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BIOPOLYMER AND *TRICHODERMA HARZIANUM* COMPATIBILITY FOR SUNFLOWER SEED COATING

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ABSTRACT

Seed production demands the progressive replacement of insecticides and fungicides with natural and easily degradable products. Biopolymers and coating technology can be combined to meet that goal. This study proposes chitosan, sodium alginate and *Trichoderma harzianum* formulations that can be applied to sunflower seeds, maintaining their quality and safe storage. The aim of this study was to analyse the effect of coating with different chitosan and sodium alginate combinations on *Trichoderma harzianum* viability and sunflower seed quality. Sunflower seeds were coated with *Trichoderma harzianum* powder mixed with different biopolymer formulations (chitosan at 1% and 3%, sodium alginate at 1.5 %). *Trichoderma* viability was evaluated over time, through colony-forming units per ml. Sunflower seed quality was determined by, radicle emergence, germination percentage, root seedling growth and field emergence. Chitosan applied as seed coating had harmful effects on *Trichoderma* viability and sunflower seed quality. Instead, sodium alginate not only improved the adherence and survival of *Trichoderma harzianum* strains but also maintained the radicle emergence, root growth and germination levels. Sodium alginate creates a protective film for *Trichoderma harzianum* strains from chitosan damaging effect, ensuring adequate storage of sunflower seeds.

Keywords: chitosan, sodium alginate, *Trichoderma,* germination.

INTRODUCTION

Seed production demands the progressive replacement of insecticides and fungicides with natural and degradable products. Biopolymers are derived from renewable resources and, can be degraded into environmentally safe molecules (Valero-Valdivieso et al. 2013). Chitosan is a biodegradable polymer of N-glucosamine and N-acetyl-D-glucosamine units that originates from the enzymatic or chemical deacetylation of chitin. Chitosan stimulates plant growth, has insecticidal and antifungal activity, triggers defensive mechanisms (elicitor effect) and induces enzymatic synthesis (Rajeswari et al. 2020). However, some beneficial microorganisms placed next to the seeds could be altered in their functionality by direct contact with chitosan (Montes Hernandez et al. 2017).

Coating technology allows the successive formation of layers that provide a buffer effect to avoid direct contact between the seeds, the chemical treatments, and the external environment (Pedrini et al. 2017). In addition, some biopolymers can be used as carriers for coating materials and also be able to ensure microorganisms' survival (Chin et al. 2021). Therefore, chitosan application to sunflower seeds, through coating technology, should include biopolymers that have a protective effect on beneficial microorganisms. Sodium alginate is a linear heteropolysaccharide of D-mannuronic and L-guluronic acids extracted from different species of algae, which has become the most common material for microorganism encapsulation and protection (Power et al. 2011). In soybean, sodium alginate

promotes the adhesion of *Bradyrhizobium sp.* on the seeds and protects them from the inhibitory action of fungicides (Romero-Perdomo et al. 2015). The protective effects of alginate on microorganisms were also demonstrated in *Brassica sp*. (Lally et al. 2017, Chin et al. 2021).

The use of biopolymers in combination with coating technology should protect seedlings from insect and fungal attack during field emergence, without detrimental effects on seed quality or shelf life. Beneficial effects of chitosan on seed germination and vigour have been detected in different crops (Prasad et al. 2020, Chin et al. 2021). However, special care should be taken with the formulations and doses used (Balla et al. 2022) because small changes in concentrations can induce or inhibit root growth (Iglesias et al. 2019). Besides, in most experiments, the seeds are soaked in chitosan solutions (priming technique), which implies some difficulty in transferring the results to real scales of production (Cho et al. 2008). Therefore, it would be necessary to investigate different formulations capable of protecting both beneficial microorganisms and seeds and ensuring their safe storage. The aim of this study was to analyze the effect of coating with different chitosan and sodium alginate combinations on *Trichoderma harzianum* viability and sunflower seed quality.

