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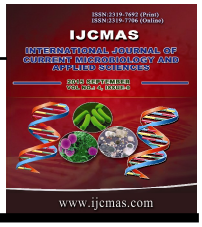

Original Research Article

Antifungal Activity of Microbiological Chitosan and Coating Treatment on Cherry Tomato (*Solanum lycopersicum var. cerasiforme*) to Post-Harvest Protection

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Keywords

Microbiological chitosan, *Botrytis cinerea*, Antifungal activity, Postharvest protection

A B S T R A C T

Chitosan is naturally found in the cell wall of fungi (Zygomycetes) and can be obtained from chitin by deacetylation to form a higher proportion of polymer chain units β1,4-2-amino-2-deoxy-D-glucose, and a smaller number of units β1,4-2-acetamido-2-deoxy-D-glucose. In this study aimed to evaluate the effect of microbial activity of chitosan (Ch), and bioactive coatings on cherry tomato to protect against to *Botrytis cinerea* infection. The chitosan was obtained from *R. arrhizus* showed degree of deacetylation (DD) of 68% and molecular weights of 1.40 x 10^4 g / mol. The spores' viability studies with the pathogen *B. cinerea* by light microscopy showed 70% of germ tubes emergency formed in 16 hours of incubation. Investigations were carried out with chitosan (Ch) evaluating its minimal inhibitory concentration (MIC) and minimum fungicide concentration (MFC) of 100μg / ml against *B. cinerea*. Bioactive coatings on cherry tomato by chitosan (Ch) at concentration 100μg / mL showed higher protection to *B. cinerea* infection. The promising results showed the potential of microbiological chitosan (Ch) to pathogen *B. cinerea*, suggesting to be an important crop protection.

Introduction

Chitosan forming a greater proportion of polymer chain β1,4-2-amino-2-deoxy-D-glucose units is a polymer derived from chitin deacetylation process β1,4-2-acetamido-2-deoxy-D- glucose, may be found in large amounts in the shells of crustaceans and insects and cell walls of fungi, in particular Zygomycetes. This biopolymer has great biotechnological importance considering its properties, such as biocompatibility, biodegradability, antimicrobial, anti-inflammatory and healing

action. It has immense structural possibilities for chemical modifications to generate novel properties, functions, and applications especially in agricultural area (Ali *et al.,* 2012; Fai *et al.,* 2011; Yogeshkumar *et al.,* 2013; Freddo, 2014).

In addition, the advances in fermentation technologies suggest that the cultivation of *Mucor alean* fungi can provide an alternative source of chitin and chitosan. The amount of these polysaccharides depends on the fungi species, carbono and nitrogen sources, and cultural conditions. Fungal mycelia from Zygomycetes are relatively consistent in composition and are not associated with inorganic materials; therefore, no demineralization treatment is required to recover fungal chitin and chitosan (Chandy and Sharma, 1990; Rinaudo *et al.* 1992; Feofilova *et al.,* 1996; Tan *et al.,* 1996; Teng *et al.,* 2001; Andrade *et al.,* 2000; Pochanavanich and Suntornsuk, 2002; Pochanavanich and Suntornsuk, 2002; Synowiecki and Al-Khateeb, 2003; Andrade *et al.,* 2003; Hu *et al.,* 2004; Campos-Takaki, 2005; Santos *et al.,* 2013; Berger *et al.,* 20014).

Microorganisms plant pathogens are considered economically a problem to agriculture around the world. They induce decay on a large number of agricultural crops during the growing season, in particular postharvest. They induce decay on a large number of agricultural crops during the growing season and postharvest. In particular, the fungus *Botrytis cinerea* infects some fruit and cause huge economic losses in various stages of harvest and post-harvest. The agent responsible for the infection is difficult to control due to their saprophytic activity and forming resistance structures. One way to control this pathogen is to use chemicals, however, tolerance for chemical residues in food, it is becoming less desirable from an ecological point of view and public health (Williamson *et al.,* 2007; Badawy and Rabea, 2011).

The cherry tomato (*Solanum lycopersicum var. cerasiforme*), one of the most important fruit in the world, is highly susceptible to necrotrophic pathogenic fungi as Botrytis cinerea and *Alternaria solani*. Alternative treatments such as the use of plant extracts, essential oils and biopolymers such as chitosan have been studied to reduce the use of synthetic fungicides which do not bring benefits to health of living beings and the environment (Smith, 2014).

