

Enhancing the Shelf Life and Stress Tolerance of the Biocontrol Agent *Trichoderma harzianum* by Encapsulation in Green Matrices of Nanocellulose and Carboxymethyl Cellulose

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ABSTRACT: Microbial inoculants offer a promising solution for reducing the environmental impact of agrochemicals while enhancing crop productivity within a bioeconomy framework. However, extending the shelf life and enhancing the stability of these beneficial microorganisms are crucial for making these biological solutions viable alternatives to chemical fertilizers and pesticides. In this study, we developed biobased encapsulation matrices using cellulose nanocrystals (CNC) and a composite of CNC and carboxymethyl cellulose (CNC:CMC) to encapsulate spores of the biocontrol fungus *Trichoderma harzianum*. Our findings revealed that encapsulation significantly increased the microorganism shelf life. After 1 year, approximately 10^8 CFU/mL of the initial 10^9 CFU/mL encapsulated spores remained viable, while nearly all free spores were no longer viable. Encapsulation also improved the microorganism resistance to stressful conditions, such as heat, UV radiation, and chemical fungicide exposure. Specifically, the CNC and CNC:CMC matrices maintained up to 4.7×10^8 CFU/mL after fungicide exposure. Furthermore, encapsulation preserved the antagonistic activity of *T. harzianum* against the phytopathogen *Fusarium solani* for up to 1 year. These results demonstrate the potential of cellulose-based matrices for developing microbial inoculant formulations that support the shift toward more sustainable agricultural practices.

KEYWORDS: cellulose nanocrystals, carboxymethyl cellulose, microbial inoculant, encapsulation, shelf life

INTRODUCTION

Chemical fertilizers and pesticides are essential products to increase the agricultural productivity, but the uncontrolled and exacerbated usage of these products can cause severe problems to the environment and human health.^{1,2} This highlights the need for more sustainable agricultural practices to enhance productivity while aligning with the concept of the bioeconomy.^{3,4} In this context, microbial inoculants have received increasing attention in recent years. These products consist of live beneficial microorganisms that are applied to soil or plants to improve nutrient availability and uptake, increase plant resistance to environmental stresses (extreme temperatures, drought, flooding, and UV exposure, etc.), and control phytopathogenic diseases. In addition to increasing crop productivity, microbial inoculants can also improve soil health.^{5–7}

The fungus *Trichoderma harzianum* is a widely reported microbial inoculant due to its beneficial effects on plant growth promotion and disease suppression.^{8–11} The biocontrol activity of *T. harzianum* is well documented and can be attributed to its ability to compete with phytopathogens for nutrients, produce secondary metabolites that inhibit pathogen growth, and stimulate the plant's defense mechanisms, thereby enhancing its resistance to pathogen attacks.^{12,13} As reported by Zhang et al.,¹³ the use of *T. harzianum* in soybean plants promoted growth by inducing the production of phytohormones such as indole acetic acid. Additionally, it reduced the severity of white mold disease, caused by the fungus *Sclerotinia sclerotiorum*, due to the formation of an oppressive structure

against the phytopathogen hyphae and by producing lytic enzymes (chitinase and β -glucanase), which degraded the cell membrane of *S. sclerotiorum*.¹³ Beyond its role as a biocontrol agent, *T. harzianum* has also been reported to enhance nutrient availability and uptake in plants^{14,15} and to release bioactive compounds such as phytohormones which contribute to plant growth and help alleviate abiotic stress conditions, including salinity and drought.^{16,17}

Although microbial inoculants have shown great potential for improving plant growth in line with sustainable agricultural practices, several challenges remain regarding their application.^{5,18} One of the main concerns is ensuring the viability and stability of the inoculant during production, storage, transportation, and field application, as these microorganisms are highly sensitive to environmental stressors.¹⁹ A well-designed formulation can enhance microbial protection and optimize product efficacy.^{5,19–21} Moreover, the formulation should be based on environmentally friendly materials, to avoid environmental issues, promote inoculant colonization and survival in the target plant, and be easy to apply, making them accessible and practical for farmers and researchers.^{5,21–23}

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In this sense, encapsulation techniques demonstrate favorable outcomes in meeting the aforementioned criteria. In addition to extending inoculant shelf life, these formulations can improve product efficacy by providing a slow and controlled release of microorganisms, while protecting them from degradation by soil enzymes or other microorganisms.^{20,22,24} The use of polysaccharides as encapsulation matrices is particularly attractive, as these materials are abundant, biocompatible, and biodegradable, making them ideal for sustainable agricultural practices.^{20,22–24} Polysaccharides can provide both physical protection and essential nutrients for microorganisms. Additionally, their properties can be modified to optimize the release of encapsulated cells, ensuring effective delivery to the target plants. The slower release rate provided by the matrices can reduce fertilization costs for farmers, as fewer applications are required throughout the growing season.^{22–24} For instance, polymers such as alginate, starch, and chitosan have been reported to enhance the shelf life of microbial inoculants, enable controlled microbial release, and improve crop yields.^{25–28}

Cellulose-based materials, including cellulose nanocrystals (CNC) and carboxymethyl cellulose (CMC) were identified as promising candidates for *T. harzianum* encapsulation due to their excellent properties.^{29,30} Their beads can be produced by simple coagulation routes using CaCl_2 as a cross-linker. However, while the physicochemical properties of these capsules have already been described, their impact on microorganism protection and shelf life remains unclear.

Here, we evaluate for the first time the effects of green matrices composed solely of CNC and a CNC:CMC composite on the protection and shelf life of the biocontrol fungus *T. harzianum*, used here as a model microorganism. Free and encapsulated spores were exposed to stressful conditions, including UV radiation, fungicide exposure, and high temperatures, and their viability was assessed. Additionally, antagonistic activity and spore viability were monitored over a 1-year period, demonstrating that these materials are highly effective candidates for microbial inoculant encapsulation.

