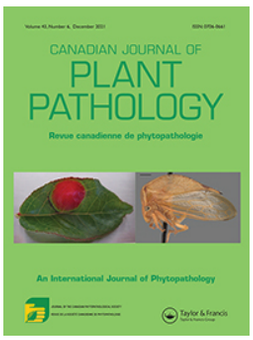

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Chitosan induces tomato basal resistance against *Phytophthora nicotianae* and inhibits pathogen development

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Abstract: Chitosan is a polymer of glucosamine with antimicrobial properties and an elicitor of plant defence responses. In this study, different chitosan concentrations were assessed for impact on the life cycle of *Phytophthora nicotianae* Breda de Haan, an oomycete pathogen of tomato and tobacco crops. Reduced mycelial growth was observed when chitosan was added to solid media, and this inhibitory activity was amplified with increasing chitosan concentrations from 0.5 g L^{-1} to 2.5 g L^{-1}. Colony morphology and hyphal diameter were unaffected by the polymer; however, the number of oospores (sexual reproductive structures) was reduced at the lowest chitosan concentration (0.5 g L^{-1}) tested. Mycelia plugs imbibed with 0.5 g L^{-1} chitosan were prone to increased sporangium formation, but the effect was subdued with higher chitosan concentrations. The length/width ratio of the sporangia did not change in the presence of the polymer. Indirect germination of the sporangia was particularly sensitive to this compound, and 1.5 g L^{-1} completely abolished this process. The zoospores treated with chitosan formed cysts that did not germinate and were unable to infect tomato plant roots. Chitosan was also found to prime tomato plantlets for *P. nicotianae* resistance; a foliar application of 0.1 and 1 g L^{-1} of chitosan 72 h before inoculation induced host resistance, which was concurrent with increased activity of phenylpropanoid pathway markers. The results presented herein show the potential of chitosan as a tool to control *P. nicotianae* inoculum and increase tomato crop resistance against this pathogen.

Keywords: antimicrobial activity, chitosan, induced resistance, mycelial growth, *Phytophthora nicotianae*, sporulation

Résumé: Le chitosane est un polymère de la glucosamine qui possède des propriétés antimicrobiennes et est également un éliciteur qui déclenche des réactions de défense chez les plantes. Dans cette étude, différentes concentrations de chitosane ont été évaluées afin d'en connaître l'influence sur le cycle de vie de *Phytophthora nicotianae* Breda de Haan, un oomycète pathogène de la tomate et du tabac. Lorsqu'on ajoutait du chitosane à un milieu solide, la croissance mycélienne était réduite, et cette action inhibitrice était amplifiée en fonction des concentrations de chitosane (de 0,5 à 2,5 g L^{-1}). La morphologie de la colonie et le diamètre des hyphes n'étaient pas influencés par le polymère. Toutefois, le nombre d'oospores (structures sexuelles de la reproduction) était réduit à partir de la plus faible concentration de chitosane testée (0,5 g L^{-1}). Des pastilles mycéliennes imbibées de 0,5 g L^{-1} de chitosane tendaient à promouvoir la formation des sporanges, mais l'effet était atténué à des concentrations plus élevées. Le rapport longueur/largeur des sporanges n'a pas été modifié par le

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polymère. La germination indirecte des sporanges était particulièrement sensible à ce composé et 1,5 g L^{-1} en a entièrement interrompu le processus. Les zoospores traitées avec le chitosane ont développé des kystes qui n'ont pas germé et qui n'ont pu infecter les racines des plants de tomate. Le chitosane a également préparé les plantules de tomate à résister à *P. nicotianae*. Une application foliaire de 0,1 et de 1 g L^{-1} de chitosane 72 heures avant l'inoculation a induit la résistance de l'hôte, ce qui coïncidait avec l'activité accrue des marqueurs de la voie des phenylpropanoïdes. Les résultats présentés ici montrent le potentiel du chitosane en tant qu'outil de lutte contre l'inoculum de *P. nicotianae* et pour accroître la résistance de la tomate à cet agent pathogène.

Mots clés: Activité antimicrobienne, chitosane, croissance mycélienne, *Phytophthora nicotianae*, résistance induite, sporulation

Introduction

The genus *Phytophthora* belongs to the fungal-like phylum Oomycota, which includes over a hundred phytopathogenic species of economic importance across the world (Brasier 2009; Kroon et al. 2012). Some of these species have a wide host range; for example, *P. nicotianae* Breda de Haan (1896) causes damage to a diverse array of solanaceous species, such as tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.), as well as citrus and ornamental plants (Erwin and Ribeiro 1996; Pérez-Sierra et al. 2012). The diseases caused by this soil-borne pathogen usually occur when high temperatures and humidity are present, resulting in root and crown rot, stem girdling, damping off, and leaf and shoot blight (Erwin and Ribeiro 1996; Jones et al. 2006). In tobacco, *P. nicotianae* is the causal agent of black shank, a devastating disease that has caused greater than 80% yield losses in infected plantations (Fernández 1998; Espino 2006). The disease was first observed in 1905 in Cuba, but the pathogen was not isolated and identified until 1957 (reviewed in Vaillant Flores and Gómez Izaquirre 2009; Fernández et al. 2012; Gonzalez Toledo 2016). In tomato plants, this pathogen affects plantations around the world and has become a problem for tomato exporters (Chowdappa et al. 2016; Boix-Ruiz et al. 2017).

Phytophthora produce asexual zoospores, which are among the most important infectious propagules of this genus of pathogens. Zoospores are formed in the sporangia when temperatures drop below 12°C, but alternatively, the sporangia have the ability to directly germinate at temperatures of approximately 20°C (Walker and van West 2007). The zoospores are then released into the soil where they can infect the roots of susceptible hosts, allowing the rapid dissemination and successful establishment of the pathogen (Hardham 2001). The mechanism of infection heavily relies on a firm adhesion of zoospores to the host surface, which occurs due to secretion of adhesive material that requires Ca^{2+} to function during zoospore encystment (Hardham 2001). Therefore, strategies to inhibit the adhesion and germination of these structures would stand as a feasible solution to hamper the infection cycle for these soilborne phytopathogens.

Exogenous chitosan treatments have emerged in recent years as alternative disease-control strategies for *Phytophthora* (Kiprushkina et al. 2017; Malerba and Cerana 2018; Esyanti et al. 2019). Chitosan is a linear polymer of partially acetylated glucosamine with β-1,4-bonds, and can be isolated from chitin obtained from crustaceous exoskeletons. This polymer is an innocuous and biodegradable compound with chelating and antimicrobial activities and is also an elicitor of plant defence responses (Lopez-Moya et al. 2019; Mukhtar Ahmed et al. 2020). Different mechanisms have been ascribed to the antimicrobial properties of chitosan, which are at least in part related to its polycationic nature resulting from the positively charged amine groups in its chain at low pH, which promote a strong electrostatic interaction with anionic cell wall/membrane constituents of microbes and lead to the loss of structural integrity, disruption, and leakage of ions and intracellular components (Xu et al. 2007a, 2007b; Palma-Guerrero et al. 2009; Kumaraswamy et al. 2018; Chakraborty et al. 2021). Additionally, chitosan molecules can inhibit glucan biosynthesis for the fungal cell wall (Kumaraswamy et al. 2018).

