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CHITOSANS INFLUENCE ON SOYBEAN (*GLYCINE MAX* L. MERRILL) NODULATION AND VEGETATIVE GROWTH

Influencia de quitosanas en la nodulación y el crecimiento vegetativo de soya (*Glycine max* L. Merrill)

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ABSTRACT. The purpose of this work was to study the *in vitro* compatibility of an induced inoculant containing of the *Bradyrhizobium japonicum* E109 strain with chitosans of different physico-chemical characteristics. The influence of chitosan *in vivo* nodulation and growth of soybean plants, when applied to seeds at planting time (A), by foliar spraying to 15 days after seedling (B) and with the combination of both forms of application (C) was also evaluated. The chitosan and inoculant combination caused a variable effect in the number of viable bacteria until the thirty days of their conservation which depended on the chitosan type, the concentration, and the evaluation time. The response in plants was influenced by the application form and concentrations of chitosan assayed, but not for the differences between them in terms of molecular mass. The 100 and 500 mg L^{-1} polymer and hydrolyzed chitosan concentrations, applied by foliar spray, exerted beneficial effect on increased number and nodule dry mass formed in soybean roots, respectively. Also, the root length was stimulated with different concentrations of partially hydrolyzed chitosan, applied by foliar spray. Our results suggest that the combination of chitosan with rhizobia inoculants can be used as organic fertilizers to enhance of soybean nodulation and growth and must be examined in future work, the application of chitosan in the field using the application forms studied.

Key words: *Bradyrhizobium*, leguminous, polymer, symbiosis

RESUMEN. El propósito de este trabajo fue estudiar la compatibilidad *in vitro* de un inoculante inducido que contiene la cepa de *Bradyrhizobium japonicum* E109 con quitosanas de diferentes características físico-químicas. Se evaluó la influencia de las quitosanas en la nodulación y el crecimiento *in vivo* de plantas de soya, al ser aplicadas a las semillas en el momento de la siembra (A), mediante aspersión foliar a los 15 días posteriores a la siembra (B) y la combinación de ambas formas de aplicación (C). La combinación de las quitosanas y el inoculante causó un efecto variable en el número de bacterias viables hasta los treinta días de su conservación, que dependió del tipo de quitosana, la concentración y del momento evaluado. La respuesta en las plantas estuvo influenciada por la forma de aplicación y las concentraciones de las quitosanas, pero no por la diferencias entre ellas en cuanto a masa molecular. Las concentraciones 100 y 500 mg L^{-1} del polímero y la quitosana hidrolizada, aplicadas por aspersión foliar, ejercieron mayor efecto benéfico en el número y la masa seca de los nódulos formados en raíces de soya, respectivamente. También, la longitud radical fue estimulada con las distintas concentraciones de la quitosana parcialmente hidrolizada, al ser asperjada foliarmente. Nuestros resultados sugieren que la combinación de quitosanas con inóculos de rizobios puede ser utilizada como fertilizantes biológicos para potenciar la nodulación y el crecimiento de soya y debe examinarse en trabajos futuros, la aplicación de las quitosanas en campo mediante las formas de aplicación estudiadas.

Palabras claves: *Bradyrhizobium*, leguminosas, polímero, simbiosis

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INTRODUCTION

Soybean grain has excellent nutritional qualities due to its content in proteins, amino acids and oil, making it the most attractive oilseed in the production of industrial products for animal and human consumption (1). It is a crop of easy and low cost of production, besides being very little demanding in cultural attention, with requirements ranging from 60 to 80 kg of N per ton of grain (2).

The nitrogen demand in the crop is covered by its absorption from the soil and through the Biological Nitrogen Fixation (FBN, according its acronyms in Spanish) mechanism, through its symbiotic association with bacteria of the *Bradyrhizobium* genus for its development (3). To this end, a recommended practice to guarantee N in this crop is the inoculation of rhizosphere bacteria incorporated by commercial preparations, which is an environmentally friendly and viable alternative in crop productivity (3).

Chitosan and its derivatives are exogenous oligosaccharins that exert proven effects on the growth and development of plants. The chitosan polymer is obtained by basic deacetylation of the chitin which forms part of the exoskeleton of crustaceans and their derivatives are mainly obtained by chemical and enzymatic hydrolysis of the glycosidic bond (4).

