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Evaluation of chitosan as an inhibitor of soil-borne pathogens and as an elicitor of defence markers and resistance in tobacco plants

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Abstract

In *in vitro* assays, a chitosan polymer caused differential growth inhibition of the following pathogens isolated from tobacco: *Phytophthora parasitica* Dastur var. *nicotianae* (*Ppn*), *Pythium aphanidermatum* (Edson) Fitzp, *Rhizoctonia solani* Kühn, and *Sclerotium rolfsii* Sacc. The most sensitive were *P. aphanidermatum* and *S. rolfsii,* the growth of which was fully inhibited at a chitosan dose of 1.5 g L^{-1}; the growth of *Ppn* was fully inhibited at 2 g L^{-1}. *In vivo* assays involving plants grown from seeds immersed in chitosan, as well as plants sprayed with this product, were performed to detect the induction of defence response markers in the leaf and consequent resistance to disease. Although defence/resistance marker enzyme activities varied, activation was greater in the chitosan-treated plants than in controls. Marker enzyme activities in the sprayed plants were generally equal to or stronger than those recorded in the seed immersion-treated plants, except for phenylalanine ammonia-lyase activity at the lowest immersion concentration tested. Although there were no statistical differences among treatments with respect to resistance against *Ppn*, the greatest protection was afforded by the spray treatments, in which the infection index was reduced between 17 and 19% compared to the controls. In conclusion, this chitosan polymer directly inhibited the growth of several tobacco pathogens and caused the induction of defence enzymes in leaves, but was not able to protect tobacco plantlets against *Ppn* infection via the activation of induced resistance. This work demonstrated the potential of chitosan in protecting tobacco plants against soil-borne pathogens.

Additional key words: antifungal activity, induced resistance, *Nicotiana tabacum*, *Phytophthora parasitica*, *Pythium aphanidermatum, Rhizoctonia solani, Sclerotium rolfsii.*

Resumen

Evaluación de quitosano como inhibidor de patógenos del suelo y elicitor de marcadores defensivos y resistencia en plantas de tabaco

En ensayos *in vitro,* se demostró la inhibición diferenciada, causada por un polímero de quitosano, sobre el crecimiento de los siguientes patógenos del suelo aislados de tabaco: *Phytophthora parasitica* Dastur var. *nicotianae (Ppn), Pythium aphanidermatum* (Edson) Fitzp, *Rhizoctonia solani* Kühn y *Sclerotium rolfsii* Sacc. Los más sensibles fueron *P. aphanidermatum* y *S. rolfsii,* ya que la inhibición total del crecimiento ocurrió con 1,5 g L^{-1} de quitosano, mientras el crecimiento de *Ppn* fue totalmente inhibido a 2 g L^{-1}. En ensayos *in vivo*, se determinó la inducción foliar de marcadores defensivos y resistencia en plántulas de tabaco previamente tratadas por inmersión de semillas y aspersión foliar del quitosano. La activación enzimática fue mayor en los tratamientos con quitosano. Al analizar las dos

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formas de aplicación, los valores de actividad en los tratamientos de aspersión fueron iguales o superiores que la simple inmersión de semillas, excepto en la actividad fenil alanina amonio-liasa a la menor concentración. Aunque no hubo diferencias significativas entre los tratamientos en la resistencia de las plántulas de tabaco contra *Ppn*, los mayores niveles de protección fueron encontrados en los tratamientos asperjados, donde el índice de infección se redujo entre 17 y 19% respecto al control. En conclusión, un polímero de quitosano inhibió directamente el crecimiento de patógenos del tabaco, causó la inducción de enzimas defensivas en las hojas, pero no protegió las plántulas contra *Ppn* por activación de resistencia inducida. El trabajo demostró el potencial del quitosano en la protección del tabaco contra patógenos de suelo.

