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Evidence on antimicrobial properties and mode of action of a chitosan obtained from crustacean exoskeletons on *Pseudomonas syringae* pv. *tomato* DC3000

A. Y. Mansilla · L. Albertengo · M. S. Rodríguez · A. Debbaudt · A. Zúñiga · C. A. Casalongué

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Abstract *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) causes bacterial speck of tomato, a widely spread disease that causes significant economical losses worldwide. It is representative of many bacterial plant diseases for which effective controls are still needed. Despite the antimicrobial properties of chitosan has been previously described in phytopathogenic fungi, its action on bacteria is still poorly explored. In this work, we report that the chitosan isolated from shrimp exoskeletons (70 kDa and 78 % deacetylation degree) exerts cell damage on *Pto* DC3000. Chitosan inhibited *Pto* DC3000 bacterial growth depending on its concentration, medium-pH, and presence of metal ion (Mg^{+2}). Biochemical and cellular changes resulting in cell aggregation and impaired bacterial growth were also viewed. In vivo studies using fluorescent probes showed cell aggregation, increase in membrane permeability, and cell death, suggesting the chitosan antibacterial activity is due to its interaction as a polycation with *Pto* DC3000 membranes. Transmission electron microscopic analysis revealed that chitosan also caused morphological changes and damage in bacterial surfaces. Also, the disease incidence in tomato inoculated with *Pto* DC3000 was significantly reduced in chitosan pretreated seedlings, revealing a promising action of chitosan as nontoxic biopesticide in tomato plants. Indeed, a wider comprehensive knowledge of the mechanism of action of chitosan in phytopathogenic bacterial cells will increase the chances of its successful application to the control of spread disease in plants.

Keywords Chitosan · Membrane permeability · *Pseudomonas syringae* pv. *tomato* DC3000 · *Solanum lycopersicum*

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A. Y. Mansilla · C. A. Casalongué (✉)
Instituto de Investigaciones Biológicas. UE CONICET-UNMDP, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC 1245 (7600) Mar del Plata, Argentina
e-mail: casalong@mdp.edu.ar

L. Albertengo · M. S. Rodríguez · A. Debbaudt · A. Zúñiga
Laboratorio de Investigaciones Básicas y Aplicadas en Quitina (LIBAQ), Instituto de Química del Sur (INQUISUR). UE CONICET-UNS, Departamento de Química, Universidad Nacional del Sur, Avenida Alem 1253 (8000) Bahía Blanca, Argentina

Introduction

Bacterial speck is an economically significant disease and representative of many bacterial plant diseases (Wilson et al. 2002). *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) is the cause of bacterial speck on tomato and *Arabidopsis*, and represents an important model in molecular plant pathology. Tomato (*Solanum lycopresicum*) is one of the most important vegetable crops worldwide. The effective controls of bacterial speck are being currently explored in crop plants. Nowadays, toxic chemicals cause resistance development in pathogens and decrease in beneficial organism population (Glick and Bashan 1997; Ji et al. 2006). For all these reasons, current knowledge is emerging on more sustainable compounds.

Chitosan is a natural nontoxic biopolymer produced by partial alkaline *N*-deacetylation of chitin, the main component of crustacean exoskeletons and fungi cell walls. Chemically, chitosan is a polycationic heteropolysaccharide made up by copolymer, (1→4)-2-amine-2-deoxy-β-D-glucan and (1→4)-2-acetamide-2-deoxy-β-D-glucan. Chitosan is a molecule of high molecular mass and rather insoluble in water but soluble in diluted aqueous acidic solutions below its pK_a (~6.5) that can convert glucosamine units into the

soluble protonated form ($-NH_3^+$) depending on its physicochemical nature. The biological properties of chitosan are attributed to several causes including the deacetylation degree, molecular mass and concentration (Aranaz et al. 2009; Goy et al. 2009; Liu et al. 2006) as well as the biological sources of chitin and protocols used to prepare chitin/chitosan (Kim and Rajapakse 2005; Kumirska et al. 2011).

Most of the agricultural applications of chitosan are for stimulation of plant defense mechanisms (El Hadrami et al. 2010; Rabea et al. 2003). In phytopathology, antimicrobial properties of chitosan have been mainly studied against fungi (Palma-Guerrero et al. 2009; Palma-Guerrero et al. 2008), and to a lesser extent against viruses and bacteria (Chirkov 2002; Kulikov et al. 2006). Notably, antibacterial action of chitosan has been widely reported for food and clinically important bacteria however, this fact has not been equally matched with phytopathogenic bacteria (Raafat et al. 2008). Otherwise, some studies on chitosan action directed towards bacterial pathogens in plants have been more recently carried out (Li et al. 2010; Li et al. 2008; Lou et al. 2011; Wang et al. 2012). Recently, Li et al. (2013) described the antibacterial activity of two chitosans from commercial source on rice bacterial leaf blight and leaf streak. Apparently, the effectiveness of a supplemented and commercial chitin extract towards the phytopathogenic bacterium, *Pseudomonas syringae* pv. *actinidiae* has been first reported by Ferrante and Scortichini (2010). The chitosan here studied was obtained from exoskeleton wastes of crustaceans that live in the Argentine seacoast. The influence of this chitosan on foods used in human diets has been previously studied (Rodríguez et al. 2008; Rodríguez and Albertengo 2005). In this work, we studied the chitosan action on Gram-negative bacteria *Pto* DC3000. These findings reveal antimicrobial properties of the tested chitosan in bacterial cells and tomato seedlings resulting in an outstanding nontoxic compound to be used as a bactericide in plants.

