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Bioefficacy of fungal chitin oligomers in the control of postharvest decay in tomato fruit

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Abstract

Tomato is one of the most commercialised and consumed fruits worldwide. However, tomatoes are highly susceptible to *Alternaria* rot. Among the safe strategies proposed to control *Alternaria* rot is the induction of defence mechanisms through biological elicitors, such as chitin. Chitin and its oligosaccharides are an activate plant defence mechanisms, but studies of fruits exposed to fungal chitin fragments are scarce. Therefore, the present work aimed to obtain and partially characterise chitin oligomers of *Alternaria alternata*, and evaluate their effect on the defence mechanism of tomato fruits and their tolerance to *Alternaria* rot. The chitin oligomers obtained had a molecular weight of ≤ 1 kDa, 12% N-acetyl-glucosamine, 0.2% residual protein, and were 94% acetylated. These oligomers markedly increased the enzymatic activity of chitinase and β-1,3-glucanase in tomato fruits, and the development of *Alternaria* rot was inhibited by 78%. Chitin oligomers of *A. alternata* represent a promising alternative to attenuate *Alternaria* rot in tomato fruits through an enzymatic defence mechanism.

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Introduction

Tomato (*Solanum lycopersicum*) is an economically significant fruit worldwide (FAO, 2019). However, its physiology and high water content make it highly perishable and susceptible to microbial damage, thus causing estimated losses ranging from 10 to 39% in developing countries (Kitinoja and Kader, 2015). When tomato fruit is harvested and stored under refrigerated conditions, it becomes highly susceptible to *Alternaria* rot (also known as black rot), a decay caused by the necrotrophic fungus *Alternaria alternata* (Sánchez-Domínguez *et al*., 2011), thus impairing the quality of the fruit and reducing its shelf life (Troncoso-Rojas *et al*., 2005). Although synthetic fungicides are the most effective method to control postharvest diseases, increasing concern exist regarding the possible negative health effects of these fungicides, and the emergence of resistant strains of pathogens caused by their excessive and prolonged use (Dukare *et al*., 2018). Therefore, to ensure food safety, some research groups have focused on identifying for less harmful preservation strategies. Among these safe strategies is the stimulation of plant defence mechanisms, that through a complex network of molecular events, protect plants against pathogen attack (Alkan and Fortes, 2015).

Chitin, a homopolymer of N-acetyl-D-glucosamine linked through β (1-4) bonds, and one of the principal components of fungal cell wall, can induce the plant defence mechanism. This polysaccharide and its derivatives are valuable due to their diverse biological and agrochemical applications (Das *et al*., 2015; Malerba and Cerana, 2019). Additionally, chitin has been approved for use as a food additive by the United States Food and Drug Administration (FDA, 2011).

Experimental evidence indicates that chitin increases the resistance to postharvest diseases by activating defence mechanisms in plants. For instance, Fu *et al*. (2016) observed that peroxidase

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and polyphenol oxidase activities increased when colloidal chitin was applied to pear fruits, and that the severity of blue mould rot caused by *Penicillium expansum* was significantly reduced. In another study, chitin isolated from the yeast *Saccharomyces cerevisiae* increased resistance against *Botrytis cinerea* in tomato fruits through the induction of defence-related enzymes (Sun *et al*., 2018). To date, no published studies have investigated the effect of fungal chitin oligosaccharides in inducing the defence mechanisms and increasing resistance against postharvest decay in tomato fruits. Therefore, the present work aimed to obtain and partially characterise chitin oligomers from the necrotrophic fungus *A. alternata*, and to evaluate their effects on the tomato infection by *A. alternata*. Furthermore, the impact on the activity of pathogenesis-related proteins in tomato fruits was also evaluated.

Materials and methods

Production of chitin oligomers from A. alternata

A strain of *A. alternata* that was previously isolated from tomato fruit and conserved in refrigeration in the Plant Biotechnology and Postharvest Laboratory of CIAD, A.C., was used. The micro- and macromorphological characteristics of the strain coincide with those reported by Pryor and Michailides (2001) and Lawrence *et al.* (2013) for *A. alternata* strain. The DNA sequences of the ITS regions of the strain were compared with those deposited in the GenBank, showing 99% of identity with *A. alternata* (GenBank accession number: AF347031.1). The reactivation and growth conditions of *A. alternata* were the same as those reported by Henry *et al*. (2019).

