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Original article

Effect of chitosan foliar spray on the vegetative development of inoculated soybean

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ABSTRACT

Chitosan is a linear polymer of glucosamines linked by β 1-4 exoskeleton, which is obtained by alkaline deacetylation of chitin and, has a wide application as an agricultural biostimulant. The objective of this work was to evaluate the effect of different concentrations (1, 10, 50, 100, 500 and 1000 mg L^{-1}) of chitosan, applied in phases V1 and V2 by foliar spray, in the nodulation, the physiology and vegetative growth indicators of IS-27 inoculated soybean with Azofert-S®, in controlled conditions. Seeds inoculated with the ICA 8001 strain of *Bradyrhizobium elkanii*, prior to sowing, were placed in pots and grown in a growth chamber until phase V4. The application of 10 mg L^{-1} chitosan stimulated both the number and the dry mass of the nodules formed and the concentrations 500 and 1000 mg L^{-1} stimulated plant growth. On the other hand, the inoculation of seeds with *Bradyrhizobium* increased the levels of the nitrate reductase (NR) enzyme activity in the V1 soybean phase, while in the V2 and V3-V4 phases, the NR activity was increased with the application of the concentrations between 10 and 500 mg L^{-1} chitosan. The benefit of the indicators of the metabolism of nitrogen by chitosan in this work could be to the stimulated vegetative development in plants related.

Key words: biostimulants, *Bradyrhizobium*, concentration, growth, nitrogen

INTRODUCTION

Chitosan is a linear biopolymer of glucosamine monomers and a small amount of N-acetyl-glucosamine monomers, derived by partial deacetylation of chitin, which is as the second most abundant polysaccharide in nature after cellulose recognized [(1)]. Chitosan is a recognized agricultural biostimulant for causing biological effects in plants such as promoting growth and development and anti-stress protection through the activation of plant metabolism. In this way, the stimulation of growth and development in plants by polymers and oligomers of chitosan is the result of the benefit of the accumulation of essential nutrients, of the photosynthesis process and the accumulation of carbohydrates, of the activation of enzymes of carbon metabolism and nitrogen and the increase in the content of secondary metabolites [(2-5)].

Some authors have reported benefits in the development of soybeans with chitosan and its derivatives with different molecular masses, concentrations, forms and times of application of these compounds [(6-9)]. In particular, most of the investigations carried out in the cultivation with foliar spraying have used high concentrations of chitosan and its derivatives (from 1 to 40 g L^{-1}), with significant consequences in its development that, even, have reduced the negative effects caused by abiotic stresses [(3,10)]. However, previous results in the cultivar IS-27 demonstrate the positive effect of concentrations lower than 1 g L^{-1} of a chitosan polymer and oligomer, on nodulation and growth *in vitro* [(11)].These concentrations have not been in this cultivar evaluated by foliar spraying of the polymer.

Therefore, the objective of this work was to evaluate the effect of different concentrations (1, 10, 50, 100, 500 and 1000 mg L^{-1}) of chitosan, applied in stages V1 and V2 by foliar spraying, on indicators of nodulation, physiology and vegetative growth of soybean *cv* IS-27 inoculated with Azofert-S®, under controlled conditions.

MATERIALS AND METHODS

Chitosan polymer

The commercial chitosan polymer (PANVO Chemicals, India) was characterized by INCA's Bioactive Products Group, by viscometry and infrared spectrometry, showing among its physicochemical characteristics a molar mass of 100 kDa and an acetylation degree of 13.7 %, respectively. From a stock solution of 10 g L^{-1} of chitosan dissolved in acetic acid (1 %) and adjusted to pH=5.2 with KOH, aliquots were taken to prepare the different concentrations to be evaluated: 1, 10, 50, 100, 500 and 1000 mg L^{-1} for foliar application in soybean plants.

Chemical soil analysis

In the experiment execution, a soil from the National Institute of Agricultural Sciences (INCA) was used, which was physically classified as typical Leachate Red Ferralitic, eutric (12). The chemical analysis of this soil was carried out in the Biofertilizers and Plant Nutrition Laboratory of the institution itself, from samples collected between 0 and 20 cm deep, using the methods of some authors (13). The soil presented low values of organic matter (16.1 g kg^{-1}), slightly acidic pH (H_2O) (6.23), high content of available phosphorus (P) (180 mg kg^{-1}) and potassium (K^+) exchangeable (1.23 Cmolc kg^{-1}), as well as high contents of interchangeable calcium (Ca^{2+}) (29.25 Cmolc kg^{-1}) and magnesium (Mg^{2+}) (9.75 Cmolc kg^{-1}), without the presence of sodium (Na^+). These values are suitable for soybean cultivation, which requires medium fertility (14).