Therefore, the objective of the present study was the production of chitosan by *Rhizopus arrhizus* and evaluates the activity of coating chitosan on cherry tomato (*Solanum lycopersicum var. cerasiforme*) against *Botrytis cinerea* infection. Previous experiments were done to evaluate the spores germination, radial growth of *B. cinerea*, and established the minimum inhibitory concentration (MIC) and the minimum fungicide concentration (MFC) of chitosan solution.

Materials and Methods

Microorganisms

The Mucoralean of *Rhizopus arrhizus* UCP/WFCC 1295 was isolated from caatinga soil of Pernambuco, PE, Brazil, and belongs to UCP (Universidade católica de Pernambuco) Collection and is registered in World Federation Culture for Collection (WFCC). The fungus was mantained in Sabouraud Dextrose agar at 5° C, and was used to production chitosan.

The fungus *Botrytis cinerea* URM 2802 was kindly obtained from Micoteca of the Mycology Department of the Federal

University of Pernambuco, Recife-PE, Brazil. The strain was isolated from plant pathogen in tomato fruits (*Solanum lycopersicum var. Cerasiforme*), and was maintained on PDA (potato dextrose agar) at 5° C.

Agroindustrial substrates

Glycerin was kindly supplied by CETENE (Center of Tecnologies of Northeast, Recife, PE, Brazil), and corn steep liquor from Corn Products (Cabo de Santo Agostinho-PE,Brazil).

Tomato

Samples of sherry tomatoes (*S. lycopersicum var. cerasiforme*) were used for experimental purposes in the test against *Botrytis cinerea*. The tomatoes fruits were obtained from informal plantation from Cabo de Santo Agostinho – Pernambuco, PE, Brazil. The tomatoes were pretreated with 1% of sodium hypochlorite solution, followed wash in sterile distilled water.

Microbiological chitosan production

The fungus *A. arrhizus* was grown in Petri dishes (9cm diameter) containing YMA [yeast malt agar], and the plates were incubated at 28° C for 120h until sporulation. Spores were collected and transferred to Erlenmeyers flasks with 125mL capacity, containing 50 ml of buffered saline with 0.9% [sodium phosphate monobasic 0.45 g sodium phosphate dibasic 5.8125 g, sodium chloride 2.25 g water 250mL distilled], counted in hematocitociometer to a concentration of 10^7 spores.mL^{-1}, as pre-inoculum.

The *R. arrhizus* spores suspension was spread on Petri dishes containing YMA and incubated at 28º C for 24h, and discs of 6mm diameter was cut using corker borer and tem discs was transferred to Erlenmeyers flasks of 250 mL capacity, containing 50 mL of medium, with pH adjusted to 5.8, containing corn steep liquor 8.82% glycerin and 6%, incubated at 28° C for 96 h in orbital shaker at 150 rpm.

The chitosan was obtained from biomass if *R. arrhizus* by methodology according to Hu (1999) which involves deproteinization by 1M sodium hydroxide (w/v), is followed by centrifugation, acid hydrolysis with 2% acetic acid (3v/w), centrifugation to obtaining chitosan by neutralization to pH 10.

Characterization of chitosan

Determination of degree of deacetylation of chitosan fungal

The chitosan was submitted to infrared spectroscopy, using Fourier Transform spectrophotometer (FTIR), IFS 66 Bruker Mod (Analytical Center - Fundamental Chemistry Department - DQF - UFPE). The degree of deacetylation was determined according to the equation described by Roberts (1992):

$$DD\% = 100 - [(A1655 / A3450)\ 100 / 1.33]$$

Molecular Weight

The molar mass was determined by viscosity, according to the methodology proposed by Santos *et al.* (2003). The intrinsic viscosity measurements were performed using a glass capillary Cannon-Fenske type (dinterno = 1,01mm) thermostated at (25 ± 0.01) ° C, in a AVS 350-Schott-Geräte viscometer. To determine the intrinsic viscosity [h], chitosan solutions were prepared (using acetic acid buffer plus acetate as solvent) at concentrations ranging from 1.0 x 10^{-3} to 8.0 x 10^{-3} g. mL^{-1}. The flow times in seconds were determined.