Chitin was isolated from *A. alternata* by enzymatic treatment as described by Henry *et al*. (2019) with some modification. Briefly, a total of 20 U/mL of commercial protease (*Bacillus licheniformis*; Sigma-Aldrich Corp., St. Louis, MO, USA) were added to *Alternaria* biomass, and incubated for 24 h at 50°C. Subsequently, 0.5 U/mL of commercial β-1,3-glucanase (*Trichoderma longibrachiatum*; Sigma-Aldrich Corp., St. Louis, MO, USA) were added to the mixture, and incubated for 72 h at 37°C. Chitin was recovered in organza fabric, washed with distilled water, and centrifuged at 15,000 *g* at 4°C. The pellet recovered was frozen and lyophilised (Freeze Dryer DC801, Yamato Scientific, Co. Ltd.) for 24 h. The lyophilised material was stored in a desiccator until subsequent resuspension. Chitin was mixed with Milli-Q water at a 5 mg/mL concentration, and then was ultrasonicated (Branson Ultrasonicator, Model 2510) at 250 W and 50°C. The sonication time was increased to 4 h instead of 3 h as reported by Henry *et al.* (2019). The suspension was filtered through organza fabric, and subsequently ultrafiltered in an AMICON stirred cell (Millipore), using a regenerated cellulose membrane with a nominal molecular weight limit (NMWL) of 1 kDa (Sigma-Aldrich Corp. St. Louis, MO, USA). The fractions containing the low-molecular-weight molecules (≤ 1 kDa) were pooled and stored at 4°C until characterised. The extraction procedure was repeated twice, obtaining different batches with similar results.

Partial physicochemical characterisation of fungal chitin oligomers

Quantification of the N-acetyl-glucosamine (GlcNAc) content

The GlcNAc content for the preparation of chitin oligomers was determined as described by Suárez *et al*. (2005) with some modifications. Briefly, 1 mL of the sample was mixed with 500 µL of 0.1 N HCl, and the mixture was stirred and allowed to stand for 5 min. Next, 1 mL of Milli-Q water was added, followed by 1 mL of 35% NaOH (m/v), and the mixture was left to stand for 5 min. The mixture was placed in a water bath at 100°C for 10 min, and allowed to cool thereafter. Its absorbance at a wavelength of 330 nm was then measured using a spectrophotometer (HACH; DR 5000). For quantification, a calibration curve (R^2 = 0.99) was constructed using standard GlcNAc (Sigma-Aldrich Corp. St. Louis, MO, USA). The measurements were made in triplicate, and the results were reported as the percentage of GlcNAc present in the solution.

Determination of the protein content

The protein content of the chitin preparation was determined using the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The reaction mixture comprised 100 µL of tomato extract, 400 µL of Bradford reagent, and 1,500 µL of water. Subsequently, the mixture was homogenised, and the absorbance at a wavelength of 590 nm was measured using a spectrophotometer. The measurements were made in triplicate, and the results were expressed as µg protein/mL.

Degree of acetylation assay

The degree of acetylation (DA) was determined through conductimetric titration using a pH meter (Model 215; Denver Instrument Co. CO, USA) as reported by Farris *et al.* (2012) and Henry *et al.* (2019). For this, a sample of the permeate (100 mL) was titrated with 0.1 N HCl. The volume of HCl needed to protonate the free amino groups of the chitin molecule was determined. A graph showing the pH and conductance values as a function of the volume of 0.1 N HCl added to the sample was constructed. From the intersection points of the linear segments of the conductivity plot and pH (or breakpoint), the volume (mL) of HCl required to deprotonate all carboxylic groups on chitin was determined. By multiplying this value by its concentration (normality) and referring to the initial polymer mass, the charge density of the polymer (mmol/g) and the percentage of acetylated groups were calculated.

Analysis of absorption bands associated with chemical bonds

The absorption bands associated with the chemical bonds in the sample were determined according to Morales-Ortega *et al*. (2013) with some modifications. Infrared spectroscopy with Fourier transform (FTIR Thermo Scientific Nicolet; Model iS50) coupled with a total attenuated reception (ATR) accessory, was performed. Dry samples of chitin and chitin oligomers were placed on top of the diamond crystal of the ATR-FTIR spectrophotometer. Mechanical pressure was applied from a rotary knob to the samples in order to acquire the proper contact with the diamond crystal. The vibrational spectra were recorded under ambient conditions. Sample scanning was performed at a resolution of 0.99 cm^{-1} with a detection range of 400 to 4000 cm^{-1}.

