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ORIGINAL PAPER

The effect of size and acetylation degree of chitosan derivatives on tobacco plant protection against *Phytophthora parasitica nicotianae*

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Abstract Enzymatic degradation of chitosan polymer with Pectinex Ultra SPL was used to obtain derivatives with biological potential as protective agents against *Phytophthora parasitica nicotianae* (*Ppn*) in tobacco plants. The 24 h hydrolysate showed the highest *Ppn* antipathogenic activity and the chitosan native polymer the lowest. The in vitro growth inhibition of several *Phytophthora parasitica* strains by two chitosans of different DA was compared. While less acetylated chitosan (DA 1%) fully inhibited three *P. parasitica* strains at the doses 500 and 1000 mg/l the second polymer (DA 36.5%) never completely inhibited such strains. When comparing two polymers of similar molecular weight and different DA, again the highest antipathogenic activity was for the less acetylated polymer. However, degraded chitosan always showed the highest pathogen growth inhibition. Additionally, a bioassay in tobacco seedlings to test plant protection against *Ppn* by foliar application demonstrated that partially acetylated chitosan and its hydrolysate induced systemic resistance and higher levels of glucanase activity than less acetylated chitosan. Similarly, when treatments were applied as seeds coating before planting, about 46% of plant protection was obtained using chitosan hydrolysate. It was concluded that, while less acetylated and degraded chitosan are better for direct inhibition of pathogen growth, partially acetylated and degraded chitosan are suitable to protect tobacco against *P. parasitica* by systemic induction of plant resistance.

Keywords Antipathogenic activity · Chitosan · Glucanase · PAL · *Phytophthora parasitica* · Systemic induced resistance · Tobacco

Abbreviations

DA	Degree of acetylation
DP	Degree of polymerization
PDA	Potato-dextrose-agar
AcK	Potassium acetate
PAL	Phenyl alanine ammonio lyase
Ppn	*Phytophthora parasitica nicotianae*

Introduction

Phytophthora parasitica var. *nicotianae* (*Ppn*) is a pathogenic oomycete that resemble a fungus both morphologically and physiologically, but is phylogenetically related to

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diatoms and brown algae. Oomycetes have a distinct physiology, which causes that most fungicides worldwide fail against them (Thakur and Mathur 2002). *Ppn* is the causal agent of a common and worldwide destructive soilborne disease of tobacco (*Nicotiana tabacum* L.) characterized by primary affectation of roots and stem basal region of the plant, although all the parts of the plant can be eventually infected. In Cuba this disease, commonly known as Black shank, is the most important problem for commercial tobacco crops and prevention against the pathogen is performed by applying chemicals and using fairly resistant varieties. However, current approaches are focused on decreasing costs of the crop production by reducing the cost of agrochemicals and by improving the ecological management in the tobacco farming. Additionally, it is now documented several cases of *Ppn* resistance against chemicals used to control it (Jaarsveld et al. 2002).

Resistance to disease in plants can be enhanced systemically by earlier infections with pathogens or by previous chemical treatments with agents called elicitors (Kessmann et al. 1994; Hahn 1996). Some known are oligosaccharins, as for instance, oligoglucans, oligogalacturonides and chitin derivatives, and they have demonstrated to be excellent inducers of defensive responses in many plant species and even of plant resistance against several pathogens (Doares et al. 1995; Shibuya and Minami, 2001; Barka et al.2004).

Chitin is a lineal polysaccharide composed of 2-acetamide-2-deoxy-D-glucopyranoside residues β-(1 $\rightarrow$ 4) linked. Chitin production from the crustacean shells has increased substantially in the last 20 years as a result of the versatility and the multiple applications of this polymer and their derivatives in the fields of medicine, nutrition, industry, cosmetic and agriculture (Majeti and Kumar 2000; Bautista-Baños et al.2006).

Chitosan is the main chitin derivative and is obtained by its partial or total deacetylation. This process produces a polymer soluble in diluted acids, which is one of the main advantages of chitosan used in agriculture. In addition, chitosan can inhibit the growth of an important number of fungal pathogens (El Gaouth et al.1992; Laflamme et al.1999; Park et al. 2002), elicit defensive responses (Kauss et al.1997; Vander et al.1998), protect against pathogens in plants (Ben-Shalom et al.2003; Molloy et al.2004) and regulates plant growth (Chibu et al.2002; Ohta et al.2004).

The degree of acetylation (DA) and polymerization (DP) of chitosan can affect its biological activities. Several authors have demonstrated that chitosan DA modulates enzymatic activities such as peroxidase activity, phenylalanine ammonia-lyase activity (PAL) in plants, and induces synthesis of hydrogen peroxide. These responses increase along with DA of the polysaccharide, achieving maximal responses with a DA above 20% (Kauss et al.1997; Vander et al.1998). On the contrary, the fungicidal activity of chitosan rises when the DA decreases (El Gaouth et al.1992; Park et al.2002).

