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Original Article

Optimizing Biopolymer Seed Coatings: Impact of Sodium Alginate and Chitosan on the Viability of Trichoderma Harzianum, Bradyrhizobium Japonicum and Soybean Seed Quality

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Abstract *- Insecticides and fungicides used for seed treatment are replaced by eco-friendly biopolymers derived from natural sources, such as sodium alginate and chitosan. Despite their promising potential, there are several knowledge gaps concerning the effects of these biopolymers on microbial viability and seed quality. This study aimed to i) evaluate the effect of different sodium alginate and chitosan coating formulations on the viability of Trichoderma harzianum and Bradyrhizobium japonicum during storage and ii) explore the impact of these biopolymers on soybean seed quality. The microorganisms and biopolymers were mixed with different formulations of sodium alginate, chitosan (in powder or acidic solution), and talc. Viability was assessed through colony-forming units per mL (CFU mL⁻¹) over time and soybean seed quality by radicle emergence, germination percentage and seedling growth. The results were analysed using ANOVA and Tukey's test. Both microorganisms, incorporated in a sodium alginate layer followed by the solid chitosan/talc mixture, maintained high CFU mL⁻¹ even after 30 days of storage. However, solid chitosan mixed with talc negatively affected radicle emergence, extending to germination and seedling growth. Chitosan in an acidic solution exhibited differential activity on the viability of microorganisms, not affecting Bradyrhizobium japonicum but inhibiting Trichoderma harzianum. It did not alter radicle emergence and maximised germination. Coating soybean seeds with a specific formulation of sodium alginate, chitosan, Trichoderma harzianum, and Bradyrhizobium japonicum maintained viability during short-term storage. Adjusting the chitosan dose is crucial to avoid negative effects on soybean radicle emergence and germination.*

Keywords *- Biopolymers, Microbial viability, Germination, Radicle emergence.*

1. Introduction

Although insecticidal and fungicidal seed treatments provide agricultural benefits, they pose phytotoxic risks, contribute to pathogen resistance, and can lead to environmental contamination [1]. Therefore, there is a growing need to replace these substances with eco-friendly natural compounds, such as biopolymers derived from plants, fungi, animals, bacteria, and other microorganisms. These natural polymers aim to mitigate soil degradation, water contamination, and biodiversity loss associated with conventional pesticides due to their biocompatibility, biodegradability, and controlled release of active compounds. System sustainability could be further enhanced by incorporating beneficial microorganisms such as *Bradyrhizobium japonicum* and *Trichoderma spp.* Both microorganisms are essential for restoring soil fertility and enhancing crop performance against diseases and stress [2, 3]. However, their effectiveness is limited by low survival rates and colonization efficiency when applied directly to the soil. Biopolymers have emerged as an innovative solution, enabling bioinoculant encapsulation increasing their viability and shelf life while protecting plants from biotic and abiotic factors [4]. Sodium alginate is particularly effective in encapsulating bacteria, protecting them from external factors and enabling their controlled release into the target medium, thereby enhancing the bacteria's survival after inoculation. Sodium alginate is a linear copolymer composed of β-D-mannuronic acid and α-L-guluronic acid residues, linked by (1

→ 4) glycosidic bonds and randomly distributed along the carbohydrate chains. It is widely used in agriculture due to its water retention capacity and non-toxic properties. Unlike sodium alginate, chitosan is derived from chitin and is extensively recognised for its insecticidal and antifungal properties [5, 6, 7]. It is a linear, semicrystalline polysaccharide composed of glucosamine ($C_6H_{13}NO_5$) and N-acetylglucosamine units linked by β (1→4) glycosidic bonds.

Additionally, chitosan promotes plant growth by increasing water and nutrient uptake, enhancing the synthesis of hormones and enzymes, and triggering the plant´s defense system [8]. Nevertheless, the use of chitosan is limited by its water insolubility, requiring dissolution in acidic solutions [9]. To overcome these limitations, researchers have developed alternative formulations, including emulsion-based films and coatings combined with other hydrophilic polymers [10], lower molecular weight polymers [8] and nanoparticles [11].

