CHITOSAN MIXED WITH BENEFICIAL FUNGAL CONIDIA OR FUNGICIDE FOR BEAN (*Phaseolus vulgaris* L.) SEED COATING

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SUMMARY

*Chitosan has been recommended for agricultural applications as an adherent, additive or coating for seeds. It has also been reported to induce endogenous defenses and promote the establishment and development of seedlings. However, these properties seem to be associated to the evaluated species. In this paper, two kinds of chitosan coatings were generated and mixed with fungicide and with beneficial fungi conidia (*Beauveria bassiana *and* Trichoderma harzianum*). Chitosan from two sources (shrimp and insect Pterophylla beltrani) was used. Coating mixtures were applied on the surface of bean (*Phaseolus vulgaris *L.) seeds by immersion. Neither coating treatment with chitosan affected seed germination. In the case of coating with fungicide, the shrimp chitosan allowed gradual release of the chemical agent on the seed surface for a longer time. Moreover, an important part of* B. bassiana *conidia incorporated into the coatings germinated, indicating that this type of biological agent can be used in coatings to provide seeds with biological protection. Little germination of* T. harzianum *conidia was observed. More studies are needed to establish times and conditions to control germination of conidia in the coatings.*

Introduction

An artificial seed coating is a layer that covers the entire seed surface. It is usually formed by inert materials to provide adequate handling, promote particular microenvironments and protect seeds against pathogens and insect damage (Giménez-Sampaio *et al*., 1992; Ziani *et al*., 2010; Zeng *et al*., 2012). Usually, seed coatings function as carriers for pesticides which protect the seeds and the emerging seedlings (Vavrina and McGovern, 1990; Kaufman, 1991). The coating agent should not be toxic or harmful for the plant or the environment. Chitosan is a natural polymer that has shown good results when applied as seed coating for some crops (Benhamou *et al*., 1994; Bhaskara Reddy *et al*., 1999; Boonlertnirun *et al*., 2008; El Hadrami *et al*., 2010). This polymer is obtained from the deacetylation of chitin, the predominant component of arthropod exoskeletons and of cell walls of several fungi. Because it is biodegradable and non-toxic, and has antimicrobial properties, chitosan is seen as a versatile material for several agricultural applications (Badawy and Rabea, 2011). It has been shown that chitosan elicits defense mechanisms in plants through induction of glucanases, chitinases, phenolic compounds, terpenoids, PR proteins, protease inhibitors, and compounds associated with oxidative burst, lignification and callose deposition (Bautista-Baños *et al*., 2006; Franco and Iriti, 2007; Mandal and Mitra, 2007; Hadwiger, 2013; Mejia-Teniente *et al*., 2013).

Chitosan has been used to coat corn, tomato, rice and wheat seeds and has been associated with several effects that include better physiological quality, increased vigor, higher germination rates and induction of plant defenses (Benhamou *et al*., 1994; Bhaskara Reddy *et al*., 1999; Boonlertnirun *et al*., 2008; Ziani *et al*., 2010; Zeng *et al*., 2012). This underlines the value of chitosan used as an additive for seed coating. The use of agrochemicals in combination with chitosan has been evaluated and has yielded good results (Kashyap *et al*., 2015; Symonds *et al*., 2016). However, compatibility of this polymer with beneficial microorganisms that are frequently incorporated in some seed coats remains to be explored in depth.

Incorporation of beneficial agents into seed coatings has yielded good results in terms of plant protection, production or endophytic fungus colonization. The most common beneficial agents used for seed coating include *Pseudomonas* spp., *Gliocladium* spp., *Glomus* spp., *Trichoderma* spp. and *Beauveria bassiana*, which are associated with mineral solubilization, plant defense promotion, insect infection and antagonism on phytopathogens. Usually, these beneficial agents are mixed with adhesive components of polymeric nature, including xanthan gum, methylcellulose, latex derivatives

KEYWORDS / *Beauveria bassiana* / Biofunctional Coating / Germination / *Trichoderma harzianum* / Ziram® /

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QUITOSANO EN MEZCLA CON ESPORAS DE HONGOS BENÉFICOS O CON FUNGICIDA PARA RECUBRIMIENTO DE SEMILLAS DE FRIJOL (*Phaseolus vulgaris* L.)

