

Article

Gelatin–Chitosan–PVA Hydrogels Incorporating *Trichoderma* and Their Application in the Control of Phytopathogens

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Abstract

The utilization of microorganisms as biocontrol agents represents a sustainable alternative to agrochemicals. *Trichoderma* spp. has been identified as a fungus that promotes plant growth and suppresses phytopathogens. Nonetheless, conventional commercial formulations are constrained by factors such as their limited shelf life, environmental sensitivity, and inadequate carrier systems. In this study, *Trichoderma harzianum* (T22) and *T. viride* (T18) strains were encapsulated in a hydrogel composed of chitosan, gelatin, and polyvinyl alcohol, which was prepared by pH-induced gelation via alkaline precipitation. The characterization of the hydrogels was conducted in several domains. Initially, the water absorption of the samples was examined at varying pH values. Secondly, the morphology of the samples was investigated using scanning electron microscopy (SEM) and stereo microscopy. Thirdly, the chemical interactions in the hydrogels were analyzed by Fourier-transform infrared spectroscopy (FTIR). The final stage of the experiment involved assessing the degradation behaviour of the hydrogels in both sterile and inoculated soils. The efficacy of the isolates in protecting chilli plants from *Phytophthora capsici* was subsequently evaluated. As demonstrated in the extant research, encapsulation techniques have been shown to preserve the viability of fungal organisms and promote their growth after 10 days of storage at ambient temperature. These effects have been observed to exhibit strain-dependent variations. It is noteworthy that hydrogels loaded with *T. viride* (HT18) induced resistance against *P. capsici*, resulting in complete symptom suppression and enhanced plant growth, whereas hydrogels loaded with *T. harzianum* (HT22) showed no protective effect. These results demonstrate the potential of the hydrogel formulated with T18 as an effective carrier, as it maintains *Trichoderma* spp. viability and protects chilli plants against *P. capsici* infection.

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Keywords: encapsulation; biopolymer; *Trichoderma*; hydrogel; gelatin–chitosan–PVA; *Phytophthora capsici*

1. Introduction

Crop production constitutes the foundation of food and agriculture. Pests and diseases pose a grave threat to food security, trade, and livelihoods worldwide, causing production losses of up to 40%. The escalating global demand for food is predominantly attributable to population growth, and projections indicate that by 2050 demand will have increased by 50% [1]. This increase in demand has led to greater reliance on agrochemicals to maintain high agricultural productivity. However, a wide variety of soil-borne phytopathogens, such as the oomycete *Phytophthora capsici*, the causal agent of wilt [2], pose a serious threat to the yield of a wide variety of solanaceous and cucurbitaceous crops, mainly chilli peppers (*Capsicum* spp.). This crop possesses remarkable horticultural, economic, medicinal, and nutraceutical value. However, it is highly susceptible to both biotic and abiotic stressors [3]. *P. capsici* possesses the capacity to persist in the soil for extended periods in the absence of host plants through the utilization of oospores, thereby complicating its eradication and management [4].

Agrochemicals, particularly fungicides, have historically played a pivotal role in preserving crop yields. However, concerns about their excessive use have emerged due to their potential to cause environmental contamination, public health risks, and the development of fungal resistance [5]. Moreover, the detrimental effects on non-target organisms have prompted the establishment of more stringent regulations and legal frameworks governing the utilization of chemical compounds [6,7]. Consequently, farmers are exploring alternative methods, including biological approaches. Biological control methods have emerged as a promising avenue for expanding regenerative agriculture practices [8].

In the context of chilli cultivation, various strains of *Trichoderma* spp. have been documented to establish associations with the root system, thereby exerting protective effects against *P. capsici* infections. This protection has been associated with biostimulation, resistance induction, and systemic response effects [9,10], but strains with both properties have also been used [11–13]. However, large-scale production of agro-formulations based on biofungicides faces considerable challenges. Formulating *Trichoderma*-based biocontrol products poses several technical and commercial challenges due to the living nature of the microorganisms. Maintaining high spore viability and stability throughout storage and distribution constitutes a significant challenge, as *Trichoderma* spp. exhibit sensitivity to environmental factors, including temperature, humidity, oxygen levels, and light exposure. The selection of formulation type (solid, liquid, or granular) significantly influences shelf life, ease of application, and field performance, often necessitating trade-offs between stability and practicality [14]. Consequently, there is an imperative to develop hydrogel-like carriers that facilitate the production of cost-effective, natural, and non-toxic biological products. The employment of such carriers would enhance the efficacy, longevity, and persistence of biological agents. Furthermore, it would minimize production risks and protect agents from environmental stressors [15].

The encapsulation of *Trichoderma* spp. within hydrogels could provide viable and sustainable solutions for large-scale agricultural applications. The encapsulation of beneficial microorganisms in protective polymer matrices has been shown to enhance the viability of conidia under adverse environmental conditions and stressors [16]. In the pursuit of sustainable alternatives, gelatin and chitosan, natural biopolymers, along with

polyvinyl alcohol (PVA), a synthetic biopolymer, have emerged as promising candidates due to their ecological benefits and cost-effectiveness as carriers for biological control agents [17].

Gelatin (Gel), a soluble protein derived from the partial hydrolysis of collagen, is widely used for its high water-holding capacity, ability to form hydrogels, emulsify systems, and stabilize macromolecules [18]. Chitosan (CTS) is a linear deacetylated polysaccharide derived from chitin and composed of varying amounts of linked residues (β 1–4) of N-acetyl-2 amino-2-deoxy-D-glucose (glucosamine, GlcN) and 2-amino residues of 2-deoxy-D-glucose (N-acetylglucosamine, GlcNAc), which are biodegradable and non-toxic, and have been the focus of extensive research due to their antimicrobial properties, ability to promote plant growth, and capacity to induce defence mechanisms in plants [19]. Polyvinyl alcohol (PVA) is a vinyl polymer that is distinguished by its inherent solubility in water, its capacity to form hydrogels, and its complete biodegradability under conditions involving metabolically acclimated microbial consortia [20]. Conversely, chitosan has demonstrated its ability to form matrix structures in combination with other polymers, thereby encapsulating biological control agents for seed coatings [21]. This property not only shields the agents from stress but also enhances their effectiveness in promoting germination. Furthermore, chitosan-coated fertilizers have been demonstrated to exhibit controlled-release and water-retention properties [22]. For instance, CTS has been shown to form beads via pH-induced gelation via alkaline precipitation with 1 N NaOH, thereby enabling control over the release of bioactive agents [23].

The development of Gel-CTS-PVA hydrogels for agricultural applications has been documented. The working group has developed a biodegradable Gel-CTS-PVA hydrogel that possesses an interconnected, highly cross-linked, porous, and biodegradable polymer network. This network can retain biomolecules and serve as a carrier [24]. These hydrogels are distinguished by their notable biocompatibility, exceptional mechanical properties, and their capacity to facilitate cellular interactions, rendering them highly suitable for a wide range of applications involving microorganisms and cells [25]. Consequently, it can be posited that incorporating biological control microorganisms, such as *Trichoderma* spp., could enhance the formulation's protective properties and viability.

To the best of our knowledge, there are no reports describing the encapsulation of *Trichoderma* spp. using a ternary Gel-CTS-PVA blend for hydrogel prepared specifically by gelation pH induction with sodium hydroxide (NaOH). The objective of this study was twofold: first, to evaluate the effect of incorporating two strains of *Trichoderma* into a hydrogel composed of Gel-CTS-PVA formed by pH-induced gelation on its physicochemical properties; second, to determine its effectiveness in protecting against *P. capsici* infection in serrano chilli seedlings under greenhouse conditions.

2. Results and Discussion

2.1. Hydrogel Preparation

In this study, the objective was to fabricate Gel-CTS-PVA hydrogels via pH-induced gelation by alkaline precipitation with 1 N NaOH, serving as a carrier for *Trichoderma* spp., as a green strategy for chilli plant protection against the *Phytophthora capsici* pathogen. The encapsulated fungus comprised two distinct strains: a commercial *Trichoderma harzianum* T-22 (T22) and a strain from the CIATEJ collection, *Trichoderma viride* T18 (T18).