Postharvest application of fungal chitin oligomers to tomato fruits

Tomato fruits were obtained from a central supply located at Sonora, Mexico. Fruits at the pink ripening stage (colour number four according to the USDA colour chart), of uniform size, and without visible damage were selected. The fruits were disinfected with chlorinated water (150 mg/L), rinsed, and allowed to dry at room temperature. The fruits were divided into three groups, two of which were inoculated by immersion in a 10^6 spore suspension of *A. alternata*. The spore suspension was prepared according to French and Herbert (1982). Briefly, a culture of *A. alternata* was inoculated in PDA culture medium and incubated for 10 d at 26°C. Subsequently, 10 mL of sterile distilled water was added to the surface colony, which was carefully scraped with a sterile Pasteur pipette. The suspension was collected in a sterile Falcon tube. The spore concentration was adjusted using a Neubauer chamber and a compound microscope.

The inoculated groups were treated with chitin oligomers at different concentrations (50 and 300 µg/mL) by immersion for 30 s; these tomato groups are hereinafter referred to as InQO-50 and InQO-300, respectively. The concentrations were selected based on a preliminary test conducted in the laboratory. The third fruit group was non-inoculated, and considered as the control. The treated and control tomato fruits were placed in a cold room at 20°C and 90% relative humidity (R.H.) for 72 h. Samples of the tomato tissue (pericarp) were taken at various times (0, 0.5, 1, 3, 6, 24, 48, and 72 h), frozen at -80°C, lyophilised (Freeze Dryer DC401; Yamato Scientific Co., Ltd.), and stored for subsequent analysis. All the experiments were repeated twice.

Determination of β-1,3-glucanase activity

The enzyme extraction procedure was conducted as described by Cota *et al*. (2007). The β-1,3-glucanase activity was determined using the 3,5-nitrosalicylic acid (DNS) method (Jiménez-Maldonado *et al*., 2018) with some modifications. The reaction mixture comprised 50 µL of protein extract, 900 µL of sodium phosphate buffer (50 mM, pH 7), and 50 µL of laminarin as substrate. The mixture was incubated for 10 min at 50°C, after which 1000 µL of DNS reagent was added, and the mixture was subjected to enzyme inactivation (immersion in boiling water for 5 min). After cooling at room temperature, the absorbance of the mixture was read in a microplate reader (FLUOstar Omega, BGM LABTECH) at a wavelength of 540 nm. The specific activity was established as the amount of enzyme that hydrolysed the release of 1 µmol of glucose per min per mg protein. The determinations were performed in triplicate, and the results were reported as U/mg protein.

Chitinase activity assay

Chitinase activity was determined using a fluorometric method (Cota *et al*., 2007) with some modifications. The protein extract (10 µL) was mixed

with 5 µL of 4-methylumbelliferyl β-D-N with N',N''-triacetylchitotriose as a substrate, and 35 µL of sodium phosphate buffer (50 mM, pH 7), and incubated in a water bath (37°C) for 5 min. The reaction was stopped with Na_2CO_3 (0.2 M). The release of 4-methylumbelliferrone (4-UM) was measured by fluorescence spectroscopy (Turner BioSystems model TBS-380) using an emission wavelength of 446 nm, and an excitation wavelength of 325 nm. Quantification was conducted using a standard curve of 4-MU; the specific activity of chitinase was defined as the release of 1 µmol of 4-MU per min per mg protein. The determinations were performed in triplicate, and the results were reported as U/mg protein.

Effect of chitin oligomers on tomato tolerance to Alternaria rot

Tomato fruits were inoculated and exposed to chitin oligomers at two different concentrations (50 and 300 µg/mL) by immersion for 30 s, as mentioned previously. Two control groups were tomato fruits inoculated by immersion in a 10^6 spore suspension of *A. alternata* (positive control), and tomato fruits inoculated and treated with a commercial fungicide (Captan (R); negative control). The experimental fruits and controls were maintained in a cold room for 21 d at 20°C and 90% R.H. The number of infected fruits, progress, and severity of the lesions caused by *A. alternata* were evaluated daily. Considering the particular symptomatology of *Alternaria* rot on tomato fruits, the best option proposed to assess and quantify this disease on infected fruit is by estimating the fruit surface showing the black spots. Thus, the diameter of each lesion per fruit was measured using an electronic calliper. Thereafter, all the lesion sizes were summed to obtain the total damage size per fruit. Measurements were made from five fruits, and the results were reported as the mean area of injury in cm^2 per treatment.

Statistical analyses

The data were analysed using a completely randomised design with factorial arrangement, in which Factor A was the treatments or control, and Factor B was the sampling time. ANOVA and comparison of means were performed using Fisher's test ($p \leq 0.05$). The data were analysed using NCSS statistical analysis software v. 2011 (NCSS, Kaysville, Utah).