The heterogeneity of chitosan depends on its physicochemical characteristics such as molecular mass, viscosity, degree of acetylation, among others (5, 6). Some of the mentioned characteristics give these compounds biological potentialities for their application in agriculture. Biological effects that are desirable in the agricultural context include inhibition of growth and development of pathogenic microorganisms including fungi, oomycetes and bacteria, as well as the induction of defensive and protective responses against other pathogens including viral infections (4, 5, 7, 8). It is also known the action of these compounds as promoters of the growth and development of several species, in some cases related to an antiperspirant and performance-enhancing activity in some crops (8-10).

In soybean, different chitosan compounds of different chemical characteristics have been studied in the symbiotic interaction *Bradyrhizobium*-soy, specifically in the nodulation and *in vitro* growth processes of seedlings inoculated with *B. elkanii*. The results with these compounds demonstrated that the degree of polymerization and acetylation are essential in the growth and formation of radical nodules and even in the viability bacteria. It was demonstrated for the first time that the reduction of molecular mass of the polymer improves the aforementioned indicators, whereas these are affected, as the concentration of chitosan increases (11, 12). However, the influence of the physicochemical characteristics of chitosan on soybean cultivated under *in vivo* conditions through other forms of application is not known. Therefore, the objective of this work was to evaluate the effect of two chitosan on the viability of *Bradyrhizobium*, nodulation and the growth of soybean inoculated with this microorganism.

MATERIALS AND METHODS

Inoculant used

The *Bradyrhizobium japonicum* E-109 strain, from the National Institute of Agricultural Technology (INTA, according its acronyms in Spanish, Castelar), cultivated at 28 °C in Jap medium (13), induced in the synthesis of nodulation factors was used(14), to a final concentration of 1x10 10 CFU mL^{-1} (colony forming units per milliliter of medium).

Characteristics and preparation of the chitosan used

Two chitosan compounds with different physicochemical characteristics were used. The chitosan polymer (Sigma-Aldrich), low molecular weight (32 kDa) and acetylation degree between 15-25 %, was prepared from a stock solution (1 %) which was dissolved in acetic acid (1 %) and water, adjusted to pH =5,6 with potassium hydroxide (KOH, 2N). Partially hydrolyzed chitosan (10 % acetylated) was obtained from the enzymatic hydrolysis of the polymer, with 1,43 g of the commercial enzyme papain (*Applichem*), at 345 rpm and at a temperature between 46 and 50 ^{0}C. After the solution was dissolved for 20 hours, it was autoclaved for 15 minutes.

Effect of chitosan on the viability of Bradyrhizobium japonicum

In order to study the effect of addition of the chitosan polymer and its hydrolyzate on the viability of *Bradyrhizobium japonicum*, solutions of 10, 100 and 500 mg L^{-1} of chitosan, previously dissolved in 100 mL of water and contained in Erlenmeyers of 250 mL, which were sterilized for 20 minutes in an autoclave at 120 ^{0}C and 152 KPa. 0,4 mL aliquots of the chitosan solutions were added and added to 100 mL of the inoculant obtained in Jap medium, with a final concentration of 1x10 10 CFU mL^{-1}. Three replicates were placed at 26 ^{0}C under static conditions for 30 days. The number of colony forming units per milliliter of medium (CFU mL-1) was then determined every three days by sowing

by the dissemination of 100 µL of each sample in plates with solid medium Manitol yeast extract. The plates were incubated at 29,5 ^{0}C for seven days, at which time the typical colony count was performed.

Effect of chitosan on nodulation and soybean growth

The influence of polymer chitosan and partially hydrolyzed chitosan on nodulation and *in vivo* growth of soybean plants was determined by three different application methods: seeds before planting (A), foliar spraying (1,5 mL) per plant) to the 15 days after planting (B) and the combination of both forms A+B, (C).

100 g of DM 3810 soybean seeds from Don Mario Seedlings were treated with 0,4 mL of the bacterial inoculum and 0,4 mL of chitosan solutions (10, 100 and 500 mg L^{-1}). Inoculated soybean seeds without addition of chitosan were used as a control treatment.

