Enhancing Stability and Efficacy of *Trichoderma* Bio-Control Agents through Layer-by-Layer Encapsulation for Sustainable Plant Protection

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Abstract

Agricultural fungicide pollution poses a significant environmental challenge and carries adverse consequences for human health. Therefore, strategies to limit fungicide usage have gained paramount importance. *Trichoderma* fungi, owing to their antagonistic activity against various pathogenic fungi, have emerged as prospective candidates for enhancing both the effectiveness and sustainability of plant protection. Nevertheless, the utilization of bio-control agents like *Trichoderma* has unveiled new challenges, notably their vulnerability to physical stimuli and diminished efficacy during prolonged storage.

To overcome these drawbacks, we present a mild and scalable encapsulation method for *Trichoderma* spores, employing a layer-by-layer (LbL) encapsulation approach using biobased lignin derivates. Our investigations demonstrate that the LbL-encapsulation technique imparts remarkable improvements in spore stability, even under adverse conditions such as variable temperature and prolonged exposure to UV irradiation compared to unencapsulated spores. Notably, encapsulated *Trichoderma* spores exhibit increased efficiency in the cultivation of tomato plants when compared to their unencapsulated counterparts. Additionally, our findings reveal that the *in planta* efficacy of encapsulated spores is contingent upon the specific *Trichoderma* strain employed.

The results outlined herein suggest that Trichoderma spores, encapsulated within lignin through the LbL approach, exhibit potential as promising and sustainable alternative to chemical fungicides and potential commercialization.

Keywords: microcapsules; lignin; *Trichoderma*; biocontrol; tomato plants.

Introduction

Fungal and bacterial infections in agriculture pose a worldwide economic burden and a threat to food security [1]. Plant pathogens result in a 30-40% loss in crop production annually, which is food loss that could have been used to feed the 1 billion people lacking sufficient food supplies [2]. As an example, tomato plants are susceptible to over 200 diseases, with *Fusarium oxysporum* being one of the most important threats to tomatoes worldwide causing 10-80% yield loss per year[3, 4]. Decreasing food loss mainly relies on heavy use of chemical fungicides and pesticides, which is both an inefficient and environmentally harmful strategy [5, 6]. Preventatively spraying plants with pesticides inevitably leads to pollution of the surroundings, damaging plants and animals [7]. Furthermore, pesticide pollution is a human health concern, since exposure can increase the risk of several diseases, posing a particular threat to the farmers who are in most direct contact to these toxic compounds.[8] However, traceable amounts of pesticides are also found in food products and drinking water causing a threat to the broader population[6, 7, 9]. Alternatives to spraying of fungicides are therefore urgently needed to address these environmental and health related issues. A strategy broadly used is delivery of fungicides to the plants by injection of fungicide-containing nanocarriers, for example using chitosan,[10] hemicellulose,[11] cellulose,[12] or lignin as encapsulation material[13, 14, 15]. It has been demonstrated that targeted delivery of fungicides reduces the needed dose and in planta transport of nanocarriers was studied.[16] Injection limits the fungicide pollution, while still showing efficient plant protection.[17, 18] However, the individual injection into each plant is time-consuming, could affect the tree and residues could be found if they are injected before blossom.[17] A completely fungicide-free alternative is biological control agents (BCAs), which are microorganisms that can control the growth of pathogens [19]. BCAs afford a sustainable alternative to pesticides, with *Trichoderma* fungi being identified as particularly promising. *Trichoderma* has demonstrated improved crop

yields, due to its ability to confer resistance to plants, by increasing access to nutrients, production of antibiotics, plant hormones and water acquisition rate [20, 21]. The use of *Trichoderma* also increased shoot and root growth in tomato plants when compared to control plants [20, 22]. Several other studies have shown improvements in plant health and disease resistance after treating with *Trichoderma* spores [3, 20, 23]. However, BCAs have significant drawbacks preventing wider applications due to their poor shelf-life and general instability [24]. These drawbacks can be addressed by developing methods for encapsulation of *Trichoderma* spores, which in turns can improve the stability.[25]

As a sustainable polymer for encapsulation, lignin is promising for a wide range of application in agriculture and health. Lignin is an underutilized bioresource with very interesting properties [26, 27]. This compound is obtained from the paper production waste making it ecofriendly and sustainable [26, 27]. Lignin has shown to be promising approach to treat Esca, a grapevine trunk disease, using various methods. Single injections of fungicide-loaded lignin nanocarriers showed fungicide efficiency of at least four years [13]. Lignin has been used to encapsulate *Trichoderma* spores and hydrophobic fungicides. As the lignin can degrade through secretion of ligninolytic enzymes, it makes possible the transfer of the spores without reducing its effectivity in protection against various pathogens [15, 28, 29]. For this type of encapsulation, lignin is interesting as it manages to self-assemble, permits controlled release of substances, offers intrinsic antimicrobial, antioxidant and possess UV-shielding properties and is widely available [26, 30].