The procedure for obtaining the hydrogels is delineated in Figure 1. The process for obtaining the hydrogel was modified and simplified compared to that reported by López-Velázquez et al. [24]. The precipitation of chitosan (CTS) occurs via an acid–base reaction. In this process, the acidified polymeric suspension containing *Trichoderma* spp. functions as a proton-donating system due to the presence of chitosan solubilized in acetic acid. In an acidic environment, the amino groups ($-\text{NH}_2$) of chitosan undergo protonation,

accepting protons (H^+) from acetic acid. This results in the conversion of the amino groups into positively charged ammonium groups ($-NH_3^+$). This process, known as protonation, increases the charge density of the chitosan chains, thereby enhancing their polarity and water solubility. Subsequent addition of NaOH results in an increase in the pH of the reaction medium (approximately 7.5), leading to deprotonation and neutralization of the ammonium groups.

Consequently, chitosan solubility decreases, leading to promoting formation of stable hydrogels in the form of spheres (beads) that physically encapsulate *Trichoderma* spp. [23]. The hydrated (wet) hydrogels devoid of fungal components exhibited a spherical morphology and a slightly transparent yellow hue. At the same time, the hydrogels containing *T. harzianum* also showed a spherical morphology and a brown coloration.

Finally, the wet hydrogels were dried by a convection process (dried hydrogels). However, a substantial decrease in size was observed across all samples, accompanied by a transition to a whiter hue and increased rigidity due to water loss. Convection drying, which utilizes hot air, has been observed to alter the structure and mechanical properties of hydrogels to a greater extent than freeze drying. This results in variations in the physicochemical properties of the hydrogel and, most notably, in the protective activity of *Trichoderma* spp. (swelling, permeability, and overall stability of the material) [26,27]. The convection-drying method has been observed to alter the three-dimensional configuration of the polymer network within the hydrogel during water removal. In the hydrated state (wet), hydrogen bonding and polymer chain entanglement increase, resulting in a more expanded structure that undergoes a more drastic collapse when water is lost during convection drying [28]. Freeze-dried hydrogels exhibit a more porous structure, thereby altering the release of loaded compounds and the absorption of water [29]. A comparison of the preparation process of López-Velázquez et al. [24] with that reported in this research reveals a reduction in the number of steps, with the elimination of freeze/thaw cycles, double freeze-drying, and the use of xylene. This optimization of the preparation process enables green routes to the preparation of Gel-CTS-PVA-based hydrogels for agricultural use.

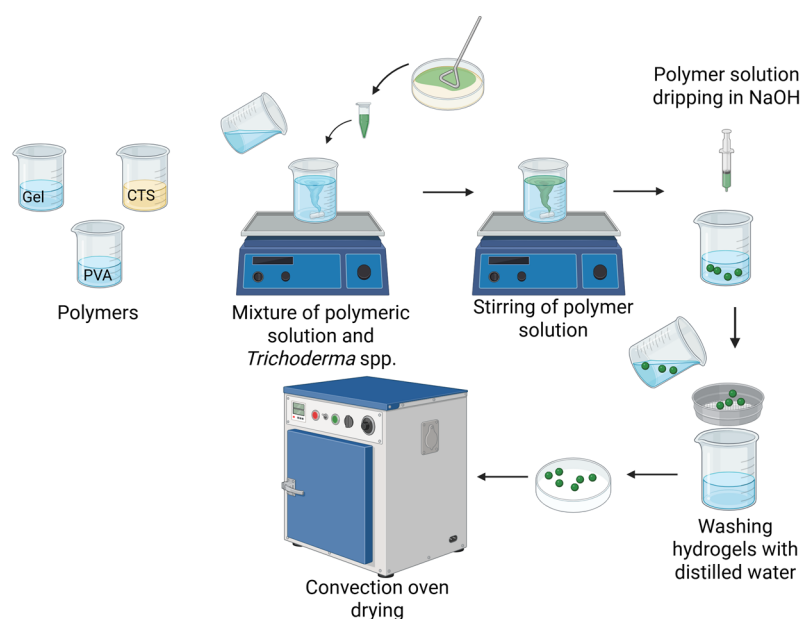


Figure 1. Process for obtaining hydrogels with *Trichoderma* spp. Created with BioRender.com.

Given that the physicochemical properties of the obtained hydrogels are primarily determined by the polymer composition and the gelation method [30], only the hydrogels

loaded with *T. harzianum* (HT22) were physicochemically characterized. Hydrogels loaded with *T. viride* (HT18) were prepared and used in crop protection assay.

2.2. Water Absorption and pH Sensitivity Study

The quality of irrigation water is a fundamental factor in crop cultivation. pH is an indicator of the acidity, neutrality, or alkalinity of the water, which, in turn, affects crop yield. Furthermore, the pH of irrigation water for chilli crops has been demonstrated to influence the water-absorption capacity of hydrogels, as reflected in their swelling [24]. The water absorption of hydrogels was evaluated meticulous measurement of the quantity of liquid absorbed by the material until saturation, also known as swelling equilibrium.

The hydrogels exhibited discrepancies in their water absorption capacity between the hydrogel devoid of microorganisms (H) and those incorporating *T. harzianum* (HT22), along with a substantial impact of the pH of the medium (Figure 2). The H treatment exhibited statistically significant disparities compared to the control at pH 5 and 6, a behaviour that contrasts with that reported by López-Velázquez et al. [24]. In the case of HT22, no significant differences were observed between the different pH ranges evaluated and the control; however, differences were detected when compared to H. These results suggest that the hydrogel structure responds significantly to pH due to changes in the ionic concentration gradient between the polymer matrix interior and the swelling medium [31]. During swelling, water molecules diffuse from the surface of the polymer network towards its interior [32]. The degree of hydrogel swelling is directly proportional to the ionic strength of the environment. At acidic pH, the amino groups of chitosan undergo protonation (NH_3^+), thereby increasing electrostatic repulsion with the ionic groups. This phenomenon has the potential to enhance the matrix's hydrophilicity, thereby promoting structural expansion [32].

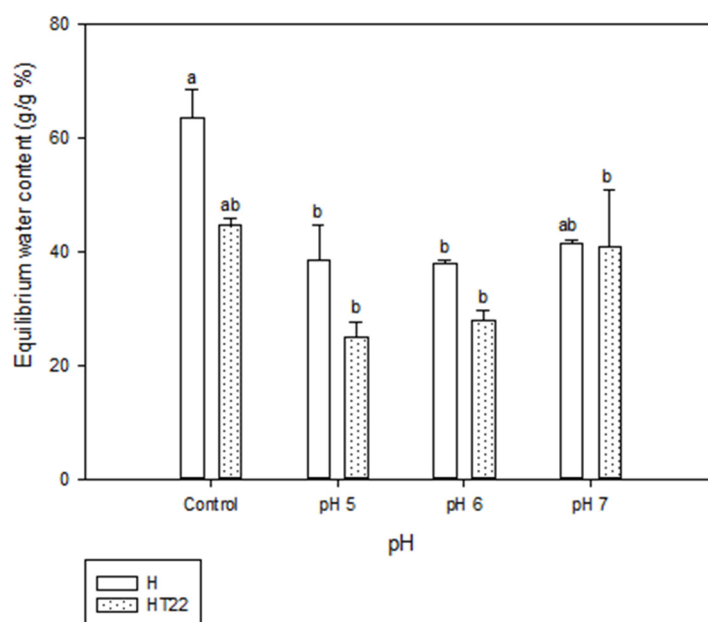


Figure 2. Swelling ratios of the hydrogel with *Trichoderma harzianum* (HT22) and without fungus (H) at different pH values. Values with the same letter are statistically equal according to Tukey's test ($p < 0.05$).

2.3. Soil Water Equilibrium Content

The equilibrium water content in the soil was identified as a critical factor in a hydrogel intended for agricultural application, given its impact on the gel's capacity to absorb irrigation and rainfall through its polymer network [33].