The treated seeds were planted in pots of 330 g, per seed per pot. The soil used was free of rhizobia, with pH = 6,50; 2,58 ppm nitrate content (N-NO_3 -phenoldisulfonic acid) (15); 19,35 ppm of assimilable phosphorus (Phosphorus available-extractable at pH <7) (16); 0,91 % of organic matter (17); 0,045 ppm of total nitrogen and 0,63 % of carbon.

The plants were grown in a growth chamber with a photoperiod of 16/8 light/dark hours at 28 ^{0}C and 60 % relative humidity for 35 days. Irrigation was performed by capillarity with deionized water, maintaining the water regime in approximately 90 % of the field capacity.

Ten plants per treatment were used to evaluate nodulation variables (number, dry mass (mg) and percentage (%) of the total nodules formed in the roots) and growth (height (cm), root length dry aerial and root (g) per plant). The total nitrogen concentration of the dry aerial part (%) per plant was determined by the Kjeldah method, and with the multiplication by 6,25 of this variable, the percentage of total protein (%) of the aerial part was estimated.

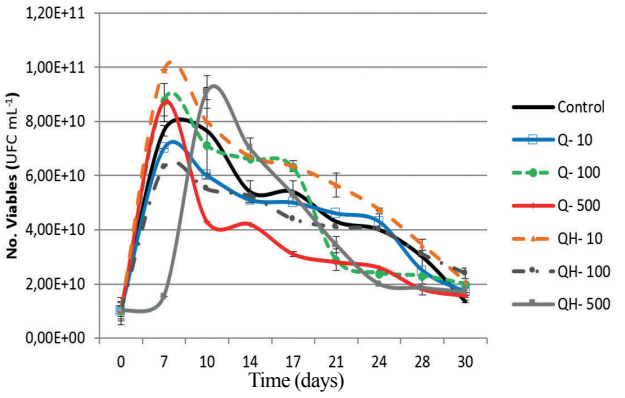
Statistical design and processing

The experiments were repeated twice, using a Completely Randomized Design and the data were subjected to the normality test and homogeneity of variance. In the experiment in plants the analysis was performed by the factorial method with the factors: form of application of chitosan and concentrations, with three levels each factor. In all cases, the Tukey HSD Multiple Rank Test ($p \leq 0.05$) was used to discriminate differences between means (18) in the Statgraphics Plus, version 5.1 (19) program package.

RESULTS

Effect of chitosan on the viability of Bradyrhizobium japonicum

The chitosan compatibility assay with the *Bradyrhizobium japonicum* inoculum showed that the presence of both chitosans in the volume and concentrations tested positively or negatively affected the viability of the bacteria at the time evaluated (Figure 1).



The confidence interval bars appear at each evaluated momentum determined with the Tukey HSD Multiple Rank Test for p≤0,05

Figure 1. Effect of polymer (Q) and hydrolyzed chitosan (QH) on the viability of *Bradyrhizobium japonicum* for 30 days

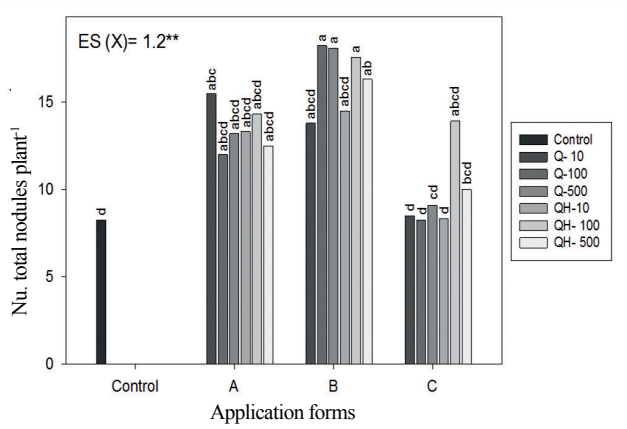
During the 30 days, the highest values of colony forming units per milliliter (UFC mL^{-1}) were obtained with the hydrolysed chitosan at the concentration of 10 mg L^{-1} in almost all the evaluated moments, whereas the polymer chitosan of 500 mg L^{-1}, significantly reduced the number of viable *B. japonicum*, from seven to twenty-eight days after inoculum addition to the control without product. All treatments, at 30 days, were matched to the control treatment that had the lowest bacterial concentration (Figure 1).

The rest of the concentrations of both chitosans did not modify the viability of the inoculant; therefore they can be used as a whole.

