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Original Article

Effect of chitosan molecular mass on germination and *in vitro* growth of soy

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ABSTRACT

Physical-chemical characteristics of chitosan influence the development of crops when applied as a biostimulants. The objective of the research was to determine the influence of chitosan of different molecular masses on the *in vitro* initial vegetative growth of soybean seedlings. The seeds were disinfected and imbibed for one hour in chitosan solutions at 10, 100 and 500 mg L^{-1}, with medium molecular mass (Q_1, 100 kDa) and low (Q_2, 25.3 kDa). After 72 hours of incubation the seeds were determined the percentage of germination and the length of the radicle, before placing in containers with sterile Hougland nutrient solution and, cultivated in a room of lights for 10 days (Phase R1). The results obtained showed the biostimulant effect of chitosan, when applied by imbibition of seeds, regardless of their molecular mass and concentration, in the percentage of germinated seeds, while the growth of the seedlings was influenced by the concentration of 500 mg L^{-1} of chitosan. The application of medium molecular mass chitosan (Q_1) increased the number of leaves, the length of the stem and roots, the radical dry mass and the leaf area of the seedlings. Not influenced on the dry mass of the aerial part was observed, however, Q_2 stimulated the dry biomass, while Q^1, increased the leaf area index (LAI) of the seedlings, by ten times with the concentration of 500 mg L^{-1}. Both chitosan polymers doubled the root-stem ratio (r/v), in relation to the control plants from seeds embedded in water. The effect of the molecular mass and concentration of chitosan on the biostimulant capacity of soybean seedlings when applied by seed imbibition was demonstrated.

Key words: biostimulant, concentration, imbibition, seeds

INTRODUCTION

Soy (*Glycine max* [L.] Merrill) is the most important oilseed in the world because of its grains, which constitute the main source of vegetable protein; various essential products are obtained in human and animal feed [1,2]. Soy cultivation ended with 348 million tons in the 2016-2017 campaign, constituting a world-wide historical record [3]. Due to its relevance, this crop has been used as a model in research aimed at the study of plant responses to the application of biological inducers. Among these, chitosan and its derivatives are recognized non-microbial biostimulants [4,5] widely used in agriculture because they improve germination, growth and crop yields under normal conditions and under abiotic stresses, in addition to being used in the coating of seeds and preservation of fruits and vegetables [5-7]. Chitosan is a copolymer of N-acetyl-D-glucosamine and D-glucosamine, which is obtained from the chitin present in the exoskeleton of crustaceans, when more than 80 % of the acetyl groups are, removed from the N-acetyl residues -D-glucosamine present in its precursor [6]. Its biological role in plants depends on its main physical-chemical characteristics (molecular mass, degree of acetylation and concentration), in addition to the form and timing of application [6,8]. The application of chitosan to seeds is one of the most important ways in the stimulation of germination, growth and yields of some crops, disease control or activation of innate plant defenses against pathogens, whether by imbibition, coating or priming of the seeds [6,9-11]. These studies have been carried out in legumes and the response in germination and plant growth has depended on the concentration of chitosan [12-14]. In soybeans, however, the influence of the chitosan molecular mass is not known when applied by imbibition of the seeds in the germination process and the early growth of the plants, so the objective of the present work was to compare the effect of two polymers of chitosan, of medium and low molecular mass, in germination and vegetative growth (V1) *in vitro*, by imbibition of seeds from the soybean cultivar IS-27.

MATERIALS AND METHODS

Seeds from the IS-27 cultivar of soybeans were disinfected with alcohol (75 %) and sodium hypochlorite (25 %) for five minutes and rinsed six times with sterile distilled water before being immersed for one hour in sterile chitosan solutions (100 mL) and distilled water, as a

control treatment. The chitosan concentrations tested were 10, 100 and 500 mg L^{-1} of polymers with molecular masses of 124 kDa (Q_1) and 25.3 kDa (Q_2) with 85 % average deacetylation. Then, the seeds were placed in Petri dishes with Phytoagar-water semi-solid vegetable medium (0.8 %) and incubated in the dark at 29 ºC for 72 hours for germination. After this time, the percentage of germination of the seeds (5 replicates of 15 seeds in each plate) and the length of the radicle (3 replicates of 10 seeds each) were determined, before being transferred to containers (0.41 kg), containing 20 mL of sterile Hougland nutrient solution, at the rate of four containers with 10 plants each, per treatment. The containers were placed in a light room: 12-hour photoperiod, temperature of 25-30 ºC and humidity of 60 % for ten days (phase R1) to perform morphoagronomic evaluations of vegetative development of seedlings. The evaluations were: number of trifoliate leaves (first pair), stem and radical length (cm), dry mass (MS) of the aerial part (PA) and roots (g) at 70 ºC for 72 hours, and the leaf area (AF, cm^2) with the portable meter AM 300, UK, to each seedling. In addition, the root-stem ratio (r/v) = root MS/stem DM was calculated and the leaf area index (IAF) = total plant AF/MS (cm^2 g^{-1}).