The swelling percentage of the hydrogels in the soil showed a statistical difference between the hydrogel alone (H) and the hydrogel with *T. harzianum* (HT22) added (Figure 3). For H, greater absorption was observed between 1 and 3 h, followed by a gradual, significant decrease in volume from 12 to 24 h. For HT22, a significant difference was observed between 3 h and 24-h absorption, and a significant difference was observed at the 3-h time point with respect to H. This phenomenon resembles the observations reported by López-Velázquez et al. [24] and Estrella [34], who attributed it to the “overshooting” phenomenon. The overshooting phenomenon is characterized by rapid, high initial absorption, which subsequently decreases gradually until reaching equilibrium. An internal redistribution of water follows the rapid initial hydration of the hydrogel as the system stabilizes [35].

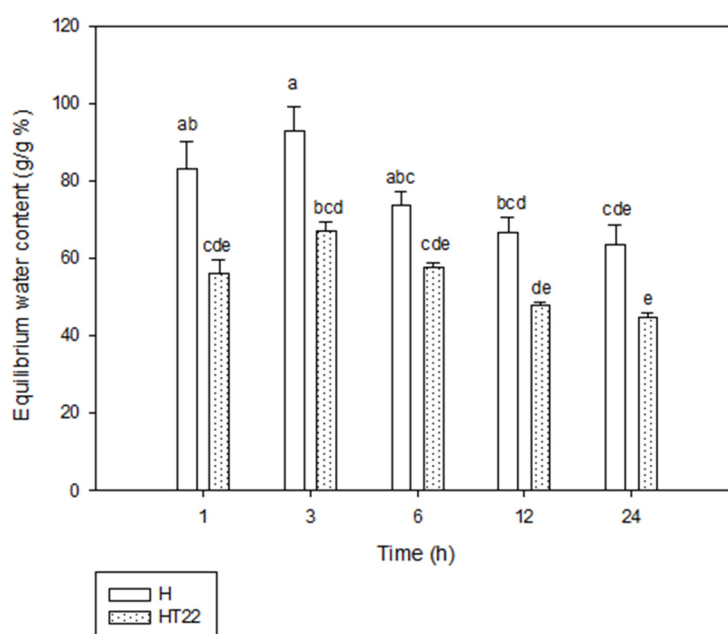


Figure 3. Equilibrium water content of the hydrogel with *T. harzianum* (HT22) and without fungus (H) in soil. Values with the same letter are statistically equal according to Tukey's test ($p < 0.05$).

The free amino groups present within the polymer network generate electrostatic repulsions between the polymer chains, thereby facilitating a more rapid swelling process. The electrostatic interactions initiated by the functional groups drive the hydrogel's expansion until equilibrium is reached [33].

2.4. Morphological Analysis

The disparities in structural morphology between hydrogels with *T. harzianum* (HT22) and without *T. harzianum* (H) were substantiated by SEM images. A series of magnifications was employed to facilitate a comprehensive examination of the freeze-dried beads, to elucidate their external and internal structural configurations.

In the micrographs obtained by scanning electron microscopy (SEM), an irregular and agglomerated surface without pores was observed (Figure 4A,B) for both hydrogels (H and HT22). The irregular morphologies observed after convection drying can be attributed to humidity gradients and polymer network collapse. These phenomena are

primarily prevented by freeze-drying [36]. Brondi et al. [16] obtained analogous results on the surface of hydrogels using a convection oven drying process (28 °C). As indicated by Bocourt et al. [37], freeze-dried hydrogels exhibit greater porosity than sun-dried hydrogels. This observation indicates that the drying method significantly impacts the morphology and porosity of hydrogels. However, no embedded spores were observed. Nonetheless, the viability assay demonstrated the presence of *Trichoderma* spp. in the hydrogel.

Furthermore, the hydrogels were observed under a stereomicroscope at 20× magnification. The hydrogels formed (wet state) exhibited a spherical shape with a diameter of 2.50 mm and a uniform, slightly yellowish colour in the fungus-free hydrogels (H) (Figure 4C). In contrast, the hydrogels containing *T. harzianum* (HT22) exhibited irregular shapes and a darker tone, attributable to the incorporation of fungal spores (Figure 4D), thereby substantiating the efficacy of the encapsulation process.

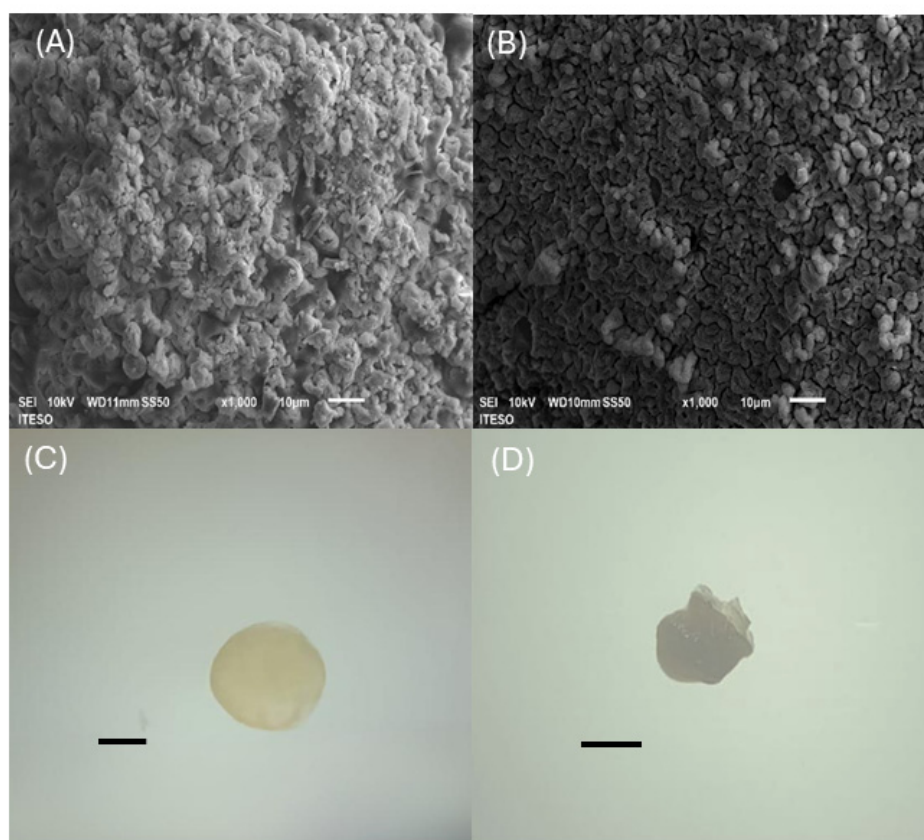


Figure 4. Morphology of hydrogels. SEM images: (A) H: Dry hydrogel at 1000×, (B) HT22: Dry hydrogel with *T. harzianum* (1×10^9 spores). Stereoscopic images: (C) H: Wet hydrogel without *Trichoderma* at 20×, (D) HT22: Wet hydrogel with *T. harzianum* (1×10^6 spores) at 20×. at 1000×. Black bar corresponds to 1 mm.

2.5. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra were then subjected to rigorous analysis to elucidate the chemical interactions between the polymers in the hydrogels (H and HT22). The spectra of the pristine polymers (Gel, CTS, and PVA) were utilized as controls and compared with those of the hydrogels. The FTIR spectra were described in the 4000–600 cm^{-1} region.

The Gel IR spectrum (Figure 5) exhibited the following peaks: 1000–950 cm^{-1} (amide III: skeletal stretch), and 1435 cm^{-1} (amide II: The following peaks have been identified: CH_2 bend at 1250 cm^{-1}), amide III: N–H bend at 1100 cm^{-1} , amide III: C–O stretch at 1625 cm^{-1} , and C=O stretching/hydrogen bonding couple with COO at 1625 cm^{-1} [25].

The analysis of the CTS spectra revealed that the amino (-NH) peak was situated within the range of 1550 to 1570 cm^{-1} , while the carboxyl (C=O) peak was positioned at approximately 1650 cm^{-1} . The characteristic bands of chitosan saccharides are located between 890 and 1150 cm^{-1} , while the amine groups are found at 1680 cm^{-1} [38]. Conversely, the bands within the range of 3500 to 3200 cm^{-1} are attributed to amide A and the amide B band at 3080 cm^{-1} , which are significant groups within the structure of gelatin [39].