Samples were prepared in triplicate and the average value was calculated.

Scanning electron microscopy

In order to observe the microstructures of chitosan used scanning electron microscopy analyze was performed. Sample of chitosan produced by *R. arrhizus* was ground and dried under vacuum using a lyophilizer and its surface was observed using a scanning electron microscope XL 30 series (Umax) ESEM (Env. Scan. Electron Micros, Jeol, Tokyo, Japan) with tungsten filament, 20 kV accelerating voltage.

***Botrytes cinerea* Spores Viability**

Rates of 0.5 ml suspension of 10^5 spores/mL^{-1} of *B. cinerea* URM 2802, were transferred to Erlenmeyer flasks containing 100 ml of medium potato dextrose, incubated orbital shaker at 28°C. Aliquots of 2.5 mL were collected in triplicate at intervals: 0h, 4h, 8h, 12h, 20h and 24h, and was observed under light microscopy at 40X to determine the emergency tubes formation, and number of cells viability.

Effect of Chitosan on Radial Growth of *B. cinerea*

Petri dishes of 6cm of diameter containing PDA cultivation medium, PDA plus 1% acetic acid, PDA added chitosan in the concentrations of 100µg.mL^{-1} (MIC), 1000µg.mL^{-1} and 2000µg.mL^{-1}. After solidification the medium discs of 6mm of *B. cinerea* grown in PDA was done in the center of the plate, incubated at 28°C during 72h. The effect of different concentrations of chitosan on radial growth of *B. cinerea* was determined.

Determination MIC and MFC

Minimal inhibitory concentration (MIC) and minimum fungicide concentration (MFC) to microbiological chitosan against to *B. cinerea* tested were performed using the broth microdillution method as described by Ahmed *et al.* (2008). The assay was carried out using 20µL of the inoculum of *B. cinerea* (10^5 spores/mL) in wells containing potato juice with chitosan concentrations between 100 to 600µg.mL. The system was incubated at 28° C for 24 hours.

The MIC was defined as the lowest concentration of chitosan capable of inhibiting the cellular multiplication. The CFM was determined based on the methodology of Santurio *et al.* (2007). From the wells in which there was no visible fungal growth pulled out a 10µL aliquot, which was inoculated on the surface of PDA medium. The plates were incubated at 28° C, and after 24 h, was determined as the lowest concentration CFM chitosan able to inhibit the growth or cause the death of fungus spore. MIC and MFC assays were performed in triplicate.

Treatment of Tomatoes with chitosan solution

To evaluate the direct effect of chitosan on the development of *B. cinerea*, the tomatoes were inoculated with a conidial suspension at a concentration of 10^5 conidia/mL^{-1}, determined by hemacytometer count, according to methodology described by Camili *et al.* (2007) after 4pm immersed in chitosan solution at CIM concentration then arranged on sterile petri dish on a tray covered with plastic wrap at 28°C.

The treatment was accompanied by the loss weight of tomatoes control without chitosan with acetic 1% and coated with chitosan acid, the results are monitoring each 24h of intervals. The weight lost was calculated as a percentage relative to the initial weight of the samples Meng *et al.* (2008).

Results and Discussion

Chitosan characterization

Deacetylation Degree

Spectroscopy analysis of the infrared region of the chitosan produced by *R. arrhizus* was performed to determine the degree of deacetylation (DD%), with the purpose of characterization, according to Roberts (1992).

The infrared spectrum absorption of the chitosan showed bands regarding OH-axial stretching between 3396 and 2931cm^{-1}, superimposed on the NH stretching band with axial deformation of amide C = O at about 1650cm^{-1}, NH angular deformation at approximately 1575cm^{-1}; axial deformation of amide-CN at around 1415cm^{-1}, symmetrical angular deformation in CH_3 at 1379 cm^{-1}, -CN axial deformation of amino groups from 1078 to 1250cm^{-1} and polysaccharide structures bands in the range of between 597 and 1150 cm^{-1} (Figure 1).