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RESUMEN

*El quitosano ha sido sugerido como un compuesto con aplicaciones agrícolas, entre ellas su uso como adherente agrícola o como recubrimiento para semillas. Se ha reportado que induce las defensas endógenas y promueve el establecimiento y desarrollo de plántulas; no obstante, parece que estos atributos están asociados a las especies evaluadas. En este trabajo, se generaron recubrimientos de quitosano en mezcla con un fungicida y con esporas de hongos benéficos (*Trichoderma harzianum *y* Beauveria bassiana*). Los recubrimientos están basados en dos tipos de quitosano, el cual proviene de camarón y de Pterophylla beltrani. Dichos recubrimientos se aplicaron sobre la superficie de la semilla de frijol (*Phaseolus vulgaris *L.) mediante inmersión. La presencia de los recubrimientos no afectó los porcentajes de germinación bajo ningún tratamiento. La incorporación de fungicida en mezclas con quitosano permitió que el agente químico se mantuviera por más tiempo sobre la superficie de la semilla. Además, una parte importante de las esporas de* B. bassiana *incorporadas a los recubrimientos germinó, lo que indica que este tipo de agente biológico se podría incorporar como parte de recubrimientos para brindar protección biológica a las semillas. Se observó poca germinación de conidia de* T. harzianum. *Se requieren estudios adicionales para establecer los tiempos y condiciones para controlar las conidias en los recubrimientos.*

QUITOSANO EM MISTURA COM ESPORAS DE FUNGOS BENÉFICOS O CON FUNGICIDA PARA REVESTIMENTO DE SEMENTES DE FEIJÃO (*Phaseolus vulgaris* L.)

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RESUMO

*O quitosano tem sido sugerido como um composto para aplicações agrícolas, incluindo o uso como um adesivo agrícola ou como revestimento para as sementes. Tem sido relatado para induzir defesas endógenas e promove a criação eo desenvolvimento de mudas; No entanto, parece que estes atributos são associados com as espécies testadas. Neste papel, revestimentos de quitosano foram gerados em mistura com um esporos fungicidas e fungos benéficos (*Trichoderma harzianum *e* Beauveria bassiana*). Os revestimentos são baseados em dois tipos de quitosana, que vem de camarão e* Pterophylla beltrani. *Estes revestimentos foram aplicados sobre a superfície da semente de feijão (*Phaseolus vulgaris *L.), por imersão. A presença dos revestimentos não afetou as taxas de germinação sob nenhum tratamento. A incorporação de misturas fungicidas com quitosano permitiu que o produto químico é retida mais tempo na superfície da semente. Enquanto um importante esporos de* B. bassiana *parte incorporados nos revestimentos germinadas, indicando que este tipo de agente biológico pode ser incorporado como parte de revestimentos para proporcionar protecção biológica para as sementes.*

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and synthetic adhesives to produce seed coatings (Mao *et al*., 1997; Tefera and Vidal, 2009; Brownbridge *et al*., 2012; Colla *et al*., 2015). Nevertheless, combinations of chitosan and beneficial microorganisms in seed coatings are less frequent. Recently, the tolerance of some beneficial microorganisms (yeast cells and *Trichoderma* spores) to chitosan and some potential applications have been demonstrated (Saifuddin and Raziah, 2007; Spasova *et al*., 2011).

Use of chitosan in seed coatings can potentially promote early plant defense responses, but in addition, the incorporation of agrochemicals and microorganisms could increase seed and seedling protection. However, chitosan varies because of the variability of its production (Lertsutthiwong *et al*., 2002; Abdou *et al*., 2008) and the nature of the agrochemical or biological agent to be incorporated, and each formulation should be evaluated. Therefore, the aim of this study was to evaluate application of various formulations of chitosan in mixtures with a fungicide or with conidia of beneficial fungi to determine their feasibility as a crop seed coating.

Materials and Methods

Preparation of seeds

Bean (*Phaseolus vulgaris* L.) 'negro Jamapa' seeds were acquired from a local supplier in Ciudad Victoria, Tamaulipas, Mexico, and damaged seeds were discarded. For fungicide retention tests and for testing coatings with conidia, seeds were disinfected with commercial 10% NaClO solution for 5min and rinsed three times for 5min with sterile distilled water. The seeds were then placed on sterile paper and air dried inside a laminar flow hood for 24h before coating and subsequent testing.

