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Effective Postharvest Preservation of Kiwifruit and Romaine Lettuce with a Chitosan Hydrochloride Coating

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Abstract: Kiwifruits and romaine lettuce, among the most horticulturally-consumed fresh products, were selected to investigate how to reduce damage and losses before commercialization. The film-forming properties, physico-chemical, and morphological characteristics, as well as the antimicrobial response against *Botrytis cinerea* and *Pectobacterium carotovorum* subsp. *carotovorum* of chitosan hydrochloride (CH)-based coatings were investigated. The results underlined the film-forming capability of this CH that maintained its physico-chemical characteristics also after dissolution in water. Morphological investigations by FESEM (Field Emission Scanning Electron Microscopy) underlined a well-distributed and homogeneous thin coating (less than 3–5 μm) on the lettuce leaves that do not negatively affect the food product functionality, guaranteeing the normal breathing of the food. FESEM images also highlighted the good distribution of CH coating on kiwifruit peels. The in vitro antimicrobial assays showed that both the mycelial growth of *Botrytis cinerea* and the bacterial growth of *Pectobacterium carotovorum* subsp. *carotovorum* were totally inhibited by the presence of CH, whereas in vivo antimicrobial properties were proved for 5–7 days on lettuce and until to 20–25 days on kiwifruits, demonstrating that the proposed coating is able to contrast gray mold frequently caused by the two selected plant pathogens during postharvest phases of fruit or vegetable products.

Keywords: chitosan hydrochloride; coating; edible film; food safety; postharvest; antimicrobial properties; *Botrytis cinerea*; *Pectobacterium carotovorum* subsp. *carotovorum*; rotting

1. Introduction

Harvested fruits and vegetables, when infected by degrading microbes, inevitably undergo a reduction of economic value, a significant reduction of the food shelf-life, and often become insecure for human safety [1]. In 2011, a FAO (Food and Agriculture Organization of the United Nations) report quantified postharvest losses for about one-third of the fresh consumables produced worldwide [2], which explains the great efforts of researchers and industries to counteract these costs in recent decades.

The main strategy to control postharvest rot provides for the use of fungicides [3]. However, their use can be both non-economical, when the cost of treatment exceeds the loss from rot, and dangerous, because of the risk to select resistant strains by reiterated applications [4]. Moreover,

the risk for human health and environment pushes urgently for new, effective, and safer control strategies against postharvest diseases. Biological control with antagonistic microorganisms is one of the most promising alternatives, within the development of technologies able to preserve fresh horticultural products from dangerous microorganisms (bacteria and fungi) during postharvest [5–7]. The use of natural compounds or less aggressive additives as chemical extracts was also recently investigated [8–11]. Alternative postharvest solutions are becoming essential to preserve the freshness and quality of food products, and the application of edible coatings lend themselves to this aim, with promising results already reported in preserving the quality of products [12–14].

Recently, the use of nontoxic materials to develop edible coatings with the idea to preserve human health was proposed [15–17]. Specifically, edible coatings are thin layers of non-toxic materials, often extracted from animal or vegetal source, and applied directly on to the food surface. The use of edible coatings to preserve food product quality is a relatively low cost and environmentally friendly strategy with several advantages, including biodegradability, as well as the possibility to obtain a semi-permeable barrier against gases and water vapor, reducing microbial attack [18]. Furthermore, edible coatings can be combined with natural or synthetic active principles to prevent microbial decay in a more effective manner [19]. Natural additives, such as essential oils and fruit seed extracts have demonstrated good antimicrobial and antifungal activity with biopolymeric compatibility, thus lending themselves to be added into edible coating formulations [20]. Plant extracts offer advantages in terms of low production costs, low toxicity, and good biodegradability [21]. Additionally, most of the extracts are rich in polyphenols which can improve the antioxidant properties of the edible coatings [22].

In this research activity, chitosan hydrochloride (CH) film-forming solution was considered as an active coating for kiwifruit and lettuce and its effect as an antimicrobial agent was investigated. Chitosan hydrochloride is used as basic substance in plant protection; it is purposed in conformity with the legal provisions of Regulation (EC) No 1107/2009 [23]. Recently, chitosan and its byproducts, like oligochitosan, have been investigated to control postharvest diseases [17]. Chitosan is a natural, nontoxic in a range of toxicity tests, biocompatible, safe, and biodegradable natural alkaline polysaccharide derived from the de-acetylation of chitin [24], widely applied in biotechnology, medicine, water treatment, food science, and agriculture [25]. In the agricultural sector, chitosan has been used in leaf, seed, vegetable, and fruit coatings, and in plant protection [26]. Chitosan is obtained by deacetylating chitin comprising copolymers of β(1,4)-glucosamine and *N*-acetyl-d-glucosamine, and it can be extracted from wastes of the food-processing industry (shrimps, shells of crabs, krill, and lobsters) by using a concentrated basic solution combined with high temperatures [27]. Chitosan was classified as safe (GRAS) by the US Food and Drug Administration (FDA) in 2001 and it presents bacteriostatic and fungistatic properties [28] that perfectly address the active edible coating concept to preserve the freshness and to avoid the microbial growth on the surface of vegetable and fruit products [11]. The scavenging ability of chitosan is associated with the presence of active hydroxyl and amino groups in the polymer chains. The hydroxyl groups in the polysaccharide units can react with free radicals, and according to free radical theory, the amino groups of chitosan can react with free radicals to form additional stable macroradicals [29].

The aim of this work is to analyze the effect of chitosan hydrochloride-based edible coating for the preservation of fresh vegetables and fruits, specifically of lettuce and kiwifruits, from soft rots and gray mold caused, respectively, by *Pectobacterium carotovorum* subsp. *carotovorum* and *Botrytis cinerea* during postharvest storage. The effectiveness of this innovative treatment was evaluated both in vitro and in vivo on kiwifruits of *Actinidia deliciosa* cv. Hayward and on heads of Romaine lettuce (*Lactuca sativa* var *longifolia*).

2. Materials and Methods

2.1. Materials

Romaine lettuce was collected in Central Italy, Lazio region, Romaine lettuce, as a dark leafy green, is rich source of vitamin K, vitamin A, antioxidants, and a moderate source of folate and iron. It is particularly requested from consumers and, so, its cultivation and production in the USA recently increased up to 40% [30].

Kiwifruits were collected in Central Italy, Lazio region (Cisterna di Latina, LT), the most important Italian kiwifruit area, where around 8000 hectares of *Actinidia* spp are cultivated. The *Actinidia* cultivation and the kiwifruit production show a trend of an exponential increase that has reached nearly 100,000 hectares, and Italy is the world largest producer of kiwifruit with commercial value, excluding China.

Chitosan hydrochloride (CH—M_w = 60,000 Da, degree of deacetylation = 80%–90%) was supplied by Sigma-Aldrich® (St. Louis, MO, USA).

2.2. Preparation and Characterization of Film Coating-Forming Solution

Chitosan hydrochloride solution (1 g/L) was prepared by dissolving the polymer in deionized water under magnetic stirring for 2 h at room temperature (RT). The chitosan solution was cast onto a Petri dish cover by Teflon® (The Chemours Company, Wilmington, DE, USA) and dried at RT for 24 h. The preparation of the CH-based film before the coating application on food product surface was useful to evaluate the morphological, chemical, and thermal characteristics.