As chitosan DP is also important in antifungal activity (El Gaouth et al.1992) and eliciting of plant defence and plant protection (Vander et al.1998; Struszczyk et al.1999), several methods to degrade the polymer and to obtain oligosaccharide mixtures had been established. The methods more frequently used are chemical or enzymatic hydrolysis. Both release chitooligomers of different size and DA when a partially acetylated chitosan is used as starting material (Zhang et al.1999; Jin and Shen, 2002; Li et al.2004; Cabrera et al.2005). Depending on the chemical structure of chitosan, the type of pathogen or the plant specie selected to carry out the bioassay, a range of responses can be obtained.

The main purpose of this work was to investigate how the DA and DP of chitosan influence the protection of tobacco plants against one of its more important pathogens. In this sense, two types of experiments were carried out using chitosans with different DA and DP: an in vitro growth inhibition of several strains of *P. parasitica*, and an in vivo bioassay to test the induction of systemic resistance in tobacco seedlings.

Materials and methods

Chemicals

Lobster chitin was supplied by ''Mario Muñoz'' Pharmaceutical Laboratories (Havana, Cuba) and used to prepare chitosan polymers (*CH-63, CH-88*) under homogeneous conditions following the methodology described by Alimuniar and Zainuddin (1991). Chitosan *CH-99* was obtained by heterogeneous desacetylation procedure as previously described (Cabrera et al. 2000). The molecular weight of chitosan was determined in a Ubbelohde capillary viscosimeter using the buffer 0.3 M acetic acid/ 0.2 M sodium acetate (Cárdena, et al.2002; Cabrera and Van Cutsem 2005). The DA of chitosan polymers was determined by potenciometry (*CH-63, CH-88*) (Cabrera and Van Cutsem, 2005) and ^{1}H NMR (*CH-99*) as previously reported (Argüelles-Monal et al.2000).

Chitosan hydrolysis

Chitosan (*CH-63*) was dissolved at 1% (w/w) in 0.2 M acetic acid and the pH adjusted to 5.6 with a solution of 2N KOH. Pectinex Ultra SP-L (Novozymes A/S, Bagsvaerd, Denmark) enzymatic preparation was added to the chitosan solution in a rate of 1:100 v/v. Hydrolysis was performed

during 24 h at 37°C with continuous magnetic stirring. Several aliquots of 20 ml were collected every two hours until 24 and autoclaved for 15 min at 121°C to stop enzymatic reaction. Each fraction was analysed by determination of viscosity and reducing sugars (Nelson 1944) using glucosamine as standard.

Biological tests

In vitro antifungal activity assay

Three experiments were carried out in the following conditions: Petri dishes of 90 mm of diameter were used containing 20 ml of Potato-Dextrose-Agar (PDA) including the treatments at the concentrations of 250, 500 and 1000 mg/l. The salt produced during chitosan neutralization (potassium acetate) was also added to PDA medium and used as control. In each case, a mycelial plug of 8 mm of diameter; taken from the margin of 7–9 days old *Phytophthora parasitica* cultures, previously grew in PDA dishes; was placed in the middle of the dish and the fungal growth was determined by measuring the diameter of each colony at intervals of 24 h until the control colony reached the dish border. Dishes were incubated in the dark at 25–27°C. Three replicates of four dishes were used by each treatment and experiments were repeated twice.

In the first experiment, a comparison between chitosan polymer *CH-63* and three of its enzymatic hydrolysates on a pathogenic strain of *P. parasitica* was carried out. In the second experiment, two chitosans (*CH-99*, *CH-63*) were tested on three strains of *P. parasitica* having different degrees of aggressiveness against tobacco: Very aggressive, moderately aggressive and tobacco non-host, according to previous plant inoculation tests performed with different *Phytophthora* isolations (Toledo 2001). In the third experiment, a general comparison among *CH-63*, *CH-88* and the hydrolysate of 24 h (*HCH-24*), at two concentrations (500 and 1000 mg/l), on the 227 strain was performed. For all experiments data was expressed as the percentage of radial growth inhibition relative to the control and analysed through a Bi-factorial and Tri-factorial ANOVA, respectively, using the statistical programme SPSS 11.0 for Windows©. Means with same letters did not differ for $P < 0.01$ in the test of Tukey.