Results

Physicochemical characteristics of chitin oligomers of Alternaria alternata

Chitin oligomers of *A. alternata* were obtained using an extraction process involving enzymatic treatment, sonication, and ultrafiltration. The chitin extracted from *A. alternata* was initially dark in colour; however, after sonication and ultrafiltration of the extract, a clear and transparent solution containing low-molecular-weight (≤ 1 kDa) chitin oligomers was obtained. Based on the molecular weight of the chitin monomer, a polymerisation degree of ≤ 5 was estimated.

The chitin oligomers in the aqueous solution contained 12% (w/v) GlcNAc (Table 1), and had a protein content of 0.2% (w/w) after protease treatment. The acetylation degree of the oligomers was 94% (Table 1), thus suggesting that the polysaccharide in the extract was chitin. This result was confirmed by FT-IR, which showed a broad absorbance band in the region close to 3300 cm^{-1} corresponding to the stretching of the O-H bond, a characteristic of polysaccharides. The region between 1654 and 1623 cm^{-1} is associated with the amide I

Table 1. Physicochemical characteristics of chitin oligomers from *A. alternata*.

Physical and chemical characteristic	Result
N-acetyl-glucosamine content	12.0 ± 1.09% (w/v)
Residual protein	0.20 ± 0.05% (w/w)
Degree of acetylation	94.0 ± 0.89%
Degree of polymerisation (estimated)	≤ 5
Solubility	Partially soluble in water
Solution appearance	Clear, transparent, colourless

group, and could be related to proteins. The band centred at 1015 cm^{-1} could be associated with the C-OH bond; it displayed shoulders at 1149 and 885 cm^{-1} that could be attributed to the C-O-C stretching mode of the glycosidic bond and to β (1,4) linkages. Additionally, the absorbance bands observed in the region between 1420 and 1320 cm^{-1} suggested the presence of chitin (Figure 1). These results are in agreement with the FT-IR spectrum corresponding to the whole chitin molecule extracted from the necrotrophic fungus *A. alternata* reported by Henry *et al*. (2019).

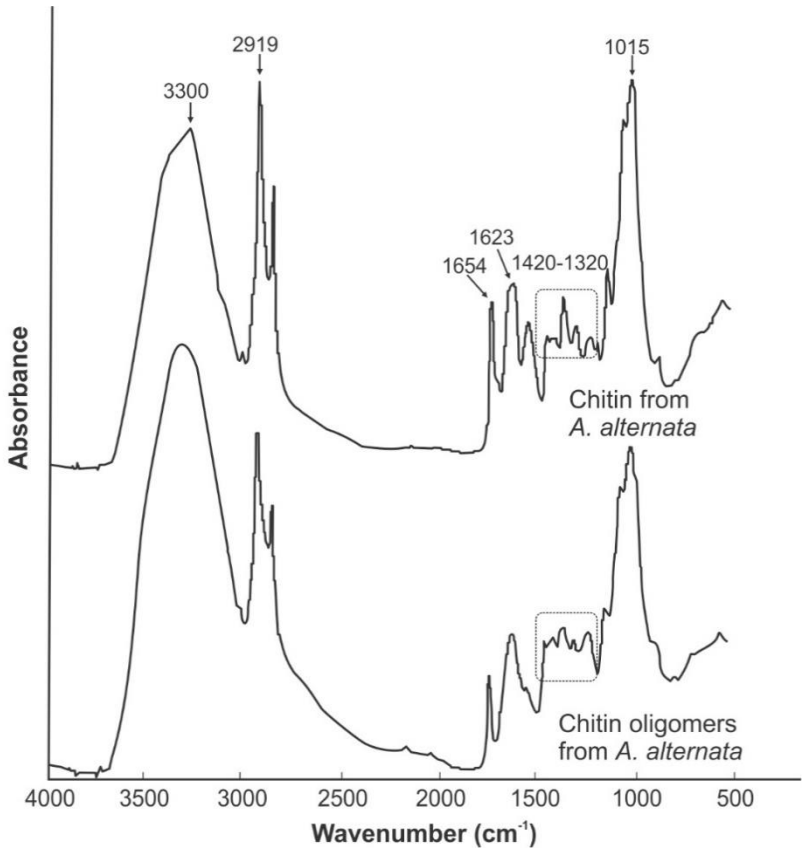

Figure 1. FTIR spectrum of chitin and chitin oligomers from *A. alternata* extracted by enzymatic treatment.

Effect of fungal chitin oligomers on the enzymatic activity

The effects of fungal chitin oligomers on the β-1,3-glucanase activity of tomato fruits are presented in Table 2. The enzymatic activity of fruits that received different treatments showed significant differences. Fungal chitin oligomers induced a significant increase ($p \leq 0.05$) in β-1,3-glucanase activity when compared with the control. However, the exposure of the inoculated fruits to a high concentration of chitin oligomers (300 µg/mL) further induced this enzymatic activity to a level of 2.24 times higher than the basal activity (2.50 U/mg protein) observed in the control.