Growing conditions

The soybean seeds *cv* IS-27 were inoculated with the commercial inoculant Azofert-S® based on the ICA 8001 strain of *B. elkanii*, in a dose of 200 mL per 50 kg of seed and with a concentration of 1x1010 forming units of colonies per milliliter (CFU mL^{-1}), at the time of sowing. The seeds were left to dry for one hour before being sown in plastic pots of 1370.25 cm^3 volume and 1.2 kg of soil capacity, at the rate of two plants per pot. The plants were grown in a growth chamber, with a cycle of 16/8 hours light/dark, temperature between 25-27 ºC and relative humidity of the air between 50-70 %. Eight treatments were made in total, six of them corresponded to chitosan concentrations, which were sprayed foliarly (1 mL per plant) in two stages (V1 and V2) of soybean growth, and two control treatments without the presence of chitosan: one inoculated (CI) and the other not (CA). Thirty plants were selected per treatment (n=30) to perform morphoagronomic and physiological evaluations related to nodulation and plant growth.

Plant evaluations

Among the nodulation variables evaluated, the number of nodules distributed in the main root (RP) and secondary roots (RS) was counted, in addition to the totals (NT) formed per plant. Likewise, the dry mass of the nodules by root sections was determined by weighing, after being placed in the oven at 75 ºC for three days, and the percentage of nodular effectiveness (%) according to the reddish coloration inside the nodules with a cross section of them.

At 15 days (V1), 22 days (V2) and 29 days (V3-V4) of cultured plants, samples of third trifoliole were collected to develop the enzymatic activity nitrate reductase (NR) (15), except in the first moment (V1) that the first trifoliole was taken to compare the control treatments (absolute and inoculated). In all cases, the leaves were cut into segments of approximately 1-1.5 cm and 0.25 g were weighed before being placed in test tubes, forming three replicates of each leaf sample and read at a wavelength of 540 nm. A standard curve of sodium nitrite ($NaNO_2$) was made in a concentration range from 2 to 24 nmol and the data were expressed in nitrite produced (µmol NO^{-2}) min^{-1} mg^{-1} of fresh mass (MF) of leaves.

The concentration of macronutrients was determined by chemical analysis in nodules and third trifoliole, such as nitrogen (N) by the Nessler method, phosphorus (P) by the formation of molybdenum blue and potassium (K) by flame photometry (mg kg^{-1} of dry sample) [(13)]. The determination of calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), zinc (Zn) and Na (mg kg^{-1}) ions was carried out by flame atomic absorption spectrophotometry (Rayleigh, model WFX-210, China), from the digestion of ground dry samples (0.5 g) of both soybean organs with concentrated nitric acid (HNO_3, 4 mL), in a microwave oven for 20 minutes. According to the ISO-11466 (1995) standard curves and dilutions were made with deionized water for each element, except for Ca and Mg, which were diluted in a lanthanum chloride solution (0.2 %). In addition, the relative content of total chlorophylls was determined in the third trifoliole with a portable meter (MINOLTA SPAD* 502 plus), therefore the values are shown as spad units.

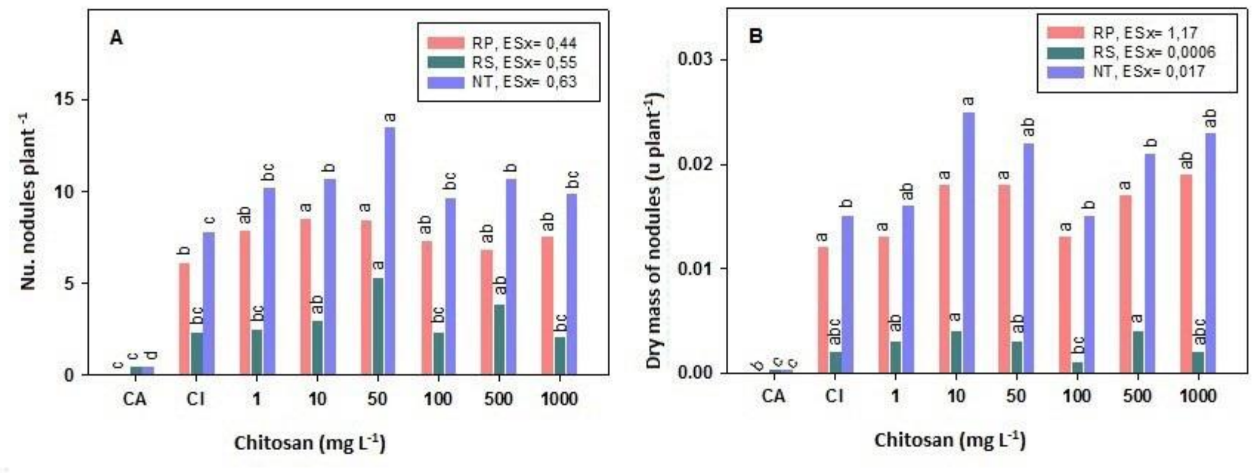
Regarding the growth variables, the number of trifoliate leaves, the stem diameter (mm) measured with a digital electronic vernier caliper (0-150 mm, China), the stem and root length (cm) were evaluated. The dry mass (g $plant^{-1}$) of the soybean organs was determined on a Sartorius CPA 3245 analytical balance, after placing the samples in a forced air oven (BINDER, USA) for 72 hours, at 75 ºC until weight was obtained constant. In addition, the plants were removed by destructive method to determine the leaf area (cm^2) of all the trifoliate leaves of each plant, which were scanned with a tabletop scanner (Canon MG2520) and all the images were processed with the Adobe Photoshop CS software, (version 8, 2003).

