

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800

Open access books available

122,000

International authors and editors

135M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Fungal Growth Control by Chitosan and Derivatives

Enio Nazaré De Oliveira Junior

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/63308>

Abstract

Chitin and chitosan are aminogluco-pyranans composed of N-acetyl-D-glucosamine (GlcNAc) and glucosamine (GlcN) residues and are renewable resources currently being studied by academic and industrial groups owing to their attractive properties and biological activities. Chitosans have been indicated for the preservation of foods, juices and other material from microbial deterioration due their action against different groups of microorganisms, such as bacteria, yeast and fungi. Studies on coating of fruits and vegetables and defensive plant mechanism studies have been described in the literature. There is a worldwide trend to explore new alternatives that can control postharvest pathogenic diseases, giving priority to methods that reduce disease incidence and avoid negative and side effects on human health as a result of the excessive application of synthetic fungicides. Thus, alternative approaches are necessary to maintain the marketable quality of fresh fruits. The antifungal activities of chitosan and its derivatives *in vitro*, preharvest and postharvest studies are reviewed in this chapter. The abilities of chitosan and its derivatives to elicit resistance reactions in plants and its action in the production and viability of fungal spores is reported. Finally, the chapter is concluded, with the possible mechanisms, suggested in the literature for the antifungal activity of chitosan.

Keywords: Chitosan, fungi, antifungal activity, plants and fruits

1. Introduction

The discovery of natural antimicrobial compounds, due to growing consumer demand for food without chemical preservatives, has been focused on numerous studies. In this context, the antimicrobial activity of chitin, chitosan and its derivatives against different types of microorganisms, such as bacteria, fungi and yeasts, has received considerable attention. In this chapter important developments concerning the application of chitosan and its derivatives as antimicrobial compound against fungi and yeasts, assumptions involved in their antimicrobial

activity and effects on the quality and storage of fresh vegetables treated with these compounds are described.

The polymers of chitin, chitosan and chito-oligomers have been extensively studied, due to their high potential for applications in food, pharmaceutical, cosmetic and agriculture areas. The applications of these compounds in several areas, especially application of chitosan, is justified by the low cost of production, which is produced from the disposal of processing crustaceans, which are an abundant and renewable source. In general, commercial chitosans are available in the range of molar masses between 50 and 2000 kDa and degree of acetylation (DA) between 0.1 and 0.4 [1].

The polymers chitosan and chitin (Figure 1) are aminoglucopyranans composed of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues (Figure 2). The polymers may be distinguished by their solubility in 1% aqueous acetic acid (v/v). Chitin containing $\geq 40\%$ GlcNAc ($FA \geq 0.4$) is insoluble, whereas soluble polymers are named chitosan [2]. Chitosan is composed of three reactive functional groups: an amino group and two hydroxyl groups in the primary and secondary carbons of the positions C-2, C-3 and C-6, respectively [3]. Chemical and biotechnological processes are currently being investigated for the production of chitosan. Industrially chitosan is produced from the alkaline deacetylation of chitin (alkaline hydrolysis), but chitosan can also be obtained from enzymatic deacetylation of chitin, a process investigated in academic studies (Figure 1).

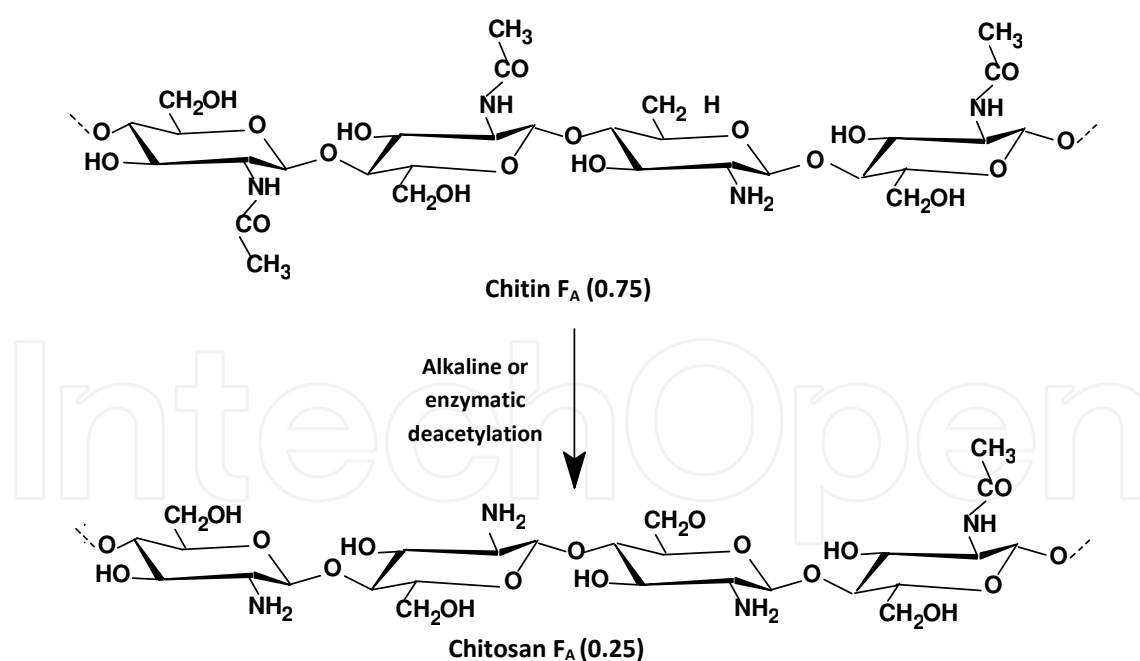


Figure 1. Chemical structures of chitin and chitosan.

Chitin is also widely distributed in fungi, occurring in *Basidiomycetes*, *Ascomycetes* and *Phycomycetes*, where it is a component of cell walls and structural membranes of mycelia, stalks and spores [4]. The amounts vary between traces and up to 45% of the organic fraction, and

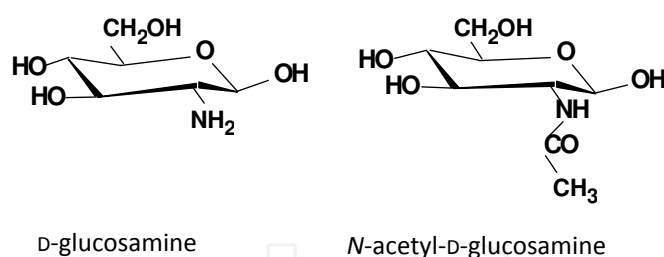


Figure 2. Chemical structures of *N*-acetyl-d-glucosamine (GlcNAc) and d-glucosamine (GlcN) residues.

the remainder is composed mainly of proteins, glucans and mannans [5]. However, not all fungi contain chitin, which may be absent in some species close to other containing chitin as a cell wall component [4]. After the discovery of antimicrobial activity of chitosan and its derivatives by Allan & Hadwiger [6], Kendra & Hadwiger [7] and Uchida et al. [8], many researchers have done studies in this field. In this context the antimicrobial activity of chitin, chitosan and its derivatives against different groups of microorganisms such as bacteria, yeast and fungi has received considerable attention.

The antifungal activities of chitosan and its derivatives *in vitro*, preharvest and postharvest studies are reviewed in this chapter. Besides the review of the antifungal activities of chitosan and its derivatives, their abilities to elicit resistance reactions in plants are also reviewed. In addition, chitosan action in the production and viability of fungal spores is reported in this chapter. Finally, the chapter is concluded, with the possible mechanisms suggested in the literature for the antifungal activity of chitosan.

2. *In vitro* and *in vivo* antifungal activity of chitosan and its oligomers

The postharvest deterioration due to the action of fungi limits the economic value of stored vegetables. Although fungicides are used extensively in control of postharvest diseases, there is a public interest in reducing these residues in food and in pathogens resistant to fungicides.

Unconventional methods of postharvest pathogens control have been reported in the literature. In addition to studies involving the control of pathogenic fungi by fungicides, other methods have been employed, such as biological control [9], biological control association of CaCl₂ [10–12] biological control of association with modified atmosphere [13], postharvest heat treatment [14, 15], heat treatment association and ethanol [16] and chitosan [17, 18].

