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ORIGINAL ARTICLE

Canola seeds coating with formulations based on sodium alginate, chitosan and *Thichoderma harzianum*

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Abstract

Seed coating technology combined with biopolymers offers an alternative method to reduce environmental contamination. However, when biological agents are incorporated, biopolymers would have diverse properties and effects. This underscores the necessity of exploring the optimal dosages and formulations of biopolymers to ensure the survival of beneficial microorganisms, seed quality, and proper storage. This study aimed to explore the effects of different sodium alginate and chitosan coating formulations on *Trichoderma harzianum* viability and canola seeds quality. The coating process involved mixing *T. harzianum* powder with sodium alginate, talc and chitosan in different doses, sequences and formulations. *Trichoderma harzianum* viability was assessed through colony-forming units per ml over time. Canola seed quality was evaluated by measuring radicle emergence, germination percentage, seedling growth, and field emergence. Sodium alginate, both alone and in combination with talc, improved *T. harzianum* viability immediately after treatment and during storage. These coatings did not impair seed germination and improved canola root growth. Among the different chitosan formulations, a 1 : 100 ratio in talc improved strain survival and root growth without affecting germination, radicle, and field emergence. Coating canola seeds is a practical alternative to the application of *T. harzianum*, sodium alginate and talc, as it preserves their viability over time and improves seedling performance. Chitosan formulations in acetic acid should be carefully developed to prevent negative effects on seeds or biological agents.

Keywords: biopolymers, field emergence, germination, growth, radicle

Introduction

Seed coating technology encompasses the application of several materials, including fungicides, insecticides, biostimulants, growth regulators, nutrients, and inoculants (Ma 2019). The basic treatment, or 'film coating', involves applying a thin material layer resulting in a weight increase of less than 10%. When weight increases range from 20 to 200%, while preserving the seed's original shape, it is referred to as 'encrusting' (Taylor 2020). Materials used in seed coating are categorized into binders and fillers. Binders are polymers applied in liquid form that offer adhesion, cohesion, ingredient retention, and integrity. The most frequently used binders include water, polyvinyl alcohol, polyvinyl acetate, methylcellulose, carboxymethylcellulose, maltodextrins, and arabic gum. Fillers consist of inert fine powders such as bentonite, calcium carbonate, talc, diatomaceous earth, sand, and wood dust (Pedrini *et al.* 2017). Seed coating reduces the risk

of environmental contamination, as the active ingredients are used in significantly lower amounts. Additionally, the application of multiple layers of binder and filler provides a physical buffer, preventing direct contact between the seed, chemicals, and other active layers (Zhang *et al.* 2022).

A potentially beneficial method for environmental protection is biopolymer-based seed coating. The term "biopolymers" derives from renewable living sources composed of monomer units, such as nucleic acids, amino acids, proteins, or monosaccharides (George *et al.* 2020). Biopolymers have the potential to protect plants against fungal pathogens by serving as carriers of active ingredients, antifungal compounds, or defense system inducers (Korbecka-Glinka *et al.* 2022). Sodium alginate, a linear polysaccharide derived from algae, is composed of β-D-mannuronate, and α-L-guluronate linked by 1–4 glycosidic bonds. It is an ideal hydrogel matrix, which can increase the water-holding capacity of agricultural land, and encapsulate biological agents or chemical pesticides (Riseh *et al.* 2022). Chitosan, a cationic polysaccharide formed from N-glucosamina and N-acetil-D-glucosamina units, is obtained through the alkaline deacetylation of chitin. The latter is found in marine invertebrates, insects, and fungi. Chitosan has multiple agricultural applications, including pest and pathogen protection, defense response induction, stress tolerance enhancement, secondary metabolite production, and plant growth improvement (Sun *et al.* 2023). However, chitosan´s specific form can significantly affect results, since deacetylation and polymerization degrees confer distinct biological properties (Coelho and Romano 2022).