Statistical analysis of the data

The experiment, with a completely randomized design, was repeated twice and the results of one of the experimental repetitions are shown. The assumptions of normality and homogeneity of variance were verified from the data obtained by the Kolmogorov - Smirnov and Levene tests, in addition to performing a Simple Classification ANOVA. Control treatments were compared by Student's t test in growth stage V1 for the determination of the enzymatic activity Nitrate reductase. The statistical package Statgraphics (version 5, 2011), was used. The comparison of the resulting means was performed by the Tukey HSD test $p < 0.05$ and the graphs were performed in SigmaPlot, (version 11, 2008).

RESULTS

When evaluating the effect of chitosan applied by foliar spray of soybeans, a superiority of concentrations 10 and 50 mg L^{-1} is observed, in the formation of nodules in the main root (RP), in addition to 500 mg L^{-1} in the roots secondary (RS), compared to CA. While in the number of total nodules (NT), the aforementioned concentrations stood out, with respect to the controls. The increases in these chitosan concentrations ranged between 32 and 74 %, with respect to the plants inoculated with *Bradyrhizobium* (Figure 1).



Means with the same letters do not differ statistically for p <0.05, according to the Tukey HSD Multiple Range Test.

ES $\bar{X}$: Standard error of the experiment (n = 30)

Figure 1. Effect of different concentrations of chitosan applied by foliar spraying on the number and dry mass of total nodules (NT), of the main root (RP) and of secondary roots (RS), of soybean plants cv IS-27 inoculated with *B. elkanii*

Even when large differences were found in the number of nodules, the differences in their dry mass were lower, between the inoculated plants (CI) and those treated with chitosan by foliar spraying (Figure 1). Chitosan applied by foliar spray of soybean did not modify the dry mass of the nodules formed on the main roots of the inoculated plants. However, the dry mass of the nodules formed in the secondary roots (RS) benefited with the 10 and 500 mg L^{-1} concentrations of chitosan. The mass of the total nodules benefited with the 10, 50, 500 concentrations and 1000 mg L^{-1} of chitosan, with increases in this variable, of approximately between 40 and 67 %, with respect to the CI (Figure 1).

Table 1 shows the nitrate reductase (NR) enzymatic activity determined in leaves collected at three moments of vegetative growth of soybean IS-27 plants. A first moment (V1), in which the chitosan foliar spraying had not been carried out, to compare the values of the control plants. The other two moments were seven days after each chitosan foliar spray was performed.

Table 1. Effect of different concentrations of chitosan applied by foliar spraying on the behavior of Nitrate reductase activity in the growth (V1-V4) of soybean plants inoculated with *B. elkanii*, under controlled conditions

Chitosan mg L^{-1}	Act. NO_3 reductase (µmol NO^{2-}) min^{-1} mg^{-1} Foliar MF)		
	V1	**V2**	**V3-V4**
CA	4.3013	11.443 e	0.851 d
CI	6.0544	13.343 cd	0.926 d
Q-1		11.733 de	0.866 d
Q-10		15.791 ab	2.327 bc
Q-50		17.119 a	3.281 b
Q-100		15.523 ab	4.816 a
Q-500		16.715 a	4.933 a
Q-1000		14.287 bc	1.389 cd
ES $\bar{X}$	*t*= - 5.62*	0.285**	0.264*

Means with the same letters do not differ statistically for $p < 0.05$, according to the Tukey HSD Multiple Range Test.