Figure 2a shows the effect of chitin oligomers on the β-1,3-glucanase activity of the tomato fruits over time. Higher β-1,3-glucanase activity was observed in inoculated fruits exposed to chitin oligomers than in the control. Immediately after exposure to chitin oligomers, increased β-1,3-glucanase activity was observed in the treated fruits. As shown in the figure, the chitin oligomers at concentrations of 50 and 300 µg/mL induced 1.81 and 1.46 times the β-1,3-glucanase activity in tomato fruits as compared to control at zero time after treatment. Additionally, the fruits inoculated and exposed to a high concentration of chitin oligomers (300 µg/mL) showed the highest enzymatic activity ($p \leq 0.05$) after 1 h of treatment, when compared with the other treatments and sampling times. Interestingly, after 3 h, the β-1,3-glucanase activity of the treated fruit significantly decreased, and remained constant until the end of the experiment.

Table 2. β-1,3-glucanase and chitinase activities in tomato fruits exposed to fungal chitin oligomers and inoculated with *A. alternata.*

Treatment	β-1,3-glucanase activity (U/mg protein)	Chitinase activity (U/mg protein)
Control	2.50 ± 1.25[c]	25.01 ± 3.67[b]
Inoculated + chitin oligomers (50 µg/mL; InQO-50)	4.60 ± 1.71[b]	32.84 ± 3.25[a]
Inoculated + chitin oligomers (300 µg/mL; InQO-300)	5.61 ± 1.13[a]	26.13 ± 2.47[b]

Values are mean ± SD (n = 24). Means followed by different lowercase superscripts in a column are significantly different ($p \leq 0.05$).