There is strong evidence that the fungal mycelium growth can be delayed or completely inhibited when chitosan is added to the yeast culture medium. When increasing the chitosan concentration of 0.75 to 6.0 mg × ml⁻¹, El Ghaouth et al. [19] observed a decrease in the radial growth of *Alternaria alternata*, *Botrytis cinerea*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. The same effect was observed against *Sclerotinia sclerotiorum* by increasing the chitosan concentration of 1 to 4% (w/v) [20]. Other studies showed a linear decrease in the growth of *Rhizoctonia solani* with gradual increase in the concentration of 0.5 to 6.0 mg × ml⁻¹ of chitosan

[21]. The mycelial growth of *Fusarium solani* f. sp. phaseoli and *F. solani* f. sp. pisi was inhibited at minimum concentrations of 12 and 18 mg × ml⁻¹ [22, 23]. Complete inhibitions of the fungi *F. oxysporum*, *R. stolonifer*, *Penicillium digitatum* and *C. gloeosporioides* were obtained at a concentration of 3% (w/v) [24, 25].

Table 1 lists some studies that evaluated the effects of chitosan *in vitro* growth of pathogenic fungi.

Fungus	Chitosan concentration % (m/v)	Effect	Author
<i>A. alternata</i> , <i>B. cinerea</i> e <i>R. stolonifer</i>	0.075 to 0.6	Reduction of radial growth	[26]
<i>Rhizoctonia solani</i>	0.05 to 0.6	Growth reduction	[21]
<i>F. solani</i> f. sp. <i>phaseoli</i>	1.2	Complete inhibition	[22]
<i>F. solani</i> f. sp. <i>pisi</i>	1.8	Complete inhibition	[23]
<i>F. oxysporum</i> , <i>R. stolonifer</i> , <i>Penicillium digitatum</i> e <i>C. gloeosporioides</i>	3	Complete inhibition	[25, 26]
<i>Mucor racemosus</i>	0.2	73% of growth reduction	[27]
<i>A. alternata</i>	0.01 to 0.08	Complete inhibition	[28]
<i>B. cinerea</i>	0.04 to 0.1	Complete inhibition	
<i>P. expansum</i>	0.08	Growth reduction	
<i>R. stolonifer</i>	0.01 to 0.02	Complete inhibition	

Table 1. *In vitro* chitosan effect on fungal growth.

Allan & Hadwiger [6] reported that chitosan has a strong antifungal activity against numerous pathogens with the exception of the class *Zygomycetes*, which contains chitosan as the major cell wall component. However, the results of Roller & Covill (1999) [27] demonstrated that the fungus *Mucor racemosus*, whose cell walls are composed of chitosan, was inhibited at a concentration of chitosan 1 g × L⁻¹, contrary to Allan & Hadwiger [6] proposition, which included strains of *Mucor spp.*

No et al. [29] examined the antibacterial activity of chitosans with different molar masses on the growth of gram-positive and gram-negative bacteria. They observed that the growth of gram-positive bacteria was nearly or completely inhibited by all samples of chitosan with different molar mass. On the other hand, for the gram-negative bacteria, the antibacterial activity appeared to increase with decrease of molar mass.

El Ghaouth et al. [30] investigated the effect of chitosan coating (1.0 to 1.5% m/v) in controlling decay of strawberries at 13° C compared to the fungicidal effect of ipridione (Rovral®) and concluded that the coating with chitosan was more effective than treatment with Rovral® fungicide in controlling postharvest decay. The antifungal effects of chitosan on *in vitro* growth of strawberries postharvest have also been studied by El Ghaouth et al. [26]. According to this study, chitosan F_A 0.83 markedly reduced the mycelial growth of the fungi *Botrytis cinerea* and

Rhizopus stolonifer with a great effect at high concentrations. These authors also confirmed the importance of the large number of positively charged groups along the polymer chain due to the fact was observed that *N, O*-carboxymethyl showed lower antifungal activity than chitosan [26]. In an *in vivo* study, El Ghaouth et al. [26] reported that signs of infection were observed in strawberry fruits after five days of storage at 13°C, while the control fruit had shown signs of infection with only one day of storage. After 14 days of storage, chitosan coatings, whose concentration was 15 mg x mL⁻¹, reduced the deterioration strawberries at 60%, caused by the same fungi, and it was observed that the coated fruit usually matured without showing obvious signs of phytotoxicity.

Oliveira Jr. et al. [28] studied the inhibitory effects of fifteen chitosans with different degrees of polymerization (DP) and different DA on the growth rates (GR) of four phytopathogenic fungi (*Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer*) by using a 96-well microtiter plate and a microplate reader. The minimum inhibitory concentrations (MICs) of the chitosans ranged from 100 µg x mL⁻¹ to 1,000 µg x mL⁻¹ depending on the fungus tested and the DP and FA of the chitosan.

Table 2 lists the MICs of chitosan samples that are more effective against fungi *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer*. Complete inhibition of the fungi *A. alternata*, *B. cinerea* and *R. stolonifer* and growth reduction of *P. expansum* were obtained with chitosan DP 45 to 1,460 and FA 0.08 to 0.22. Chitosans with smaller values of F_A 0.1 and larger values of DP 3,780 promoted the maximum fungistatic activity against the fungi *B. cinerea* and *A. alternata* [28].

Fungus	Chitosan samples		MIC (µg x mL ⁻¹)
	DP	F _A	
<i>A. alternata</i>	190	0.01	200
	320	0.15	400
	121	0.49	800
	3,726	0.10	100
	3,726	0.30	100
	3,850	0.50	300
<i>B. cinerea</i>	190	0.01	800
	3,726	0.10	400
	3,726	0.30	800
	3,850	0.50	800
<i>R. stolonifer</i>	1,383	0.22	200
	45	0.22	200
	1,171	0.08	100
	1,089	0.16	100

Table 2. MICs of the chitosans with different DP and different F_A (fraction of acetylation) against *A. alternata*, *B. cinerea* and *R. stolonifer* [28].

Oliveira-Jr et al. [31] have observed that chito-oligosaccharides of DP ≤ 8 are not notably inhibitory to any of the fungi *A. alternata*, *B. cinerea*, *P. expansum* or *R. stolonifer* and high-DP chito-oligosaccharides (DP ≤ 12) showed initially inhibitory effects. However, the complete inhibition for all fungi was not obtained by using chito-oligosaccharides. In contrast, as reported by Oliveira-Jr et al. [28], *A. alternata*, *B. cinerea* and *R. stolonifer* were completely inhibited and growth reduction for *P. expansum* was observed by high-DP chitosans (DP 45 to 2,608).

Fruit, vegetable or plant	Fungus	Chitosan concentration % (m/v)	Effect (infection reduction %)	Author
Strawberry ^a	<i>B. cinerea</i>	1.0 to 1.5	77	[30]
Strawberry ^a	<i>B. cinerea</i> and <i>R. stolonifer</i>	1.5	60	[19]
Carrots ^a	<i>Sclerotinia sclerotiorum</i>	2.0 to 4.0	68	[20]
Cucumber plant ^b	<i>B. cinerea</i>	0.1	65	[32]
Strawberry ^b	<i>B. cinerea</i>	0.2 to 0.6	45 to 62	[33]
Papaya ^b	<i>C. gloeosporioides</i>	1.5	60	[24]

Table 3. Effect of chitosan coating formed on the surface of fruits and vegetables postharvest^a immersed in acidic solutions of chitosan or fruits and plants pre harvest^b sprayed with chitosan. The fruits, vegetables and plants were inoculated with the respective pathogenic fungi.

Cuero et al. [34] reported that *N*-Carboxymethyl reduced by 90% of aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*, while the fungal growth was reduced to less than 50%. Chitosan coating was effective in the inhibition of *Sclerotinia sclerotiorum* on carrots (*Daucus carota* L.) whose incidence was significantly reduced from 88 to 28% in the roots, covered with 2 and 4% (m/v) chitosan [20]. Microscopic studies revealed that the mycelium of *Sclerotinia sclerotiorum* exposed to chitosan appeared to be dead and deformed, since the control mycelium was normal.

The control of gray mold caused by *B. cinerea* in cucumber plants by chitosan oligomers was studied by Ben-Shalom et al. [32], who suggested that the primary effect of the oligomers in the control of disease was due to a fungistatic effect on the germination of the conidia of *Botrytis* because chitosan is a positively charged polymer that can prevent binding of conidia somewhere. Bhaskara Reddy et al. [33] evaluated the preharvest spraying effect of chitosan on postharvest quality of strawberry and incidence of the pathogen *B. cinerea* and observed that preventive spraying of chitosan was effective in controlling infection of *B. cinerea* on strawberries.

The relationship between molar mass of chitosans and chito-oligomers and antifungal activity has been analyzed in several studies. Kendra and Hadwiger [23] observed that monomers and dimers of chitosan showed no antifungal activity against *Fusarium solani*, while heptamers had

antifungal activity equivalent to chitosan. Uchida et al. [8] reported that a mixture of chito-oligomers with DP 2 to 8 (average of 5 DP) and concentration of 1% (m/v) were inactive against three species of the genus *Fusarium*.