The microstructure of CH pellet and produced film (surface and fractured surfaces) were investigated by FESEM (Supra 25-Zeiss, Carl Zeiss Microscopy GmbH, Jena, Germany) after gold sputtering and by using an accelerating voltage of 5 kV. Fourier infrared (FTIR) spectra of chitosan powder and chitosan film were analyzed by using a Jasco FTIR 615 spectrometer (Jasco Inc, Easton, MD, USA) in the 400–4000 cm^{-1} range, in transmission mode. The chitosan hydrochloride pellet was analyzed using KBr discs while a few drops of chitosan solution were casted on a silicon wafer and investigated. Thermogravimetric measurements (TGA) were performed by using a Seiko Exstar 6300 (RT Instruments Inc., Woodland, CA, USA). Heating scans from 30 to 900 °C at 10 °C·min^{-1} under nitrogen atmosphere were performed for the chitosan pellet and film.

2.3. In Vitro Antimicrobial Assays

The in vitro antimicrobial activity of chitosan hydrochloride was assayed by preliminary incorporation of the active ingredient in respective media for each of the two considered pathogens (both bacterium and fungus). Chitosan hydrochloride was dissolved at 1% (w/v) concentration in sterile distilled water under magnetic stirring for 2 h at RT. Then, 100 mL of the solution was added to 1 L of nutrient agar (NA) medium immediately before it was poured into the Petri dishes at a temperature of 40–45 °C, to obtain a final concentration of 1 g/L in the medium. Parallel controls were maintained with corresponding amounts of sterile distilled water mixed with NA medium.

Antimicrobial assays were developed by using aggressive micro-organisms on cultivated plants and able to cause severe damage and economic losses, especially during postharvest phases. Specifically, a high virulent strain of the fungal pathogen *Botrytis cinerea*, able to colonize host tissue by a relevant mycelium development and causing extended gray mold, and the collapse of kiwifruits during their storage/commercialization [31]. Additionally, a highly virulent bacterial strain of *Pectobacterium carotovorum* subsp. *carotovorum* able, due to its enzymatic properties, to destroy external and internal tissues of several vegetables, such as lettuce, was selected and used [32].

Concerning *B. cinerea*, the known strain CBS 120091 (obtained from Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) was stored in the microbial collection of the Department of Agriculture and Forestry Science (DAFNE) at the University of Tuscia, Viterbo, Italy. After the revitalization on potato dextrose agar (PDA) medium, plugs 5 mm in diameter were excised from a

freshly-grown culture and positioned at the center of the Petri dishes filled with the PDA medium incorporated with CH, and then incubated at 24 ± 1 °C for seven days. The treatment, as for control without CH, was tested in triplicate. The radial growth of mycelium was measured along perpendicular axes from inoculum point at the center of the plate (four measures per each plate) after one, two, three, four, and seven days after inoculation.

With respect to *P. c.* subsp. *carotovorum*, the known bacterial strain used (DSM 30184 from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in the experiment was stored in the same microbial collection of DAFNE. After revitalization on NA supplemented by 5% of sucrose (NAS), bacterial colonies were collected and suspended in sterile distilled water (SDW) at a concentration of 1×10^6 CFU/mL. Then, 100 µL aliquots of the bacterial suspension were homogeneously distributed on the surface of Petri dishes filled with the NA medium incorporated with CH, as described, and then incubated at 27 ± 1 °C for 48 h. The treatment, as for control without CH, was tested in triplicate. The growth of bacterial colonies was assessed 48 h after inoculation.

2.4. Coating Application and in Vivo Tests on Kiwifruits and Lettuce

The in vivo tests, both those on kiwifruit and on lettuce, have been set to reproduce, at best, what is provided in common postharvest treatments. The application of CH coating, indeed, was simply obtained by full immersion in chitosan hydrochloride solution (1 g/L) after a previous washing in tap water. Then kiwifruits and vegetables were dried at room temperature.

More specifically, for kiwifruit, the wounds, normally caused by detachment from the plant during harvest, were simulated by a cut with a sterile scalpel at the petiole. Then, fruits were immediately immersed for 30 s in a bath containing the chitosan hydrochloride solution, or the other active ingredients chosen for the comparison, or pure water as a control. According to the market analysis, Fenhexamid, as chemical active ingredient present in the most used commercial formulation (Teldor® Plus, New Boston, NH, USA) was selected and utilized, dissolved in water at the commercially-suggested dose (1.2 g/L) for comparative treatment. Then, 100 µL aliquots of a suspension containing 1×10^6 conidia/mL of *B. cinerea* in sterile distilled water, as assessed by a Thoma cell counting chamber, were distributed by pipette on the surface of the cut petiole. Inoculated fruits were then transferred into a storage box at 4 °C. Six homogenous fruits were used per each treatment: chitosan solution, one commercial formulation, and SDW for both positive (inoculated fruits) and negative (not inoculated fruits) controls, for a total of 30 fruits. The entire experiment was repeated twice. The effectiveness of treatments was evaluated by means of a quantitative scale for gray mold severity, in which 0 is for totally healthy fruits, 1 is for fruits with 1%–20% surface damage, 2 is for fruits with 21%–40% surface damage, 3 is for fruits with 41%–60% surface damage, 4 is for fruits with 61%–80% surface damage, and 5 is for fruits with >81% surface damage. Fruits were examined and data collected at 1, 7, 14, 17, 20, 23, and 25 days after inoculation. Data were statistically analyzed by Tukey's HSD multiple comparison test with $p = 0.01$.

On Romaine lettuce (*Lactuca sativa* L. var. *longifolia*), five heads were first cut off by sterile scalpel at the taproot, to imitate what happens during harvest, washed in a water bath, and treated with chitosan hydrochloride solution. As a reference for the effectiveness of the treatment (positive control), five heads were alternatively treated with a sodium hypochlorite solution (1 mL/L), which is a common postharvest treatment for lettuce, or not further treated, as negative control (five heads). After the treatments, the lettuce heads were dried at room temperature and then inoculated on the fresh cut by spraying about 500 µL of a bacterial suspension (1×10^6 CFU/mL). A set of five plants were not subjected to inoculum as an additional control. After the inoculation, lettuce heads were kept at 90% RH and 26 °C to ensure environmental conditions ideal for bacterial proliferation. The entire experiment was repeated twice. The disease progression on lettuce heads was checked at one, three, and five days after inoculation, by means of a scale of damaging on the taproot cut surface, in which 0 is for totally healthy cut, 1 is for 1%–20% surface damage, 2 is for 21%–40% surface damage, 3 is

for 41%–60% surface damage, 4 is for 61%–80% surface damage, and 5 is for >81% surface damage. Data were statistically analyzed by Tukey's HSD multiple comparison test with $p = 0.01$ by DSAASTAT software (Version 1.022).

Finally, the presence and microstructure of chitosan coatings on lettuce leaves and cores, and on kiwifruit peel, were investigated by FESEM (Supra 25-Zeiss, Carl Zeiss Microscopy GmbH, Jena, Germany) after gold sputtering and by using an accelerating voltage of 5 kV.

3. Results and Discussion

3.1. Morphological, Chemical, and Thermal Properties of Polymer Pellet and Chitosan-Based Film Coating

The bioactivity of chitosan is a function of its physico-chemical properties which also have an effect on its film-forming characteristics [33]. For this reason, morphological, chemical, and thermal properties of a polymer pellet and chitosan-based coating film were investigated to study the applicability of the selected commercial polymer grade as a coating with the final goal to preserve the food quality and safety during the storage, transportation, and market period.