MATERIALS AND METHODS

Materials

Seeds from on especific sunflower hybrid, obtained during 2021/2022 in Venado Tuerto, Santa Fe (33º 44' S; 61º 58' O), Argentina, were evaluated. The seeds were stored for 3 months at 10°C, before the beginning of the experiments. A commercial powder formulation of *Trichoderma harzianum* was used at a dose of 3 g/Kg of seed (Nitrur Trichogen Cergen S.R.L, Buenos Aires, Argentina). This formulation was evaluated before the trials were performed and contained 3.3 x 10^6, colony-forming units per millilitre (CFU/ml).

The biopolymers used were chitosan (medium molecular weight) and sodium alginate, both from Sigma-Aldrich®. Chitosan solutions were prepared by dissolving 1 and 3 g in100 ml of water (Cho et al. 2018), previously acidified with acetic acid "1%" (v/v), to obtain a final chitosan concentration of 1% and 3% (w/v). These solutions were prepared 24 h before being applied to the sunflower seeds and placed on a shaker overnight at room temperature prior to use. Sodium alginate solution was prepared by dissolving 1.5g in100 ml of distilled water to obtain a final concentration of 1.5 % (w/v), and freshly applied to sunflower seeds (Chin et al. 2021). The study was done in Faculty of Agricultural Sciences, University of Lomas de Zamora, "Buenos Aires", "Argentina" "during" "2022".

Treatments

The coating technique consisted of mixing 40 ml in each biopolymer solution in successive layers and *Trichoderma harzianum* powder to obtain the following treatments:

- Chitosan 1% (CH1)
- Chitosan 3% (CH3)
- *Trichoderma harzianum* + water (THW)
- Chitosan 1% + *Trichoderma harzianum*+ water (CH1 + THW)
- Chitosan 3% +*Trichoderma harzianum* + water (CH3+THW)
- Sodium Alginate (SA)
- *Trichoderma harzianum* + Sodium Alginate (THSA)
- Chitosan 3% +*Trichoderma harzianum* + Sodium Alginate (CH3+THSA)

The coating was applied progressively to 300 g of sunflower seeds in continuous rotation (Cortés-Rojas et al. 2021) for 3 m, to ensure homogeneous distribution, adhesion and absorption. In the control treatment (W), seeds were coated with 40 ml of sterile distilled water. Seeds were treated with chitosan and dried out for 24 h before the rest of the treatments were applied. Finally, seeds were dried out again for 24 h at room temperature (25 °C) and stored in brown paper bags at 10°C.

Laboratory Test

Trichoderma Harzianum Viability

One hundred seeds were placed on Erlenmeyer with 90 ml of Tween solution (1 % v/v) and vortexed for 10 min, to extract the conidia from their surface. Samples of 1 ml were extracted from each washing suspension and

placed in tubes containing 9ml of the Tween solution. Subsequently, the serial dilution methodology (Báez et al. 2019) was applied and 3 replicates of 0.1 "ml" were seeded in Petri dishes with Trichoderma selective media (TSM) (Elad et al. 1981), by replacing chloramphenicol with ampicillin (0.2 g/l). The Petri dishes were incubated at 25 °C for 7 days, at which time, the colony count was performed (Báez et al. 2019). The results were expressed in colony-forming units per millilitre (CFU/ml). This variable was analyzed at 1, 30 and 60 days after seed coating.

Radicle Emergence (RE) Test

Radicle emergence was evaluated by placing three 50-sunflower seed replicates of each treatment in 9-cm-diameter Petri dishes on two pieces of Whatman N°1 filter paper, moistened with 2.5 ml distilled water. Afterwards, the Petri dishes were wrapped up in plastic film and placed in a germination chamber at continuous 25°C, with 12 h of alternating light/dark cycles. The number of seeds with emerged radicals > 2mm (Paoloni and Hernández, 1998) was counted at 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, and 68 h after sowing. These times were determined by observing the time from which the radicle emergence in sunflower seeds was evident, according to previous work (Szemruch and Ferrari, 2013). The time required for the emergence of 50% of radicles (RE50) was calculated according to Ranal and García de Santana's formula (2006), expressed in hours for 50% of maximum radicles emergence (1).

