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CHITOSAN AND ITS HYDROLYSATE AT TOBACCO-*Phytophthora parasitica* INTERACTION

A. B. Falcón✉, M. A. Ramírez, Ramona Márquez and Marta Hernández

ABSTRACT. Chitosan is the non-acetylated derivative from chitin, a polymer of N-acetyl-glucosamine that is extracted from crustacean exoskeleton. Both polymers as well as their oligomers protect several species of plants against fungal diseases by means of the induction of histological and biochemical defensive mechanisms that stop pathogen advance in the plant. In our work a chitosan preparation was carried out by means of basic desacetylation of chitin of pharmaceutical quality. Afterwards, chitosan was exhaustively hydrolyzed with a commercial enzymatic preparation (celluclast) to obtain small-sized oligomers. Chitosan polymer and its hydrolysate were studied regarding their potentialities to induce systemic resistance in tobacco plants by means of assays under controlled conditions where the capacity of both elicitors was determined for the induction of resistance markers (chitinase, glucanase and PAL activities) and in the hydrolysate case, its capacity to induce tobacco plantlet protection against the invasion of pathogen *Phytophthora parasitica* var. *nicotianae* was also tested. Results demonstrated an induction of chitinase and glucanase activities by chitosan at the concentrations of 50 and 500 mg.L^{-1}, much higher in the case of the topmost concentration of the elicitor. Tobacco plantlet protection against Ppn was also observed when plants were treated through the roots with the enzymatic hydrolysate at the concentrations between 5 and 500 mg.L^{-1} with an induction of differentiated PAL and β 1-3 glucanase response during plant exposition to the pathogen.

Key words: chitosan, elicitors, *Phytophthora parasitica*, PR-proteins, fungal disease, induced resistance, enzymatic hydrolysis

RESUMEN. La quitosana es el derivado desacetilado de la quitina, un polímero de N-acetil-glucosamina que se extrae del exoesqueleto de los crustáceos. Ambos polímeros así como sus oligómeros protegen varias especies de plantas contra enfermedades fungosas, mediante la inducción de mecanismos defensivos histológicos y bioquímicos que detienen el avance del patógeno en el vegetal. En nuestro trabajo se realizó la preparación de quitosana mediante desacetilación básica de quitina de calidad farmacéutica. La quitosana, a su vez, fue hidrolizada exhaustivamente con un preparado enzimático comercial (celluclast) para obtener oligómeros de pequeño tamaño. Tanto al polímero como a su hidrolizado enzimático se les estudiaron sus potencialidades para inducir resistencia sistémica en plantas de tabaco mediante bioensayos en condiciones controladas, donde se determinó la capacidad de ambos elicitores para la inducción de marcadores de resistencia (actividad quitinasa, glucanasa y PAL) y en el caso del hidrolizado, la protección del tabaco contra la invasión del patógeno *Phytophthora parasitica* var. *nicotianae*. Los resultados demostraron una inducción de actividad quitinasa y glucanasa por quitosana a concentraciones de 50 y 500 mg.L^{-1} mucho más elevada en el caso de la más alta concentración del elicitor. Se observó además protección contra Ppn cuando se trataron plantas de tabaco vía raíz con el hidrolizado enzimático a concentraciones entre 5 y 500 mg.L^{-1} con una inducción de respuesta PAL y β 1-3 glucanasa diferenciada en el tiempo de exposición de las plantas al patógeno.

Palabras clave: quitosana, elicitores, *Phytophthora parasitica*, PR-proteínas, enfermedades fungosas, resistencia inducida, hidrólisis enzimática

INTRODUCTION

Chitosan, a β (1-4)-glucosamine polymer, is an important structural component of the cell wall of some plant-pathogenic fungi, especially Zygomycets (1). It is produced from the chitin components of either fungal walls or arthropod exoskeletons by fragmentation and deacetylation.

Chitosan was shown to be fungicidal against a wide range of fungi (2, 3). According to histochemical results in some pathogens it was demonstrated that the antifungal activity of chitosan is caused by the deposition of this molecule within the fungal cell preventing pathogen

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Abbreviations: H_2O d: distilled water; DP: Polymerization degree; DA: Acetylation degree; Ppn: *Phytophthora parasitica* var. *nicotianae*; PAL: Phenyl-alanine-ammonialyase

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growth (4). Additionally, the maximal antifungal activity of chitosan derivatives was exhibited by chitosan oligomers of seven or more units.