Coating soybean seeds with a combination of chitosan, alginate/polyethene glycol, and *B. japonicum* had no significant impact on nodule formation or yield compared to inoculation alone [12]. Conversely, some studies showed a deterioration in the viability of *T. harzianum* spores when seeds were coated with chitosan. In peanut crops, the effectiveness of a chitosan-based double-layer seed coating with fungicide, *T. harzianum*, and *B. japonicum* demonstrated high compatibility among the components, improved survival in soil, and enhanced seed quality and seedling growth [13]. On the other hand, chitosan's effectiveness varies with concentration, application method, plant species, cultivars, and when combined with other treatments [14]. Therefore, a deeper analysis of the relationship between carriers, additives, microorganisms, and plant systems can provide valuable insights for understanding immobilised biostimulants' functional characteristics and determining strategies for their application [15]. This study aimed to i) evaluate the effect of different sodium alginate and chitosan coating formulations on the viability of *Trichoderma harzianum* (*T. harzianum*) and *Bradyrhizobium japonicum* (*B. japonicum*) during storage and ii) explore the impact of these biopolymers on soybean seed quality.

2. Materials and Methods

2.1. Materials

Seeds of soybean (*Glycine max* L. Merrill) from maturity group IV were evaluated. The commercial strains of *T. harzianum* and *B. japonicum* used in the study contained 1.0 x 10^9 conidia g^{-1} and 1.5 x 10^9 CFU mL^{-1} (colony-forming units per millilitre), respectively. *T. harzianum* was applied in powder form at a dose of 6 g per 100 g of seeds, while *B. japonicum* was used in a liquid solution at 0.3 mL per 100 g of seeds. Sodium alginate (Sigma-Aldrich®) was used as an adherent by dissolving it in distilled water to obtain a final concentration of 1.5% (w/v). Two chitosan-based formulations (medium molecular weight, Sigma-Aldrich®) were prepared: a liquid formulation obtained by dissolving chitosan in an acetic acid solution 1% (v/v) and a solid formulation created by mixing chitosan with talc ($Mg_3Si_4O_{10}(OH)_2$) in a 1:100 ratio to reach a final concentration of 1% (w/w). Chitosan was employed as an adherent and combined with talc as a solid filler.

2.2. Treatments

Seed coating involves the application of active ingredients in successive layers, supplemented by a binder and filler. In this study, 20 mL of the biological agents and biopolymer solutions were applied in successive layers on 400 g of seeds, which were continuously rotated for 3 minutes. Each layer was separated from the next by a drying period of two hours at room temperature to obtain the treatments listed in Table 1. In the control treatment (W), seeds were coated with 20 mL of sterile distilled water. After treatment, the seeds were air-dried for an additional 24 hours at room temperature (25 °C) and then stored in brown paper bags at 10 °C. The seed treatment techniques used in the control and treatments without talc are classified as film 'coating', as the weight increase is less than 10% (Figure 1a). In contrast, treatments involving talc are more accurately described as 'encrusting', with a weight increase of 13.3%, while maintaining the original shape of the seeds (Figure 1b). These techniques differ from seed priming, which involves the controlled hydration of seeds before sowing, initiating the germination process but then re-drying before the radicle extension occurs [16]. One treatment (Ts+B[CH/talc]) was discarded because the talc layer detached from the seed coat after 24 hours, indicating that the chemical nature of the products hindered proper adhesion.

2.3. Laboratory Tests

2.3.1. Compatibility and Viability of B. japonicum and T. harzianum

Compatibility between *T. harzianum* and *B. japonicum* strains was verified by culturing both on YEM (Yeast Extract Mannitol) medium, as described by [20]. Both microorganisms grew without inhibition halos (Figure 2a) and continued to proliferate when inoculated again on the same culture medium (Figure 2b, c). To determine viability, conidia were recovered from the seed surface as follows: 100 seeds from each treatment were transferred to Erlenmeyer flasks containing 12.6 mL of washing solution (0.85 g of NaCl and 0.4 mL of a 0.01 % v/v Tween solution per 100 mL of water) and vortexed for 20 minutes. Samples of 1 mL were extracted from each washing suspension and placed in tubes with 9 mL of the same solution. Subsequently, the serial dilution method was employed [17]. Three replicates of 0.1 mL were inoculated onto Petri dishes containing *Trichoderma spp*. selective medium (TSM), and onto *B. japonicum* selective medium [19] supplemented with fungicide [20]. In the TSM, ampicillin (0.2 g/L) was used instead of chloramphenicol [18].