The effect of chitosan on fungal development has been investigated in different genera, including *Rhizopus, Mucor, Fusarium, Alternaria, Botrytis, Colletotrichum, Rhizoctonia*, and *Sclerotinia* (Sánchez-Dominguez et al. 2007; Guerra-Sánchez et al. 2009; reviewed by Malerba and Cerana 2018; Silva-Castro et al. 2018; El-Mohamedy et al. 2019; Mohammed et al. 2019). However, in *Phytophthora* species, most of the available research has primarily addressed the asexual (vegetative) growth phase, with a few studies also showing the effect of chitosan on the ultrastructure of these pathogens (Xu et al. 2007a, 2007b; Ramírez-Benítez et al. 2019). It has been previously demonstrated that chitosan protects tobacco and tomato plantlets by activating basal induced

resistance (Atia et al. 2005; Falcón-Rodríguez et al. 2007, 2011); nevertheless, the potential of this polymer to protect tomato plants from *P. nicotianae* infection has not been evaluated. Therefore, the aim of this study was to evaluate the antifungal effect of chitosan on *P. nicotianae* during its life cycle, and the ability of chitosan to induce resistance against this pathogen in tomato plantlets.

Materials and methods

Chitosan polymer

The chitosan polymer (82.9 kDa, 12% degree of acetylation) was obtained by basic deacetylation of lobster chitin (Falcón et al. 2010), which was supplied by Mario Muñoz Pharmaceutical Laboratories (La Habana, Cuba). A stock solution was prepared by dissolving 10 g of chitosan in 1 L of 1% acetic acid and the pH was readjusted to 5.6 with KOH (Falcón-Rodríguez et al. 2007). The stock solution was further diluted with double distilled water as needed.

Pathogens and culture medium

Four *P. nicotianae* isolates, Pn9, Pn227, PnSAL, and Pn148, derived from tomato, tobacco, *Salvia*, and citrus, respectively, were used in this study. These isolates were identified and characterized in previous studies (Fernández 1998; Machado 2009) with the first three belonging to the fungal collection of the Instituto de Investigaciones de Sanidad Vegetal (INISAV), and the latter to the Instituto de Investigaciones de Fruticultura Tropical (IIFT), La Habana, Cuba. The isolates were grown on V8 agar: 20% clarified V8 juice (a mixture of tomato, celery, spinach, beet, lettuce, parsley, cress, and carrot juice; Del Frutal Company, Guatemala) with 15 g L^{-1} agar, 2.0 g L^{-1} calcium carbonate and 2.0 g L^{-1} Asparagine, pH 5.6 (adjusted with HCl).

Antimicrobial activity on vegetative growth

The effect of chitosan on vegetative growth was measured on V8 agar (20-mL in 9-cm-diam. Petri dishes) supplemented with 0, 0.5, 1.0, 1.5 and 2.5 g L^{-1} chitosan. A total of seven plates per treatment (chitosan concentration) were inoculated by placing 1-cm *Phytophthora* plugs (mycelial side down) at the centre of each Petri dish. The dishes were incubated in the dark at 27°C and radial growth was measured every 24 h. When the mycelia of the control treatment reached the edge of the Petri dishes, colony morphology was described according to Erwin and Ribeiro (1996). To measure hyphal diameter, three samples per treatment were stained with lactophenol blue and were examined under an Olympus BH optical microscope at 1000X magnification.

Antimicrobial activity of chitosan on sexual reproduction

To analyze the effect of chitosan on the formation of oospores, it is necessary first to induce the appearance of these sexual structures. *Phytophthora nicotianae* is a heterothallic organism, which means two different mating types (A1 and A2) are required for sexual reproduction assays. Since the mating type of Pn9 was not yet determined, a previously identified A2 strain (Pn227; Fernández 1998) was challenged against Pn9 and two other strains of undetermined mating types. This allowed us to determine whether Pn9 was sexually compatible for mating with an A2 strain, and also to identify A1 types.

Pn227, known as A2, was challenged against Pn9, Pn148, and PnSAL. Each isolate was cultured on Petri dishes with 20% V8 juice, 2 g L^{-1} $CaCO_3$ and 15 g L^{-1} agar, and 10 mm mycelial discs from the actively growing region were transferred to fresh Petri dishes after 4 days of growth. Two discs were placed onto each plate, spaced 2 cm apart, one Pn227 disc, and the other disc from one of the three challenged isolates. Three plates per treatment were incubated in the dark at 27°C for 7 days, and the experiment was repeated twice.

Samples were taken from the confrontation line and were placed on slices with Polyvinyl-Lacto-Glycerol (PVLG). They were visualized with an optical microscope (Carl Zeiss) equipped with an Axiocam HR, and the images were analyzed using the AxioVision 3.1 software. Pn148 and PnSAL produced oospores when challenged with Pn227 and were therefore deemed A1 mating types. Subsequently, Pn9 was also screened against these two A1 mating types.

To assess chitosan-driven inhibition of sexual reproduction, the positive assays were repeated on Petri dishes supplemented with different concentrations of chitosan (0, 0.5, and 1.0 g L^{-1}). After 10 days of incubation, the oospores were quantified in three optic fields per plate, and in five plates per treatment.

Antimicrobial activity of chitosan on asexual reproduction

Mycelia discs of Pn9 colonies were placed on 6 cm-diam. Petri dishes with 5 mL of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 g L^{-1} chitosan solutions. The dishes were

incubated under continuous light for 48 h at 25°C, after which sporangia formation was observed and counted at 200X with an optic microscope. Sporangial dimensions, length/width ratio, and indirect germination percentage were calculated in three optic fields per plate and three plates per treatment, using an ocular micrometric scale.

The velocity and pattern of zoospores movement were estimated after the sporangia germinated in the different chitosan solutions. A previously established qualitative evaluation method was employed, where a reduction in the number of positive signs is indicative of a reduced speed, and negative signs indicate the absence of locomotion (Fernández 1998).

To analyze the effect of chitosan on cyst germination, a zoospore suspension (0.2 mL) was combined with different chitosan solutions (0.2 mL) in 0.5 mL Eppendorf tubes, reaching final concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 g L^{-1}. For each treatment, eight 20-µL drops of the zoospore-chitosan solutions were placed onto two slides in a moistened chamber at 27°C (Fernández 1998). The number of zoospores in movement, encysted and germinated cysts were evaluated at 60 and 180 min after incubation, using an optic microscope at 100X magnification. The cyst was considered germinated when the germ tube was longer than the cyst.

Disease assessment of stem-base inoculated tomato plants and the effect of chitosan on zoospore infectivity

Seeds of the tomato (*Solanum lycopersicum* L.) cultivar 'Amalia' were disinfected with 2.5% sodium hypochlorite for 45 s and then washed with abundant distilled water. Seeds were germinated on moistened filter paper in covered Petri dishes at 25°C for 72 h. Seedlings were transplanted into pots containing sterilized substrate with equal proportions of organic matter and soil at pH 6.8 and were kept in a growth chamber at 25 ± 2°C with a photoperiod of 16 h.