Trichoderma spp., an antagonistic fungus with diverse species, plays a significant role in protecting crops from a range of diseases through mechanisms like competition, mycoparasitism, and antibiosis (Tyśkiewicz *et al.* 2022). Additionally, it produces lytic enzymes and secondary metabolites derived from agroindustrial waste (Hamrouni 2019). However, its efficacy can vary based on different doses, molecular weights and methods of chitosan application (Kappel *et al.* 2022; Szemruch *et al.* 2022).

Chitosan and alginate, two biopolymers with considerable potential for encapsulating beneficial microorganisms, can offer synergistic advantages when applied together (Riseh *et al.* 2022). When these biopolymers are mixed with biological agents, through coating technology, it is essential to test their compatibility to prevent any adverse effects on seed and microorganism performance. The aim of this study was to explore the effects of different sodium alginate and chitosan coating formulations on *Trichoderma harzianum* viability and canola seed quality.

Materials and Methods

Materials

Seeds from canola (*Brassica napus* L.) belonging to a spring hybrid of Australian origin were evaluated. The seeds were stored for 3 months at 10°C prior to the beginning of the experiments. A commercial powdered formulation of *Trichoderma harzianum* [*T. harzianum*] containing 1×10^9 conidia · g^{-1} was used at a dose of 3 g/50 g of seed. Sodium alginate from Sigma-Aldrich® was prepared by dissolving 1.5 g in 100 ml of distilled water to obtain a final concentration of 1.5% (w/v) and this solution was freshly applied to canola seeds. Two chitosan-based formulations (medium molecular weight, Sigma-Aldrich®) were prepared. A liquid formulation was obtained by dissolving chitosan in a 1% (v/v) aqueous solution of acetic acid to achieve a final chitosan concentration of 3% (w/v) (Szemruch *et al.* 2022). This solution was prepared 24 hours in advance of its application to canola seeds. The other formulation, in a solid state, was prepared by mixing chitosan with talc [$Mg_3Si_4O_{10}(OH)_2$] used as a carrier, at two different ratios, 1 : 100 and 3 : 100 of chitosan: talc, respectively. The adhesive capacity of alginate was compared with the most commonly commercial adherent used in seed technology, in an 8% (w/v) aqueous solution.

Treatments

The seed treatment technique included a biological control agent (*T. harzianum* powder), an organic control agent (chitosan - CH), two binders (sodium alginate – SA; commercial adherent – CA), and a filler (talc). These were applied in successive layers to obtain the treatments listed in Table 1. To ensure uniform distribution, adhesion, and absorption, 10 ml of each biopolymer solution was progressively applied to 50 g of canola seeds in continuous rotation for 3 minutes. For the control treatment (W), seeds were coated with 10 ml of sterile distilled water. Subsequently, all treatments were air-dried for an additional 24 hours at room temperature (25°C) and stored in brown paper bags at 10°C. To maintain terminological precision it is important to consider that, the control and treatments without talc correspond to the "film coating" technology, since the increase in weight was less than 10%. In contrast, treatments involving talc are more accurately described as 'encrusting,' as their weight increases by 44.7% while preserving the original shape of the canola seed.

Table 1. Biopolymers and *Trichoderma harzianum* [*T. harzianum*] application sequences in canola seed coating

Seed treatment code	First layer	Second layer
SA	sodium alginate	–
ThSA	*T. harzianum* + sodium alginate	–
ThSA + Talc	*T. harzianum* + sodium alginate + talc	–
CA	commercial adherent	
ThCA + Talc	*T. harzianum* + commercial adherent + talc	–
AA	acetic acid solution	–
CH3 + ThSA	chitosan in acetic acid solution (3%)	*T. harzianum* + sodium alginate
ThSA + TalcCH1	*T. harzianum* + sodium alginate	talc + chitosan solid 1%
ThSA + TalcCH3	*T. harzianum* + sodium alginate	talc + chitosan solid 3%

Laboratory test

Trichoderma harzianum viability

To recover conidia from the seed surface, the weight equivalent of 1000 seeds from each treatment was transferred to Erlenmeyer flasks with 6.84 ml of Tween solution (1% v/v) and vortexed for 20 minutes. Samples of 1 ml were extracted from each washing suspension and placed in tubes containing 9 ml of the Tween solution. Subsequently, the serial dilution method (Báez *et al*. 2019) was employed. Three replicates of 0.1 ml were seeded in Petri dishes containing *Trichoderma* spp. selective media (TSM) (Elad *et al*. 1981), with ampicillin (0.2 g · l^{-1}) used as a substitute for chloramphenicol. The Petri dishes were incubated for 7 days at 25°C, at which moment, colony count was performed. The results were quantified in terms of colony-forming units per millilitre (CFU · ml^{-1}) and assessed at 1, 30, and 60 days post-seed coating.