ES $\bar{X}$: Standard error of the experiment (n = 4)

In phase V1 of plant growth, the student's t test showed significant differences between the controls (CA and CI), highlighting the inoculation of *B. elkanii* on the non-inoculated plants in a higher NR activity (40 % increase) determined in sheets (Table 1). In the following stages, the plants sprayed with chitosan produced higher values of nitrite (NO^{-2}), which immediately became ammonia (a form assimilable by the plants). Chitosan concentrations from 10 to 500 mg L^{-1} contributed to the accumulation of this activity at both times. However, in phase V2, there were increases between 10 and 28 %, with respect to the inoculated control0 while in V3 the activity increases with chitosan were between 0.5 and 4.3 times higher than the activity value of the inoculated control despite having the lowest absolute values (Table 1).

The foliar application of chitosan did not modify the response found in the concentration of N in the soybean organs beyond the increase obtained with the inoculation of the seeds with the bacteria. It is excepted with the concentration of 1000 mg L^{-1} in the nodules that increased in 10 % the content of N, with respect to the CI (Table 2 and 3).

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Table 2. Effect of chitosan foliar spraying on the concentration of nutrients determined in nodules of soybean plants inoculated with *B. elkanii*

Chitosan (mg L^{-1})	Nutrient concentration in nodules (mg kg^{-1})								
	N	**P**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Zn**	**Na**
CA	Nd	Nd	Nd	1.28 b	0.258 c	0.150 e	16.83 c	0.290 d	0.0018 b
CI	6.85 bc	0.63 a	1.81 ab	1.50 ab	0.660 a	0.112 f	17.11 c	0.292 cd	0.0090 a
Q- 1	6.26 d	0.61 ab	1.79 ab	1.55 ab	0.420 b	0.105 f	19.17 bc	0.319 bcd	0.0011 b
Q- 10	6.13 d	0.59 ab	1.78 ab	1.60 ab	0.130 d	0.151 e	20.78 b	0.333 abc	0.0021 b
Q- 50	6.73 c	0.60 ab	1.84 ab	1.65 a	0.040 d	0.307 a	26.98 a	0.352 ab	0.0070 a
Q- 100	7.25 ab	0.49 ab	1.90 a	1.70 a	0.090 d	0.250 c	19.50 bc	0.297 cd	0.0015 b
Q- 500	6.94 bc	0.57 ab	1.69 b	1.83 a	0.099 d	0.282 b	19.00 bc	0.366 a	0.0016 b
Q- 1000	7.56 a	0.45 b	1.88 ab	1.80 a	0.091 d	0.218 d	18.80 bc	0.341 ab	0.0016 b
ES $\bar{X}$	0.079	0.021**	0.034	0.045**	0.021	0.003	0.42**	0.007	0.0003**

Means with the same letters do not differ statistically for p <0.05, according to the Tukey HSD Multiple Range Test. ES $\bar{X}$: Standard error of the experiment (*n* = 2). **Nd: the concentration of the macronutrients N, P and K was not determined in the nodules of the control plants (CA) because there was not enough sample for its determination*

Table 3. Effect of chitosan foliar spraying on the concentration of nutrients determined in leaves of soybean plants inoculated with *B. elkanii*

Chitosan (mg L^{-1})	Nutrient concentration in leaves (mg kg^{-1})								
	N	**P**	**K**	**Ca**	**Mg**	**Mn**	**Fe**	**Zn**	**Na**
CA	4.65 c	0.41	2.29	1.49 c	0.131 cd	0.141 e	18.22 b	0.319 b	0.0038 c
CI	6.56 a	0.41	2.24	1.60 c	0.418 b	0.158 de	18.00 b	0.351 ab	0.0030 c
Q- 1	5.36 bc	0.42	2.44	1.60 c	0.187 c	0.471 ab	17.50 b	0.354 ab	0.0071 b
Q- 10	5.57 b	0.42	2.33	2.00 bc	0.515 a	0.480 ab	19.00 ab	0.383 a	0.0017 d
Q- 50	7.25 a	0.48	2.39	2.50 ab	0.149 cd	0.268 c	21.85 ab	0.236 c	0.0088 a
Q- 100	5.67 a	0.42	2.57	2.75 a	0.129 cd	0.436 b	27.50 a	0.240 c	0.0089 a
Q- 500	5.57 b	0.41	2.45	3.00 a	0.099 d	0.490 a	22.32 ab	0.259 c	0.0086 a
Q- 1000	7.21 a	0.41	2.20	3.00 a	0.089 d	0.195 d	22.82 ab	0.245 c	0.0080 ab
ES $\bar{X}$	0.13	0.03	0.078	0.10	0.008	0.0083	1.61	0.007	0.0002

Means with the same letters do not differ statistically for p <0.05, according to the Tukey HSD Multiple Range Test. ES $\bar{X}$: Standard error of the experiment (n = 3)

In soybean trifoliole, the concentration of P and K did not show significant differences between the treatments (Table 2). In general, there were no differences at the level of the nodules, except for the concentrations of 100 and 1000 mg L^{-1}, which reduced the content of K and P with respect to the CI, by 5 and 40 %, respectively (Table 3).