In this work, spores of three *Trichoderma* strains were encapsulated into lignin and the effect of this encapsulation on the spores stability was investigated. The encapsulated spores were prepared by applying alternate layers of cationic and anionic lignin polyelectrolytes implementing the protective properties of this polymer to the spores. Naked- and encapsulated spores were exposed to high (50°C) and low (-20°C) temperatures, UVC and UVB light, and

long-term storage and analyzed by a germination test to determine if the spore stability was improved by encapsulation. Finally, the naked and encapsulated spores were used as treatment of tomato plants in a greenhouse study to examine whether the encapsulated spores performed superior plant protection compared to naked spores. Overall, the encapsulated spores outperformed the naked spores but also strain type influenced their efficiency. In summary, our study reveals that LbL encapsulation of *Trichoderma* spores into biobased lignin capsules holds significant promise as a stabilizing formulation, offering a sustainable alternative to traditional fungicides. This research enhances the potential of BCAs by extending their shelf-lives and confirming their activity following soil application

Results and discussion

Synthesis and characterization of modified biopolymers

Cationic and anionic polyelectrolytes were needed for the layer-by-layber (LbL) encapsulation of the *Trichoderma* spores [29]. To ensure biocompatibility and sustainability, lignin-based polymers were chosen as biopolymers, which are available as a waste material from the paper production [26, 27, 31]. Cationic lignin was prepared from alkaline Kraft lignin by a substitution reaction with glycidyl trimethylammonium chloride (**Figure 1A**). This modification was performed using an improved literature protocol, which provided a high yields (above 85%) and opportunity for large scale production (more than 15 g in the university lab, which should not be regarded as a maximum)[32]. Lignosulfonate (**Figure 1B)** is commercially available, however, it contains minor impurities from various carbohydrates and was therefore purified by dialysis before use. Anionic- and cationic lignin were characterized by nuclear magnetic resonance spectroscopy (^{1}H NMR), fourier transform infrared (FTIR), and gel permeation chromatography (GPC) demonstrating the isolation of pure polymers with the intended chemical functionalities. The results can be found in the Supporting Information (SI). The molar masses of the lignin-based polymers were in the

range of 1-3 kDa with relatively broad molar mass distributions as expected for lignin-based polymers.[33] The easy access and straightforward production of both polymers suitable for a LbL encapsulation underline their potential as sustainable materials for biobased plant protection products.

