ABRATES

Journal of Seed Science

ISSN 2317-1545
www.abrates.org.br/revista

*ARTICLE*

Journal of Seed Science, v.43, e202143036, 2021

http://dx.doi.org/10.1590/2317-1545v43254286

# Chitosan coating on bean and maize seeds: release of agrochemical fungicide and post-storage condition

**Nancy Araceli Godínez-Garrido[1], Juan Gabriel Ramírez-Pimentel[1], Jorge Covarrubias-Prieto[1], Francisco Cervantes-Ortiz[1], Artemio Pérez-López[2], Cesar Leobardo Aguirre-Mancilla[1]***

**ABSTRACT:** Chitosan is a biopolymer obtained from deacetylation of chitin; it has multiple applications in agriculture as an antifungal, soil conditioner, inducer of defense mechanisms, fruits postharvest coating, leaves and seeds, among others. The objective in this research was to evaluate the effect of chitosan coatings mixed with fungicide (dithiocarbamate) on the germination and germination speed of bean and maize seeds in storage and to determine the retention capacity of the fungicide in the coated seeds under different times of imbibition. Two coating treatments at concentrations of 0.1 and 0.5% chitosan in water, two coatings treatments at 0.1 and 0.5% chitosan supplemented with 0.5% fungicide and a coating without chitosan using only 0.5% fungicide in water were used in bean and maize seed; and as control seeds imbibed in distilled water were used; after treatments, germination percentage and germination speed were determined, also fungicide release were determined at 0, 1, 2 and 6 h of imbibition, and the effect of storage time on germination and germination speed was determined at 30, 60, 90, 120, 150 and 180 days of storage at 4 °C and 45% relative humidity. The fungicide release effect was determined by inhibiting *Fusarium oxysporum* conidia germination. There were no negative effects of coatings on seed germination after storage. The treatment that provided both greater retention of the fungicidal agent and released it gradually, was 0.5% chitosan mixed with fungicide concentration. Chitosan coating seeds mixed with fungicide do not cause negative changes in seed germination or germination rate.

Index terms: film, germination, storage, *Fusarium oxysporum.*

**RESUMO:** A quitosana é um biopolímero obtido a partir da desacetilação da quitina, tem múltiplas aplicações na agricultura como antifúngico, condicionador de solo, indutor de mecanismos de defesa, revestimento pós-colheita de frutos, folhas e sementes, entre outros. O objetivo desta pesquisa foi avaliar o efeito de revestimentos de quitosana misturados com fungicida (ditiocarbamato) na germinação e velocidade de germinação de sementes de feijão e milho armazenadas e determinar a capacidade de retenção do fungicida nas sementes revestidas sob diferentes tempos de embebição. Dois tratamentos de revestimento usando quitosana nas concentrações de 0,1 e 0,5% de quitosana em agua, dois tratamentos de revestimento usando quitosana nas concentrações de 0,1 e 0,5% de quitosana suplementados com fungicida 0,5% e um revestimento usando apenas 0,5% de fungicida em água foram aplicados como recobrimento em sementes de feijão e milho; e como controle foram utilizadas sementes embebidas em água destilada; após os tratamentos, a porcentagem de germinação e a velocidade de germinação foram determinadas, também a liberação de fungicida foi determinada em 0, 1, 2 e 6 h de embebição, e o efeito do tempo de armazenamento na germinação e na velocidade de germinação foi determinado a 30, 60, 90, 120, 150 e 180 dias de armazenamento a 4 °C e 45% de umidade relativa. O efeito da liberação do fungicida foi determinado pela inibição da germinação de conídios de *Fusarium oxysporum*. Não houve efeitos negativos dos revestimentos na germinação da semente após o armazenamento. O tratamento que proporcionou maior retenção do agente fungicida e liberou-o gradativamente foi a concentração de 0,5% de quitosana misturada ao fungicida. O recobrimento de sementes com quitosana misturadas com fungicida não causam mudanças negativas na germinação das sementes ou na taxa de germinação.

Termos para indexação: filme, germinação, armazenamento, *Fusarium oxysporum.*

***Corresponding author**
E-mail: cesar.am@roque.tecnm.mx

**Received:** 05/22/2021.
**Accepted:** 10/19/2021.

[1]Tecnológico Nacional de México / I.T. de Roque, km 8 Carretera Celaya-Juventino Rosas. C.P. 38110, 38010, Celaya, Guanajuato, México.

