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EVALUATION OF INDICATORS CHARACTERIZING THE PROTECTIVE ACTION OF CHITOSAN IN TOBACCO (*Nicotiana tabacum* L.) vs *Phytophthora nicotianae* BREDA DE HAAN

Evaluación de indicadores que caracterizan la acción protectora del quitosano en *Nicotiana tabacum* L. vs *Phytophthora nicotianae* Breda de Haan

Dianevys González-Peña Fundora✉, Daimy Costales Menéndez and Alejandro Falcón Rodríguez

ABSTRACT. Chitosan is a glucosamine polymer with significant properties as antimicrobial compound and elicitor of defense response on plants, which emerges as an ecological alternative to pesticides uses. The aim of the research was to evaluate the effect of this polymer on the growth and sporulation of an isolate of *Phytophthora nicotianae* Breda de Haan; as well as in the glucanase activity on tobacco (*Nicotiana tabacum* L.) plants infected with the pathogen. For this purposes, a disc with *P. nicotianae* mycelium was placed in Petri dishes with PDA-V8 culture medium and different chitosan concentrations. The inhibition of the mycelium growth was determined by the chitosan dissolvent and the concentrations used. A direct relationship between the inhibitory effect and the levels of the polymer in the culture medium was observed. In addition, *P. nicotianae* sporulation decreased when added up to 3,0 g L^{-1} and there was a biostatic effect in the colony, but not biocide. In the other hand, tobacco plants of 30 days old, treated with chitosan and/or inoculated through the root with the SS-11 isolate, changed their protein concentration and glucanase activity over time. Moreover, a 26 % of plant protection was observed when 0,5 g L^{-1} of the polymer was applied to tobacco plants. The results show the effect of the chitosan as inhibitor of *P. nicotianae* development and it's potential to increase glucanase activity and crop protection against this pathogen.

Key words: glucosamine, oomycete, glucanase, tobacco, priming

RESUMEN. El quitosano es un polímero de glucosamina con importantes propiedades como compuesto antimicrobiano e inductor de respuestas defensivas en plantas, el cual emerge como una alternativa ecológica al empleo de pesticidas. El objetivo del presente trabajo fue evaluar el efecto que ejerce este polímero en el crecimiento y la esporulación de un aislado de *Phytophthora nicotianae* Breda de Haan, así como en la actividad glucanasa en plantas de tabaco (*Nicotiana tabacum* L.) infectadas con el patógeno. Para ello se inoculó un disco con micelio de *P. nicotianae* en placas Petri con medio de cultivo PDA-V8 y diferentes concentraciones de quitosano. La inhibición del crecimiento del micelio estuvo marcada por el tipo de ácido disolvente y las concentraciones empleadas. Se observó una relación directa entre el efecto inhibitorio y los niveles del polímero en el medio de cultivo. Además, disminuyó la esporulación de *P. nicotianae* al adicionar hasta 3,0 g L^{-1} y hubo un efecto biostático del crecimiento de la colonia, pero no biocida. Por otra parte, las plantas de tabaco de 30 días de sembradas, tratadas con quitosano o inoculadas con el aislado SS-11 a través de la raíz, variaron la concentración de proteínas y la actividad glucanasa en el tiempo. También se alcanzó un 26 % de protección vegetal al aplicar 0,5 g L^{-1} del polímero a plantas de tabaco. Los resultados muestran el efecto del quitosano como inhibidor del desarrollo de *P. nicotianae*, así como su potencialidad para incrementar la actividad glucanasa y la protección del cultivo ante la presencia de este patógeno.

Palabras clave: glucosamina, oomycete, glucanasa, tabaco, priming

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is severely affected at the seedbed stage by a disease known as Tobacco black shank, caused by the pathogen

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Phytophthora nicotianae Breda de Haan (1896). The disease is characterized by necrosis in the basal region of the stem that advances to all parts of the plant till killing it (1). In Cuba, the disease is controlled with integrated management practices, the selection of less sensitive tobacco varieties and the application of biological and chemical products (2). However, the incidence of this disease persists so it is necessary to find new alternatives to the use of pesticides that in most cases are contaminant to the ecosystem.

Under these circumstances chitosan emerges, a polymer of acetylated glucosamine produced in industrial quantities through deacetylation of chitin that forms part of the exoskeleton of crustacean. Its polycationic nature grants it important antimicrobial and eliciting properties of defensive responses in plants that vary depending on the acetylation, polymerization degree and the pH (3, 4, 5). In general, it is dissolved in acetic acid though other authors have reported the use of ascorbic, hydrochloric and lactic acids; they could influence the potential use of polymer (6, 7).