MATERIALS AND METHODS

Materials. Commercial cellulose nanocrystals (CNC, Celluforce, Canada—crystallinity of 70–90%, sulfur content of 5000–9000 mg/kg, aspect ratio of 31, and a ζ potential of approximately -40 mV)^{31,32} and sodium carboxymethyl cellulose (CMC, Synth, Brazil), with a degree of substitution of 0.7 and medium viscosity, were used as the polymeric matrix for the beads production. The cross-linking agent was calcium chloride (CaCl_2 , Synth, Brazil). *T. harzianum* LQC-99 (donated by Embrapa Environment, Brazil) was used as a model microorganism for the encapsulation experiments. The fungus spores were germinated for 7 days at 28°C in Petri dishes containing potato dextrose agar (PDA, Acumedia, Brazil). Spores were extracted by a 0.85% (w/v) NaCl solution, and the final dispersion was centrifuged to increase the spore's concentration. A Neubauer chamber was utilized to determine the final spore concentration.

Preparation of CNC and CNC:CMC Beads. For beads production, all materials (except the polymers) were first autoclaved at 121°C for 15 min, and the encapsulation process was carried out under sterile condition in a laminar flow cabinet. Briefly, CNC (5% w/v) and CMC (1.5% w/v) were separately dispersed in distilled water, following the methodology reported by Brondi et al.²⁹ Two polymeric matrices were used for the capsules production. The first consisted of a pure CNC dispersion (5% w/v), while the second was a mixture of CNC/CMC in a volume ratio of 3:1 (with a final mass concentration of 3.75% (w/v) CNC and 0.375% (w/v) of CMC). A

T. harzianum spore dispersion was added to all matrices at a concentration of approximately 10^9 spores/g dry polymer. These dispersions were then dripped into a 1 M CaCl_2 coagulation bath. The polymer contents and the cross-linking agent concentration were set at these values based on our previous study.²⁹ The selection criteria included the evaluation of the bead stability, morphology, and resistance to handling.

The produced beads were kept in a salt solution under mild agitation for 30 min, followed by distilled water washing to remove the salt excess. The spheres were then stored in a refrigerator at 4°C for further experiments of cell viability, stress tolerance, and antagonism.

Scanning Electron Microscopy (SEM). A morphological study of the lyophilized CNC and CNC:CMC beads containing the fungus spores was performed using a scanning electron microscope (SEM—JEOL JSM-6510) with an accelerating voltage of 5.0 kV. For the SEM analysis, the samples were coated by sputtering with gold.

Evaluation of the Fungus Growth over the Beads. The fungus growth over the beads placed in the PDA medium was evaluated by photos taken over time. For that, the Petri dishes containing the nutrient medium and the beads were incubated at 28°C for up to 7 days. The photos were taken using the camera of a Samsung Galaxy S22 smartphone.

Viability Experiments. To determine the free and encapsulated spore survivability after the beads production and after being stored under refrigeration (4°C) for 9 and 12 months, viability experiments were carried out. For that, 0.5 mL of the free spores solution and 20 beads of CNC and CNC:CMC matrices were added to an Erlenmeyer flask containing 10 mL of NaCl 0.85% (w/v). Then, 20 μL of the commercial enzymatic cocktail (Cellic CTec3, from Novozymes) was added to speed up the release of spores from the beads. The Erlenmeyer flask was then incubated in an orbital shaker at 30°C , 200 rpm, for 24 h. The resulting solution was then serially diluted with NaCl (0.85%) and inoculated in a Petri dish with PDA medium, which was incubated at 28°C for 24 h. After that, the colony-forming units (CFU/mL) were determined visually. Additionally, the total amount of spores released in the Erlenmeyer flask was determined in a Neubauer chamber. All experiments were carried out in biological and technical triplicates. The concentrations in CFU/mL or viability percentage were reported as mean $\pm$ standard deviation. The total initial concentration of the spores was standardized as 10^9 spores/mL.

Stress Tolerance Experiments (Heat, UV Radiation, and Fungicide Exposure). The ability of the encapsulation matrix to protect the microorganisms against some stressful conditions was also evaluated. For that, free and encapsulated *T. harzianum* were exposed to three different situations: (1) Temperature of 40°C for 24, 48, and 192 h; (2) ultraviolet (UV-C) radiation for 30 and 90 min, at room temperature, and at a distance of 20 cm from the light source, in a dark room; (3) direct contact to a commercial fungicide solution (difenoconazole, 0.0167% (w/w), Forth, Brazil) for 24 h. After being submitted to these conditions, free and encapsulated fungi were placed in an Erlenmeyer flask in a 0.85% NaCl solution with 20 μL of the commercial enzymatic cocktail (Cellic CTec3, from Novozymes) for the release of spores from the matrices and incubated in a shaker at 200 rpm, 30°C for 24 h. The resulting solution was then serially diluted and inoculated in a Petri dish with PDA medium, followed by colony-forming units (CFU/mL) determination. All experiments were carried out with biological and technical triplicates. Values were expressed as the mean $\pm$ standard deviation. For all experiments, the total initial concentration of spores was standardized as 10^9 spores/mL.

Antagonism Experiments. *Fusarium solani* is a phytopathogenic fungus that can infect plant roots (e.g., tomatoes, soybeans, eggplants, etc.), decreasing their productivity. This phytopathogen was used to evaluate the antagonistic activity of *T. harzianum*, in its free and encapsulated form. *F. solani* strains were cultivated in PDA medium at 28°C for 10 days. For the antagonism's experiments, a 5 mm disk containing the mycelia of *F. solani* was placed on one side of a 9 cm Petri dish with PDA medium. On the opposite side of the dish, *T. harzianum* was placed (free spores (20 μL) or one bead of the