[2]Universidad Autónoma Chapingo, Departamento de Ingeniería Agroindustrial, km 38.5 Carretera Federal México-Texcoco, C.P. 56230, 56100, Texcoco, México.

## INTRODUCTION

Seed treatments with polymer-based coatings have helped to improve the applications of agrochemicals, such as nutrients and plant protectants to increase the quality of the seeds and assist in maintaining crop yield potential (Freiberg et al., 2017b). Furthermore, this type of treatment allows reducing the amounts of the components carried by the coating by focusing the effect of the components to the sowing site, with a controlled release of the coating components, reducing undesirable impacts to the environment (Ludwig et al., 2020).

For this reason, in recent years, research has increased in the development of less aggressive materials, more efficient application strategies and more effective treatments for the phytopathogens reduction. Besides, additional commitment to reduce environmental contamination (Ramos-Campos et al., 2015; Marroquín-Cardona et al., 2016; Guerra-Fuentes et al., 2019). New strategies for agrochemicals application have been reported where controlled release mechanisms are proposed which allows more efficient application dose; part of these strategies is the use of natural polymers, which, unlike synthetic polymers, are degraded into the soil, so they do not represent a contamination source (Timilsena et al., 2015).

One of the most versatile polymers that has recently been incorporated into agricultural area is chitosan, which can be obtained by chitin deacetylation. This polymer has various agriculture applications, since it is biodegradable, non-toxic, polycationic, has antimicrobial and nematicidal properties; in addition, it induces endogenous plants defense, which causes low pest infestation rates (Rodríguez-Pedroso et al., 2009).

There are several studies where the potential use of chitosan and its compatibility with various chemical substances have been demonstrated. The effect of chitosan and hydrogen peroxide applied as seed coating and as aspersion on maize seedling was demonstrated, which was associated with increase in germination rate, plant height, ear length and protein seed quality without crop phenology affection (Lizárraga-Paulín et al., 2013). In another study, the effect of soybeans coating was demonstrated to control soybean borer (*Agrotis ipsilon*) and soybean aphid (*Aphis glycines*); the study showed that chitosan stimulated plants to produce systemic defense responses, which produced repellent effects to deter pests (Zeng et al., 2012). Chitosan coating application on tomato seeds delayed infection symptoms with *F. oxysporum*; furthermore, chitosan incorporation into substrate favored injured roots reduction by the infection (Benhamou et al., 1994).

One of the most versatile and impactful chitosan applications in agriculture is the release of active substances. Ferreira et al. (2019) formulated a chitosan nanosphere with *Siparuna guianensis* essential oil as an active ingredient, in order to control *Aedes aegypti* larvae, which is the main vector of virus that causes dengue; their results showed a sustained release for at least 8 days, significantly reducing the insect larvae survival.

Wu and Liu (2008) reported gradual nitrogen release, phosphorus and potassium (NPK) coated with acrylic acid-co-acrylamide and chitosan. In another study, Ruiz-de-La-Cruz et al. (2017) used two different chitosan sources (shrimp and insects) coatings in mixture with fungicide on black bean seeds; they found out that at 1 and 0.25% chitosan concentrations under infection conditions, 35% of sampled seeds retained the fungicide and prevented rapid release into the medium.

Maize and bean are a source of carbohydrates and proteins for human consumption mainly in developing countries; both species are the basis of the diet and economy in México from pre-Hispanic times to the present. Therefore, it is important to propose strategies that contribute to improving seed storage conditions and also protection in sowing with the use of coatings such as chitosan and chitosan mixed with fungicides.

Therefore, this research objective was to evaluate the effect of chitosan coatings mixed with fungicide (dithiocarbamate) on the germination and germination speed of bean and maize seeds in storage and to determine the retention capacity of the fungicide in the coated seeds under different times of imbibition.

## MATERIAL AND METHODS

### *Seed preparation*

Bean seeds (*Phaseolus vulgaris* L.) 'Pinto Americano' were obtained from the spring summer 2015 production season, from San Luis Potosí (México) and those of white maize QPM (*Zea mays* L.) were obtained from the spring summer 2015 production season, from the 'Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Bajío'; Celaya (México). Seeds were disinfected following the ISTA (2004) methodology, and were left to dry at room temperature (25 °C) for 24 h.