Materials and methods

Isolation and chemical characterization of chitosan

Chitin was first isolated from shrimp (*Pleoticus mülleri*) waste as described previously (Rodríguez et al. 2008). Chitosan was prepared directly by heterogeneous deacetylation of chitin with 50 % (*w*/*w*) NaOH. For the biopolymer characterization, moisture and ash contents were determined at 100–105 °C and 500–550 °C, respectively. Deacetylation degree (DD) was obtained using FT-IR spectroscopy (Nicolet iS10 FT-IR Spectrometer, Thermo Fisher Scientific, Waltham, USA) with samples in the form of KBr at ratio of 1:2. Viscosity of 1 % (*w*/*v*) chitosan in 1 % (*v*/*v*) acetic acid solution was measured with a Brookfield model DV-IV + viscosimeter (Brookfield, Stoughton, MA, USA) with spindle 21. Viscosity average molecular weight (Mv) was determined by using an Ubbelohde viscometer. The characteristics of the chitosan used in this study are 70 kDa, DD 78 %, viscosity 80 m Pa s, 6.7 g % (*w*/*v*) moisture and 0.67 g % (*w*/*v*) ash. Stock solution of 10 mg ml^{-1} chitosan was prepared in 1 % (*w*/*v*) acetic acid, pH 5.5 adjusted with 1 M KOH, and sterilized by autoclaving.

N-propyl-*N*-methylene phosphonic chitosan derivative (PNMPC) was obtained and characterized as described by Zuñiga et al. (2010).

Bacterial strain and culture conditions

Pto DC3000 (NCPPB, USA, collection number 1008), a well-characterized Gram-negative bacterium, was used (Cecchini et al. 2011). The bacterium was maintained on King's B (KB) agar medium (King et al. 1954) containing suitable antibiotics, 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Single colonies grown at 30 °C for 48 h were isolated from agar plates and transferred to KB broth and grown overnight at 30 °C with shaking. Fifty-milliliter cultures were grown aerobically at 30 °C with shaking at 150 rpm. A 10 % (*v*/*v*) inoculum from an overnight starter culture was used.

Effects of pH and $MgCl_2$ on chitosan-treated *Pto* DC3000 cells

Aliquots of overnight starter cultures of *Pto* DC3000 were inoculated into fresh KB liquid medium to nearly 10^6 cells ml^{-1} and mixed with different concentrations of chitosan (50 and 100 μg ml^{-1}) as indicated.

The effect of 100 μg ml^{-1} chitosan on *Pto* DC3000 cells was analyzed under different pH conditions and in the presence and absence of $MgCl_2$. In parallel, two sets of identical experiments were conducted by using KB broth adjusted with diluted acetic acid solution to pH 7.0 or 6.5. For the metal ion assay, KB medium containing or not 100 μg ml^{-1} chitosan was supplemented with 25 mM $MgCl_2$ at pH 6.5. The OD_{600} was checked in cell suspension cultures at 0, 6, and 16 h by using an Ultrospec 1100 pro UV/visible spectrophotometer (GE Healthcare, USA). Each value of OD_{600} indicated the average of triplicate samples. The OD_{600} values allowed us to assess the cells concentration by the equation OD_{600} $0.2=2\times10^8$ cells ml^{-1} (Nemchinov et al. 2007). The *Pto* DC3000 cell wet weight was assessed as the cell pellet weight after centrifugation of culture aliquots at 16 h after onset of initial treatments.

Colony-forming units from chitosan-treated *Pto* DC3000 cells

For determination of colony-forming units (CFU), dilution series of the cell suspensions treated or nontreated with chitosan for 16 h were prepared. Twenty-five microliters of each

dilution were plated on KB agar plates containing 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Before counting the number of colonies plates were incubated overnight at 30 °C.

Plant material and inoculation

The experiments with plants were performed using tomato seeds of cv. Platense. Seedlings were grown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 0.8 % (*w*/*v*) agar. After 4 days of germination at 25 °C in darkness seedlings were placed on MS-agar plates supplemented with 10 μg ml^{-1} of chitosan or 0.001 % (*v*/*v*) acetic acid solution as control. Eight days old seedlings were infected with *Pto* DC3000 according to Uppalapati et al. (2008). Briefly, an overnight culture of the *Pto* DC3000 was diluted with a sterile solution of 10 mM $MgCl_2$ and 0.025 % (*v*/*v*) SILWET L-77 (OSI Specialities Inc., Danbury, CT, USA) as surfactant until reaching an OD_{600}=0.1. To achieve uniform inoculation, the cell suspension was dispensed into Petri dishes until seedlings were submerged. Then, seedlings were exposed to bacterial cells with gentle mixing for 5 min. After the inoculum was discarded, Petri dishes containing inoculated seedlings were sealed with parafilm M and incubated at 18 °C with light intensity of 120 μmol photons m^{-2} s^{-1} with 16:8 h light/dark cycles. The symptoms were noted in 7-day-old seedlings. As mock control, seedlings were flooded in 10 mM $MgCl_2$ and 0.025 % (*v*/*v*) SILWET L-77. Nearly, 15 seedlings were examined in each experiment. Independent experiments were repeated at least three times.