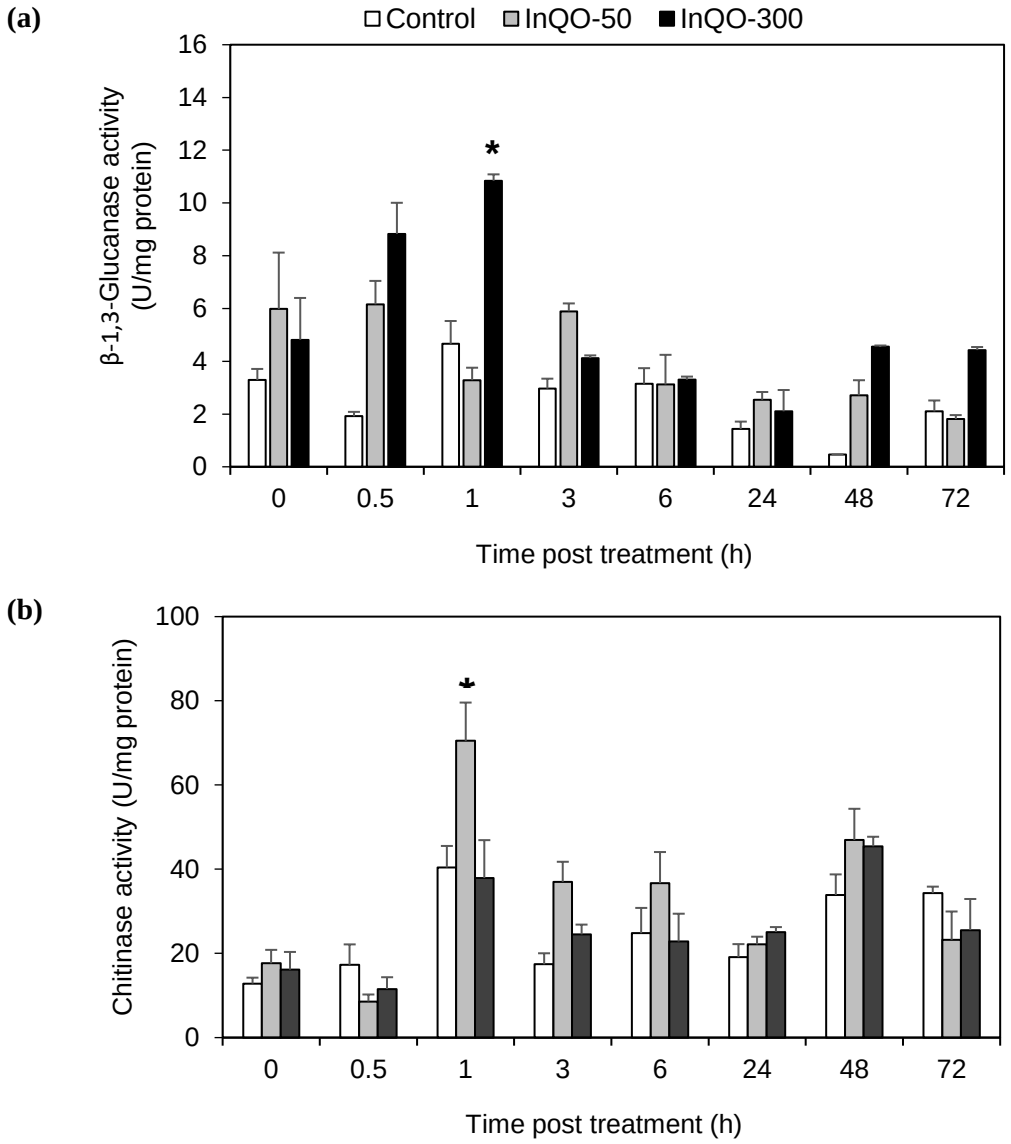

Figure 2. Effect of chitin oligomers from *A. alternata* on the enzymatic activity of inoculated tomato fruits evaluated at different post-treatment times: **(a)** β-1,3-glucanase activity; and **(b)** chitinase activity. The columns represent the mean + SD (n = 3), and the bars represent the standard errors of the means. The asterisk (*) above the columns indicate significant differences ($p \leq 0.05$).

Exposure to chitin oligomers obtained from *A. alternata* also induced an increase in chitinase activity in tomato fruits (Table 2). Significant differences were found between treatments; fruits inoculated with *A. alternata* and exposed to a low concentration of chitin oligomers (50 µg/mL) showed the highest ($p \leq 0.05$) chitinase activity. The chitin oligomers induced a significant increase in chitinase activity immediately after exposure, particularly at the lowest concentration of chitin oligomers used (50 µg/mL; Figure 2b). At that time, chitinase activity was induced to a level of 2.6-fold higher than that of the control. The highest activity was induced after 1 h of exposure to chitin oligomers, and this behaviour was similar to that observed for β-1,3-glucanase activity.

Effect of chitin oligomers on the development of Alternaria alternata disease in tomato fruits

Figure 3 shows the effect of the fungal chitin oligomers in preventing the development of *A. alternata* lesions on the surfaces of the fruits. The first visible symptoms of infection appeared as small dark spots in the inoculated control fruit at 8 d post-inoculation, whereas the fruits exposed to chitin oligomers at a concentration of 300 µg/mL showed the first fungal lesions after 14 d of inoculation. No significant differences were observed between the inoculated control fruits and inoculated fruits exposed to chitin oligomers at a concentration of 50 µg/mL, thus indicating that exposure to chitin oligomers at this concentration had no significant effect ($p \geq 0.05$) on the control of *Alternaria* rot in the fruits. The tomato fruits treated with the highest concentration of fungal chitin oligomers had the most evident effect on the control of *Alternaria* rot. This treatment reduced the area of injury by more than 78% when compared with the inoculated control at 21 d after treatment.