In order to determine the ability of Pn9 zoospores to cause disease in tomato plants, zoospores were obtained by incubating three mycelium discs on 9-cm-diam. Petri dishes containing 10 mL of distilled water. Ten Petri dishes were incubated under constant light at 25 ± 2°C for 48 h. All suspensions were collected in a pool and counted in a Neubauer chamber. Fifteen-day-old plants were inoculated at the base of the stem with a Pn9 zoospores suspension (7 x 10^4 zoospores mL^{-1}), or with water as a negative control. Two methods of application were employed: 1 mL of zoospore suspension or distilled water was applied with a pipette to an injured (with a sterile scalpel) or uninjured stem base. Plants were rated for disease severity 5 days after inoculation, according to the following scale of pathogen invasion: 0 = healthy plant; 1 = roots and stem base affected; 2 = stem and cotyledons affected; 3 = first and second leaves pair affected; and 4 = dead plant.

To assess the impact of chitosan on zoospore infectivity, the experiment was repeated with a chitosan-zoospore suspension as the inoculum and without injuring the stem. The suspension was composed of a 14×10^4 zoospores mL^{-1}, combined at a 1:1 ratio with a chitosan solution to a final concentration of 0 (positive control), 0.5, 1.0, 1.5, 2.0, or 2.5 g L^{-1}. The final concentration of the Pn9 zoospores in the chitosan-zoospore suspension was 7×10^4 zoospores mL^{-1}. The mixture was incubated for 60 and 180 min at 25°C ± 2°C prior to use. Once again, distilled water (without zoospores or chitosan treatment) was used as a negative control.

Disease assessment and metabolic analysis of spray-inoculated, chitosan-treated tomato plants

Twenty-one days after sowing, tomato plants were sprayed with approximately 1 mL of chitosan solution (0.1, 1.0, 2.5 g L^{-1}) per plant. A 50-mM potassium acetate solution was sprayed onto control plants. After 72 h, plants were gently removed from the substrate and placed in contact through the roots with a pathogenic strain (Pn227) of *P. nicotianae* in glass tubes (Falcón-Rodríguez et al. 2011). After 4 days of incubation with the microorganism (7 days after chitosan was sprayed onto the plants), a plant infection index was determined for each plant using the scale of pathogen invasion and the following formula: $Infection\ index = \left[\frac{\sum(G \times F)}{N \times 4}\right] \times 100$, where G is the pathogen invasion value (according to the scale described above), F is the frequency, N is the total number of plants in each group, and 4 is the maximum degree in the scale.

For metabolic analysis (flavonoid and phenolic compounds), samples of tomato true leaves were collected 3 days after chitosan spray treatments (just before plants were placed in contact with the pathogen) and at day-7 after treatments (4 days after plant—pathogen incubation) to determine total flavonoid content using the aluminium chloride method (Quettier-Deleu et al. 2000) and total phenol content according to the method described by Singleton et al. (1999).

Statistical analysis

The experiments were performed using a completely randomized design. For disease assessment of spray-inoculated plants, data were analyzed with the Kruskal–

Wallis non-parametric test, and all means were independently compared with the Mann–Whitney test for $p \leq 0.05$, using the Bonferroni correction that allows comparison of more than two samples, through the statistical softwareSPSS v. 21 for Windows. Fifteen plants were tested per treatment, and experiments were conducted twice with similar results.

For all other experiments, data were processed through one-way ANOVA and means were compared using a Tukey test with a significance level of $p \leq 0.05$. For the variable percentage of germinated sporangia, an arcsin $\sqrt{x}$ scale transformation was carried out on the data prior to one-way ANOVA. After data normality and homoscedasticity were verified, a Tukey test with a significance level of $p \leq 0.05$ was used. All assays were conducted twice. Data were analyzed in SPSS Statistics v. 21 with graphs generated in SigmaPlot 2001 v. 7.0.

Results

Chitosan has a fungistatic effect on the mycelium of P. nicotianae

Chitosan inhibited *P. nicotianae* mycelial growth (Fig. 1*a*, *b*) and this vegetative growth reduction was inversely correlated with the concentration of chitosan. The colony diameter was smaller when higher chitosan

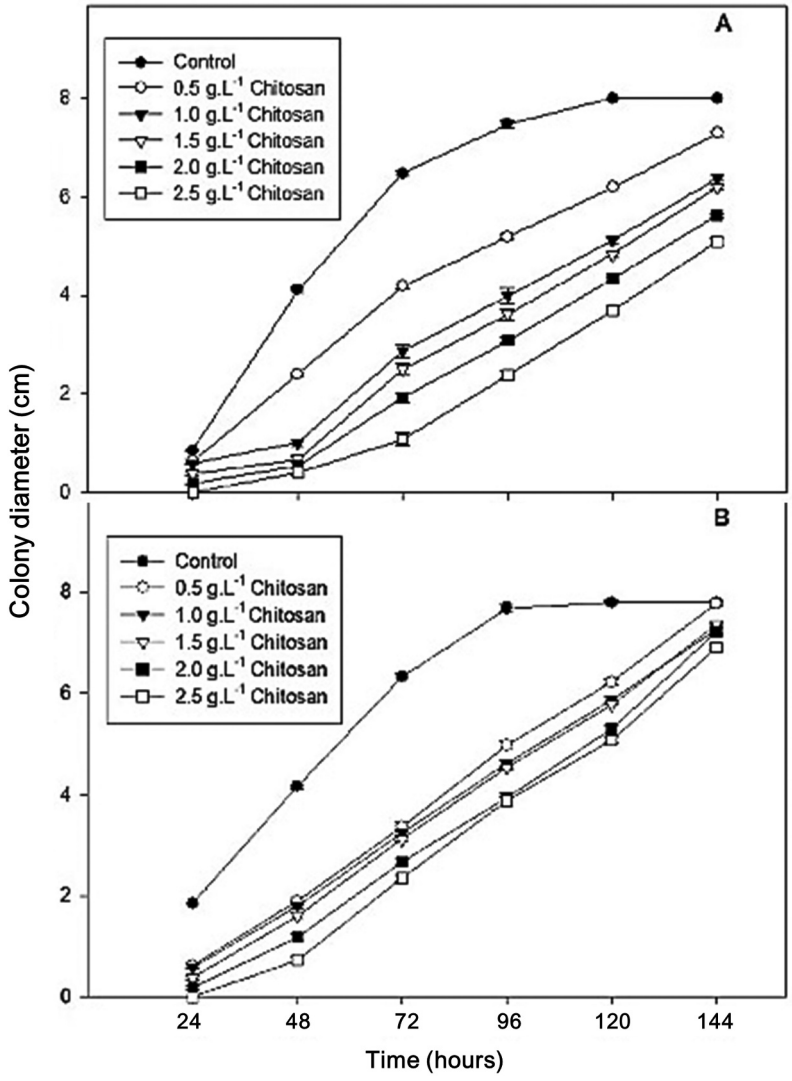

Fig. 1 Colony diameter of isolates Pn227 **(a)** and Pn9 **(b)** of *Phytophthora nicotianae* on V8 culture medium amended with different chitosan concentrations. Data were analyzed through one-way ANOVA and means were compared using the Tukey test with a significance level of $p \leq 0.05$ (Pn9 SEM = 0.07803 and Pn227 SEM = 0.09818).

concentrations were included in the culture medium. A fungistatic, but not fungicidal, effect was observed for all concentrations tested. The Pn9 isolate was less sensitive to chitosan concentration changes than Pn227; the growth diameter of Pn9 did not differ among treatments with 0.5, 1.0 or 1.5 g L^{-1} of chitosan (Fig. 1*b*). The strongest inhibition of mycelial growth for either strain was observed with 2.5 g L^{-1} chitosan.