The microstructure of the CH pellet and produced film (surface and cross-section) were investigated by FESEM in order to prove the film-forming capability of the selected grade of polymer in water and investigate the morphological and physico-chemical properties of the obtained film-coating before its application on the fresh food. Chitosan hydrochloride powder is a white or off-white odorless, semitransparent, and amorphous material. FESEM micrographs of the CH pellet at different magnifications are shown in Figure 1. The CH powder appeared agglomerated into flakes with irregular shape and having dimensions ranging between 10 and 100–200 μm (Figure 1a,b) [34]. Chitosan hydrochloride solution was then prepared by dissolving the polymer, at a specific concentration (1 g/L), in deionized water, by magnetic stirrer. As demonstrated by the inset in Figure 1b, this grade of polymer presented a perfect ability to dissolve in water, forming a clear and stable solution characterized by a pH value of 6.22 [17]. For this ability to easily dissolve in a sustainable solvent, chitosan hydrochloride is widely used in the food industry as a preservative and can keep fruits and vegetables fresh. After the dissolution, the obtained CH solution was cast onto a Petri dish and dried in order to test the film-forming capability of the selected commercial polymer. Figure 1c,d show the surface topography of the obtained film at different magnifications. The film surface was characterized by a well-defined porous structure, with interconnected pores that are also present on the cross-sections of the obtained film, as shown in Figure 1e,f. FESEM investigation of the film fractured surfaces, in fact (Figure 1e,f), underlined a tri-dimensional porous structure on the film thickness, whereas thin films (4.8 ± 0.3 μm thick), typical of medium-low molecular weight polymer, were obtained. This morphology will guarantee the breathing of fruit-vegetable products of fundamental importance during the storage, transportation, and market period for their correct conservation.

Furthermore, before the application of the CH coating on fruit and vegetable products, chemical characterization by FTIR and the investigation of the thermal stability by TGA, before and after the dissolution in water of CH powder, were performed and compared. Figure 2a shows the FTIR spectra for the chitosan pellet and film, whereas Table 1 shows the assignment of the main peaks at each wavenumber. The results underlined that not particular alterations on the chemical properties of CH powder were induced by the dissolution in water since all the main characteristic bands are present in both the CH pellet and film spectrum. The main characteristic bands were assigned to saccharide structures at 900, 1020, and 1155 cm^{-1} and strong amino characteristic bands at around 1635 and 1570 cm^{-1} assigned to amide I and amide II bands, respectively. Furthermore, the peak at about 1250 cm^{-1} corresponds to the amino group, whereas 2884 cm^{-1} corresponds to the C–H stretching [35,36]. All these cited peaks, characterizing both the CH pellet and film spectra, underlined that the film-forming procedure does not affect the chemical properties of the selected polymer; however, a shift of the OH stretching, centered at 3430 cm^{-1} for the chitosan pellet and at 3370 cm^{-1}

for the film was registered (Table 1), and a more intense associated band (Figure 2a) was highlighted for the CH film, induced by the solvent casting procedure in water. This result was also confirmed by TGA analysis.

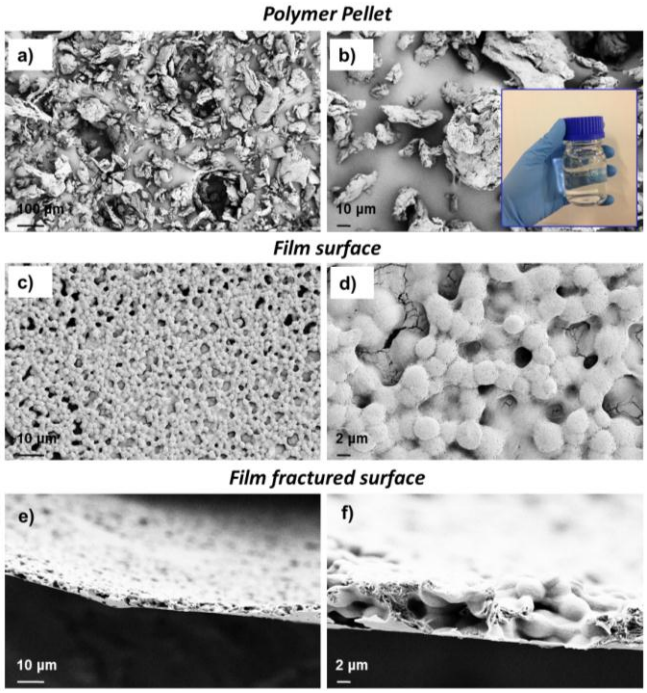

Figure 1. FESEM investigation of (**a**,**b**) chitosan pellet, (**c**,**d**) surface, and (**e**,**f**) fractured surface of the chitosan hydrochloride-based coating. Visual image of chitosan hydrochloride solution (inset in **b**).

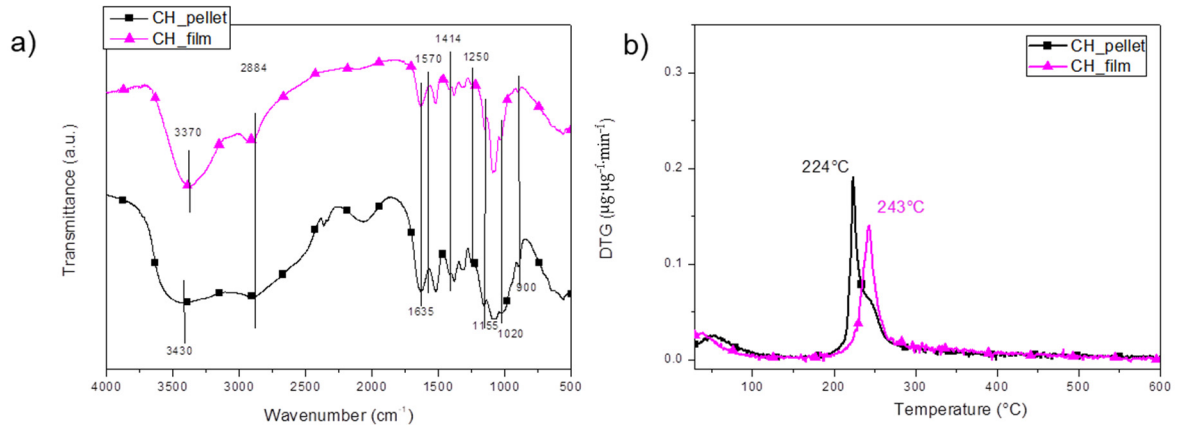

Figure 2. (**a**) FTIR spectra and (**b**) DTG thermograms of the chitosan hydrochloride pellet and film.

The derivate (DTG) curves of weight loss for the TGA tests of the chitosan pellet and film are reported in Figure 2b. Two significant weight loss stages were observed in the DTG curve for CH powder. The small weight loss at 50–120 °C (about 10%) is due to the loss of adsorbed and bound water, while the second main weight loss step between 200 and 300 °C and centered at 224 °C (showing a shoulder at 240–250 °C) was attributed to the thermal degradation of CH, as previously reported by several authors [36,37]. The CH film thermogram showed a similar behavior with the first small signal (at around 100 °C) and the second main peak always in the same region (200–300 °C). However, a shift to a higher temperature of the main degradation peak (centered at 243 °C for chitosan film) was

registered and due to a re-arrangement of the polymer chain during the film-forming procedure that induced and increased in the final thermal stability of the obtained structure.

Table 1. FTIR bands of chitosan hydrochloride.