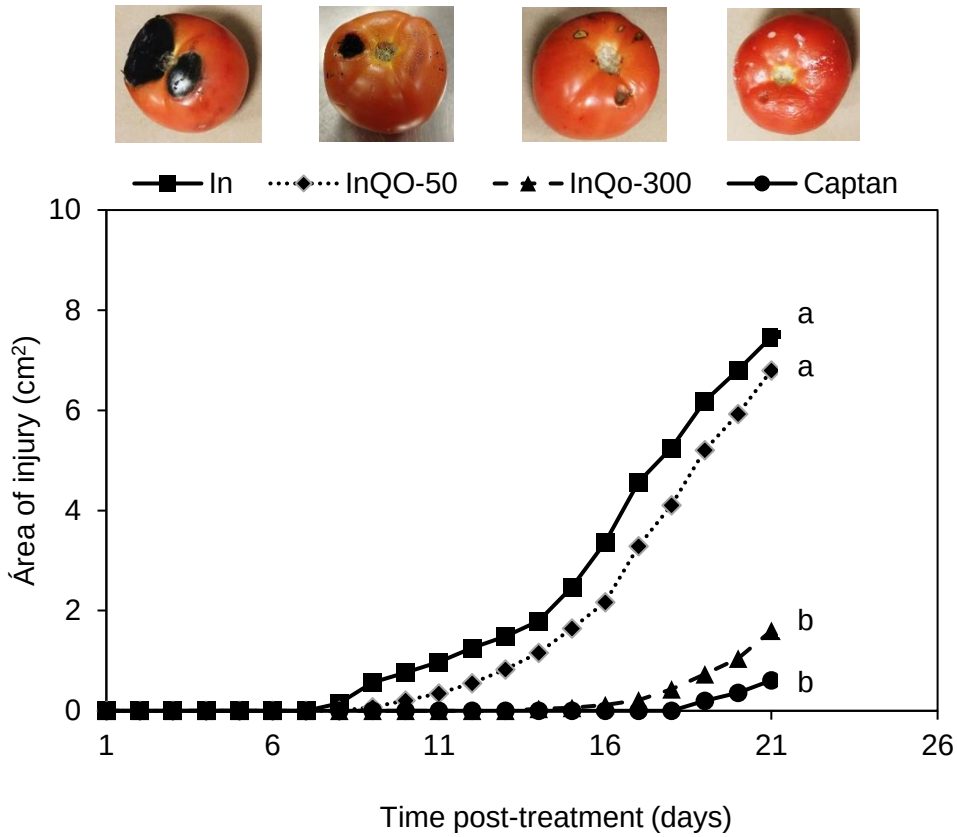

Figure 3. Development of *Alternaria* rot in tomato fruits exposed to fungal chitin fragments. In: inoculated fruits with *A. alternata*; InQO-50: inoculated fruits with *A. alternata* and exposed to fungal chitin oligomers at concentration of 50 µg/mL; InQO-300; inoculated fruits with *A. alternata* and exposed to fungal chitin oligomers at concentration of 300 µg/mL. Different lowercase letters indicate significant differences ($p \leq 0.05$).

Discussion

Chitin oligomers are recognised as molecular patterns associated with microbes (PAMPs), particularly fungi, which induce immune responses in plants. The present work demonstrated that chitin oligomers of *A. alternata* were capable of protecting tomato fruits against *Alternaria* rot, and this effect was partly attributed to the increase in β-1,3-glucanase and chitinase enzymatic activity.

In the present work, chitin oligomers of molecular weight ≤ 1 kDa were obtained from *A. alternata*, a necrotrophic fungus that causes postharvest decay in tomato fruits. Importantly, the increase in the sonication time used in the present work, when compared with the extraction method previously reported by Henry *et al*. (2019), improved the protocol and a higher yield of N-acetyl-glucosamine was obtained. According to previous studies (Mislovičová *et al*., 2000), sonication makes it possible to obtain water-soluble polysaccharide fractions of an insoluble polymer (chitin-glucan), with yields of 13.6 to 24.4% with respect to the concentration of the initial insoluble complex. This result is consistent with the results of the present work, in which the percentage of water-soluble N-acetyl-glucosamine in the extracted polysaccharide fraction was approximately 12% (w/v) (Table 1).

Chitin oligomers from *A. alternata* showed a high degree of acetylation. The chitin molecule contains more than 50 - 60% acetylated units, while fewer than 50% of acetylated units correspond to the chitosan molecule. Wu *et al*. (2005) reported that fungal chitin obtained from *Aspergillus niger* by alkaline treatment presented 76.53% of acetylation degree. These acetylation percentages are lower than those found in the present work. The difference might have been due to the different extraction methods used. The traditional extraction method which involves treatment with high concentrations of sodium hydroxide at high temperatures, causes deacetylation of the molecule (Rojas *et al*., 2014). However, the enzymatic treatment allows the production of chitin with a high degree of acetylation (Henry *et al*., 2019).

It is widely reported that plants activate defence mechanisms in response to various types of stimuli and elicitors. In the present work, the capacity of chitin oligomers from *A. alternata* to act as an elicitor of defence mechanisms was evaluated based on the activity of pathogenesis-related proteins and development of *Alternaria* rot in tomato fruits. From the results obtained, it was clear that chitin oligomers induced a significant increase in β-1,3-glucanase and chitinase activities in tomato fruits immediately after treatment. Although the first sampling time used in the present work was marked as 0 min, the tissue samples were actually taken 3 - 5 min after the fruits were treated and processed. Some authors have reported that the first molecular interactions involved in activating the biochemical events occur approximately 5 min after contact of the invading agent with the plant (Shen *et al*., 2017). Although early interaction times are not well defined or studied, it is clear that the defence mechanism in plants is activated within the first few minutes after contact with an elicitor. However, scientific studies published to date on the inductive effect of chitin in fruits are scarce to non-existent. Therefore, the results of the present work are particularly relevant, and clearly showed that the activation of the chitin-related defence mechanism in fruits occurred within a few minutes after contact with the elicitor.