Analysis of *P. nicotianae* colonies on solid culture media in the presence or absence of chitosan did not result in significant phenotype changes; the colonies were white and cottony in appearance, with regular edges and a star-shaped morphology. *P. nicotianae* hyphal diameter mean values ranged from 4.9 µm to 5.1 µm with no significant differences among treatments.

Chitosan-inhibited sexual reproduction of P. nicotianae

Confrontation screening between *P. nicotianae* isolates showed that PnSAL and Pn148 were both A1 mating types since oospores were observed when challenged with the A2 isolate Pn227. Moreover, the Pn9 isolate was not compatible with Pn227, as determined by the lack of oospore formation at the interception line. However, when Pn9 was challenged with the A1 mating types, Pn148 or PnSAL, oospores of approximately 20 µm in diameter were observed, establishing Pn9 as an A2 mating type (Fig. 2*a, b*).

The presence of chitosan in the culture media, at 0.5 or 1.0 g L^{-1}, reduced the number of oospores formed in both interactions (Pn9 *vs* Pn148 and Pn9 *vs* PnSAL) by more than 40% (Fig. 3). No changes in oospore morphology were observed under these conditions when chitosan was added to the culture medium.

Chitosan inhibited asexual reproduction of P. nicotianae

The asexual reproduction of *P. nicotianae* isolate Pn9 was affected by increments in chitosan levels in the solution (Table 1 and Fig. 4). Specifically, the number of sporangia decreased when chitosan concentration was equal to or greater than 1.5 g L^{-1}; however, the sporangial length/width ratio was not affected (Table 1). Interestingly, the number of *P. nicotianae* sporangia increased compared with the negative control, in the presence of 0.5 g L^{-1} chitosan (the lowest concentration tested). In addition, the indirect sporangia germination was reduced by roughly 50% when 0.5 g L^{-1} was added and was completely inhibited with 1.0 g L^{-1} (Fig. 4).

Phytophthora nicotianae zoospores were around 10 µm in length and moved at high speed with a rectilinear movement (Table 2). When chitosan was added to the zoospore suspensions, their behaviour markedly changed. Their movement patterns became helicoidally instead of linear, and their speed decreased as chitosan concentrations were augmented (Table 2).

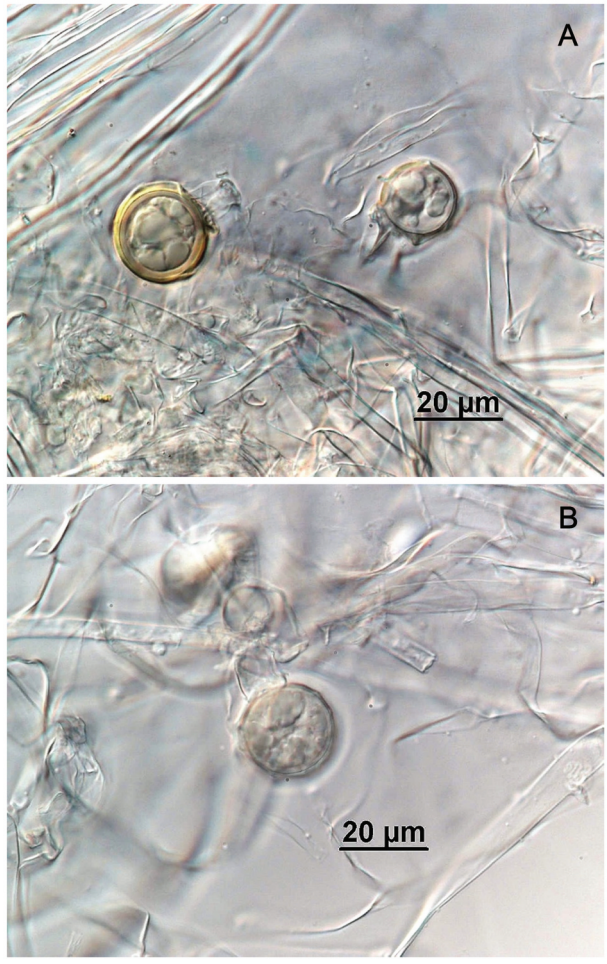

Fig. 2 Oospores formation on Petri dishes 7 days after challenge with (**a**) *Phytophthora nicotianae* Pn9 X PnSAL and (**b**) *P. nicotianae* Pn9 X Pn148.

The *P. nicotianae* cyst germination process was considerably affected when chitosan was included in the zoospore suspension. Complete inhibition of germ tube emergence was observed with 0.5 g L^{-1} at 60 and 180 min of incubation (Table 2). After 180 min of incubation, all of the *P. nicotianae* zoospores in chitosan solution had become encysted, whereas in the control treatment, 45% had already germinated at this time point. The germ tube length reached five times (in a few cases seven times) the cyst dimensions. Incubation with chitosan caused a total inhibition of the

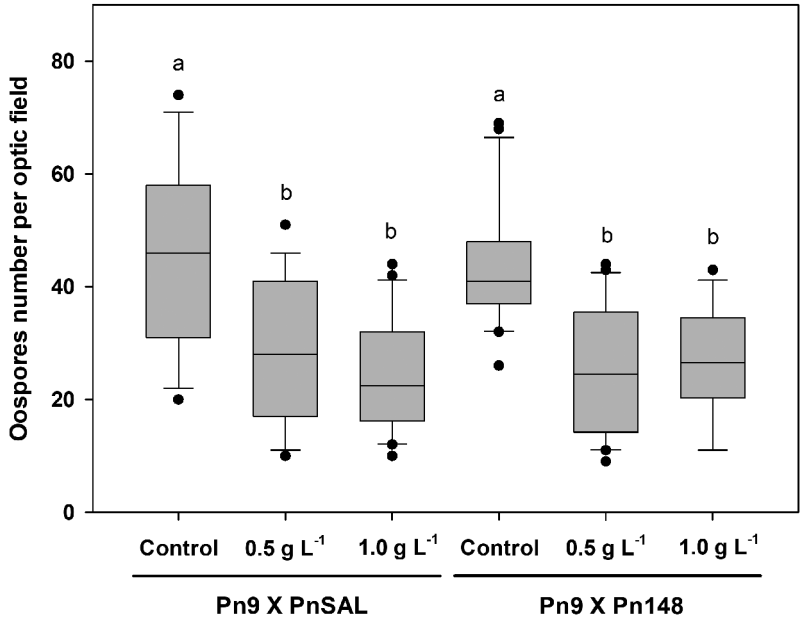

Fig. 3 Effect of chitosan on *Phytophthora nicotianae* oospore formation. Data were analyzed through one-way ANOVA and means were compared using the Tukey test ($p \leq 0.05$). Same letters did not differ statistically.