Assessment of disease resistance in tobacco plants induced with chitosan and derivatives

All experiments were performed using tobacco (*Nicotiana tabacum*) plants of ''Corojo'' Cuban variety (susceptible to *P. parasitica*) cultivated in a mixture of soil and organic matter 1:1 v/v under semi-controlled conditions with a light/dark and a temperature regime of 16/8 h and 28°C/23°C, respectively. In one, tobacco plants were grown during 35 days before being sprayed with solutions containing chitosan polymers (*CH-63,CH-88*) or the hydrolysate of 24 hours (HCH-24), dissolved at 500 mg/l in 50 mM potassium acetate buffer pH 5.5, 0.01 % Tween 80. As control, Tween 80/ potassium acetate solution was sprayed. After 120 h, plants were gently removed from the substrate and placed in eppendorf tubes containing distilled water and an agar culture plug bearing mycelium of a pathogenic strain of *P. parasitica* placed at the bottom, allowing contact between plant roots and pathogen (Ricci et al. 1992). Plant pathogen interaction was allowed to proceed for five days before determining plant infection. In the second experiment tobacco seeds were immersed during 4 hours in chitosan polymers and the hydrolysate of 24 h at 500 mg/l in the conditions previously described. As control, tobacco seeds were immersed for the same period in the potassium acetate solution mentioned before. Then, the seeds were dried before planting and cultivated during 32 days. Finally, the same bioassay was performed with the same pathogen described in the previous experiment.

Data processing of plant protection

Infection degree was determined in each plant using the scale of pathogen invasion described by Ricci et al. (1992) and modified for our purposes (Table 1). Twelve and fifteen plants by treatment (respectively, for spray or seed coat assay) were tested and experiments were repeated twice with similar results. Data were processed according to the Kruskal-Wallis non-parametric test and all means compared in an independent way through the Mann–Whitney test for $P < 0.05$, by using the statistical programme SPSS 11.0 for Windows©. Results were also presented as percentage of plant infection compared to controls (100 % infection).

Plant proteins extraction

Before placing plants with the pathogen, the leaves from plants treated by spraying (and not used in the pathogen bioassay) were collected and ground in a porcelain mortar and pestle in presence of liquid nitrogen. Powdered leaves

Table 1 Scale of pathogen invasion in tobacco plantlets

Degree	Description
1	Healthy plant
2	Roots affected
3	Hypocotyls and cotyledons affected
4	First and second leaves pair affected
5	Dead plant

were extracted in 50 mM sodium acetate buffer pH 5.2 containing 5 mM EDTA, 14 mM β-mercapto-ethanol and 1.0 M NaCl to the rate of 1 g per 2 ml of buffer. The extract was then centrifuged at 12000*g* for 15 min at 4°C in a Sigma 2–15 centrifuge. The supernatant was collected in clean eppendorf tubes and stored at –10°C for subsequent analysis.

Plant enzymatic determinations

Enzymatic activities were determined at the beginning of the infection. Glucanase activity was determined using CM-Curdlan-RBB (Loewe Biochemica GmbH, Sauerlanch, Germany) as substrate and according to the methodology described by Wirth and Wolf (Wirth and Wolf 1990). The absorbance at 600 nm was recorded and results were expressed as the change in optical density: ΔOD mg/protein/min. The PAL activity was determined using L-Phenyl-Alanine (Merck, Germany) at 1 mg/ml as substrate in buffer solution of 0.1 M sodium borate pH 8.8. To 0.9 ml of substrate was added 0.1 ml of plant extract and the mixture was incubated at 40°C for 30 min. Reaction was stopped by addition 0.25 ml of 5N HCl and allowed to cool in ice for 5 min. Finally, 5 ml of water were added and the absorbance was recorded at 290 nm in an UV-Spectrometer. Results were expressed as units of transcinnamic acid formed per minutes per mg of protein (u/min/mg) and one unit of enzymatic activity was stated as 1 mM of transcinnamic acid formed. Protein determinations were performed following a μLowry assay described (Sun 1994). For practical reasons results presented on graph were expressed as times increment of enzymatic activity related to the control for each derivative.

Results and discussion

Due to the importance of the chemical structure of chitosan on biological activity, the average molecular weight and the acetylation degree of polymers were determined. In Table 2 a summary of chitosan characterization is shown.

Table 2 Chemical characterization of chitosan polymers

Chitosan	Mv[a] (×10 ^{5} Da)	DPv[b]	DA[c] (%)
CH-63	1,27	794	36.5
CH-88	1,22	813	12
CH-99	3.0	1863	1[d]

[a] Average molecular weight determined by viscosimetry

[b] Average degree of polymerization determined by viscosimetry

[c] Degree of acetylation by Potentiometry

[d] Degree of acetylation by ^{1}H Nuclear Magnetic Resonance

Hydrolysis of chitosan

The chitosan enzymatic degradation by a commercial enzymatic preparation is shown in Fig. 1. As chitosans are relatively rigid linear polysaccharides, any change of their viscosity reflects modifications of their DP. A marked endochitosanase action can be deduced from the abrupt decrease of the viscosity (around 60%), and from the not significant increment of reducing sugars after 2 h of hydrolysis. It is probable that a low or none exo-chitosanase activity was occurring during this time due to either the lacking of this activity in the enzymatic preparation or to a low concentration of its substrates at the beginning of hydrolysis. However, from 2 to 24 h the viscosity was reduced just about 20% while the increment of reducing sugars was higher. During this period the availability of substrates for chitosanase activity rose due to new fragments generated by chitosan hydrolysis.