Wavenumber (cm^{-1})	Assignment
3430 (pellet) 3370 (film)	–OH stretching
2884	C–H stretching
1635	Amide I
1570	Amide II
1414	$–CH_2$ bending
1250	amino group
900, 1020, and 1155	Saccharide structures

After the characterization, the CH solution at the specific selected concentration of 1 g/L was applied on lettuce and kiwifruits as described. The ability of chitosan hydrochloride to form a homogeneous film, its appearance and distribution on both vegetables (here, lettuce as an example) and fruit (kiwi) were investigated by FESEM at different magnifications and compared with the same food products treated with water as a control. Furthermore, in the case of lettuce, evidence of both leaves and taproot were reported since the taproot surface represents a preferential method for pathogen attack.

Figure 3A reports the evidence of lettuce leaves treated with water characterized by the typical stomatal aperture (Figure 3A(a,b)).

The arrows in Figure 3A(c,d) indicated the stomatal aperture partially covered by the CH coating. The FESEM images underlined a well distributed and homogeneous coating on the lettuce leaves, as well as the lettuce taproot (Figure 3B(c,d)) with the typical tri-dimensional porous structure as reported and discussed in Figure 1c–f (see inset in Figure 3B(d)). The FESEM images also underlined that, although the CH coating was well distributed on the vegetable's surface, it should not negatively affect the food product functionality since the stomatal aperture was quite evident after the application of the coating, guaranteeing the normal breathing of the food due to the porous structure of the applied CH coating.

Comparable results were also obtained for kiwifruit peel treated with the CH coating and always compared with water-treated kiwifruit products, used as a control (Figure 4). FESEM images underlined the well-obtained distribution of CH coating that perfectly adhered and covered the kiwifruit peels (see arrows in Figure 4c,d) maintained, also in this case, the porous structure (see inset in Figure 4d). Furthermore, although in all cases a thin film (less than 3–5 μm) was obtained due the low molecular weight of the selected commercial-grade polymer, the coating was able to thoroughly seal lettuce and kiwifruit peels, potentially guaranteeing good conservation of the food products.

In any case, the main goal of the present work was to prove the film-forming characteristics of the selected chitosan hydrochloride-grade polymer and to prove its potential efficiency, in vitro and in vivo, against specific pathogens causing fresh food deterioration, as following discussed. No direct evidence of the shelf-life or food qualities and vegetable/fruit organoleptic characteristics during storage were addressed here or studied, which could represent the main goals for future work.

3.2. Antimicrobial In Vitro Activity of Chitosan Hydrochloride

The results of in vitro radial growth of *B. cinerea* on PDA medium (control) or on PDA amended with chitosan hydrochloride (CH) are shown in Figure 5. The differences between CH treatment and control in terms of radial growth of the fungus (*B. cinerea*) at different times are reported, showing an inhibition of about 50%.

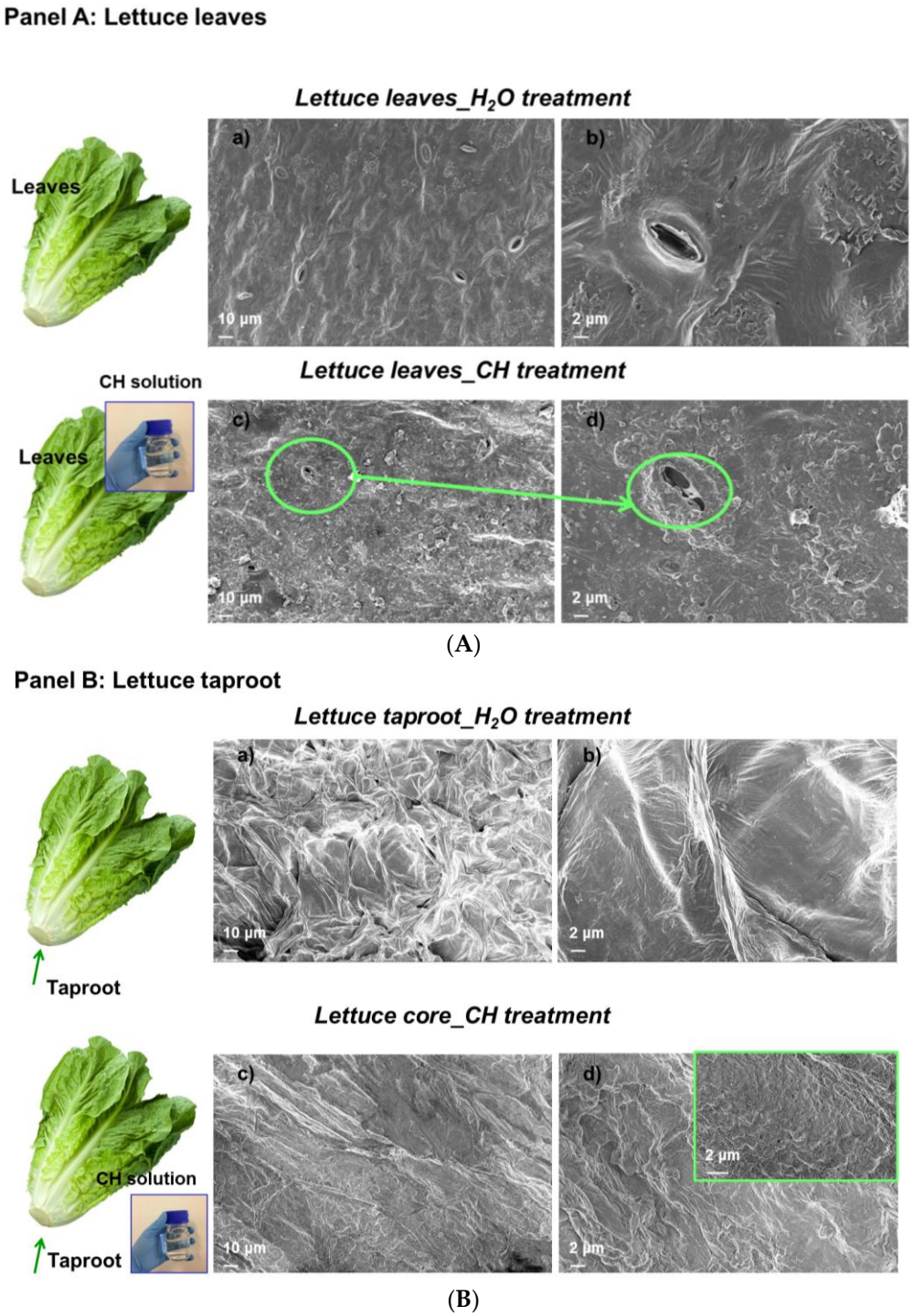

Figure 3. Evidence of CH coating on (**A**) lettuce leaves and (**B**) lettuce taproot treated with (**a**,**b**) water as control and with (**c**,**d**) CH solution.