### *Chitosan Preparation*

Chitosan was obtained from commercial chitin (Sigma-Aldrich, St. Louis, MO, U.S.A.), following the procedure of Ruiz-de-La-Cruz et al. (2017). Chitin deacetylation was carried out in 70% NaOH solution (1:4 w/v ratio) for 1 h; the mixture was left to stand for 12 h, then it was washed using distilled water. Once the chitosan was obtained, it was dried at room temperature (28-30 °C), dissolved in 2% acetic acid aqueous solution by constant stirring and adjusted to pH 6 with 2M NaOH. Final chitosan concentration was adjusted to 2%; finally, the mixture was dialyzed by changing distilled water every 12 h, for three days. A standard Spectra / Por® 6 membrane with a molecular weight cutoff of 25 kDa (SpectrumLabs, CA, U.S.A.) was used for dialysis. To determine the deacetylation degree, the potentiometric valuation method was used (Yuan et al., 2011).

### *Treatments Preparation*

Six treatments were evaluated. Sterile distilled water as a control (W); 0.5% of dithiocarbamate fungicide solution (Ziram ®FMC Agroquímica de México, Zapopan, Jalisco, México) in distilled water (FS); 0.1% chitosan in distilled water (C0.1); 0.5% chitosan in distilled water (C0.5); 0.1% chitosan mixed with 0.5% of the fungicide (C0.1FS); and 0.5% chitosan mixed with 0.5% of the fungicide (C0.5FS); volume of slurry was 500 $mL.kg^{-1}$ seeds.

### *Seed coating*

The disinfected and dried seeds were submerged in each treatment for 30-45 s, and then they were drained and left to dry at room temperature (25 °C) for 48 h.

### *Coated seed germination*

To identify the possibility of some of the coatings toxicity, germination percentage carried out on filter paper moistened with distilled water in Petri dishes in germination chamber (ISTA, 2004) considering germinated seeds those that presented the protruded radicle at 4th day, germination speed (dimensionless value) (Maguire, 1962) was also evaluated; to this last trait, samples were taken each 24 h until $4^{th}$ day (sampling time). The experiment was carried out under controlled conditions in a germination chamber with 12 h of light at 25 °C and 12 h in darkness at 18 °C. A complete randomized experimental design with four replications, 100 seeds were used for both, beans and maize.

### *Fungus inoculum Preparation*

In order to evaluate the fungicide release from the seed coatings treatments, before germination, conidia of *Fusarium oxysporum* were used to infect seeds of all treatments; the strain was provided from the collection of the Biotechnology and Genetics laboratory of the Institute of Applied Ecology of the Autonomous University of Tamaulipas (México). The fungus was grown for a week in Petri dishes with PDA medium (Bioxon México); subsequently, conidia were harvested, adding 10 mL of sterile 0.01% Tween 80 solution (Sigma-Aldrich, St. Louis, MO, U.S.A) to each Petri dish; recovered suspension was used as a source of conidia. Conidia suspension was counted and adjusted to a concentration of $1 \times 10^5$ $conidia.mL^{-1}$ (Ruiz-de-La-Cruz et al., 2017).

*Fungicide Retention in seed coatings*

To evaluate fungicide retention by the coatings, 100 seeds from each treatment (included treatments without fungicide) were soaked in 1 L of sterile distilled water for different periods (0, 1, 2 and 6 h, with water changes every 2 h); after imbibition, seeds were drained out and allowed to dry for 30 minutes at room temperature (25 °C) to eliminate water excess. Subsequently, the permanence of fungicidal effect on germination of *F. oxysporum* conidia was evaluated for each treatment; seeds were placed in Petri dishes with PDA medium and each seed was inoculated with 10 µL of the conidia suspension of *F. oxysporum*, ($1 \times 10^5$ conidia.mL$^{-1}$) Petri dishes were kept at room temperature (25 °C). The process was repeated for each treatment with four-imbibition times and three replications (100 seeds). Finally, seeds number with the presence of mycelium were counted, 72 h after inoculation. The experiment used for both, beans and maize was a bifactorial with six treatments, subjected to four rinsing times with three replications; the treatments were laid out in a completely randomized design, with an a x b arrangement, considering factor a: the treatments; factor b: imbibition times.