Effect of chitosan on nodulation and growth of soybean plants

The soil used was characterized by having a near neutral pH that is suitable for the development of the crop and the establishment of the *Bradyrhizobium* population. The content of organic matter and of total nitrogen is very low, while the levels of assimilable phosphorus and the percentage of carbon are high.

The results of the statistical analysis of the experiment in plants showed that there was interaction between the form of application and the chitosan concentration used in the nodulation processes and most of the soybean growth variables (Figures 2, 3, 4, 5, 6 and 8).



Equal letters do not differ statistically according to the Tukey HSD Multiple Rank Test

Figure 2. Effect of the application of chitosan (Q and QH) on the number of total nodules per soybean plant, when they were added to seeds (A), leaf sprayed (B) and the combination of both forms (C) in soybean

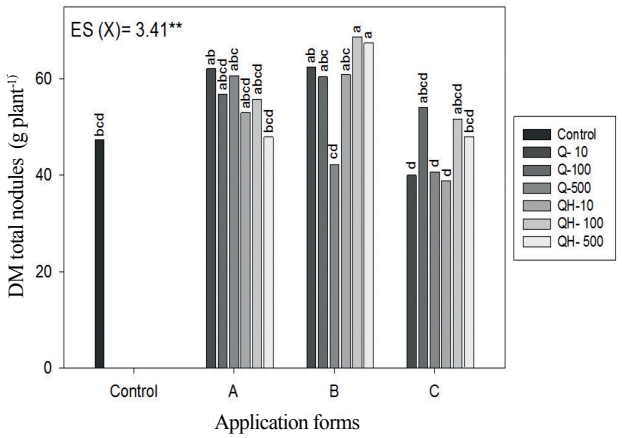


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Figure 3. Effect of chitosan (Q and QH) application on dry mass of total nodules per soybean plant, when they were added to seeds (A), leaf sprayed (B) and the combination of both forms (C) in soy

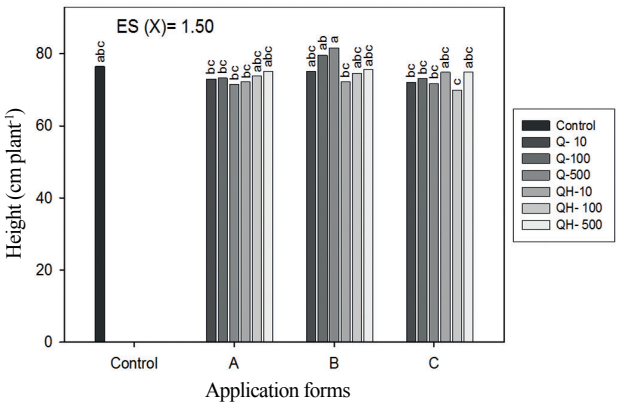

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Figure 4. Effect of chitosan (Q and HH) application on plant height, when added to seeds (A), leaf sprayed (B) and the combination of both forms (C) in soybean

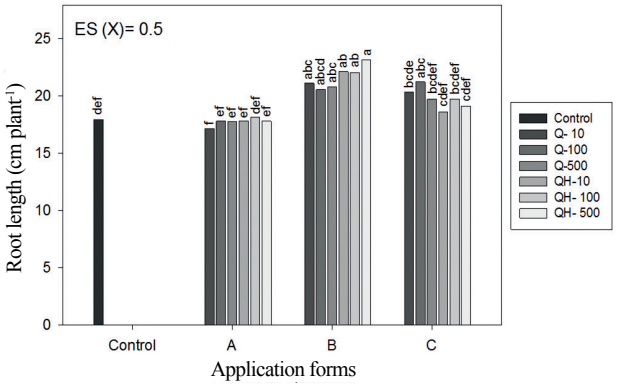

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Figure 5. Effect of the application of chitosan (Q and QH) on the root length of the plants, when they were added to seeds (A), leaf sprayed (B) and with the combination of both forms (C) in soybean