The PVA spectra exhibited peaks at 915 cm^{-1} (CH_2 rocking), 820 cm^{-1} (C-C stretching), 1085 cm^{-1} (C-O stretching and O-H bending), 1350 cm^{-1} (scissoring O-H , rocking with C-H , wagging), and 1400 cm^{-1} (CH_2 bending) [25].

The lyophilized powder of *T. harzianum* exhibited the major infrared absorption bands that are characteristic of lipids, proteins, and phosphate. The range of wavelengths between 600 and 4000 cm^{-1} encompasses a broad spectrum of molecular compounds, including but not limited to fatty acids (lipids), proteins and peptides (amides), and carbohydrates (polysaccharides present in the cell wall). Peaks of higher absorbance were observed at ~ 880 , ~ 1050 , and $\sim 1100\text{ cm}^{-1}$. These peaks correspond to vibrations of lipids, amide I and amide II, nucleic acids, ribose, and glycogen, respectively [40].

The FTIR spectra of the lyophilised hydrogels loaded with *Trichoderma*, with and without spores, reflected hydrogels loaded with *Trichoderma* and showed similar chemical structures, overlapping in most regions due to their high inherent similarity. Consequently, distinguishing between them using rudimentary methods proved challenging. The presence of -OH bonds from the interaction between chitosan and polyvinyl alcohol is revealed by the spectra in the range of $3000\text{--}3500\text{ cm}^{-1}$, associated with hydrogen bonds. The functional groups of chitosan interact with those of PVA through hydrogen bonds between the -NH_2 and -OH groups of chitosan with the -OH groups of PVA, keeping both chains close together [37,41]. The band observed between 3000 and 2800 cm^{-1} is attributed to the stretching of -CH alkane groups, while the band at 1680 cm^{-1} corresponds to the C=O bonds of the acetate group. The peaks observed at 1550 cm^{-1} and 1460 cm^{-1} are attributed to the ionization of the primary amino groups [41].

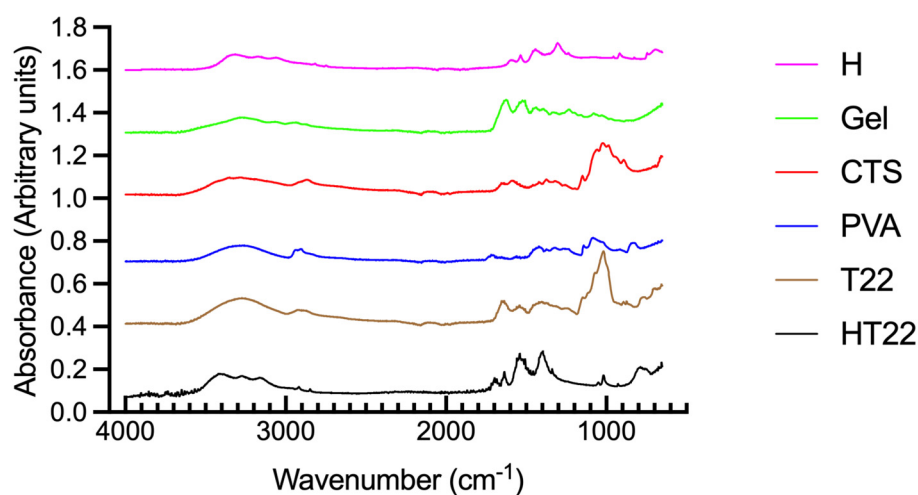


Figure 5. FTIR spectra of the hydrogel (H), hydrogel with *T. harzianum* (HT22), gelatin (Gel), polyvinyl alcohol (PVA), chitosan (CTS), and *T. harzianum* (T22).

2.6. Degradation Study Using the Burial Technique

The samples were buried at a depth of 4 centimetres and placed in containers with autoclave-sterilized substrate (degradability). The sphere's surface undergoes a transformation over time, becoming increasingly irregular, with brown spots and a general yellowish hue (Figure 6). These changes indicate degradation of the polymer matrix.

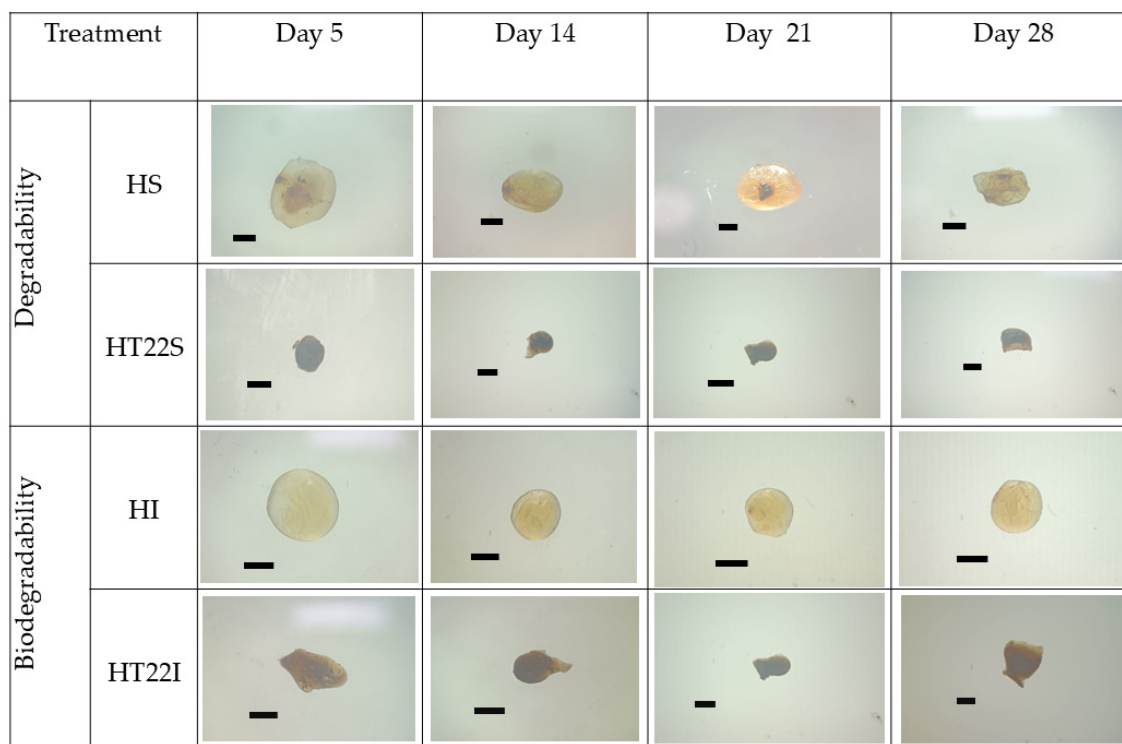


Figure 6. Stereoscopic microscope photographs of biodegraded hydrogels using the soil burial method. HS (hydrogel) and HT22S (hydrogel with *T. harzianum*) in sterile soil; HI (hydrogel) and HT22I (hydrogel with *T. harzianum*) in inoculated soil. Bar corresponds to 1 mm.

A reduction in the mass of the hydrogels was observed as the days of exposure in the soil increased (Figure 7). The hydrogels that incorporated *Trichoderma* spp. exhibited a greater loss of mass compared to the hydrogels devoid of fungal elements. Furthermore, in soil inoculated with microorganisms, a significant difference in the recorded weight of HT22I was observed compared to the other treatments. López-Velázquez et al. [24] reported that soil bacterial load is associated with hydrogel degradation, suggesting that microorganisms can accelerate this process. It has been documented that alterations in the physical and biological environment of the soil can impact the activity and efficiency of beneficial microorganisms and biocontrol agents within the soil microbiome, as well as their interactions with plants [42]. Moreover, it has been documented that hydrogels composed of polyacrylamide, a synthetic polymer, exhibit minimal degradation in soil when used as a standalone material. However, when chitosan is incorporated into the formulation, the hydrogels undergo degradation in soil in the presence of enzymes produced by fungi such as *T. viride* [43]. We hypothesize that the accelerated hydrogel degradation (HT22I) is due to the fungus's activation and germination, as well as its enzymatic machinery, which accelerate the degradation of the HT22I hydrogel compared to the other treatments.