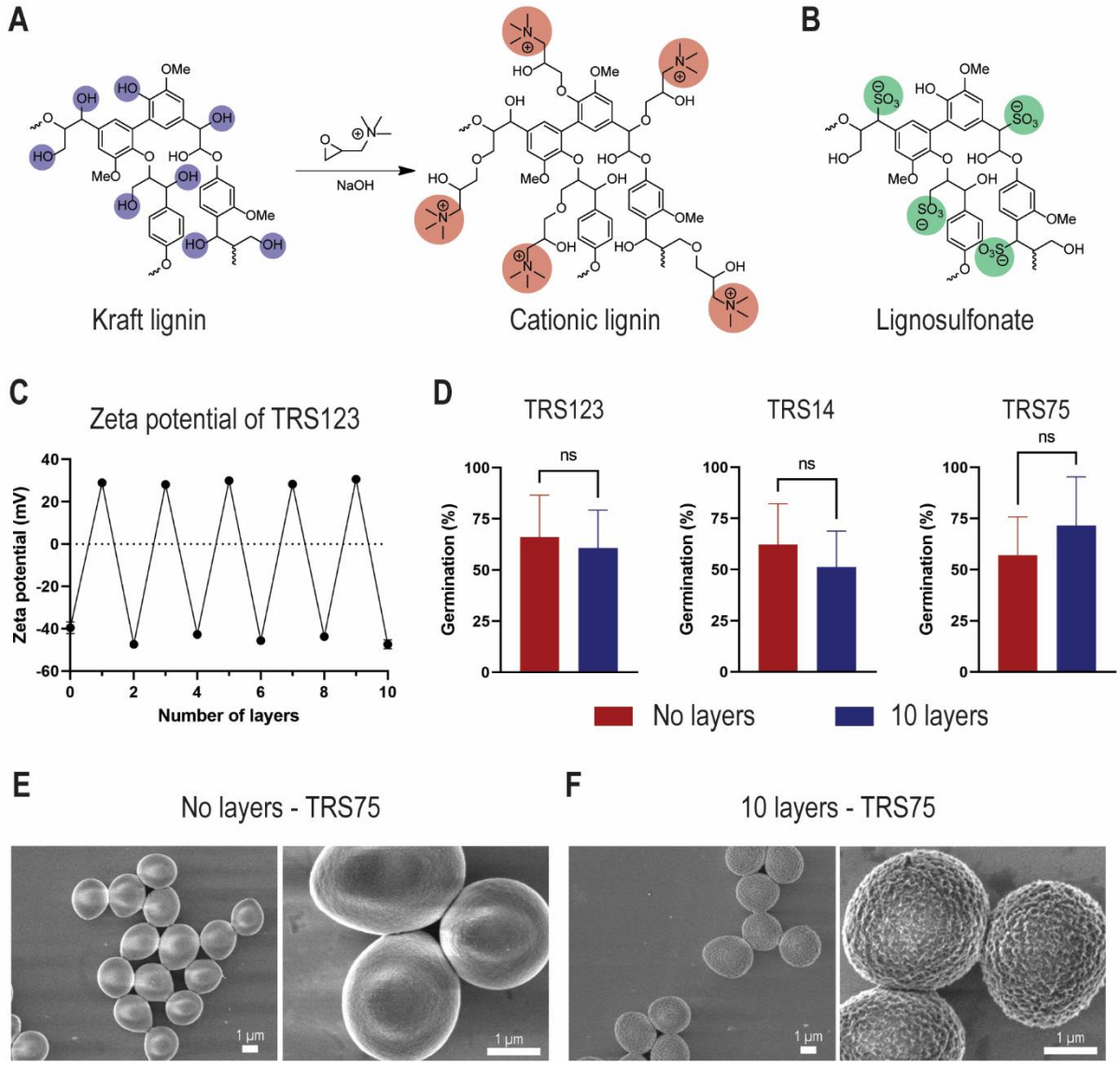

Figure 1: A) Schematic illustration of the synthesis of cationic lignin from Kraft lignin. Blue: Reactive hydroxyl groups. Red: Cationic functionality of lignin after modification. B) The structure of lignosulfonate with the anionic functionality marked in green. C) Zeta potential measurements after the absorption of each lignin layer of the TRS123 Trichoderma strain (see supporting material for the two other strains). The measurement was performed in

triplicates. D) Germination of naked spores (No layers-red) and lignin encapsulated spores (10 layers-blue). The results are shown for all three strains used: TRS123, TRS14, and TRS75. The experiment was performed in triplicates (n=3) and repeated three times on different days (N=3). P(TRS123) = 0.7567, P(TRS14) = 0.5170, P(TRS75) = 0.4547. P-values > 0.05 are non-significant (ns). E) SEM of naked TRS75 (no layers) with two different magnifications and E) SEM of encapsulated spores with 10 lignin polymer layers with two different magnifications.

Preparation and characterization of encapsulated *Trichoderma* spores by layer-by-layer assembly

The encapsulation of microorganisms, spores and cells is challenging due to the poor stability of the spores when exposed to organic solvents, chemical compounds, and heat [34]. Therefore, it was necessary to choose a mild strategy for encapsulation of *Trichoderma* spores, which could remain anoxic to the spores. In our previous study, we found that a mild layer-by-layer technique did not affect the viability of *Trichoderma* spores of a different strain [29]. These results laid the foundation for this study, in which we wanted to investigate the stability of encapsulated spores and their efficiency against infected tomato plants.

Before the encapsulation was performed, an experiment was conducted to assess if the lignin polymers showed any toxicity toward the *Trichoderma* spores chosen for this study. All three strains used in this study (called TRS14, TRS75, and TRS123, see Supp. Info. (Table S1) for details) were treated with cationic lignin or lignosulfonate and compared to an untreated sample before a germination test was performed. The results demonstrated no toxicity from the lignin polymers to the strains making them suitable to use for layer-by-layer encapsulation (**Figure S1**).

The layers were applied by suspending the spores in pure water followed by addition of a polymer solution (0.2wt%). Since the spores' surface is negatively charged, the first polymer applied was the cationic lignin. After each layer, the spores were analyzed with zeta potential measurements to ensure that the polymer was absorbed onto the spores. **Figures 1C and S2** shows how the zeta potential varies between positive and negative values by addition of the cationic and anionic polyelectrolyte layers. The last layer applied was lignosulfonate to ensure an outer negative charge similar to the naked spores. A total number of 10 layers were absorbed, which created enough protection of the spores while still being an easily scalable process. After the encapsulation, the effect of the 10 polymer layers on spores germination ability was analyzed by a germination test. The results of the germination test on all three strains are shown in **Figure 1D**, which illustrates that germination did not significantly differ between the encapsulated and naked spores. This demonstrates that LbL using lignin-based polymers is a successful and mild strategy for encapsulation of *Trichoderma* spores. The encapsulated spores were further characterized by scanning electron microscopy (SEM) and compared to naked spores. The original *Trichoderma* spores had a smooth surface (**Figure 1E**), however, upon addition of the polymer layers, the surface became rough indicating successful adsorption of the polymers (**Figure 1F**, the other trains are shown in **Figure S3**).

Stability when exposed to physical stimuli

The successful encapsulation of the *Trichoderma* spores using three different strains demonstrated the mild and reproducible LbL method was effective with lignin-based polymers. We hypothesized that the encapsulation of the spores would improve the stability making them more suitable as BCAs. One major drawback of using *Trichoderma* to treat plant diseases is the poor shelf-life and instability when exposed to various physical stimuli such as heat, cold and ultraviolet (UV) light [18, 19]. For BCAs like *Trichoderma*, it is

therefore important to improve the stability when exposed to those stimuli. Experiments were conducted to investigate if the LbL-encapsulated spores would have improved stability to those stimuli.

Temperature stability. We investigated how temperature variation affects the viability of the spores. Here, the spores were exposed to either heating (50°C) or freezing (-20°C) for 2 hours and the viability of the naked and encapsulated spores were compared to untreated samples. The viability of the spores was measured by comparing the germination of untreated and treated spores, assuming that the untreated spores were 100% viable. The results are shown in **Figure 2A** and **Figure 2B**. In both experiments encapsulated spores were significantly more stable than naked spores. While spores with 10 layers of polymer maintained almost full viability upon treatment, viability of the naked spores decreased to around 50% both when exposed to heat or freezing. The improved stability to temperature variation is a huge advantage making spore storage much simpler and thereby more accessible as a possible treatment of plant diseases.