Radicle emergence rate

The radicle emergence rate was evaluated according to ISTA (2023) on four replicates of 100 seeds for each treatment. The numbers of seeds with radicle breaking through the seed coat were counted at 6, 9, 12, 15, 18, 21, 24, 27 and 30 hours from sowing. These time points were determined according to a previous trial with the same genotype. The time required for the emergence of 50% of radicles (RE50) was calculated according to the Ranal and García de Santana formula (2006) and expressed in hours for 50% of maximum radicle emergence. Lower RE50 values indicate higher seed vigour (ISTA 2023).

Germination percentage

Germination was calculated through the counting of normal seedlings, 7 days after sowing and expressed as a percentage. Three replicates of 100 seeds were sown on top paper, pre-chilled and placed in a germination chamber at 20°C and 12 hours, alternating light/dark cycle (ISTA 2023).

Seedling growth

Seedling growth was evaluated by measuring the length of roots and hypocotyls of 10 seedlings from each germination test replicate and expressed in mm.

Field emergence

The field emergence tests were performed shortly after seed coating. One hundred seeds were sown in each 1 × 0.8 m plot with five rows separated by 0.20 m and at 2 cm soil depth. These plots were free from weeds, diseases and pests and without fertilization. Field emergence was evaluated by counting the cotyledons at intervals of 1 or 2 days after sowing. Time for 50% of maximum seedlings emergence (SE50) was calculated using the same formula for RE50, but replacing hours per days, and number of radicles per number of seedlings.

Statistical analysis

Laboratory and field tests were studied by means of a complete randomized design (CRD). *T. harzianum* viability also included two factors, the seed coating treatments and storage time. Percentage values were transformed using angular transformation. Analysis of variance and Tukey's (honestly-significant-difference) test were performed with a 5% significance level.

Results

Trichoderma harzianum viability

Immediately after seed treatments, sodium alginate obtained the highest viability of *T. harzianum* with 2.04 ×10^6 CFU · ml^{-1} (Fig. 1). This was significantly higher than the rest of the treatments. This biopolymer maintained high *T. harzianum* conidia viability even when combined with talc (ThSA + Talc) (Fig. 1). Incorporating chitosan in acetic acid solution as the

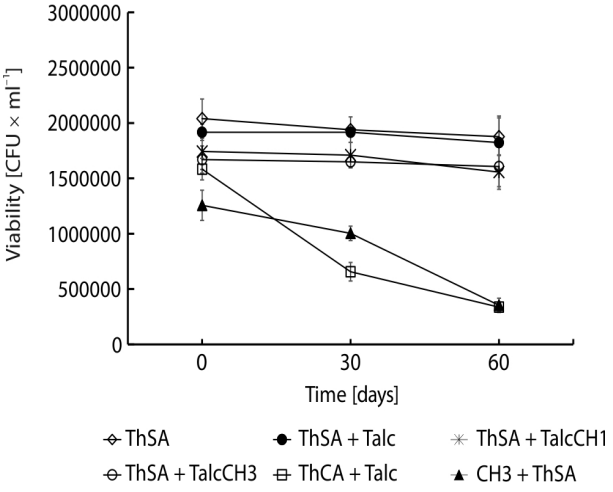

Fig. 1. Evolution of *Trichoderma harzianum* (*T. harzianum*) viability during storage time in different treatments
ThSA: *T. harzianum* + sodium alginate; ThSA + Talc: *T. harzianum* + sodium alginate + talc; ThSA + TalcCH1: *T. harzianum* + sodium alginate + talc + chitosan 1%; ThSA + TalcCH3: *T. harzianum* + sodium alginate + talc + chitosan 3%; ThCA + Talc: *T. harzianum* + commercial adherent + talc; CH3 + ThSA: chitosan acetic acid solution 3% + *T. harzianum* + sodium alginate. Vertical bars indicate ± 1 SD. Two points differ significantly when the standard deviation bars do not touch each other