$$RE\ 50 = \frac{\left[\left(ER_{MAX}/2\right) - R_1\right] X\ (H_2 - H_1)}{R_2 - R_1} + H_1$$

Where RE is the final percentage of seeds with emerged radicles, H_1 refers to the number of hours from the beginning of the radicles emergence period, H_2 corresponds to the number of hours that elapsed until the end of the radicles emergence period, R_1 is the number of emerged radicles counted at H_1, and $R_2$is the number of radicles emerged counted at H_2.A high number of hours required for RE50 involves lower sunflower seed vigour.

Germination percentage

Germination was calculated by counting the normal seedlings on the tenth day after sowing and expressed as a percentage (ISTA, 2022). Three replicates of 50 seeds were sown in sterilized sand boxes and placed in the germination chamber (IDE, TIPO 40S12, Córdoba, Argentina) at 25°C for 12 h, with alternating light/dark cycles (ISTA, 2022). This variable was evaluated at 1 and 60 days after seed coating.

Root growth

Root length was measured according to Lakshman and Ghodke (2018), on 10 washed seedlings, from each germination test box and expressed in cm.

Field emergence

The field emergence tests were performed near seed coating on a Typical Argiudoll soil in the experimental field of the Faculty of Agronomy, University of Lomas de Zamora (34º 45º S; 58º 29' W). One hundred seeds were sown in each 1 × 1 m plot with 4 rows separated by 0.25 m and at 5 cm soil depth. These plots were free from weeds, diseases and pests and without fertilization and supplementary irrigation. Field emergence was evaluated by counting the emerged seedlings (Schneiter and Miller, 1981) at intervals of 2 or 3 days after sowing. Time for 50% of maximum seedlings emergence (SE50) was calculated using the same formula as for RE50, but replacing hours with days, and number of radical with number of seedlings.

Statistical analysis

Laboratory and field tests were studied by means of a complete randomized design (CRD). *Trichoderma harzianum* viability also included two factors: the seed coating treatments and storage time. Percentage values were transformed using angular transformation. Analysis of variance and LSD Fisher tests were performed with a 5% significance level. Determination coefficients were calculated between laboratory and field data. Infostat statistical software was used (Di Rienzo et al. 2008).

RESULTS

Trichoderma harzianum viability

Near the seed treatments, the highest viability of *Trichoderma* was obtained in sodium alginate coating with 8.2 x 10^4 CFU/ml (Figure 1). Both chitosan doses (1% and 3%) in an aqueous combination significantly reduced the amount of CFU/ml (3.6 – 4.3 x 10^3). Chitosan with sodium alginate coating showed an intermediate level of colony viability (3.5 x 10^4 CFU/ml). Although the amount of CFU/ml was reduced throughout the storage time, sodium alginate reached maximum viability after 30 days (4.9 x 10^4CFU/ml) in