In the present work, two concentrations (50 and 300 µg/mL) of chitin oligomers were applied, but no significant differences were found in the β-1,3-glucanase activity of the fruits treated with chitin oligomers at these two concentrations. These results are similar to those reported by other authors who observed the induction of β-1,3-glucanase activity in barley cells after treatment of the plants with chitooligosaccharides at a concentration of 0.3 µg/mL. However, when plants were treated with oligosaccharides at a concentration of 4 µg/mL, the enzymatic activity remained constant (Kaku *et al*., 1997). This result suggested that the eliciting capacity does not depend on the concentration of the elicitor used, although more experimental evidence is required to support this hypothesis.

Importantly, scientific studies are lacking, in which the early events that occur during fruit infection are evaluated. In published studies, enzymatic activities were evaluated beginning at 12 or 24 h after inoculation. However, the results obtained in the present work suggested that fruit responses begin within a few minutes after the spore comes into contact with the fruit surface or cuticle. At that time, pathogens secrete various molecules that allow them to modulate the host's physiology, prepare the colonisation site, or provide protection

(Rodriguez-Moreno *et al*., 2018). *A. alternata* secretes enzymes, polysaccharides, and toxins that facilitate the development of infection. Additionally, during the first 3 h of contact with the host, germination and penetration through the cuticle occur (Troncoso-Rojas and Tiznado-Hernández, 2014), thereby triggering a series of biochemical and molecular events that coincide with the induction of β-1,3-glucanase activity in the early stages after inoculation, as observed in the present work. However, the enzymatic activity decreased after 3 h, thus suggesting that the fruit cells responded to counteract the effect of germination and penetration of the fungus by initiating other biochemical and molecular events, or that the β-1,3-glucanase enzyme can be inhibited, sequestered, or blocked by fungal effectors, as reported by Pusztahelyi (2018).

Mild symptoms of *Alternaria* rot were observed after 14 d in fruits exposed to fungal chitin treatment, while in inoculated fruits, the first symptoms were observed after 8 d. According to the literature, the first symptoms of *A. alternata* are typically observed on the third day post-inoculation (Adhikari *et al*., 2017; Encinas-Basurto *et al*., 2017). These differences might have been related to the tomato variety used as factors such as plant variety, preharvest conditions, and susceptibility associated with the stage of maturity could influence the development of the infection (Cota *et al*., 2007; Alkan and Fortes, 2015).

No evidence in the literature exists regarding the effect of chitin oligomers from *A. alternata* in reducing fungal decay in horticultural products. Few studies are available in which the antifungal activity of chitin from crustaceans or yeasts has been studied (Zhang *et al*., 2016). A recent study reported that chitin obtained from the cell wall of *Saccharomyces cerevisiae* at a concentration of 0.5% significantly reduced the disease incidence of *Botrytis cinerea* in the fruits (Sun *et al*., 2018). This treatment reduced the incidence of fungal infection after two days of incubation and storage at 20°C by more than 28%. This inhibition was lower than that observed in the present work, thus suggesting that the chitin oligomers of *A. alternata* have a strong inductive effect on tomato defence mechanisms, thereby leading to significant inhibition of infection caused by *A. alternata*.

The chitin molecule does not present antifungal activity by itself, rather it acts by inducting the plant defence mechanism (Kecheng *et al*., 2020). This mechanism, which is very complex, begins with the recognition of PAMPs by RLK or RLP-type membrane receptors such as CERK1 (reported in *Arabidopsis thaliana*) or CEBiP (reported in *Oryza sativa*) (Miya *et al*., 2007; Kishimoto *et al*., 2010; Buendia *et al*., 2018; Abdul *et al*., 2020). However, little or no information is available on possible receptors in tomato that recognise the fungal chitin molecule and its oligosaccharides. This recognition activates a complex network of biochemical events that include changes in pH; reactive oxygen species production; increased synthesis of ethylene, jasmonic acid, and salicylic acid; production of secondary metabolites; and the synthesis of enzymes, among others (Iizasa *et al*., 2010; Hayafune *et al*., 2014; Pusztahelyi, 2018); these events contribute to the protection of the fruit against fungal infection. The findings reported herein confirmed that the enzymes evaluated contributed to the defence mechanism. Furthermore, these enzymes were likely induced through fruit recognition of the chitin oligomers of *A. alternata.*

Conclusion

Chitin oligomers isolated from *A. alternata* with low molecular weight (≤ 1 kDa) and a high degree of acetylation (94%) induced the tomato fruit defence mechanism against *A. alternata* attack by activating β-1,3-glucanase and chitinase enzymatic activities. Additionally, these chitin oligomers significantly inhibited the development of the infection caused by *A. alternata* in tomato fruit. Chitin oligomers of *A. alternata* are a promising alternative to control *Alternaria* rot in tomato fruit through the enzymatic defence mechanism.

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