*Chitosan-fungicide coated seed storage*

This test was carried out to analyze the effect on seeds germination for both species, associated with the coatings; seeds were subjected at 30, 60, 90, 120, 150 and 180 days of storage. After coating and drying, 300 seeds per treatment were packed into waxed sachets and stored at 5 °C, 45% relative humidity in dark. Each month, 150 seeds of each treatment were sampled (3 replications of 50 seeds). For evaluation, the seed germination methodology on paper, according to ISTA (2004) was followed, which allowed determining the germination percentage and the germination speed (dimensionless value) (Maguire, 1962). For this test, a trifactorial experiment of a x b x c was carried out and considering 3 replications; the treatments were laid out in a completely randomized design considering factor a: treatments, factor b: storage time and factor c: sampling time. Data obtained were subjected to analysis of variance and means comparison by Tukey test ($p \leq 0.05$) using Statistical Analysis System package version 9.4 (SAS Institute, 2013).

## RESULTS AND DISCUSSION

*Germination percentage and germination speed*

The chitosan coating mixed with fungicide did not negatively affect the bean and maize seeds germination percentage, radicle protrusion at 4th day (Figures 1A and 1B). For bean seeds, it was observed that there was an increase of 7% and 9% on the seeds germination of coated with all the treatments with chitosan as compared to the treatment with fungicide only (FS) and control (W), respectively (Figure 1A). Additionally, the germination speed augmented between 49 and 66% in treatments with chitosan (C0.1, C0.5) and chitosan mixed with fungicide (C0.5FS), respectively, as compared to the control; It was also observed that the presence of fungicide in the coating (FS) managed to increase the germination speed by 28% as compared to the control (W) (Figure 1C).

No statistical differences were observed in maize germination percentage for any treatment (Figure 1B); however, the germination speed increased between 12 and 14% in the treatment with chitosan (C0.1) and chitosan in mixture with fungicide (C0.1FS and C.05FS), as compared to the control (W); however, C0.5 was similar to W and FS and resulted in a lower germination speed than the rest of the chitosan treatments (Figure 1D).

Germination is an important parameter to evaluate the seed physiological quality and the presence of fungicide in mixture with chitosan did not negatively affect germination nor the germination speed stimulated by chitosan in both types of coated seeds.