Several studies show that chitosan inhibits the growth and formation of vegetative structures of some pathogens of plants and animals by destabilizing cell walls and membranes and inducing cell lysis; however, it does not equally affect all organisms, and its action mechanism is not fully clarified (4, 8, 9, 10).

Some authors also suggest that this compound can induce a priming status that allows the plant responds faster to the future presence of a stress (11, 12). Likewise, a higher activity **P**athogenesis **R**elated Proteins (PRs) has been proven, whose expression decreases the sensitivity of plants to the attack of biological agents (13, 14). Among the PRs there are glucanase, quitinase and peroxidase, just to mention some of them (15).

Glucanase are essential to protect tobacco plants against some of the main diseases like the blue mold, black shank, caused by *Peronospora tabacina* and *P. nicotianae*, respectively, if it is taken into account that the enzyme degrades glucan polymers and both microorganisms have this polysacharid on the cell wall (16).

It has been proven that different chitosan byproducts induce defensive responses in tobacco plants even without being in contact with pathogens, and they vary depending on the acetylation degree, molecular weight, via and application time of the polymer (17); however, the defensive response of the plant to the inoculation with this microorganism has not been evaluated, not even in combination with seedlings elicited with chitosan.

From the above, this research has the objective of determining the chitosan effect on the growth and formation of spores of an isolate of *P. nicotianae*, and evaluating the induction of the glucanase activity and the protection ot tobacco plants inoculated with this isolate.

MATERIALS AND METHODS

Chitosan polymer

A chitosan polymer of molecular mass 1,35*10^{5} (viscosimetry) and acetylation degree 12% (spectroscopy IR), produced by the basic chitin deacetylation of Cuban lobster, supplied by the Pharmaceutical Laboratories Mario Muñoz. Three separate acids were used to compare their effect as dissolvent, they were: lactic, hydrochloric and acetic acid at 1%, while in the rest of the experiments acetic acid was only used. The pH was adjusted to KOH at 5,6 in all cases.

Microbial culture

An isolate of *Phytophthora nicotianae* (Pn), SS-11, breed 0, group 1, from the cultivar Habana 92, from the Tobacco Research Institute (IIT) was used. For culturing microorganisms the medium potato-dextrose-agar (PDA), which calcium carbonate (2,0 g L^{-1}), Asparagine (2,0 g L^{-1}), and vegetable juice (V8) at 20 % (tomato, celery, spinachs, beet, lettuce, parsley, cress and carrot), from the commercial line Del Frutal (Guatemala) were added to. The pH was adjusted at 5.6 with HCl and KOH. The culture medium was sterilized in autoclave for 15 minutes and was poured in Petri dishes of 90mm of diameter.

Dishes were inoculated with 10 mm of diameter disks containing mycelium SS-11 and were incubated at 27 °C under dark conditions.

Tobacco plants culture

Tobacco plants (*Nicotiana tabacum* L.) from seeds of the cultivar Corojo 99 of the IIT, were planted on a substrate at the rate of 1:1(v/v) of soil and acid peat. Seedlings were kept under semicontrolled conditions at 25°and under a regime of 16/8 hours light/darkness.

Thirty days old germinated tobacco plants with nearly 5cm height and a pair of true leaves already developed, were removed from the substrate, washed several times with distilled water and then placed on Eppendorf tubes of 2ml containing 1ml of distilled water, Hogland nutritive solution (1:50 v/v) or chitosan at different concentrations, depending on the experiments. Twenty plants per treatment were used.

Chitosan effect on the mycelial growth of *P. nicotianae*

Essays were done in glass Petri dishes (90mm of diameter) that contained 25ml of culture medium with different chitosan concentrations. The medium and the solutions were sterilized separately in autoclave at 120°C for 15 minutes and then mixed before being poured. Disks of 10 mm of diameter were placed at the center of the dishes that contained mycelium of SS- 11 from 7 to 9 days of growth. Dishes were incubated at 27°C in darkness. Ten replicates per treatments were made.

Effect of chitosan dissolvents on the mycelial growth of *P. nicotianae*

Chitosan was dissolved in acetic, lactic and hydrochloric acids at 1 %. Concentrations of 0, 0,5 and 1,0 g L^{-1} of chitosan corresponding to each dissolvent were used. Controls were established for each acid used and the diameter of the colony was determined when the control reached the edge of the dish. Data were processed through a bifactorial design with decomposition of the factors acid dissolvent and concentration, each of them with 3 levels.