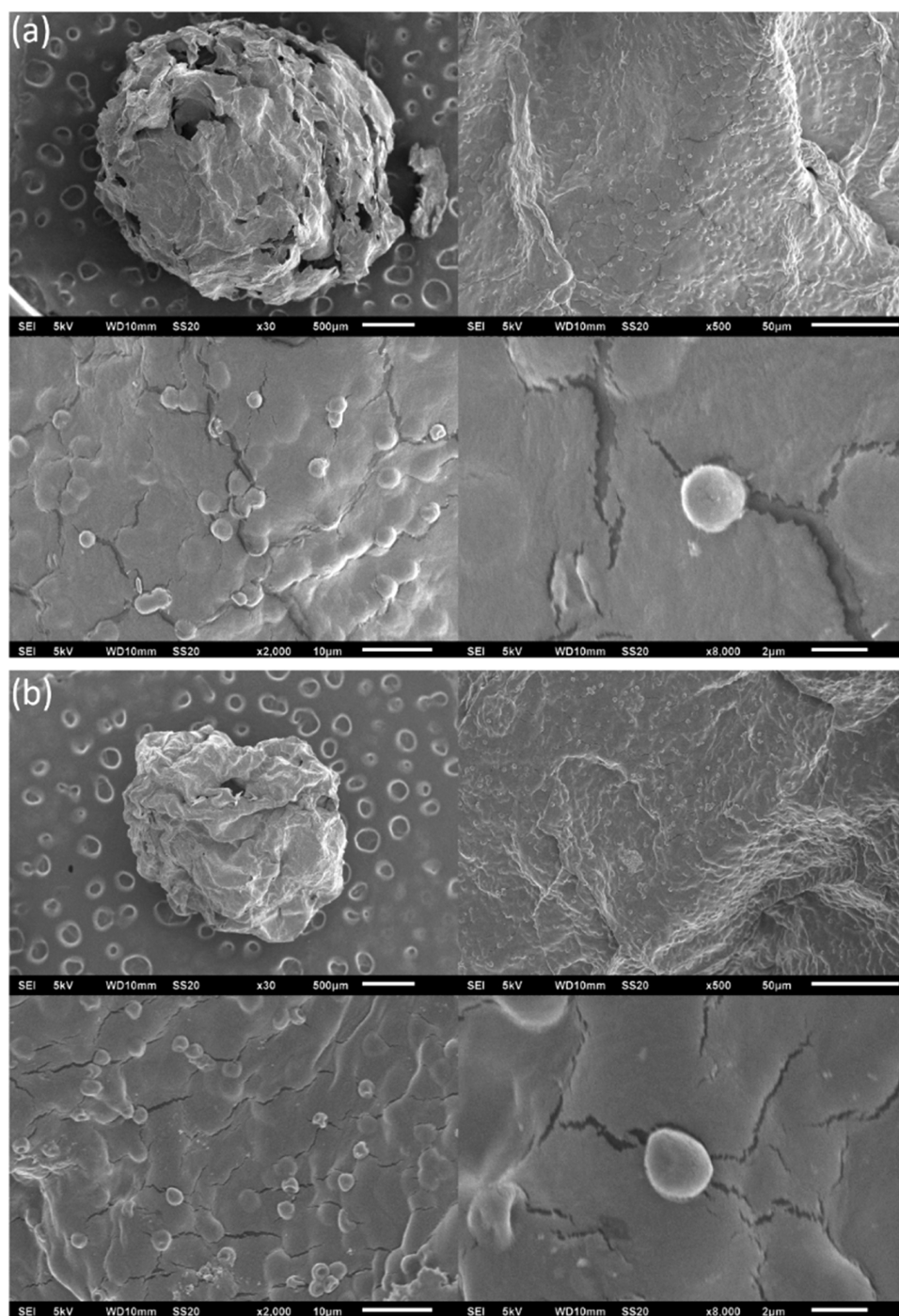


Figure 1. Morphological characterization by SEM of lyophilized CNC beads (a) and CNC:CMC beads (b) with different magnifications.

encapsulated fungus in the CNC or CNC:CMC matrices). The encapsulated *T. harzianum* was evaluated right after its production and after being stored for 1 year. The Petri dishes containing both fungi were then kept at 28 °C, and the microorganisms' growth across the PDA medium over time was evaluated by daily photos. This test was carried out in triplicate.

Water Retention Capacity. To determine the water retention capacity of the beads, a set of 10 wet capsules was weighed after their production. The material was then freeze-dried to remove water and weighed again. The water content of the beads was calculated by subtracting the initial mass (water + polymers) from the mass of the freeze-dried material. The water retained by the materials was expressed as the mass of water per unit mass of dry polymer (g H₂O/g

polymer). This experiment was carried out in triplicate, and the data were expressed as mean $\pm$ standard deviation.

Statistical Analysis. Data of cell viability were subjected to analysis of variance (ANOVA) and Tukey's test (p -value <0.01), performed using Excel.

RESULTS AND DISCUSSION

Characterization of the Beads. This study selected the dripping and ionic cross-linking process in CaCl₂ to obtain the beads due to its simplicity, mild conditions for the microorganism, and ease of scalability. As reported in a previous study, the use of a CNC-based matrix was necessary to ensure the formation of structurally stable beads through CaCl₂ ionic

gelation.²⁹ An attempt was made to produce a matrix in which CMC was the main constituent; however, CMC alone did not form stable beads when cross-linked with Ca^{2+} , as it lacks the structural integrity provided by CNC.

Both the CNC and CNC:CMC lyophilized beads containing the microorganism were characterized by SEM (Figure 1). The beads were lyophilized only for this characterization assay to preserve the three-dimensional structure of the material for micrograph's acquisition. As shown, the CNC bead (Figure 1a) exhibited a higher number of macropores on its surface compared to the composite CNC:CMC (Figure 1b). Besides these pores, the CNC bead's surface seemed to be smoother with some surface cracks. The occurrence of cracks and pores can be a beneficial feature, as it may facilitate the release of microorganisms into the soil by increasing the material's surface area and, consequently, enhancing its interaction with the external environment. For both matrices, fungal spores were visible on the bead surfaces, as highlighted in the higher magnification micrographs. Figure S1 provides a cross-sectional view of the beads, confirming the presence of spores inside the capsules.