Table 1. Number and length/width ratio of *Phytophthora nicotianae* Pn9 sporangia in different chitosan solutions. Optic field at 200X magnification. Mean values ± SD are presented.

Chitosan Concentration (g L^{-1})	Sporangia Number	Length/width Ratio
0	10.2 ± 4 **b**	1.28 ± 0.08 **ab**
0.5	13.3 ± 7 **a**	1.24 ± 0.17 **b**
1.0	12.2 ± 3.3 **ab**	1.39 ± 0.14 **ab**
1.5	7.1 ± 1.9 **c**	1.36 ± 0.17 **ab**
2.0	6.7 ± 1.1 **c**	1.46 ± 0.26 **a**
2.5	6.8 ± 2.4 **c**	1.38 ± 0.25 **ab**

germination process, even at the lowest polymer concentration tested.

In some cases, when the suspensions were incubated with 2.0 or 2.5 g L^{-1} of chitosan, the edges of the cysts were not defined, suggesting that the cell wall had ruptured, and some vacuole-like structures were observed near those ruptured cysts.

Effect of chitosan on the ability of P. nicotianae *zoospores to infect tomato plants*

In the absence of chitosan, the tomato plants inoculated with a zoospore suspension of 7×10^4 zoospores per mL became infected at the base of the stem. The stems turned brown and putrefied after 5 days. In some cases, the leaves retained some green colour but the whole plant died shortly thereafter. No differences were observed in the disease response between the wounded and unwounded inoculation methods. In both cases, symptoms were observed, characterized by a stem with necrosis and reduced diameter. Plants from the control treatment, not treated with zoospores, were healthy and vigorous (Fig. 5). By contrast, in experiments with chitosan treatments, no symptoms of infection were observed when inoculated with *P. nicotianae*, regardless of chitosan concentration, whereas the positive control (inoculated with the pathogen but no chitosan treatment) plants died within 5 days. Negative controls treated only with water or with chitosan solutions did not show any symptoms (Fig. 5).

Exogenous chitosan treatment reduces disease symptoms in P. nicotianae *inoculated tomato plants by inducing host defences responses*

Foliar spray of chitosan polymer caused a reduction of the infection index in tomato plants inoculated with *P. nicotianae* compared with control plants (Table 3). Plant protection was higher (46–48.5%) at 0.1 and 1.0 g L^{-1}, respectively, whereas a higher dose (2.5 g L^{-1}) reduced the infection index by only 21.8% and with no significant difference with the inoculated control. It is not understood why the higher dose, which would be expected to have a stronger effect, did not have any significant outcome. Nonetheless, these results suggest that chitosan

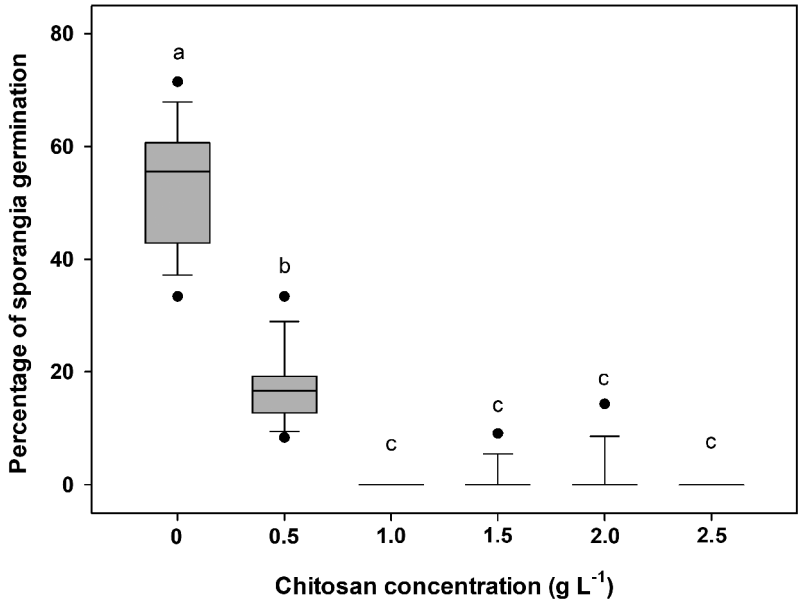

Fig. 4 Percentage of *Phytophthora nicotianae* Pn9 sporangia germinating under different chitosan concentrations. Data were analyzed through one-way ANOVA and means were compared using the Tukey test with a significance level of $p \leq 0.05$. Same letters did not differ statistically.

Table 2. The effect of different chitosan concentrations on movement, cyst formation, and germination of zoospores of *Phytophthora nicotianae* Pn9. More + symbols indicate faster movement. Values given represent the percentages after 60 min and 180 min of incubation.

Chitosan Concentration (g L^{-1})	Movement Speed	Movement Pattern	Zoospores in movement (%)	Cyst (%)	Germinated cysts (%)
0	+++	Linear	24/0	61/55	15/45
0.5	++	Helicoidally and Linear	0/0	100/100	0/0
1.0	+	Helicoidally	0/0	100/100	0/0
1.5	-	-	0/0	100/100	0/0
2.0	-	-	0/0	100/100	0/0
2.5	-	-	0/0	100/100	0/0

+, movement; -, no movement

offers protection to tomato plants from *P. nicotianae* attack by activation of induced resistance, since in the chitosan treatment applied, the polymer did not come into contact with the pathogenic mycelium at any time.

To investigate possible causes for this induced host resistance, determinations of total flavonoids and phenols were carried out in tomato leaves following the different treatments. Three days after foliar spray, chitosan increased the flavonoid content of leaves that were treated with 1 g L^{-1} of the polymer by about 28% above the control, while 0.1 and 2.5 g L^{-1} were not statistically different from the control (Fig. 6*a*). However, when plantlets were placed in contact with the pathogen, inoculation alone (in the absence of chitosan) augmented flavonoid content by about 80% with respect to the uninoculated control, whereas the chitosan treatment (0.1 g L^{-1}) alone significantly raised flavonoid content by ~ 27% of the level in the inoculated control, and more than doubled the content compared with the uninoculated control (Fig. 6*b*).

Concerning the phenolic content, at 3 days after foliar spray, chitosan at 0.1 and 1.0 g L^{-1} significantly augmented the phenol content above that of the control by ~ 18 and 36%, respectively (Fig. 7*a*). However, 4 days after pathogen inoculation (7 days after foliar spray), the phenol content in the inoculated control surpassed that of the uninoculated control by more than two times. Furthermore, the phenol content of tomato plants pre-treated with either 0.1 or 1.0 g L^{-1} chitosan significantly increased at 4 days post-