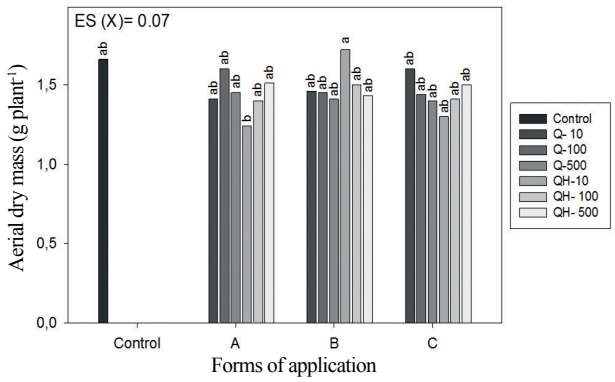

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Figure 6. Effect of the application of chitosan (Q and QH) on aerial dry mass of plants, when they were added to seeds (A), sprinkled leaf (B) and with the combination of both forms (C) in soybean

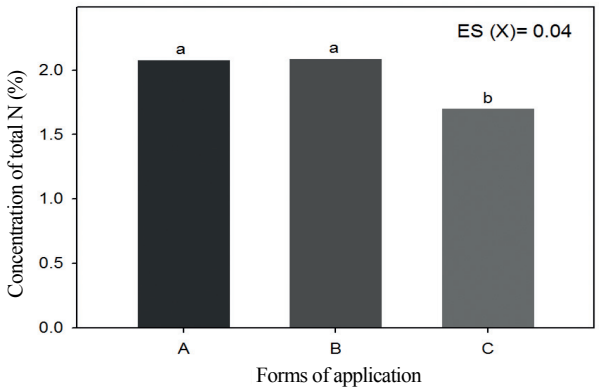

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Figure 7. Effect of the chitosan application (Q and QH) on the total nitrogen concentration of the aerial part when they were added to seeds (A), leaf sprayed (B) and with the combination of both forms (C) in soybean

The number of nodules in soybean plants was stimulated by the concentrations of 100 and 500 mg L^{-1} of both chitosan sprinkled on the 15th day of planting (B), with values higher than the control treatment (Figure 2). All nodules were effective.

The dry mass of the nodules formed was only benefited by the foliar spray of 100 and 500 mg L^{-1} of the hydrolyzed chitosan (Figure 3).

The height of the plants was not positively influenced by any of the treatments as they did not differ from the control treatment (Figure 4).

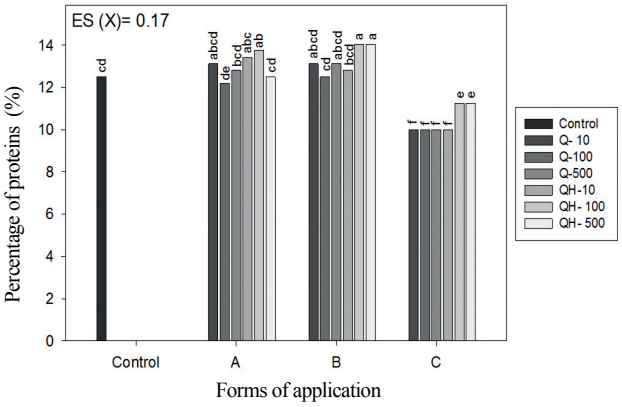
The highest values of radical length were obtained with the foliar spraying of the chitosans (B) at the different concentrations, except with the polymer at the concentration of 100 mg L^{-1}. However, this treatment did benefit the variable in question when combined was used on seeds and then foliar (C) (Figure 5).

No significant differences were observed in chitosan and its concentration in relation to the control, but in the application of the hydrolyzed chitosan to the concentration of 10 mg L^{-1}, as applied by foliar (B) spray differs from that added to seeds (A) (Figure 6).

In the dry mass, no interaction of the evaluated factors (forms of application and concentration of the chitosan) was presented, nor did they influence independently. The values of this variable ranged from 0, 35 to 0, 44 g per plant.

The concentration of total nitrogen in the aerial part of the soybean depended on the application form of the chitosan and not on the concentration of each one of them (Figure 7). Application to seed (A) and foliar (B) did not show differences; however, the combination of both forms of application (C) decreased the total nitrogen content. The values of total nitrogen content were low in all treatments, when compared with other results obtained in this oilseed.

The percentage of proteins estimated from the nitrogen concentration of the aerial part was influenced by the interaction of the form factors of application and chitosan concentration (Figure 8).