Effect of the chitosan concentration on the development of *P. nicotianae*

Treatments consisted in different chitosan concentrations: 0, 0,25, 0,5, 1,0, 1,5, 2,0, 2,5 and 3,0 g L^{-1}, the radial growth of SS-11 was determined when colonies reached the edge of the dishes. A zoospore counting in Neubauer chamber was done for which 10 ml of distilled water per dish were added and the colony was smoothly macerated with a Drygalski spatula, without destroying the agar. Dishes were incubated at 4 °C for 2 hours followed by a thermal shock for 30 minutes at 37 °C, for the release of the zoospores.

Defensive responses in tobacco plants

Activation of defensive responses in tobacco leaves before the application of chitosan. Tobacco plant roots were exposed at concentrations of 0,05, 0,5 and 1,0 g L^{-1} of chitosan, while controls were dipped into water. After 2 hours, they were washed and placed into the vials with the nutritive solution. After 72 hours of being in contact with the solutions, the plant material was extracted to determine the protein concentration and the glucanase activity.

Dynamics of defensive responses in tobacco leaves inoculated with de respuestas defensivas en hojas de plantas de tabaco inoculadas con P. nicotianae. Tobacco plants roots were exposed for 120 hours to 10 mm diameter disks with mycelium of SS-11 that were placed on the vials with nutritive solution. Controls were wekp under equal conditions, except that they were not contact with the pathogen. The extraction of the plant material to evaluate de protein concentration and the glucanase activity was made at 24, 48, 72 and 120 hours of being in contact with the pathogen.

Defensive response in tobacco leaves inoculated with P. nicotianae and previously treated with chitosan. Tobacco plants were exposed for 2 hours at 1,0 g L^{-1} of chitosan, while controls were kept in distilled water this time. Plants were washed and except those that later on were the positive control, the rest was put into contact with the microorganism. Protein determination and the glucanase activity was made to extracts from the plant material at 48 and 96 hours of plants interaction with the pathogen.

Extraction of the plant material. The extraction of the plant material was made at the rate of 1 g of leaflet foliol/1,2 mL of extraction buffer ($Ac^{-}Na^{+}$ 0,05M pH 5,2 + NaCl 1M + EDTA 0,005 M + Mercaptoetanol 0,005 M), by macerating the first pair of true leaves on a porcelain mortar.

The extract was centrifuged for 15 minutes at 5000 rpm at 5 °C, the pellet was wasted and the supernatant was centrifuged again at 12000 rpm for 10 minutes. The supernatant was collected and kept at -10 °C till making the protein and enzymatic determinations.

Determination of proteins and enzymatic activities. The protein content was determined by the methodology of micro- Lowry, described by Sun[A], with the use of a patter curve of BSA and was expressed as mg of protein per gram of fresh plant tissue.

The β-1,3 glucanase activity was evaluated according to the methodology described by Falcón (18) and the release of reducing sugars of laminarine (Sigma) was quantified by the action of extract enzymes. The total of reducing sugars was determined by the colorimetric method of Somogyi (19) and the results were expressed as specific activity (UAE min^{-1}. mg $protein^{-1}$), defining a unit of enzymatic activity as the release of 1 microgram of glucose equivalent per minute per mg of protein. In so doing, a patter curve of glucose at 520 nm was used.

Protection of tobacco plants against P. nicotianae treated with chitosan. Tobacco plant roots were dipped into water, 05 g L^{-1} and 0,5 g L^{-1} of chitosan for 2 hours and after being washed with distilled water, were passed to vials with 1ml of nutritive solution. After 16 hours, plants entered in contact with 10mm of diameter disks of mycelium SS-11. The infection degree was evaluated after 72 hours of exposure to the pathogen, for which a modification of the infection scale used by Falcón (17) (Table I) was used.

[A] Sun, S.M. *Methods in plant molecular biology and agricultural biotechnology: A laboratory training manual*, Asian Research and Development Center, Shanhua, Tainan, Taiwan (ROC), 1994, p. 94.

Table I. Infection scale of *P. nicotianae* in tobacco plants

Degree	Description
1	Healthy plant
2	Affected roots
3	Affected hypocotils cotiledons
4	First and second affected pair of leaves
5	Dead plant

Data from the infection scale served to calculate the infection index of each treatment with the formula I.I. = [∑ (infection degree x № of observations)/N x 5] x 100, where N is the total number of plants in the treatment and 5 is the maximum degree of damage on the invasion scale. From the infection index, the protection percentage of the treatments in relation to the control was also determined.