A visual characterization was conducted to determine whether the encapsulated fungus could grow within the encapsulation matrix. Fungal growth was assessed on the matrices (CNC and CNC:CMC) placed in a PDA medium by photos taken in different time periods (Figure 2). After 3 days

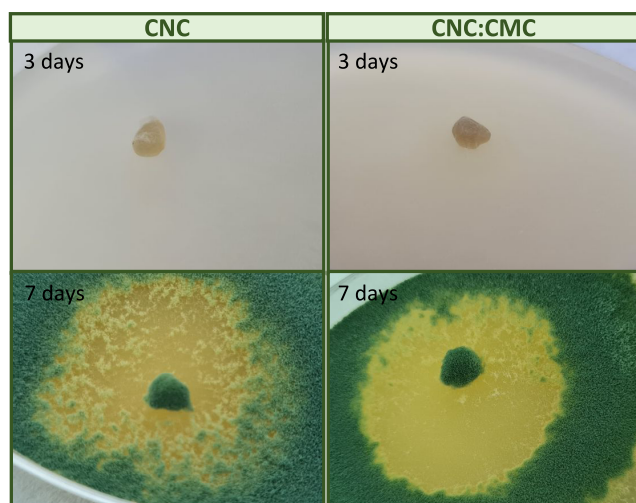


Figure 2. Evaluation of the encapsulated *T. harzianum*'s growth over the beads after 3 and 7 days in a Petri dish with PDA medium at 28 °C.

of incubation, fungal hyphae were observed on both matrices. After 7 days, both the CNC and CNC:CMC composites were completely covered by the *T. harzianum* spores, suggesting that the microorganism could also utilize these matrices as a carbon and energy source. This characteristic may be advantageous, as it could facilitate fungal growth and improve adaptation when the material is applied to agricultural crops.

Effect of Encapsulation on Microorganism Shelf Life.

The effect of encapsulation in CNC and CNC:CMC matrices on the fungus viability over time was evaluated and compared to the free microorganism stored under the same conditions. Initially, the entrapped fungus was released from both beads by an enzymatic hydrolysis method. This approach was chosen to accelerate the spore release from the beads, as diffusion in

0.85% NaCl alone was significantly slower. *T. harzianum* viability was assessed immediately after encapsulation and after storage for 9 and 12 months at 4 °C.

As reported in Figure 3, after 9 and 12 months, the beads stood out for their great effect in preventing spore inactivation.

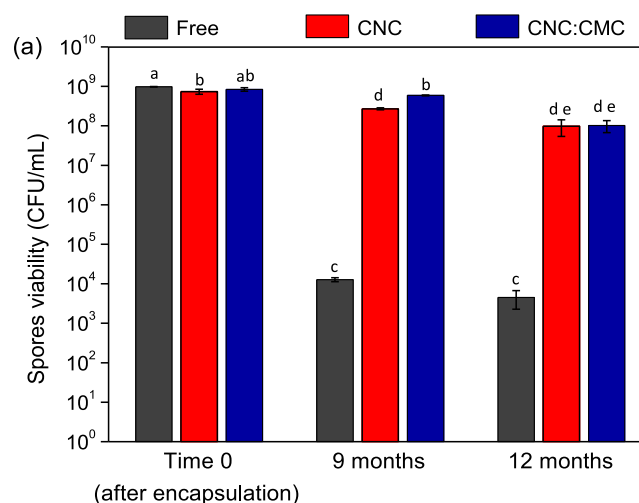


Figure 3. Shelf life (spores' viability—UFC/mL) of the free and encapsulated *T. harzianum* in CNC and CNC:CMC beads kept under refrigeration (4 °C) immediately after the cross-linking process (time zero) and after 9 and 12 months of storage. Different lowercase letters indicate statistically significant differences according to Tukey's test ($p < 0.01$).

While the viability of free microorganisms decreased to $\sim 1.3 \times 10^4$ CFU/mL after 9 months and 4.5×10^3 CFU/mL after 12 months, CNC beads maintained viability at 2.7×10^8 and 9.9×10^7 CFU/mL, respectively. The composite performed even better, with viability levels of 5.9×10^8 and 1×10^8 CFU/mL, after 9 and 12 months, respectively. These results were remarkable, as the encapsulated spores exhibited a viability reduction of less than 1 order of magnitude (a 10-fold decrease) over 1 year, whereas the free spores demonstrated a reduction of approximately 6 orders of magnitude (a million-fold decrease).

These results were particularly interesting since wet formulations of biocontrol agents usually suffer from short shelf life, which ends up impacting their storage and effect after application by farmers.³³ When compared to other encapsulation matrices reported in previous studies (Table 1), survival rates ranged from 10 to 40% when *T. harzianum* was encapsulated in maltodextrin, gum Arabic, or a combination of these polymers and stored at 4 °C for 8 weeks (~ 2 months). After 12 weeks under the same conditions, the survivability was nearly zero.³⁴ Similarly, when alginate was used as encapsulating matrix for *T. harzianum* spores (coagulated in a CaCl_2 cross-linking bath and stored wet under refrigeration), viability decreased from 10^8 CFU/mL immediately after encapsulation to 10^6 CFU/mL after 120 days (~ 4 months).³⁵ In another study using an alginate-montmorillonite matrix for *T. harzianum* encapsulation,³⁶ the capsules stored at 5 °C exhibited a viability loss of approximately 5 orders of magnitude over 6 months, decreasing from 10^8 to 10^3 CFU/mL.