Chitosan preparation

For coatings, two types of chitosan were used. Chitosan from shrimp was prepared from commercial chitin (Sigma-Aldrich, St. Louis, MO, USA) by deacetylation in 70% NaOH solution at 120ºC for 1h and left to rest for 12h at room temperature before extensive washing with distilled water. Chitosan was dried at 60ºC for 12h and then dissolved to a concentration of 2% in 5% acetic acid by constant stirring. The solution pH was adjusted to 6 with 2M NaOH, and dialyzed for salt removal using distilled water for 3 days with three daily water changes. Dialysis was done with a standard RC membrane Spectra/Por® 6 and pre-wetted dialysis tube with 25kDa molecular weight cutoff (SpectrumLabs, USA). After this, the solution was adjusted to pH 6 using 1% HCl. This solution was used to formulate treatments, adjusting it to the required concentration

in each experiment. The degree of deacetylation was determined by the potentiometer titration method (Yuan *et al*., 2011).

Insect chitosan was obtained from adult corpses of *Pterophylla beltrani* (Bolivar & Bolivar), which were processed with adaptations to the procedure of Torres-Castillo *et al*. (2015). Ground insects were first washed with 250mM NaOH and then with 500mM NaOH at 90ºC, followed by five rinses with distilled water. After this, the resulting material was subjected to deacetylation as indicated above. This chitosan solution was also subjected to dialysis and the degree of deacetylation was determined by the potentiometer titration method (Yuan *et al*., 2011).

Seed germination with commercial chitosan and mixtures of chitosan with fungicide

Six treatments were prepared under aseptic conditions. DW: distilled water (control); FS: 0.5% fungicide solution Ziram® (FMC Agroquímica of Mexico, Zapopan, Jalisco, Mexico) in distilled water; C0.25: 0.25% chitosan in distilled water; C1: 1% chitosan in distilled water; C0.25F: 0.25% chitosan solution mixed with 0.5% Ziram® fungicide; and C1F: 1% chitosan solution mixed with 0.5% Ziram® fungicide. The fungicide was dissolved in water, to be later incorporated into the mixture of chitosan, and stirred periodically to prevent aggregate formation or sedimentation. Previously disinfected and dried seeds were individually immersed in their respective solution for 2-5s and then dried for 48h at room temperature (28-30 C). In each treatment, a sterile glass beaker with 200ml of solution was used to immerse the seeds. To evaluate germination, 100 seeds per treatment were tested in Petri dishes with Whatman No. 1 filter paper moistened with 5ml of sterile distilled water. Seeds were considered germinated when roots at least 5mm long were present; the number of germinated seeds was recorded daily and germination percentage was calculated after 72h. The experiment was performed in triplicate.

Fungicide retention test

Because chitosan has been suggested as an agent for retention and gradual release of various compounds, the ability of shrimp chitosan to retain Ziram® fungicide on the surface of bean seeds was assessed. A total of 200 seeds per treatment were coated by individual immersion in the corresponding solution for 2-5s and dried for 24h at room temperature (28-30ºC). A group of 50 seeds per treatment was reserved until use, the remaining 150 per treatment were washed in sterile distilled water. The fungicide retention test was related to permanence of the fungicide effect on seeds from treatments FS, C0.25F and C1F after washing with sterile water. Groups of 150 seeds per treatment were placed in containers with 500ml of sterile distilled water, which was changed every 2h over an 8h period. After 1h, 50 washed seeds per treatment were removed; after 2h, another 50 seeds per treatment were removed; and finally, after 8h, the last 50 seeds per treatment were removed. All seeds were dried for 1h at room temperature (28-30ºC) inside a laminar flow hood. Each 50-seed treatment had different exposure times to rinses. To determine permanence of the fungicide, all rinsed and dried seeds were placed in Petri dishes with potato dextrose agar (PDA, Bioxon) culture medium forming two groups of 25 seeds (experiment in duplicate). Then, each seed was inoculated individually with 10µl of a solution of *Fusarium oxysporum* conidia at a concentration of 1×10^5 conidia/ml. Conidia were harvested by washing the surface of a 5 days old *F. oxysporum* culture with 15ml of 0.05% Tween 80 (Sigma-Aldrich) sterile solution. Conidia concentration was adjusted to 1×10^5. Petri dishes with inoculated seeds were incubated 72h at room temperature (28-30ºC). Presence of mycelial growth on seeds was related to conidia germination, and seeds with mycelial growth from treatments FS, C0.25F and C1F were considered to have lost fungicidal effect.