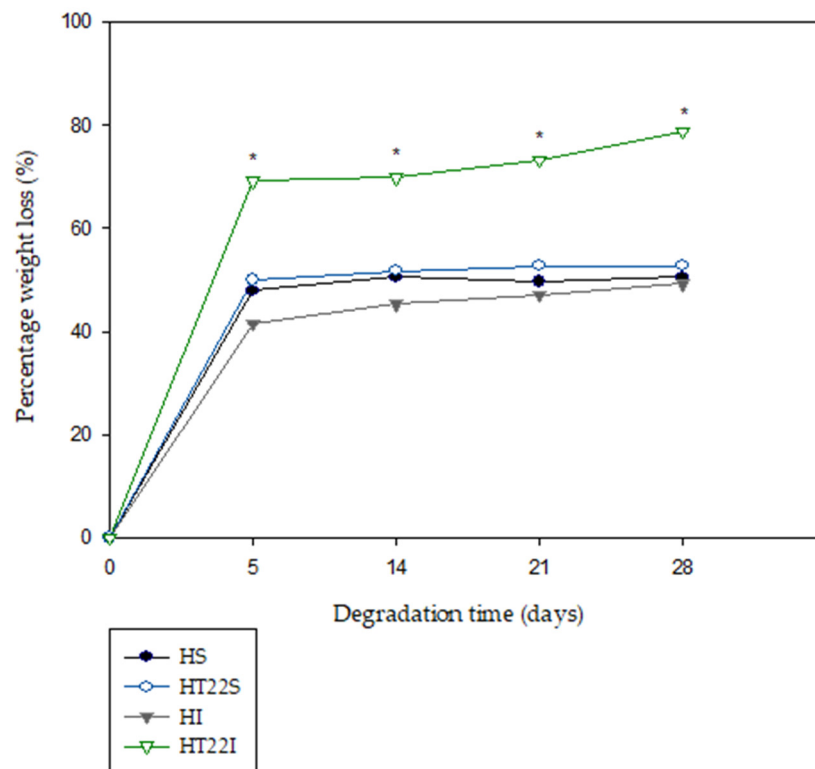


Figure 7. Biodegradation studies. Sterile soil: HS (hydrogel) and HT22S (hydrogel with *T. harzianum*); Inoculated soil: HI (hydrogel) and HT22I (hydrogel with *T. harzianum*). Values with (*) are statistically significant differences according to Tukey's test ($p < 0.05$).

2.7. Effect of Encapsulation on *Trichoderma* Growth

The present study was conducted to evaluate the effect of hydrogel encapsulation on the germination and growth of *Trichoderma* spores. To this end, the germination and mycelial development times of recently recovered free spores (T22, T18) were compared with those of encapsulated free spores (T22, T18) and with those of encapsulated spores in the polymer matrix (HT22, HT18). The proliferation of free spores in PDA medium was observed beginning on the third day, consistent with the HT22 hydrogel; conversely, HT18 hydrogels exhibited growth until the fifth day (Figure 8). These results suggest that the viability of *Trichoderma* spp. spores are maintained after the encapsulation process, although with different growth rates depending on the encapsulated strain. The decline in microbial growth rate could be associated with the restricted water availability within the polymer matrix and its compact structure, which may impede spore distribution [16]. Alternatively, this phenomenon could be associated with the induction of spore dormancy following the drying process. Dormancy is seen as a physiological state of inactivity resulting from a significant reduction in humidity and temperature. This allows for the prolonged survival of dehydrated spores and their subsequent reactivation in the presence of water and nutrients [44]. In the context of *Trichoderma* spore encapsulation, Dogaru et al. [45] observed that spore growth in culture medium was evident only after 7 days, a finding attributed to encapsulation within a gelatin-based nanocomposite matrix. This component is also present in the formulation utilized in the present study.

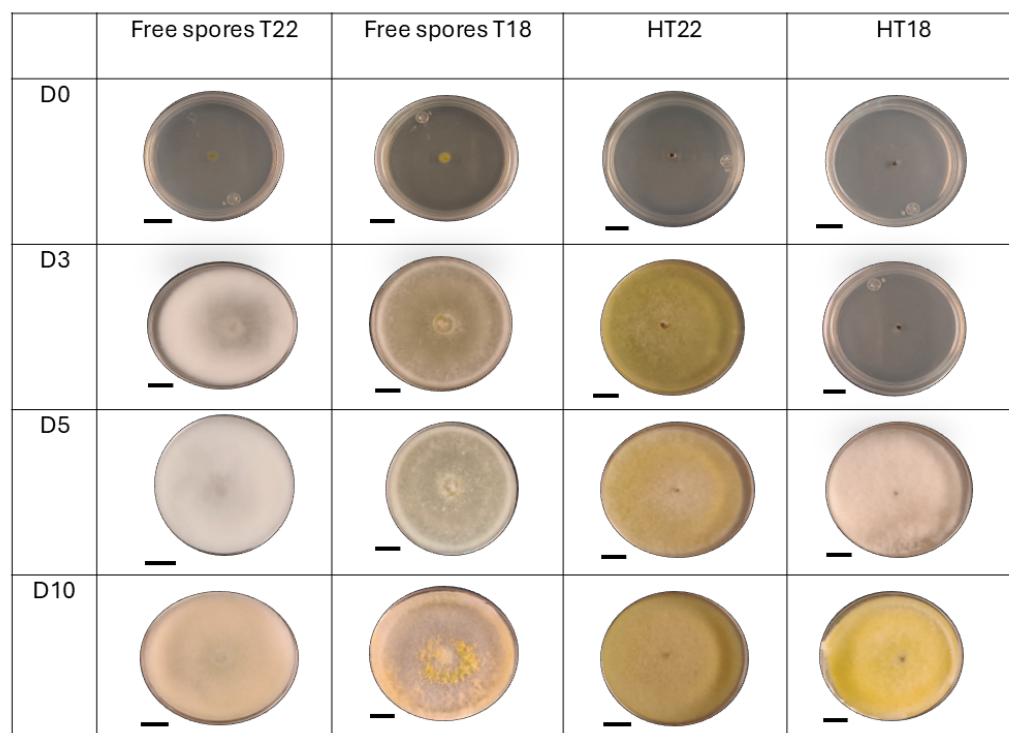


Figure 8. Growth on PDA medium of free and encapsulated spores. Letters D represent the days after sowing the microorganisms, HT22 and HT18 are hydrogels with *Trichoderma* strains. Bar corresponds to 1 cm.

2.8. Crop Protection

The present study was conducted to evaluate the effect of *Trichoderma* spore encapsulation on the ability to control *Phytophthora capsici* infection in chilli seedlings. The results for plant growth parameters, disease incidence, and root viability are presented in Table 1; the effects on plant and root protection are presented in Figure 9. Statistically significant differences were observed for the growth parameters with respect to the PHC treatment. These differences were specifically noted in plant height for treatments T22, H, and HT18, and in fresh weight only for treatment HT18.

Table 1. Parameters of plant growth, incidence, and root damage at the end of the experiment.

Treatment	Plant Height (cm)	Fresh Weight (g)	Incidence (%)	Root Viability (mg g ⁻¹ h)
T0	31.92 ± 6.11 ^{AB}	0.552 ± 0.1647 ^B	0	0.907 ± 0.536 ^A
PHC	23.35 ± 7.22 ^C	0.442 ± 0.355 ^B	46	0.367 ± 0.202 ^A
T18 + PHC	27.58 ± 6.13 ^{BC}	0.596 ± 0.268 ^B	31	0.978 ± 0.115 ^A
T22 + PHC	31.77 ± 7.47 ^B	1.094 ± 0.471 ^{AB}	8	0.656 ± 0.354 ^A
H + PHC	32.77 ± 8.31 ^A	0.993 ± 0.381 ^{AB}	15	0.419 ± 0.268 ^A
HT18 + PHC	31.85 ± 5.77 ^B	1.350 ± 0.529 ^A	0	0.779 ± 0.421 ^A
HT22 + PHC	23.77 ± 7.32 ^{BC}	0.729 ± 0.704 ^{AB}	54	0.715 ± 0.360 ^A

T0: control plants; PHC: plants inoculated with *P. capsici*; T22: plants inoculated with T22 spore solution; T18: plants inoculated with T18 spore solution; H: plants with hydrogels; HT22: plants with hydrogels + T22; HT18: plants with hydrogels + T18. Values with different letters show statistically significant differences according to Tukey's test ($p < 0.05$).