Palabras clave adicionales: actividad antifúngica, *Nicotiana tabacum, Phytophthora parasitica, Pythium aphanidermatum,* resistencia inducida, *Rhizoctonia solani, Sclerotium rolfsii.*

Introduction

Tobacco (*Nicotiana tabacum* L.) is a crop of great economic importance worldwide. Its cultivation involves a nursery phase lasting around 40 days, after which the most viable plantlets are moved to the field where they grow until they reach commercial size. Protection in the nursery is essential for obtaining healthy plants. During this phase, the plantlets suffer the attack of many soil-borne pathogens which need to be controlled by chemical means (Erwin and Ribeiro, 1996). However, current thinking is focused on reducing crop production costs by cutting those associated with the use of agrochemicals, and on the use of more ecologically sound management systems (García and Andino, 2002; García *et al*., 2002). In addition, several soil-borne pathogens have developed resistance to the chemicals usually used to control them (Jaarsveld *et al*., 2002), making alternative protection systems necessary.

The use of natural compounds to control plant pathogens may lead to a reduction in the use of fungicides. Chitosan, a partially N-acetylated polymer of β-1-4-glucosamine and an important structural component of the cell wall of certain fungal phytopathogens (mainly Zygomycetes) (Bartnicki-García, 1970), appears to have great potential in this area. Commercially, this biodegradable material is produced by fragmentation and deacetylation of the chitinous components of fungal cell walls and crustacean exoskeletons (Alimuniar and Zainuddin, 1992; Majeti and Kumar, 2000; Ramírez *et al*., 2000). Chitosan and its derivatives are reported to have an antimicrobial effect against a broad range of pathogens (Laflamme *et al*., 1999; Park *et al*., 2002; Zheng and Zhu, 2003; Badawy *et al*., 2004; Devlieghere *et al*., 2004, Bautista-Baños *et al*., 2006). Histochemical studies performed with fungi and bacteria suggest its antimicrobial activity to be due to direct damage caused to cell membranes (Liu *et al*., 2004), and to its deposition inside the cells of microorganisms, where it causes morphological and cytological alterations that finally prevent growth (Hadwiger *et al*., 1981; El Gaouth *et al*., 1992). Additionally, chitosan and its derivatives can elicit a number of defensive responses in plants, including the synthesis of low molecular weight compounds known as phytoalexins, the production of histological barriers that inhibit pathogen entry, and the production of resistance proteins (Van Loon, 1999; Shibuya and Minami, 2001; Agrawal *et al*., 2002).

The efficacy of chitosan as a crop protection agent rests on its solubility, degree of acetylation and molecular weight (Kauss *et al*., 1997; Vander *et al*., 1998; Cabrera *et al.,* 2006). In a study involving whole tobacco plants, Falcón *et al.* (2007) showed that the mycelial growth of *Phytophthora parasitica nicotianae* (*Ppn*) was more greatly inhibited by polymers with a low level of acetylation, while the induction of glucanase activity and consequent plant protection was favoured by a high degree of acetylation. However, it is not known how different doses of low acetylation polymers might enhance the defence responses of tobacco plantlets against this pathogen.

The aim of this work was to evaluate the potential antimicrobial effect of a low acetylation level chitosan polymer against different soil-borne phytopathogens isolated from tobacco, and to determine its capacity to induce the production of defensive response markers in tobacco seedlings and their consequent resistance to disease.

Material and Methods

Chitosan preparation

Lobster chitin was supplied by Mario Muñoz Pharmaceutical Laboratories (Havana, Cuba) and used to

prepare chitosan following the method of Alimuniar and Zainuddin (1992) with some modifications (Ramírez *et al*., 2000). According to previous determinations (Cabrera and Van Cutsem, 2005), the degree of acetylation of this chitosan is 12% and its molecular weight 82.9 kDa. The chitosan polymer powder was then stirred for 2 h (to achieve dissolution) in 1% acetic acid before adjusting the pH to 5.6 by adding 2N KOH. The resulting solution of chitosan in potassium acetate was then diluted to 1% with dH_2O.

In vitro assay

An antifungal assay was performed on potato dextrose agar (PDA) plates amended with chitosan at different concentrations following the method of Falcón *et al*. (2004). For this, the chitosan solution was autoclaved and added to sterile PDA to obtain chitosan concentrations of 0.5, 1, 1.5 and 2 g L^{-1}. Aliquots (20 mL) of these solutions were then poured into 90 mm diameter Petri dishes, each of which was seeded with a 6 mm diameter mycelial disc taken from the margin of 3-9 day old fungal cultures (depending on the growth rate of the strain in question). Three replicates of five plates were employed for each strain at each chitosan concentration. The plates were incubated at 28 ± 1°C in the dark. Pathogen growth was recorded daily until the controls (no chitosan added) reached the edge of the plate. This test was repeated twice and the results expressed as the percentage of inhibition of radial growth (relative to the control). This was calculated using the formula:

$$1\text{-}ds/dc \times 100$$

where *ds* is the diameter of radial growth in the chitosan-amended plates, and *dc* the diameter reached in the control plates. The following pathogen species (isolated from tobacco) were used in the above tests: *Phytium aphanidermatum* (Edson) Fitzp*, Sclerotium rolfsii* Sacc., *Rhizoctonia solani* Kühn and *Phytophthora parasitica* Dastur var. *nicotianae* (Strains SJ-44). Data were analysed by bifactorial ANOVA using SPSS 11.0 software for Windows.

Disease assessment in pathogen-inoculated, chitosan-treated tobacco plants

This experiment was performed using tobacco (*Nicotiana tabacum* L.) plants of the Cuban «Corojo» variety (susceptible to *P. parasitica nicotianae*) cultivated in a substrate mixture of red ferralitic soil and organic matter (filter press mud-peat, 1:1 v/v) (1:2 v/v), pH 6.0, previously sterilised twice in an autoclave at 120°C for 1 h. Plants grew under semi-controlled conditions with a light/dark regime of 16/8 h and a temperature of 28°C/23°C d/n.

Different doses of the chitosan polymer (0.1, 0.5, 1 g L^{-1}) dissolved in potassium acetate plus 0.01% Tween 80 were applied either via seed immersion for 4 h before planting or as foliar spray 30 days after planting. Thirty five day-old tobacco plants (five days after spraying in the case of the spray treatment) were gently removed from the substrate and placed in Eppendorf tubes containing diluted (1:50 v/v) nutrient solution (Hoagland and Arnon, 1950) and an agar culture plug (placed at the bottom) bearing a mycelium of a pathogenic strain (227) of *P. parasitica*. This allowed contact between the plant's roots and the mycelium (Ricci *et al*., 1992); plant-pathogen interactions were allowed to proceed for five days before determining the degree of plant infection. For control experiments, tobacco seeds were immersed or plants sprayed only with the potassium acetate solution.

Plant protein extraction

Before exposing plants to the pathogen, the true leaves from a separate set of tobacco plants (five per treatment) previously treated as described above (but not used in the pathogen bioassay) were collected and ground with a porcelain pestle and mortar in the presence of liquid nitrogen. Proteins were extracted from the powdered leaves in 50 mM sodium acetate buffer pH 5.2 containing 5 mM EDTA, 14 mM β-mercaptoethanol and 1.0 M NaCl (1 g per 2 mL of buffer). The extract was then centrifuged at 12,000 g for 15 min at 4°C in a Sigma Microcentrifuge. The supernatant was collected in clean Eppendorf tubes and stored at –10°C until analysis.

Plant enzyme determinations

Enzyme activities were determined at the beginning of infection. Glucanase (EC 3.2.1.6) and chitinase (EC 3.2.1.14) activities were determined using CM-curdlan-remazol brilliant blue and CM-chitin-remazol brilliant violet (Loewe Biochemica GmbH, Sauerlanch, Germany) as substrates respectively, following the

method of Wirth and Wolf (1990). The absorbance at 600 nm (glucanase) and 550 nm (chitinase) was recorded and the results expressed as the change in optical density (ΔOD) per mg of protein per hour.

Chitosanase (EC 3.2.1.132) activity was assessed using the experimental chitosan at 0.25% as a substrate. The reaction mixture was incubated at 40°C for 24 h before stopping the reaction with Schales reagent (Schales and Schales, 1945). Results were expressed as µg of glucosamine per mg of protein per hour.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was determined using L-phenyl-alanine (Merck, Germany) at 1 mg mL^{-1} as a substrate in a buffer solution of 0.1 M sodium borate (pH 8.8). Plant leaf extract (0.1 mL) was added to 0.9 mL of substrate and the mixture incubated at 40°C for 30 min. The reaction was stopped by adding 0.25 mL of 5N HCl and cooling on ice for 5 min. Finally, 5 mL of water were added and the absorbance recorded at 275 nm using a UV-spectrometer. Results were expressed as µg of *trans*-cinnamic acid per mg of protein per min.