These findings indicate that under similar conditions CNC and CNC:CMC beads were significantly more effective in preserving fungal viability than alginate (one of the most

Table 1. Viability Data Reported for the Encapsulation of *T. harzianum* in Different Matrices

matrix	viability result	reference
sodium alginate (2% w/v) beads formed by ionic gelation in CaCl_2 (0.1 M)	encapsulated spores kept in the wet matrix at 5 °C lost 22% of their viability after 90 days encapsulated spores kept in a dried matrix at 5 °C lost 20% of their viability after 90 days free spores kept at the same condition lost 32% of their viability after 90 days	43
sodium alginate (2% w/v) beads formed by ionic gelation in CaCl_2 (0.05, 0.1, and 0.15 M)	after 2 years of storage at room temperature, the fungus was able to grow in nutrient medium	44
sodium alginate (2% w/v) beads formed by emulsion with corn oil		
sodium alginate (2% w/v) beads formed by ionic gelation in CaCl_2 (0.1 M)	wet microparticles viability stored at 5 °C went from 10^8 to 10^6 CFU/mL after 120 days wet microparticles viability stored at 30 °C went from 10^8 to 10^7 CFU/mL after 30 days dried microparticles stored at 30 °C presented no viable spores after 90 days	35
sodium alginate and montmorillonite cross-linked with 0.5 M of CaCl_2	beads stored at 30 °C presented no viability after 4 months encapsulated spores viability (stored at 5 °C) went from 10^8 to 10^3 CFU/g after 6 months	36
microencapsulation with maltodextrin and gum arabic by spray-drying	no viability was observed for free cells after 8 weeks of storage at 4 and 29 °C no viability was observed for encapsulated spores after 12 weeks of storage at 4 and 29 °C	34
core/shell matrix and osmoprotectant	encapsulation significantly increased the microorganism shelf life in an organic–inorganic NPK fertilizer stored at 25 °C. Best results were obtained when trehalose was used	45
core: alginate + spores + osmoprotectant (trehalose/xylitol)-cross-linked with CaCl_2 shell: CMC cross-linked with FeCl_3 granules were prepared by ionic gelation with CaCl_2 (2 M). Different matrices were evaluated:	after 6 months, the spores viability was log 5.72 CFU/g for free cells, log 6.78 CFU/g for alginate/CMC matrix and log 7.30 CFU/g for the alginate + trehalose/CMC matrix	
(1) alginate/glycerol	viability after storage at 28 °C: the only alginate, the alginate/glycerol, and alginate/starch matrices presented no viability after 4 months	46
(2) alginate/polyphosphate	alginate/polyphosphate: no viability after 2 months	
(3) alginate/starch		
(4) alginate/polyphosphate/starch/glycerol	alginate/glycerol/polyphosphate/starch/pectin/wheat bran: no viability after 9 months	
(5) alginate/pectin		
(6) alginate/pectin/polyphosphate/glycerol	alginate/pectin and alginate/wheat bran: no viability after 14 months	
(7) alginate/wheat bran		
(8) alginate/glycerol/polyphosphate/starch/pectin/wheat bran	alginate/pectin/polyphosphate/glycerol: spores remained viable after 14 months. Viability went from $\sim 10^{10}$ CFU/g (time 0) to 10^6 CFU/g after 14 months	
(9) only alginate	alginate/polyphosphate/starch/glycerol: spores remained viable after 14 months. Viability went from $\sim 10^9$ CFU/g (time 0) to 10^5 CFU/g after 14 months	

commonly used matrices for microbial inoculant encapsulation) and other natural polymers. This improved performance can be attributed to the fact that alginate beads produced via CaCl_2 coagulation are typically fragile and prone to disintegration during handling, storage, and processing, leaving the microorganisms more exposed. To address these limitations, CNC has been incorporated into alginate-based matrices to enhance mechanical properties.³⁷ Additionally, CNC increases matrix tortuosity, thereby slowing down diffusion processes and improving microbial protection.³⁷ In this sense, a CNC-based matrix resulted in less fragile and denser beads, as highlighted in our previous work by SEM and X-ray microtomography analyses,²⁹ which ended up increasing the protection and viability of the microorganism, as demonstrated in the present study. Another advantage of CNC over alginate is its higher surface area-to-volume ratio, which allows for greater cell loading capacity.^{38,39} While CNC is commonly reported to have surface areas exceeding 50 m²/g, alginate typically falls within the range of 1–10 m²/g.^{40–42} This larger surface area might enhance the retention of microbial cells within the encapsulation matrix, contributing to the improved inoculant stability.

Effect of Abiotic Stressful Conditions on Free and Encapsulated *T. harzianum*. Ensuring microbial inoculant protection during manufacturing, storage, transportation, and after application on crops is essential for the product's success. Some field conditions that these products encountered include pH and temperature variations, excess or lack of water, UV-

light exposure, contact with other agrochemical products, and varying soil salinity. Here, the protection provided by the CNC and the CNC:CMC matrices over the spores was evaluated during the following stressful situations: (a) a heat stress of 40 °C, maintained constant for 24, 48, and 192 h; (b) exposure to UV–C radiation for 30 and 90 min; (c) exposure to a commercial fungicide for 24 h. The results are presented in Figure 4. For comparison, free spores were also subjected to the same conditions.

Thermal Stress. Thermal stress significantly impacts the viability of *T. harzianum*. When exposed to high temperatures, the microorganism can face protein denaturation, enzyme inactivation, and DNA and RNA oligonucleotides rupturing, all of which affect its survival.⁴⁷ Our results (Figure 4a) showed that continuous exposure of free spores to 40 °C severely affected their survivability. While free spores initially had a viability of 9.8×10^8 CFU/mL, its incubation at this temperature for 24, 48, and 192 h reduced the number of viable cells to 3.4×10^8 , 2.8×10^8 , and 3.9×10^5 CFU/mL, respectively. For the spores encapsulated in the CNC matrix (which presented an initial viability of 7.4×10^8 CFU/mL), the remaining viable cells were 5.4×10^8 , 4.8×10^8 , and 2.6×10^7 CFU/mL after 24, 48, and 192 h, respectively. For the fungus encapsulated in the CNC:CMC composite (initial survivability of 8.4×10^8 CFU/mL), viability decreased to 6.3×10^8 , 6.1×10^8 , and 2.1×10^7 CFU/mL over the same time intervals.