Regarding disease incidence and its impact on plant protection, the use of unencapsulated *Trichoderma* spores at the plant base showed variable protective effects. Treatment T18 resulted in a 23% reduction in incidence (see Figure 9D), while T22 achieved an 83%

reduction (see Figure 9C). Conversely, when the spores were applied encapsulated in hydrogel, differential behavior was observed. Treatment HT18 provided 100% protection of the plants (Figure 9F), while HT22 showed no protective effect (Figure 9G).

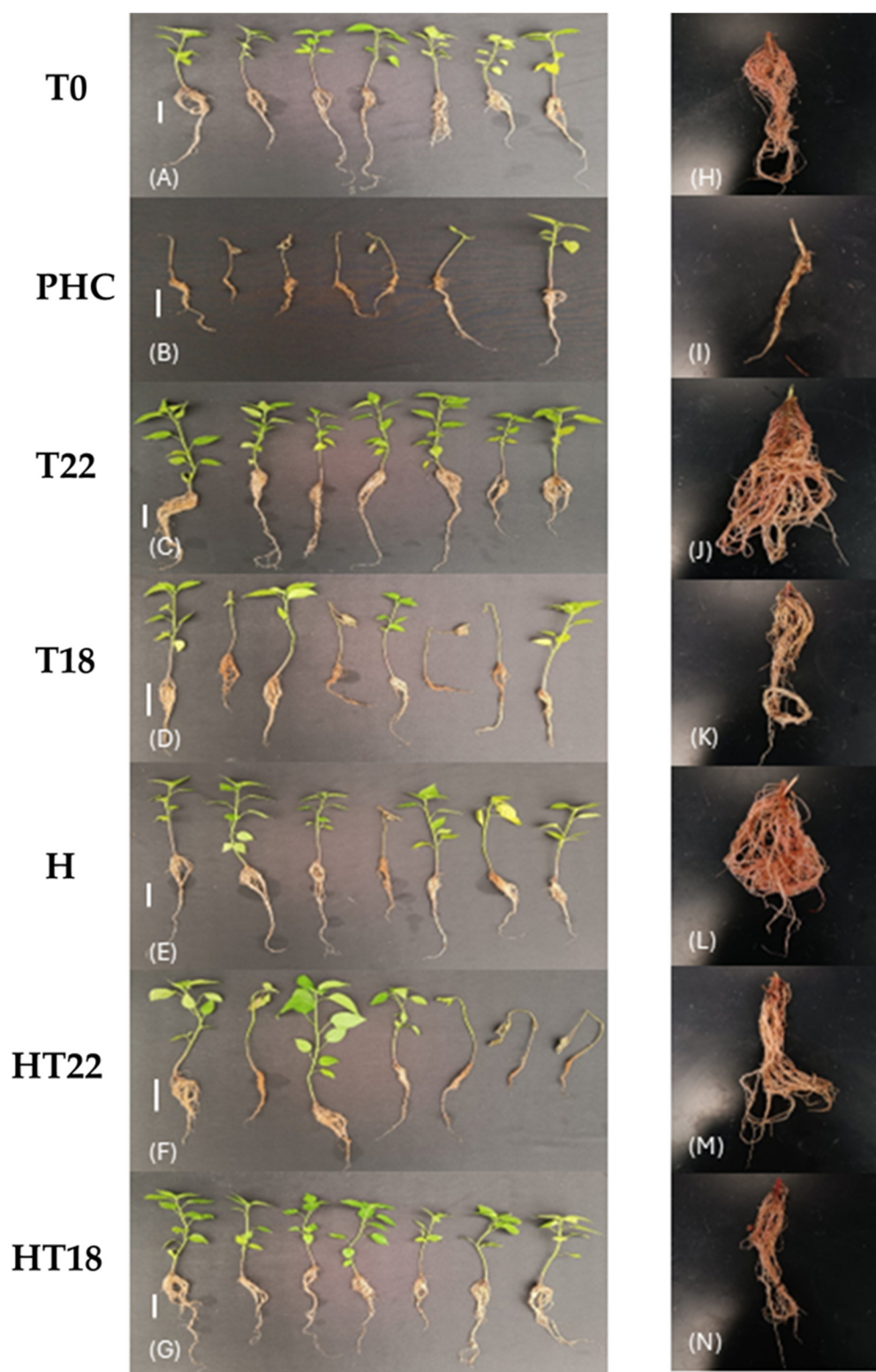


Figure 9. Effect of hydrogels on crop protection: (A) T0: control plants; (B) PHC: plants inoculated with *P. capsici*; (C) T22: plants inoculated with T22 spore solution; (D) T18: plants inoculated with T18 spore solution; (E) H: plants with hydrogels; (F) HT22: plants with hydrogels + T22; (G) HT18: plants with hydrogels + T18; (H) T0: viability of control roots; (I) PHC: viability of roots inoculated with *P. capsici*; (J) T22: viability of roots inoculated with T22 spore solution; (K) T18: viability of roots

inoculated with T18 spore solution; (L) H: viability of roots with hydrogels; (M) HT22: viability of roots with hydrogels + T22; (N) HT18: viability of roots with hydrogels + T18. Bar corresponds to 5 cm.

These results contrast with those reported by López-Velázquez et al. [24], who observed up to 80% mortality using hydrogels with analogous components. However, there are significant methodological discrepancies between the two studies, particularly regarding the type of chitosan used. In the study by López-Velázquez et al. [24], reactive-grade chitosan (Sigma-Aldrich, St. Louis, MO, USA) was utilized, while in the present study, high-density food-grade chitosan was employed. It has been reported that the origin and physicochemical properties of chitosan significantly influence the induction of plant resistance. López-Velázquez et al. [46] reported that high-density food-grade chitosan induced greater protection of coffee against coffee leaf rust infection by increasing the activity of β -1,3 glucanases and peroxidases and by increasing the accumulation of phenolic compounds. This could explain the protective effect observed even with hydrogel treatment alone [46].

It is noteworthy that the HT18 treatment, corresponding to a native strain of *T. viride*, showed a more favourable interaction with encapsulation in the hydrogel for plant protection than HT22. This observation is particularly interesting given that HT22 was able to activate, germinate, and emerge from the hydrogel in a shorter time (Figure 8). It has been documented that *T. harzianum* possesses a diverse array of biological control mechanisms, including the production of antimicrobial metabolites and antifungal lytic enzymes, in contrast to *T. viride*. However, it has also been documented that certain strains of *T. viride* may exhibit greater enzymatic activity than some strains of *T. harzianum*. In this regard, Gajera and Vakharia [47] demonstrated that five strains of *T. viride* exhibited significantly higher lytic enzyme activity (i.e., chitinases, β -1,3 glucanases, and proteases) compared to six strains of *T. harzianum* during interactions with *Aspergillus niger*. This background could explain the enhanced response observed with the HT18 encapsulation treatment, in which the hydrogel components, particularly chitosan, would have promoted metabolic activation of the fungus, thereby facilitating more efficient root colonization and, consequently, a higher level of plant protection compared to HT22 [48]. In a similar manner, strain T22 (*T. harzianum*) displays a predominantly intracellular root colonization pattern via the symplastic pathway, whereas strain T18 (*T. viride*) exhibits apoplastic or extracellular colonization [12]. These differences suggest that different mechanisms of action are engaged during biocontrol. Specifically, the activity of T22 has been predominantly linked to competition for nutrients and colonization sites, as well as to the production of antifungal metabolites and plant growth-promoting effects. In contrast, T18 is characterized by increased production of hydrolytic enzymes, especially β -1,3 glucanases and chitinases, which play a key role in the degradation of the cell walls of phytopathogenic fungi. In subsequent studies, the validation of both hypotheses must be conducted through assays that assess the enzymatic activity of the two *Trichoderma* strains, evaluate the hydrogel's impact on the germination of *Trichoderma*, and the elucidation of the specific mechanisms of action exhibited by each strain.