UV stability. The aromatic structure of lignin makes it efficient as protection against UV light, which was demonstrated in coatings, cremes, and other lignin-containing polymer films[35]. We anticipated a similar protective effect towards *Trichoderma* of the lignin layer against UV light: first, the spores were treated with UVB light at a wavelength of 302 nm at different energies (**Figure 2C**). Here, the viability of the spores decreased for both encapsulated and naked spores, however, the decrease was more severe for naked spores. Furthermore, the decrease in viability of the naked spores seemed to drop immediately (0.1 J/cm^2) to 50% while encapsulated spores were still almost fully viable at that timepoint. The decrease in viability of the naked spores was significant compared to encapsulated spores. A similar trend was observed when the spores were treated with UVC light at a wavelength of 235 nm for 3 minutes (**Figure 2D**). UVC light is normally used for sterilization, hence being germicidal [36].

Under UVC irradiation, viability of both samples significantly decreased. However, the encapsulated spores seemed to be more resistant to the UVC light and maintained more than 20% viability. The viability of naked spores was significantly lower and decreased to less than 5%. Combined, these data suggest that lignin provide a protective layer towards exposure to UV light.

Shelf-life improvement. Trichoderma spores exhibit good shelf-lives for months if kept dry.[37] However, wet formulations of BCAs show a poor shelf-life due to unwanted germination making them less suitable for farmers to store and use effectively.[37] Several strategies to increase their shelf-life were investigated but with only limited success.[37, 38] We studied how the lignin LbL-encapsulation strategy affected storage time of *Trichoderma* spores. After 2 months, no differences were observed between encapsulated and naked spores when stored in saline solution at room temperature (**Figure S4**). However, after 9 months of storage a significant decrease in germination was observed for naked spores while the encapsulated spores still maintained similar degree of germination (**Figure 2E**). This is of high importance and especially the fact that the encapsulated spores were stable in saline solution is highly interesting. Until now formulations of *Trichoderma* spores have been based on freeze-dried powders or granules, which are all processes that are inconvenient and damaging to spores. The possibility of simply suspending the spores in saline solution is therefore both simpler and suggests it is possible to avoid costly and damaging processing techniques. The *Trichoderma* spores were also analyzed with SEM after 9 months of storage (**Figure 2F**) and showed that the lignin layers are intact, and it would therefore be expected to maintain protective properties against the various physical stimuli assessed.