Evaluation of *Pto* DC3000 in tomato cotyledons

Bacterial growth in tomato seedlings was measured by determining the remaining bacterial inoculums in the leaf tissue at the end of the experiment. To remove epiphytic bacteria before sampling, seedlings were surface-sterilized with 70 % (*v*/*v*) ethanol for 1 min. Nearly 50 mg of green tissue from seedlings were washed twice with sterile distilled water and then homogenized in 500 μl of cold and sterile distilled water using a pestle in sterile microcentrifuge tubes. The homogenates were vortexed for 5 s, 10-fold serially diluted and cultured on KB medium plates supplemented with 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} rifampicin. Plates were placed at 30 °C for 2 days. For each dilution, the number of CFU was counted.

The lesion area in tomato cotyledons was measured using the image-processing software ImageJ (NIH, Maryland, USA) and related to 100 % as the total cotyledons area.

Cell permeabilization assay

Bacteria with compromised cell membranes were detected by recording the fluorescence of the DNA-binding dye SYTOX Green (Roth et al. 1997). Permeabilization of the bacterial outer and/or cytoplasmic membranes allows the dye to cross the membranes and to intercalate into the DNA.

Pto DC3000 cultures treated with 100 μg ml^{-1} chitosan or 0.01 % acetic acid (*v*/*v*) for 16 h were diluted to an OD_{600}= 0.5. For fluorescence assays, aliquots of 100 μl of cell suspension were mixed with the fluorescent dye to a final concentration of 5 μM SYTOX Green (Molecular Probes Inc., Eugene, Oregon, USA) and incubated at room temperature for 15 min in the dark. When excited at 495 nm, the dye binding to DNA resulted in an increase in emitted fluorescence at 538 nm, which was measured in a microtitre plate reader (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland). As a positive control, the fluorescence emission from 70 % (*v*/*v*) ethanol-treated cells was analyzed. Background readings were obtained from cell-free wells containing KB media and SYTOX Green. An additional control of chitosan and fluorescent probe solution without bacteria was included. The values were expressed as the means of four independent experiments. Each independent experiment was performed in duplicate. Qualitative detection of SYTOX Green uptake was done with a Nikon Eclipse TiU inverted microscope equipped with an epifluorescence unit and B-2E/C filter set (Nikon Corp., Tokyo, Japan) containing an excitation filter at 480/30 nm, suppressor filter at 535/40 nm, and a 505-nm dichroic mirror.

Cell viability assay

Cell viability was determined by propidium iodide exclusion (Novo et al. 2000). Propidium iodide is used as a DNA stain to evaluate cell viability or DNA content. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20–30-fold. Propidium iodide (Sigma–Aldrich, St. Louis, MO, USA) was added in each well to a final concentration of 120 μM containing 100 μl of *Pto* DC3000 suspension (OD_{600}=0.5) and kept at room temperature for 15 min in the dark. Five microliters of the stained cell suspensions were deposited onto glass slides and covered with coverslips. Cells were noted and photographed in a Nikon Eclipse TiU inverted microscope equipped with an epifluorescence unit and G-2E/C filter set (Nikon Corp., Tokyo, Japan) containing an excitation filter at 540/25 nm, suppressor filter at 630/60 nm, and at 565 nm dichroic mirror.

Transmission electron microscopy

Pto DC3000 sensitivity to chitosan was also evaluated using transmission electron microscopy (TEM; Jeol 100 CXII, Peabody, MA, USA). After 16 h of incubation with KB liquid medium supplemented or not with 100 μg ml^{-1} of chitosan at 30 °C aliquots of cell cultures were collected. After centrifugation, the pellet was suspended in 0.1 M phosphate buffer pH 7.0 containing 2.5 % (*v*/*v*) of glutaraldehyde and incubated at 4 °C for 16 h. Then, cells were washed three times with

0.1 M phosphate buffer pH 7.0. The pellet was fixed with 1 % (*w*/*v*) osmium tetroxide at 4 °C for 1.5 h, dehydrated in graded ethanol, and embedded in epoxy resin. Finally, the pellet was sectioned, stained with 1 % (*w*/*v*) uranyl acetate and 1 % (*w*/*v*) lead citrate, and examined in a transmission electron microscope. Photomicrographs were taken with a digital camera Gatan model 785 ES1000W (Gatan Inc., Pleasanton, CA, USA). Cellular ultrastructures of chitosan-treated and nontreated cells were compared.

Statistical analysis

The values shown in each figure are mean values ± SD. Data were subjected to analysis of variance (one-way ANOVA) and post hoc comparisons were done with Tukey's multiple range test at $P<0.05$ level. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used as statistical software program.