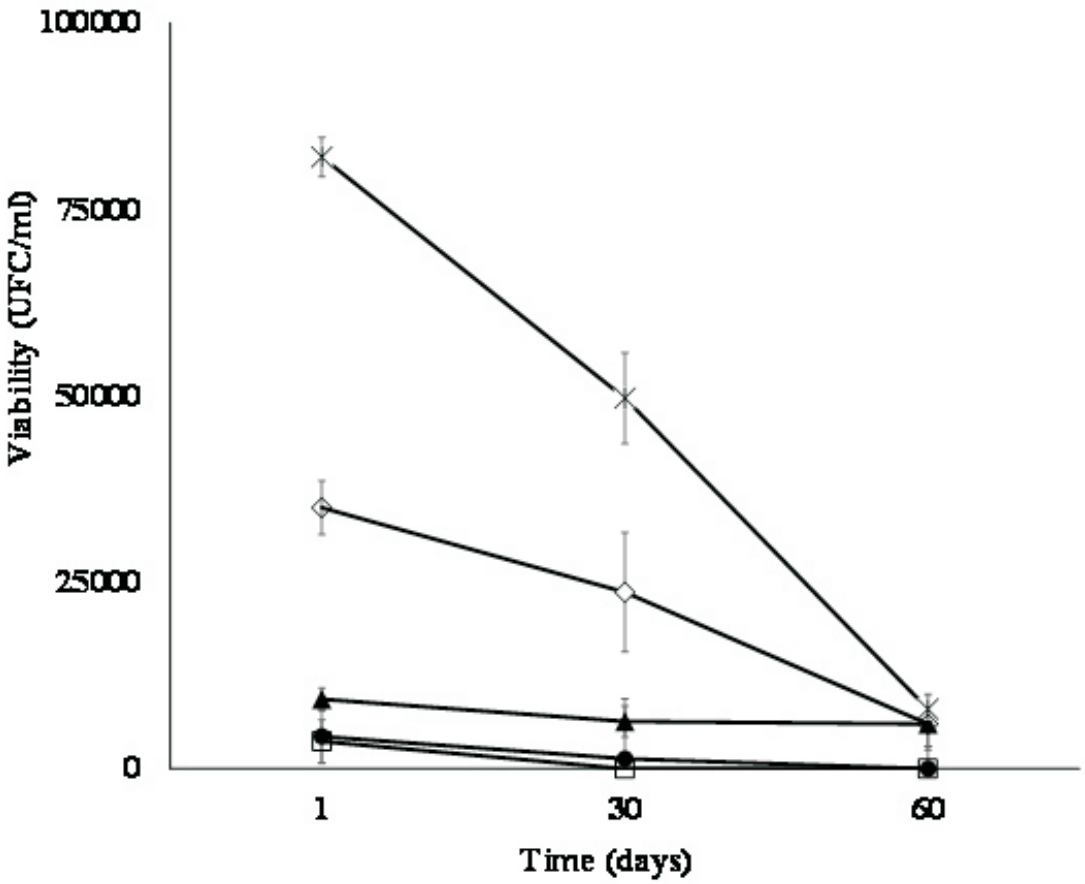

Figure 1. Evolution of *Trichoderma harzianum*(TH) viability during storage time in the following combinations: TH + water (▲), *TH* + sodium alginate (×), chitosan 1 % +TH + water (□), chitosan 3 % +TH + water (●), chitosan 3 % + TH + sodium alginate (◇). Vertical bars indicate ± 1 SD. Two points differ significantly when the standard error bars do not touch each other. L.S.D test ($p < 0.05$)

Radicle Emergence (RE)

Compared with the control, both chitosan doses significantly increased the time required for the emergence of 50% of radicles (RE50) (35.7–36 h). Moreover, its combination with *Trichoderma* in an aqueous solution increased the emergence time even further (41.7-43.1h), thus showing, in these experimental conditions, a reduction in sunflower seed vigour. Instead, sodium alginate coatings maintained radicle emergence at levels similar to those of the control (29.1 h), also when combined with chitosan (30.5 h) (Figure 2).