In the experiment repeated twice, a 3 x 2 bifactorial analysis was used, between the factors: concentration and chitosan compound, with three and two levels, respectively, compared with a reference control (imbibition control in water). The comparison of means was made with the use of the Tukey HSD Multiple Range Test ($p < 0.05$) to discriminate differences between the means, in the Statgraphics Plus program package, version 5.1.

RESULTS

In the process of germination of soybeans and the length of the radicle there was no interaction of the factors: concentration (10, 100 and 500 mg L^{-1}) and molecular mass of chitosans (124 kDa (Q_1), 25.3 kDa (Q_2), nor significant differences between the levels of each factor separately (Data not shown).

The percentage of germination that was determined after 72 hours of embedding the seeds with both polymers, showed higher values (82 and 87 %) and different values compared to those embedded in water (76.2 %). The radicle length values achieved with all treatments ranged between 5.3 and 5.7 cm long.

In the morphoagronomic response of soybean seedlings, despite having interaction between the factors studied in all the growth variables evaluated, a greater influence of the concentration than of the molecular mass of the chitosan polymers was observed (Table 1).

Table 1. Effect of seed imbibition for one hour with chitosans (Q_1 and Q_2) on the vegetative growth of soybean seedlings after ten days after treatment.

Chitosan (mg L^{-1})	Nu. leaves	Stem length (cm)	Root length (cm)	Dry mass PA (g)	Radical dry mass (g)	Foliar area (cm^2)
Control	0.56	7.41	12.84	0.094	0.022	5.41
Q_1- 10	0.59 a	10.95 b	16.90 b	0.086 a	0.032 b	5.49ab
Q_1- 100	0.32 b	9.66 c	16.51 b	0.075 b	0.031 b	5.08 ab
Q_1- 500	0.40 b	12.27 a	22.04 ab	0.079 ab	0.037 a	6.26 a
Q_2- 10	0.29 b	10.25 bc	17.99 ab	0.078 ab	0.035 ab	4.80 b
Q_2- 100	0.60 a	11.49 ab	23.03 a	0.080 ab	0.038 a	5.04 ab
Q_2- 500	0.66 a	11.16 ab	20.40 ab	0.084 a	0.038 a	5.18 ab
ES $\overline{X}$	0.06*	0.42*	1.79*	0.003*	0.001*	0.36*

Equal letters do not differ statistically for $p < 0.05$, according to the Tukey HSD Test

The number of trifoliate leaves was stimulated with 10 mg L^{-1} of the highest mass chitosan (Q_1) and the 100 and 500 mg L^{-1} concentrations of the lowest molar mass chitosan (Q_2), but only with the highest concentration of Q_2 they obtained higher and different values of the seedlings that were embedded in water (Table 1).

The chitosan of greater mass (Q_1) at the concentration of 500 mg L^{-1} stood out in the stem length, but without differences with the chitosan of smaller mass (Q_2) at the higher concentrations, while the latter chitosan, at the concentration of 100 mg L^{-1}, it was who increased the radical length, with significant differences of 10 and 100 mg L^{-1} of chitosan Q_1 (Table 1).