Table 1. Biopolymers, *Trichoderma harzianum* (*T. harzianum*) and *Bradyrhizobium japonicum* (*B. japonicum*) application sequences and formulation in soybean seed coating

Treatment	1° Layer	Dry	2° layer	Dry	3° Layer
CHa	Chitosan in acetic acid solution	-	-	-	-
Ts	T. harzianum in sodium alginate	-	-	-	-
Bs	B. japonicum in sodium alginate				
Bs/talc	B. japonicum in sodium alginate + talc	-	-	-	-
TBs[CH/talc]	T. harzianum and B. japonicum in sodium alginate (chitosan solid + talc)	-	-	-	-
B+Ts[CH/talc]	B. japonicum in water solution	2	T. harzianum in sodium alginate (chitosan solid + talc)	-	-
Ts+B[CH/talc]	T. harzianum in sodium alginate	2	B. japonicum in water solution (chitosan solid + talc)	-	-
CHa+B+Ts	Chitosan in acetic acid solution	2	B. japonicum in water solution	2	T. harzianum in sodium alginate

Fig. 1 Soybean seeds treated with film coating (a) Encrusting (b) technologies

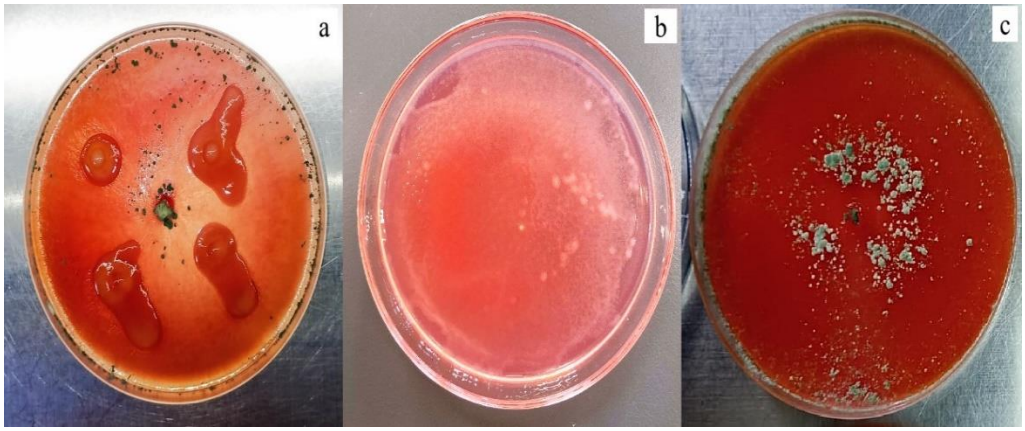

Fig. 2 Strains from *T. harzianum* and *B. japonicum* co-cultured on YMA medium (a) inoculated individually: B. japonicum (b) T. harzianum (c)

For YEM, cycloheximide was replaced by thiabendazole at a dose of 16.6 mg l^{-1}, as tested in previous trials. The Petri dishes were incubated at 28°C for 7 days for *T. harzianum* and 10 days for *B. japonicum*, after which final counts were recorded. The results were assessed in terms of CFU mL^{-1} at 1, 30, and 60 days post-seed coating.

2.3.2. Radicle Emergence Rate

This variable was assessed through four replicates of 100 seeds per treatment in accordance with the methodology defined in [21]. The results were quantified in hours required for 50% of radicle emergence [22]. A shorter duration is indicative of higher seed vigor [23]. Seed counts with radicles breaking the seed coat were recorded at consistent intervals, spanning from 24 to 76 hours post-sowing.

2.3.3. Germination Percentage

Germination was evaluated by sowing between the paper of three replicates of 50 seeds, which were placed in the germination chamber at 25°C with a 12-hour light/dark cycle [21]. After 8 days of sowing, the number of normal seedlings was counted and expressed as a percentage. This variable was evaluated at 1 and 60 days of storage.

2.3.4. Seedling Growth

Seedling growth was evaluated by measuring the length of roots and hypocotyls of 10 seedlings from each germination test replicate, with results expressed in centimetres (cm).

2.4. Statistical Analysis

The laboratory tests utilized a Completely Randomized Design (CRD). The study centered on the viability of *T. harzianum* and *B. japonicum*, with two principal variables: seed coating treatments and storage duration. Germination data were subjected to angular transformation, while viability data were log-transformed (Log_{10}) to ensure normality and homogeneity of variances. Subsequently, an Analysis of Variance (ANOVA) was performed to detect statistically significant treatment differences [35]. Tukey's Honestly Significant Difference (HSD) test, at a 5% significance level, was applied for post-hoc analysis to identify specific group differences.