On the other hand, chitosan and its derivatives have been claimed to elicit an immune response in plants by inducing low molecular weight compounds (phytoalexins), histological barriers, enzymes and resistance proteins through interactions with receptors in plant cell membranes (4, 5).

In plant tissues, extracellular chitinases and chitosanases in concert with β 1-3 glucanases are likely to partially degrade fungal cell wall polysaccharides (6). At least the elicitation of some resistance markers and consequently the plant protection effectiveness depend on the degree of polymerization and acetylation of the chitosan and its hydrolysates (7, 8).

In this work, a preparation of chitosan from chitin of Cuban lobster was made, characterizing chemically and biologically this chitosan and its enzymatic hydrolysate regarding their potentialities to elicit resistance markers, and plant protection against a pathogen of tobacco in a bioassay modified for our purposes.

MATERIALS AND METHODS

Chitosan preparation. Chitosan preparation from lobster chitin of pharmaceutical quality was carried out following a methodology (9), washing with distilled water and centrifuging until remaining salts were discarded. This last step purifies the resulting chitosan.

Acetylation and polymerization degrees of the chitosan obtained were determined by potentiometry and viscosimetry techniques respectively following protocols described in the lab (10).

Chitosan hydrolyzed preparation. A chitosan hydrolyzed was prepared by performing an enzymatic hydrolysis with celluclast, an enzymatic complex from novozymes rich in cellulolytic activities. A 20 $g.L^{-1}$ of chitosan in sodium acetate solution pH 5 was allowed to be hydrolyzed with celluclast at a rate of 1/500 v/v for 24 hours and after the enzymatic complex was added again to a final rate of 1/300 v/v during 24 additional hours.

Elicitation of defense markers by chitosan in tobacco. Plants were cultivated under semi-controlled conditions with a light/dark regime of 16/8 hours and temperature 28°C/23°C respectively for all bioassays. Tobacco plants were grown during approximately 25 days before being placed in solutions containing the different treatments (50 and 500 $mg.L^{-1}$) dissolved in potassium acetate buffer with less than 50 mM of ionic force. Solutions with pH 5.7-6.0 were applied through plant roots.

Plants were in treatment solutions or their controls for at least 24 hours. Afterwards, plants were placed in eppendorf containing distilled water for additional 24 hours, then the true leaves of each treatment were harvested and extracted with sodium acetate buffer 0.05 M + NaCl 0.2 M in presence of liquid nitrogen. The extract was centrifuged at 10000g for 15 minutes and the supernatant was used for the enzymatic determinations of glucanase and chitinase activities according to a methodology (11).

Bioassay of tobacco plant protection. To test induction of tobacco plantlet protection against *Phytophthora parasitica* var. *nicotianae* (Ppn) by a differential concentration of chitosan hydrolysate, the following bioassay (12), lightly modified for our purpose, was performed. When the second pair of leaves was growing, 30-day-old tobacco plants were placed in contact with treatment solutions and controls (in eppendorf tubes) through the roots for 24 hours. Afterwards, they were placed in a spore suspension (10^3 $spores.mL^{-1}$) of Ppn, except a control containing water during seven days. After this period the infection degree was determined in each plant using the scale described and modified (12). 10 plants per treatment were tested and three times replicated. Results were processed according to Kruskal-Wallis' non parametric test and the means compared using Duncan´s test at the signification level of 1 %.

Treatments:

1. H_2O d + fungal spores
2. Elicitor (500 $mg.L^{-1}$) + fungal spores
3. Elicitor (100 $mg.L^{-1}$) + fungal spores
4. Elicitor (50 $mg.L^{-1}$) + fungal spores
5. Elicitor (5 $mg.L^{-1}$) + fungal spores
6. Elicitor (500 $mg.L^{-1}$)
7. H_2O d

Table I. Scale of disease evaluation

Degree	Description
0	Healthy plant
1	Roots affected
2	Hypocotyls and cotyledons affected
3	First leaf pair affected
4	Second leaf pair affected
5	Dead plant

In order to know how some resistance markers behave during plant infection, a bioassay similar to that done for plant protection was performed, but leaf extraction for enzymatic determinations was done the second day (48 hours after elicitation and 24 hours after placing the plant in the spore solution) and the seventh day of the experiment. In this experiment glucanase and PAL (phenyl-alanine-ammonialyase) activities were determined following the method described for PAL enzymatic activity (13).

RESULTS AND DISCUSSION

Starting from the determinations with viscosimetry and potentiometry methods, the chitosan resulting from our preparation had 720 and 36.5 % of DP and DA respectively. A summary of the procedure to perform the chitosan and hydrolysate preparation is shown in Figure 1.