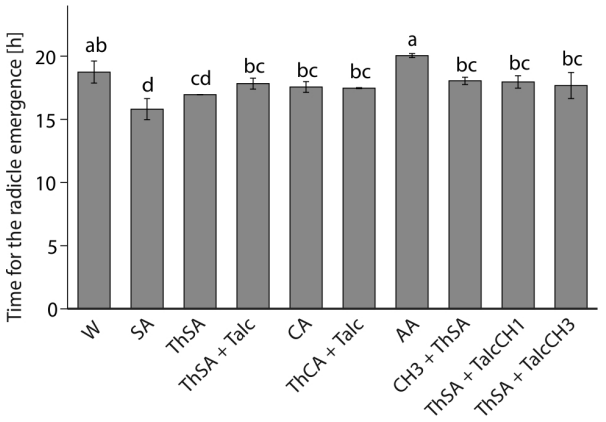

Fig. 2. Time required for the emergence of 50% of radicles (RE50) in canola after different seed coating treatments
W: water; SA: sodium alginate; ThSA: *T. harzianum* + sodium alginate; ThSA + Talc: *T. harzianum* + sodium alginate + talc; CA: commercial adherent; ThCA + Talc: *T. harzianum* + commercial adherent + talc; AA: acetic acid; CH3 + ThSA: chitosan acetic acid solution 3% + *T. harzianum* + sodium alginate; ThSA + TalcCH1: *T. harzianum* + sodium alginate + talc + chitosan 1%; ThSA + TalcCH3: *T. harzianum* + sodium alginate + talc + chitosan 3%. Different letters indicate significant differences ($p < 0.05$). Vertical bars indicate ± 1 SD

first layer on the seeds (CH3 + ThSA) resulted in the lowest counts (1.2×10^6 CFU · ml^{-1}). However, adding chitosan as a solid mixed in talc (ThSA + TalcCH1, ThSA + TalcCH3) improved *T. harzianum* survival (Fig. 1). Although there were no significant differences between them, the 1% mixture had a higher level of colony viability.

Throughout the storage time, *T. harzianum* CFU · ml^{-1} decreased, however sodium alginate treatment maintained the highest viability (1.80×10^6 CFU · ml^{-1}) compared to the rest. After 60 days, treatments that included chitosan solid mixed in talc showed higher viability than those with acetic acid solution and the commercial adherent ($3.30–3.50 \times 10^5$ CFU · ml^{-1}) (Fig. 1).

Radicle emergence

In comparison to the control, the time for radicle emergence was significantly faster in coatings that included sodium alginate alone (SA) or its mixture with *T. harzianum* (ThSA). This indicates an increase in canola seed vigour (Fig. 2). Sodium alginate + talc and both solid chitosan formulations had the same radicle emergence time between them and compared to the control, which did not harm the vigour of the seeds (Fig. 2).

Table 2. Germination percentage of canola after different seed coating treatments

Treatments	Germination [%]
W	72 b
SA	96 a
ThSA	97 a
ThSA + Talc	97 a
CA	98 a
ThCA + Talc	98 a
AA	98 a
CH3 + ThSA	98 a
ThSA + TalcCH1	98 a
ThSA + TalcCH3	96 a

W: water; SA: sodium alginate; ThSA: *T. harzianum* + sodium alginate; ThSA + Talc: *T. harzianum* + sodium alginate + talc; CA – commercial adherent; ThCA + Talc: *T. harzianum* + commercial adherent + talc; AA: acetic acid; CH3 + ThSA: chitosan acetic acid solution 3% + *T. harzianum* + sodium alginate; ThSA + TalcCH1: *T. harzianum* + sodium alginate + talc + chitosan 1%; ThSA + TalcCH3: *T. harzianum* + sodium alginate + talc + chitosan 3%. Different letters indicate significant differences ($p < 0.05$)

Germination percentage

Immediately after seed coating, treatments combining *T. harzianum*, sodium alginate and chitosan in all its formulations (acetic acid solution and solid mixture) increased germination levels by 25% on average compared to the control (Table 2). Additionally, the talc carrier had no adverse effect on germination.