The chitosan hydrolysis behaviour could be explained from two points of view. It is possible that, as molecular weight of chitosan decreases during hydrolysis, enzymes with endochitosanase action could lose their affinity for hydrolysis products or even they could be inhibits by excess of products formed, as it has been found by some authors (Shin-Yaa et al. 2001; Kittur et al. 2003). This agree with the flatter viscosity in the decreasing behaviour obtained after 2 h. At the same time, hydrolysis could result in an increment of exochitosanase substrate concentration. Such activity could be due to the action of other enzymes of the preparation. This would explain the increasing of reducing sugar with little change of viscosity after 6 h of incubation.

Another explanation could be that as hydrolysis progress, the low molecular size of the oligomers obtained does not change the viscosity. This could be happening after 2 h, in which the hydrolysis continues (it is confirmed by increasing of reducing sugars) while the viscosity decreases to reach a plateau around 10 h.

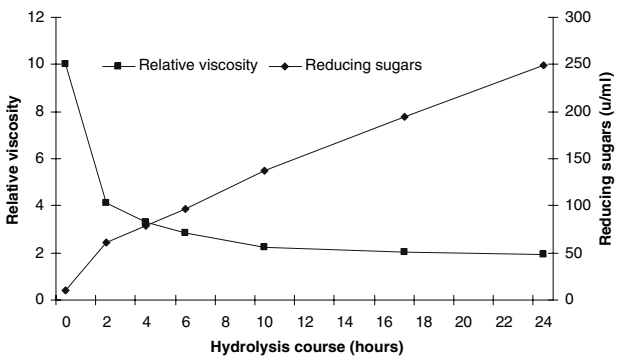

Fig. 1 Time course of *CH-63* enzymatic hydrolysis with Pectinex Ultra SP-L

Several authors have studied chitosan degradation due to the biological activity found on its oligomers (Majeti and Kumar 2000). At present, the enzymatic hydrolysis is preferred regarding to chemical to prepare chito-oligomers since it has the advantage of being highly specific and also because of the unusual susceptibility to different enzymes observed in chitosan (Pantaleone et al. 1979). Several enzymes with pectinolytic activity have been recently used to hydrolyse chitosan (Shin-Yaa et al. 2001; Kittur et al. 2003; Cabrera and Vat Cutsem 2005). We decided to take advantage of either, the availability and price of a commercial preparation, which contains such enzymatic activity, and the possibility of preparing hydrolysates enriched in chitosan fragments with DP higher than six, taking into account recent results with this enzymatic preparation (Cabrera and Van Cutsem 2005). The hydrolysis behaviour showed in Fig. 1 suggested that chitosan was degraded with Pectinex in an endo manner. Thus, it was possible to obtain chitosan with different DP and biological activity in a cheap way using this pectinolytic preparation. Since chitinase and chitosanase are currently unavailable in bulk quantities for commercial exploitation, the used of Pectinex represent an inexpensive way for chitosan modification with agricultural purposes.

Antifungal activity

To compare the growth inhibitory activity of chitosan polymer *CH-63* and its hydrolysates, an experiment using a *P. parasitica* strain (227) isolated from tobacco was carried out and results are shown in Fig. 2. It was observed that hydrolysates maintained and showed higher antipathogenic properties than the native chitosan. There were significant differences among treatments, mostly at 250 mg/l. An increase in the *Phytophthora* growth inhibition at lower concentration was observed as the hydrolysis of chitosan progressed. When products were compared at a concentration of 1000 mg/l it was found that full control was reached only by *HCH-24* treatment. This suggests that hydrolysis of *CH-63* for 24 h is a suitable mean to generate new fragments with effective antipathogenic activity.

Regarding to the influence of the polymer size in the control of *P. parasitica* in vitro, Kendra and Hadwiger (1984), working with *Fusarium solani*, demonstrated that the antifungal activity was produced from DP ≥ 7. According to recent studies (Cabrera and Van Cutsem 2005) and from our results, it is thus tempting to speculate that, at 24 h of hydrolysis, chitosan fragments with high DP are still present and they improve the control *P. parasitica* growth regarding to the chitosan polymer from origin.