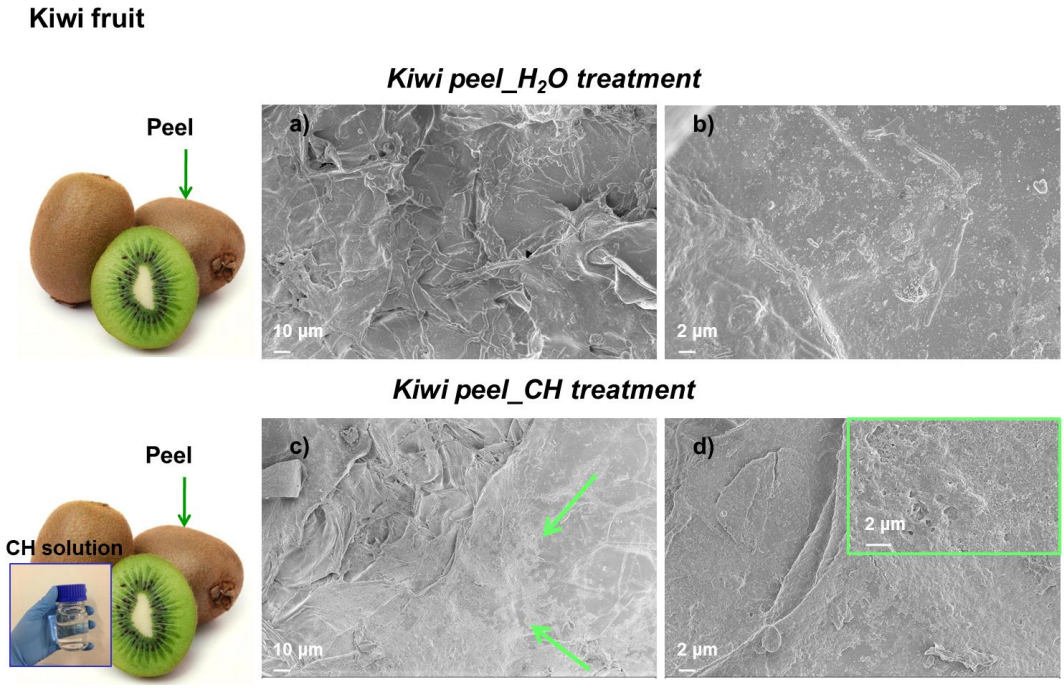

Figure 4. Evidence of CH coating on kiwifruit peel treated with (**a**,**b**) water as control and with (**c**,**d**) CH solution.

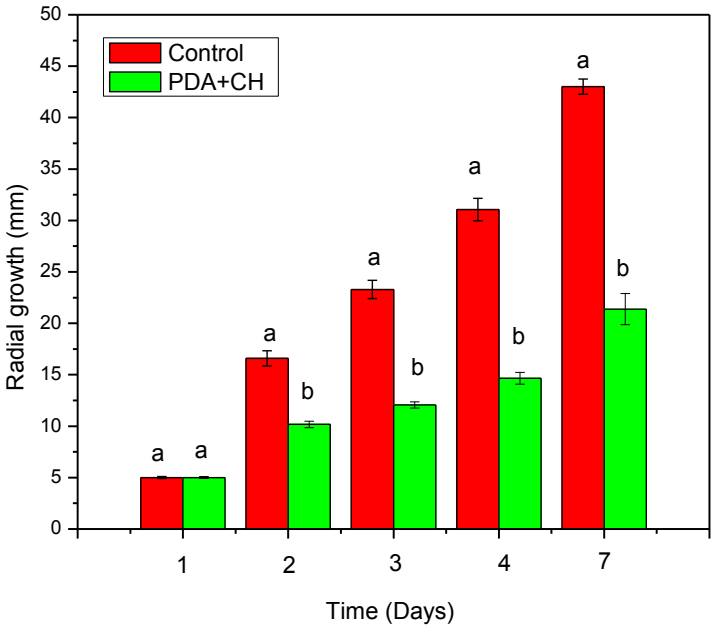

Figure 5. In vitro radial growth of *Botrytis cinerea* on PDA medium (control) or on PDA amended with chitosan hydrochloride (CH). For each evaluation date, columns with different letters are significantly different according to Tukey's HSD test ($p = 0.01$). Significant differences between two bars are marked with different letters; if there is no significant difference between two bars they get the same letter.

Figure 6 shows, visually, the antimicrobial effect of chitosan hydrochloride against *B. cinerea*. The image (Figure 6b) well underlined as the mycelial growth of *B. cinerea* was almost totally inhibited by the incorporation of chitosan hydrochloride solution in the medium until the end of the test.

The mycelial mat was rarefied and weak due to the CH presence (Figure 6b) whereas it appears completely homogeneous in the control (Figure 5a).

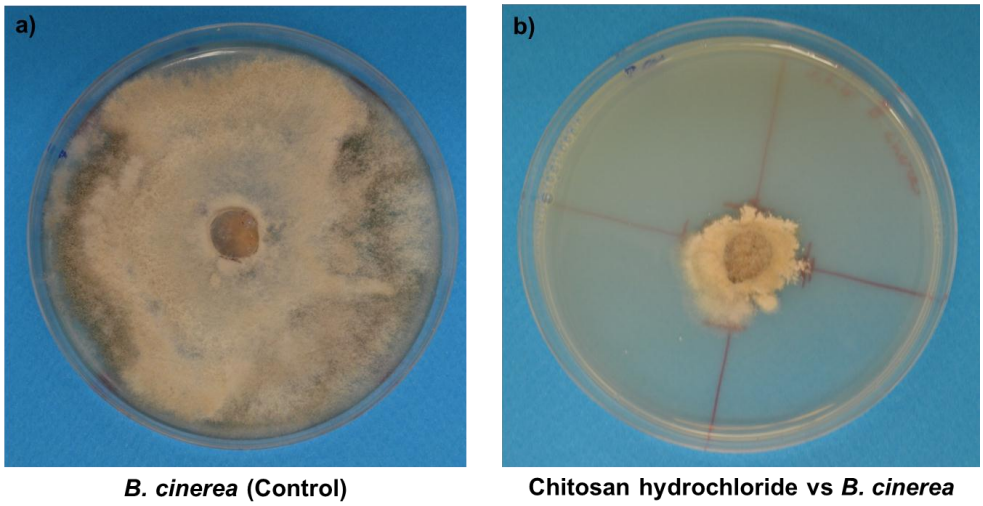

Figure 6. In vitro results after 7 days, with an evident inhibition of radial growth of *B. cinerea* by chitosan hydrochloride. *Botrytis cinerea* (**a**) and Chitosan hydrochloride vs *Botrytis cinerea* (**b**).

Figure 7 shows the antimicrobial effect of chitosan hydrochloride in vitro against *P. c.* subsp. *carotovorum* bacterium. As previously discussed for the fungal pathogen, the bacterial growth of *P. c.* subsp. *carotovorum* was also totally depleted by CH (data not shown), showing a very important activity to inhibit the growth of the colonies of this dangerous bacterial plant pathogen (Figure 7b).

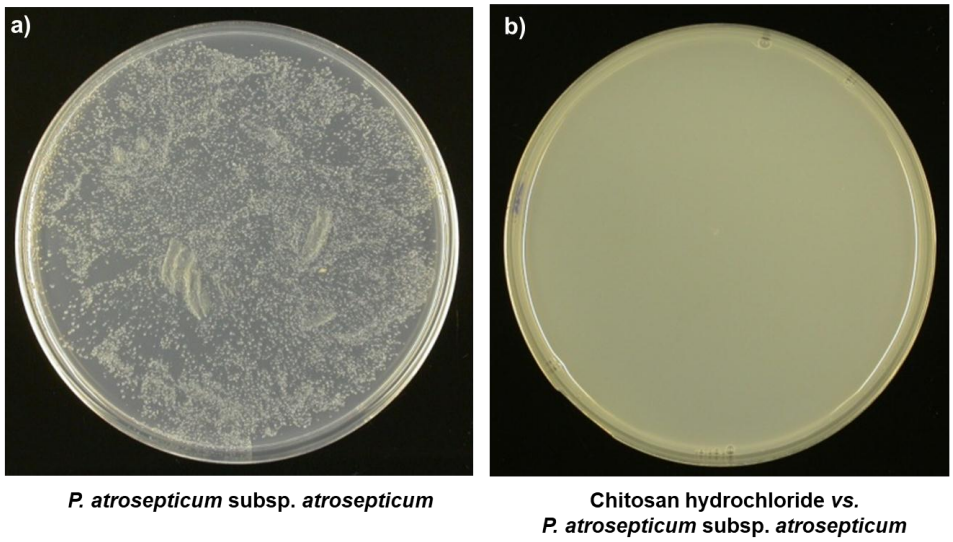

Figure 7. In vitro growth of colonies of *Pectobacterium carotovorum* subsp. *carotovorum* (**a**) in NA medium (control) or (**b**) in NA amended with CH (p = 0.01).