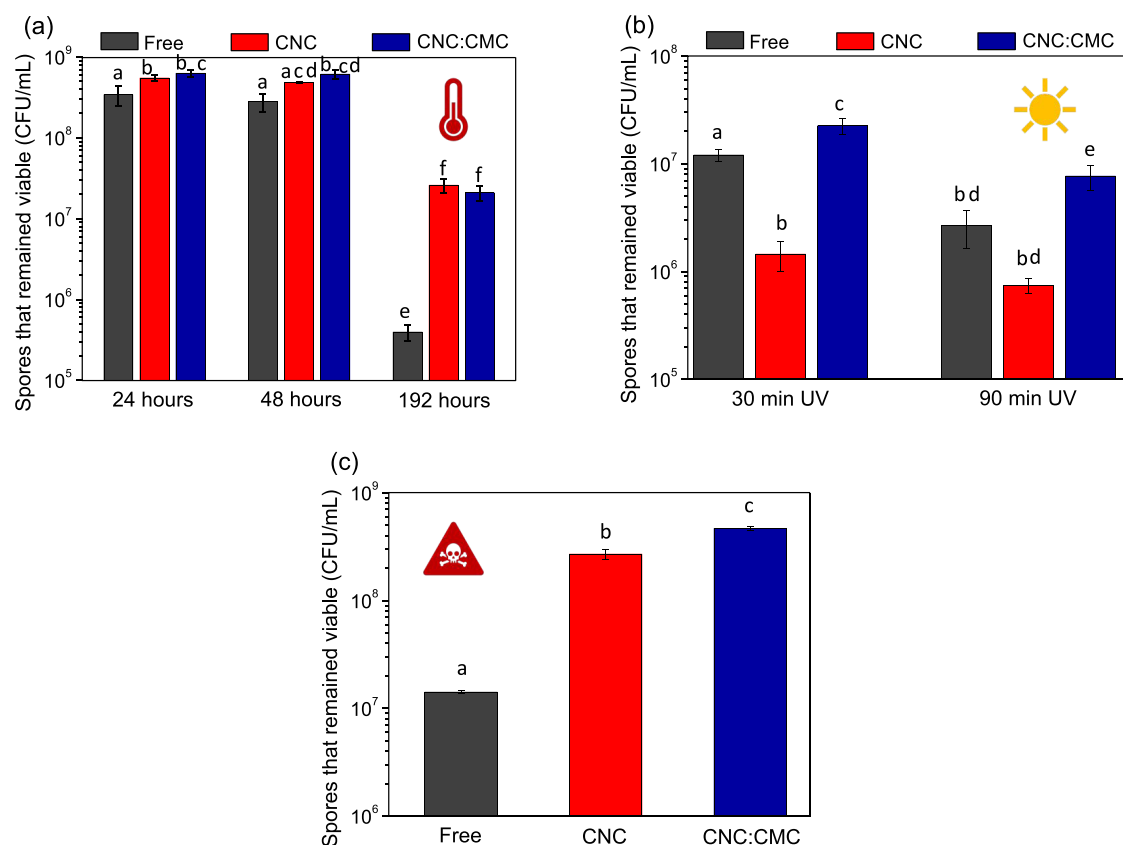


Figure 4. Viability of the free and encapsulated *T. harzianum* when submitted to stressful conditions. (a) Exposed to the temperature of 40 °C for 24, 48, and 192 h. (b) UV–C light exposure for 30 and 90 min. (c) Commercial fungicide incubation for 24 h. Different lowercase letters indicate statistically significant differences according to Tukey's test ($p < 0.01$).

In summary, while free microorganism survivability decreased by 4 orders of magnitude after 192 h, encapsulated spores exhibited only a 1 order decrease. This clearly demonstrates that encapsulation acted as a protective barrier, reducing heat transfer across the beads and mitigating fungal inactivation due to thermal stress. The dense structure formed by polymeric chains cross-linked by Ca^{2+} ions around the microorganism likely reduced heat transfer from the external environment to the cells' interior.^{29,48}

The impact of heat stress on spore viability was previously assessed using an alginate matrix obtained by dripping in a CaCl_2 solution. In that study, spores were stored at a constant temperature of 35 °C for up to 90 days.⁴³ Complete loss of viability occurred for free fungus after the 90-day period, whereas those encapsulated in the wet matrix retained a survival rate of 51%. While this outcome appears more favorable than our findings, it is important to consider that a 5 °C temperature difference can significantly impact fungal viability, given that the optimal growth temperature for *T. harzianum* is approximately 28 °C. Additionally, differences in *T. harzianum* strains used in each study may also contribute to variations in results.

UV Radiation Stress. UV radiation is one of the main environmental factors that can affect fungi survivability.⁴⁹ When spores were encapsulated in the CNC or the composite CNC:CMC matrix, the polymers acted as a UV-blocker. It happened since the CNC crystals hindered the light passage across the matrix, acting as a physical barrier that reduced UV-light transmittance.^{50,51} For instance, while a 2% (w/v) CMC film presented a UV-light transmittance (280 nm) of 77%, a

CNC addition (10% w/w) reduced the transmittance to 32% at the same wavelength.⁵¹

In this context, our results (Figure 4b) showed that *T. harzianum* encapsulated in the composite matrix was relatively less sensitive to UV-light exposure than its free form. Although the survivability decreased greatly for all conditions after 30 and 90 min under UV exposure, spores entrapped in the CNC:CMC matrix exhibited a survivability of 2.3×10^7 and 7.7×10^6 CFU/mL, while the free form had 1.2×10^7 and 2.6×10^6 CFU/mL, respectively.

Unexpectedly, CNC-only beads resulted in lower survival rates than free spores, with viabilities of 1.5×10^6 and 7.5×10^5 CFU/mL for 30 and 90 min, respectively. Despite CNC's reported UV-blocking properties, its lower protective effect in this study can be attributed to the structural characteristics of the CNC matrix. Unlike the composite, CNC beads exhibited a more porous structure, which may have allowed greater UV penetration, reducing their overall protective effect and more significantly affecting fungal survival. This observation is supported by our SEM analysis (Figure 1), which revealed a higher presence of surface cracks and macropores in CNC beads compared to CNC:CMC.