However, the same results were observed by Mario *et al.* (2008) who described the *N*-deacetylation is associated with a progressive weakening of the band occurring at 1,655 cm^{-1}(amide I vibrational mode), and the disappearance of the band at 1,550 cm^{-1} (amide II vibrational mode). Our results showed deacetylation degree of microbiological chitosan of 68%, and it can be seen that in the fingerprint region. The DD% is an important parameter that determines the physicochemical properties of the chitosan because it is linked to the cationic properties of chitosan (Fai *et al.*, 2011).

Molecular weight

The viscosimetry evaluation of chitosan obtained from submerged fermentation of *R. arrhizus* in medium containing corn steep liquor and glycerin from biodiesel production corresponded to lower molecular weight (1.40 x 10^{4}g. mol^{-1}). The results obtained from of *R. arrhizus* is similar to reported by Pochanavanich and Suntornsuk (2002) and Berguer *et al.* (2014) for molecular weight from fungal chitosan which ranging from 5.00 × 10^3 to 9.4 × 10^5 (Table 1).

Scanning electron microscopy

The figure 2 (A and B) showed the morphology of chitosan produced by *R. arrhizus* and chitosan from crustaceous by scanning electron microscopy. The chitosan from fungus also exhibited an amorphous structure that was more compact and dense, and nor porous was observed. This biopolymer also showed crumbling layers of flakes was detected as similar morphology was observed in crustacean chitosan and few aggregated flakes formed a dense and firm structure, without porosity, as described to fungal chitosan by the literature (Yen and Mau, 2007; Yen *et al.*, 2009; Berger *et al.*, 2014).

Biochemical studies with *Botrytis cinerea*

Spore viability

Previous studies of cell viability by light microscopy showed that the culture of *B. cinerea* grown on potato broth at concentration of 10^5 conidia/mL^{-1} appeared to be feasible for use as a phytopathogenic fungus. After 8 to 12h of cultivation 100% of the spores increase in the size was observed.

The emergency of the germinated tubes were formed corresponding to 70% of viability at 16h. However, at the end of cultivation the spores (30%) germinated between 20 and 24 h of cultivation.

Table.1 Properties of fungal chitosan and crab shell chitosan standard

Chitosan sample	DD* (%)	MW**(Da)	Reference
Rhizopusarrhizus UCP/WFCC1295	68.00± 0.9	1.4 $\times 10^4$	This study
Cunninghamella elegans UCP/WFCC 0542	82.24 ± 2.0	5.00 × 10^3	Berguer et al. (2014)
Crab shell (Sigma)	97.9 ± 0.9	9.4 × 10^5	Pochanavanich and Suntornsuk (2002)
Aspergillus niger TISTR3245	90.9 ± 2.1	1.4 × 10^5	Pochanavanich and Suntornsuk (2002)

***** DD (%) = Degreeofdeacetylation (%), ****** MW= Molecular Weight

Figure.1 Infrared absorption spectra of microbiological chitosan obtained from *Rhizopus arrhizus*