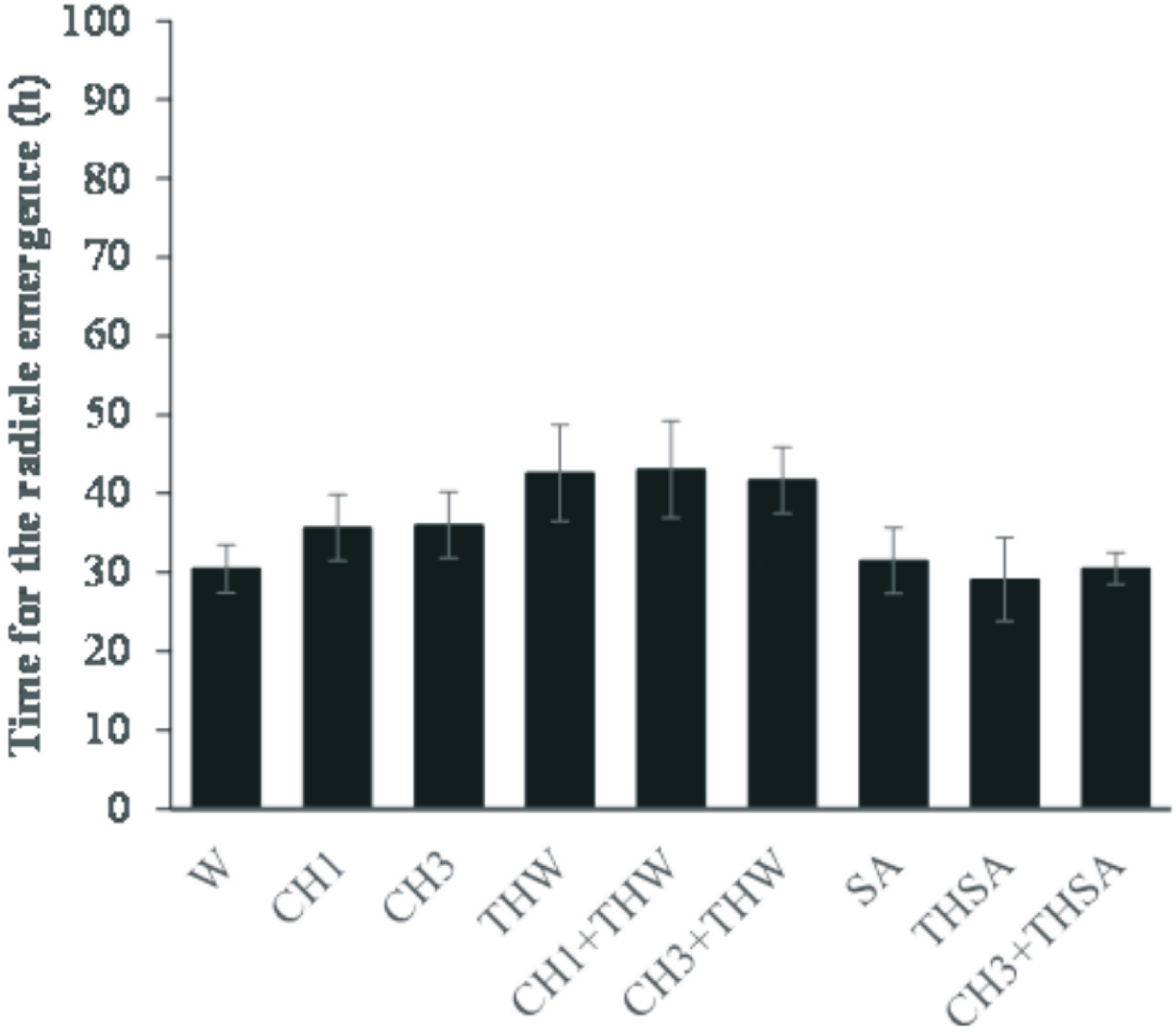

Figure 2. Time required for the emergence of 50% of radicles (RE50) in sunflower seeds after the following treatments water (W), chitosan 1 % (CH1), chitosan 3 % (CH3), *Trichoderma harzianum (*TH*)* + water (THW), chitosan 1 % +TH+ water (CH1+THW), chitosan 3% + *TH* + water (CH3+THW), sodium alginate (SA), TH + sodium alginate (THSA), chitosan 3 % +TH + sodium alginate (CH3+THSA). Vertical bars indicate ± 1 SD. Two points differ significantly when the standard error bars do not touch each other. L.S.D test ($p < 0.05$)

Germination Percentage (GP)

Near seed coating, all treatments with sodium alginate maintained high germination levels in the range of 90% to 93% (Table 1). In contrast, combinations with chitosan and *Trichoderma* in an aqueous solution reduced the germination percentage by 25.5 % on average (Table 1). In addition, this disadvantage of chitosan treatments persisted throughout the storage time (Table 1).