Equal letters do not differ statistically according to the Tukey HSD Multiple Rank Test

Figure 8. Effect of the chitosan application (Q and QH) on the total protein content from the aerial part dry mass when they were added to seeds (A), leaf sprayed (B) and with the combination of both Forms (C) in soybean

Chitosan applied in combination with seeds and foliar spray (C) decreased drastically this variable when applied individually, with a behavior similar to the untreated control. The differences with respect to the control are due to the concentrations used, where the doses of 100 and 500 mg L^{-1} sprinkled in plants and 100 mg L^{-1} on seeds of the hydrolyzed chitosan are highlighted (Figure 8).

DISCUSSION

The antimicrobial activity of chitosan has been inferred by several mechanisms previously reviewed, but is mostly attributed to the polycationic character of chitosan at pH ≤ 6, which accentuates the presence of positive charges in the biopolymer chain (4, 17, 20). Therefore, polymers have a greater possibility of interacting with the negative charges of phospholipids on the cell membrane of microorganisms compared to their derivatives, because they have a greater number of non-acetylated amino groups in the chitosan molecule and, therefore, its antimicrobial activity. In this sense, it has been reported that unlike chitosan oligomers, polymers can surround microbial cells and cause their death, in addition to starvation by interacting and chelating the metals needed in enzymatic processes of the bacterium and inhibiting bacterial growth (7, 20).

The results obtained in the present work, with the combination of a polymer chitosan and its derivative, on the viability of an inoculant based on *B. japonicum* confirm that the antibacterial properties of the chitosan are determined to a great extent by the molecular mass (Figure 1). In general, partially hydrolyzed chitosan to a lesser extent harms viability of the strain, as compared to the polymer.

Chitosan had a variable effect on the number of viable bacteria until thirty days of its conservation, which, in addition to being dependent on the type of chitosan, was also influenced by the concentration and the time evaluated. There are numerous studies that refer to chitosan as an antimicrobial substance by direct inhibition of microorganisms and indirect induction of plant defense enzymes (6, 21), which depend on the above factors, among others (22-24).

The concentrations used in the chitosan compatibility test with the inoculant are in the range of minimum inhibitory concentrations of chitosan studied in bacteria ranging from 0,01 % to 1 % (25). In this sense, the highest concentration (500 mg L^{-1}) of the chitosan represents 0,05 %, and it was this concentration of the polymer, which significantly reduced the number of colony forming units per milliliter of *Bradyrhizobium* in most of the period Evaluated (Figure 1). This result agrees with that obtained in viability studies of the *B. elkanii* strain, which demonstrated bacteriostatic activity of a polymer with a molecular mass of 81,3 kDa and degree of acetylation of 12 % with that concentration. However, the reduction of its molecular mass to oligomers (5-9 kDa), decreased the inhibitory action on the microorganism when the chitosan was added to the microbial culture medium (12). Another result indicates that the application of this concentration decreased the growth rate of *Escherichia coli* strains incubated in chitosan microparticles (26).

In spite of the inhibitory action and protection against phytopathogens exerted by chitosan (4, 7, 8), comparative studies of formulations based on the combination of chitosan and plant growth promoting bacteria (BPCV) have been established, in the growth of plants (27) and in disease suppression (28). However, in the use of inoculants for legumes there are no results in this regard. The results of the application of these products in inoculated soybean plants showed that, in general, the most influential form of application in nodulation and in some growth variables was foliar spraying (B) followed by addition to seeds prior to planting (A), and the least that was the combination of both forms of application of chitosan (C).

The foliar spray of the polymer and its derivative (B) at different concentrations (10, 100 and 500 mg L^{-1}), benefited nodulation and some growth variables, compared to the other two forms of application of chitosan: addition to seeds (A) and the combination of both (C). The response in the activation of both processes varied in dependence of the concentration of chitosan; however, was not influenced by the difference of their molecular mass, since they behaved in a similar way.

In the nodulation of soybean plants, the concentrations of 100 and 500 mg L^{-1} leaf-sprinkled were observed in the number and the dry mass of the nodules, with the polymer and the hydrolyzed respectively (Figure 2 and 3). However, nodulation of soybean and peanut (*Arachis hypogaea*) was reported to have a greater positive effect when combined with the imbibition of seeds before planting and leaf spray of chitosan (29). Other authors were able to stimulate nodulation, acetylene reduction (ARA) and soybean yield by supplementing the soil with chitin and chitosan at the concentration of 1 g L^{-1} (30). The results of this work and those cited above confirm that the application of chitosan

in legumes can stimulate nodulation of plants, depending on the mode of application, concentration and type of chitosan, in addition to plant species, among others factors.