Using the continuous enzyme assay of Ben-Shalom *et al.* (2003) (slightly modified), peroxidase (EC 1.11.1.6) activity was assayed using pyrogallol as a substrate. Results were recorded every 15 s for 4 min. Enzyme activity was expressed as µmoles of H_2O_2 per mg of protein per min. Protein determinations were performed according to the micro Lowry assay (Sun, 1994).

Statistical analysis of plant protection data

The degree of infection was determined in each plant using the scale of pathogen invasion described by Falcón *et al.* (2007). Plants were visually scored on a scale of 1 to 5 to estimate the extent of fungal damage (1 = no symptoms, 2 = roots affected, 3 = hypocotyls and cotyledons affected, 4 = first and second leaf pair affected, 5 = dead plant). Fifteen plants per treatment were tested; the experiments were repeated twice with similar results. The data were processed according to Achuo *et al.* (2004) using the Kruskal-Wallis non-parametric test. All means were compared independently using the Mann-Whitney test. Significance was set at $p < 0.05$. All calculations were performed using SPSS v. 11 software for Windows. An infection index was calculated using the following formula:

$$\text{Inf. Index} = \sum (d \times f) / (N \times D)$$

where d is the degree of infection according to the above scale, f the respective frequency, N the total number of plants examined in the treatment, and D the highest degree of infection on the scale (Romanazzi *et al.,* 2002). For ease of understanding, the results were also expressed as a percentage of plant protection compared to controls (0%).

All enzyme data were analysed by ANOVA and the resulting means compared using the Tukey test. Significance was set at $p < 0.05$.

Results

In vitro antifungal assay

The results of this test showed that chitosan inhibited mycelial growth in the four species analysed, but to different degrees (Fig. 1). *Phytium* and *Sclerotium* were the most sensitive to the action of the polymer; their growth was fully inhibited at 1.5 g L^{-1} chitosan, while the growth of *Phytophthora* was fully inhibited at 2 g L^{-1}. The least sensitive pathogen was *Rhizoctonia*; at 2 g L^{-1} chitosan the growth rate was still 70% that of the control. At the lowest chitosan concentration *Phytophthora* and *Sclerotium* were the most sensitive (significantly more so than either *Phytium* or *Rhizoctonia*; Fig. 1).

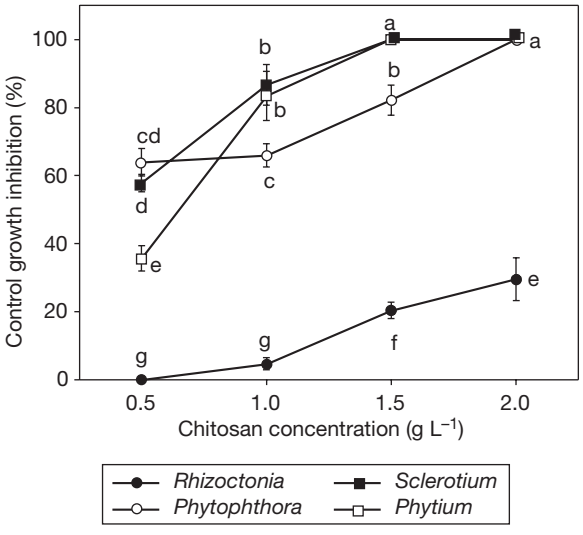

Figure 1. Chitosan inhibition of mycelium growth in four soil-borne pathogens isolated from tobacco. Different letters among treatments indicate significant differences ($P \leq 0.05$; as determined by the Tukey test). Bars represent standard deviations of the mean.

Table 1. Infection index and percentage protection achieved in tobacco plants (*Nicotiana tabacum* L. cv. Corojo) treated with different chitosan concentrations either by seed immersion before planting or by foliar spraying five days before inoculation with *Phytophthora parasitica* Dastur var. *nicotianae*. The data were analysed using the Kruskal Wallis non-parametric test. The Mann-Whitney test was used to compare means ($P < 0.05$)

Treatments	Seed inmersion			Foliar spray		
	N	Infection index (%)	Protection (%)	N	Infection index (%)	Protection (%)
Control (KAc^-)	14	78.6 a	0	15	78.9 a	0
Chitosan 0.1 g L^{-1}	15	73.3 a	6.5	15	65.3 a	17.2
Chitosan 0.5 g L^{-1}	15	70.7 a	10.1	15	64.0 a	18.9
Chitosan 1 g L^{-1}	15	72.0 a	8.4	15	64.0 a	18.9