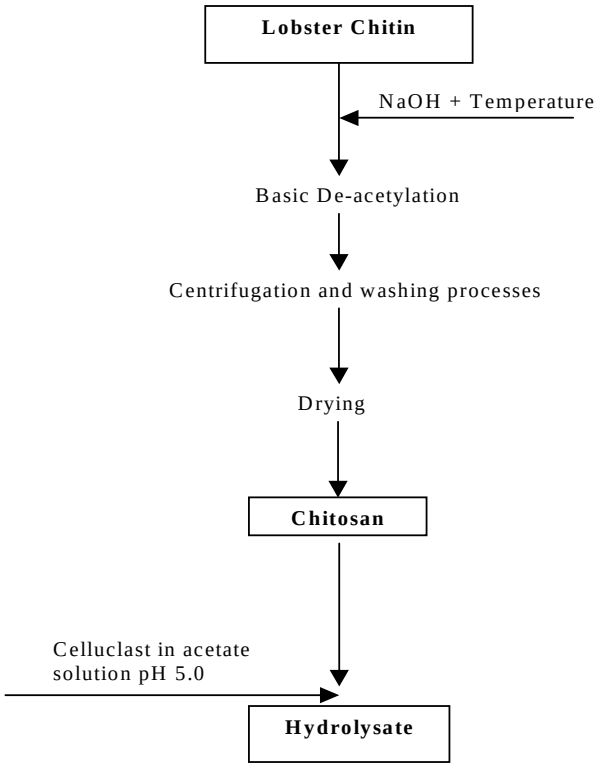

Figure 1. Experimental procedure to obtain the elicitors. Chitosan preparation was performed (2) by starting from lobster chitin. Hydrolysate was prepared from the chitosan obtained (DA=36 %) using the enzymatic complex called celluclast

According to the results of several authors (7, 8), chitosans with acetylating degrees between 20 and 60 % have the biggest potentialities of induction of defensive markers in several plant families such as cucurbitaceous, solanaceous and leguminous. Taking into account both obtained, a chitosan polymer of 36.5 % of acetylation as well as the yield in the process for quantity of departure chitin (data not shown), the methodology used is considered suitable for our purposes.

Beginning with the results presented in Figure 2, the chitosan obtained at the concentrations between 50 and 500 mg.L^{-1} elicits defensive markers reported as very important in the control of fungal pathogen attack (4, 14). It demonstrates chitosan potentiality as a resistance elicitor in plants. Nevertheless, starting from the results obtained and according to the bioassay carried out, it is advisable to optimize the biological assay concerning the timing to apply the treatment, the timing of plant exposition to the treatment and the suitable moment to do extraction and marker determinations, something that will positively redound on plant protection in the experiments with pathogens.

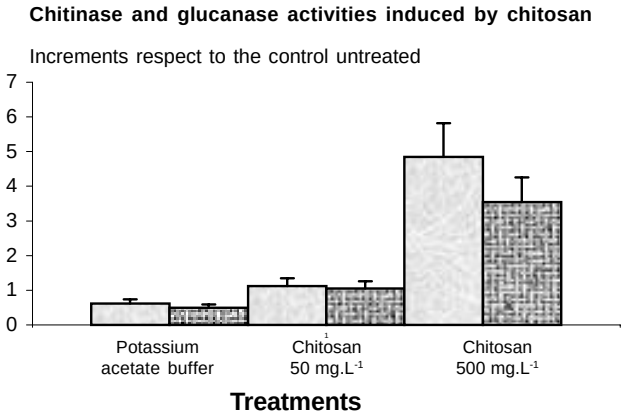

Figure 2. 30-day-old tobacco plants were placed in contact with treatment solutions and acetate buffer (in eppendorf tubes) through the roots for 24 hours. After that they were placed in distilled water for 48 hours before being extracted with sodium acetate buffer 0.05 M + NaCl 0.2 M in presence of liquid nitrogen. Chitinase (diagonal lines) and glucanase (vertical lines) activities were performed according to methodologies (11)

According to the results of Table II and Figure 3, chitosan hydrolysate induces protection against the invasion of the pathogen *Phytophthora parasitica* var. *nicotianae* at concentrations between 5 and 500 mg.L^{-1}, the best protection being at 50 mg.L^{-1}. The increment of the concentration starting from 50 mg.L^{-1} probably damages the physiological state of the plant under the treatment conditions followed (immersion via root for at least 24 hours in elicitors).