It is important highlight that, while solar radiation can only penetrate the soil to a limited extent, reaching no more than 10 mm in depth,⁵² the beads developed in this study may not always be located beneath the soil's surface. In scenarios where they are applied to the soil surface, the capsules and, consequently, the fungus become more vulnerable to solar radiation. This highlights the importance of evaluating how such stress conditions affect their efficacy.

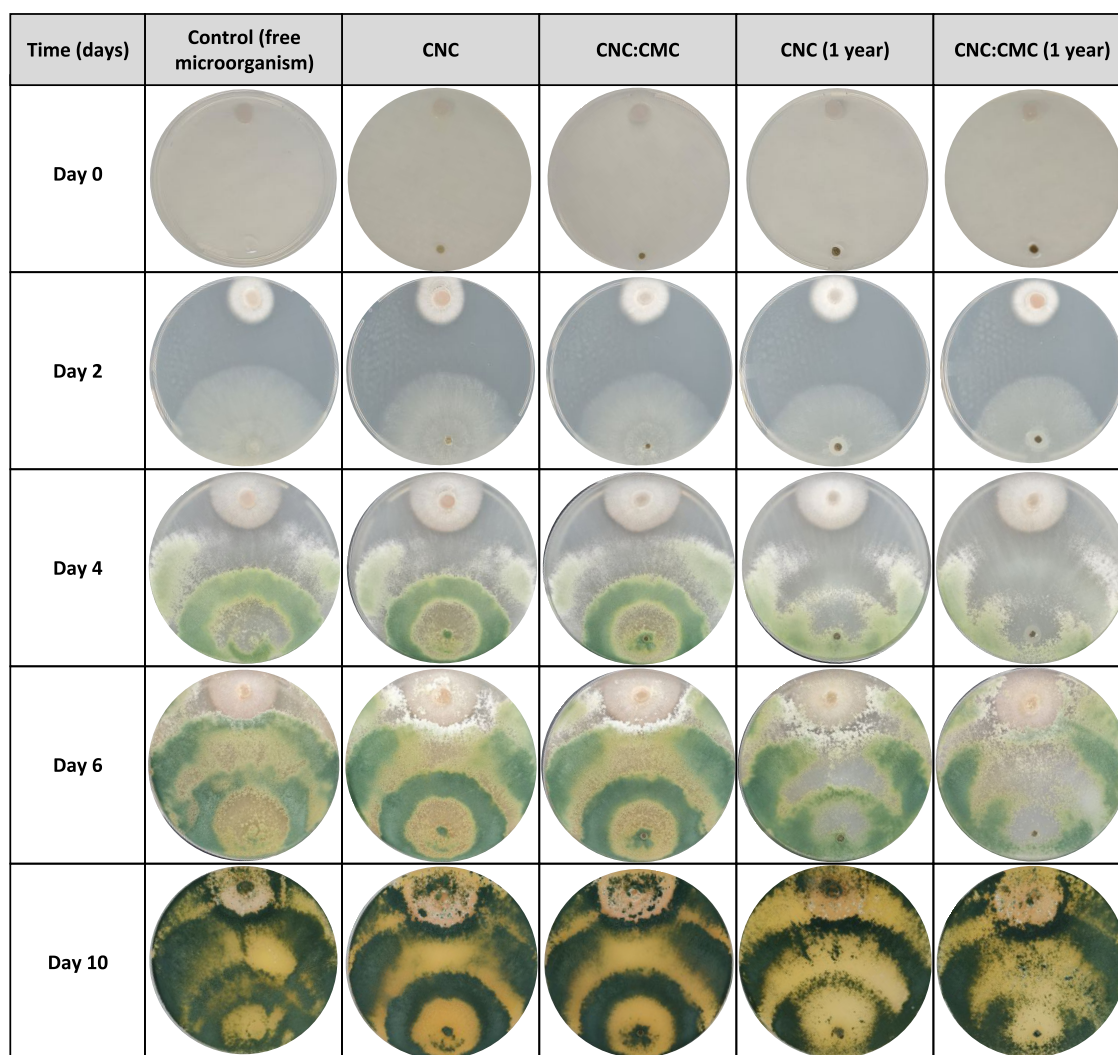


Figure 5. Antagonistic test of the biocontrol fungus (free and encapsulated, stored for up to 1 year) *T. harzianum* (bottom of the Petri dish) against the phytopathogen *F. solani* (top of the Petri dish) evaluated over time by photos.

Other biopolymers, such as alginate, have already been reported to enhance *T. harzianum* spore protection against UV exposure.⁴³ For instance, wet alginate capsules obtained via ionic gelation with CaCl_2 improved the microorganism survival when exposed to UV radiation. After 24 h, microorganism survivability decreased by 93%, whereas free spores showed no viable cells.⁴³ These results, along with our findings, reinforce the idea that encapsulation acts as a protective shield, increasing microorganism resistance to environmental stressors.

Exposure to a Commercial Fungicide. The use of agrochemicals is often necessary to increase agricultural productivity; however, these products generally have low compatibility with microbial inoculants, posing a limitation for the application of these bioproducts.⁵³ For instance, seeds inoculated with *Bradyrhizobium* strains and treated with fungicides exhibited bacterial mortality rates of up to 62% after 2 h and 95% after 24 h.⁵⁴ Therefore, an effective microbial inoculant formulation should be capable of protecting the microorganism from chemicals' pesticide exposure.