The assessment of root viability using 2,3,5-triphenyltetrazolium chloride (TTC) reduction revealed that plants treated with encapsulated *Trichoderma* spores exhibited viability values comparable to those in the PHC treatment, but lower than those in the control group. However, despite the absence of statistically significant differences between treatments (see Table 1), notable variations in root development were observed (see Figure 9H–N). The present findings are consistent with those reported by López-Velázquez et al. [49], who evaluated the root viability of plants treated with selenite against infection by *P. capsici*. The researchers observed lower values in infected plants than in selenite-treated plants.

The contrast in biological response between the protected plants and the diseased ones has been highlighted. The majority of diseased plants exhibited greater disease severity (Figure 10). As previously shown in other studies, the efficacy of biological control by individual *Trichoderma* species or strains varies significantly. The successful implementation of this control depends on a meticulous evaluation of prevailing environmental conditions and the specific interactions between the plants and the pathogens involved [50].

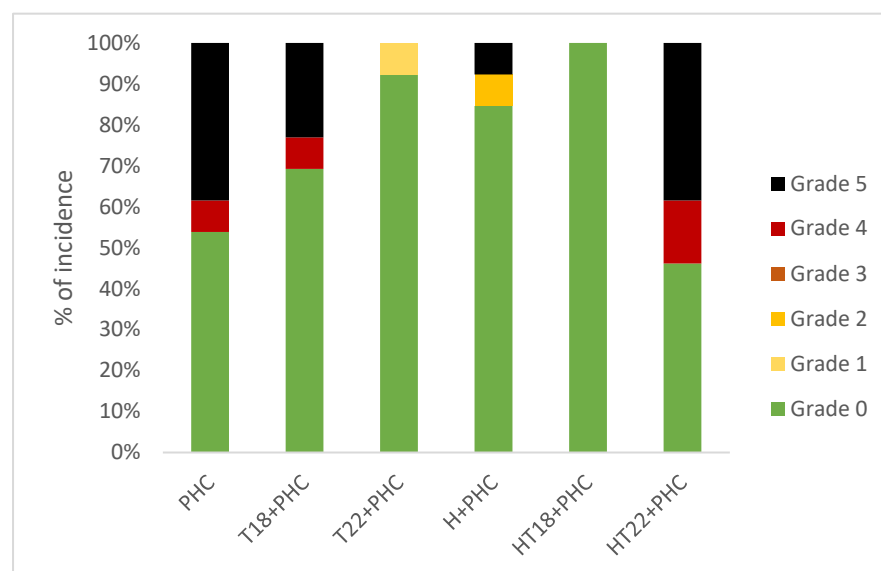


Figure 10. Disease severity in chilli seedlings infected with *Phytophthora capsici*. PHC: plants inoculated with *P. capsici*; T18: plants inoculated with T18 spore solution and inoculated with *P. capsici*; T22: plants inoculated with T22 spore solution and inoculated with *P. capsici*; H: plants with hydrogels and inoculated with *P. capsici*; HT22: plants with hydrogels + T22 and inoculated with *P. capsici*; HT18: plants with hydrogels + T18 and inoculated with *P. capsici*—disease severity scale from grade 0 (healthy plants) to grade 5 (dead plants).

Therefore, it is imperative to enhance the encapsulation process's efficacy to ensure the preservation or augmentation of the protective mechanisms triggered by *Trichoderma*. The present study revealed modifications in the physicochemical properties of the hydrogel, associated with its composition, manufacturing process, and incorporation of *Trichoderma* spores. These modifications were observed to occur without compromising the hydrogel's viability or its plant protection capacity. Nonetheless, several issues must be examined in subsequent studies. These include the function of the inherent characteristics of *Trichoderma* strains in regulating protective activity, as well as the processes involved in the appropriate reactivation of spores and the dynamics of activation and growth of the fungus within the hydrogel matrix.

3. Conclusions

The present study demonstrated that the formulated hydrogel can incorporate and maintain *Trichoderma* spp. spores viable after the manufacturing process. The hydrogel exhibited a dense, agglomerated structure, an irregular surface, and a water absorption capacity of 1.40–1.63 (g/g). Furthermore, it demonstrated degradation in burial tests in sterile soil and soil inoculated with microorganisms. HT18 hydrogels demonstrated 100% inhibition of *P. capsici* symptoms, while concurrently promoting vegetative growth compared to control plants. Taken together, these results suggest a potential use of hydrogel as a delivery and application system for beneficial microorganisms in agricultural systems.

4. Materials and Methods

4.1. Reagents

Food-grade gelatin (Gel, Bloom 290 Duche®, Mexico City, Mexico), high-density food-grade chitosan (CTS, America Alimentos, Guadalajara, Mexico), medium viscosity polyvinyl alcohol (PVA, $\leq 98\%$ hydrolysis, medium viscosity, POCHTECA®, Mexico City, Mexico), phosphate-buffered saline (PBS), hydrochloric acid (HCl, Sigma-Aldrich, St. Louis, MO, USA).

4.2. Biological Material

The encapsulated fungi were *Trichoderma harzianum* T-22 (T22) and *Trichoderma viride* T18 (T18). *T. harzianum* was obtained from the commercial product PHC® T-22® (Plant Health Care Mexico, Mexico City, Mexico). *T. viride* T18 (T18) was supplied by the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, AC. (CIATEJ). The strain was selected from a collection of *Trichoderma* strains from various regions of Mexico for its inhibitory effects against multiple pathogenic fungi [12]. The subjects were cultivated on potato dextrose agar (PDA) medium for 7 days at 26 ± 2 °C.

A pathogenic strain of *Phytophthora capsici* (JAL-M60) from the CIATEJ strain collection was used, grown in clarified V8 medium for 5 days in darkness at 26 °C $\pm$ 2 °C, followed by 5 days in continuous light at 26 °C $\pm$ 2 °C. The container was then filled with sterile distilled water and maintained at 4 °C for 30 min to facilitate zoospore release. The quantification was conducted using a Neubauer chamber. The samples were resuspended in sterile distilled water to a concentration of 1×10^4 zoospores per millilitre [51].

4.3. Plant Material

The experiment utilized serrano pepper var. Camino Real F1 seeds (Harris Moran®, Seed Company, Davis, CA, USA). The seeds were then placed in seed trays for germination in substrate (Mix No. 3, Sunshine®), with a 16/8 light/dark photoperiod at 26 °C $\pm$ 2 °C.

4.4. Preparation of Hydrogels

Ternary polymeric mix: Solutions of chitosan (CTS) at 3% (w/v) and two polymers at concentrations of 2.5% (w/v) were prepared. The CTS was dissolved in 0.4 M acetic acid, while the polymers were dissolved in distilled water at 37 °C and 85 °C, respectively, with stirring for 2 h. The polymer mixture was stirred at room temperature for 1 h.

Spore suspension: The encapsulated fungi were identified as *T. harzianum* (T22) and *T. viride* (T18). The spore suspension was obtained by cultivating the *Trichoderma* strains on PDA for seven days at 26 °C $\pm$ 2 °C. Subsequently, 10 mL of sterile distilled water was added, the samples were homogenized, and the conidia were recovered and quantified using a Neubauer chamber. The suspension was concentrated by centrifugation at $11,000 \times g$ for 14 min (Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). This procedure was undertaken until a concentration of 1×10^9 spores per milliliter was attained.