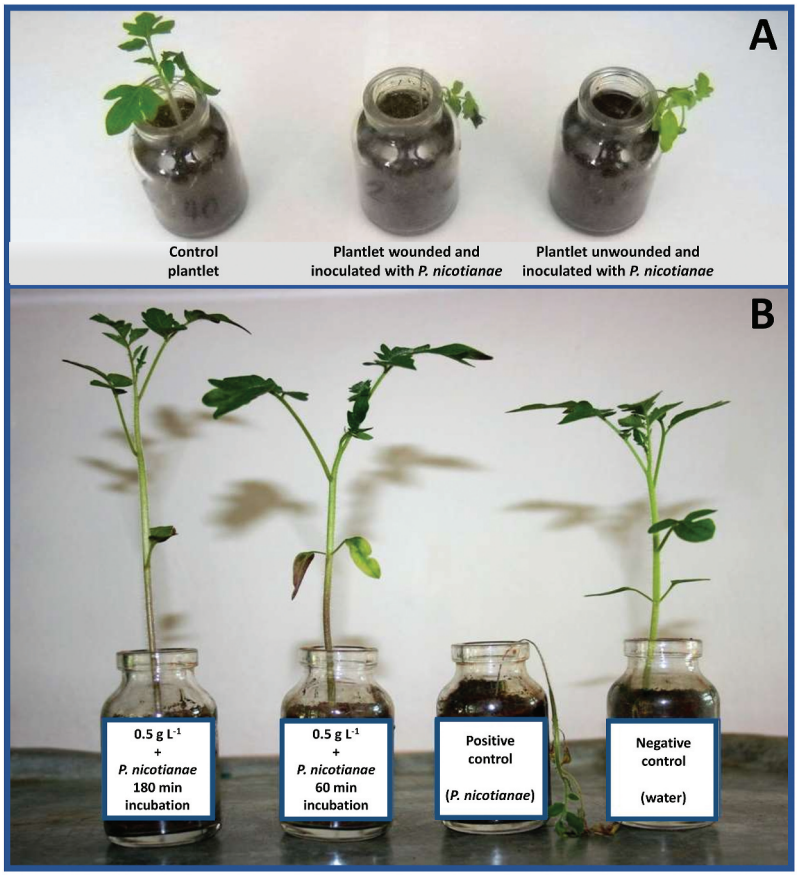

Fig. 5 Tomato plantlets 5 days after inoculation with a suspension of *Phytophthora nicotianae* zoospores alone **(a)** or in combination with 0.5 g L^{-1} chitosan **(b).**

Table 3. Effect of chitosan foliar spray on infection of 28-day-old tomato plantlets by *Phytophthora nicotianae* (Pn9). Results are shown as the mean of the ranks in the non-parametric Kruskal–Wallis test, and as infection index for each treatment.

Treatments	Mean of rank	Infection index
1. Inoculated control	34.54 **b**	100
2. Chitosan 0.1 g L^{-1}	18.67 **a**	54.05
3. Chitosan 1 g L^{-1}	17.79 **a**	51.5
4. Chitosan 2.5 g L^{-1}	27 **ab**	78.2

Different letters after the mean of rank indicate significant differences among treatments in the test of Mann–Whitney with the Bonferroni correction ($p \leq 0.05$).

inoculation by 8–10% compared with the inoculated control, and more than doubled compared with the uninoculated control (Fig. 7*b*).

Discussion

The antimicrobial effect of chitosan has been studied in many plant pathogens, especially in fungi and oomycetes (Qin et al. 2014; Kumaraswamy et al. 2018; Martín-López et al. 2020; Mukhtar Ahmed et al. 2020). There are few reports, however, regarding its effects in the *Phytophthora* genus, and the available studies primarily evaluated its action on mycelial growth, with only a handful pertaining to other aspects of its life cycle (Xu et al. 2007a, 2007b; Falcón et al. 2008, 2010).

The effect observed on mycelial growth, primarily reported as inhibition of radial growth, varies among genera and species. While *Fusarium oxysporum* f. sp. *radicis-lycopersici* requires up to 1 g L^{-1} to achieve over 50% growth inhibition (Benhamou 1992), the same concentration resulted in less than 50% inhibition in *Aspergillus ochraceus* (Meng et al. 2020). Even isolates of the same species, such as Pn9 and Pn227 (Fig. 1) differed in their sensitivity to chitosan, where Pn227 was more resistant than the former to the effects of chitosan in axenic cultures. In another example, a chitosan concentration of 25 g L^{-1} caused a 100% suppression of *Penicillium digitatum* and *Rhizopus stolonifer* mycelial growth, whereas only 15 g L^{-1} had the same effect on

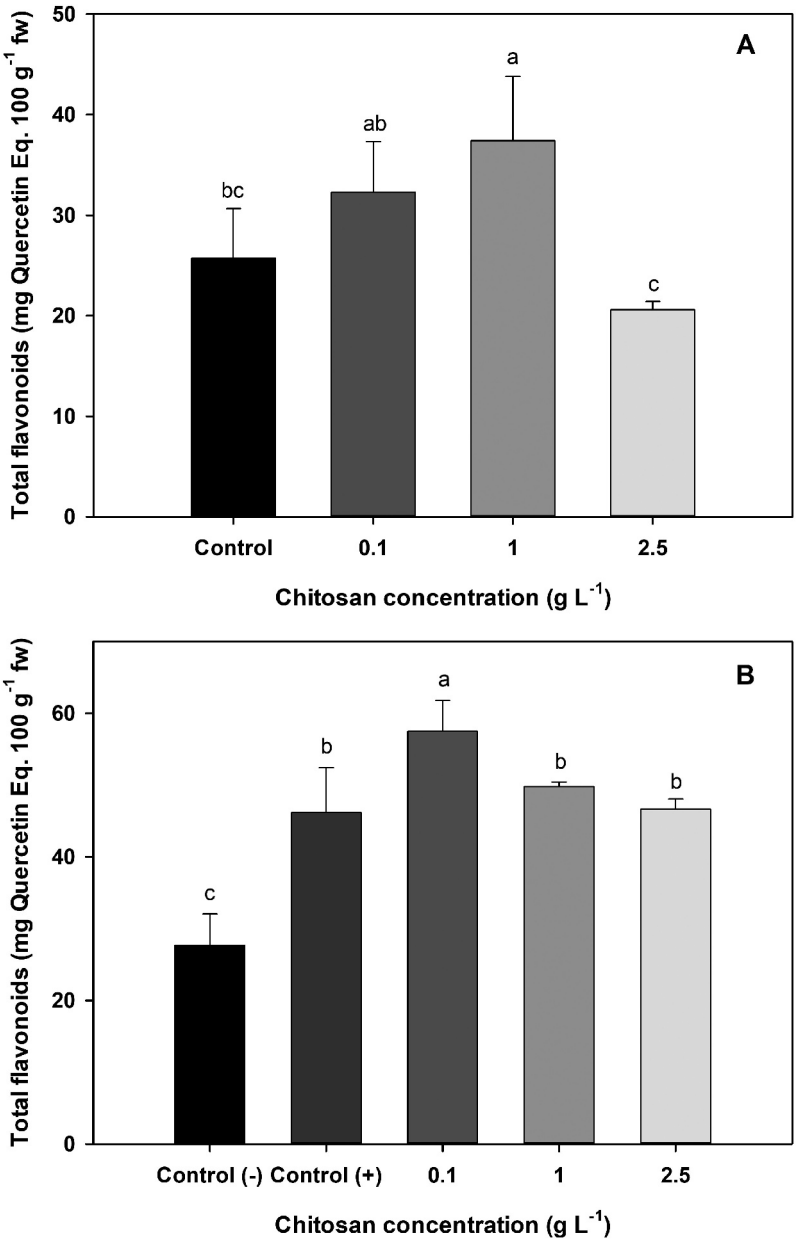

Fig. 6 Total flavonoids content in tomato seedling leaves at 72 h after treatment with different doses of chitosan before inoculation with *Phytophthora nicotianae* **(a)** and at 96 h after inoculation **(b)** (7 days after chitosan application). Inoculated (+) and non-inoculated (-) controls were included. Bars represent the mean values ± SD. The Tukey test with a significance level of $p \leq 0.05$ was used to compare treatments mean values. Same letters did not differ statistically.