3.3. Antimicrobial Activity on Stored Fruits and Vegetables: In Vivo Assays

The results of in vivo antimicrobial tests against the *B. cinerea* by using CH on kiwifruits, are summarized in Figure 8. Until the 25th day after artificial inoculation, kiwifruits treated with chitosan hydrochloride showed a lower level of disease severity with respect to the control kiwifruits and to those treated with chemical compounds, both at external and internal levels (Figure 8).

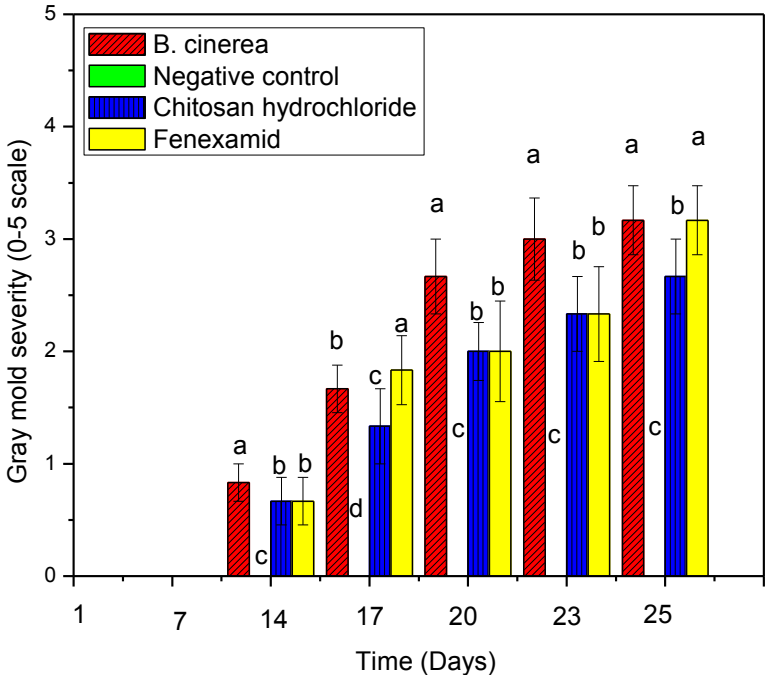

Figure 8. Gray mold severity on kiwifruits artificially inoculated with *B. cinerea* after treatment with the fungicide fenhexamid or CH. Negative controls were not inoculated. For each evaluation date, columns with different letters are significantly different according to Tukey's HSD test ($p = 0.01$). Green histograms related to negative controls (only sterile distilled water, SDW) are absent because non-microbial growth was recorded in this thesis. Significant differences among the bars are marked with different letters; if there is no significant difference among the bars they get the same letter.

The kiwifruits, inoculated with this fungal pathogen, but not protected by any chemical product, started showing the typical gray mold at the petiole end, where the inoculum was deposited, starting from day 10. After that, the tissue rotting continued to expand reaching, after 25 days, an average of 3.2 in the symptom scale, which corresponds to about 70% of the entire surface of the fruit. The initial symptoms on kiwifruits treated with chitosan was slightly delayed, appearing around the 15th day but, interestingly, the rotting process was limited and was markedly less than those observed on kiwifruit controls and to those on kiwifruits submitted to chemical treatment.

The in vivo antimicrobial assays of the CH coating applied on lettuce surfaces with respect to *P. c.* subsp. *carotovotum* bacterial plant pathogen were investigated. The results, after seven days (data not shown), demonstrated an important reduction of damage (value 2–3), induced by chitosan hydrochloride-based coating. The damage, in the presence of the active coating, appeared (see Figure 9), in fact, much less severe that those recorded for the controls (only inoculated by *P. c.* subsp. *carotovotum*, 10^6 CFU/mL) even if the resulted in being much less effective with respect to the damage level developed on/in the samples treated with chemical compounds (value 1–2).

All the results obtained by in vitro and in vivo antimicrobial tests were statistically significant. On the basis of our evidence, the selected grade of chitosan hydrochloride, able to form a stable solution in a green solvent as water and to form a homogeneous coating on food, could be preventively applied to prolong the postharvest shelf-life of important fresh products. Considering the high concentrations used for fungal and bacterial artificial inoculations (1×10^6 conidia/mL and 1×10^6 CFU/mL, respectively) it is reasonable to consider the effectiveness of the chitosan hydrochloride-based coating against these plant pathogens up to five days on Romaine lettuce and up to 20 days on kiwifruits. Mechanisms by which chitosan hydrochloride coating reduced the decay of lettuce and kiwifruits with respect to *P. c.* subsp. *carotovorum* and to *B. cinerea*, not even studied here in depth, seems to be related to its bacteriostatic/fungistatic properties. Until now, antimicrobial activity of chitosan has been

widely studied against clinically-important microorganisms; this can be considered a new contribution with respect to dangerous foodborne (bacteria and fungi) plant pathogens as other active principles of natural origin recently resulted in being able to control plant pathogens in greenhouses and in open fields [38–40]. Finally, it is well known that gray mold of kiwifruits is mainly caused by latent or wound infections that are produced in the orchard [41]; thus, effective postharvest control means need to provide curative activity (the treatment should be applied after the pathogen inoculation). Here the potentiality of the chitosan hydrochloride treatment, as a preventive strategy, in contrast to *B. cinerea*, was considered and studied to reduce further treatments. Future research activities will be also addressed for curative activity.