3. Results

3.1. T. harzianum and B. japonicum Viability

Immediately after seed treatments, chitosan diluted in acetic acid ([CHa+B+Ts]) significantly reduced the viability of *T. harzianum* (Figure 3a), while *B. japonicum* was most adversely affected by treatment with sodium alginate and talc (Figure 3b). The viability of *T. harzianum* with sodium alginate (Ts) coating was high (1.85×10^7 CFU mL^{-1}) and dropped to 1.15×10^7 CFU mL^{-1} after 30 days (Figure 3a). *B. japonicum* combined with sodium alginate (Bs) resulted in 1.6×10^7 CFU mL^{-1}, significantly reduced after 30 days of storage (Figure 3b). The viability of both microorganisms improved with the combination of sodium alginate, solid chitosan, and talc in two layers on the seeds (B+Ts[CH/talc]). However, in *B. japonicum*, it was significantly reduced after 30 days (Figure 3a, b). In contrast, when *T. harzianum* and *B. japonicum* were applied with these biopolymers in a single layer (TBs[CH/talc]), their viability remained high even after 30 days of storage (Figure 3a, b).

3.2. Radicle Emergence Rate

Formulations containing solid chitosan significantly delayed radicle emergence by an average of 25 hours compared to the control, as shown in Figure 4. This delay suggests a reduction in soybean seed vigor following treatment with solid chitosan. In contrast, other seed coatings, which included beneficial microorganisms (B. japonicum and T. harzianum) and sodium alginate and chitosan dissolved in an acidic solution, did not show any adverse effects on the radicle emergence rate. These alternative formulations maintained a normal radicle emergence process, indicating that the presence of microorganisms and using acid-soluble chitosan may mitigate any potential negative impacts on seed vigor.

3.3. Germination Percentage

Chitosan in acetic acid solution (CHa) had the highest germination percentage (Table 2). Meanwhile, T. harzianum and B. japonicum were applied with sodium alginate and maintained germination percentages above 80%, with no significant differences compared to the control (Table 2). However, incorporating talc and solid chitosan reduced germination to below 80%, a trend also observed with the three-layered formulations (CHa+B+Ts) even after 60 days of storage (Table 2).

3.4. Seedlings Growth

The coating process that involved mixing both microorganisms with sodium alginate or chitosan in an acid solution did not negatively affect the size of the seedlings, as shown in Figure 5. This indicates that the application of these coatings is safe for seedling development. In contrast, the formulations that included solid chitosan and talc resulted in the shortest lengths for both the root and hypocotyl (Figure 5a, b). This suggests that while some coatings may be beneficial, others can significantly hinder the growth of seedling structures.

4. Discussion

4.1. T. harzianum and B. japonicum Viability

Co-inoculation in soybean crops is typically performed on different substrates [20]. Consequently, the coating offers a new technique for incorporating both microorganisms. To maintain their viability after coating and over time, it is important to consider the application sequence and the number of layers. Immediately after the seeds were treated, two coating strategies analysed in this study maximised the viability of both microorganisms. One consists of a first layer of *B. japonicum* in water solution, followed by a second layer

of sodium alginate containing *T. harzianum*, which is applied after two hours of drying. Sodium alginate functioned as an adherent for the subsequent solid chitosan/talc mixture (B+Ts[CH/talc]). The other option involves applying a single layer of sodium alginate with both microorganisms, followed without delay by the solid chitosan/talc mixture (BTs[CH/talc])

In this treatment, after 30 days of storage, the CFU mL^{-1} remained at levels of 1.4 x 10^7 and 3.9 x 10^7 for *T. harzianum* and *B. japonicum*, respectively. Similar findings were observed in the coating of sunflower and canola, where sodium alginate maintained the viability of *T. harzianum* in the range of 4.9 x 10^4 to 1.8 x 10^6 CFU mL^{-1} over the same storage period. These results are also comparable to liquid inoculants combining *B. japonicum* and sodium alginate, which maintained concentrations between 10^7-10^8 cells/mL after 6 months of storage [24]. The higher viability observed in the BTs[CH/talc] formulation compared to the individual treatments suggests a possible synergistic effect between both microorganisms when combined in the sodium alginate matrix on the seeds. A synergistic effect was observed when both microorganisms were inoculated into a mixture of sand and perlite, possibly related to the production of auxins by *T. harzianum* [20]. Traditional treatments with *B. japonicum* are typically applied at the time of sowing [25]. Alternatively, inoculation using the seed coating technique offers a significant advantage by allowing seeds to be stored with viable microorganisms for up to 30 days. *Bradyrhizobium sp.* cells encapsulated in alginate beads exhibited high viability for 12 months, likely due to the protective barrier that mitigated dehydration [26]. Chitosan applied in an acid solution significantly reduced the viability of *T. harzianum*, which is in line with previous research. However, this formulation did not harm the viability of the bacteria, maintaining it at intermediate levels immediately after treatment and after 30 days.