Zhang et al. [35] reported that chito-oligomers with an average DP of 20 inhibited the growths of 16 plant pathogens. Torr et al. [36] suggested that higher antifungal activity against certain fungi may be obtained with chito-oligomers (DP 5, DP 9 and DP 14) when compared to those obtained with chitosan (310 kDa to >375 kDa; DP 1,925 to 2,329). Chitosan acetate and mixtures of chito-oligomers, cited above, were tested against *Leptographium procerum*, *Sphaeropsis sapinea* and *Trichoderma harzianum*. The average GR of *T. harzianum* decreased with the increase of concentration of chitosan acetate and chito-oligomers 0.1 to 0.4% (m/v), which caused an initial period of fungistase and eventually overcome by the fungus. *Sphaeropsis sapinea* and *Leptographium procerum* were more likely to chitosan and chito-oligomer activities than *T. harzianum*, whose growth was inhibited at the concentration of 0.4% (m/v) within 35 days. The antifungal activities of the three mixtures of chito-oligomers were higher at pH 4.0 than at pH 6.0, in which chito-oligomers DP 9 and 14 were more effective against *S. sapinea* and *L. procerum* than the mixture DP 5.

2.1. Action of chitosan in the production of fungal spores

The chitosan effect on spore production by the fungi *F. oxysporum*, *R. stolonifer*, *C. Gloeosporioides*, *A. alternata* f. sp. *lycopersici* and *A. Niger* was analyzed by Bhaskara Reddy et al. [33], Bautista-Baños et al. [24, 25] and Plascencia-Jatomea et al. [37]. The sporulating fungi treated with chitosans are generally lower than the untreated mold. Moreover, in some studies sporulation was completely inhibited when treated with chitosan. However, in some cases it was observed that chitosan stimulates sporulation. The formation of spores of *A. alternata* in the presence of chitosan at concentrations of 100 and 500 $\mu\text{g} \times \text{mL}^{-1}$ (sub-lethal dose) was significantly higher than the control without chitosan [25, 33]. These authors indicated that high sporulation may have been due to a stress response induced by this polymer.

2.2. Action of chitosan in the fungal spore viability

The viability of fungal spores has been analyzed after treatment with chitosan. Concentrations of 0.75 $\text{mg} \times \text{mL}^{-1}$ reduced the viability of fungal spore germination and tube growth of *B. cinerea* and *R. stolonifer* [19]. In another study, low chitosan concentrations (20-30 $\mu\text{g} \times \text{mL}^{-1}$) caused 50% inhibition of germination and 50 $\mu\text{g} \times \text{mL}^{-1}$ promoted almost complete inhibition of spore germination [32]. These authors also observed that chitosan has promoted the size reduction of the germination tubes, which had a mean size of $\sim 15 \mu\text{m}$ in the presence of water and 2 μm in the presence of 10 $\mu\text{g} \times \text{mL}^{-1}$ of chitosan, and both treatments were incubated for 24 hours. Sathiyabama and Balasubramanian [38] evaluated the effect of chitosan concentration on the viability of fungal spores of *Puccinia arachidis* incubated for 4 hours and observed that with increasing chitosan concentration from 100 to 1,000 $\mu\text{g} \times \text{mL}^{-1}$, a reduction percentage of the number of germinated spores was observed from 24 ± 3 to $6 \pm 0\%$, respectively. On the other hand, untreated spores with chitosan had a germination of $96 \pm 1\%$.

2.3. Changes in hyphal morphology due to chitosan treatment in some fungal species

Microscopic observations of fungi treated with chitosan showed that the polymer can affect the hyphal morphology. Changes in hyphal morphology, such as excessive mycelial branching, abnormal shapes, swelling and hyphae size reduction, were observed in *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *R. stolonifer* and *S. sclerotiorum* treated with chitosan [20, 26, 39]. Similarly, chitosan caused morphological changes as large vesicles or empty cells devoid of cytoplasm in the mycelium of *B. cinerea* (Figure 3) and *F. oxysporum* f. sp. *albedinis* [40, 41]. In further studies, the morphology of fungi *C. gloeosporioides*, *R. stolonifer*, *P. digitatum* and *F. oxysporum* treated with chitosan was evaluated. Bautista-Baños et al. [24, 25] reported that area, size and shape of conidia of each fungi tested were affected according to the fungal species and incubation time exposed in the chitosan solutions. Plascencia-Jatomea et al. [37] reported that the morphology of the spores of *A. niger* was also affected when treated with chitosan.

Changes in hyphal morphology due to chitosan treatment in *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer* were analyzed by scanning electron microscopy [42]. The micrographs revealed mycelial aggregation and morphological structural change as excessive branching, cell wall swelling and reduction in hyphal length (Figures 4 and 5).

Aggregation, excessive mycelial branching and hyphae size reduction of all fungi treated with chitosan were observed by Oliveira Junior et al. [42]. *A. alternata*, *B. cinerea* and *R. stolonifer* treated with chitosan, besides to have the morphological changes mentioned before, also showed abnormal shapes and swelling in their mycelia (Figures 4 and 5).

The micrographs of *P. expansum* previously treated with chitosan viewed in high magnification of 10,000× showed the chitosan coating formed on surface of the mycelia (Figure 6 A).

The results demonstrated that chitosan acetate was effective in restricting the fungal growth of filamentous fungi [42] by causing a fungistatic inhibition effect as observed by the scanning electron microscopy. In case of *A. alternata*, it was common to observe some spores with germ tube inhibition as shown in Figure 6 B.

Chitosan coating observed on the surface of the mycelia suggested that the fungal growth inhibition could be explained by a direct interaction of chitosan on the fungal cell wall as a consequence of polycationic nature of chitosan. Oliveira-Jr et al. [28] have observed that chitosan samples with low FA (high concentration of free amino groups protonated) and large DP were most effective against the phytopathogenic fungi tested, while chitosan with high FA did not have the ability to inhibit the fungal growth *in vitro*. In another study, Oliveira Jr. et al. [31] have demonstrated that chito-oligosaccharides of eight DP were not notably inhibitory to any of the fungi. On the other hand, higher chito-oligosaccharides (DP 10 and DP 12) showed initially inhibitory effects, which seemed to be more pronounced at a lower F_A .

3. Chitosan as inducer of response mechanisms in plants

Stimulants are substances (oligosaccharides, glycoproteins, peptides and lipids) that can induce defense responses when applied on plant tissue or plant cell culture. Oligosaccharides