The effectiveness of chitosans *CH-99* and *CH-63*, at different concentrations, to inhibit the growth of three

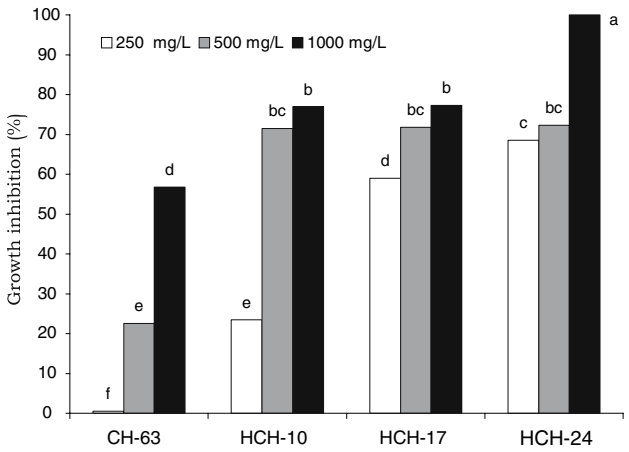

Fig. 2 Antipathogenic activity against *Phytophthora parasitica* (strain 227) of chitosan (*CH-63*) and its hydrolysates of 10 (*HCH-10*), 17 (*HCH-17*) and 24 h (*HCH-24*) of hydrolysis at different concentrations. Data was expressed as the percentage of radial growth inhibition relative to the control and analysed through a bi-factorial ANOVA using the Statistical programme SPSS 11.0 for Windows©. Means with same letters did not differ for $p < 0.01$ in the test of Tukey

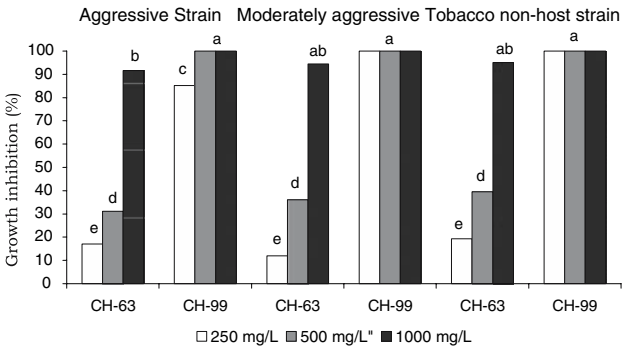

Fig. 3 Antipathogenic activity against three strains of *Phytophthora parasitica* of two chitosans (*CH-99, CH-63*) with different DA. Data was expressed as the percentage of radial growth inhibition relative to the control and analysed through a Tri-factorial ANOVA using the Statistical programme SPSS 11.0 for Windows©. Means with same letters did not differ for $p < 0.01$ in the test of Tukey

strains of *P. parasitica* were tested (Fig. 3). Both products effected antipathogenic activity against all strains of *P. parasitica* tested, however, no relationship between the level of strain aggressiveness to tobacco and the antipathogenic activity of the products was observed. The highly deacetylated polymer (*CH-99)* showed full control over all strains, except in the aggressive strain at 250 mg/l, which was inhibited in 85%. The behaviour of less deacetylated polymer (*CH-63)* was almost the same for all strains tested. The antipathogenic activity of *CH-63* at concentrations of 250 mg/l was near 20%, at 500 mg/l reached less than 50% of growth inhibition and at the highest concentration inhibited more than 90%. These results show the

susceptibility of *P. parasitica* strains to highly desacetylated chitosan polymers at the concentration tested.

When comparing two chitosans of same origin and molecular weight (*CH-63, CH-88*) but, with different DA (36.5% and 12%, respectively), it was found statistical difference at the lowest concentration tested (Fig. 4). The most deacetylated chitosan polymer (*CH-88*) showed twice antipathogenic activity than the three times more acetylated one (*CH-63*), this difference was not statistically important at the highest concentration tested. However, in all cases the hydrolysate of 24 h (*HCH-24*) was the best inhibiting the mycelial growth of *Ppn*.

In this work, we confirmed for *Ppn* that the antifungal activity is inversely correlated with chitosan DA, as previously described for other fungi (El Gaouth et al.1992; Park et al. 2002). The pathogen growth inhibition was higher for the most deacetylated chitosans tested (*CH-99, CH-88*). These chitosans possesses a higher polycationic character, in acid medium, because of a lower number of acetyl groups on its structure. It seems that, chitosan antifungal activity is associated with the presence of free amine groups on its structure, which could form polyelectrolyte complexes with negatively charge carboxyl groups present in the fungi or microbacteria cell walls (Allan and Hadwiger 1979). It is likely that more than one mechanism is involved on its activity because several damages occurring in fungus cells when treated with chitosan. These include the thickness and weakness of the pathogen cell wall, cellular leakage of aminoacids and proteins (El Gaouth et al.1992a; Laflamme et al.1999) and the accumulation of chitosan fragments inside the cell that could hinder the replication of the nuclear material (Hadwiger et al.1986). This last mechanism can justify the increment of antipathogenic activity found when degrading the chitosan polymer.