Table 1 Sunflower seed germination (%) during storage time after the following coatings treatments: water (W), chitosan 1 % (CH1), chitosan 3 % (CH3), *Trichoderma harzianum* (TH*)* + water (THW), chitosan 1 % + TH + water (CH1+THW), chitosan 3 % + *TH* + water (CH3+THW), sodium alginate (SA), *TH* + sodium alginate (THSA), chitosan 3 % + *TH* + sodium alginate (CH3+THSA). Different uppercase letters indicate significant differences within each line between coating treatments and lowercase letters within each column between days of storage.

	Time (days)	
	1	60
W	95 ± 3,1 Aa	93 ± 2,3 ABa
CH1	87 ± 4,2 Ba	85 ± 8,5 BCDa
CH3	91 ± 4,2 ABa	89 ± 5,0 ABCa
THW	93 ± 4,2 ABa	94 ± 2,0 ABCa
CH1+THW	73 ± 6,1 Ca	74 ± 2,2 Ea
CH3+THW	77 ± 6,1 Ca	79 ± 1,2 DEa
SA	91 ± 4,2 ABa	83 ± 4,5 CDEa
THSA	90 ± 5,3 ABa	89 ± 6,4 ABCa
CH3+THA	93 ± 2,0 ABa	89 ± 8,3 ABCa

Mean values ± 1 SD. L.S.D test ($p < 0.05$)

Seedlings growth

The maximum root length (above 10 cm) was obtained in *Trichoderma* + water and *Trichoderma* + sodium alginate coating (Figure 3), followed by treatment with *Trichoderma* + sodium alginate+ chitosan 3%.

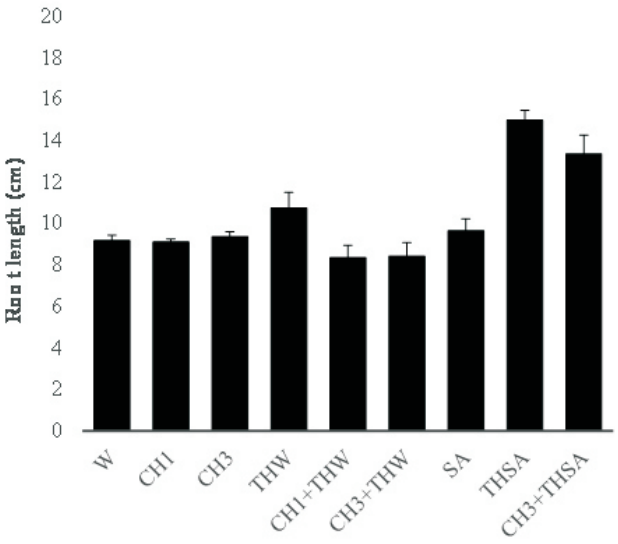

Figure 3. Root length in sunflower seedlings after the following coating treatments: water (W), chitosan 1 % (CH1), chitosan 3 % (CH3), *Trichoderma harzianum* (TH) + water (THW), chitosan 1 % + TH + water (CH1+THW), chitosan 3 % + TH + water (CH3+THW), sodium alginate (SA), TH + sodium alginate (THSA), chitosan 3 % + *TH* + sodium alginate (CH3+THSA). Vertical bars indicate ± 1 SD. Two points differ significantly when the standard error bars do not touch each other. L.S.D test ($p < 0.05$)

The rest of the chitosan treatments showed significantly lower root size (under 10 cm) but without significant differences from the control (Figure 3).

Field Emergence

Sunflower seeds coated with chitosan and their *Trichoderma* combination delayed the field emergence time (SE50) significantly until 32 to 35 days (Table 2). The faster field emergence time was measured in treatments that included *Trichoderma* and sodium alginate with 26.7–30.7 days (Table 2). SE50 had a significant and positive association with ER50 (determination coefficients of 0.57) and showed that the differences in vigour found in the laboratory were also observed in field conditions.