F. oxysporum (Bautista-Baños et al. 2004). In agreement with our results, 2.5 g L^{-1} chitosan caused up to 100% radial inhibition of the oomycete *Pythium ultimum* over 24 h, while at 120 h, the percentage of growth suppression declined to 64%—72% compared with the untreated controls (Martín-López et al. 2020). The response observed in these microorganisms when chitosan is present might be related to the polymer's capacity to alter membrane permeability and cause osmotic imbalances, which result in structural destabilization and cell lyses, as has been previously reported (El Ghaouth et al. 1992a; Kumaraswamy et al. 2018). Furthermore, chitosan has chelating activity and interferes with metal ions, thereby immobilizing nutrient uptake by microorganisms (Rhazi et al. 2002; Kumaraswamy et al. 2018; Lopez-Moya et al. 2019). Hence, the fungistatic effect against

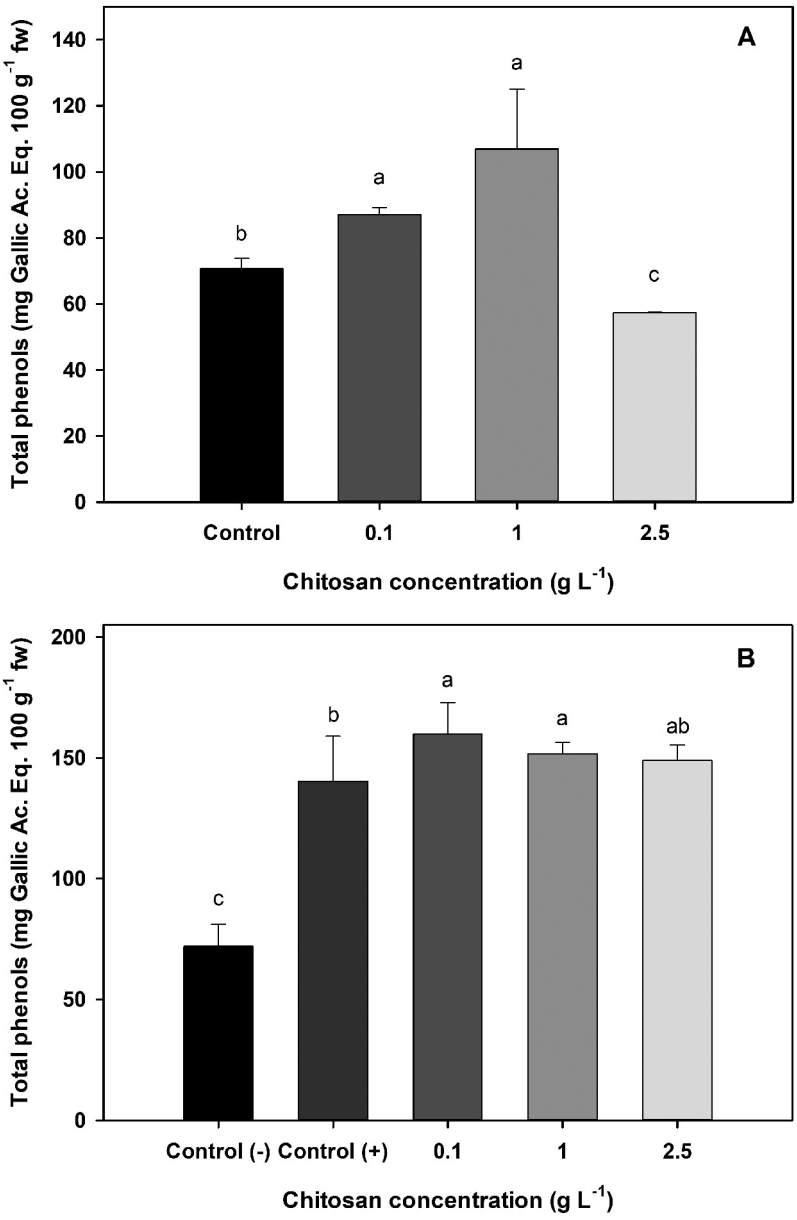

Fig. 7 Total phenols content in tomato seedling leaves at 72 h after treatment with different doses of chitosan before inoculation with *Phytophthora nicotianae* **(a)** and at 96 h after inoculation **(b)** (7 days after chitosan application). Inoculated (+) and non-inoculated (-) controls were included. Bars represent the mean values ± SD. The Tukey test with a significance level of $p \leq 0.05$ was used to compare treatments mean values. Same letters did not differ statistically.

P. nicotianae observed in this study may have occurred due to a combination of such mechanisms, resulting in a limited colony diameter that was evident from the lowest chitosan concentration (0.5 g L^{-1}) used in our assay, but where the highest concentration (2.5 g L^{-1}) did not reach 100% inhibition.

Common chitosan-induced damages in different fungal species, such as *F. oxysporum* f. sp. *lycopersici, R. stolonifer, Pythium aphanidermatum, A. alternata, B. cinerea*, and *Penicillium expansum*, include mycelial aggregation, excessive branching, swelling of the cell wall, hyphae size reduction, vacuolation, and protoplasm disintegration (Benhamou 1992; El Ghaouth et al. 1992b, 1994; de Oliveira et al. 2012). Moreover, chitosan has been shown to damage the vacuole system and plasmalemasomes of *P. capsici* hyphae (Xu et al. 2007a). Plasmalemasome structures are involved in cell wall synthesis, suggesting that chitosan could retard mycelial

growth by interfering with cell wall biogenesis (Xu et al. 2007a). To determine whether chitosan affected the *P. nicotianae* hyphal dimensions, hyphal diameter was measured using an optical microscope, but no effect was observed. However, it is not known whether a higher resolution instrument would have revealed cellular changes within the hyphae. Interestingly, discrepancies in the antifungal effectiveness of chitosan are most likely related to the particular strain studied or to the physical-chemical properties of the polymer.

The effect of chitosan on fungal sporulation has been widely reported (Bautista-Baños et al. 2004; Hernández-Lauzardo et al. 2008; Palma-Guerrero et al. 2008, 2009), but only a few studies pertaining to sporulation have been conducted on oomycetes, especially within the *Phytophthora* genus (Xu et al. 2007a, 2007b). *Phytophthora nicotianae* A1 isolates are endemic to Cuba, and A2 isolates, which are also prevalent, were originally detected in the 1990s (Fernández 1998). Sexual reproduction is key for the emergence of new and more aggressive isolates, as different mating types exchange genetic information. Therefore, the incidence of both mating types within the same region increases the pathogenic potential of these species. In order to limit the emergence of more virulent isolates, it is necessary to evaluate new products that can target sexual reproduction. This study demonstrates for the first time that chitosan decreases *Phytophthora* oospore formation, as observed at concentrations of 0.5 and 1.0 g L^{-1} (Fig. 3). This result has a high practical significance, since the control of oospore formation could contribute to decreasing the opportunities for the emergence of new and more virulent strains.