Germination of seeds coated with shrimp or insect chitosan and mixtures of each with conidia

Since chitosan is associated with the natural development of entomopathogenic and antagonistic fungi, we included *B. bassiana* and *T. harzianum* conidia in the formulations of shrimp and *P. beltrani* chitosan to coat bean seeds. Conidia were harvested by washing the surface of a 10 days old solid fungal culture with a sterile solution of 0.05% Tween 80 (Sigma-Aldrich) and concentration was adjusted to 2.5×10^5. Treatments for the experiment with *B. bassiana* included DW: distilled water control; C0.25: shrimp chitosan 0.25%; C1: shrimp chitosan 1%; C0.25Bb: shrimp chitosan 0.25% with *B. bassiana* conidia; C1Bb: shrimp chitosan 1% with *B. bassiana* conidia; CP0.25: *P. beltrani* chitosan 0.25%; CP1: *P. beltrani* chitosan 1%; CP0.25Bb: *P. beltrani* chitosan 0.25% with *B. bassiana* conidia; and CP1Bb: *P. beltrani* chitosan 1% with *B. bassiana* conidia. For the experiment with *T. harzianum* conidia, the treatments were: DW: distilled water control; C0.25: shrimp chitosan 0.25%; C1: shrimp chitosan 1%; C0.25Th: shrimp chitosan 0.25% with *T. harzianum* conidia; C1Th: shrimp chitosan 1% with *T. harzianum* conidia; CP0.25: *P. beltrani* chitosan 0.25%; CP1: *P. beltrani* chitosan 1%, CP0.25Th: *P. beltrani* chitosan 0.25% with *T. harzianum* conidia; and CP1Th: *P. beltrani* chitosan 1% with *T. harzianum* conidia. Seeds previously disinfected and dried were subjected to individual immersion in the respective solution during 2-5s and then deposited onto clean waxed paper and dried for 48h at room temperature (28-30ºC). To evaluate germination, 100 seeds were used for each treatment in Petri dishes with Whatman No. 1 filter paper. Seeds were moistened with 5ml of sterile distilled water; moisture was kept by adding 1.5ml of sterile distilled water every other day. Germination was recorded daily and germination percentage for each treatment was calculated after 72h. The experiment was performed in triplicate.

Statistical analysis

The data were analyzed using a completely randomized design by analysis of variance using the Statistical Analysis System version 6.2 (SAS Institute, Inc., Cary, North Carolina). Means were compared by Tukey ($p<0.05$).

Results and Discussion

Effect of coatings on germination

Coatings from the two sources of chitosan formed a continuous translucent film with a dusty appearance when dry. FS, C0.25F and C1F produced seeds with dusty whitish coatings; seeds treated with *B. bassiana* and *T. harzianum* had dusty translucent coatings. Viability of conidia mixed with chitosan was related to mycelial growth on some seeds; abundant mycelial growth for *B. bassiana* but scarce for *T. harzianum* was observed.

Despite the wide diversity of applied treatments and the 70% germination in some treatments, no significant differences in seed germination percentage were observed ($p<0.05$). This confirmed that chitosan can be useful as an adherent agent (Figure 1) since it did not affect the germination process. Seeds coated with 0.25% to 1% chitosan mixtures and their respective mixtures with fungicides showed no significant difference in germination rate (Figure 1a). Furthermore, germination of seeds coated with the two types of chitosan and at different concentrations