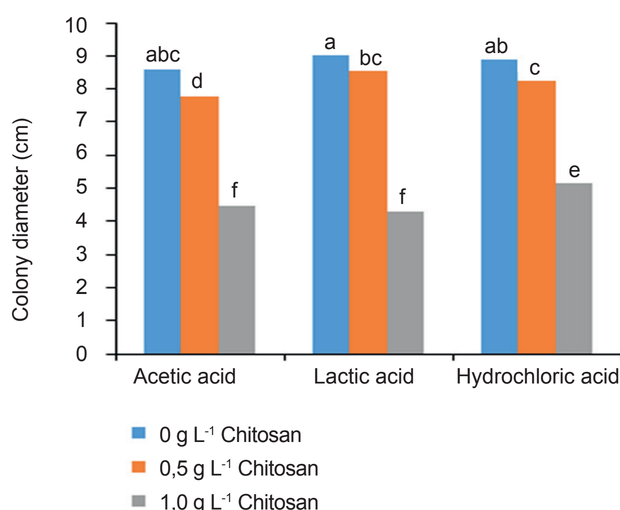
Statistical analysis

Results were statistically processed through bifactorial ANOVA to evaluate the effect of chitosan dissolvents on the growth of *P. nicotianae* colonies; and also through a simple ANOVA for the rest of the antimicrobial essays. Confidence intervals were also used in the assays with plants and the means were compared by Tukey's test for p<0.05. Data from the protection assay were processed through the non-parametric test of Kruskal Wallis, and Mann-Whitney of means comparison for p<0.05. The statistical package SPSS 11.5 version for Windows was used too (20).

RESULTS AND DISCUSSION

The growth of the SS-11 isolate of *P. nicotianae* in the culture medium was affected by the presence of chitosan. The bifactorial reflected a significant interaction between the dissolvent acid and the concentration of acetic and lactic acids (4,47 and 4,28 cm of growth, respectively) without differences among them at 1,0 g L^{-1}. By using 0,5 g L^{-1} acetic acid resulted the most effective one in stopping the development of the microorganism, a reason for which it was used as dissolvent in the rest of the essays.

Experiments showed that the influence of the concentration of chitosan and the synergistic action with the acid used as solvent is significant in the inhibition of the microorganism (Figure 1). Similar results were obtained Romannazi *et al*., (6) to buy the effect of various solvents which it was obtained that acetic allowed greater inhibition than others such as hydrochloric acid, L-ascorbic acid, formic acid, L-glutamic acid, lactic, maleic, malic, and succinic. Antifungal activity of chitosan must then be carefully compared taking into account that its potential varies depending on the acid dissolve, among other factors.



Same letters did not differ statistically in Tukey test (p≤0,05)
ESx= 0,14

Figure 1. Effect of different chitosan dissolvent on *P. nicotianae* (SS-11) growth

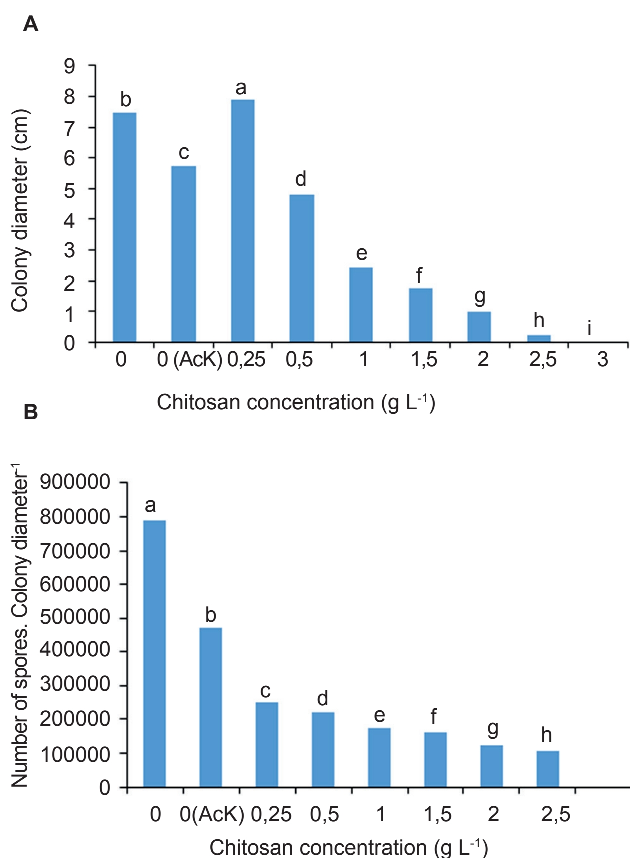
Effect of chitosan concentration on the development of P. nicotianae. During the development of the *P. nicotianae* colony in the presence of chitosan, it was observed that the first treatment in reaching the edge of the dish was the equivalent to the lowest concentration (0,5 g L^{-1}), followed by the positive control (Figure 2 A). Growth values decreased as chitosan concentrations increased. There were significant differences among all treatments and the concentration of 3,0 g L^{-1} was the one that completely inhibited the growth of SS-11; nevertheless, when non-grown disks were moved to a fresh medium without chitosan, the microorganism developed a new colony which proved the fungistatic effect of the polymer, but not its fungicide effect.