Disease assessment in pathogen-inoculated, chitosan-treated tobacco plants

Table 1 shows the effect of chitosan against *Ppn* infection in tobacco plants. Overall, no significant differences were seen among the treatments tested. However, foliar spraying led to a 17-19% reduction in the infection index. In the forty day-old plants grown from seeds immersed in the chitosan solution, the reduction in the infection index was between 6 and 10% compared to controls. Although no significant differences were found among the treatments, the application of chitosan did modify the frequency of pathogen invasion in tobacco plants (Fig. 2), especially when they had been sprayed. Spraying reduced the percentage of dead plants compared to those obtained with the immersion treatment and no treatment (control); a greater percentage of the plants remained healthier and showed only root damage.

The induction of resistance to pathogens by a compound is the result of its activating a wide range of defence responses. Figure 3 describes the activation of defensive hydrolytic enzymes (ß 1-3 glucanase, chitinase and chitosanase) in the leaves of tobacco plants treated with different doses of chitosan polymer (by seed immersion and foliar spraying).

Enzyme activation was stronger in most of the chitosan treatments than in the controls. For all the hydrolytic enzymes tested, the activities achieved with the spray treatment were equal to or greater than those obtained with seed immersion. In terms of glucanase and chitosanase activities, most of the spray treatments achieved better results than seed immersion. For both enzymes, the highest activation values were obtained with the lowest chitosan concentration spray treatment (0.1 g L^{-1}). With respect to chitinase activity, the results for all treatments were statistically different to those obtained for the control treatment, but did not differ significantly among themselves (Fig. 3).

The PAL and peroxidase activities were different to those shown by the hydrolytic enzymes. They differed according to the chitosan concentration applied. No pattern was detected with respect to the way in which the product was applied. For example, for seed immersion, the two lowest doses led to greater PAL activity than that recorded for the controls, while for the foliar spray, the two highest dose were associated with the

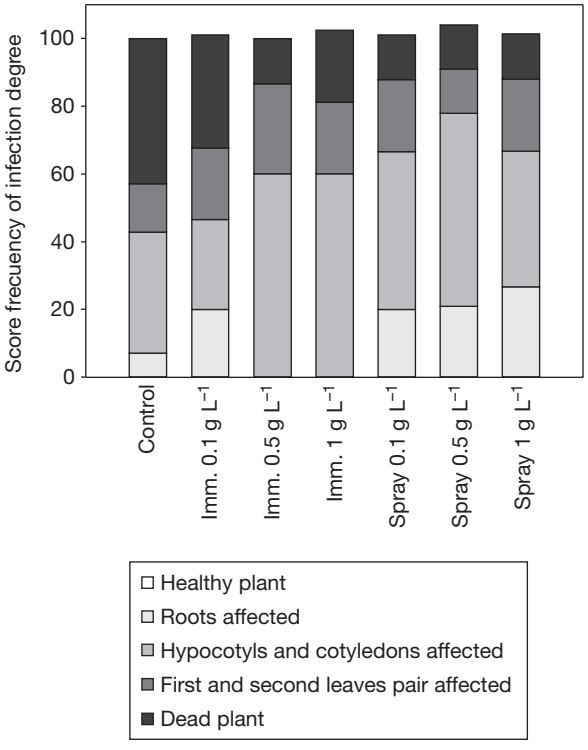

Figure 2. Infection score frequency (% pathogen invasion of the tobacco plantlets in each treatment). Each stack in the columns represents the frequency of each degree of infection according to the scale described in Material and Methods.