Difenoconazole is a systemic triazole fungicide commonly used to prevent and treat fungal plant diseases. It can be applied to seeds, roots, or leaves, and it usually has a high

efficiency.^{55,56} However, its application has been reported to decrease soil microbial community.⁵⁶ Additionally, when *T. harzianum* was exposed to concentrations higher than 64 ppm, this fungicide completely inhibited fungal growth.⁵⁷

In this context, encapsulated and free spores of *T. harzianum* were exposed for 24 h to a liquid solution with a commercial difenoconazole fungicide, and its effect on fungus viability was evaluated (Figure 4c). Free microorganisms were significantly affected by the fungicide, presenting a final viability after the inoculation of 1.4×10^7 CFU/mL. However, encapsulation matrices effectively reduced spore mortality by acting as a physical barrier that limited direct contact between the fungus and the fungicide. The interaction between polymer chains, induced by ionic cross-linking during bead coagulation with Ca^{2+} ions, resulted in the formation of a dense structure around the fungal spores, particularly those located in the inner regions of the beads, thereby enhancing the protection of the microorganism.²⁹ While CNC beads resulted in a viability of 2.7×10^8 CFU/mL, the composite provided even greater protection, with approximately 4.8×10^8 CFU/mL remaining viable. These results highlight the effectiveness of both matrices in enhancing fungal protection, emphasizing nano-

cellulose as an excellent material for microbial inoculant formulation.

Antagonistic Activity of the Encapsulated *T. harzianum*. Plant pathogens can significantly decrease crop production around the world.⁵⁸ *F. solani* is a well-reported phytopathogenic fungus that can cause root rot and other plant diseases in economically important crops such as soybean, potatoes, peas, tomatoes, among others, decreasing productivity.^{59–62} It has been widely used as a model pathogen in studies evaluating the biocontrol potential of *T. harzianum*. Several reports highlighted the effectiveness of *T. harzianum* against *F. solani* through mechanisms such as nutrient competition, mycoparasitism, antibiosis, production of hydrolytic enzymes (e.g., chitinases and β -glucanases), and induction of plant defense responses.^{63–66} In this sense, an in vitro antagonism test was carried out to evaluate if the encapsulated *T. harzianum*, after the beads production and after being stored for one year, was still able to suppress the growth of the phytopathogen *F. solani*.

As reported by Figure 5, in all evaluated samples, *T. harzianum* was able to efficiently inhibit the growth of *F. solani* in the PDA medium, completely dominating the Petri dish after 10 days. Even the beads stored under refrigeration for 1 year were able to prevent the phytopathogen from spreading across the Petri dish, showing that both the CNC and CNC:CMC matrices preserved the antagonistic activity of the fungus. The free spores exhibited a faster growth rate compared with the encapsulated forms. This was expected, as the spores did not need to overcome the matrix barrier to begin their development in the nutrient medium. Furthermore, as shown in Figure 3, the free fungus exhibited slightly higher initial viability, which could also explain the initial faster growth rate. However, after 6 days, the growth rates appeared to be very similar for all of the samples.

Beads' Additional Positive Effects. It is important to highlight that after these beads' application to crops, besides releasing the microorganisms, they could also be a source of water, since these capsules were kept wet. This water content could be important for fungus development, and it could also improve the water retention in the soil, providing water for plants. This is an interesting resource for areas where irrigation is difficult or where drought conditions prevail.^{67,68} Several studies have shown the positive effect of CNC and CMC as hydrogels to increase the water retention capacity in soils.^{39,69–71} For instance, a CMC:CNC hydrogel was reported to have a water absorption capacity of 150 times its dry weight.⁷² Here, both the CNC and CNC:CMC beads exhibited a water absorption capacity of 20 times that of their dry weight (Figure 6).

These hydrogels could effectively enhance plant growth and development by increasing water availability.^{39,69–71} Furthermore, the fact that these beads were not dried can be an advantage for biocontrol applications, where microorganisms need to be released and grow quickly to suppress phytopathogen development. Dried beads could provide lower delivery and diffusion rates, which may affect the inoculant's effectiveness. Moreover, the bead application could help increase soil organic matter due to polymer degradation, providing more nutrients for soil microorganisms.⁶⁸

The results reported here demonstrated that cellulose-based matrices, including CNC-only and composite CNC:CMC, were highly effective in enhancing the shelf life and protection of the biocontrol fungus *T. harzianum*, one of the most well-

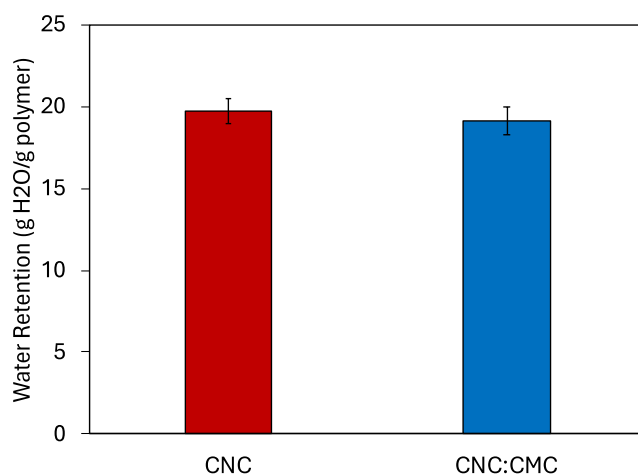


Figure 6. Water retention capacity (g H₂O/g polymers) of the CNC and CNC:CMC beads after the coagulation process.

known microbial inoculants. These biopolymers were able to enhance the fungus's protection when exposed to stressful conditions commonly encountered after application on agricultural crops, such as heat, UV-light exposure, and contact with chemical fungicides. Additionally, the encapsulation using these green, renewable, and biodegradable matrices was able to maintain the fungus activity as a biocontrol agent even after 1 year of storage. These properties are crucial for making these biobased products more competitive compared to chemical fertilizers and pesticides, which can help reduce the carbon footprint associated with agrochemical applications. Moreover, since the technique employed is simple and operates under mild conditions, it is likely suitable for encapsulating other beneficial plant microorganisms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsagstech.5c00189>.

SEM micrographs showing cross-sectional views of the CNC beads and the CNC:CMC composite beads (PDF)

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Notes

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