Seedlings growth

Treatments without chitosan, which included *T. harzianum*, increased the root length by 35.4 mm on average compared to the control (Fig. 3A). This trend was also observed when *T. harzianum* was combined with talc, sodium alginate (ThSA, ThSA + Talc), and commercial adherent (ThCA + Talc) (Fig. 3A). The 1%

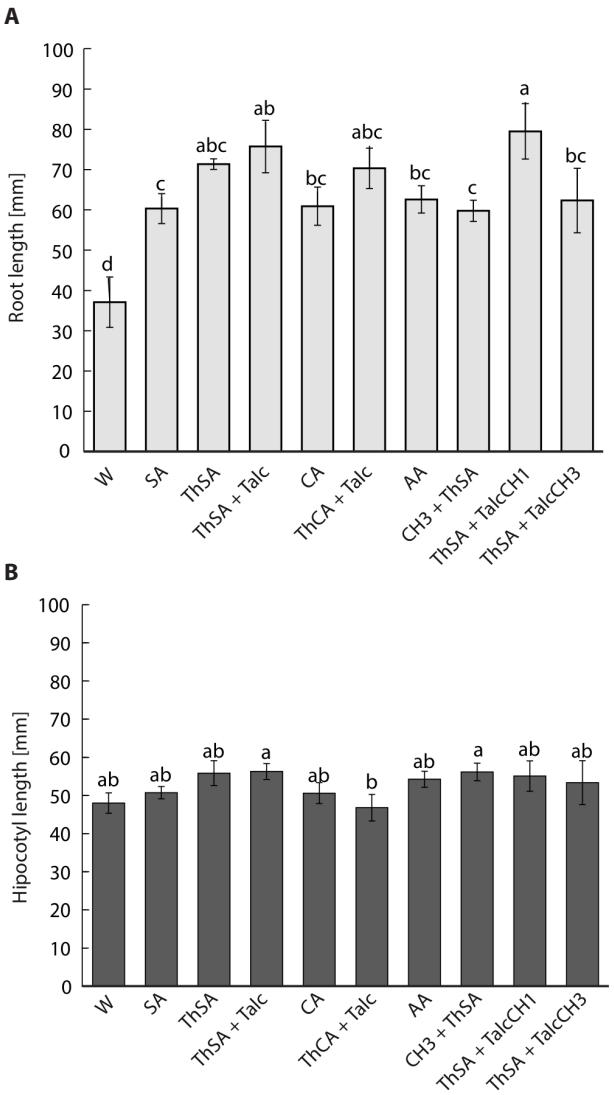

Fig. 3. Root (A) and hypocotyl (B) length in canola seedlings after different coating treatments W: water; SA: sodium alginate; ThSA: *T. harzianum* + sodium alginate; ThSA + Talc: *T. harzianum* + sodium alginate + talc; CA: commercial adherent; ThCA + Talc: *T. harzianum* + commercial adherent + talc; AA: acetic acid; CH3 + ThSA: chitosan acetic acid solution 3% + *T. harzianum* + sodium alginate; ThSA + TalcCH1: *T. harzianum* + sodium alginate + talc + chitosan 1%; ThSA + TalcCH3: *T. harzianum* + sodium alginate + talc + chitosan 3%. Different letters indicate significant differences ($p < 0.05$). Vertical bars indicate ± 1 SD

chitosan solid formulation (ThSA + TalcCH1) resulted in the maximum root length (79.5 mm), which was 42.4 mm greater than the control (Fig. 3A). In contrast, incorporating chitosan in the acetic acid solution as the first layer, significantly reduced the growth to 59.7 mm. Smaller differences were observed in hypocotyl length, with combinations of *T. harzianum* with sodium alginate and talc (ThSA + Talc), as well as the acetic acid chitosan formulation (CH3 + ThSA), showing a favorable effect. Both chitosan solid mixtures in talc (ThSA + TalcCH1, ThSA + TalcCH3) maintained the same hypocotyl length and was comparable to the control (Fig. 3B).