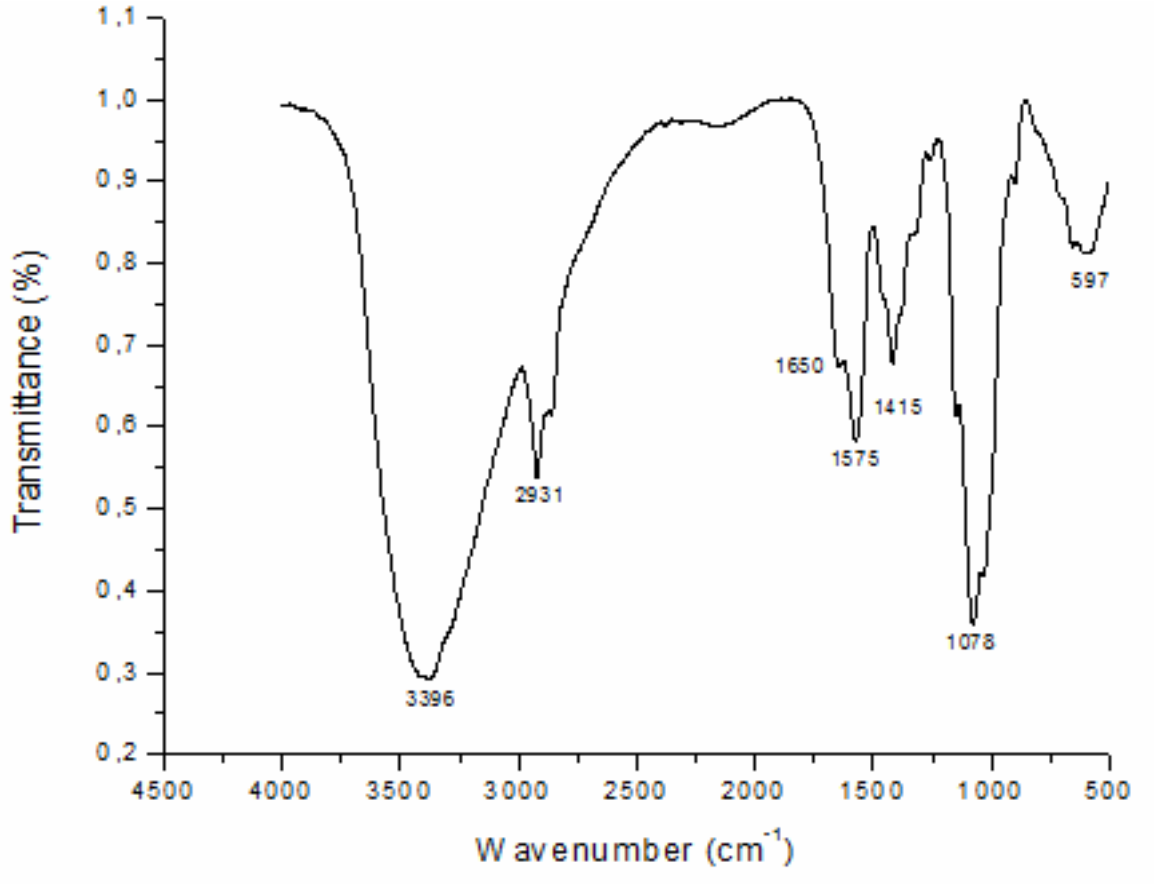
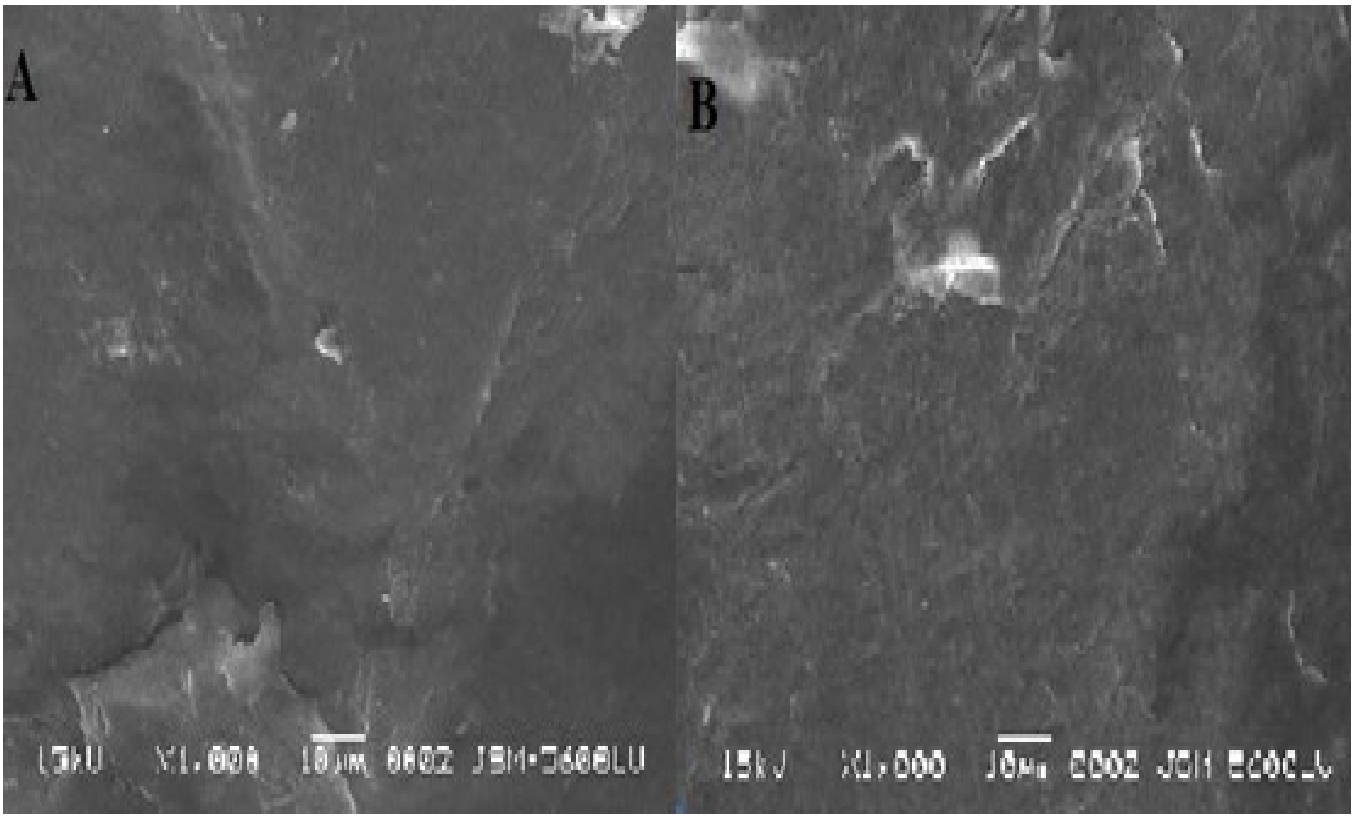