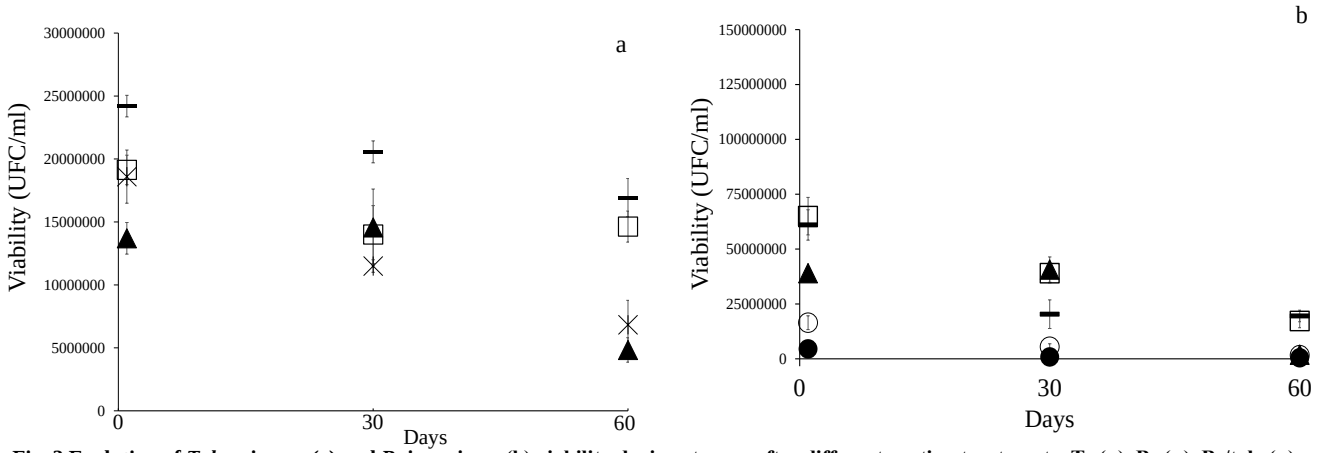

Fig. 3 Evolution of *T. harzianum* (a) and *B. japonicum* (b) viability during storage after different coating treatments: Ts (×), Bs (○), Bs/talc (●), TBs[CH/talc] (□), B+Ts[CH/talc] (—), CHa+B+Ts (▲). Vertical bars indicate ± 1 SD. Two points differ significantly when the standard error bars do not overlap. Tukey test ($p < 0.05$)

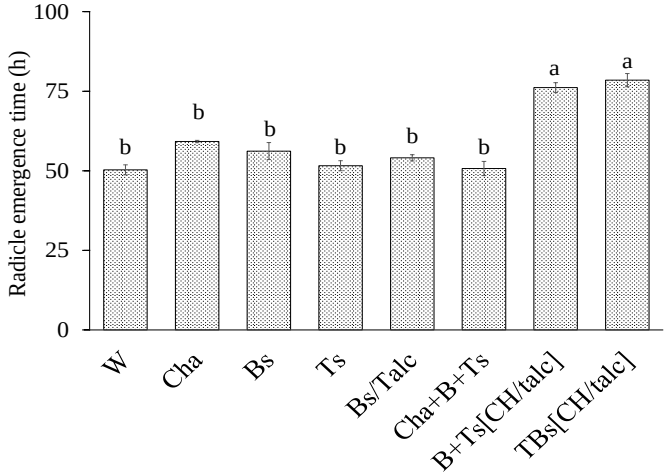

Fig. 4 Radicle emergence rate (hours) in soybean seeds after coating treatments. W = Control; Cha = chitosan in acetic acid solution; Bs = *B. japonicum* in sodium alginate; Ts = *T. harzianum* in sodium alginate; Bs/talc = *B. japonicum* in sodium alginate + talc; CHa+B+Ts = chitosan in acetic acid solution + *B. japonicum* in water solution + *T. harzianum* in sodium alginate, B+Ts[CH/talc]= *B. japonicum* in water solution + *T. harzianum* in sodium alginate (chitosan solid + talc), TBs[CH/talc] = *T. harzianum* and *B. japonicum* in sodium alginate (chitosan solid + talc). Vertical bars indicate ± 1 SD. Different letters indicate significant differences between coating treatments according to the Tukey test ($p < 0.05$).