Table II. Protection of tobacco plants by different concentrations of chitosan hydrolysate. Data were processed according to the nonparametric test of Kruskal-Wallis and the means compared using Duncan´s test at the signification level of 1 %.

Treatments	Scale run	Range
H_2O d + Ppn spores	0-5	536 a
Elicitor (500 mg.L^{-1}) + Ppn spores	0-5	492 a
Elicitor (100 mg.L^{-1}) + Ppn spores	0-5	385 b
Elicitor (5 mg.L^{-1}) + Ppn spores	0-5	373 b
Elicitor (50 mg.L^{-1}) + Ppn spores	0-3	329 b
Elicitor (500 mg.L^{-1})	0-0	185 c
H_2O d	0-0	185 c
H: 31.3158***		

Below 50 mg.L^{-1}, it is probable that the concentration of the active compound is not sufficient to protect plants totally and this is directly linked to the conditions of exhaustive hydrolysis to which the chitosan was subjected with celluclast. This hydrolysate contains oligomers fundamentally with an average of 2-7 remains of glucose,

that is to say, from dimer to heptamer, according to a determination performed by means of precipitation and separation in thin layer chromatography of the hydrolysate (data non shown). Some authors state that the size of chitosan oligomers is fundamental for the resistance induction and defensive markers in the systems tested (7, 15). According to these references, the inducing activity of resistance markers and protection occurred from tetramer and pentamer oligomers to higher, the monomer and dimer being totally inactive. Taking into account our results and those from literature, it can be concluded that hydrolysate is not too rich in the activate oligomers, probably because of the exhaustive hydrolysis with celluclast made; consequently, it is suggested to follow lighter conditions in next chitosan hydrolysis in order to obtain higher oligomers. For instance, less time of hydrolysis or a lower concentration of the enzymatic complex will be recommended if taking into account that celluclast is a very rich cellulolytic complex as stated by the manufacturers and this is an important hydrolytic activity for chitosan.

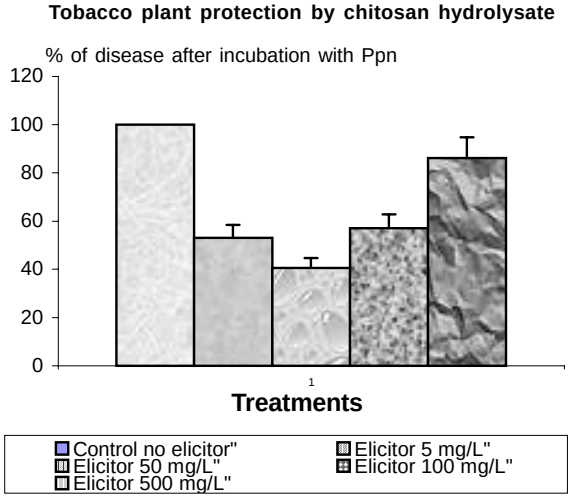

Figure 3. Bioassays of protection against Ppn were performed following a procedure reported and modified (12). 30-day-old plants were placed in contact with treatment solutions and controls (in eppendorf tubes) through the roots for one day. After that they were placed in a spore suspension (10^3 spores.mL^{-1}) of Ppn, except a control containing water during seven days. Determination of the infection degree using the scale described (12) and modified, was performed in each plant after the incubation time and 10 plants were used per treatment

Regarding the resistance markers tested during infection, the behavior of PAL and β 1-3 glucanase activities in tobacco plants treated before with the hydrolysate and next challenged with the pathogen are shown in Figures 4 and 5. In both cases, the values of enzymatic activity at 48 hours since elicitor treatment and 24 hours after the plants challenged with the pathogen are below the control. These results are in agreement with a conclusion drawn few years ago for the activity of β 1-3 glucanase and chitinase (16). These authors speculated that resistance may need a minimum activity of defense enzymes at the time of infection, but higher levels developed later are ineffective for protection. Indeed, they found higher increments of chitinase and glucanase activity in pathogen inoculated and non-induced cucumber leaves than in the case of pathogen inoculated and previously induced leaves even 10 days after the treatment with elicitors. However, in this case, seven days after the challenge the situation is reversed and PAL and glucanase activities are in higher levels than their controls.