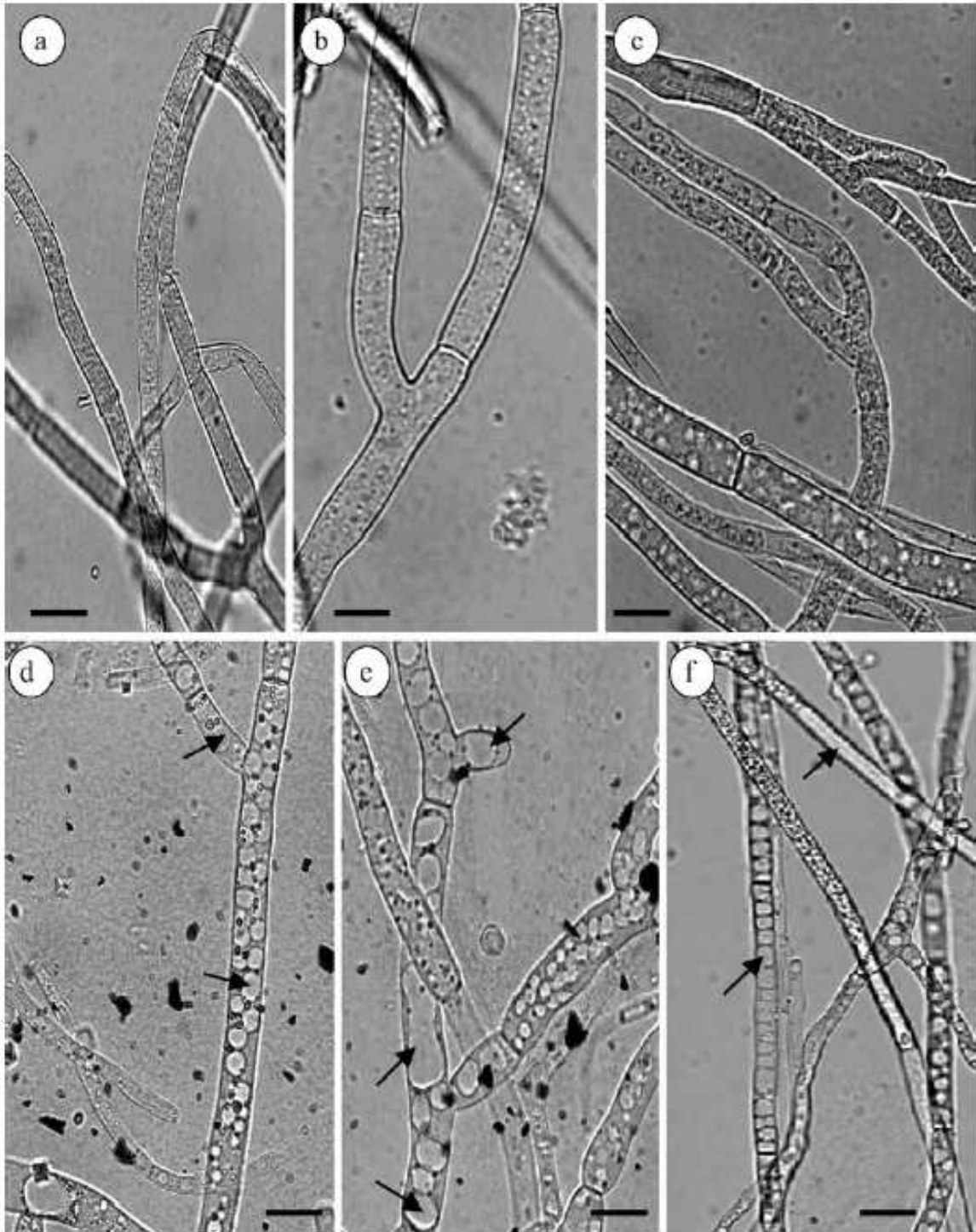


Figure 3. Microscopic structural changes of hyphal fragments of *B. cinerea* in response to the presence of (a) chitosan and (b) control mycelium; (c–f) Mycelia of fungal cultures grown on PDA containing 1.75% (v/v) of chitosan (Chito-gel®). Bars: 40 μm. Small and large vesicles appeared in the samples treated with chitosan and in some cases the cytoplasm was free of any organelle (arrows). (Reproduced from Ait Barka et al. [40]. Copyright of Plant Cell Reproduction 2004.)

most studied as inducers are oligomers of glucan, chitin, chitosan and galacturonic acids. When a plant is attacked by a pathogen, fast defense mechanisms are activated in the infected site and various biochemical defense responses occur around the dead cells. Among the biochemical defense responses include the production of reactive oxygen, structural changes in the cell wall, protein accumulation related to defense and biosynthesis of phytoalexins [43].

The stimulatory abilities of chitosan in the natural plant defense responses have been extensively studied. Physiological and biochemical changes that occur in plants due to stimulation by chitosan have been described in several studies [45–53]. Primary physiological changes were observed in plants treated with chitosan, whose openings of the stomata were decreased impeding the fungal access inside the leaf tissues. Lee et al. [44] observed that guard cells of plant leaves produce H_2O_2 , which is a mediator compound promoted by chitosan stimulus, which induces a decrease in stomatal openings (Figure 7).

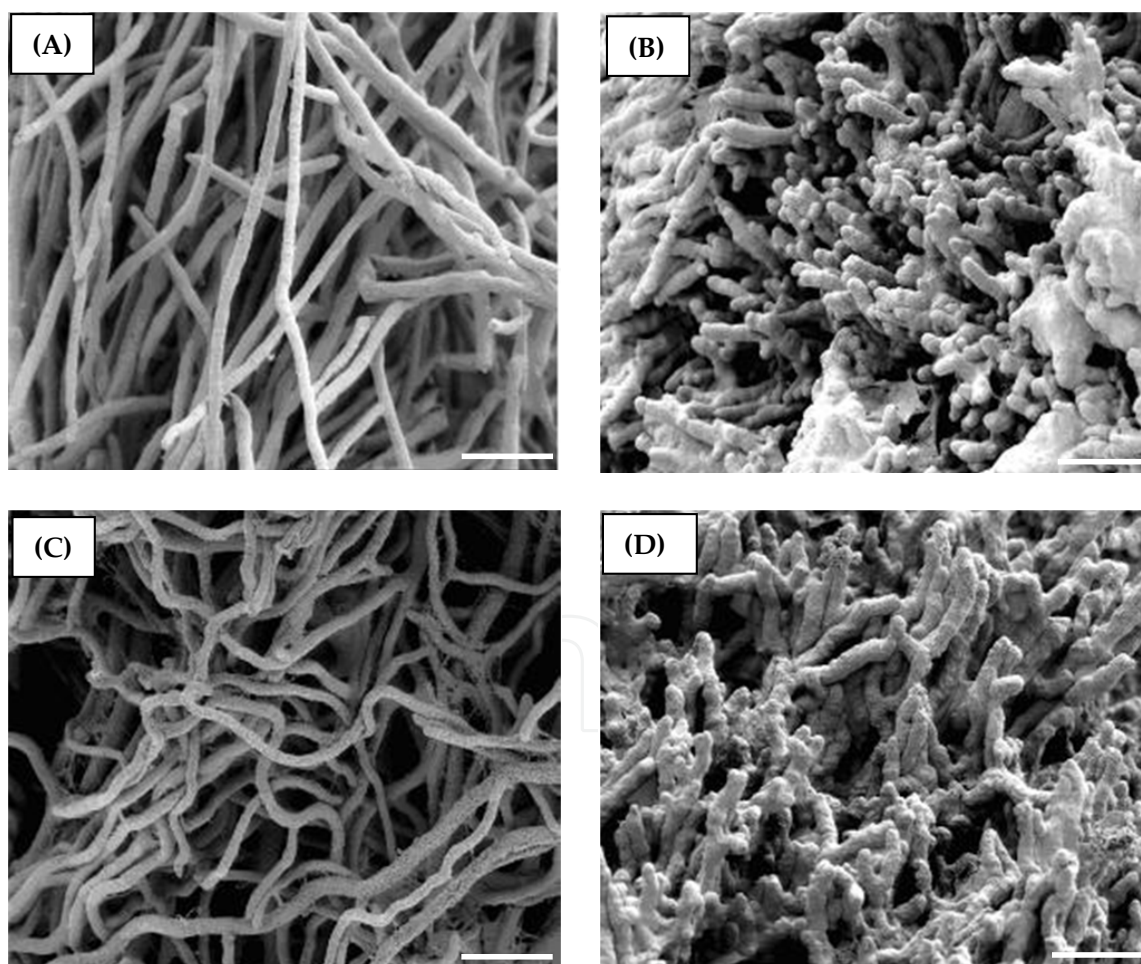


Figure 4. Scanning electron micrographs of mycelia after 5 days of cultivation at 25°C. *Alternaria alternata* (A) control media and (B) medium amended with chitosan ($500 \mu\text{g} \times \text{mL}^{-1}$). *Botrytis cinerea* (C) control media and (D) medium amended with chitosan ($500 \mu\text{g} \times \text{mL}^{-1}$). Bars = 20 μm . Reproduced from Oliveira Junior et al. [42]. Copyright of Brazilian Archives of Biology and Technology 2012.

Chitosan oligosaccharides lignin stimulated accumulation of callose, phytoalexins, and/or protease inhibitors in various plant tissues. The mechanism of action by which induces this lignification chitosan have been studied in different types of plants [46, 54].

Induction of several enzymes related to plant defense process has been studied [45, 46]. These enzymes participate in the initial defense mechanisms and prevent infection by pathogens. Oligomers of chitin and chitosan have been associated with stimulation of other systems involved in resistance as the activity of lipoxygenase and phenylalanine ammonia lyase and the formation of lignin in wheat leaf [45, 46].