Figure.2 Scanning electron microscopy (SEM) of chitin (A) and (B) chitosan produced by *Rhizopus arrhizus* . (500 × magnification 200/1000). The measurement bar = 10 µM



These results are similar to those presented by Gull and Trinci (1997) which reveal that there was spore germination with a germ tube from *Botrytis cinera* cultivation, the germination remaining stable in values of 50% from 20h to 24h. Tratci and Beettiol (1997) described in their studies the action of biofertilizers on mycelial growth and germination of pathogenic fungi, obtained spore germination of *B. cinerea* (10^4 spores mL^{-1}) in water resulting in values of 50% after 20h of incubation at 20°C.

Determination MIC and MFC to microbiological chitosan

B. cinerea showed higher inhibition by microbiological chitosan and the minimum inhibitory concentration (MIC) and the minimum fungicide concentration (MFC) was found 100µg mL^{-1}. Studies carried out by Oliveira *et al.* (2014) with chitosan obtained from *Cunninghamella elegans* UCP 0542 versus grapes phytopathogen infection, exhibited an MIC value OF 15

mg.mL^{-1} against *B. cinerea* and *Penicillium expansum*. However, Coqueiro and Piero (2011) was evaluated the effect of commercial chitosan isolate from crustaceous with different molecular weights to *Alternaria solani* showed fungistatic effect inhibiting the mycelial growth and spore germination in the concentrations of 0.125 and 0.5 mg mL^{-1}, respectively. The antimicrobial effect of fungal chitosan to *R. arrhizus* and *Cunninghamella elegans* was observed in the concentrations of 300 µg mL^{-1}.

Recently studies with the MIC and MFC of chitosan was observed similar values (500 µg.mL^{-1}) to inhibit the growth and death of *Salmonella enterica* and *Staphylococcus aureus* (Berger *et al.,* 2014). The chitosan from crustaceous with 85% DD showed increasing inhibition rates in amounts ranging from 0.25 to 0.5 gL^{-1} according to the polymer concentration against *Aspergillus flavus* and *Aspergillus parasiticus* (Souza *et al.,* 2013).

Tests were carried out using potato dextrose agar (PDA) added the concentration of chitosan based in the CMI (100 mgmL^{-1}) and inoculated discs of *B. cinerea,* incubated at 28°C and the radial growth was evaluated in comparison with control (without chitosan), however, in the solid medium the fungus was totally inhibited the growth.

The results obtained with *B. cinerea* inhibiton of growth by chitosan are corroborated by Liu *et al.* (2007), Camili *et al.* (2007), Liu (2007) and Botelho *et al.,* (2010). Recently, Freddo *et al.* (2014) showed that chitosan at 0.25, 0.5, 1 and 2% concentrations reduced the mycelial growth of *R. solani* in vitro culture, and progressive ecrease of mycelial growth related to the increase of chitosan concentration.