Table 2: Time for 50% of maximum for sunflower seedling emergence (SE50) after the following coatings treatments: water (W), chitosan 1% (CH1), chitosan 3% (CH3), *Trichoderma harzianum* (TH) + water (THW), chitosan 1% + TH + water (CH1+THW), chitosan 3% + TH + water (CH3+THW), sodium alginate (SA), TH + sodium alginate (THSA), chitosan 3% + TH + sodium alginate (CH3+THSA). Different uppercase letters indicate significant differences within each column between coating treatments.

	Time (days)
W	29,3 ± 1,78 ABC
CH1	32,4 ± 2,57 CD
CH3	31,4 ± 3,24 BCD
THW	29,5 ± 1,16 C
CH1+THW	35,0 ± 4,02 D
CH3+THW	32,9 ± 2,30 CD
SA	30,7 ± 0,79 BCD
THAS	26,7 ± 1,74 A
CH3+THA	28,0 ± 2,25 AB

Mean values ± 1 SD.L.S.D test ($p < 0.05$)

DISCUSSION

Trichoderma harzianum viability

Viability was reduced in coating treatments that involved direct contact between chitosan and *Trichoderma*. This could be due to the fact that chitosan does not diffuse, that is, its antimicrobial activity occurs without molecule migration (Montes Hernandez et al. 2017). When *Trichoderma* powder was incorporated into an additional layer mixed with sodium alginate (1.5%), the strains' viability increased significantly, verifying the results of Costales et al. (2019) in soybean. This is due to the ability of this substance to form a gel on the surface of the seeds (Romero Perdomo, 2015). In this way, sodium alginate may generate a protective layer from chitosan' sharmful effect on *Trichoderma* strains. Similar observations were made in groundnut (peanut) and safflower by Prasad et al. (2020) who reported that blends with polyethyleneglycol-chitosan were efficient at maintaining the viability of *Trichoderma* spore.

In addition, the alginate gelling capacity (Lee and Mooney, 2012) may facilitate the adherence of *Trichoderma* spores on sunflower seeds. A higher *Trichoderma* spore viability was obtained with sodium alginate for Chin et al. (2021), although under the seed priming methodology. Szopa et al. (2022) consider that the optimal concentration of sodium alginate is between 1% and 3%, because high doses cause increased viscosity and poor results in matrix cross linking. For these reasons, a concentration of 1.5% is recommended for sunflower seeds. Employing other biopolymers (gelatine and pectin) on rice seed coating, Cortés-Rojas et al. (2021) also observed the highest protection of *Trichoderma koningiopsis* at 60 days. These substances act as water and gas barriers creating a microenvironment that isolates the conidia from drying. Based on the ranges obtained in other species (Astiz Gassó, 2017), the application of *Trichoderma* + sodium alginate may maintain a quantity of 4.9 x 10^4 CFU/ml on sunflower seeds for 30 days. This could ensure the sunflower producer an adequate concentration of inoculum in sowings delayed by a month.

Radicle emergence (RE)

The coating with both chitosan doses (1% and 3 %) reduced the radical emergence rate and, therefore, the sunflower seed vigour. Using a different application technology from the one used in our study, Jabeen et al. (2012) found that high chitosan concentrations could obstruct water absorption due to its high stickiness. This, in turn, could interfere with seminal covering permeability, limiting water absorption and oxygen transfer and affecting embryo development (Peña-Datoli et al. 2016, Cortés-Rojas et al. 2021). The delay in sunflower radicle emergence could be explained then, by the lower water or oxygen absorption rate by chitosan-coated seeds. Further studies are required to determine the water and oxygen consumption of sunflower seeds coated with chitosan. In addition, high chitosan doses stopped root growth in *Arabidopsis*, *Lycopersicum* and *Hordeum*, due to an alteration in auxin synthesis, transport and signaling (Lopez Moya et al., 2017).