The formation of structural barriers on the affected areas by fungi is the most common process response to pathogen invasion. Cell suberization and lignification and other defense processes are stimulated during the process of infection in some organs of plants. Reports describe that chitosan restricted in some cases, the fungal penetration and it induces the formation of different structural barriers. A moderate lignification on wheat leaf, as a result of chitosan

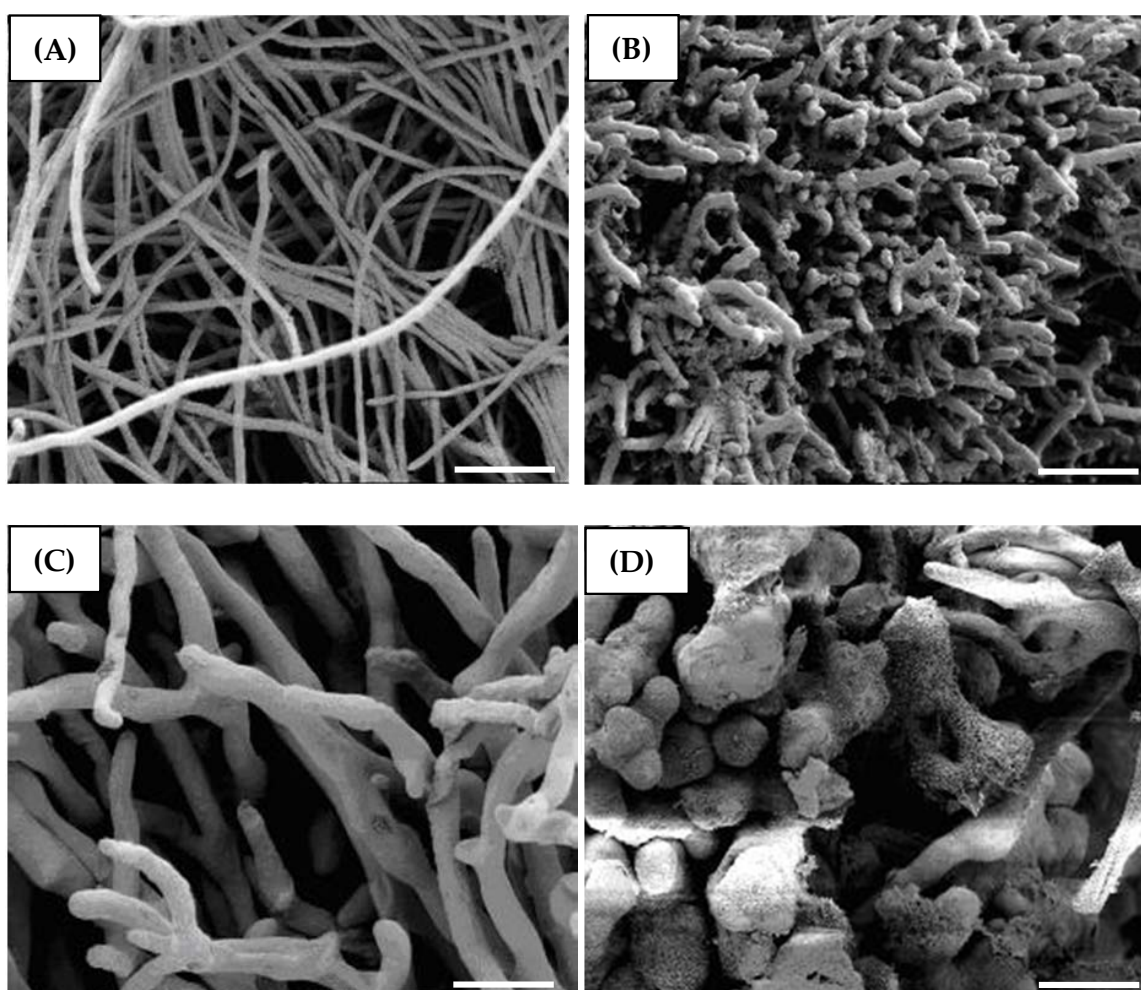
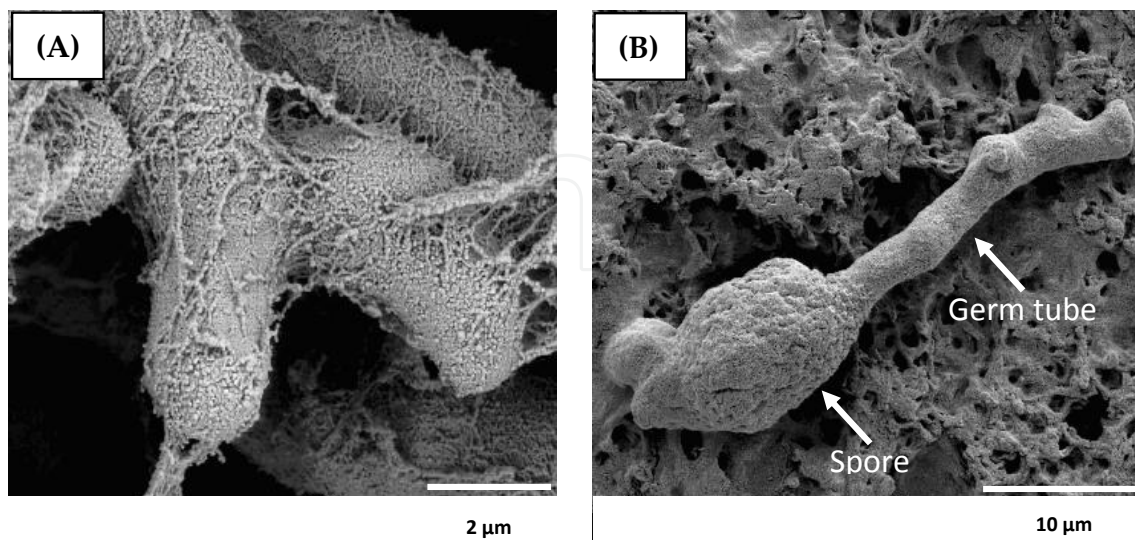


Figure 5. Scanning electron micrographs of mycelia after 5 days of cultivation for *Penicillium expansum* and after 3 days for *Rhizopus stolonifer* at 25 °C. *Penicillium expansum* (A) control media and (B) medium amended with chitosan ($500 \mu\text{g} \times \text{mL}^{-1}$). *Rhizopus stolonifer* (C) control media and (D) medium amended with chitosan ($500 \mu\text{g} \times \text{mL}^{-1}$). Bars = 20 μm . Reproduced from Oliveira Junior et al. [42]. Copyright of Brazilian Archives of Biology and Technology 2012.



(A) bar = 2 μm; (B) bar = 10 μm. Reproduced from Oliveira Junior et al. [42]. Copyright of Brazilian Archives of Biology and Technology 2012.

Figure 6. (A) Scanning electron micrograph of *Penicillium expansum* mycelia after 5 days of culture at 25°C with medium amended with chitosan D (1,000 μg × mL⁻¹). (B) Spore and germ tube of *Alternaria alternata* with medium amended with chitosan P (500 μg × mL⁻¹).

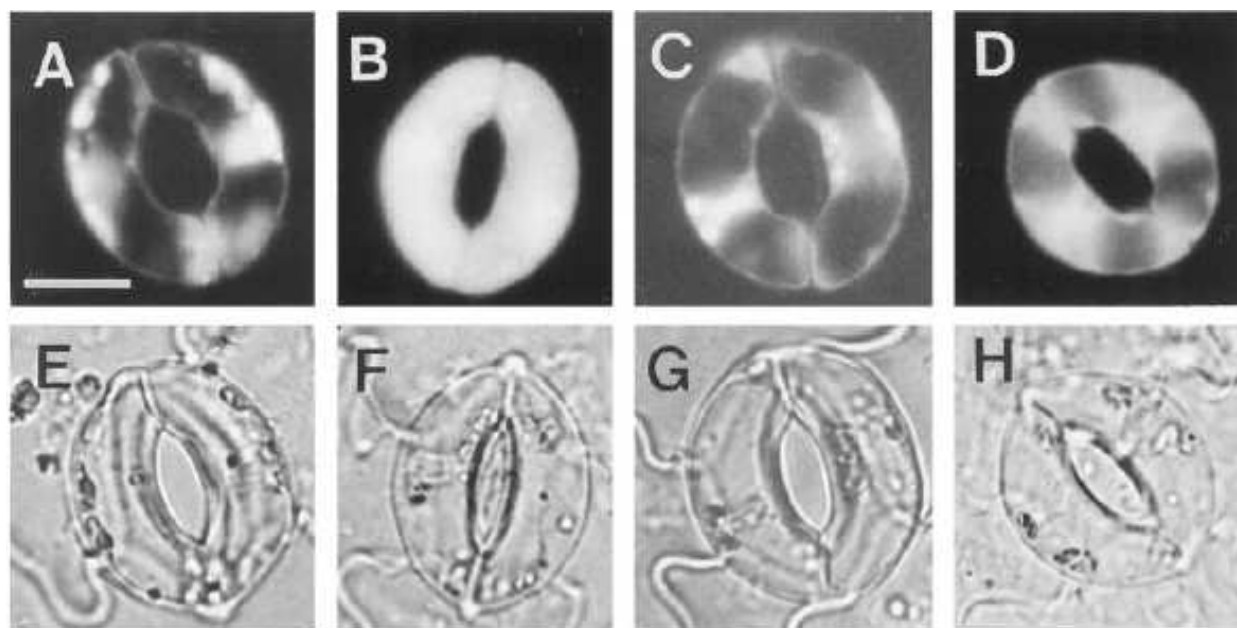


Figure 7. Induction of H₂O₂ production by chitosan in the guard cells of tomato leaves. Epidermal chunks of tomato leaves without chitosan (Controls A and E) or treatments of 30 minutes with only chitosan (B and F), chitosan and catalase (C and G) or with chitosan and ascorbic acid (D and H). Fluorescence microscopy are shown in A–D and optical microscopy are shown in E–H. The bar in A is 10 μm and applies to all figures. Reproduced from Lee et al. [44]. Copyright Plant Physiology (1999).