Chitosan also affected asexual reproduction, where increasing concentrations of this compound reduced the number of sporangia formed; however, it was observed that in the presence of 0.5 g L^{-1} (the lowest concentration tested) the number of sporangia increased compared with the control (Table 1). This type of result has been observed in the fungal species *P. digitatum* and *Alternaria alternata*, and it is hypothesized that the stress caused by low doses of chitosan induces the formation of reproductive structures to ensure survival (Bautista-Baños et al. 2004; Sánchez-Domínguez et al. 2007). It is important to note that even though the number of *P. nicotianae* sporangia could be reduced, their length/width ratio was not affected (Table 1), which agrees with results reported by Martínez-Camacho et al. (2010) in which chitosan did not affect *Aspergillus Niger* spore size but did inhibit its mycelial growth.

Sporangia can germinate to produce a germ tube (direct germination) or zoospores (indirect germination) in a temperature-dependent manner (Erwin and Ribeiro 1996). The release of the zoospores is an important phase in the oomycete life cycle since each sporangium contains about a dozen of these structures, which can swim long distances in search of host tissues, resulting in a bigger impact on crop health. In this study, the lowest chitosan concentration tested prevented more than half of *P. nicotianae* sporangia from germinating, and higher concentrations resulted in complete inhibition (Fig. 4). Previous research also demonstrated that oligochitosans can cause intracellular damage in the sporangia of other *Phytophthora* species, such as *P. capsici*, and can thereby decrease the number zoospores released (Xu et al. 2007a).

Phytophthora nicotianae zoospore movement (Table 2) was also affected by chitosan. It has been shown that chitosan can alter the membrane permeability and efflux/influx of certain ions, such as potassium, on *R. stolonifer* (García-Rincón et al. 2010). It is also known that potassium is involved in regulating zoospore movement and it is thought to be the cause of erratic motility patterns observed in the zoospores of different *Phytophthora* species (*P. palmivora, P. megakarya*, and *P. infestans*), where the rectilinear pattern becomes undulating and circling when potassium homoeostasis is altered (Appiah et al. 2005). Similar observations were made in this study with respect to the motility of *P. nicotianae* in response to chitosan treatments. It is hypothesized here that erratic zoospore movement is caused by changes in potassium homoeostasis consequent to chitosan-induced altered membrane permeability. Nevertheless, a solution with a higher viscosity than water could also influence the motility of the zoospores across solutions with different chitosan concentrations.

The inhibition of spore germination in the presence of chitosan has been observed in many studies of different fungi, such as *A. Niger, A. alternata, R. stolonifer, Mucor* spp., *F. concentricum* (Plascencia-Jatomea et al. 2003; Hernández-Lauzardo et al. 2007; Liu et al. 2007; Sánchez-Domínguez et al. 2007) and a small handful of studies on *Phytophthora* species (Xu et al. 2007a; Falcón et al. 2010). In this work, it was observed that *P. nicotianae* cyst germination was completely arrested by 0.5 g L^{-1} of chitosan after 3 h of incubation (Table 2). However, longer experiments will be necessary to determine whether this effect is irreversible or if germination is simply delayed. Other authors reported that *P. digitatum* spore germination was completely inhibited after 12 h of incubation with chitosan, but reached a 56%

germination rate by 24 h (Pacheco et al. 2008). The mechanism of delayed spore germination is not known, but it is worth noting that chitosan can chelate different metal ions, especially calcium, which, incidentally, is necessary for correct development at this stage of the life cycle (reviewed by Cota-Arriola et al. 2013).

The cyst germination assay presented in Table 2 was first carried out *in vitro*. An *in planta* assay was then performed to determine whether the effect observed, regarding the damage or delay on cyst germination, could influence *P. nicotianae* virulence. A cyst solution was inoculated onto tomato plantlets in the presence or absence of chitosan. As a result, no disease was observed on tomato plantlets treated with the cyst suspension incubated with chitosan for either 60 or 180 min, in correspondence with the *in vitro* assay. It could be expected that root exudates triggered cyst germination (Suo et al. 2016), and that chitosan simply delayed germination of these *P. nicotianae* structures rather than completely inhibiting it. In any case, the chitosan treatment offered protection to the tomato plantlets at lower concentrations (Fig. 5). This result provides a foundation for devising the use of chitosan in fertigation systems, since the zoospores spread through the water.

To determine whether the protection offered by chitosan is a direct consequence of its antimicrobial, elicitor activities or both, another assay was performed in which the chitosan did not directly contact the pathogen. The leaves of tomato plantlets were first sprayed with chitosan, and then 72 h later, the roots were placed in contact with the mycelial pathogen out of the substrate (in glass tubes). Again, chitosan offered protection to the tomato plantlets against infection, suggesting that the effect was the unique result of a systemic induced resistance elicited in the host (Table 3). These results are like the observations made in a similar assay using tobacco plantlets (Falcón-Rodríguez et al. 2011); therefore, it is possible to expect that chitosan offers general protection to solanaceous species against this pathogen.

In an effort to validate these observations of systemic induced resistance, two important defence markers in solanaceous plants, related to the phenylpropanoid pathway, were evaluated both before and after pathogen inoculation. The results suggested that the protection against *P. nicotianae* afforded by 0.1 and 1.0 g L^{-1} chitosan was related to an enhancement of basal resistance, as evidenced by the highest increments of both the phenol and flavonoid defences (Figs 6, 7). This supports a previous hypothesis suggesting that an incompatible plant–pathogen interaction is related to the plant's ability to respond, in a timely manner, with a suitable defence against the pathogen. An incompatible interaction could be achieved in susceptible plants through the application of exogenous elicitors prior to infection (Walters 2011). In this regard, induced resistance to *P. nicotianae* was achieved in tobacco plants treated with chitosan derivatives (Falcón-Rodríguez et al. 2011). Similarly, the induction of disease resistance markers and a decreased susceptibility to viral and fungal agents, such as *Cucumber mosaic virus* (Rendina et al. 2019) and *Fusarium* (Chun and Chandrasekaran 2019), was observed in tomato plants treated with chitosan and chitosan nanoparticles. These results suggest that the preventive application of chitosan can elicit defence reactions in tomato against diverse phytopathogens.

In conclusion, chitosan inhibited the growth and development of *P. nicotianae*. Despite the high concentrations used in this work (2.5 g L^{-1}), no fungicidal effect on mycelial growth was detected, but fungistatic activity was observed. The reproductive structures were more sensitive to the polymer than the vegetative structures, and zoospores incubated with chitosan did not infect tomato plants. Additionally, the ability of chitosan to elicit systemic induced resistance against this pathogen was demonstrated in tomato plantlets. According to secondary metabolite behaviour, observed both herein and in previous reports on the protection of tobacco plantlets, preventive application of chitosan could be highly effective in reducing *Phytophthora* infection both by direct antifungal activity and indirectly, via elicitation of protective responses in tomato plants and in other solanaceous species.

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