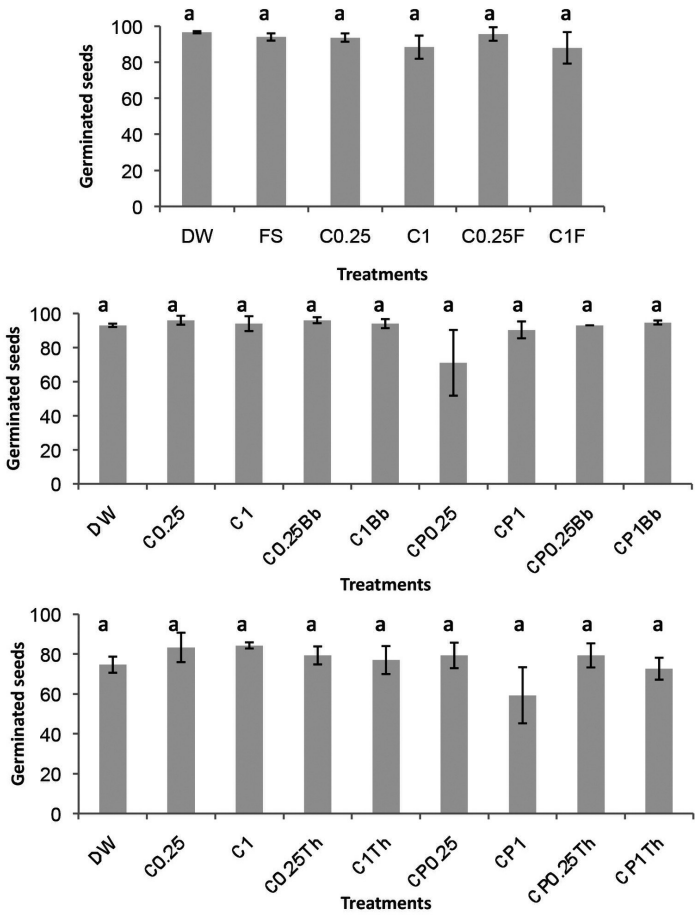

Figure 1. Germination percentage. a: germination percentages of seeds treated with chitosan coatings and fungicide, and control treatments. b: germination percentages of seeds with chitosan coatings, chitosan mixtures with *Beauveria bassiana* conidia, and control solution. c: germination data of seeds coated with chitosan mixtures with *Trichoderma harzianum* conidia, and control solution. Same letters indicate no significant differences (P <0.05).

was not statistically different from the control. In addition, none of the two types of chitosan mixed with conidia of fungal species negatively affected seed germination (Figure 1b and c). These results show that application of these chitosan coatings and derivatives did not affect the hydration process or germination percentages of bean seeds. Regarding the effects of chitosan on germination, different reports indicate an inductive effect of germination of some species, such as *Sorghum* or Egyptian anise, but can inhibit germination of lettuce seeds, while others report that it may or may not affect germination rates compared to control treatments. These reports indicate that the effects are variable and will depend on the nature of the chitosan, its molecular size, crop characteristics and growth conditions (Lizárraga-Paulín *et al.*, 2011; Goñi *et al.*, 2013; Mahdavi and Rahimi, 2013; Hameed *et al.*, 2013, 2014). In our study, the degree of deacetylation was 68.5% for shrimp chitosan and 72.4% for chitosan from *P. beltrani*; and the molecular weight was >25kDa, relative to the dialysis membrane used.

Retention of fungicide on the surface of seeds treated with chitosan

Application of chitosan for gradual release of drugs and agrochemicals has been reported (Teixeira *et al.*, 1990; Bansal *et al.*, 2011). For this reason, a fungicidal agent was included as part of the chitosan coating formulation.

Coating formulations with chitosan allowed functionality and retention of the fungicide on the seed surface. Rinse times of seeds were related with the absence or presence of *F. oxysporum* mycelia on the seed surface as an indication of either prevalence or loss of fungicidal effect. Presence of mycelia at 72h in most seeds in DW, C0.25 and C1 was observed. In contrast, in the case of FS, C0.25F and C1F, the presence of mycelia was minimal for water immersion times of 0 and 1h. However, as immersion time increased, more mycelia-covered seeds were observed. Seeds from FS showed gradual mycelial growth in accord with rinse time. When comparing the three formulations with fungicide, most of the seeds with the same rinse times had mycelia after 8h. Therefore, the longer the contact with the medium, the greater the release of the agrochemical when no adherent is present. In the case of C0.25F and C1F, the fungicidal effect remained on most of the seeds, even after immersion in water for 8h (Figure 2), confirming that chitosan coatings retained the fungicide and prevented fast release into the medium, as suggested by Roy *et al.* (2014). This theoretically would increase the protection time on the seed and in its surroundings and decrease the impact on soil microbiota by preventing diffusion into the soil, due to confined fungicide application.