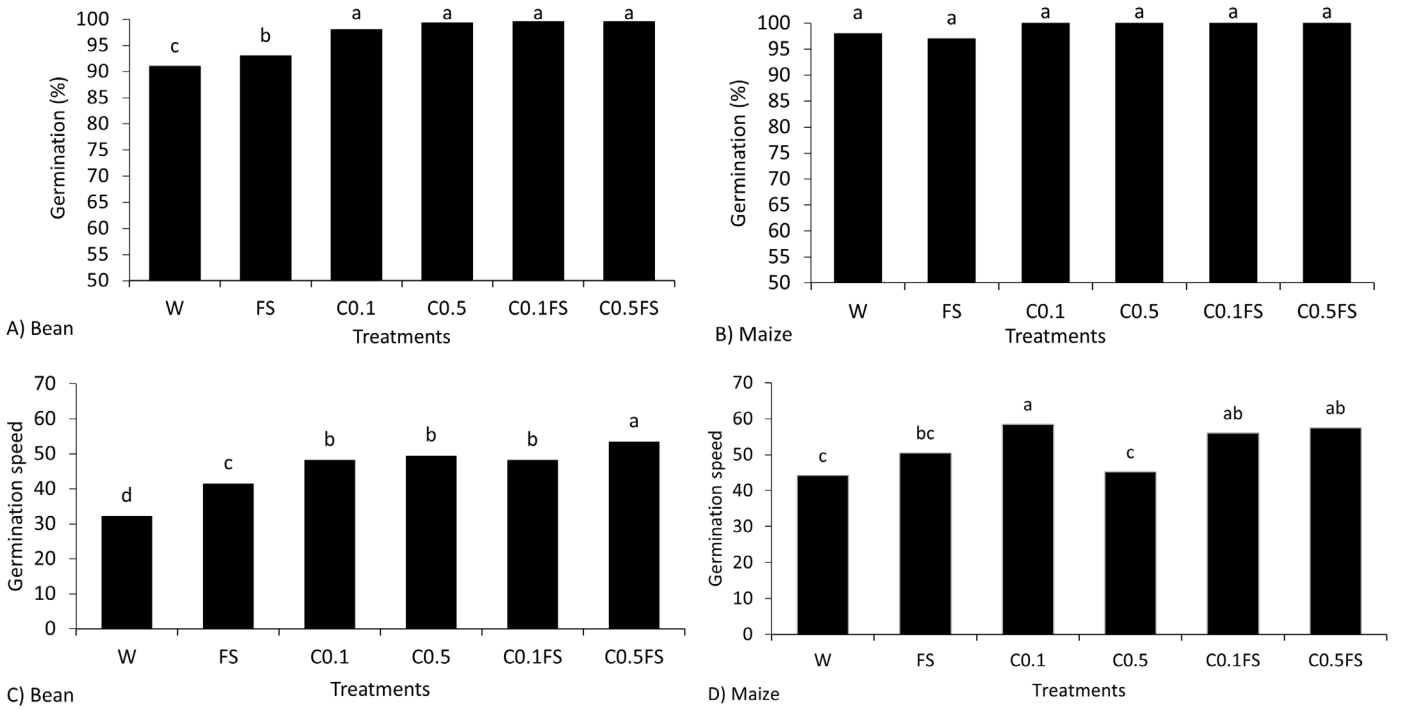


Figure 1. Means comparison of germination and germination speed of bean and maize seeds coated with chitosan, chitosan-fungicide mixture, fungicide and control. A) Bean seeds germination. B) Maize seeds germination. C) Bean seeds germination speed. D) Maize seeds germination speed. W: Distilled water as a control; FS: 0.5% solution of fungicide in distilled water; C0.1: 0.1% chitosan in distilled water; C0.5: 0.5% chitosan in distilled water; C0.1FS: 0.1% chitosan mixed with 0.5% fungicide; and C0.5FS: 0.5% chitosan mixed with 0.5% fungicide. Treatments with same letter are statistically equal, according to Tukey's test ($p \leq 0.05$).

### *Fungicide retention in seed coatings*

The treatment that showed the highest effectiveness (70 and 76% growth inhibition as compared to the control) against the development of *F. oxysporum* on coated beans and maize seeds was C0.5FS (Figures 2A and 2B). This same coating also showed a gradual release of the fungicide from both seeds followed by the C0.1FS treatment (Figures 2C and 2D). In the case of maize seeds coated with C0.5FS treatment, the retention of fungicidal activity was observed (46% reduction in the presence of mycelium in the seeds) even at 6 h of imbibition (Figure 2D). In beans case, with the C0.5FS treatment, a 38% decrease in the presence of mycelium was observed on the coated seeds at 2 h of imbibition (Figure 2C); however, C0.5FS coating failed to retain the antifungal activity with 6 h of imbibition of the bean coated seeds.

Results indicate that the higher chitosan concentration allowed the fungicidal activity retained for a longer time. The ability to retain the fungicide and inhibit *F. oxysporum* development was directly related to the amount of chitosan present in the coating and no significant effect was observed on the fungus growth of the treatment that contained the same concentration of chitosan, but without the fungicide (C0.5). Due to the structure and chemical characteristics of chitosan, which has hydrophilic groups, it can retain water, thus allowing the release of compounds present in mixture with it.

Presence of *F. oxysporum* was related to imbibition time. For the bean seeds longest imbibition time case (6 h) conidia germination was similar to the control for all treatments. The lower proportion of conidia germination was observed at the imbibition times 0 and 1 h, for chitosan and fungicide coatings, that is, the fungicide content was reduced in the coatings with longer rinsing time. Beans and maize testa showed anatomical and structural differences.

Beans testa is smooth while maize testa presents reliefs and a less smooth texture than bean seed. This condition allowed obtaining differences in the results of fungicide retention; maize case, the film coating allowed to fill the reliefs, which allowed a greater fungicide retention. But more studies are needed to confirm this hypothesis.

The chitosan obtained from shrimp exoskeleton through a process similar to that carried out in this research did not affect the *Beauveria bassiana* growth (Bals.-Criv.) Vuill fungi. and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Torres-Castillo et al., 2015). Previous research related to chitosan and its potential use for gradual release in admixture with other polymers showed prolonged release times. For example, high chitosan concentrations (3% to 5%) were used in mixture with starch to generate macrospheres of the chitosan-starch-sodium triphosphate matrix to release $KNO_3$ fertilizer, which released up to 93% of the fertilizer in a 14-day period (Perez and Francois, 2016). These results are interesting, however, this mixture does not interact directly with living tissue (seeds) so more complex matrices can be used, higher chitosan concentrations for prolonged releases of the desired components. In seeds case, it is necessary to consider the particle size and chitosan concentration, in addition to the seeds characteristics, such as morphological features, time of emergence, vigor and strength to ensure that they can break the coating, and not affect the emergence or the development during the initial germination stages. This research results agree with those reported by Ruiz-de-La-Cruz et al. (2017) where the retention action of chitosan was verified in seed coatings with agrochemicals and biological agents in concentrations of 0.25% and 1%.