treatment, as well as the inoculum of cell walls of *B. cinerea* after 48 and 72 hours were reported by Pearce and Ride [47]. Transmission electron microscopy showed the formation of particular structures and new materials. The main reactions observed in the host cells of tomato roots and leaves treated with chitosan and infected by *F. oxysporum* f. sp. *radicis-lycopersici* were as follows: (1) blockage of xylem vessels by an opaque fibrous or granular material or blister-shaped structure; (2) coating the secondary membrane making it thicker and characterized by lesions (3) forming papillae (affixing wall) within the cortex and endothermic tissues [48, 49]. Other reactions of the specific host plant roots of tomato plants treated with chitosan showed deformed epidermal cells [50]. In pepper fruits of bell pepper, structural defense responses were observed only in the first layer of fabric next to broken cells, as thickening of the cell walls, forming spherical and hemispherical protrusions along the cell walls, and blocking of cellular spaces was also observed due to the formation of fibrillar material [51, 52]. Other studies have shown that the combination of two control methods (chitosan and biological control with *Bacillus pumilus*) increased defense reactions of the host plant [53]. Cucumber plants grown in nutrient solutions containing chitosan, and inoculated with *P. aphanidermatum*, had similar reactions to those observed in tomato roots treated with chitosan as obstruction of cellular spaces with opaque and fibrillar materials and lastly it was observed the formation of buds along the host cell wall [51].

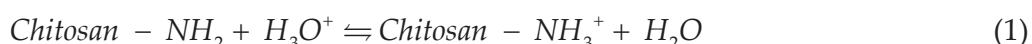
4. Effect of chitosan on postharvest quality of plant products

Plant products have their shelf life extended when coated with chitosan. Chitosan forms a semi-permeable film that regulates gas exchange and reduces losses by transpiration; therefore, the ripening of the fruit is delayed. Different fruits coated with chitosan, usually have their respiration rates and reduced water losses, among them tomatoes, strawberries, longan, apples, mangoes, bananas and bell peppers [55–60]. The efficacy of chitosan in reducing internal CO₂ production is described in tomatoes and pears [56, 57]. Chitosan coatings associated with storage temperature may be associated with a reduction in CO₂ production. Cucumbers and peppers had lower respiration rates at 13°C than at 20°C [55]. Besides the inhibition of CO₂ resulting of chitosan coating, the ethylene production of fruits is also reduced. Both inhibitory effects were observed on peaches and tomatoes coated with chitosan [56, 61]. Fruits such as strawberries, raspberries, tomatoes, peaches, papaya and other fruits had their firmness loss delayed during storage when treated with chitosan [24, 30, 61]. Sprays of chitosan preharvest at concentrations 2, 4 and 6 g × L⁻¹ on strawberry plants did not cause phytotoxicity and the fruits treated with chitosan were firmer than the control fruits [33]. In general, the anthocyanin degradation on fruits treated with chitosan is delayed, which has been demonstrated in lychee, strawberry and raspberry [62–64]. On the other hand, it was observed by El Ghaouth et al. [30] anthocyanin synthesis in strawberries treated with chitosan. Strawberries, tomatoes and peaches treated with chitosan after storage showed higher acidity compared to the control fruits, while other fruits like mangoes and longan had reduced acidity slowly [56, 59, 61, 65]. Mangoes and bananas coated with chitosan showed lower total soluble solids than fruits untreated; however, higher levels were reported in

peaches treated with chitosan. In another study it was not observed difference of soluble solid values of papayas treated with chitosan and untreated [24, 57, 60, 65]. The contents of reducing sugar of fruits are also affected by chitosan coating. Reducing sugar contents in bananas treated with chitosan were lower than contents in untreated fruits [60]. However, contradictory reports regarding to the reducing sugar contents of mango fruits treated with chitosan have been described in the literature. A possible explanation for this could be related to the chitosan application method on the surface of the fruit. In the first study, mango fruits were packed in cardboard boxes and covered with chitosan film; in this case the levels of reducing sugars were higher than those of control fruits, while in the second study, mango fruits were immersed in a solution of chitosan, and these fruits had lower levels of reducing sugars than the control fruits [60, 65]. These results indicate that the immersed fruits had decreased metabolism compared to untreated fruits with chitosan. Ascorbic acid content in mangoes and peaches treated with chitosan were also evaluated [61, 65]. In these studies, the content of this vitamin in mango fruits treated with chitosan gradually decreased during the storage period and it was lower than in fruits untreated. But in peaches, ascorbic acid levels were higher in fruits treated with chitosan than in fruits untreated, as well as treated with fungicide Prochloraz after 12 days of storage. Although few studies report the effect of chitosan on sensory attributes of plant products treated with chitosan, some reports showed that flavor and taste remain unchanged. Mangoes and strawberries treated with chitosan had higher scores in the sensory attributes compared to untreated fruit stored for 21 and 15 days, respectively [60, 62]. In other studies, strawberries coated with chitosan and stored for 12 days at 7° C had a slightly bitter taste only on day zero [66].

5. Mode of action of chitosan

Numerous possible mechanisms for the antimicrobial action of chitosan have been proposed, mostly based on the positive charge conferred by protonation of free amino groups at acidic pH, although the exact mechanism of action is still unknown. A polycationic chitosan or oligomer can potentially interact with negatively charged fungal cell membrane components (i.e., proteins, phospholipids), thus interfering with the normal growth and metabolism of the fungal cells [17, 18, 67]. Roller and Covill [27] reported that amino groups in chitosan have the ability to interact with a multitude of anionic groups on the yeast cell wall surface, thereby forming an impervious layer around the cell. Because of its property to form films, chitosan may thus act as a barrier (i.e. anionic groups) and consequently, reducing their availability to a level that will not sustain growth of the pathogen (4). This important property of the polymer chitosan, the ability to protonate at acidic solutions is due to the presence of amines in the molecule that bind to protons as shown in equation (1).



The pKa value of chitosan is approximately 6.3. The chitosan is solubilized when more than 50% of the amino groups are protonated [68]; thus, the solubility of chitosan sharply decreases when the pH increases above 6.0 to 6.5 [18].

Sudarshan et al. [70] and Papineau et al. [71] observed the bacterial agglutination using low concentrations of chitosan lower than $0.2 \text{ mg} \times \text{mL}^{-1}$ probably due to binding of the polycationic polymer to the negatively charged bacterial surface; However, at high concentrations agglutination was observed, which according to the authors may be linked to the high number of positive charges that can be formed a positive net charge on the bacterial surface keeping them in suspension.

The interaction between chitosan and the cell can also alter the permeability of the cell membrane. For example, fermentation of yeast used in baking is inhibited by certain cations that act at the cell surface and prevent glucose entry [43]. The interaction between chitosan and *Pythium oarocandrum* cells was studied by Leuba & Stossel [72], who used a UV technique, and they found that there was considerable release of protein material from the cells at pH 5.8.

Chitosan also acts as a chelating agent that selectively binds to trace metals and thus inhibits toxin production and microbial growth [34].

Liu et al. [73] reviewed the antibacterial activity of chitosan acetate solution against *Escherichia coli* and *Staphylococcus aureus*. The integrity of the cell membrane of both species was investigated by determining the release of intracellular materials which absorb at 260 nm. It has been observed a gradual increase in intercellular material suspensions of bacteria treated with chitosan acetate (0.5 and 0.25% w / v) for two hours of monitoring. The authors also noted that according to the results of infrared spectroscopy and thermogravimetric and differential thermogravimetry profiles, there was ionic bond formation between the NH_3^+ group of the chitosan acetate and the phosphoryl group of phosphatidylcholine. This result appears to confirm the electrostatic interaction between chitosan and ethyl phosphatidylcholine, which is a component of bacterial cell membrane.