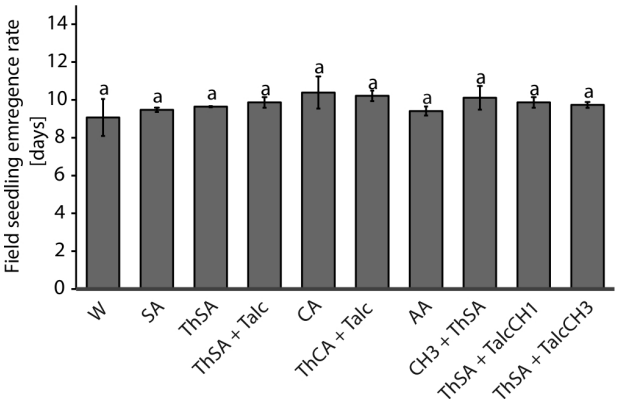

Fig. 4. Time required for the emergence of 50% of canola seedlings (SE50) after different seed coating treatments
W: water; SA: sodium alginate; ThSA: *T. harzianum* + sodium alginate; ThSA + Talc: *T. harzianum* + sodium alginate + talc; CA: commercial adherent; ThCA + Talc: *T. harzianum* + commercial adherent + talc; AA: acetic acid; CH3 + ThSA: chitosan acetic acid solution 3% + *T. harzianum* + sodium alginate; ThSA + TalcCH1: *T. harzianum* + sodium alginate + talc + chitosan 1%; ThSA + TalcCH3: *T. harzianum* + sodium alginate + talc + chitosan 3%. Different letters indicate significant differences ($p < 0.05$). Vertical bars indicate ± 1 SD

Field emergence

Canola seeds coated with *T. harzianum,* sodium alginate and chitosan solid mixture, maintained stable field emergence rates (Fig. 4). Commercial adherent alone and chitosan in acetic acid solution slightly delayed the field emergence time (SE50), although not significantly (Fig. 4).

Discussion

Coating canola seeds with the mixture of sodium alginate and *T. harzianum* improved the viability of the strains immediately after treatment and during 60 days of storage. In a different application technique, biopriming of *Brassica rapa* seeds with sodium alginate (1.5% w/v) also improved the viability of *T. asperellum* spores (Chin *et al.* 2021). Cortés-Rojas *et al.* (2021) demonstrated that certain biopolymers (such as gelatine and pectin) provide desiccation tolerance to *T. koningiopsis* conidia for up to 60 days by creat-

ing a microenvironment that isolates the conidia from water vapor or reactive oxygen agents. The improved survival of *T. harzianum*, when the coating technique was applied, could be due to the properties of alginate. Its gelling and water absorption capacity could protect conidia from dehydration, similar to encapsulated granules (Locatelli *et al.* 2018).

The coating examined in this research was comprised of different layers, including not only sodium alginate and *T. harzianum* conidia but also a carrier such as talc. Arsyadmunir *et al.* (2023) investigated a biopolymeric formulation for coating maize seeds using *Trichoderma* spp. as the active ingredient. They found that a composition of 5% xanthan gum, 5% sodium alginate and talc could reduce the viability of *Trichoderma* and the seeds 60 days after application. However, in our study, talc appeared to act as an inert agent that maintained the viability of *T. harzianum* conidia for 60 days and the physical integrity of the canola seeds. This is consistent with Aguirre *et al.* (2023), who mentioned that talc did not decrease significantly the *T. harzianum* viability, but its use must be accompanied by other adjuvants such as carboximetilcelulosa, to avoid conidia dehydration. In *B. juncea* L. seeds, formulations based on *T. harzianum* and talc, at a 1 : 9 ratio, maintained viability at levels of 1.0×10^6 up to 150 days of storage (Meena *et al.* 2014). Polymeric coating of soybean seeds with *T. viride* and talc improved plant growth and endophytic colonization, although no detailed information on the coating used is provided (Kuchlan *et al.* 2018). Therefore, talc may also serve as a suitable *T. harzianun* carrier when applied in seed coating.