The number of zoospores by diameter of the colony gradually decreased with the increased levels of chitosan, showing significant differences among all treatments. The lowest value was reached with the highest concentration of the polymer: 2,5 g L^{-1} ($1,1*10^5$spores/mL).

The antimicrobial activity of chitosan is reported against a wide variety of microorganisms and it is stated that the minimum necessary concentration to inhibit growth varies depending on several factors like the acetylation degree, polymerization, pH of the solution and target organism, among others (3, 10, 21).

The results of this research showed that in the presence of 3,0 g L^{-1} of chitosan, the growth of the SS-11 isolate of *P. nicotianae* was significantly inhibited unlike other authors that achieved a similar reduction of

isolates of this specie with a lower concentration (22), which shows the importance of the target organisms for the evaluation of the antimicrobial properties of this polymer (3). This concentration does not exert a biocide action which was proven by passing the disk with the mycelium to the medium PDA-V8 where a new colony was formed.



Same letters did not differ statistically in Tukey test (p≤0,05)

Figure 2. Mycelium growth (A) ESx=0,064 and sporulation (B) ESx= 577,35 of *P. nicotianae* (SS-11) in different chitosan concentrations

The differences with Falcón's report (22), where 2.0 g L^{-1} of chitosan completely inhibited the growth of an isolate of *P. nicotianae*, can be due to the use of different isolates even when they were of the same species, and the use of different culture medium. The PDA medium supplemented with juice V8 used in this assay, allowed the microorganism a greater development that can induce a higher activation of enzymes for substrate degradation and the uptake of nutrients from the medium. Due to this, the growth inhibition of SS-11 could need a higher concentration of the polymer compared to other isolates from the same species.

Differences have been observed in the growth of various fungi isolated and oomycetes when placed in different culture media with chitosan (23). In that case, the growth in PDA was higher than the growth on agar-water medium and the inhibition of the latter was higher, where a less dense aereal mycelium was observed. In most of the cases, it coincided with the reduction in diameter of the colony with increased chitosan concentrations from 0,5g L^{-1} to 2g L^{-1}, particularly for *Pythium ultimum* and *Fusarium oxysporum*, though for *Pochonia rubescens* and *Beauveria bassiana* showed a mycelium increased, standing out that the target organism is a determining factor to evaluate the effect of this polymer over the microbial development.

Other authors have evaluated the growth of various microorganisms and also observed inhibition of their development, although with different concentrations of chitosan, ranging from 0,01 mg L^{-1} to 5 g L^{-1}, as shown in the subject several reviews (3 , 21, 24).

An interesting result was that with the lowest concentration (0,25 g L^{-1}) growth surpassed that of the control (Figure 2A). It could be explained because in the presence of a stress, as chitosan is, the microorganism manages its metabolism in an attempt to perpetuate the specie by accelerating growth; however, when these concentrations are high, the cell damage can be greater or the microorganism could detect these changes as a very negative environment and in turn readdresses its metabolism towards slowering down till reaching a new environment with better conditions.

Similar behavior was reported in previous research on the growth of *Fusarium dimerium* when concentrations between 500 and 2000 ppm were used chitosan and sporulation of *Alternaria alternata* (Fr.) Keissl., with doses from 5 to 15 g L^{-1} wherein increased growth over the control (25, 26) was observed.

Sporulation, evaluated through the release of zoospores to the environment, was also affected by using chitosan in the culture medium. Results showed that as chitosan concentration increased, this process substantially decreased, even in the case when the polymer favored the growth of *P. nicotianae* mycelium (Figure 2B). This is a very important fact because, zoospores in particular, are considered the most important infectious propagule of Oomycetes, since these structures can travel great distances over the water.