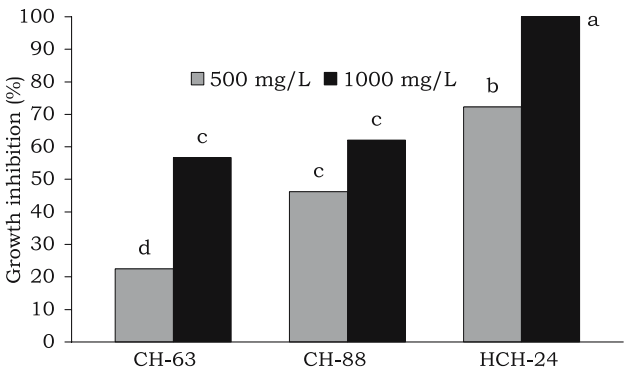

Fig. 4 Antipathogenic activity against *Phytophthora parasitica* (strain 227) of chitosan polymers (*CH-63, CH-88*) and the hydrolysate of 24 h (*HCH-24*) from *CH-63*. Data was expressed as the percentage of radial growth inhibition relative to the control and analysed through a bi-factorial ANOVA using the Statistical programme SPSS 11.0 for Windows©. Means with same letters did not differ for $p < 0.01$ in the test of Tukey

Tobacco plant protection against fungal pathogen

To compare the ability to induce protection in tobacco seedlings against the soilborne pathogen *P. parasitica*, in vivo bioassays were performed in 35 and 32 days old tobacco plants. It was observed a tobacco plant protection near 55% with chitosan polymer (*CH-63*) and 59% with its hydrolysate of 24 hours (Fig. 5A). However, treatments did not show statistical differences among them but, indeed there were significant differences with the control used in

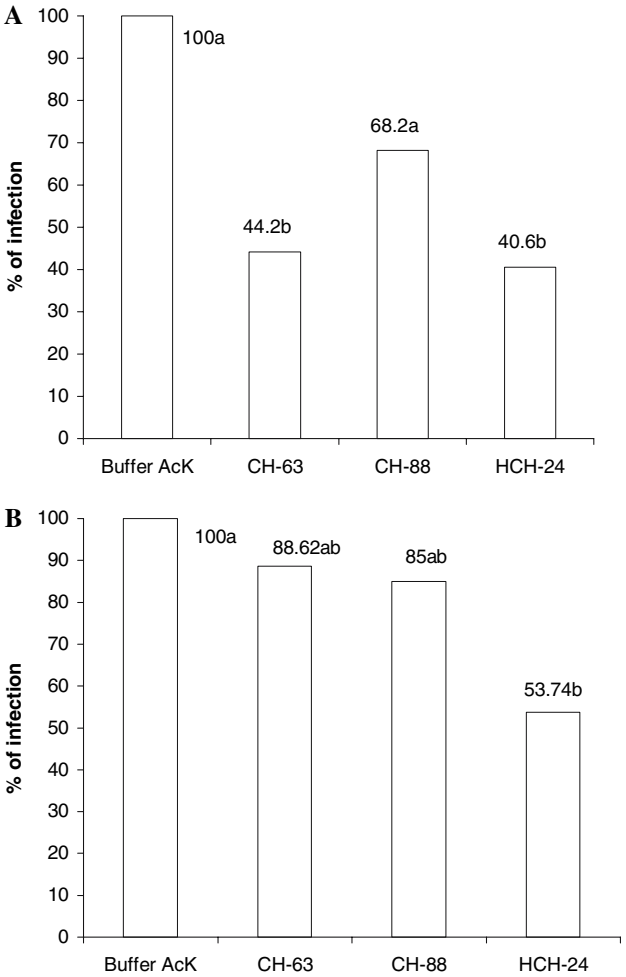

Fig. 5 Tobacco plantlets protection against *Phytophthora parasitica* (strain 227) by foliar spraying (**A**) and seed coating (**B**) treatments. In the foliar spraying experiment, tobacco plants 35 days old were sprayed with 500 mg/l of treatments (*CH-63, CH-88, HCH-24*) and control (Potassium acetate solution) and five days later, plants were placed in contact with the pathogen for five days, following a methodology described in the materials and methods. In the seed coating experiment, tobacco plants, 32 days after having treated the seeds, were placed in contact with the pathogen following the same methodology. Data were processed according to the Kruskal-Wallis non parametric test and all means compared in an independent way through the Mann-Whitney test for $p < 0.05$, by using the statistical programme SPSS 11.0 for Windows©. Results were shown as percentage of plant infection compared to controls (100 % infection)