The impact of these formulations on germination and seedling growth processes can differ. In soybean, an increase in the concentration of sodium alginate above 4% reduced germination, seed vigour and seedling emergence (Scarsi *et al.* 2020). Introducing *T. guizhouense* in a starch-based coating improved the germination, and growth of maize and watermelon (Xie *et al.* 2023). A more complex formulation, using a gelatin film reinforced with cellulose nanocrystals as a matrix for *T. harzianum* spores in seed coatings, enhanced the germination speed and root length of maize seeds (Dogaru *et al.* 2021). Immersing *B. rapa* L. seeds in sodium alginate (1.5% w/v) and *T. viride* had no adverse effect on radicle length or germination (Chin *et al.* 2021). In canola seeds, sodium alginate (1.5%) combined with *T. hazianum* did not impair germination and improved root growth. These benefits could depend on the species used, as *T. harzianum* showed significant increases in germination percentage, root length and stem growth, while *T. atroviride* did not exhibit a significant difference compared to the control, despite an increase in germination rate (Ghasemialitappeh *et al.* 2018). In *Arabidopsis thaliana*, the release of volatile organic compounds from *T. harzianum* delayed germination, but at the same time had a positive effect on seedling growth and defense system (Rubio *et al.* 2023). These authors found it challenging to interpret the variation in growth as different *Trichoderma* spp. strains can exert different stimulations depending on the type of volatile compound released. Further research on the interactions between sodium alginate and *T. harzianum* is needed to identify which substances altered the growth of canola seedlings.

The effects of chitosan on microorganism viability and germination depend on the species, the application technique and the combinations of biopolymers used (Chen *et al.* 2023). The chitosan and polietilenglycol blended filmogenic solution with glycerol can be used as a carrier for *Trichoderma* spp. strains to be applied as castor seed coating material. These polymers form a uniform film on seeds and can regulate gas exchange and water vapor transmission without inhibiting germination. They provide good compatibility and protection for *Trichoderma* spp. during storage (Chandrika *et al.* 2019). Chitosan film coating diluted with water, increased germination by 26% in bean and 16% in sesame compared with the control. These treatments also enhanced the germination speed index and the root length (Godínez-Garrido *et al.* 2022). Formulations of alginate, nanocellulose and chitosan in microcapsules maintained 100% *T. longibrachiatum* conidia viability for 2 months (Arias-Chavarría *et al.* 2023). The coating with chitosan dissolved in acetic acid reduced sunflower seed germination (Szemruch *et al.* 2022) and maize field emergence (Peña-Datoli *et al.* 2016). In this study, *T. harzianum* viability was also reduced when chitosan was dissolved in acetic acid. For these reasons, to avoid direct contact of the chitosan with the *T. harzianum* strains, a new mode of coating chitosan was proposed. This involved applying chitosan in a solid form mixed with talc as a second layer on the seeds that had been previously treated with sodium alginate + *T. harzianum*. This 1% chitosan formulation improved strain survival both 24 hours after treatment and during storage. Furthermore, it did not impair radicle emergence speed, germination, field emergence and enhanced root growth. These results are in agreement with Turkan *et al.* (2023) who found that coating canola seeds with a mixture of chitin, methylcellulose and *T. viride* spores did not affect seed germination rate and increased seedling growth.

Conclusions

Among the possible combinations of biopolymers and biological agents, this research provides specific information on doses and sequences for canola seed coating

with sodium alginate, chitosan and *T. harzianum*. The results can not only help to reduce the environmental impact but also enhance the efficiency of these substances during application and storage. Mixing *T. harzianum* with sodium alginate (1.5%) maintains its viability over time and improves seedling performance. However, chitosan formulations in acetic acid should be carefully developed to prevent negative effects on seeds or biological agents. An alternative approach, demonstrated in this study, involves the incorporation of solid chitosan mixed with talc, on a second coating layer.

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