6. Final Remarks

Chitin, chitosan, derivatives and their oligomers have been widely studied and the existence of great number of scientific papers that have been published in the literature reflects the great potential applications of these polymers, derivatives and oligomers. Considering the global trend of consumer preference for foods without chemical preservatives, the chitosan and other natural compounds have shown to be alternative compounds to control fungi and bacteria, although chemical preservatives are also used extensively in the control of these microorganisms, especially the fungicides used in the control of postharvest diseases of fruits. Pre- and postharvest studies of plants, vegetables and fruits have shown that the polymer chitosan has triple effect in the treatment of these; it controls pathogenic microorganisms, it activates various defense responses, inducing and/or inhibiting different biochemical activities during plant-pathogen interaction and it increases the storage time of fresh vegetable due to film formation properties.

Acknowledgements

Financial support by the Brazilian funding agency FAPEMIG is gratefully acknowledged.

Author details

Enio Nazaré De Oliveira Junior

Address all correspondence to: eniobio@ufsj.edu.br

Department of Chemistry, Biotechnology and Bioprocess Engineering, Federal University of São João del Rei, Ouro Branco, Brazil

References

- [1] Rege, P. R., Block, L. H. Chitosan processing: influence of process parameters during acidic alkaline hydrolysis and effect of the processing sequence on the resultant chitosan's properties. *Carbohydrate Research*. 321, n.3-4, 235–245, 1999.
- [2] Peter, M.G. Chitin and Chitosan from Animal Sources. In: *Biopolymers, Polysaccharides II: Polysaccharides from Eukaryotes*, (A. Steinbüchel series ed.; E. J. Vandamme, S. De Baets and A. Steinbüchel volume eds), 2002a, 6, pp 481–574. Weinheim: Wiley-VCH.
- [3] Furusaki, E.; Ueno, Y.; Sakairi, N.; Nishi, N.; Tokura, S. Facile preparation and inclusion ability of a chitosan derivative bearing carboxymethyl- β -cyclodextrin. *Carbohydrate Polymers*. 9, 29–34, 1996.
- [4] Peter, M.G. Chitin and Chitosan in Fungi. In: *Biopolymers, Polysaccharides II: Polysaccharides from Eukaryotes*, (A. Steinbüchel series ed.; E. J. Vandamme, S. De Baets and A. Steinbüchel volume eds), 2002b, 6, pp 123–157. Weinheim: Wiley-VCH.
- [5] Roberts, G. A. F. Chitin chemistry. Ed Macmillan, 1992, 350 p.
- [6] Allan, C.R., Hadwiger, L.A. The fungicidal effect of chitosan on fungi of varying cell composition. *Experimental mycology*. 3, 285–287, 1979.
- [7] Kendra, D.F., Hadwiger, L.A. Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation by *Pisum sativum*. *Experimental mycology*. 8, 276–281, 1984.
- [8] Uchida, Y., Izume, M., Ohtakara, A. Preparation of chitosan oligomers with purified chitosanase and its application. In: Skjak-Braek, G., Anthonsen, T., Sandford, P. (Eds.). *Chitin and chitosan: sources, chemistry, biochemistry, physical properties and applications*. Elsevier, London, 373–382, 1989.

- [9] Melo, I. S. Recursos Genéticos Microbianos. In: Melo, I. S., Valadares-Inglis, M. C., Nass, L. L., Valois, A. C. C. *Recursos Genéticos e Melhoramento-Microrganismos*. Jaguaruina: Embrapa Meio Ambiente, 2002. Cap. 1, pp 1–48.
- [10] Wisniewski M., Droby S., Chalutz E., Eilam Y. Effects of Ca^{2+} and Mg^{2+} on *Botrytis cinerea* and *Penicillium expansum* *in vitro* and on the biocontrol activity of *Candida oleophila*. *Plant Pathology*. 44, 1016–1024, 1995.
- [11] Tian, S. P., Fan Q., Xu, Y., Wang, Y. Effects of *Trichosporon* sp. in combination with calcium and fungicide on biocontrol of postharvest diseases in apple fruits. *Acta Botanica Sinica*. 43, 501–505, 2001.
- [12] Tian, S. P., Fan, Q., Xu Y., Jiang, A. L. Effects of calcium on biocontrol activity of yeast antagonists against the postharvest fungal pathogen *Rhizopus stolonifer*. *Plant Pathology*. 51, 352–358, 2002.
- [13] Qin G., Tian S., Xu Y. Biocontrol of postharvest diseases on sweet cherries by four antagonistic yeasts in different storage conditions. *Postharvest Biology and Technology*. 31, 51–58, 2004.
- [14] Couey, H.M. Heat treatment for control of postharvest diseases and insect pests of fruits. *HortScience*, Alexandria, 24, n.2, pp198–202, 1989.
- [15] Fallik, E., Grienberg, S., Gambourg, M., Klein, J. D., Lurie, S. Prestorage heat treatment reduces pathogenicity of *Penicillium expansum* in apple fruit. *Plant Pathology*. 45, 92–97, 1995.
- [16] Karabulut, O. A.; Gabler, F. M.; Mansour, M.; Smilanick, J. L. Postharvest ethanol and hot water treatments of table grapes to control gray mold. *Postharvest Biology and Technology*. 34, n.2, p.169–177, 2004.
- [17] Shahidi, F., Arachchi, J. K. V., Jeon, Y. J. Food applications of chitin and chitosans. *Trends in Food Science and Technology*. 10, 2, 37–51, 1999.
- [18] Bautista-Baños S, Hernandez-Lauzardo A.N, Velázquez-Del Valle M.G., Hernández-López M., Bravo-Luna L, Ait Barka E., Bosquez-Molina E., Wilson C.L. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop protection*. 25, 108–118, 2006.
- [19] El Ghaouth, E.A., Arul, J., Grenier, J., Asselin, A. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology*. 82, 398–402, 1992.
- [20] Cheah, L.H.; Page, B.B.C. *New Zealand Journal of Crop and Horticultural Science*. 25, 89–92, 1997.
- [21] Wade, H.E., Lamondia, J.A. Chitosan inhibits *Rhizoctonia fragariae* but not strawberry black root rot. *Advanced Strawberry Research*. 13, 26–31, 1994.
- [22] Hadwiger, L.A., Beckman, J.M. Chitosan as a component of pea-*Fusarium solani* interactions. *Plant Physiology*. 66, 205–211, 1980.

- [23] Kendra, D.F., Hadwiger, L.A. Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation by *Pisum sativum*. *Experimental Mycology*. 8, 276–281, 1984.
- [24] Bautista-Baños, S., Hernández-López, M., Bosquez-Molina, E., Wilson, C.L. Effects of chitosan and plant extracts on growth of *Colletotrichum gloeosporioides*, anthracnose levels and quality of papaya fruit. *Crop Protection*. 22, 1087–1092, 2003.
- [25] Bautista-Baños, S., Hernández-López, M., Bosquez-Molina, E. Growth inhibition of selected fungi by chitosan and plant extracts. *Mexican Journal of Phytopathology*. 22, 178–186, 2004.
- [26] El Ghaouth, A., Arul, J., Asselin, A. Benhamou, N. Antifungal activity of chitosan on post harvest pathogens: induction of morphological and cytological alterations on *Rhizopus stolonifer*. *Mycology Research*. 96, 769–779, 1992.
- [27] Roller, S., Covill, N. The Antifungal Properties Of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*. 47, 67–77, 1999.
- [28] Oliveira-Jr, E. N.; El Gueddari, N. E.; Moerschbacher, B. M.; Franco, T. T. Gr Inhibition of phytopathogenic fungi by characterized chitosans. *Brazilian Journal of Microbiology*. 43, 800–809, 2012.
- [29] No, H. K., Park, N. Y., Lee, S. H., Meyers, S. P. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*. 74, 65–72, 2002.
- [30] El Ghaouth, A., Arul, J., Ponnampalam, R., Boulet, M. Chitosan coating effect on storability and quality of fresh strawberries. *Journal of Food Science*. 56, 1618–1620, 1991.
- [31] Oliveira-Jr E.N.; El Gueddari, N.E.; Moerschbacher, B.M.; Peter, M.G.; Franco, T.T. Growth of phytopathogenic fungi in the presence of partially acetylated chitoooligosaccharides. *Mycopathologia*. 166, 163–174, 2008.
- [32] Ben-Shalom, N., Ardi, R., Pinto, R., Aki, C., Fallik, E. Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan. *Crop Protection*. 22, 285–290, 2003.
- [33] Bhaskara Reddy, M.V., Belcacemi, K., Corcuff, R., Castaigne, F., Arul, J. Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit. *Postharvest Biology and Technology*. 20, 39–51, 2000.
- [34] Cuero, R.G., Osuji, G., Washington, A. N-Carboxymethyl chitosan inhibition of aflatoxin production: role of zinc. *Biotechnology Letters*. 13, 441–444, 1991.
- [35] Zhang, M., Tan, T., Yuan, H., Rui, C. Insecticidal and fungicidal activities of chitosan and oligo-chitosan. *Journal of Bioactive Compatible Polymer*. 18, 391–400, 2003.