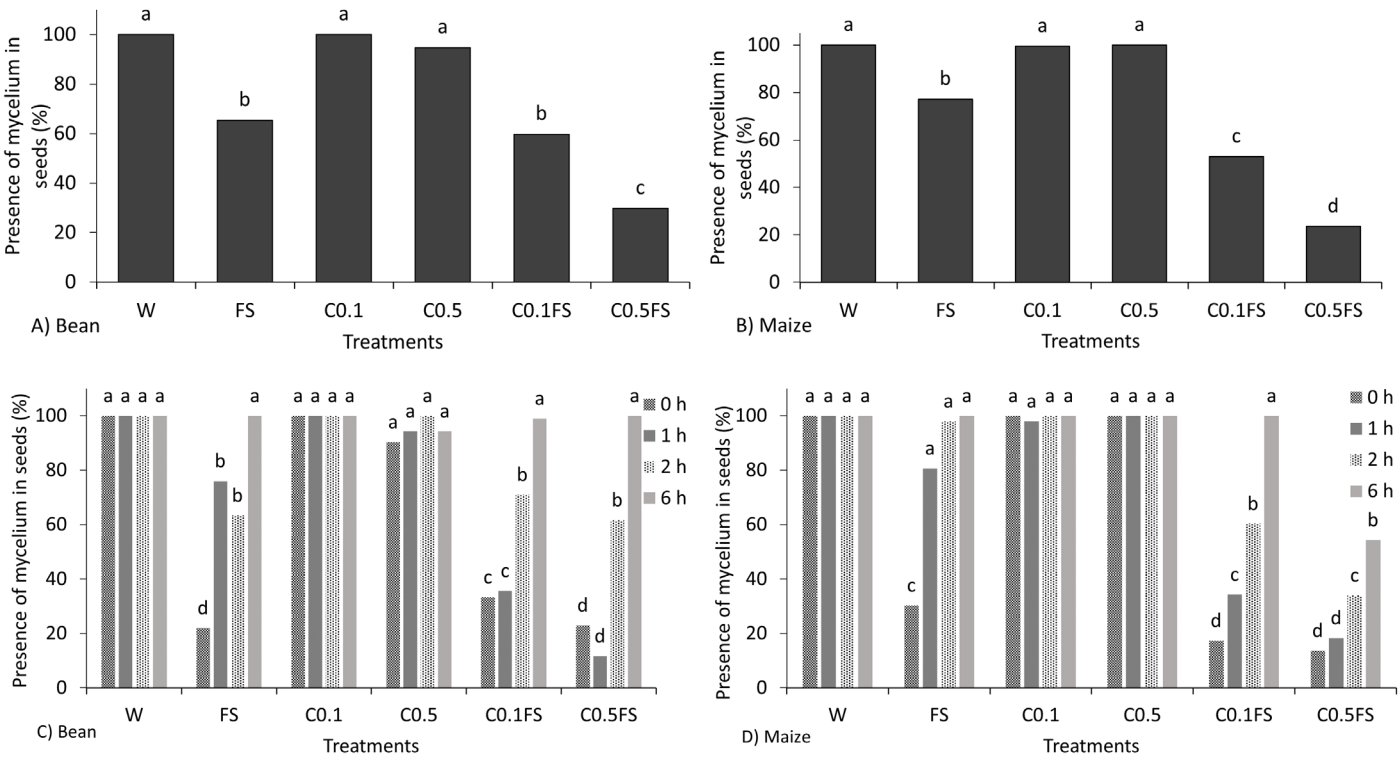


Figure 2. Means comparison of *F. oxysporum* mycelium presence on bean and maize seeds coated with chitosan, chitosan-fungicide mixture, fungicide and control. A) Presence of mycelium on bean seeds coated considering all imbibition times for each treatment. B) Presence of mycelium in maize seeds coated considering all imbibition times for each treatment. C) Presence of mycelium on bean seeds coated: retention of the fungicide respect to the time of imbibition (0, 1, 2 and 6 h). D) Presence of mycelium on maize seeds coated: retention of the fungicide respect to the time of imbibition (0, 1, 2 and 6 h). Equal letters indicate that they are statistically equal according to Tukey's test ($p \leq 0.05$).