Results

Inhibition of *Pto* DC3000 cell growth depends on chitosan concentrations, pH, and metal ion binding

Growth cell suspension cultures were initially used to assess the effect of different concentrations of chitosan on *Pto* DC3000. Aliquots of cell culture were taken at various times after onset of first treatment. OD_{600} was quantified as a measure of bacterial growth. Treatments with different concentrations of chitosan (0, 50, 100, and 250 μg ml^{-1}) showed that 40.8 μg ml^{-1} chitosan inhibited cell growth by 50 % (IC50) in KB liquid medium (data not shown). For working, the chitosan stock solution was dissolved in 0.1 % (*v*/*v*) acetic acid and then, diluted until get the used concentration. Since chitosan has different biological properties depending on environmental conditions, cell suspension cultures adjusted to neutral and lightly acidic pH were also analyzed. At pH 7.0 the bacterial growth after 16 h of incubation at either 50 or 100 μg ml^{-1} chitosan decreased 60 and 70 %, respectively, compared with controls (Fig. 1a; Online Resource 1). However, when cell culture media were adjusted to pH 6.5 the bacterial growth was completely reduced within the first 6 h independently of chitosan doses (Fig. 1b). Equivalent dilutions of acetic acid used as controls did not have any adverse effect on bacterial growth (Fig. 1a, b).

The assumption that chitosan may inhibit cell viability led us to count the number of distinct colony-forming units as estimate of the bacteria amount that can grow under each treatment. To calculate the number of CFU a wide series of dilutions in KB medium from cell suspension cultures adjusted to pH 7.0 or pH 6.5 and containing or not 100 μg ml^{-1} chitosan were combinatorial plated on Petri dishes containing KB-agar and proper antibiotics, 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} rifampicin. Again, bacteria grown in KB medium containing chitosan exerted a clear decrease in the number of CFU compared with nontreated cells. However, a significant lower number of CFU corresponded to bacteria grown at pH 6.5 (Fig. 2)

In trying to explore the influence of a divalent metal cation on the susceptibility of *Pto* DC3000 to chitosan, 25 mM $MgCl_2$ was added to the KB liquid medium containing 100 μg ml^{-1} chitosan. After time, aliquots of bacteria grown under combinatorial conditions at pH 6.5 were harvested for cell wet weight quantification (Fig. 3). In the presence of chitosan, the cell wet weight decreased 50 % compared with control. However, the addition of $MgCl_2$ rescues the values of the control treatment.

Chitosan permeabilizes cell plasma membranes

The tested chitosan had a clear inhibitory effect on *Pto* DC3000 growth since the number of surviving cells was drastically reduced within the first 6 h of initial treatments (Fig. 1). Then, the in vivo action of chitosan on the integrity of cell plasma membranes was examined using the SYTOX Green dye. Bacteria grown under different conditions were stained with 5 μM SYTOX Green and subjected to microscopy analysis (Fig. 4). A positive control of cells treated with ethanol 70 % (*v*/*v*) for 5 min and stained with SYTOX Green was also analyzed. At the optimal excitation for SYTOX Green probe fluorescence emissions from ethanol- (Fig. 4b) and chitosan-treated cells (Fig. 4c) were compared with the background of control cells (Fig. 4a). Individual bacteria with compromised plasma membranes were easily distinguished from cells with intact membranes for ethanol and control treatments, as shown Fig. 4b and a, respectively. The intensity of photon emission was strongest from chitosan-treated cells (Fig. 4c). Chitosan-treated cells also showed a clear effect of aggregation as was observed by bright field microscopy (Fig. 4c, inset).

For in vivo measurements, fluorescence intensities overtime were quantified in *Pto* DC3000 cells treated with acetic acid, ethanol, and chitosan. After 15 min of incubation with SYTOX Green dye, cells were analyzed by fluorometry. As shown in Fig. 4d the strongest shift of wavelength of the fluorescence emission was observed in 100 μg ml^{-1} chitosan-treated cells within the first 25 min compared with ethanol. Meanwhile, no changes were noted when the cells were exposed to acetic acid solutions. No changes were observed when chitosan was incubated with fluorescein dye in cell-free medium as an additional control (data not shown).

Chitosan affects *Pto* DC3000 cell viability

Propidium iodide an intercalating agent generally excluded from viable cells was also used to validate cell viability. For

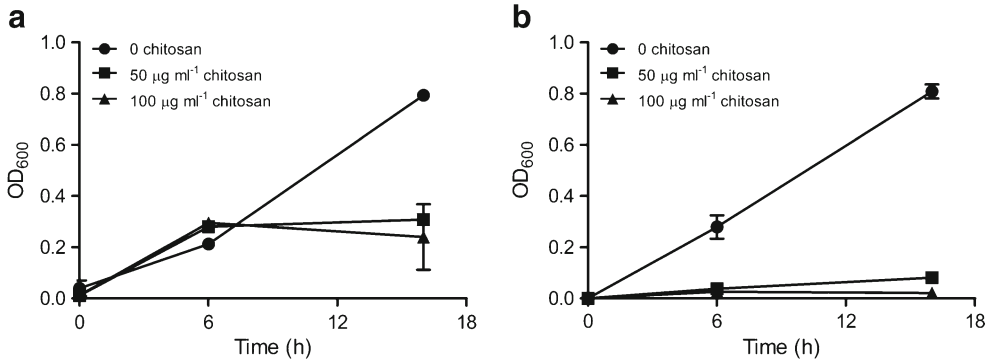

Fig. 1 Effect of different chitosan concentrations on the growth kinetics of *Pto* DC3000 cells. None (*black circle*), 50 μg ml^{-1} chitosan (*black square*), and 100 μg ml^{-1} chitosan (*black triangle*). Cells were grown at 30 °C with shaking in King's B liquid medium adjusted to pH 7.0 (**a**) or 6.5 (**b**). Each data represents the average of three independent experiments. *Bars* indicate the standard deviation of each value

a better estimation, propidium iodide staining was executed in cells from exponential phase cultured in the presence or absence of chitosan. Again, the highest fluorescence intensity compared with controls was observed from chitosan-treated cells (Fig. 5), which support that chitosan may disturb cell viability.