This result is consistent with the findings of [27], which demonstrated that chitosan coating did not compromise bacterial viability on soybeans nor adversely affect seed survival. Therefore, chitosan in an acidic solution exhibits differential activity concerning the viability of a both microorganisms. This could be attributed to the fact that the effective concentration of chitosan varies significantly depending on the type of microorganism, its origin, molecular weight, and degree of acetylation. Therefore, further exploring the antimicrobial mode of action of chitosan in liquid media remains crucial, particularly when it is applied through coating technology.

4.2. Seed Quality

The film generated by sodium alginate, even when combined with the microorganisms, did not impair radicle emergence but reduced germination, although not significantly. Similar results were obtained in peanuts in which Trichoderma and Bradyrhizobium co-inoculated slightly reduced germination 10 days after sowing [28]. The effects of chitosan on seed quality vary by formulation and are challenging to compare, as most literature discusses its application to seeds through priming technology. According to the results obtained, applying chitosan in an acidic solution did not alter radicle emergence and maximised soybean germination. This aligns with [27], who also found that chitosan concentrations between 100 and 1000 mg L^{-1} favoured soybean germination when applied before the inoculant.

Coating with chitosan diluted in glacial acetic acid and beeswax in the same crop did not modify germination until 90 days after storage [11]. Soybean seed germination is enhanced by the acidic chitosan solution, which creates a semi-permeable film on seeds. This film protects them from moisture, facilitates the imbibition process, and improves water absorption by the embryonic axis [29].

Other researchers indicate the chitosan film can prevent oxygen ingress, restrict CO_2 loss, and maintain a high CO_2 concentration. These gas-exchange mechanisms have been observed in chitosan coatings on fruits and vegetables, as properly regulating the respiration rate is beneficial for prolonging shelf life [30]. On the other hand, changes in some physiological mechanisms have been observed following applying chitosan using the priming technique.

For example, a significantly increased vitamin C, total phenols, flavonoid content, and antioxidant properties in soybean sprouts [31]. Germination, hypocotyl length, and radicle length in the same crop were significantly enhanced after 3 hours of priming with nano-chitosan due to regulating gibberellins and abscisic acid expressions [32]. To corroborate the mentioned effects, it is essential to analyse the mechanisms involved when chitosan is applied in an acidic solution for coating soybean seeds.

Table 2. Soybean seed germination (%) during storage time after the coatings treatments W = Control; Cha = chitosan in acetic acid solution; Bs = *B. japonicum* in sodium alginate; Ts = *T. harzianum* in sodium alginate; Bs/talc = *B. japonicum* in sodium alginate + talc; CHa+B+Ts = chitosan in acetic acid solution + *B. japonicum* in water solution + *T. harzianum* in sodium alginate, B+Ts[CH/talc]= *B. japonicum* in water solution + *T. harzianum* in sodium alginate (chitosan solid + talc), TBs[CH/talc] = *T. harzianum* and *B. japonicum* in sodium alginate (chitosan solid + talc). Different uppercase letters indicate significant differences within each row between coating treatments and lowercase letters within each column between days of storage.

	Storage time (days)	
	1	**60**
W	85 ± 5,0 ABCa	85 ± 1,2 ABa
CHa	92 ± 3,5 Aa	91 ± 4,2 Aa
Ts	87 ± 6,4 ABa	87 ± 1,2 ABa
Bs	82 ± 3,5 ABCa	83 ± 1,2 ABa
Bs/talc	76 ± 2,0 BCa	67 ± 4,2 Cb
CHa+B+Ts	73 ± 4,6 Ca	80 ± 4,0 Ba
B+Ts[CH/talc]	60 ± 4,0 Da	37 ± 2,8 Db
TBs[CH/talc]	56 ± 4,0 Da	61 ± 3,1 Ca