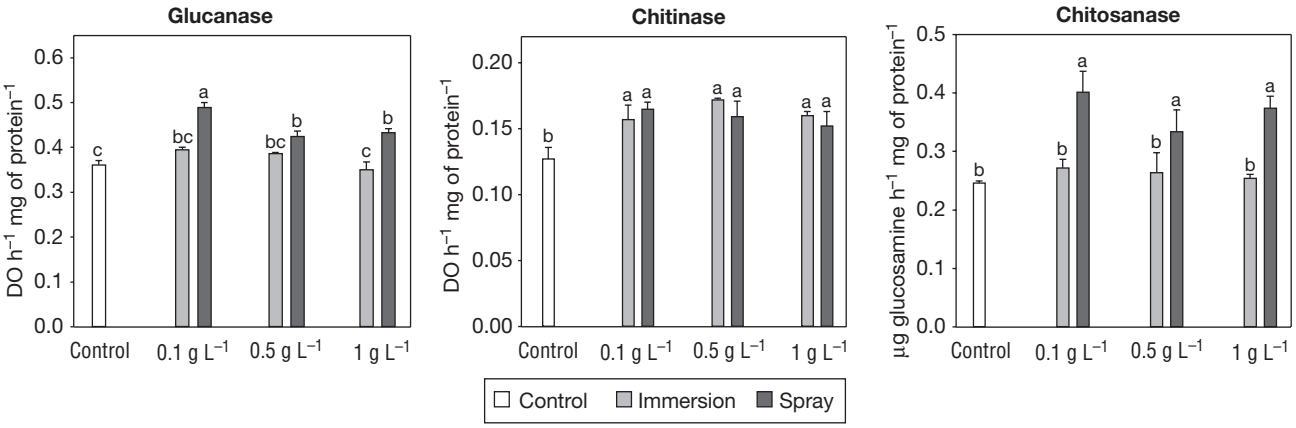

Figure 3. Induction of hydrolytic enzyme activities in leaves of plants grown from seeds immersed in chitosan and leaves treated by spraying (0.1, 0.5, 1 g L^{-1}). Different letters among treatments indicate significant differences (P = 0.05). Bars represent standard deviations of the mean.

greatest activity. With respect to peroxidase activity, the lowest and the highest doses of sprayed chitosan led to activities significantly different to that recorded for the control (Fig. 4). In general, the best glucanase, chitosanase and peroxidase activities were obtained with the spray treatments at the 0.1 g L^{-1} dose, whereas the highest PAL activity was achieved at the same dose but with seed immersion.

Discussion

Crop protection with chitosan and its derivatives, due both to induced resistance and direct inhibition of pathogen growth and germination, has been extensively investigated in recent years (Bhaskara Reddy *et al*., 2000; Li and Yu, 2000; Romanazzi *et al*., 2002; Bautista-Baños *et al*., 2003; Ben-Shalom *et al*., 2003; Molloy *et al*., 2004). Some studies have even involved commercial chitosan-based products (Ait Barka *et al.,* 2004; Sharathchandra *et al*., 2004). Tobacco, besides being a model species in plant-pathogen interaction studies, is also an economically important crop in Cuba; basic and applied research into its protection are therefore justified. This study provides new information regarding the inhibitory effect of chitosan on the growth of different tobacco pathogens. It also shows that chitosan, applied in two different ways, increases tobacco plantlet defences.