Although inoculation with *Bradyrhizobium* did not increase Ca and Mn levels with respect to CI, both nutrients were increased with several of the chitosan treatments in both organs evaluated (Table 2 and 3). Concentrations between 50 and 1000 mg L^{-1} increased the Ca content between 56 and 87 % in the leaves and 10 and 22 % in the nodules, with the highest increase being 500 mg L^{-1}.

On the other hand, the increase in the content of Mn in the leaves occurred with the applications of chitosan between 1 and 500 mg L^{-1} with increases between 70 and 200 % above the controls, with the best treatment being 500 mg L^{-1}. In the nodules the increases occurred between 50 and 1000 mg L^{-1}, the best performance being with 50 mg L^{-1} and with increases between 94 and 170 % with respect to the CI, which, in turn, reduced the content of Mn obtained with the CA.

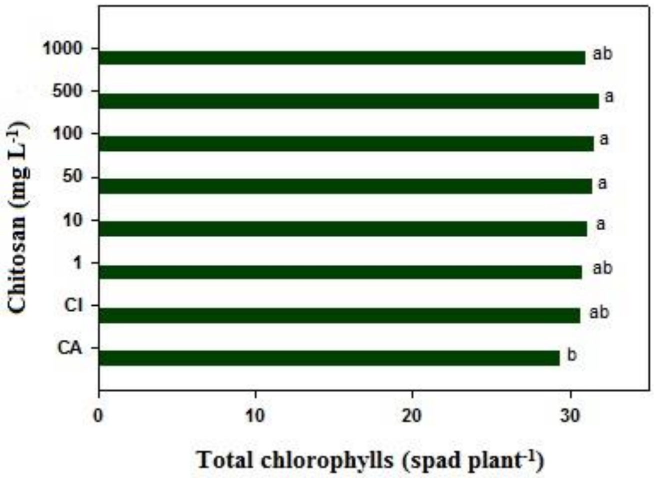
The inoculation with the symbiont bacteria increased the Mg content by 219 and 156 % in leaves and nodules of the plants respectively, however, the application of all concentrations of chitosan reduced between 123 and 370 % in the leaves, except the concentration of 10 mg L^{-1} that increased the Mg value of the inoculated control by 23 %. Similar behavior occurred in the nodules. All the chitosan treatments without exception reduced the Mg content, with respect to the inoculation between 57 and 1550 % (Table 2 and 3).

Fe levels were not affected by inoculation with *Bradyrhizobium* in both organs and were only increased with respect to CA with the application of 100 mg L^{-1} of chitosan in the leaves with a 53 % increase and with 10 and 50 mg L^{-1} in the nodules that increased 21 and 58 %, respectively (Table 2 and 3).

In soy leaves, concentrations from 50 to 1000 mg L^{-1} of chitosan reduced the concentration of Zn and increased the concentration of Na, while Zn increased in the nodules, except for 100 mg L^{-1}. The inoculation did not affect the behavior of Zn in both organs (Table 2 and 3).

Chitosan affected the concentration of macronutrients and trace elements determined in soybean organs inoculated with *Bradyrhizobium*, depending on the concentration of the polymer and the plant organ. In general, the nutrients in the nodules were increased with 50 mg L^{-1} of chitosan and in the third trifoliole, with the lowest concentrations (1 and 10 mg L^{-1}), except for nitrogen in both organs of the plants.

The relative content of total chlorophylls benefited with the application of concentrations of 10 to 500 mg L^{-1} of chitosan, which increased the variable between 6 and 8.4 %, with respect to the non-inoculated plants, although without differences with the control inoculated (Figure 2).