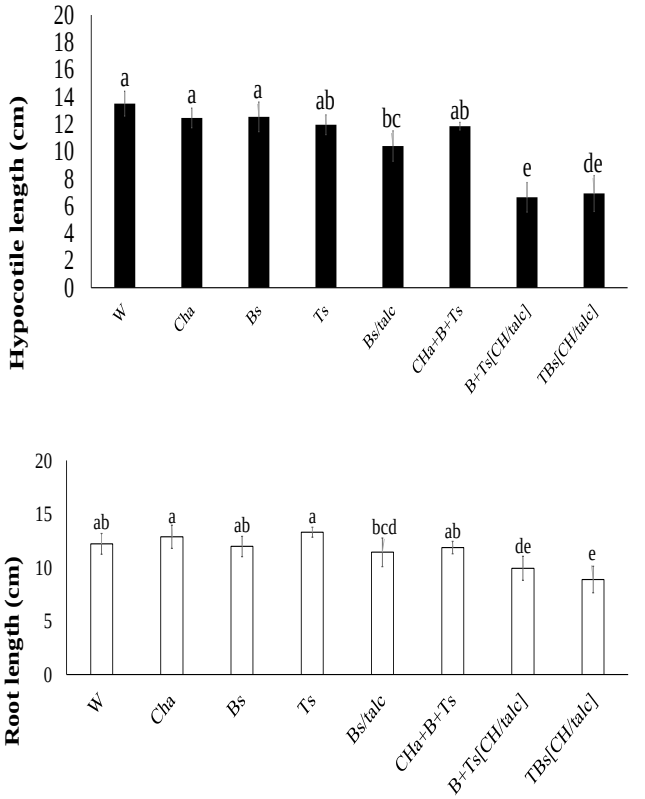

Fig. 5 Hypocotyl (a) and root length (b) in soybean seedlings after the coating treatments. W = Control; Cha = chitosan in acetic acid solution; Bs = *B. japonicum* in sodium alginate; Ts = *T. harzianum* in sodium alginate; Bs/talc = *B. japonicum* in sodium alginate + talc; CHa+B+Ts = chitosan in acetic acid solution + *B. japonicum* in water solution + *T. harzianum* in sodium alginate, B+Ts[CH/talc]= B. japonicum in water solution + *T. harzianum* in sodium alginate (chitosan solid + talc), TBs[CH/talc] = *T. harzianum* and *B. japonicum* in sodium alginate (chitosan solid + talc). Vertical bars indicate ± 1 SD. Different letters indicate significant differences between coating treatments according to the Tukey test ($p < 0.05$).

An unexpected result emerged when analysing formulations containing solid chitosan mixed with talc, as they delayed radicle emergence. Furthermore, the negative effect on radicle emergence extended to germination and seedling growth. This finding aligns with reports indicating that seeds coated with chitosan + alginate/PEG and *B. japonicum* experienced a significant delay in field emergence compared to control and inoculated seeds, leading to slight plant stress [12]. The soybean responded positively to chitosan at the tissue culture level, evidenced by increased callose deposition [33]. However, higher concentrations of chitosan (above 200 µg mL^{-1}) resulted in severe cellular damage, ultimately leading to complete cell destruction. Furthermore, it has been proposed that chitosan may activate different signaling pathways in soybean, depending on its concentration and the equilibrium of cellular substrates and cofactors [34]. This could explain the varied responses observed in this study based on the chitosan formulation. When chitosan was applied in an acidic solution, its concentration was significantly lower than the lethal dose for soybean cells (10 µg mL^{-1}). However, even though chitosan was mixed with talc (1% w/w), its solid particles could generate concentrations that exceed the lethal dose when exposed to water around the radicle. This can result in cell death and consequently delay radicle emergence. It is crucial to study specific biopolymer formulations with microorganisms, as they can significantly alter many system components. Small changes in concentrations and application sequences can enhance the performance of microorganisms but may also negatively affect soybean seed quality. This highlights the importance of selecting the appropriate formulation and application methods to ensure the effectiveness and safety of soybean seed coatings.

5. Conclusion

The specific formulation of sodium alginate, chitosan, *Trichoderma harzianum*, and *Bradyrhizobium japonicum* in soybean seed coatings effectively preserves microbial viability during short-term storage. However, precise *adjustment* of the solid chitosan dose is crucial to mitigate any adverse effects on radicle emergence and germination. The findings of this study underscore the potential for optimizing the application of these biopolymers and microorganisms in seed treatment and storage, thereby fostering more sustainable and reliable agricultural practices.

Acknowledgments

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