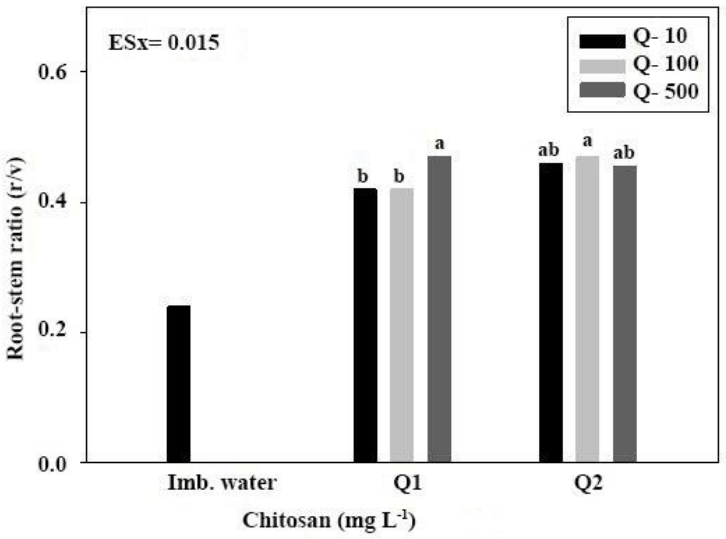
Despite the dry mass of the aerial part being stimulated with the concentration of 10 mg L^{-1} of Q_1 and 500 mg L^{-1} of Q_2, they only differed from the concentration of 100 mg L^{-1} of the larger mass chitosan; these did not surpass the control plants (seeds embedded in water). On the contrary, the radical dry mass increased due to the imbibition of the seeds with the chitosans, specifically with the chitosan Q_2 at the concentrations of 100 and 500 mg L^{-1} in addition to the latter concentration of Q_1 (Table 1).

As for the leaf area, only the concentration of 500 mg L^{-1} of the largest mass chitosan stimulated this variable, with respect to the control seedlings (Table 1).

In general, the morphoagronomic response of soybeans to seed imbibition depended on the concentration of the polymers, obtaining the greatest increases with 500 mg L^{-1}, which in some variables did not differ from 100 mg L^{-1} with both chitosans.

Soybean growth can be quantified by dry mass and leaf area

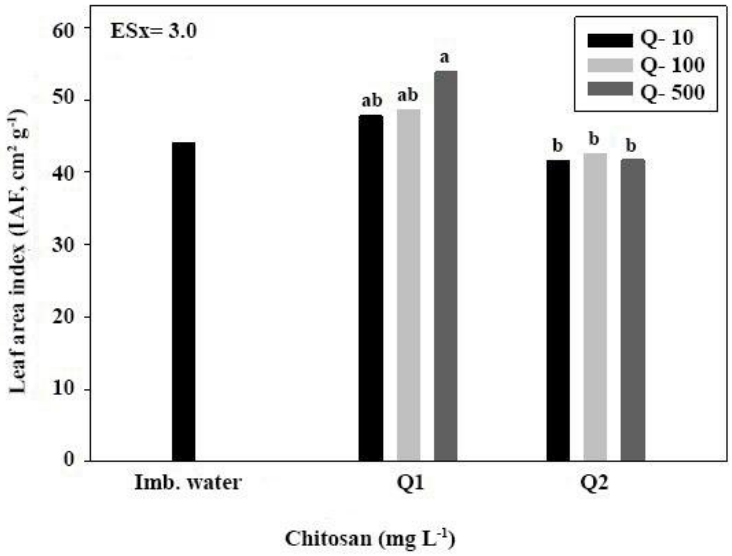
Total of the plant, the latter being the most relevant variable to quantify the production per unit area [15]. For this reason, in this work, the proportions obtained with the dry biomass gain and the leaf area index (IAF) of the seedlings, caused by the interaction between concentrations and chitosan compounds (Figures 1 and 2) were determined.



Equal letters do not differ statistically for p <0.05, according to the Tukey HSD test

Figure 1. Root-stem ratio (r/v) of soybean seedlings ten days after the imbibition of seeds with chitosans of different molecular mass (Q_1 and Q_2)

Figure 1 shows an increase in the proportion of the biomass of the seedlings (roots and stem) due to the application of both chitosan polymers, which doubles the value obtained in control plants from seeds embedded in water. The concentrations of 500 mg L^{-1} of the polymer of greater molecular mass (Q_1) and 100 mg L^{-1} of Q_2 were more effective in increasing the ratio (r/v) with respect to the effect caused by the concentrations of 10 and 100 mg L^{-1} of Q1.



Equal letters do not differ statistically for p <0.05, according to the Tukey HSD Test

Figure 2. Foliar area index (IAF) of soybean seedlings ten days after imbibition of seeds with chitosans of different molecular mass (Q_1 and Q_2)

The leaf area index (IAF) of soybeans increased with the highest molecular mass chitosan (Q_1), with increments of ten percent more with the concentration of 500 mg L^{-1} when applied by seed imbibition (Figure 2).

