned, but showed the significantly highest values of total phenolic compounds among all treatments in fruits infected with *B. cinerea* at the same storage conditions (see Table 4). Sun et al. [80] reported that the antioxidant activity of chitosan is strongly related to its MW, with antioxidant activity increasing with decreasing MW. In addition, El Ghaouth et al. [81] asserted that chitosan can play a double function by interfering directly with fungal growth and also by activating several defense processes in the host. In agreement with both of these studies, altogether, our results seem to strengthen our view that PEND could act directly against the fungus, with its effects predominantly associated with fungitoxic properties, while PEDEC might principally provide viable alternatives, such as the elicitation of biochemical defense responses in fruits, for example through total phenols [60,64]. Indeed, TPC and TFC are indicators of natural bioactive compounds considered favorable and promoting plant health in strawberry [82]. Therefore, the main impact of PEDEC on the interaction between the fruit and the fungus could be related, among the other things, to phenolics and flavonoids, which exert significant antioxidant activities [83]. In particular, antioxidant capacity in strawberry fruit was supposed to be dependent on the level of flavonoid groups [83]. So, this might be the reason why total flavonoids concentration in presence of *B. cinerea* in fruits treated with PEDEC always resulted not significantly different from that of the uninfected fruits (see Table 4). Furthermore, no wonder that TFC values were higher in mixed storage condition with 3 days at room temperature than that only refrigerated, considering that high temperatures, close to 30 °C, seem to intensify and promote the antioxidant activity in strawberry, mainly regard the phenolic and anthocyanin compounds content [84]. More recently, Ioannou et al. [85] demonstrated that heat processing of the flavonoids induces an increase of the *in vitro* and *in vivo* antioxidant activities without any negative cytotoxicity effect by the products of degradation obtained.

5. Conclusions

This study investigated the antifungal activity of two chitosans derived from *H. illucens* pupal exuviae and evaluated the efficacy of coatings made from these chitosans, PEND (not decolored) and PEDEC (decolored), for the first time, against the main strawberry decay responsible, the fungus *Botrytis cinerea*. The activity of these insect-chitosans was influenced from various factors, such as molecular weight, deacetylation degree and type of organism from which it derives. The results indicated that PEND was the best chitosan to control *B. cinerea* when used at 0.5 %, both *in vitro* and *in vivo*, and that it could act directly against the fungus, with its effects predominantly associated with fungitoxic properties. Meanwhile, PEDEC was able to control *B. cinerea* at the highest percentage of use *i.e.* 1 %, by providing viable alternatives, such as the elicitation of biochemical defense responses in fruits, through total phenols, and in particular flavonoids. Both chitosans were able to preserve the health of fruits up to 14 days in refrigerated storage condition, but to reduce the gravity of the disease better than commercial chitosans up to 10 days in mixed storage conditions (7 days at 4 °C + 3 days at room temperature).

Based on the current findings, further study could be addressed to the modification of some chitosan properties or to the variation of its concentrations for enhancing the effect of coating to protect the fruits. Moreover, studies on the elucidation of mechanisms involved in the interaction between strawberry, *B. cinerea* and chitosan from *H. illucens* would help better utilization of this biopolymer. Induction of gene expression in strawberries related secondary metabolites production and biochemical analysis of compounds involved should be the major target of further study. Surely, the current work undoubtedly seemed to prove, for the first time, that both chitosans derived from the black soldier fly showed equal or better potential application than chitosan derived from crustaceans in controlling gray mold induced by *B. cinerea* and, therefore, usable for sustainable production of high-quality strawberry.

CRediT authorship contribution statement

Antonella Vitti: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation. **Leonardo Coviello:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Micaela Triunfo:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Anna Guarnieri:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Carmen Scieuzo:** Writing – review & editing, Formal analysis, Data curation. **Rosanna Salvia:** Writing – review & editing, Investigation, Data curation. **Patrizia Falabella:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation. **Maria Nuzzaci:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Data curation, 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 presented in this study are available on request from the corresponding authors.

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