There are results in soybean with the application of different concentrations, molecular masses, forms and moments of application of chitosan in seed germination, quality of shoots, growth, physiology and biochemistry of plants and crop yield (4, 29, 31). However, there are hardly any results of the use of chitosan and its derivatives in the symbiotic interaction of the soybean with its microsymbiont.

In soybeans, growth and crop yields have been stimulated with various applications of chitosan (8, 29, 31). Our results only showed a regulatory action of the chitosan, showing no differences due to its structural characteristics, nitrogen concentration, root length and percentage of plant proteins. The last two variables were affected by the interaction of the studied factors, with the partially hydrolyzed chitosan being sprinkled foliarly in the root length and by the A and B forms in the percentage of proteins (Figure 5, 7 and 8).

Previous authors have reported the positive action of foliar spraying because of its influence on growth, physiology and legume yields under normal conditions and under water stress in the field (32-34). Results similar to those of this work regarding a greater effect with foliar spraying compared to seed imbibition were previously reported in different lentil genotypes in terms of increased crop yields (35).

Recently the increase in nutrient uptake in plants has been reported with the application of chitosan (29). In the research the increase of the nitrogen (N) concentration of the aerial part of soybean (Figure 7) was dependent on the application form, being the seed addition (A) and foliar (B). However, the total nitrogen concentration in the aerial part of the plants was not elevated with the three forms of application of the chitosan, since other authors have found values higher than 2 % with the inoculation of *Bradyrhizobium* strains in soybean (36). Therefore, the percentage of protein estimated from N is also low (Figure 8), when compared to protein values in complete soybean plants reporting up to 20 % crude protein (37).

This could be due to the fact that the determinations of this work were made 35 days after the sowing and it is considered that the stage of maximum accumulation of nitrogen occurs in the physiological maturity with 330 kg ha^{-1} (38). In addition, the soil used for plant cultivation had a low N content (0,045 ppm), although the plants showed no visual symptoms of nutrient deficiency, since the plants nodulated and grew properly during the vegetative phase, so that the contribution of N could be covered with the symbiotic fixation of atmospheric dinitrogen (FBN) efficiently. Previous work corroborates the positive effect of *Bradyrhizobium* inoculation; not only because it significantly increases the availability and uptake of N and other macronutrients in plants, but also because it contributes to increased growth and soy yields (39).

The polymer and hydrolyzated chitosan could be used as additives or additives to the *Bradyrhizobium*-based inoculant for soybean cultivation; Provided that adequate concentration of them is taken into account, which does not affect microbial growth. Consequently, concentrations of the chitosan compounds (Q-500 mg L^{-1} and QH-100 mg L^{-1}) that negatively influenced the viability of the bacteria did not similarly affect plant nodulation. This is perhaps due to the fact that they were applied foliarly. Thus, the beneficial influence of this form of application (B) of the chitosan could be due to the fact that it does not interact directly on the bacterium but it does stimulate the FBN process.

The application of biofertilizers and biostimulants to crops is an interesting strategy to improve or preserve the physical-chemical and biological conditions of soils and, at the same time, increase the protection and the potential for agroproduction. It is important to take into account the most convenient application of these products. Therefore, the use of oligosaccharins or other bioactive compounds, as additives or additives of inoculants, is useful to increase the nitrogen fixation process in the soybean.

Future studies regarding the use of chitosan, its concentrations and forms of application, in the soybean crop are necessary to verify the results found. In addition, it is suggested for the next work to evaluate the physico-chemical characteristics of the chitosan, fundamentally, the differentiation in its molecular mass, as well as the effect of this bioproduct over time, its incidence on the components of the yield in addition to the nodulation and growth in field conditions.

CONCLUSIONS

The present study demonstrated the influence of a chitosan polymer and its hydrolyzated on the symbiotic interaction *Bradyrhizobium-soy*. The effect of both chitosans on the viability of strain E109 depended on the molecular mass and concentration of these compounds; the use of the polymer at 500 mg L^{-1} being negative. Foliar spraying was the best form of application, with positive results in nodulation and vegetative growth of soybean plants.

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