DISCUSSION

Results of investigations with chitosan as a stimulator of the growth of several plant species and culture conditions, refer to the influence of molecular mass and the concentration of chitosan in the development of plants [6,8,9,16]. The effect of molecular mass and the form of application of chitosan has been demonstrated in the stimulation of rice growth (*Oryza sativa* L.) and the yield of flowers and strawberry corms (*Fragaria* sp.) [17,18]. In soy, the response in nodulation and plant growth has been affected by the influence of concentration, molecular mass and the mode of application of chitosan compounds [17,19,20].

Although beneficial effects on its development have been found in legumes with the application of chitosan by different methods of treatment to seeds [12,21,22], it is not known how the molecular mass differences of chitosans influence soy germination by imbibition of the seeds. In this sense, when comparing the chitosans of different molecular masses by imbibition of seeds, slight differences were found between the polymers of medium (Q_1) and low (Q_2) molecular mass, and these differences were due rather to the concentration of

chitosan, in the initial *in vitro* vegetative development of soy (Table 1, Figures 1 and 2). The germination percentage was stimulated with the application of both polymers with respect to the seeds embedded in water, while the length of the radicle of the germinated seeds was not modified neither by the molecular mass nor by the chitosan concentrations evaluated (Data not shown).

In all the variables of growth of the cultivar IS-27 of soybean there was a stimulating effect of the concentration of 500 mg L^{-1}, except in the emission of leaves with the polymer of greater molecular mass (Q_1), in addition to increasing the index of leaf area with Q_1 (Table 1, Figure 1). Zeng & collaborators [12] proposed that the stimulating effect of seed treatment with chitosan on germination, growth, yields and protection of soybeans against insects is caused by the formation of a semipermeable film on the surface of the seed, it maintains the humidity of the same and absorbs moisture from the soil, thus promoting germination. In addition, it prevents the entry of oxygen and restricts the loss of carbon dioxide (CO_2) in the seed, maintaining a high concentration of CO_2 in the film that prevents breathing and decreases the consumption of nutrients inside the seed. On the other hand, chitosan can increase soluble sugars and reinforce the proteolytic activity that generates the release of free amino acids that have an inhibitory effect on many plant pathogenic fungi.

In legumes, as in other crops, the r/v ratio has been determined to know the influence of multiple factors such as genotypes, growth stages, tillage system, and water deficit in the soil, among others [23-25]. In the R1 phase of seedling growth, the root-stem biomass ratio showed differences in favor of the application of chitosan, with twice the increase of the control plants (Figure 1). An increase in root development in length and dry mass was observed that could favor the absorption and translocation of nutrients from the root to the area part of the plant (Table 1). Several works refer to the stimulating effect of chitosan on the growth of crops as a result of increasing the availability and absorption of nutrients and the process of photosynthesis through the accumulation of metabolites and the increase of foliar pigments [6,11,26,27]. In particular, the treatment of seeds with chitosan improves the assimilation of nitrogen in soybean IS-27 plants due to an increase in the nitrate reductase enzyme activity and the accumulation of foliar nitrogen [22] and unpublished results.

The leaf area index (IAF) can be related to the processes of photosynthesis, respiration, and crop productivity [15]. In soybeans, the leaf area of the plants differs for different genotypes and planting moments [28]. At work, the leaf area of the seedlings was increased with the

concentration of 500 mg L^{-1} of the polymer with the highest molecular mass (Q_1), as well as the IAF, with the three concentrations evaluated (10, 100 and 500 mg L^{-1}) (Table 1, Figure 2). This stimulation in the foliar area of soybeans has been previously informed, when applied to the seeds together with the Azofert® inoculant, prior to sowing and, by foliar spray in the V2 and R2 phases in the field, with the concentration of 1 g L^{-1} of the polymer of medium molecular mass used in this work, with increases of 62.85 % with respect to the control plants [22]. This effect has also influenced the photosynthetic activity (leaf area and pigments) of bean plants (*Phaseolus vulgaris* L.), with the application of chitosan nanoparticles of low molecular mass (27 kDa) combined with gibberellic acid [29].

Taking into account the above and the results of this work it can be concluded that polymers of medium and low molecular mass cause slight biological differences in the initial stages of seedlings grown in Hougland solution

CONCLUSIONS

- The *in vitro* development of the IS-27 soybean seedlings, until the R1 phase, was influenced by the imbibition of the seeds with the chitosan polymers.
- The differences in molecular mass of chitosans (124 kDa (Q_1) and 25.3 kDa (Q_2) influenced germination and the majority of morphoagronomic growth variables.
- The concentration of 500 mg L^{-1} of both chitosans was highlighted in the initial development of soybean seedlings.

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