Sporulation inhibition of other pathogens has also been reported by other authors through the concentration ranges of chitosan are necessary to reduce spore levels which considerably vary depending on the target organism, on the concentrations and physico-chemical properties of this compound. In the case of *Alternaria alternata*,

was proved that chitosans of different molecular weights could affect sporulation and germination of an isolate of this specie (26). In that research, they used much higher concentrations than those used in this one (5-25 g L^{-1}) and observed that 25 g L^{-1} totally inhibited sporulation of *A. alternata*. Also*,* the effect of different concentrations (1- 2 g L^{-1}) and molecular weights of chitosan reducing the sporulation of *Rizopus stolonifer* (Ehrenb.:Fr.) was compared in the culture medium (27), though in these cases it depended more on the molecular weight of chitosan than on concentrations used.

In general, both colony growth and *in vitro* sporulation of the isolate SS-11 depended on chitosan concentration. It seems to be due to the polycationic nature of this polymer, since it is suggested that free amino groups should interact with the negative charges of the membrane and triggers off ionic unbalances leading to the altered pass of nutrients to the microorganism and therefore, to cell lysis, as observed in *Neurospora crassa* (8).

Activation of defensive responses in tobacco leaves with the chitosan application. The protein concentration in tobacco leaves varied according to the evaluated rates of chitosan (Figura 3 A). Chitosan treatments showed statistical significant differences of protein levels in relation to the control after 72 hours of having dipped roots in the solutions of the polymer, standing out the effect of the concentrations 0,5 and 1,0 g L^{-1}. The glucanase activity, however, reduced as compared to the control for such treatments and with 0,05 g L^{-1} the lowest levels of this enzyme were reached (Figure 3B).

The increased concentration of proteins in the presence of an elicitor like chitosano, can be due to the reorganizations of the plant functions towards the formation of compounds that allow to stop the advance of the supposed pathogen. Particularly, the glucanase enzyme does not increase this activity since this PR protein is significant to microorganism with cell walls composed of glucans, though it can create a status of priming that facilitates the plant to respond quicker and strength to the attack of pathogens (28). It will not necessarily consist in increasing its levels of enzymatic activities when in contact with the elicitor, but triggering them off when the presence of the pathogen be detected, which would increase the recognition speed, response and magnitude of the resistance to the new biotic stress.

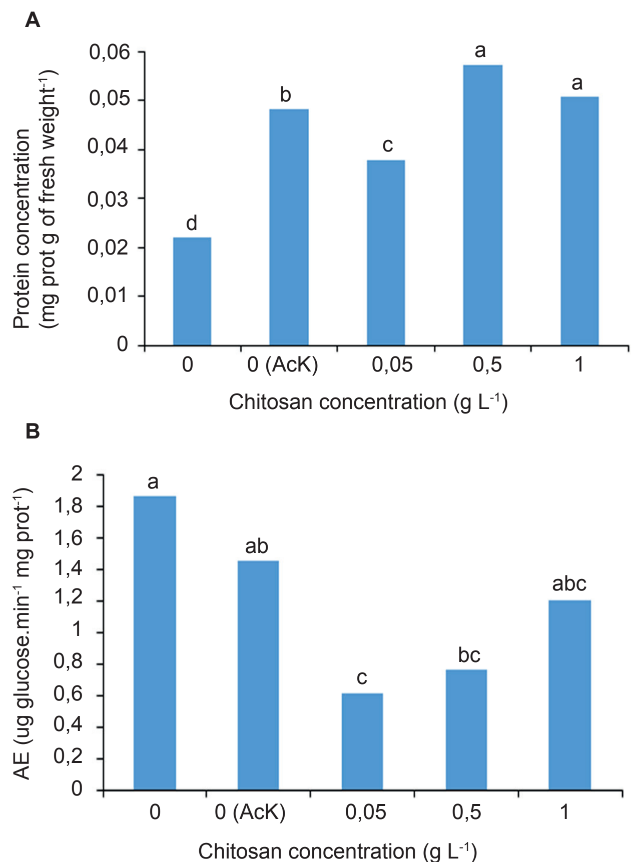


Figure 3. Variation of protein concentration (A) ESx= 0,0028 and glucanase activity (B) ESx= 0,1382 in tobacco leaves which roots imbibed on chitosan

Dynamics of the glucanase activity in tobacco plants leaves inoculated with P. nicotianae. The protein concentrations in the treatments with and without contact with the SS-11 isolate were similar among themselves, except after 120 hours when there was an increase of infected plants compared to the control (Figure 4A).

The glucanase activity varied during the evaluated moments. Most of the time, the enzymatic activity was higher in inoculated plants with SS-11 than in the control, recording the maximum value (2,13µg glucose.min^{-1}.mg $prot^{-1}$) after 48 hours when values quadrupled compared to the control (Figure 4B).