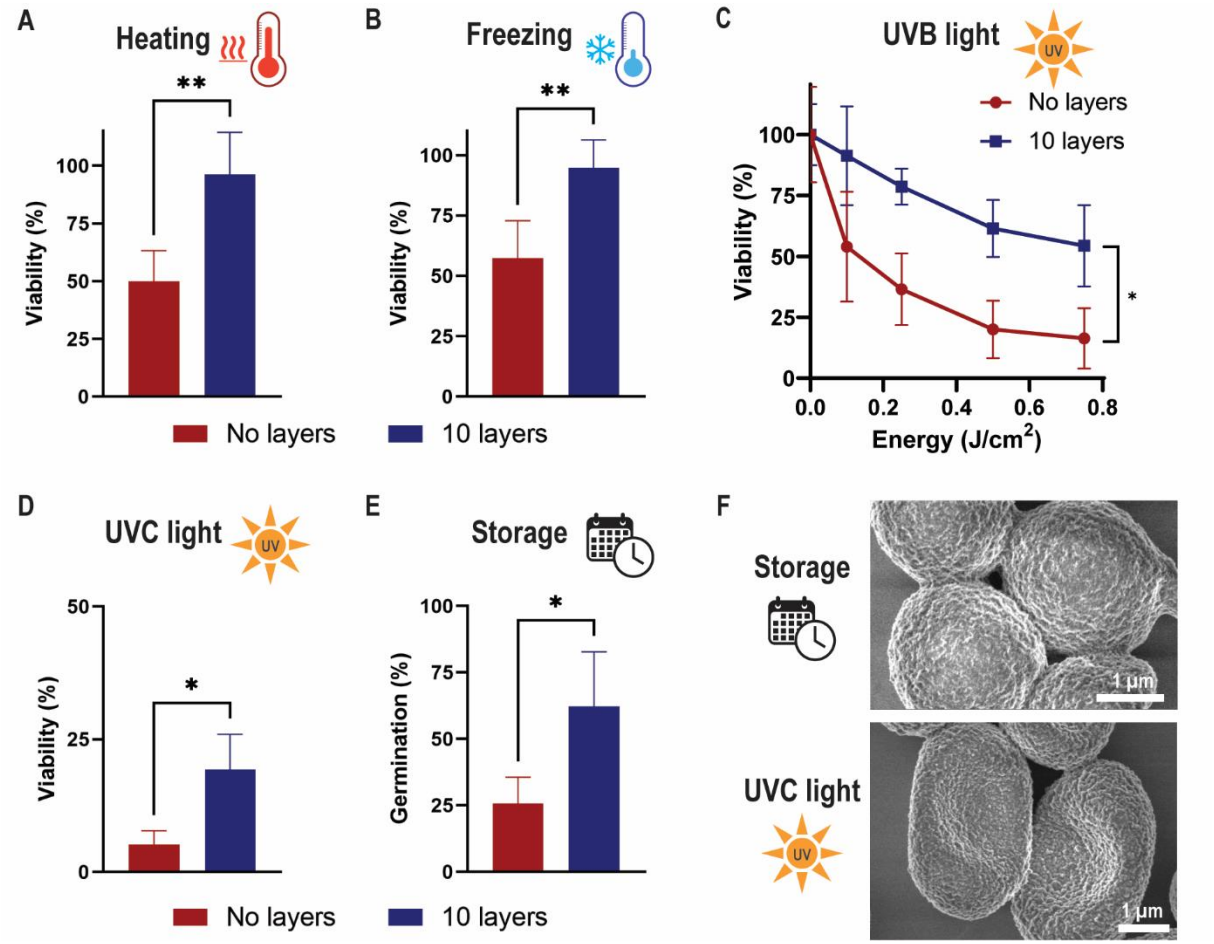

Figure 2: A-B) TRS123 Trichoderma spores naked (red) and encapsulated with (blue) polymer layers were treated either with A) heating at 50°C for 2 hours or B) freezing at -20°C for 2 hours. C-D) TRS123 Trichoderma spores naked (red) and encapsulated with (blue) polymer layers were treated with C) UVB light at different energies (in J/cm^2) or D) UVC light for 3 minutes. A-D) The treated samples were compared to non-treated samples that were normalized to 100% viability. Each experiment was performed in triplicates (n=3) and repeated three times on different days (N=3). The statistical significance was determined by a t-test (A, B, D) or a two-way ANOVA analysis (C) in GraphPad Prism. The P-values were as follows: A) P = 0.0064(**), B) P = 0.0079(**), C) see all P-values in supporting information, D) P = 0.0269(*). E) TRS14 Trichoderma spores without (red) and with (blue) polymer layers were stored in 0.85% saline solution for 9 months followed by a germination test. The experiment was performed in triplicates (n=3) and repeated three times on different days

(N=3). The statistical significance was determined by a t-test giving P = 0.0182(*). F) SEM of TRS14 with polymer layers after 9 months storage (top) and SEM of TRS123 with polymer layers after exposure to UVC light for 3 minutes (bottom).

Plant experiments

The effectiveness of encapsulated *Trichoderma* spores was examined in three, consecutive greenhouse experiments with tomato plants (photographs of the plants are to be seen in Figure S5). There were differences in the performance between studied strains. Overall, the best effect on plant growth indicated strain of *T. simmonsii* TRS75, and especially its encapsulated spores (**Figure 3)**. Application of TRS75 to the growing medium significantly increased plant weight and height compared to control (**Figure 3 A, B**). Encapsulated spores of this strain strongly reduced fusarium wilt symptoms (**Figure 3 C**). In the case of TRS75, the encapsulation of the spores resulted in remarkably better performance compared to not encapsulated ones, i.e. faster plant growth. Positive effect on plant growth was also detected when strain of *T. atroviride* TRS14 was used, which significantly increased plant height (**Figure 3 B**) and reduced fusarium wilt, compared to plants growing in the medium infested with FOL **(Figure 3C)**. However, unlike TRS75, encapsulation of TRS14 spores did not improved their efficacy. The lowest effect was obtained using spores of *T. gamsii* TRS123. This fungus positively affected tomato height compared to control, but not plant weight and fusarium wilt. Although, it was found that encapsulation of its spores significantly increased plant biomass in the medium infested with pathogenic *Fusarium* (**Figure 3 A**).

The appropriate formulation is essential for successful performance of microbial agents in commercial agricultural conditions. First of all, it should maintain viability and functional properties of the active propagules for prolonged storage. The other important subject is to

ensure uniform, consistent and free from contamination product. The biotechnological approaches to develop formulations containing *Trichoderma* are broadly discussed in the review by Martinez et al.[25]. There are also numerous reports on the effectiveness of different *Trichoderma* formulations in biocontrol and plant biostimulation [39]. Our studies underline, that the encapsulation of *Trichoderma* spores into lignin capsules by the layer-by-layer technique ensured their viability in different stress conditions. The *in planta* experiments revealed further, that the effect of encapsulated spores depends also significantly on the selected strain of *Trichoderma*. There were differences in the germination of encapsulated spores (**Figure 1 D**), and the highest germination exhibited TRS75. This strain also resulted in the highest plant growth of the tomato plants. It suggests, that the germination rate of encapsulated spores compared to bare spores may be a good indicator their further performance of *Trichoderma* preparation in growing conditions.

Our data also supports the results of microbial analyses of the growing substrate, in which encapsulated spores of TRS75 were the best colonizers of the substrate among other tested strains, and they significantly reduced *Fusarium* spp. (**Table 1**), as it is described below.