Means with different letters indicate significant differences between treatments, according to the Tukey HSD Multiple Range Test. Standard error of the experiment (ES $\bar{X}$ = 0.451, *n* = 30)

Figure 2. Effect of different concentrations of chitosan applied by foliar spraying on the relative content of total chlorophylls (spad) of third trifoliole of soybean *cv* IS-27 inoculated with *B. elkanii*

The response in the vegetative growth of soybeans with the foliar application of chitosan showed significant differences in all the morphoagronomic variables evaluated in phase V4 (Table 4). All chitosan concentrations favored the emission of leaves in the plants, although without significant differences with CI. The higher concentrations (500 and 1000 mg L^{-1}) of chitosan stimulated the diameter of the stem of the plants, with increases between 10 and 16 %, with respect to the CA and CI controls; as well as the radical dry mass, which differed only from the CA, with increases of approximately 44 % (Table 4).

Table 4. Morphoagronomic response of soybean inoculated with *B. elkanii*, with the application of different concentrations of chitosan by foliar spraying of the plants

Chitosan mg L^{-1}	Number of leaves	Stem diameter (mm)	Stem length (cm)	Root length (cm)	Aerial dry mass (g)	Radical dry mass (g)	Leaf area (cm^2)
CA	3.46 b	2.39 b	43.62 b	33.65 b	0.480 c	0.170 b	192.00 c
CI	3.82 ab	2.47 b	50.75 a	35.39 ab	0.581 bc	0.175 ab	198.65 bc
Q-1	3.86 a	2.37 b	43.27 b	38.47 ab	0.614 ab	0.190 ab	199.68 bc
Q-10	3.89 a	2.47 b	46.05 ab	34.21 ab	0.647 ab	0.199 ab	195.60 bc
Q-50	3.93 a	2.54 b	47.03 ab	35.99 ab	0.680 ab	0.185 ab	203.40 abc
Q-100	3.93 a	2.51 b	49.69 a	38.94 a	0.600 ab	0.177 ab	201.57 abc
Q-500	4.11 a	2.75 a	50.84 a	34.81 ab	0.674 ab	0.245 a	297.00 a
Q-1000	4.00 a	2.79 a	48.45 ab	38.93 a	0.715 a	0.240 a	218.73 ab
ES $\bar{X}$	0.074**	0.045	1.06	1.17	0.03	0.017	5.56

Means with the same letters do not differ statistically for p <0.05, according to the Tukey HSD Multiple Range Test.

ES $\bar{X}$: Standard error of the experiment (n = 30)

The length of the stem was stimulated with the concentrations 100 and 500 mg L^{-1}, with differences of the lower concentration (1 mg L^{-1}) sprayed and the CA. A similar result was found in the root length, but with the concentrations 100 and 1000 mg L^{-1} (Table 1). However, all chitosan concentrations applied by foliar spraying stimulated the dry mass of the aerial part, between 48.96 and 23.06 %, in relation to the CA and CI controls. The concentration of 1000 mg L^{-1} was the one who most stood out in increasing this indicator (Table 4).

The higher concentrations of chitosan favored the soybean leaf area, specifically, the concentration of 500 mg L^{-1} that raised the variable by 49.24 %, differing from 1 mg L^{-1} and the two controls (Table 4).

Chitosan applied by foliar spraying benefited the morphoagronomic response of soybeans inoculated with *B. japonicum*. The highest stimulating response to plant growth was found with concentrations of 100 to 1000 mg L^{-1} of chitosan, mainly in the diameter of the stem, the dry biomass and the foliar area.

DISCUSSION

In this work, the effect of different concentrations of a chitosan polymer on nodulation, the enzymatic activity of foliar nitrate reductase, the concentration of macro and trace elements in nodules and third trifolioles and the content of chlorophylls was evaluated; as well as the vegetative growth of soybean *cv* IS-27 inoculated with *Bradyrhizobium elkanii*, under growth chamber conditions. In the aforementioned processes, a beneficial effect of the foliar application of the polymer (phases V1 and V2) was observed in most of the variables analyzed in phase V4 of the plants (Figures 1 and 2, Tables 1, 2, 3 and 4).

The different concentrations of chitosan applied by foliar spraying in soybeans inoculated with *B. elkanii* had a positive influence on the nodulation of the plants, with the greatest increases in the number and the nodular dry mass. All this with respect to the plants that were only inoculated, with the concentrations 10, 50 and 500 mg L^{-1} of the polymer (Figure 1 A and B). The increase in both variables translates into a higher content of bacteroides, which would increase the rate of nitrogen fixation [(16)]. These results confirm what other authors in the number of nodules obtained and acetylene reduction activity (ARA) in soybeans, through other forms of application of chitosan compounds, in concentrations of 40, 50 and 1000 mg L^{-1} [(17-19)].