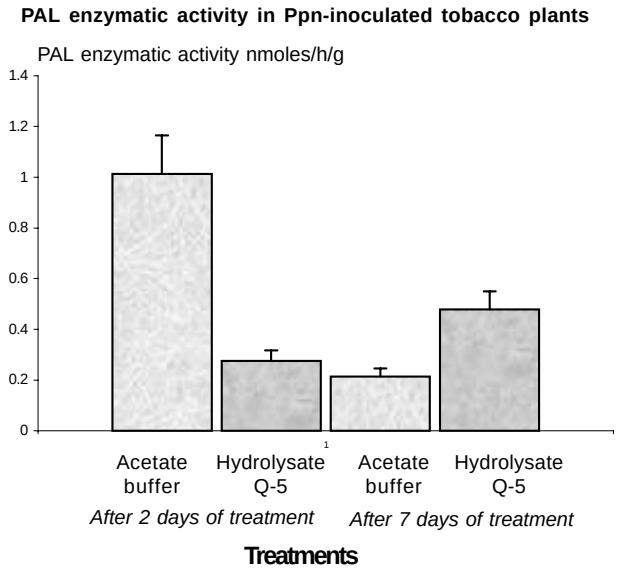

Figure 4. Enzymatic activity of Phenyl-alanine-ammonialyase in tobacco leaves placed through the roots in sterile water containing mycelium of Ppn in agar. Elicitor treatment (chitosan hydrolyzed 500 mg.L^{-1}) was applied 24 hours before the contact with the pathogen. Determination of PAL enzymatic activity was performed two and seven days after applying the elicitor treatment using L- Phenyl alanine as a substrate

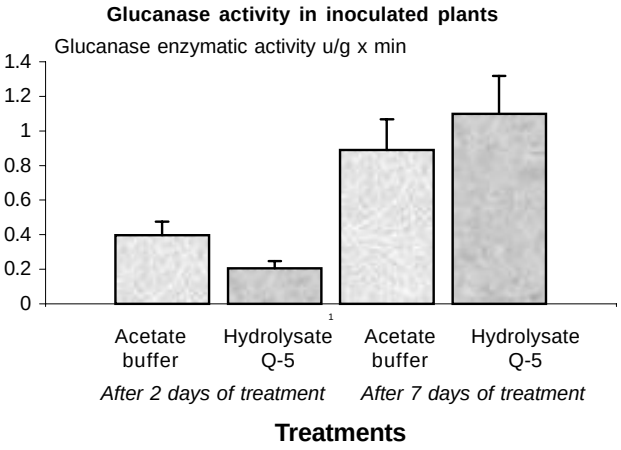

Figure 5. Enzymatic activity of β 1-3 glucanase in tobacco leaves placed through the roots in sterile water containing mycelium of Ppn in agar. Elicitor treatment (chitosan hydrolyzed 500 mg.L^{-1}) was applied 24 hours before the contact with the pathogen. Determination of enzymatic activities were performed two and seven days after applying the elicitor treatment using laminarin as a substrate

So far, this is the first time this behavior is reported in PAL activity. It is considered that the different activities achieved in both markers at the end of the experiment could contribute to the difference in resistance between elicited plant and plant controls without any elicitor treatment, despite these two defense markers are not by themselves the only responsible of protection. Plant resistance against pathogens is the result of a wide range and coordinated process of pre-formed, locally and systemically induced, physical and chemical defenses (4, 17). Moreover, it should be also taken into account that any kind of plant-microorganism interaction, compatible or incompatible (including symbiotic), have reported induction of enzymatic activities and PR-proteins (17).

Both PAL and β 1-3 glucanase are important defense enzymes; the first one is a branchpoint in metabolic pathways leading to production of phenolic structures and phytoalexins in some species, which are important antipathogenic compounds (18). PAL 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 (19). Glucanase is an important PR-protein that can degrade fungal cell walls (6) and it can be *de novo* induced in plant-pathogen interactions or as a result of treatments with elicitors in a coordinate expression with some other PR-proteins (20, 21, 22).

Taking as a whole all results, further studies are recommended following bioassays where a longer periods should be established between treatments with elicitors and challenge with pathogen or protein extractions for marker determinations. Since resistance induction on plants works as a multifactor response against avirulent microorganism, predators and elicitors, where structural and biochemical defenses are combined, preformed and induced, there is a lag phase between stimulus and response where *a novo* synthesis of many proteins takes place, they being enzymes (as for instance PAL) or PR-proteins (17, 23).

According to results from literature, at least 72 hours as lag phase are needed to get the peaks of some PR-proteins when working in plants treated with elicitors (20) while some responses are extremely fast as, for example, hydrogen peroxide which is involved in the oxidative burst that precedes synthesis of PR-proteins, phytoalexins, etc (24). Interestingly, chitosan oligosaccharide induces β 1-3 glucanase peaking after several days even when working with suspension cells (15).

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