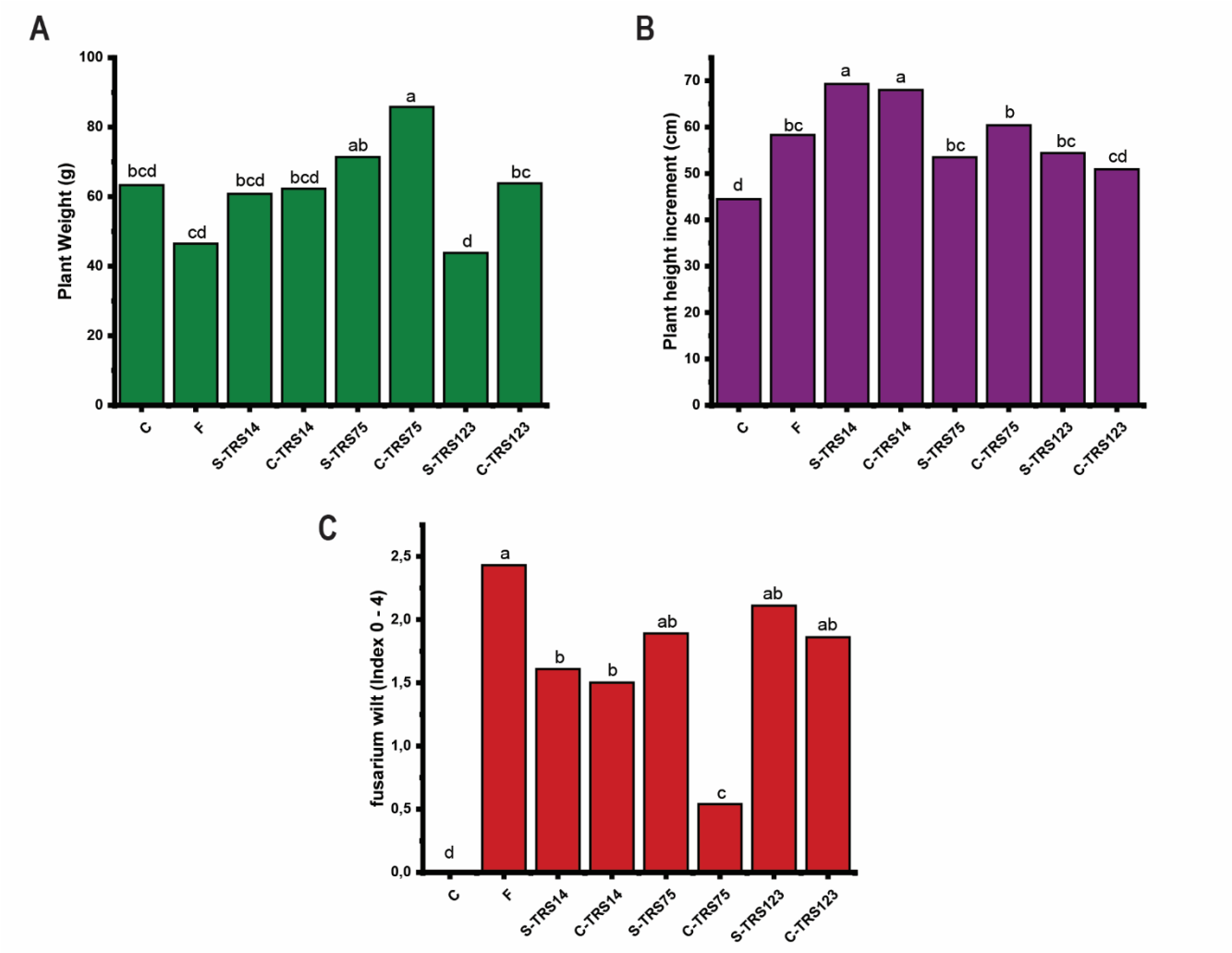

Figure 3 Growth parameters and fusarium wilt severity of tomato plants treated with encapsulated spores of Trichoderma strains). A) weight, B) height increment and C) fusarium wilt severity. C – control not treated; F – growing medium infested with Fusarium FOL; S-TRS14, S-TRS75, S-TRS123 – treatments with not encapsulated spores of Trichoderma; C-TRS14, C-TRS75, C-TRS123 – treatments with encapsulated spores of Trichoderma. The figures represents average values of three experiments. The same letters above the bars indicate no statistical difference according to Duncan test (p = 0.05).

Microbiological analyses of growing substrates added with fungi, and then plant rhizosphere, revealed that the number of *Trichoderma* propagules increased during two weeks after the application, compared to its initial added quantity of 10^4 cfu g^{-1} of the medium (**Table 2**). This was despite the fact, that the substrate (not sterilized before use) already contained indigenous *Trichoderma* fungi. The encapsulation of the spores of TRS75 and TRS123 significantly increased their viability in the growing substrate and stimulated their multiplication compared to other treatments. Two weeks after the application, the number of *Trichoderma* propagules in the substrates inoculated with TRS75 and TRS123 microcapsules increased 3 and 2.5-fold respectively (compared to the control). In the case of the encapsulated TRS14 no significant effect on their viability in the substrate compared to the naked spores of this strain was observed. However, in both substrates the number of *Trichoderma* was higher than in the control, although the differences were not significant (**Table 1**). We believe that the lignin-encapsulated spores show higher effectivity in most cases due to promotion of expressing cell wall-degrading enzymes, which are involved in biocontrol activities by Trichderma in the field, as reported earlier [25, 29]. In ongoing studies regarding the antagonistic properties of the *Trichoderma* spp. strains TRS14, TRS75 and TRS123, it was indicated that strain TRS75 exhibited a high ability to enzymatic activity, especially for cellulase, chitinase and protease, while TRS14 produced mostly glucanase, and TRS123 for chitinase and protease (not published).