Evaluation of microbiological chitosan coated protection on cherry tomato

The cherry tomato was treated with MIC/MFC concentration against to *B. cinerea* crop infection and was accompanied during 19 days at environment temperature (28°C). The coated cherry tomatoes were evaluated and control of the tests was done contamination with *B. cinerea* spores (10^5 /mL) during this period and evaluated visually and the lost weight each 24 h.

It is believed that the cationic nature collaborate for the actuation of the chitosan since their positive charges can interact with the negatively charged residues of macromolecules exposed on the surface of pathogen cell causing changes in permeability of the cell membrane, Benhamou (1996). Through experiments Olsen *et al.* (1989) reported that the amine groups of chitosan into contact with the intracellular fluids are protonated and bind strongly to the anionic groups of microorganisms, which raise the process of bonding between them, preventing their development.

Camili *et al.* (2007) using chitosan originating from crustaceans against infection of the pathogen *B. cinerea* on grapes mentions that chitosan could bind to DNA and mRNA synthesis inhibition occurs via penetration of the product in the nucleus of the microorganism also interfere with the synthesis of proteins. The mechanism of inhibition of growth of pathogens by chitosan differs from the used mechanism by the synthetic fungicides.

Besides being applied alone, there are many reports on applications of chitosan combined with other antifungal compounds. Yu *et al.,* (2007) found that chitosan applied alone or *Cryptococcus laurentii* can effectively

inhibit infection caused by *Penicillium expansum* on apple fruit after seven days of incubation at 20°C. When applied alone, treatment with chitosan at the highest concentration (1%) and low viscosity (12 cP) was the most effective. Used in combination, the treatment of *Cryptococcus laurentii* with chitosan at a concentration of 0.1% and low viscosity (12 cP) was the most effective.

As mentioned by Zang *et al.* (2011), the low molecular weight chitosan has been studied to control the fruit postharvest diseases. The results indicated that low molecular weight chitosan significantly inhibited the decomposition of fruits caused by *Penicillium digitatum*, *Penicillium italicum*, *Botrydiplodia lecanidion* and *Botrytis cinerea* after 14 days storage at 25°C being more effective than the high molecular weight chitosan.

Results presented in Liu *et al.* (2007) have shown that the application of chitosan of low molecular weight crustacean showed a greater effect on inhibition of growth of *Penicillium expansum* and *Botrytis cinerea*. Lauzardo *et al.* (2008) also reported a greater inhibitory effect when applied to low molecular weight chitosan on *Rhizopus stolonifer* with modifications in fungal reproductive structures.

The monitoring of cherry tomato during 19 days at a temperature of 25°C, was related to the initial weight and showed loss weight of the control sample (without chitosan and with 1% acetic acid), with values of 3.36% (control) and 3.58% (acetic acid treated),respectively.

However, were observed that coating treatment to cherry tomatoes with chitosan the loss weight was very lower (2.75%). Our results are corroborated by Oliveira *et al.* (1997) findings which reported that chitosan from *R. arrhizus* grapes coating, realized by controlling the weight that the coated fruits with chitosan had a lower percentage loss wet weight than the control samples.

Similar studies carried out by Pastor *et al.*, (2011) found that less weight loss with grapes treated with propolis extract and hydroxypropyl methylcellulose was evidenced.

According to edible coating's ability to prevent the fruit weight loss is based on their hygroscopic properties, which allows the formation of a water barriers between the fruit and environment (Morillon *et al.*, 2002; Xu *et al.*, 2007).

The recourse to naturally-occurring products with interesting antifungal activity as chitosan and their derivatives has been getting more attention in the recent years to make profits while guaranteeing a sustainable agriculture. Strategies were used for microbiological chitosan production by *Rhizopus arrhizus* and application against the pahytopathogenic fungi *Botrytes cinerea*.

The results obtained showed that *R. arrhizus* is a chitosan polymer producer with 68% of degree deacetylation and lower molecular weight. The polymer shown antifungal activity and the CMI and CMF are similar (100 $mgmL^{-1}$). The CMI chitosan solution was able to inhibit the mycelial growth of the fungus *Botrytis cinerea*. The coating application of chitosan treatment provided effective protection for post-harvest cherry tomato (*S. lycopersicum var. cerasiforme*) to infection against *B. cinerea*. The results obtained provide additional support to crop protection.

Acknowledgments

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