On the other hand, several studies on legumes report the beneficial effect of inoculation with biofertilizers with respect to plants that have not been inoculated [(20-23)], which is consistent with the results obtained in this study. The plants that were not inoculated barely noduled, which shows the effectiveness of the *Bradyrhizobium* strain, the cell concentration and dose of the inoculum used, with respect to the resident population of bradyrizobia in the soil used (Figure 1). This corroborates what has been reported by other authors [(23,24)].

The plants of the different inoculated treatments presented a number of nodules suitable for the vegetative stage, of uniform size and were located, fundamentally, in the crown and the main root of the plants (Figure 1 A and B), which is attributed to an effectiveness of the inoculation. This was corroborated with 100 % effective nodules found in all treatments. Some authors suggest that the occurrence and distribution of nodules in host plants depend on root development, soil conditions and the legume species. In the case of soybeans, the nodules are located mainly in the main root (25,26). Based on the information reported by these authors, the increase in the number of nodules in the present study is perhaps the result of the increase in root development due to the inoculation of plants and favorable soil conditions.

Nitrate reductase (NR) enzymatic activity can occur in different organs and provides an estimate of the N content in the plant and, very often, is correlated with its growth and yield (27,28). At work, the inoculation of the seeds with *B. elkanii* caused increases in the NR activity in the leaves, as has been demonstrated for this crop and other legumes inoculated with *Bradyrhizobium* (29,30). The highest NR values were reached in phase V2, which later decreased in V3-V4, inclusive, much lower than those obtained in V1 (Table 1). This could be because in this last phase the contribution of N is more related to the fixation of symbiotic nitrogen (31).

For its part, the foliar spraying of chitosan stimulated the NR enzyme in soybeans, depending on the concentration used. Increases in enzyme activity have been reported in various crops (32,33), which depended on the concentration and the plant species treated, with foliar spraying of the chitosan concentrations evaluated in our work. The concentrations of 10 and 50 mg L^{-1} of chitosan in this work favored the number and dry mass of the nodules formed, but reduced the NR activity in leaves during the V3-V4 stage of soybean growth. However, in this stage, the NR activity benefited with the different concentrations of chitosan from 10 mg L^{-1}, to a lesser extent 1000 mg L^{-1}, in relation to the control plants (Table 1). The above shows that the contribution of N with the lowest concentrations of chitosan could be due more to the process of biological nitrogen fixation, while with the medium concentrations it could be more related to the activity NR.

It is known that plants inoculated with *Bradyrhizobium* favor the nutritional status of soybeans in relation to plants that are not inoculated (23,34) because they activate metabolic changes in the plant, specifically the increase in the content of proteins, nitrogen and other nutrients (35), which translates into improved legume yields and has been exploited in agriculture for years. The effect that some authors attribute to inoculation is verified at work, with increases in the concentration of magnesium, manganese and sodium in nodules; as well as nitrogen and magnesium in leaves, with respect to non-inoculated plants (Tables 2 and 3). These mineral nutrients influence nodular development, BNF and plant growth, in different amounts, times and ways (36,37).

In turn, the lower concentrations of chitosan also stimulated the concentration of some nutrients, fundamentally the trace elements (Tables 2 and 3), which coincided in stimulating the formation and dry mass of nodules in soybean roots, which suggests that said concentrations favored the symbiotic process of the culture. This approach is the reason for future research to elucidate the possible stimulation mechanism of chitosan. Similar results of increases in the content of nutrients in legumes have been reported with the joint application of chitosan and biofertilizers based on free diazotrophic bacteria, with 40 % increases in nitrogen content in cowpea (*Vigna unguiculata*) and corn (*Zea may*) (34,38). Chitosan has been reported to increase nutrient availability and absorption in plants with other forms of application (5,19,39).

In this study, phosphorus was found in higher concentrations in nodules than in leaves (Tables 2 and 3). Nodules have been shown to be important sinks for this nutrient because it is required for symbiotic interaction, nodulation, and biological nitrogen fixation (BNF) (36). These processes are strongly influenced by the availability of phosphorus in the soil. Taking into account the above, the availability of phosphorus in the soil where the soybean plants were grown was high (Table 1), which could favor its absorption by the plant.

For its part, chitosan at a concentration of 1000 mg L^{-1} increased the concentration of nitrogen in the nodules, but decreased the concentration of phosphorus (Table 2), perhaps because of the energy expenditure required to maintain the BNF, the translocation of photosynthates to the leaves from the nodules and their assimilation in this organ.