Table 1 The number of Trichoderma spp. and Fusarium spp. in the growing media after two weeks of incubation, before tomato planting. The values expressed as cfu g^{-1} of dry weight of the growing medium (g.m.).

Treatments	*Trichoderma* spp. 10^5 cfu g^{-1} g.m.	*Fusarium* spp. 10^5 cfu g^{-1} g.m.
control	4.63 ± 2.69 b	$<10^5$ d
Fusarium control	4.48 ± 2.52 b	4.70 ± 0.79 a
S-TRS14	7.07 ± 2.94 b	2.07 ± 0.27 bc
C-TRS14	6.55 ± 3.81 b	2.77 ± 0.46 b
S-TRS75	4.32 ± 1.37 b	1.22 ± 0.17 cd
C-TRS75	15.47 ± 8.45 a	1.54 ± 0.40 c
S-TRS123	3.82 ± 1.58 b	1.16 ± 0.29 cd
C-TRS123	11.68 ± 5.21 ab	0.98 ± 0.24 cd

The data represents average values of three experiments. Means followed by the same letter in columns are not significantly different according to Duncan test (p = 0.05).

The analyses indicated further that all *Trichoderma* treatments significantly reduced the density of *Fusarium* spp. in the growing substrates infested with FOL. The strongest inhibitory effect was obtained with TRS75 and TRS123, which both decreased the number of *Fusarium* ca. 4-fold, while TRS14 resulted in a ca. 2-fold reduction. All encapsulated spores had no significant influence on the effectiveness of *Fusarium* mitigation in the substrate. Th encapsulation of the spores had no effect on the *Fusarium* incidence in tomato rhizosphere at the end of the experiment (**Table 2**). The rhizosphere was less colonized by *Fusarium* (10^4 cfu g^{-1} of roots) than the growing substrates (10^5 cfu g^{-1} of the substrate). Similarly, substantially less *Trichoderma* colonies were isolated from tomato roots than from the

substrate (**Table 1 and 2**). Markedly more *Trichoderma* were isolated from the roots of plants grown in TRS-inoculated substrates than from the substrate infested with FOL alone, but these differences were not as significant. There were also no significant differences between roots colonization by encapsulated and bare spores, except for TRS123, where encapsulated spores were superior.

Trichoderma fungi are being studied and used commercially to control many pathogenic fungi, among them *Fusarium* [40]. According to the review by Sharma and Sharma, a mechanism most involved in the interactions between *Trichoderma* and *Fusarium* in soil and rhizosphere is mycoparasitism, supported by production of antimicrobial volatiles and nonvolatile compounds [41]. Those activities of *Trichoderma* are also involved in resistance induction in host plants as well as help the plant for better growth and productivity. These mechanisms of *Fusarium* control by *Trichoderma* fungi have also been previously researched [42]. Previous studies on the herein used strains (**Table S1)** had shown that TRS14 indicated strong antagonism *in vitro* toward *Fusarium*, related to antibiotic compounds production, but also plant growth promotion and induction of systemic resistance. In the case of TRS75, high abilities to produce of lytic enzymes not only could have a positive effect on the spores germination, but also suggests the mycoparasitism as a mechanism to suppress FOL in the growing substrate. TRS123 mostly produced volatiles, which might contribute to resistance induction in tomato plants, however, this effect was not significant. Nevertheless, these theories require additional research.

Table 2 The number of Trichoderma spp. and Fusarium spp. in the rhizosphere of tomato plants growing in the media infested with Fusarium oxysporum f.sp. lycopersisi and inoculated with Trichoderma encapsulated or bare spores. The values expressed as cfu g^{-1} of the roots.

Treatments	*Trichoderma* spp. 10^5 cfu g^{-1}	*Fusarium* spp. 10^5 cfu g^{-1}
control	0.98 ± 0.26 b	$<10^5$ c
Fusarium control	0.71 ± 0.20 c	0.64 ± 0.12 b
S-TRS14	0.87 ± 0.22 bc	0.52 ± 0.09 b
C-TRS14	1.01 ± 0.26 b	0.54 ± 0.08 b
S-TRS75	1.13 ± 0.29 b	0.70 ± 0.09 b
C-TRS75	1.00 ± 0.27 b	0.33 ± 0.06 bc
S-TRS123	1.13 ± 0.32 b	0.57 ± 0.15 b
C-TRS123	1.39 ± 0.36 a	0.85 ± 0.30 a

The data represents average values of three experiments. Means followed by the same letter in columns are not significantly different according to Duncan test (p = 0.05).

Conclusion

The encapsulation of *Trichoderma* spores with cationic and anionic lignin significantly improved the stability of the spores when exposed to high and low temperatures, retaining almost full viability. When exposed to UV light the stability was more affected, however, encapsulated spores were better protected against UVB and UVC light compared to naked spores. Furthermore, shelf-life was improved when left in saline solution for up to 9 months without compromising germination as in the naked spores. During the greenhouse experiments, encapsulated spores proved to be more effective than naked spores in general.