*Storage of chitosan coated seeds mixed with fungicide*

Regarding for bean and maize seeds coated storage time with different formulations, it was observed that chitosan coatings and those mixed with fungicide (C0.1, C0.5, C0.1FS and C0.5FS) and fungicide alone (FS) were higher than control (W), and did not negatively affect germination, nor a delay in germination time was observed (Figures 3A and 3B). For maize seeds, germination decreased until 180 days in FS treatment. Chitosan coatings treatments were not higher than FS.

For bean germination speed, at 30, 60 and 120 days of storage, all treatments were higher as compared to W; at 90 days of storage no differences were observed between coated seeds and control. At 150 days of storage C0.5 and C0.1FS were higher compared to control, but similar to W were FS, C0.1 and C0.5FS. At 180 days of storage germination speed of C0.1, C0.5, C0.1FS and C0.5FS were higher compared to W but similar to FS. FS was different from chitosan treatments only at 30 days of storage for beans only. At related to storage time, FS, C0.1, C0.5, C0.1FS and C0.5FS germination speed decreased at 150 and 180 day of storage (Table 1).

In the case of maize germination speed, at 30 days of storage C0.1, C0.5, C0.1FS and C0.5FS were higher compared to W, however C0.5FS was similar to FS, and only at this time of storage FS and W were similar. At 60 days of storage FS, C0.5, C0.1FS and C0.5FS were higher compared to W, but C0.1 and W were similar. At 90 days of storage only C0.5FS was higher to W, and the rest of treatments (FS, C0.1, C0.5 and C0.1FS) were similar to W. At 120, 150 and 180 days of storage all treatments were higher compared to W (Table 2).

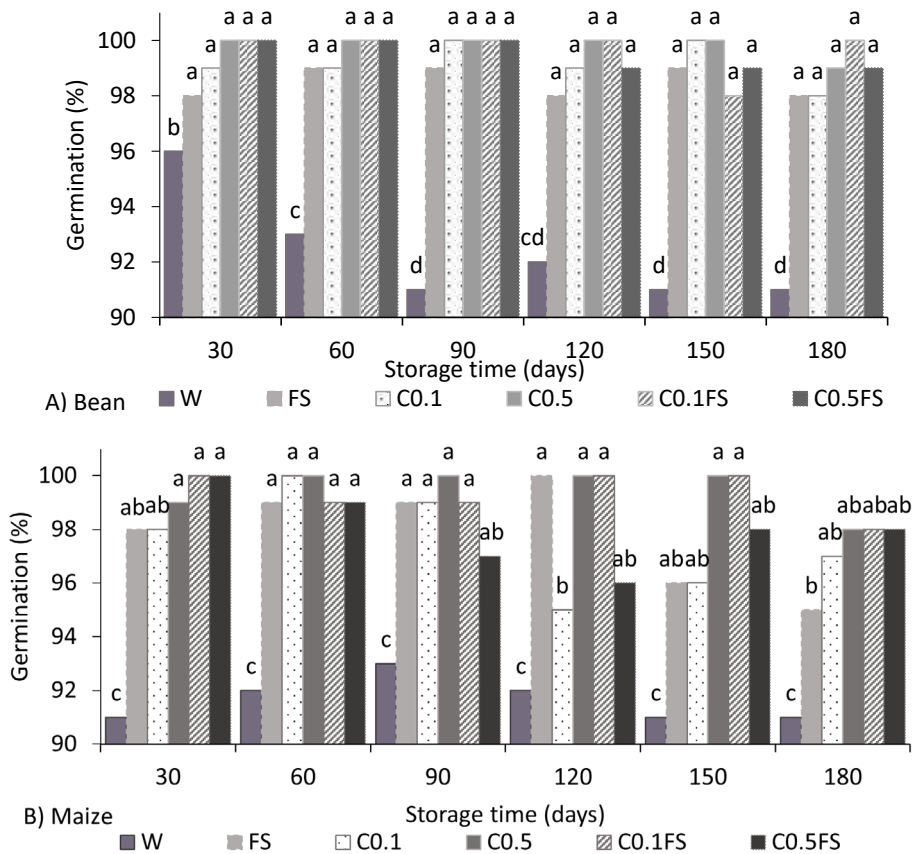


Figure 3. Means comparison of storage effect on bean and maize seeds germination percentage-coated with chitosan, chitosan-fungicide mixture, fungicide and control. A) Coated bean seeds germination under different storage times (30, 60, 90, 120, 150 and 180 days). B) Coated maize seeds germination under different storage times (30, 60, 90, 120, 150 and 180 days). Equal letters indicate that they are statistically equal according to Tukey's test ($p \leq 0.05$).