Transmission electron micrographs reveal morphological changes in *Pto* DC3000 chitosan-treated cells

To get insights on chitosan action on *Pto* DC3000 morphology, chitosan-treated cells were examined by TEM. The electron micrographs showed that control cells had compact surface, seemed normal with an electronically dense cytoplasm (Fig. 6a). However, chitosan-treated cells evidenced ruptures and disaggregation on their surfaces (Fig. 6b). A weak leakage of intracellular content could be released from chitosan-treated cells (Fig. 6c). Several chitosan-treated cells changed morphology turning more rounded. Clearly, chitosan-treated cells also showed a cytoplasm much more translucent and less dense compared with controls.

Chitosan counteracts *Pto* DC3000 infection in tomato seedlings

An in planta bioassay was performed to evaluate the chitosan action on *Pto* DC3000-infected tomato seedlings. First, to optimize the chitosan action on tomato , 4-day-old seedlings were grown in MS agar plates supplemented with different concentrations of chitosan (0.1, 1.0, 10, and 100 μg ml^{-1}). Then, seedlings were inoculated by flooding MS agar plates with 1×10^8 cells ml^{-1} bacterial suspension for 5 min. The symptoms of bacterial damage were evaluated after 7 days postinfection. As a result, 10 μg ml^{-1} chitosan strongly reduced the *Pto* DC3000 infection symptoms, but lower doses (0.1 and 1.0 μg ml^{-1}) had no effect (data not shown). Notably,

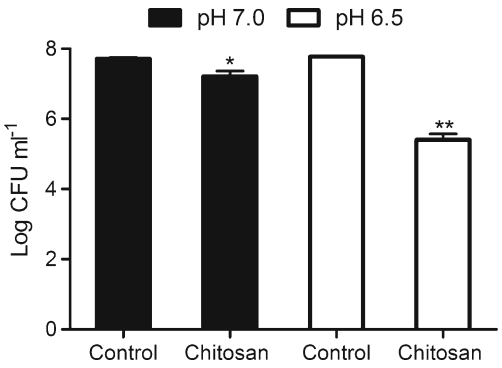

Fig. 2 Chitosan disturbs *Pto* DC3000 cell viability. Cells suspended in KB medium containing 0.001 % (*v*/*v*) acetic acid (*control*), 100 μg ml^{-1} chitosan at pH 7.0 (*black bars*), or pH 6.5 (*white bars*) were incubated for 16 h at 30 °C, and harvested. The number of CFU for each experimental condition was then quantified. Data are mean values (±SD) of three independent experiments. Statistical analysis was performed by one-way ANOVA analysis of variance; *p* values of less than 0.05 were considered as statistically significant (Tukey's Test). *$p<0.05$; **$p<0.01$

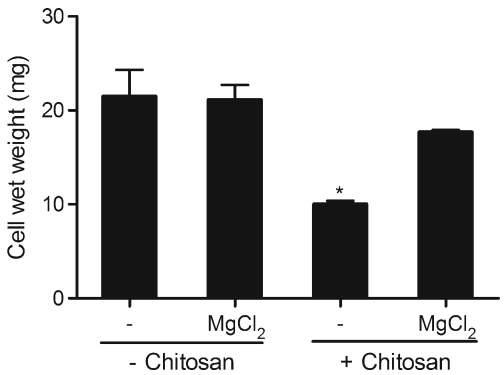

Fig. 3 $MgCl_2$ affects the susceptibility of *Pto* DC3000 to chitosan. Bacteria were grown at pH 6.5 for 16 h in KB liquid medium supplemented or not with 100 μg ml^{-1} chitosan and with or without 25 mM $MgCl_2$. The cellular wet weight for each combinatorial treatment was measured. Data are mean values (±SD) of three independent experiments. *Asterisk* indicates a significant difference at $p<0.05$ (Tukey's test)