It should be noted that the polymer also increased the concentration of calcium and manganese in both soybean organs (Tables 2 and 3). Increases in these nutrients have been reported in cucumber (40) and other crops (41,42), with the application of chitosan. Calcium acts as a secondary messenger in metabolic processes related to morphogenesis, development and protection of plants against (biotic) stresses (43). Similarly, manganese induces the synthesis of polyamides that are required for plant growth and the detoxification of reactive oxygen species, mainly in the initial stages of symbiotic interaction (36).

In leaves of different plant species, an improvement in the efficiency of the use of light, the stability of chlorophylls and increases in the diameter of chloroplasts with the application of chitosan has been verified (5). In addition, researchers reported increases in chlorophylls and stomatal density in soy leaves with the previous application of chitosan (44). In addition, there is a positive relationship between chlorophyll content and carbon assimilation in soybeans (45), because of a linear increase in the photosynthetic rate and the activity of the enzymes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Ribulose-bisphosphate carboxylase (Rubisco).

Therefore, the increase in the relative content of chlorophylls in trifoliole between 6 and 8 %, with concentrations between 10 and 500 mg L^{-1} of chitosan with respect to the absolute control (CA), and of the leaf area up to 49 % (500 mg L^{-1}). It must favor a greater photosynthesis of the plants and therefore, the availability of sugars for the synthesis of new assimilates, which can be an agro-productive advantage of the plants sprinkled with the polymer. Soy, as a typical C3 plant, is more dependent on the dual function of the Rubisco enzyme, so chitosan treatments must shift the photosynthesis-respiration balance in favor of the first process and therefore towards better vegetative growth. It shows with the gain of a greater aerial and radical dry mass in the plants of this work with certain concentrations.

Although the mechanism of action through which chitosan increases crop growth and productivity is not fully understood, an antiperspirant effect has been reported when applied by foliar spray [42]. Chitosan causes stomatal closure in the leaves [46] and prevents water loss through perspiration (antiperspirant effect), which reduces water consumption by the plant and regulates its availability for the different processes of the plant, this being essential in C3 plants to benefit photosynthesis. To this must be added the increase found, with the application of chitosan, in the content of some essential nutrients related to vegetative development such as calcium, iron and manganese.

Chitosan stimulated morphoagronomic variables, relative chlorophyll content and root development, mainly at concentrations of 100 to 1000 mg L^{-1}, specifically; the application of 500 mg L^{-1} of chitosan improved the number of leaves by 19 %, the aerial dry mass in more than 40%, the root dry mass and the foliar area in 55 %. These results confirm what was obtained by other authors, with the foliar spraying of chitosan in legumes and other plant species [22,23,47,48]. However, the single inoculation with the symbiont improved the general behavior of the plants, the foliar nitrogen concentration (40 %), the number of leaves (10 %) and the aerial dry mass (21 %).

The effect on the growth of soybean plants from the results obtained, found in this work, was due to an increase in the content of nutrients available in the plant. Both as a result of inoculation with *Bradyrhizobium* and by the additional application of chitosan which, depending on the concentration used, improved the biological fixation of N and the activity of the NR enzyme beyond the effect of inoculation, which potentially improves the protein content and nitrogen compounds available to the plant for its growth. To the above, an improvement in the photosynthetic process of the evaluated plants must be added. That is why, future studies on this topic should be aimed at knowing how both forms of nitrogen entry and transport behave in soybeans, in different organs (nodules, roots and leaves) and time dynamics, when chitosan is applied by aspersion foliar, and inoculated with *Bradyrhizobium*. All this to elucidate the contribution of each pathway to the growth and productivity of IS-27 and in indicators related to photosynthesis.

Taking into account the results achieved in the nodulation, physiology and vegetative growth of plants inoculated with *Bradyrhizobium* and foliar sprayed with chitosan, these could have an impact on the increase in the yields of soybean *cv* IS-27, something to be demonstrated on a productive scale.

In this direction, some studies showed that there is a positive correlation between chlorophylls (0.85) and leaf area (0.743) with the photosynthetic rate of soybean plants in the V4 growth stage (49) and of this, in turn, with the crop yield.

CONCLUSIONS

- The foliar spraying of chitosan in stages V1 and V2 of the cultivar IS-27 stimulated nodulation, the concentration of macronutrients and trace elements in nodules and leaves of plants and the growth of soybeans inoculated with *B. elkanii*, under controlled conditions until stage V4, depending on the concentration of the polymer.
- The enzymatic activity of foliar nitrate reductase in stages V2 and V3 increased with the application of concentrations of 10 to 500 mg L^{-1} of chitosan, which also increased the content of total chlorophylls. In turn, the plants only inoculated with *Bradyrhizobium elkanii* favored the nitrate reductase activity of the soybean plants up to stage V3, in relation to the plants that were not inoculated.

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