Seed coating with chitosan and conidia

The feasibility of a biofunctional coating based on chitosan

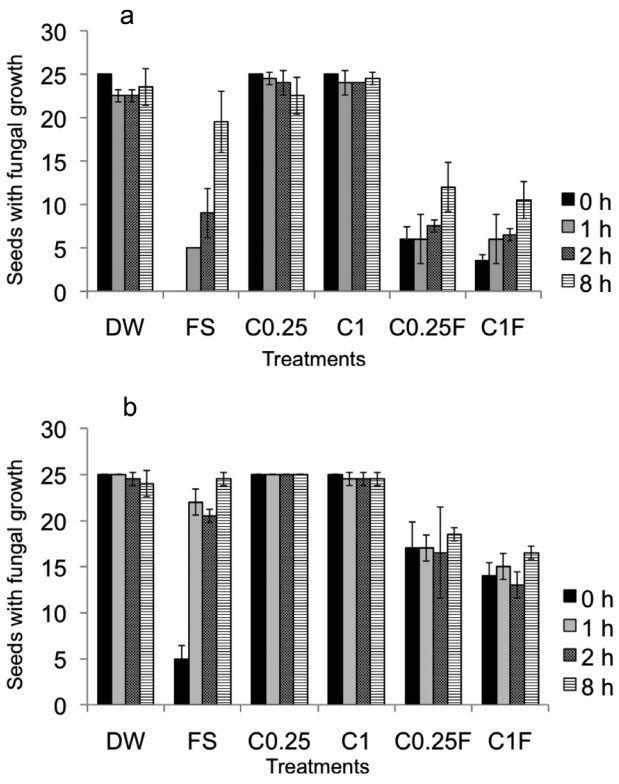

Figure 2. Retention of fungicide in chitosan coating. a: total number of seeds with mycelial growth of *Fusarium oxysporum* after 72h; b: total number of seeds with mycelial growth of *Fusarium oxysporum* after 96h. DW: treatment with water, FS: fungicide treatment dissolved in water, C0.25: treatment with chitosan 0.25%, C1: treatment with chitosan 1%, C0.25F: treatment with chitosan 0.25% mixed with the fungicide, C1F: treatment with chitosan 1% mixed with fungicide. Rinse times indicated in hours (h).

and *Trichoderma* conidia for plant protection was explored *in vitro* with the combination of chitosan and *T. harzianum* spores against sapstain fungi, and also on controlling *Fusarium* and *Alternaria* strains (Chittenden and Singh, 2009; Spasova *et al.*, 2011). Therefore, the possibility of forming seed coatings that allow germination and retention of conidia of beneficial fungi on the seed surface was evaluated.

B. bassiana and *T. harzianum* conidia were incorporated into coatings based on the two types of chitosan and were applied homogeneously. During the tests, no changes were observed in percentage of seed germination as shown above (Figure 1b and c). In the case of *B. bassiana,* mycelial growth in 45% of the treated seeds was recorded; while *T. harzianum* mycelia appeared on only 2% of the treated seeds. The fact that *B. bassiana* conidia could generate mycelia in the coating on a significant percentage of the seeds can be linked to the ability of the fungus to take advantage of the chitosan as a source of C and N (Palma-Guerrero *et al.*, 2010). In the case of *T. harzianum*, evaluation is required to determine whether the conidia remained viable in the coating or were unable to germinate. For both fungi, it would be of interest to conduct extensive tests for handling times and conditions that trigger germination when incorporated into the coating to increase success of *in situ* biocontrol.

Chitosan is considered an trigger agent of plant defenses, as well as having antimicrobial effect (Bautista-Baños *et al.*, 2006). These properties have made chitosan one of the most widely recognized agents for defense induction in several crops (Thakur and Sohal, 2013). Chitosan concentrations used in this study did not affect germination of *B. bassiana* conidia, similar to results reported previously. Nor was *F. oxysporum* conidial germination inhibited, a result opposite to other reports (Palma-Guerrero *et al.*, 2008), but this may be related to the nature of chitosan used. However, an inhibitory effect on the germination of conidia of *T. harzianum* cannot be ruled out. Palma-Guerrero *et al.* (2008) observed inhibition of *T. harzianum* and *T. atroviridae* exposed to 0.01 and 1mg·ml^{-1}, which could be indicative of generalized sensitivity of some *Trichoderma* species. Although concentrations of up to 1% chitosan can be used to include active conidia of some beneficial fungi such as *B. bassiana*, the viability of seed coatings requires conidia tolerance, which will depend on the fungal species or strains. Therefore, evaluation of each case is recommended. Like the inclusion of a fungicide agent and conidia, it is possible to include compounds such as *Bacillus thuringiensis* toxins, antimicrobial peptides or bioactive proteins (protease inhibitors, cyclotides, chitinases), phytohormones, nitrogen-fixing bacteria or growth promoters, among other components that could protect the seeds or seedling establishment (Pérez-Quiñones *et al.*, 2010; Fan *et al.*, 2012).

The success of chitosan as a retaining agent for the development of seed coatings with agrochemicals and biological agents was confirmed. Germination of bean seeds was not affected using chitosan coatings. Conidia germination of *B. bassiana* was higher than that of *T. harzianum*, which indicates differential sensitivity to chitosan. Success of seed coatings depends on many factors, including surface characteristics, architecture of seed surface, presence of trichomes, seed physiology, additive nature of chitosan, seed genetics and propagules or microorganism responses. Therefore, it is important to emphasize the need for a holistic perspective in generating coatings and studying their effect on germination stages subsequent to determining their impact on the establishment of the seedlings and on the rhizosphere.

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