The defensive response of tobacco to *P. nicotianae* has not been so much studied so far and the implementation of dynamics of the glucanase activity in response to a virulent isolate of this pathogen remains unknown.

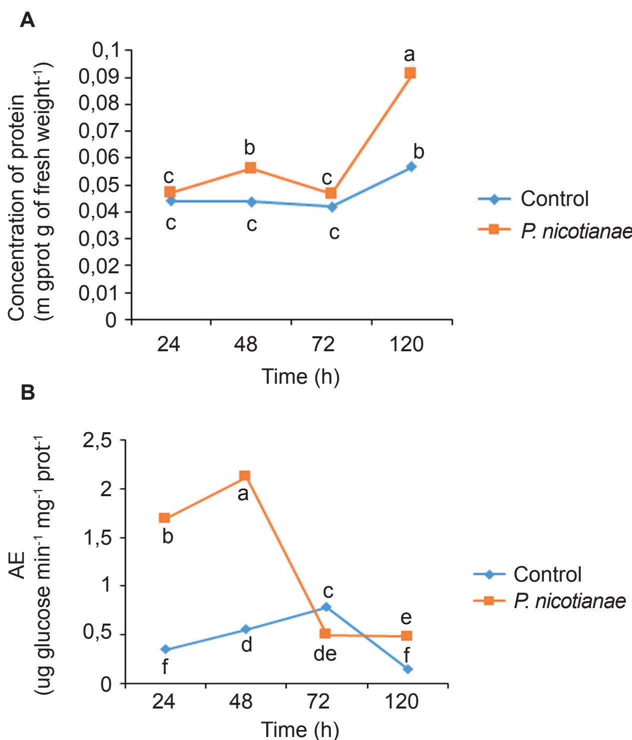


Figure 4. Variation of protein concentration (A) ESx= 0,0009 and glucanase activity (B) ESx= 0.035 in tobacco leaves when roots were in contact with *P. nicotianae* SS-11

At 120 hours there was a high protein concentration; however, the infectious process continued (data not shown). Glucanase activity, at that time, was less than that observed at 48 hours, which may suggest that other enzymes and proteins of various types could be involved in this response. Another hypothesis may be related to the state of priming expected, which could not be sufficient in magnitude and speed required to induce resistance able to stop the advance of the pathogen, as indicated Balmer *et al*. (29).

On the contrary, the induction of the glucanase activity was immediate to the inoculation with SS-11 (Figure 4B) which proves a quick recognition of the pathogen by the plant, because the cell walls of the microorganism are mainly of glucan. Nevertheless, though the enzymatic activity increased in the early days, it decreased in subsequent days. This behavior could be due to a silencing of the defensive response of the plant by the pathogen because the first increased glucanase levels were not enough to stop infection and also because other defensive responses to highten the basal resistance of the plant were not so quick and sufficiently activated.

Defensive response of tobacco seeedling leaves inoculated with P. nicotianae and previously treated with chitosan. Plants that interacted with SS-11 statistically increased the foliar concentration of proteins above the control after 48 and 96 hours of the inoculation with the pathogen. On the other hand, the treatment involving the presence of chitosan and *P. nicotianae*, showed increased protein levels after 96 hours though the values did not differ from the control 48 hours after the plants were treated. These results were similar to those of the treatment of the pathogen alone (Figure 5A).

The glucanase activity was different at every evaluated moment (Figure 5B). After 48 hours plants in contact with the microorganism showed higher levels of activity than the rest of the treatments. After 96 hours activity values of both groups of inoculated plants (with and without chitosan) were above the control.