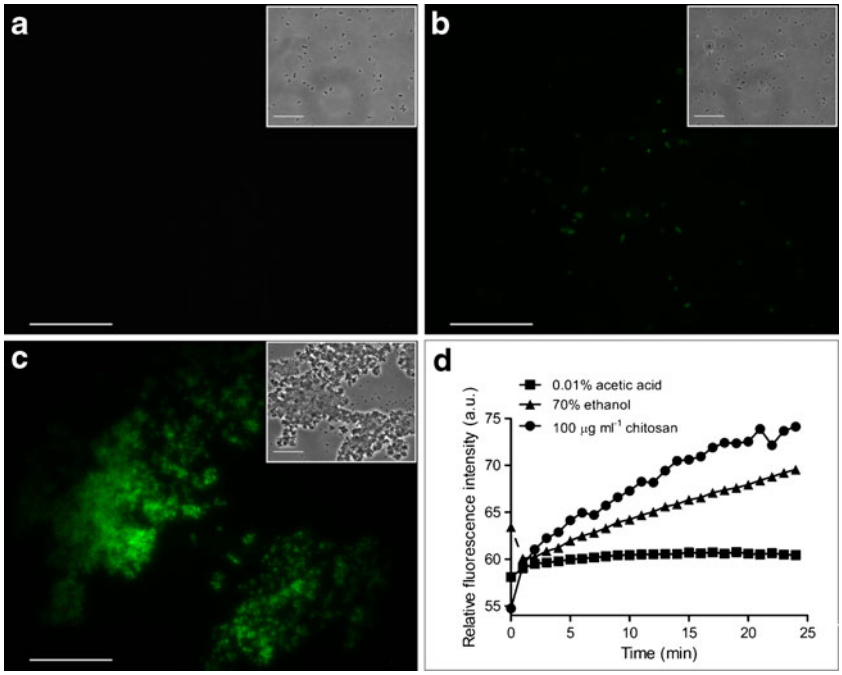

Fig. 4 Chitosan triggers bacterial aggregation and plasma membrane permeabilization. *Pto* DC3000 cells from exponential phase grown with **a** 0.01 % (*v*/*v*) acetic acid solution **c** 100 μg ml^{-1} chitosan were stained with SYTOX Green probe and analyzed with a fluorescence microscope. **b** As positive control, cells from exponential phase grown were treated with ethanol 70 % (*v*/*v*) for 5 min and stained with SYTOX Green. *Bar*=20 μm. Cells from each treatment were observed under bright field microscopy (*insets in each panel*). **d** The uptake of SYTOX Green probe by *Pto* DC3000 over time. Data of a representative experiment are shown (*n*=4). *a.u.* arbitrary units

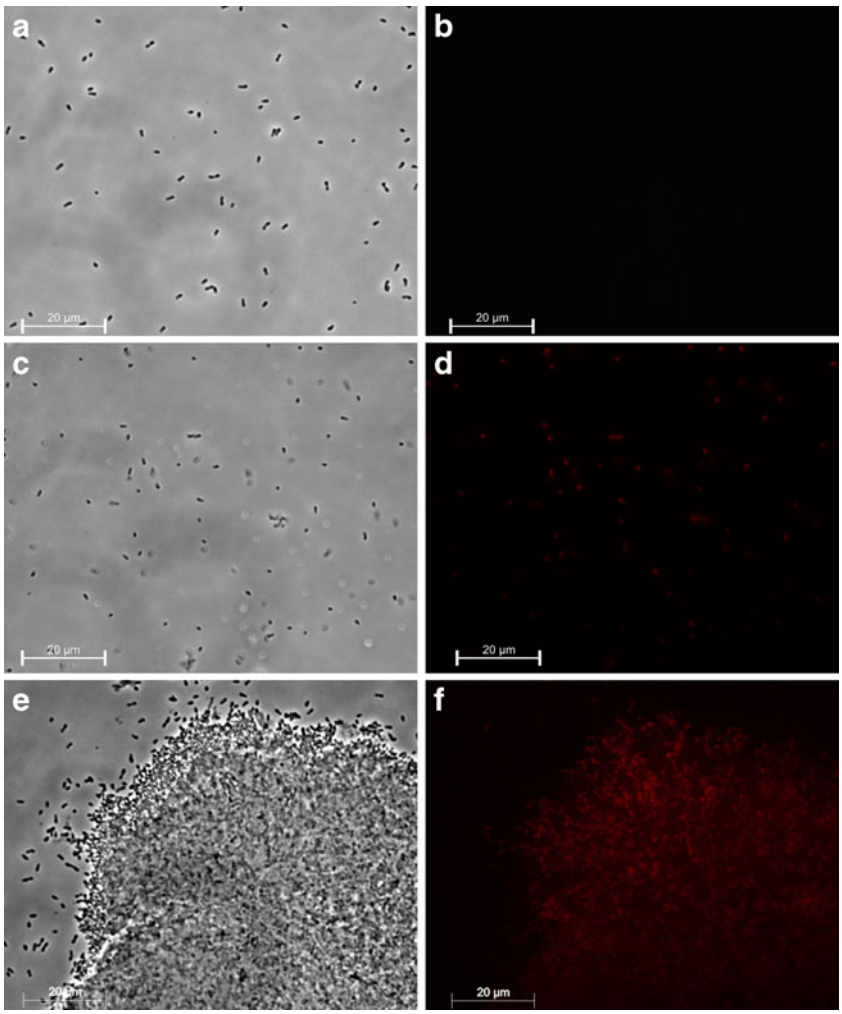

Fig. 5 Chitosan affects cell viability. *Pto* DC3000 cells from exponential phase grown with **a**–**b** acetic acid solution (negative control) or **e**–**f** 100 μg ml^{-1} chitosan were harvested. In addition, 70 % (*v*/*v*) ethanol-treated cells were analyzed as a positive control (**c**–**d**). Then, bacteria were stained with propidium iodide dye and analyzed by bright-field microscope (*left panel*) and fluorescence microscope (*right panel*). Results are representative of three separate experiments. *Bar*=20 μm