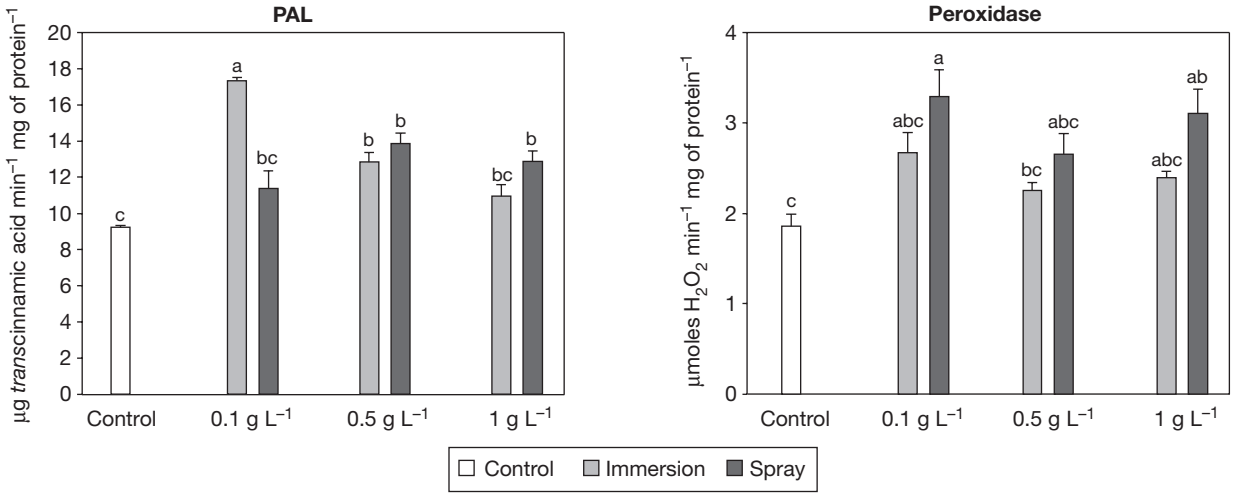

Figure 4. Induction of PAL and peroxidase activities in leaves of plants grown from seeds immersed in chitosan and leaves treated by spraying (0.1, 0.5, 1 g L^{-1}). Different letters among treatments indicate significant differences (P ≤ 0.05). Bars represent standard deviations of the mean.

The growth inhibition of soil-borne and post-harvest fungal phytopathogens has been widely studied (Laflamme *et al*., 1999; Park *et al*., 2002; Devlieghere *et al*., 2004). However, the literature contains no reports of chitosan dose response assays involving the direct inhibition of the growth of *Phytium aphanidermatum* and *Sclerotium rolfsii*. Both these species are common pathogens in Cuban tobacco nurseries; this study opens up the possibility of using this polymer to protect nursery stage tobacco plants against these and other fungi.

According to the results obtained in the induced resistance bioassay, the chitosan polymer appears unable to protect tobacco plantlets against *Ppn* by inducing systemic resistance - at least at the concentrations and with the application methods tested. However, the chitosan treatments did modify pathogen invasion (Fig. 2), leading to partial protection. Compared to the immersion treatment and controls, the spray treatments reduced the percentage of dead plants and increased the percentage of less infected plants.

This work shows the present polymer not to protect tobacco seedlings against *Ppn* via the induction of systemic resistance, unlike that achieved with a chitosan polymer of similar size and origin but three times more acetylated (Falcón *et al.,* 2007). However, the potential of the present polymer to protect tobacco against *Ppn* in nurseries should not be overlooked. The protection bioassay used in this work favoured seedling infection since the plants were exposed to the pathogen in aqueous solution for five days. The literature contains very few reports concerning crop protection in which chitosan applications provide strong protection only by inducing local and systemic resistance (Benhamou *et al.,* 1994; Sharathchandra *et al.,* 2004). Most simply report a reduction in the number of lesions (Molloy *et al.,* 2004) or the direct antifungal activity of chitosan as the main factor responsible for protection (Ben-Shalom *et al.,* 2003). Based on the present *in vitro* results, it might be expected that polymer application to the plants or to the substrate in which they are growing would cause the partial or total inhibition of soil pathogens. The results regarding the activation of defensive enzymes support this idea. Although, the resulting enzyme activities were generally higher in the sprayed plants than in the untreated controls, they were not very high compared to those obtained in a previous study in which a more acetylated chitosan polymer was used (Falcón *et al.*, 2007). Since stronger induction of plant defences is seen with more acetylated polymers (Kauss *et al*., 1997; Vander *et al*., 1998), it might be expected that this should translate into greater resistance against pathogens.

The strongest activation of defence responses —and therefore the best protection— was seen when chitosan was sprayed on the plants. According to earlier reports, concentrations above 0.5 g L^{-1} are needed to protect different plant species against their main pathogens (Ben-Shalom *et al*., 2003; Molloy *et al*., 2004; Ait Barka *et al*., 2004; Sharathchandra *et al*., 2004). Future studies should further investigate the doses and forms of chitosan applied in order to better understand the triple interaction among chitosan, plant and pathogen.

In conclusion, this study shows that a low acetylation level chitosan polymer can directly inhibit the growth of tobacco pathogens *in vitro*. However, protection against *Ppn* via the induction of resistance seems not to be achieved, neither when seeds are immersed in this product, nor when it is sprayed onto the leaves. Future research should focus on understanding the activation time associated with the defence responses of tobacco and its correlation with plant resistance when chitosan derivatives of the same origin, but with different physicochemical features, are applied. The tobacco-*Phytophthora* interaction bioassay used in this work could play an important role in this respect.

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