When chitosan and *Trichoderma* were combined, the negative effect on the radicle emergence rate was increased. *Trichoderma* coating on wheat inhibited root growth probably due to competition for nutrients or to plants expressing resistance in response to interactions with this fungus (Couto et al. 2021). Pelagio-Flores et al. (2017) found that medium acidification by *Trichoderma atroviride* may explain the loss of root meristem functionality in *Arabidopsis*. This trend was recently confirmed in soybean trials when the soil surface was sprayed with *Trichoderma* spp., resulting in their acidification (Conte et al. 2022). Additionally, Sing *et al*. (2016) indicate that the positive or negative effect of *Trichoderma asperellum* on radicle growth depends on the exact dose of spores required for the seed. Similarly, Chin *et al*. (2021) detected that high concentrations of *Trichoderma* reduced the length of the radicals in *Brassica sp.* Then is important to know the relationship between *Trichoderma* dose and the pH of the medium surrounding the sunflower radicles, especially in the first hours of the emergence (30–60 h).

On the other hand, under the circumstances of these experiments, radicle medium growth may have become more acidic because chitosan was diluted in acetic acid before it was applied it to the sun flower seeds. Unfortunately, despite chitosan's advantages, its solubility is limited at a

pH higher than 6.5 where it starts to lose its cationic nature. This problem is probably the major limiting factor for chitosan utilization (Amine et al. 2021).

In contrast, the formulations that combined sodium alginate with *Trichoderma* and chitosan maintained the radicle emergence rate at levels similar to those of the control. These results agree with Chin et al. (2021), who observed that sodium alginate did not generate adverse effects on the radicle growth of *Brassica rapa* L.

Germination percentage (GP) and seedlings growth

The treatments that included chitosan in combination with *Trichoderma* in anaqueous solution reduced the sunflower germination percentage. This means that, the harmful effects at radicle level were also transferred to seedling growth. Using chitosan as seed priming, Cho et al. (2018) detected sunflower germination enhancement due to the increased phenolic, melatonin and isoflavone contents. However in our experiments, chitosan was placed by the coating technique in direct contact with the seeds, which may explain the differences observed.

In contrast, the treatments that included sodium alginate, *Trichoderma* and *Trichoderma* + chitosan maintained germination at similar levels to those of the control. As mentioned above, the chemical characteristics of sodium alginate provide a film (Oliveira et al. 2009) that protects sunflower seeds from the harmful effects of chitosan. These results are in agreement with those obtained by Prasad et al. (2020) using alginate as a coating on safflower and peanut seeds.

The seedlings growth response to chitosan coating was similar to previous variables, with a significantly lower root length. Instead, all treatments that included *Trichoderma* showed an improvement in root growth. These beneficial effects of *Trichoderma* on root growth by the seed priming technique were observed in sunflower (Lakshman and Ghodke 2018). However, there are relatively few studies using *Trichoderma* sp. as an active agent in seed coating (Cortés-Rojas et al. 2021). In soybean seeds, some strains of *Trichoderma virens* applied by coating maintained or reduced germination and increased root size (Yusnawan et al. 2019). For Couto et al.(2021), the positive or negative effects on wheat plant growth depend on *Trichoderma* doses. At higher doses, greater production of phytohormones may have been enough to compensate for the effects of additional energy expenditure due to the endophytic interaction.

Field emergence

The presence of chitosan, alone or in combination with *Trichoderma,* reduced the sunflower seed field emergence rate. These results are in agreement with Peña Datoli et al. (2016), who found that chitosan and sodium alginate reduced maize field emergence by 40% and 14%, respectively. According to these authors, and as mentioned above, chitosan may form insoluble complexes in water, which reduces the imbibition rate and field emergence.

Treatments with *Trichoderma* alone or combined with sodium alginate had a higher field emergence rate. This is due to *Trichoderma's* promoting effect on root growth, which would generate an increase in soil water and nutrient uptake (Anis et al. 2013). Seedlings field emergence was significant and positively related to ER50, showing that the detrimental effects of chitosan and the neutral effects of sodium alginate manifest even under field conditions.

CONCLUSION

The application of sodium alginate through coating technology creates a protective film that improves the adherence and survival of *Trichoderma harzianum* strains and protects them from the chitosan damaging effect. It also improves radicle emergence, maintains germination levels and ensures adequate storage time of sunflower seeds.

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CONFLICT OF INTEREST

No conflict of interest declared

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