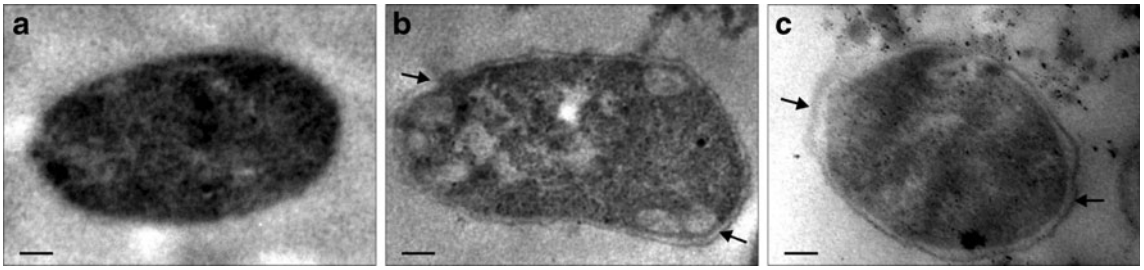

Fig. 6 Transmission electron micrographs of *Pto* DC3000 treated with 100 μg ml^{-1} chitosan until exponential phase. *Bar*=100 nm

100 μg ml^{-1} chitosan produced root shortening and negative effects on growth seedlings (data not shown). Thus, subsequent experiments were performed with the optimal chitosan dose. As shown in Fig. 7a, pretreatment of tomato seedlings with 10 μg ml^{-1} chitosan before *Pto* DC3000 inoculation significantly decreased bacterial damages in cotyledons compared with control. To evaluate the bacterial inoculum remaining in seedlings, cotyledon disks were cut and homogenized in sterile distilled water. Then, a set of serial dilutions from disk corresponding to each treatment were plated onto KB agar medium to measure the number of CFU. Revealing that chitosan contributes to counteract bacterial growth in tomato seedlings, it was much lower in chitosan-pretreated cotyledons compared with control (Fig. 7c).

Discussion

The high molecular versatility in the chitosan molecule as well as the limited number of studies on bacteria, led us to examine the antimicrobial properties of the chitosan isolated from waste crustaceans exoskeletons collected in the Southwest Atlantic Ocean against *Pto* DC3000 cells. Treated with chitosan, as proved by light microscopy and fluorometric analysis in whole-cell tests, these bacteria showed increased membrane permeability with loss of viability. Also, a decrease of the CFU number was associated to chitosan-treated cells at similar doses that have been previously described for a commercial chitosan in *Pseudomonas aeruginosa* and *Staphyloccocus aureus* (Tao et al. 2011).

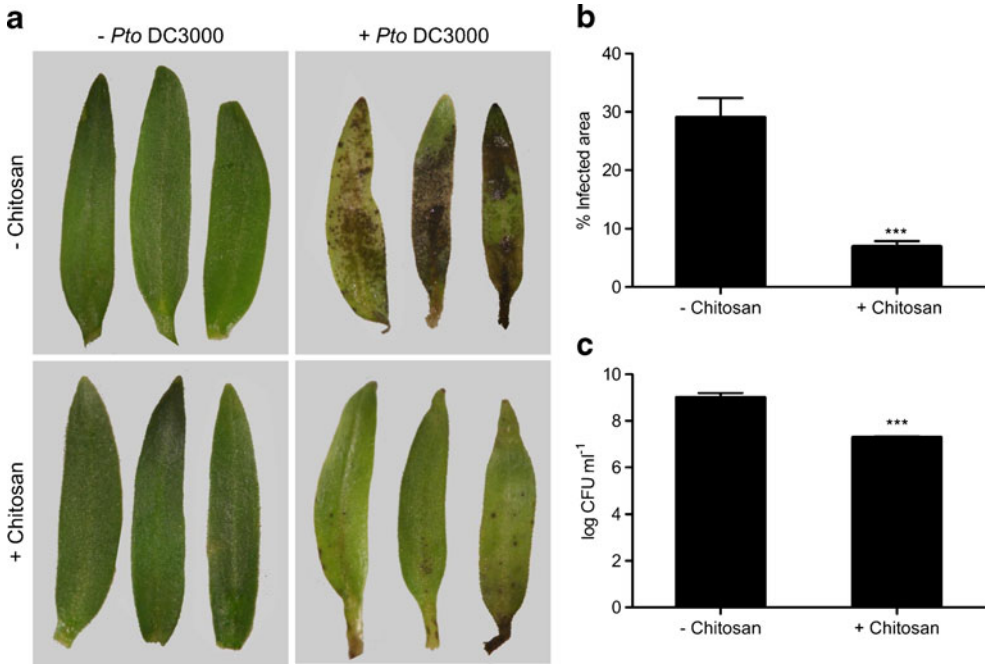

Fig. 7 Chitosan counteracts *Pto* DC3000 bacterial colonization in tomato seedlings. **a** Disease phenotype of seedlings pretreated with 10 μg ml^{-1} chitosan or 0.001 % (*v*/*v*) acetic acid and then immersed in *Pto* DC3000 cell suspension (+*Pto* DC3000) or sterile distilled water containing 0.025 % (*v*/*v*) SILWET L-77 plus 10 mM $MgCl_2$ (−*Pto* DC3000). Photographs were taken at 7 days post-inoculation. **b** Quantification of the cotyledon area affected by *Pto* DC3000 in nontreated or chitosan-treated seedlings was measured; 100 % represents the total cotyledons area. **c** At 7 days post inoculation, quantification of *Pto* DC3000 remaining inoculum in tomato seedlings cotyledons previously nontreated or treated with chitosan and expressed as the logarithm of CFU. Data are mean values (±SD) of three independent experiments. Statistical analysis was performed by one-way ANOVA analysis of variance. ***$p<0.001$ (Tukey's test)