Table 1. Means comparison of germination speed of beans seed coated in storage.

| Storage time (days) | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | W | FS | C0.1 | C0.5 | C0.1FS | C0.5FS |
| 30 | 32 Da | 41 Cb | 48 Bb | 49 ABb | 53 Ab | 51 ABA |
| 60 | 38 Ba | 51 Aa | 55 Aa | 58 Aa | 58 Aa | 52 AA |
| 90 | 38 Aa | 40 Ab | 40 Ac | 38 Ac | 43 Ad | 39 Abc |
| 120 | 32 Ca | 50 ABa | 46 Bb | 56 Aa | 47 Bc | 45 Bab |
| 150 | 33 Ba | 35 ABbc | 35 ABcd | 37 Ad | 37 Ae | 35 ABbc |
| 180 | 29 Bb | 32 ABc | 33 Ad | 33 Ad | 34 Ae | 33 AC |

Means with the same capital letter are statistically equal by treatment at each storage time. Means with the same small letter are statistically equal by storage time at each treatment. According to Tukey's test ($p \leq 0.05$).

Table 2. Means comparison of germination speed of maize coated in storage.

| Storage time (days) | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | W | FS | C0.1 | C0.5 | C0.1FS | C0.5FS |
| 30 | 46 Ca | 49 BCb | 58 Aa | 62 Aa | 58 Aa | 56 ABa |
| 60 | 39.5 Cb | 55 ABa | 48 BCb | 61 Aa | 60 Aa | 62 Aa |
| 90 | 37.3 Bb | 39 ABd | 40 ABc | 40 ABb | 40 ABb | 43 Ab |
| 120 | 37.1 Bb | 42 Ac | 42 ABc | 43 Ab | 42 Ab | 43 Ab |
| 150 | 29.8 Bc | 33 Ae | 33 Ad | 33 Ac | 33 Ac | 33 Ac |
| 180 | 26.9 Bc | 32 Ae | 31 Ad | 31 Ac | 32 Ac | 32 Ac |

Means with the same capital letter are statistically equal by treatment at each storage time. Means with the same small letter indicate that they are statistically equal by storage time at each treatment. According to Tukey's test ($p \leq 0.05$).

Several authors have reported the chitosan germination-promoting effect on cereal seeds (Lan et al., 2016; Fu et al., 2019), oleaginous (Hai et al., 2019), ornamental (Kananont et al., 2010), among others. Those results agree with what was found out with some of this research coatings, besides to stimulating seed germination in storage, it did not alter its physiological quality during the 180 days storage time (Tables 1 and 2). Baldini et al. (2018) reported negative effects in treated seeds with of agrochemicals mixtures, such as fludioxonil + metalaxyl-M, pyrethroids and carbamates. They pointed out that their active ingredients interacted with the embryo, which resulted in a significant decrease in seed vigor and seed germination percentage, which was not observed in this research with use of the fungicide or with the chitosan coatings with fungicide. Freiberg et al. (2017a) reported toxicity in a coating with the polymer 'Colorseed He' mixed with fungicide (Vitavax® Thiram 200 SC) and an insecticide (Thiamethoxam) in wheat seeds in storage at 180 days showing a low percentage of germination and an increase in the number of abnormal seedlings, unlike what was observed in this research. In general, insecticides have greater phytotoxicity than fungicides in soybean stored seeds (Carvalho et al., 2020; Rocha et al., 2020). Treating seeds with large slurry volumes is detrimental to maintain seeds vigor during storage, especially with water predominance (Santos et al., 2018) as could be seen for W treatment in storage (Figure 3).

Results presented here highlight the positive effect of chitosan on seeds coating, in addition to being useful as a gradual release agent. Its physico-chemical properties allowed it to adhere to the seed coat, generating a barrier that prevents the rapid release of fungicide and maintains its effect on the surface, increasing the seed protection time. This is of interest for sowing in conditions where the seeds are exposed to various problems, such as phytopathogenic soil fungi.

## CONCLUSIONS

Chitosan coatings and chitosan-fungicide coatings do not cause negative changes in seed germination or germination rate. When changes occur, tendency is to improve the values of these traits.

Chitosan coatings in mixture with fungicide conserve the protective effect of the fungicide for a longer time without negative effects on seed germination.

Chitosan coatings or in mixture with fungicide (Dithiocarbamate) are not harmful to the germination of stored corn and bean seeds.

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