The precipitation process of Gel-CTS-PVA was an acid-base reaction. The spore suspensions were then incorporated into the polymer mixture, which was stirred for 2 h. Thereafter, the suspension was gradually added to a 1.0 N NaOH aqueous solution under constant stirring until hydrogels with a spherical or bead-like morphology were formed. The beads/spheres obtained were washed with distilled water until the pH reached 7 [52] and then dried in a convection oven at 60 °C for 90 min (Yamato DX402C, manufactured by Yamato Scientific Co., Ltd., Tokyo, Japan). The dried samples were stored in vacuum-sealed bags and maintained at ambient temperature to facilitate subsequent characterization. The nomenclature assigned to the hydrogels is as follows: H, Hydrogel without

Trichoderma; HT18, Hydrogel encapsulating *T. viride* T18; HT22, Hydrogel encapsulating *T. harzianum* T-22.

4.5. Water Uptake and pH Sensitivity

Dry hydrogels (convection-dried) were weighed (15.9 mg). The hydrogels were immersed in 1 mL of 0.1 M sodium phosphate buffer at pH 5.0, 6.0, and 7.0, and in distilled water at pH 6.5. The samples were maintained at 28 °C for 24 h under constant agitation (Corning PC-620D) [24]. This procedure was performed in triplicate for each of the treatments with the different pH values evaluated. The hydrogels were extracted from the various solutions, dried superficially with filter paper, and weighed (BP121S Sartorius). The total water absorption was determined using the following equation:

$$S(\%) = \frac{W_s - W_d}{W_d} (100\%)$$

where S is the total water absorption percentage (%); W_s is the initial weight of the sample and W_d is the final weight of the sample.

4.6. Water Equilibrium Content

Pre-weighed dry hydrogels (convection dried) were utilized at a mass of 15.9 milligrams. These hydrogels were placed in 1.5 mL of distilled water, maintained at ambient temperature, and then extracted from the liquid at various time points (1, 3, 6, 12, and 24 h) over the course of 24 h. Each treatment at a different time was evaluated in triplicate. The excess water was then extracted using filter paper and subsequently weighed. The following equation was used to calculate the equilibrium water content (EWC):

$$EWC(\%) = \frac{W_s - W_d}{W_d} (100\%)$$

where W_s represents the mass of the hydrogel after hydration (%) and W_d corresponds to the mass of the samples in the dry state.

4.7. Morphological Analysis

The hydrogel samples (HT22 and H) were analyzed using stereomicroscopy (Leica EZ4 HD Digital Stereo Microscope, Leica Microsystems, Wetzlar, Germany) and scanning electron microscopy (SEM, JEOL JSM-6010LA, JEOL Ltd., Tokyo, Japan). For the scanning electron microscope (SEM), the samples were sputter-coated with gold (Denton vacuum desk IV, Denton Vacuum, LLC, Moorestown, NJ, USA) [53].

4.8. Fourier Transform Infrared Spectroscopy (FT-IR)

The experiment was conducted using attenuated total reflectance (ATR) spectroscopy. The lyophilized samples (designated HT22 and H) were then placed on the ATR crystal. The spectra were obtained with 24 scans using the Agilent MicroLab software, version 5.8 on the Cary 630 FTIR spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA), and the average spectrum was generated [24].

4.9. Degradation Study Using the Burial Technique

This evaluation was conducted using a soil burial test under controlled conditions [54]. A sterilized substrate (Mix No. 3, Sunshine®) was used, inoculated with microorganisms (5 mL at a concentration of 2×10^6 CFU mL⁻¹). The substrate was placed in plastic containers, maintained at approximately 70% moisture by irrigation every third day, and subsequently placed in a greenhouse for 28 days. The experiment involved three replicates, each comprising three hydrogels (H and HT22) in a sterile, inoculated soil medium,

buried at a depth of 5 centimetres. The samples were extracted on days 5, 14, 21, and 28, washed with distilled water, and analyzed by stereoscopy and by hydrogel degradation using weight-loss analysis [24].

The weight-loss analysis entailed the triplicate evaluation of the samples, with sample degradation assessed, and the weight-loss ratio calculated from the average weight change in nine samples. The mathematical formula used to calculate the weight-loss ratio of the samples is given by the following equation [55].

$$WL (\%) = \frac{W_o - W_f}{W_o} (100\%)$$

where WL: Weight loss (%); W_o : Initial weight of the sample; W_f : Final weight of the sample.

The hydrogels were examined using stereoscopic microscopy (Leica EZ4 HD Digital Stereo Microscope, Leica Microsystems, Wetzlar, Germany) at 5, 14, 21, and 28 days after the initiation of the experiment [24].

4.10. Effect of Encapsulation on *Trichoderma* Growth

The impact of encapsulation on *Trichoderma* germination and growth was assessed 24 h after encapsulation. Five hydrogels in the form of beads were used, which were immersed in 1 mL of sterile distilled water for 3 h. The samples immersed in water were then placed in the centre of Petri dishes containing PDA medium. The samples and spore suspensions (T18 and T22) were incubated at 26 ± 2 °C for 10 days at a concentration of 1×10^9 spores mL⁻¹. For comparison, the same concentration was used in the free-spore solutions as in the encapsulated spores. A comparative analysis was conducted to assess the duration required for germination and growth across all treatment groups [16].

4.11. Crop Protection

The impact on the biocontrol capacity of the two *Trichoderma* strains was assessed in a greenhouse experiment. In this study, the strains were incorporated into hydrogels in serrano chilli (*Capsicum annuum*) seedlings. The experiment involved using 30-day-old plants, followed by transplanting seedlings into bags containing sterile substrate (Mix No. 3, Sunshine®). Subsequently, five hydrogels were applied to the base of each plant. The experimental design comprised a randomized block design with 13 replicates of the following treatments: hydrogel (H), hydrogel with *T. harzianum* (HT22), hydrogel with *T. viride* (HT18), and spore solution at 1×10^6 spores mL⁻¹ of *Trichoderma* spp. strains (T22 and T18), distilled water (O) was used as a control, plants inoculated only with *P. capsici* (PHC) and with hydrogel (H). Ten days after transplanting, the plants were inoculated with *P. capsici* by applying 5 milliliters of a zoospore suspension (1×10^4 zoospores mL⁻¹) [56]. Finally, the incidence of disease, seedling height and fresh weight, and root viability were evaluated [24]. The severity of the disease was also evaluated using a scale from grade 0 (healthy plants) to grade 5 (dead plants) [12].

Root viability was assessed by measuring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) to 1,3,5-triphenylformazan (TTF) [50]. At the conclusion of the experiment, five root samples were collected, thoroughly rinsed with distilled water, and their initial weight was documented. The samples were immersed in a solution of 0.01 M TTC dissolved in 0.01 M sodium phosphate buffer, pH 7, and maintained at 37 °C for 1 h. After this interval, the reaction was terminated by adding 1 mL of 1 M sulfuric acid. The tissue was then macerated with pure ethyl acetate (Sigma-Aldrich) to recover the TTF, and the mixture was diluted to 10 mL with the same solvent. Subsequently, the degree of light absorption was ascertained by means of a spectrophotometer (Genesys 10UV Thermo Spectronic, Thermo Fisher Scientific) at a wavelength of 485 nanometers. Root viability was obtained by calculating the TCC reduction

intensity (mg g h) = absorbance of reduced TTC/PF h, where PF is the fresh root mass and h is the incubation time.

4.12. Statistical Analysis

The data obtained during the experiments were subjected to a one-way analysis of variance (ANOVA) using Minitab® 19.1 software (Minitab LLC, State College, PA, USA) and a comparison of means using Tukey's method ($p < 0.05$).

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Abbreviations

The following abbreviations are used in this manuscript:

CTS	Chitosan
PVA	Polyvinyl alcohol
Gel	Gelatin
T18	<i>Trichoderma viride</i> T18 strain
T22	<i>Trichoderma harzianum</i> T-22 strain
Gel-CTS-PVA Hydrogel	Gelatin–chitosan–polyvinyl alcohol hydrogel
H	Hydrogel without <i>Trichoderma</i> spp.
HT22	Hydrogel with <i>Trichoderma harzianum</i> T-22 strain
HT18	Hydrogel with <i>Trichoderma viride</i> T18 strain
PHC	<i>Phytophthora capsici</i>
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared
NaOH	Sodium hydroxide

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