The antimicrobial activity of the chitosan to *Pto* DC3000 cells was dependent on doses, pH, and metal ion, suggesting the charge density in the biopolymer molecule may have influence in its antimicrobial activity. Thus, *Pto* DC3000 growth was strongly reduced by chitosan in a weakest acidic condition. According to Helander et al. (2001), the inhibitory activity of chitosan towards Gram-negative bacteria should be considered in terms of chitosan chemical and structural properties. Thus, the positively charged backbone of chitosan (pK_a~6.5) would be controlled by the pH. Also, more positively charged NH_3^+ groups of glucosamine at slight acidic pH such as, pH 6.5 might increase chitosan interaction with cell surface (Badawy and Rabea 2011; Kong et al. 2010; Tang et al. 2010). Additionally, some models have been proposed to explain chelating ability of chitosan toward transition metal (Goy et al. 2009; Wang et al. 2005). Magnesium chloride may form complexes with chitosan and consequently, reduction of available amino groups could weaken its bactericidal effects in *Pto* DC3000 (Badawy and Rabea 2011; Chung et al. 2003; Tsai and Su 1999). Gram-negative bacteria outer membrane functions as an efficient outer permeability barrier against macromolecules including chitosan (Helander et al. 2001). In sum, chitosan could exert its bactericide effect by cell membrane permeabilization unlike of other types of polycationic molecules which impacts on cell permeability but lacks of direct bactericidal activity. In this same direction, the chitosan here tested might influence on negatively charged components present in *Pto* DC3000 membranes resulting in changes of permeability preceding bacterial cell death. Although the bacterial cell wall could be an important protective barrier against chitosan action (Chung and Chen 2008), it has been demonstrated that causes plasma membrane permeabilization in spores and fungal cells (Palma-Guerrero et al. 2009; Palma-Guerrero et al. 2010). Hence, we agree with Raafat et al. (2008) that more evidences are needed to prove the direct action of chitosan on bacterial cell membrane. Also, to reinforce the idea that the antibacterial mechanism of chitosan is due to the electrostatic interaction, assays with a derivative of chitosan called *N*-propyl-*N*-methylene phosphonic chitosan were performed (Zúñiga et al. 2010). PNMPC proves to be an amphiphilic system in which the hydrophobic moiety counterbalances the electrostatic repulsion, getting more tensioactive properties. That PNMPC did not show effect on cell permeability suggested that chitosan action on cell surface may be due its interaction with negatively charged microbial cell membranes (Online Resource 2). Thus, the aggregation effect mediated by chitosan on *Pto* DC3000 might also due to previous permeabilization of cell membrane, and/or a putative effect of cell to cell contact might contribute to cell permeabilization. Formation of aggregates for protection is an attractive concept because bacteria in biofilms are known to be more resistant against biocides than suspended cells (Gilbert et al. 2002; Lewis 2001). However, by the whole-cell assays the fluorescent staining of aggregated cells was a clear signal of membrane permeabilization and damage in *Pto* DC3000 aggregated cells. Consistent with our findings, cell aggregation and antibacterial action to the Gram-negative bacteria, *Pseudomonas fluorescens* and *Escherichia coli* was demonstrated for a functionalized chitosan-arginine (Tang et al. 2010). However, different grades and intensity of damage on cell membrane could be exerted by diverse chitosans and bacterial cells (Didenko et al. 2005). Also, adhesion of chitosan to *Pto* DC3000 cells was not observed suggesting that 100 μg ml^{-1} chitosan is active although it might not have strong adhesion on the *Pto* DC3000 surface. Reinforcing our findings, bioassays noted a strong decrease of the bacterial speck disease in chitosan-treated tomato seedlings before inoculation with *Pto* DC3000. Overall, our findings pointed out that the tested chitosan has a great potential for controlling bacterial speck disease in tomato by, at least, two different mechanisms. It means that might counteract bacterial growth by *Pto* DC3000 membrane permeabilization and eliciting at low doses defense mechanisms against bacteria in plants (Amborabé et al. 2008; El Hadrami et al. 2010). In plants, H^+-ATPase plasma membrane seems to be a primary site of chitosan action to elicit defense mechanisms against biotic stress (Amborabé et al. 2008).

Notably, the chitosan here studied has antimicrobial action to *Pto* DC3000 and properties in planta at slightest acidic pH and relatively low doses, which is a clear advantage for its use in biological systems. The elucidation of the complete mechanism of the chitosan action seems rather complex. However, the observed sensitivity of *Pto* DC3000 cells to the tested chitosan encourage to further evaluate its application as a sustainable and nontoxic biopesticide in soils and horticultural crops.

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