this experiment (potassium acetate solution). Results not shown demonstrated not differences between water and potassium acetate solution used as control. Conversely, the less acetylated chitosan polymer (*CH-88*) activated resistance against the pathogen, but with not statistical difference to control. Taking into account the bioassay performed, in which plants are sprayed in their aerial parts or seeds are elicited before planting, and the infection is carried out later on through the plant roots, we can conclude that the protection observed is due to the induction of systemic resistance in tobacco plants for the chitosan derivatives and not as result of direct antipathogenic activity of derivatives on pathogen growth.

A second protection experiment was performed to test if chitosan derivatives can induce time resistance in tobacco seedlings against *P. parasitica* by means of previous treatment to seeds. In Fig. 5B it is shown that it was possible to protect 32 days old tobacco seedlings by coating seeds with *HCH-24*, since about 46% of plant protection was observed regarding to the control. Treatment with both chitosan polymers showed a lower protection, although with not statistical differences to *HCH-24* and control.

The results of systemic resistance induction in tobacco seedlings, both by means of foliar spray and seed coating, demonstrate the role of chitosan as protective agent against *P. parasitica*. As far as we know, this is the first report of tobacco seedlings protected against *P. parasitica* by inducing systemic resistance with polymer and low molecular weight chitosan. As a matter of fact, reports concerning crop protection against pathogens with chitosan, where the protection is only the result of the induction of local or systemic resistance in the plant, are few or very recent (Benhamou et al.1994; Molloy et al.2004; Sharathchandra et al.2004). Most of them, reported the antifungal activity of chitosan as the main responsible of plant protection (Bautista-Baños et al.2003) or the resistance induction in the plant as not enough to protect it against pathogens (Ben-Shalom et al.2003).

However, the resulting resistance in our experiments was uncompleted in both experiments. A similar behaviour has been observed in almost all experiments under greenhouse or growth chamber conditions where highly susceptible cultivars, or high inoculum loads were used or in situations favouring the development of pathogens. In this report, the host-pathogen interaction assays were conducted under above conditions because the tobacco variety used was susceptible to *P. parasitica* and also due to the conditions of plant-pathogen contact. This allows us to assume better results in less controlled experiments or on field trials.

In both assays the best protection results were obtained with the partially hydrolyzed chitosan. It means that, plant signal perception is improved when using lower molecular size of chitosan, probably due to steric impediments of high molecular weight chitosans to penetrate plant covers. In seed coating experiments, chitosan hydrolysate produced near 50% of protection in seedlings. It is interesting to note that at 32 days of growth, half of tobacco plants were protected even using high inoculums load of the pathogen. This reinforces the potential of low molecular weight chitosan to become a useful agent for controlling *P. parasitica* disease in nursery stage, by using it as seed coating of tobacco plants.

From our results, it is possible to conclude that, highly deacetylated chitosan polymers induce less resistance than partially acetylated ones. The DA (36.5%) of *CH-63* was in the range reported by several authors with high potential in the induction of defensive markers in several plant families such as the cucurbitaceous, solanaceous, leguminous and poaceous (Kauss et al. 1989; Kauss et al., 1997; Vander et al., 1998).

Eliciting of defensive responses by chitosan derivatives

Tobacco plants sprayed with polymeric and partially hydrolysed chitosan (500 mg/l) elicited defensive enzymatic activities and higher concentrations of total soluble proteins (data not shown) in comparison with their controls after five days of treatments (Fig. 6). All chitosan derivatives improved enzymatic activities several times above the control levels for both enzymes tested. Although for PAL activity there were not statistical differences among derivatives, for glucanase activity the chemical characteristics of derivatives influence the level of activation. The highest activity was induced by the degraded chitosan (*HCH-24*) and the lowest by the less acetylated polymer (*CH-88*).

Among plant defence responses the increase in glucanase and PAL activity are often studied as defensive markers. Glucanases are PR-proteins that can degrade fungal cell walls and inhibit fungal growth in a synergistic way with chitinase enzymes (Mauch et al. 1988). Consequently, glucanases could be considered important tobacco defences against *Ppn* whose cell wall is made up of cellulose and glucan (Wessels et al. 1981). On the other hand, PAL enzyme is a branchpoint in metabolic pathways leading to production of phenolic structures as lignins and phytoalexins in some species, which are important antipathogenic compounds (Dixon et al.2002). It is also involved in the synthesis, through benzoic acid, of salicylic acid, that has been considered an important signal in the amplification of the systemic plant defensive response (Ribnicky et al. 1998; Dixon et al. 2002).