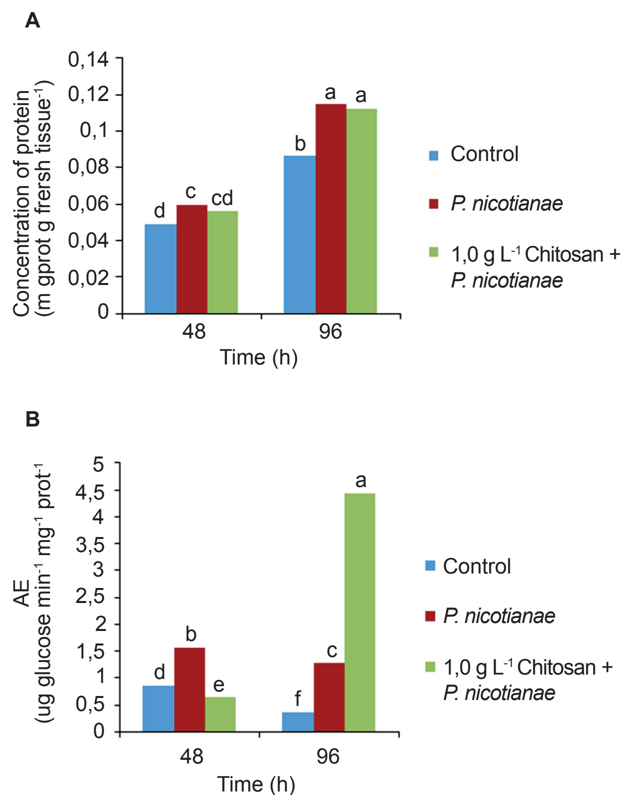


Figure 5. Variation of protein concentration (A) ESx= 0.0033 and glucanase activity (B) ESx= 0.0386 in tobacco leaves which roots embebed first on chitosan and then with *P. nicotianae* SS-11

Glucanase increases in activity observed in the inoculated plants and pretreated with chitosan show that could induce a state of priming that had not yet reached its full potential after 48 hours but the 96 increased enzyme levels almost 10 times. These results agree with those reported by Falcon *et al.*, (30) cultivate plants snuff Criollo 98, which after 72 hours treated with 1.0 g L-1 of the same polymer chitosan increased to twice the values of glucanase activity.

Tobacco plant protection agains P. nicotianae treated with chitosan. The previous treatment with chitosan reduced the infection index of tobaco plants inoculated with *P. nicotianae* (Table II). The lowest concentration (0,05 g L^{-1}) reached a reduction of the infection index in 17 % though without differences with the control, while the highest concentration of the polymer (0,5 g L^{-1}) reduced the infection in 26 % and was statistically different to the control.

Table II. Infection index and protection percentage of tobacco plants inoculated with *P. nicotianae* and previously treated with chitosan through the root

Treatments	N	Infection index	Protection %
Control H_2O d	19	67 4 b	0
Q88 0,05 g L^{-1}	20	56 ab	17
Q88 0,5 g L^{-1}	19	50 a	26

The protection differences were also reflected in the frequency percentages of the different degrees of infection according to the scale used (Figure 6). Thus, the concentration of the polymer 0,5 g L^{-1} reduced the precentage of dead and infected plants to the leaf levels and increased the percentage of plants only affected at the root level, which influenced on the reduction of the infection index reached. Likewise, the smallest concentration of the polymer reduced the percentage of affected plants to the leaf level at the expense of an increased number of affected plants to the roots, hypocotils and cotiledons (Figure 6).

The results shown on Figure 6 prove that chitosan concentrations of 0,5 g L^{-1} reduce the pathogen infection index in tobaco plants under the conditions of the assay practic ed and protect them in 26 % (Table II).

The protection of tobacco plants against *P. nicotianae* observed seems to be the result of stimulating the induced systemic resistance into the plant and not the direct microbial activity that such byproducts have on this pathogen. Such statement is possible if the application form executed via roots to the plants is taken into account.

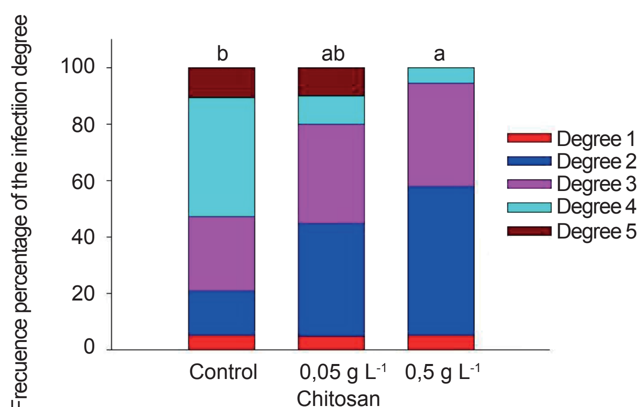


Figure 6. Percentage of frequency of tobacco plants infection grade with *P. nicotianae* SS-11 treated with chitosan

The inclusion of chitosan in the compatible interaction of tobacco with *P. nicotianae* allowed confirming that it can inhibit the growth and sporulation of microorganisms and induce systemic resistance in plants sensitive to their pathogens.

The results of this research allow assuming that under natural conditions of tobacco growing the advantages of the biological effects of chitosan can be used to achieve a high protection level agains *P. nicotianae* and other soil pathogens. It should also be considered that the combination of different forms and application times of chitosan byproducts should cause both a reduction of pathogen levels in the rhizosphere, either by the antimicrobial action and the stimulation of defensive and growth responses in plants.

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