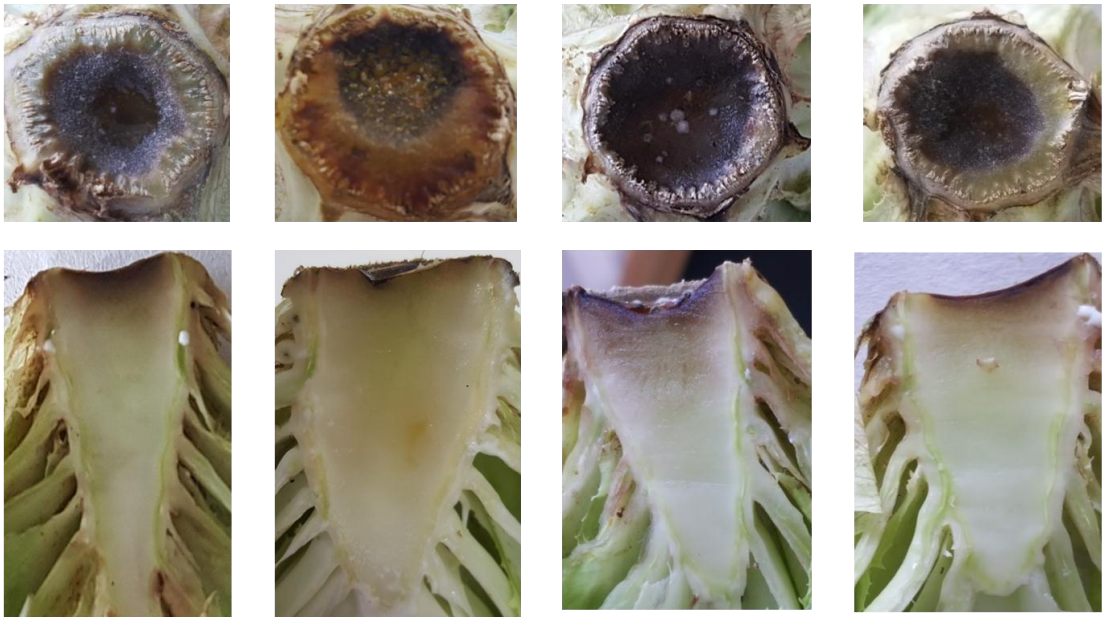

Figure 9. In vivo symptoms (external and internal damages) developed after five days due to artificial inoculation by *Pectobacterium carotovorum* subsp. *carotovorum* (DSM 30184 [Leibniz Institute German Collection of Microorganisms and Cell Cultures, Germany]) bacterial plant pathogen on Romaine lettuce after preventive treatments by chitosan hypochloride and chemical compound (Sodium hypochlorite) respect to the control thesis (treated only by *P. c.* subsp. *carotovorum* and, as control negative, only by SDW). (**a**) Control positive: *P. c.* subsp. *carotovorum* bacterial plant pathogen at 1×10^6 CFU/mL; (**b**) Control negative, SDW; (**c**) Chitosan hydrochloride (1 g/L) solution; and (**d**) Sodium hypochlorite (1 mL/L) solution (p = 0.01). Chitosan hydrochloride and Sodium hypochlorite (Sigma-Aldrich®, St. Louis, MO, USA).

4. Conclusions

The present work revealed interesting results of chitosan hydrochloride-based coatings on the safety of horticultural fresh products, such as lettuce and kiwifruits. Commercial grade chitosan hydrochloride was selected here as a polymer for food coating and its film-forming properties, physico-chemical and morphological characteristics, as well as antimicrobial response against *B. cinerea* and *P. c.* subsp. *carotovorum* were investigated. The results underlined the film-forming capability of this grade of chitosan, which maintained its physico-chemical characteristics after dissolution in water and which formed a thin and well-distributed coating on both kiwifruit and lettuce. The in vitro antimicrobial assays showed that both the mycelial growth of *B. cinerea* and the bacterial growth of *P. c.* subsp. *carotovorum* were totally inhibited by the presence of CH, whereas in vivo antimicrobial properties were proved for 5–7 days on lettuce and until 20–25 days on kiwifruits demonstrating

that chitosan-based coating is able to contrast gray mold frequently caused by the two selected plant pathogens during the postharvest phases of both fruit or vegetable products. On *B. cinerea*, chitosan has also shown to inhibit the spore germination and this is an additional positive function that this organic material can express with respect to this dangerous pathogen [42]. Chitosan applications, in combination with essential oils, were also recently tested with respect to different bacterial plant pathogens and so our study assumes a particular relevance to improve sustainable strategies able to reduce the negative impact of different plant pathogens during postharvest phases [43]. The obtained results contribute to the idea and need of novel greener strategies and approaches for food quality and safety preservation. By using natural compounds, like chitosan hydrochloride, interesting opportunities emerge to limit the damage caused by dangerous plant pathogens on vegetable and fruit production after harvesting, reducing or avoid remarkable economic losses and preserving final products.

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References

1. Lugtenberg, B.; Rozen, D.E.; Kamilova, F. Wars between microbes on roots and fruits. *F1000Research* **2017**, *6*, 343. [CrossRef] [PubMed]
2. Gastavsson, J.; Cederberg, C.; Sonesson, U. *Global Food Losses and Food Waste*; Food and Agriculture Organization (FAO) of the United Nations: Rome, Italy, 2011.
3. Eckert, J.W.; Ogawa, J.M. The chemical control of postharvest diseases: Deciduous fruit, berries, vegetables and root/tuber crops. *Annu. Rev. Phytopathol.* **1988**, *26*, 433–469. [CrossRef]
4. Spotts, R.A.; Cervantes, L.A. Populations, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and Mucor piriformis in packinghouses. *Plant Dis.* **1986**, *70*, 106–108. [CrossRef]
5. Wilson, C.L.; Pusey, P.L. Potential for biological control of postharvest plant diseases. *Plant Dis.* **1985**, *69*, 375–378. [CrossRef]
6. Wilson, C.L.; Wisniewski, M.E. Biological control of postharvest diseases of fruits and vegetables: An emerging technolygy. *Annu. Rev. Phytopathol.* **1989**, *27*, 425–441. [CrossRef]
7. Sharma, R.R.; Singh, D.; Singh, R. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biol. Control* **2009**, *50*, 205–221. [CrossRef]
8. Yang, W.; Fortunati, E.; Dominici, F.; Giovanale, G.; Mazzaglia, A.; Balestra, G.M.; Kenny, J.M.; Puglia, D. Synergic effect of cellulose and lignin nanostructures in PLA based systems for food antibacterial packaging. *Eur. Polym. J.* **2016**, *79*, 1–12. [CrossRef]
9. Mazzaglia, A.; Fortunati, E.; Kenny, J.K.; Torre, L.; Balestra, G.M. Nanomaterials in Plant Protection. In *Nanotechnology in Agriculture and Food Science*; Axelos, M.A.V., Van de Voorde, M., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2017; pp. 115–134.
10. Fortunati, E.; Benincasa, P.; Balestra, G.M.; Luzi, F.; Mazzaglia, A.; Del Buono, D.; Puglia, D.; Torre, L. Revalorization of barley straw and husk as precursors for cellulose nanocrystals extraction and their effect on PVA_CH nanocomposites. *Ind. Crops Prod.* **2016**, *92*, 201–217. [CrossRef]
11. Luzi, F.; Fortunati, E.; Giovanale, G.; Mazzaglia, A.; Torre, L.; Balestra, G.M. Cellulose nanocrystals from *Actinidia deliciosa* pruning residues combined with carvacrol in PVA_CH films with antioxidant/antimicrobial properties for packaging applications. *Int. J. Biol. Macromol.* **2017**, *104*, 43–55. [CrossRef] [PubMed]
12. Badawy, M.E.I.; Rabea, E.I. Potential of the biopolymer chitosan with different molecular weights to control postharvest gray mold of tomato fruit. *Postharvest Biol. Technol.* **2009**, *51*, 110–117. [CrossRef]
13. Davila-Avina, J.E.J.; Villa-Rodríguez, J.; Cruz-Valenzuela, R.; Rodríguez-Armenta, M.; Espino-Díaz, M.; Ayala-Zavala, J.F.; Gonzalez-Aguilar, G. Effect of edible coatings, storage time and maturity stage on overall quality of tomato fruits. *Am. J. Agric. Biol. Sci.* **2011**, *6*, 162–171. [CrossRef]