In the foliar spray assay, partially acetylated chitosan and its hydrolysate induce near 60% of protection in tobacco seedlings against *P. parasitica*. Interestingly, results

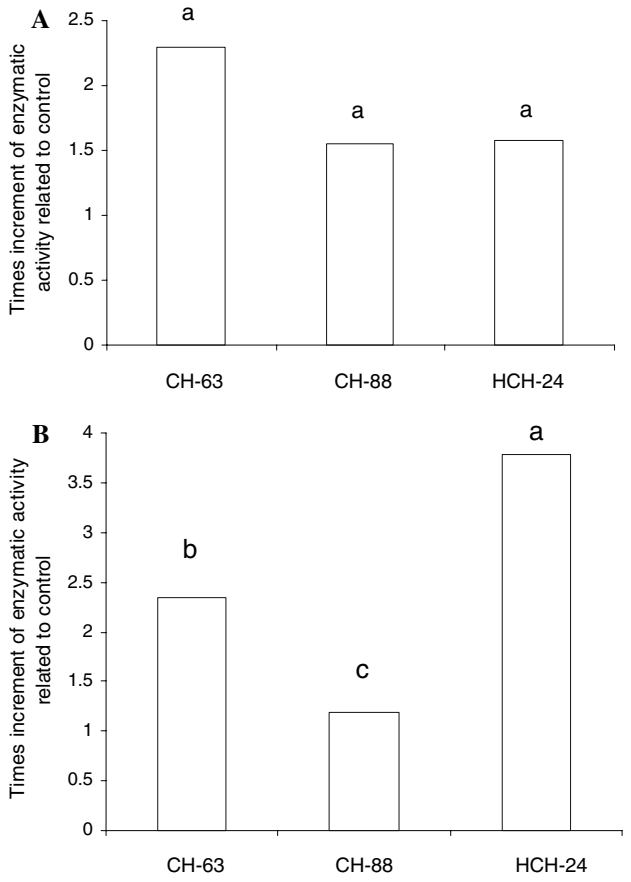

Fig. 6 Enzymatic activities PAL (**A**) and Glucanase (**B**) induced in tobacco leaves sprayed with chitosan derivatives. Enzymatic determinations were performed five days after treatments. Means with same letters does not differ for $p < 0.05$ in the test of Tukey and results were expressed as times increment of enzymatic activity related to the control for each derivative

of glucanase activity were similar than those of protection, supporting the idea that stated glucanase as an important defence against *Ppn* in tobacco. Additionally, protective and enzymatic results allow concluding that the molecular weight and the DA of chitosan derivatives are critical for activation of induced resistance in tobacco plants. Vander et al. (1998) demonstrated that, polymeric chitosan with different DP (540–1100) and DA (1–60%) were active as PAL elicitor in wheat leaves, being chitosan with DA 35% the most powerful inducer. This finding agrees with our results because *CH-63* has a DP (794) and DA (36.5%) in the same range as they used. Although the exact mechanism by which these products operate are not fully understood, the enzymatic results presented here support the view that the beneficial effect of chitosan and its derivative in reducing disease incidence is associated with an enhance of plant resistance to infection. Klosterman et al. (2001) also observed disease resistance induced by chitosan by foliar spraying to potato plants prior inoculation with *Phytophtora infestans*. However, the level of protection was inadequate to curtail potato late blight epidemics. Other authors reported that the application of chitosan and its oligosaccharides, as foliar sprays or seed treatment, to tomato plants resulted in enhanced seedling protection against *Fusarium oxysporum radicis- lycopersici* attack (Benhamou et al. 1994). This protection induced was found to correlate with the rapid expression of a number of defence responses, including the accumulation of phenolic compounds and formation of structural barriers at sites of attempted fungal penetration.

Conclusions

We have shown, by first time that the application of soluble chitosan and derivatives to tobacco plants as foliar sprays can induce systemic resistance against the attack of the pathogen *Ppn*. Besides, the use of chitosan hydrolysate as seed coating also provides disease resistance in tobacco seedlings. Induced resistance against *Ppn*, as well as, the direct inhibition of this pathogen growth in vitro was affected by DP and DA of chitosan derivatives. Both results evidence that chitosan and its low molecular weight derivatives could be used in field trials as protective agents against the tobacco disease known as Black shank.

The suitable DP could be reached by enzymatic hydrolysis of chitosan using a commercial pectinolytic preparation. In view of our results it is recommended to deep in the studies of tobacco protection using polymers and mixtures of well-known size chito-oligomers. These will contribute to understand the resistance reaction in tobacco plants elicited by partially acetylated chitosan and its oligomers.

Finally, it is clear that chitosan, with different chemical features regarding to its DA and polymerisation, is another tool in the integrated pest management.

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