- [36] Torr, K.M.; Chittenden, C.; Franich, R.A.; Kreber, B. Advances in understanding bioactivity of chitosan and chitosan oligomers against selected wood-inhabiting fungi. *Holzforschung*. 59, 559–567, 2005.
- [37] Plascencia-Jatomea, M., Viniegra, G., Olayo, R., Castillo-Ortega, M.M., Shirai, K. Effect of chitosan and temperature on spore germination on *Aspergillus niger*. *Macromolecular Bioscience*. 3, 582–586, 2003.
- [38] Sathiyabama, M., Balasubramanian, R. Chitosan induces resistance components in *Arachis hypogaea* against leaf rust caused by *Puccinia arachidis* Speg. *Crop Protection*. 17, 307–313, 1998.
- [39] Benhamou, N. Ultrastructural and cytochemical aspects of chitosan on *Fusarium oxysporum* f. sp. *radicis-lycopersici*, agent of tomato crown and root rot. *Phytopathology*. 82, 1185–1193, 1992.
- [40] Ait Barka, E., Eullaffroy, P., Clément, C., Vernet, G. Chitosan improves development, and protects *Vitis vinifera* L. against *Botrytis cinerea*. *Plant Cell Reports*. 22, 608–614, 2004.
- [41] El Hassni, M., El Hadrami, A., Daayf, F., Barka, E.A., El Hadrami, I. Chitosan, antifungal product against *Fusarium oxysporum* f. sp. *albedinis* and elicitor of defence reactions in date palm roots. *Phytopathologia Mediterranea*. 43, 195–204, 2004.
- [42] Oliveira-Jr, E. N.; Melo, I. S.; Franco, T. T. Changes in hyphal morphology due to chitosan treatment in some fungal species. *Brazilian Archives of Biology and Technology*. 55, 637–646, 2012.
- [43] Rabea, E.I., Badawy M.E.T., Stevens, C.V., Smagghe, G., Steurbaut, W. Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules*. 4, 1457–1465, 2003.
- [44] Lee S; Choi H; Suh S; Doo I S; Oh K Y; Choi E J; Schroeder Taylor A T; Low P S; Lee Y. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant physiology*. 121(1), 147–52, 1999.
- [45] Bohland, C., Balkenhohl, T., Loers, G., Feussner, I., Grambow, H.J. Differential induction of lipoxygenase isoforms in wheat upon treatment with rust fungus elicitor, chitin oligosaccharides, chitosan, and methyl jasmonate. *Plant Physiology*. 114, 679–685, 1997.
- [46] Vander, P., Varum, K.M., Domard, A., El Gueddari, N. E. Moerschbacher, B. M. Comparison of the ability of partially N-acetylated chitosans and chito oligosaccharides to elicit resistance reactions in wheat leaves. *Plant Physiology*. 118(4), 1353–1359, 1998.

- [47] Pearce, R.B., Ride, J.P. Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. *Physiology and Molecular Plant Pathology*. 20, 119–123, 1982.
- [48] Benhamou, N., Thériault, G. Treatment with chitosan enhances resistance of tomato plants to the crown and root pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Physiological and Molecular Plant Pathology*. 41, 34–52, 1992.
- [49] Lafontaine, P.J., Benhamou, N. Chitosan Treatment: an emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Biocontrol Science and Technology*. 6, 11–124, 1996.
- [50] Benhamou, N., Lafontaine, P.J., Nicole, M. Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants by seed treatment with chitosan. *Phytopathology*. 84, 1432–1444, 1994.
- [51] El Ghaouth, E.A., Arul, J., Wilson, C., Benhamou, N. Ultrastructural and cytochemical aspects of the effect of chitosan on decay of bell pepper fruit. *Physiological and Molecular Plant Pathology*. 44, 417–432, 1994.
- [52] El Ghaouth, A., Arul, J., Wilson, C., Benhamou, N. Biochemical and cytochemical aspects of the interactions of chitosan and *Botrytis cinerea* in bell pepper fruit. *Postharvest Biology and Technology*. 12, 183–194, 1997.
- [53] Benhamou, N., Klopper, J.W., Tuzun, S. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. *Planta*. 204, 153–168, 1998.
- [54] Lesney, M. S. Polycation-like behavior of chitosan on suspension-culture derived protoplasts of slash pine. *Phytochemistry*., 29(4), 1123–1125, 1990.
- [55] El Ghaouth, A., Arul, J., Ponnampalam, R. Use of chitosan coating to reduce water loss and maintain quality of cucumber and bell pepper fruits. *Journal of Food Processing and Preservation*, 15, 359–368, 1991.
- [56] El Ghaouth, A., Ponnampalam, R., Castaigne, F., Arul, J. Chitosan coating to extend the storage life of tomatoes. *HortScience*. 27, 1016–1018, 1992.
- [57] Du, J., Gemma, H., Iwahori, S. Effects of chitosan coating on the storage of peach, Japanese pear, and kiwifruit. *Journal of the Japanese Society for Horticultural Science*. 66, 15–22, 1997.
- [58] Du, J., Gemma, H., Iwahori, S. Effects of chitosan coating on the storability and on the ultrastructural changes of ‘Jonagold’ apple fruit in storage. *Food Preservation Science*. 24, 23–29, 1998.
- [59] Jiang, Y.M., Li, Y.B. Effects of chitosan coating on postharvest life and quality of longan fruit. *Food Chemistry*. 73, 139–143, 2001.

- [60] Kittur, F.S., Kumar, K.R., Tharanathan, R.N., 2001. Functional packaging of chitosan films. *Z. Lebensmitteluntersuchung und forschung A.* 206, 44–47, 2001.
- [61] Li, H., Yu, T. Effect of chitosan on incidence of brown rot, quality and physiological attributes of postharvest peach fruit. *Journal of the science of food and agriculture.* 81, 269–274, 2000.
- [62] Li, C-F., Chung, Y-C., 1986. The benefits of chitosan to postharvest storage and the quality of fresh strawberries. In: Muzarelli, R., Jeuniaux, C., Graham, G.W. (Eds.), *Chitin in Nature and Technology*. Plenum Press, New York, pp. 908–913.
- [63] Zhang, D., Quantick, P.C. Effects of chitosan coating on enzymatic browning and decay during postharvest storage of litchi (*Litchi sinensis* Sonn.) fruit. *Postharvest Biology and Technology.* 12, 195–202, 1997.
- [64] Zhang, D., Quantick, P.C. Antifungal effects of chitosan coating on fresh strawberries and raspberries during storage. *Journal of Horticultural Science and Biotechnology.* 73, 763–767, 1998.
- [65] Srinivasa, P.C., Baskaran, R., Armes, M.N., Harish Prashanth, K.V., Tharanathan, R.N. Storage studies of mango packed using biodegradable chitosan film. *European Food Research and Technology.* 215, 504–508, 2002.
- [66] Devlieghere, F., Vermeulen, A., Debevere, J. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology.* 21, 703–714, 2004.
- [67] Fang, S.W., Li, C.F., Shih, D.Y.C. Antifungal activity of chitosan and its preservative effect on low sugar candied kumquat. *Journal of Food Protection,* 56, 136–140, 1994.
- [68] Rinaudo, M., Pavlov, G., Desbrieres, J. Influence of acetic acid concentration on the solubilization of chitosan. *Polymer.* 40, 7029–7032. 1999.
- [69] Varum, K. M., Ottoy, M. H., Smidsrod, O. Water-solubility of partially N-acetylated chitosans as a function of pH: effect of chemical composition and depolymerization. *Carbohydrate Polymers.* 25, 65–70, 1994.
- [70] Sudharshan, N. R., Hoover, D. G., Knorr, D. Antibacterial action of chitosan. *Food Biotechnology.* 6, 257–272, 1992.
- [71] Papineau, A., Hoover, D. G., Knorr, D., Farkas, D. F. Antimicrobial effect of water-soluble chitosans with high hydrostatic pressure. *Food Biotechnology.* 5, 45–57, 1991.
- [72] Leuba, S.; Stossel, P. In: *Chitin in Nature and Technology*; Muzzarelli, R. A. A., Jeuniaux, C., Eds.; Plenum: New York, 1985; p 217.
- [73] Liu, H., Du, Y., Wang, X., Sun, L. Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology.* 95, 147–155, 2004.