14. Perdones, A.; Escriche, I.; Chiralt, A.; Vargas, M. Effect of chitosan–lemon essential oil coatings on volatile profile of strawberries during storage. *Food Chem.* **2016**, *197*, 979–986. [CrossRef] [PubMed]
15. Lee, S.Y.; Lee, S.J.; Choi, D.S.; Hur, S.J. Current topics in active and intelligent food packaging for preservation of fresh foods. *J. Sci. Food Agric.* **2015**, *95*, 2799–2810. [CrossRef] [PubMed]
16. Romanazzi, G.; Feliziani, E.; Santini, M.; Landi, L. Effectiveness of postharvest treatment with chitosan and other resistance inducers in the control of storage decay of strawberry. *Postharvest Biol. Technol.* **2013**, *75*, 24–27. [CrossRef]
17. Jongsri, P.; Wangsomboondee, T.; Rojsitthisak, P.; Seraypheap, K. Effect of molecular weights of chitosan coating on postharvest quality and physicochemical characteristics of mango fruit. *LWT Food Sci. Technol.* **2016**, *73*, 28–36. [CrossRef]
18. Dhall, R.K. Advances in edible coatings for fresh fruits and vegetables: A review. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 435–450. [CrossRef] [PubMed]
19. Falguera, V.; Quintero, J.P.; Jimenez, A.; Munoz, J.A.; Ibarza, A. Edible films and coatings: Structures, active functions and trends in their use. *Trends Food Sci. Technol.* **2011**, *22*, 292–303. [CrossRef]
20. Kanmani, P.; Rhim, J.W. Antimicrobial and physical-mechanical properties of agar-based films incorporated with grapefruit seed extract. *Carbohydr. Polym.* **2014**, *102*, 708–716. [CrossRef] [PubMed]
21. Maswada, H.F.; Abdallah, S.A. In vitro activity of three geophytic plant extracts against three post-harvest pathogenic fungi. *Pak. J. Biol. Sci.* **2013**, *23*, 1698–1705. [CrossRef]
22. Silva-Weiss, A.; Bifani, V.; Ihl, M.; Sobral, P.J.A.; Gomez-Guillen, M.C. Polyphenol-rich extract from murta leaves on rheological properties of film-forming solutions based on different hydrocolloid blends. *J. Food Eng.* **2014**, *140*, 28–38. [CrossRef]
23. *Review Report for the Basic Substance Chitosan Hydrochloride Finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 20 March 2014—In View of the Approval of Chitosan Hydrochloride as Basic substance in Accordance with Regulation (EC) No 1107/2009*; European Commission: Brussels, Belgium, 2014.
24. Carlson, R.P.; Taffs, R.; Davison, W.M.; Stewart, P.S. Anti-biofilm properties of chitosan coated surfaces. *J. Biomater. Sci. Polym. Ed.* **2008**, *19*, 1035–1046. [CrossRef] [PubMed]
25. Kumar, R.M.N.V. A review of chitin and chitosan applications. *React. Funct. Polym.* **2000**, *46*, 1–27. [CrossRef]
26. Devlieghere, F.; Vermeulen, A.; Debevere, J. Chitosan: Antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiol.* **2004**, *21*, 703–714. [CrossRef]
27. Tharanathan, R.N.; Kittur, F.S. Chitin—The undisputed biomolecule of great potential. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 61–87. [CrossRef] [PubMed]
28. Benhabiles, M.S.; Salah, R.; Lounici, H.; Drouiche, N.; Goosen, M.F.A.; Mameri, N. Antibacterial activity of chitin, chitosan and its oligomers prepared fromshrimp shell waste. *Food Hydrocoll.* **2012**, *29*, 48–56. [CrossRef]
29. Yen, M.T.; Tseng, Y.H.; Li, R.C.; Mau, J.L. Antioxidant properties of fungal chitosan from shiitake stipes. *LWT Food Sci. Technol.* **2007**, *40*, 255–261. [CrossRef]
30. Davidson, A. *The Oxford Companion to Food*; Oxford University Press: Oxford, UK, 1999.
31. Elad, Y.; Williamson, B.; Tudzinski, P.; Delen, N. *Botrytis: Biology, Pathology and Control*; Springer: Berlin, Germany, 2007; p. 403.
32. Ma, B.; Hibbing, M.E.; Kim, H.S.; Reedy, R.M.; Yedidia, I.; Breuer, J.; Breuer, J.; Glasner, J.D.; Perna, N.T.; Kelman, A.; et al. Host range and molecular phylogenies of the soft rot enterobacterial genera *pectobacterium* and *dickeya*. *Phytopathology* **2007**, *97*, 1150–1163. [CrossRef] [PubMed]
33. No, H.K.; Meyers, S.P.; Prinyawiwatkul, W.; Xu, Z. Applications of chitosan for improvement of quality and shelf life of food: A Review. *J. Food Sci. Technol.* **2007**, *72*, 87–100. [CrossRef] [PubMed]
34. Bonilla, J.; Fortunati, E.; Vargas, M.; Chiralt, A.; Kenny, J.M. Effects of chitosan on the physicochemical and antimicrobial properties of PLA films. *J. Food Eng.* **2013**, *119*, 236–243. [CrossRef]
35. Lawrie, G.; Keen, I.; Drew, B.; Chandler-Temple, A.; Rintoul, L.; Fredericks, P.; Grøndahl, L. Interactions between Alginate and Chitosan Biopolymers Characterized Using FTIR and XPS. *Biomacromolecules* **2007**, *8*, 2533–2541. [CrossRef] [PubMed]
36. Bonilla, J.; Fortunati, E.; Atarés, L.; Chiralt, A.; Kenny, J.M. Physical, structural and antimicrobial properties of poly vinyl alcohol–chitosan biodegradable films. *Food Hydrocoll.* **2014**, *35*, 463–470. [CrossRef]

37. Chen, C.H.; Wang, F.Y.; Mao, C.F.; Liao, W.T.; Hsieh, C.D. Studies of chitosan: II. Preparation and characterization of chitosan/poly(vinyl alcohol)/gelatin ternary blend films. *Int. J. Biol. Macromol.* **2008**, *43*, 37–42. [CrossRef] [PubMed]
38. Quattrucci, A.; Ovidi, E.; Tiezzi, A.; Vinciguerra, V.; Balestra, G.M. Biological control of tomato bacterial speck using Punica granatum fruit peel extract. *Crop Prot.* **2013**, *46*, 18–22. [CrossRef]
39. Fortunati, E.; Rescignano, N.; Botticella, E.; La Fiandra, D.; Renzi, M.; Mazzaglia, A.; Balestra, G.M. Effect of poly(DL-lactide-co-glycolide) nanoparticles or cellulose nanocrystals-based formulations on *Pseudomonas syringae* pv. *tomato* (Pst) and tomato plant development. *J. Plant Dis. Prot.* **2016**, *123*, 301–310. [CrossRef]
40. Rossetti, A.; Mazzaglia, A.; Muganu, M.; Paolocci, M.; Sguizzato, M.; Esposito, E.; Cortesi, R.; Balestra, G.M. Microparticles containing gallic and ellagic acids for the biological control of bacterial diseases of kiwifruit plants. *J. Plant Dis. Prot.* **2017**, *124*, 269–278. [CrossRef]
41. Michailides, T.J.; Morgan, D.P. New technique predicts gray mold in stored kiwifruit. *Calif. Agric.* **1996**, *50*, 34–40. [CrossRef]
42. El Gaouth, A.; Arul, J.; Grenier, J.; Asselin, A. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology* **1992**, *82*, 398–402. [CrossRef]
43. Badawy, M.E.I.; Rabea, E.I.; Taktak, N.E.M.; El-Nouby, M.A.M. The antibacterial activity of chitosan products blended with monoterpenes and their biofilms against plant pathogenic bacteria. *Hindawi Publ. Corp. Sci.* **2016**, *2016*, 1796256. [CrossRef] [PubMed]