However, the *in planta* performance of encapsulated spores was depended on the strain of *Trichoderma*. Analysing the results of three consecutive experiments, significant, positive differences between naked and encapsulated spores showed strain of *T. simmonsii* TRS75. Encapsulated spores of this strain strongly reduced fusarium wilt symptoms and improved tomato growth in growing substrate infested with FOL. Spores encapsulation improved also the performance of *T. gamsii* TRS123, but to lower extent than in the case of TRS75, as their positive effect has only been observed on the biomass of tomato plants. It was found, that both: TRS75 and TRS123, intensively colonized growing substrate, when encapsulated spores were used. *T. atroviride* TRS14 indicated positive influence on tomato plant growth, but its effect was not affected by capsulation. The diverse ability of the tested strains to produce lytic enzymes is a probable cause, or one of the reasons for their different performance, but this hypothesis requires more studies. Consequently, carrier substances such as lignin can enhance efficacy and activity in biological control, a crucial attribute when dealing with a highly aggressive target pathogen. However, in conclusion, encapsulation of *Trichoderma* spores with cationic and anionic lignin is a very promising formulation, and may be superior to others, due to its uniform and stabile quality and lack of contamination with other microorganisms.

Data availability statement

Data will be made available upon request from the authors.

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References

[1] M. C. Fisher, D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw, S. J. Gurr, Nature 2012, 484, 186; S. Savary, L. Willocquet, S. J. Pethybridge, P. Esker, N. McRoberts, A. Nelson, Nature Ecology & Evolution 2019, 3, 430.

[2] N. Evans, M. H. Butterworth, A. Baierl, M. A. Semenov, J. S. West, A. Barnes, D. Moran, B. D. L. Fitt, Food Security 2010, 2, 143; J. Flood, Food Security 2010, 2, 215.

[3] A. Singh, N. Shukla, B. C. Kabadwal, A. K. Tewari, J. Kumar, International Journal of Current Microbiology and Applied Sciences 2018, 7, 2382.

[4] M. Ma, P. W. J. Taylor, D. Chen, N. Vaghefi, J.-Z. He, Microorganisms 2023, 11, 263.

[5] D. J. Hughes, J. S. West, S. D. Atkins, P. Gladders, M. J. Jeger, B. D. Fitt, Pest Management Science 2011, 67, 1082.

[6] L. Rani, K. Thapa, N. Kanojia, N. Sharma, S. Singh, A. S. Grewal, A. L. Srivastav, J. Kaushal, Journal of Cleaner Production 2021, 283, 124657.

[7] M. W. Aktar, D. Sengupta, A. Chowdhury, Interdisciplinary toxicology 2009, 2, 1.

[8] F. H. M. Tang, M. Lenzen, A. McBratney, F. Maggi, Nature Geoscience 2021, 14, 206.

[9] A. Grewal, J Med Chem Toxicol 2017, 2.

[10] L. Xu, L.-D. Cao, F.-M. Li, X.-J. Wang, Q.-L. Huang, J. Dispersion Sci. Technol. 2014, 35, 544.

[11] S. J. Beckers, L. Wetherbee, J. Fischer, F. R. Wurm, Biopolymers 2020, 111, e23413.

[12] T. O. Machado, S. J. Beckers, J. Fischer, C. Sayer, P. H. H. de Araújo, K. Landfester, F. R. Wurm, J. Colloid Interface Sci. 2021, 601, 678.

[13] T. O. Machado, S. J. Beckers, J. Fischer, B. Müller, C. Sayer, P. H. H. de Araújo, K. Landfester, F. R. Wurm, Biomacromolecules 2020, 21, 2755.

[14] T. O. Machado, J. Grabow, C. Sayer, P. H. H. de Araujo, M. L. Ehrenhard, F. R. Wurm, Adv Colloid Interface Sci 2022, 303, 102645; S. Beckers, S. Peil, F. R. Wurm, ACS Sustainable Chemistry & Engineering 2020.

[15] J. Fischer, S. J. Beckers, D. Yiamsawas, E. Thines, K. Landfester, F. R. Wurm, Advanced Science 2019, 6, 1802315.

[16] S. J. Beckers, A. H. J. Staal, C. Rosenauer, M. Srinivas, K. Landfester, F. R. Wurm, Advanced Science 2021, 8, 2100067.

[17] C. Berger, F. Laurent, Crop Prot 2019, 124, 104831.

[18] A. VanWoerkom, S. Aćimović, G. Sundin, B. Cregg, D. Mota-Sanchez, C. Vandervoort, J. Wise, Crop Prot 2014, 65, 173.

[19] P. A. Shah, J. K. Pell, Applied Microbiology and Biotechnology 2003, 61, 413; S. L. S. Velivelli, P. De Vos, P. Kromann, S. Declerck, B. D. Prestwich, Trends in Biotechnology 2014, 32, 493.

[20] N. S. Abdullah, F. Doni, M. S. Mispan, M. Z. Saiman, Y. M. Yusuf, M. A. Oke, N. S. M. Suhaimi, Agronomy 2021, 11, 2559.

[21] J. Poveda, D. Eugui, P. Abril-Urías, P. Velasco, Symbiosis 2021, 85, 1.

[22] A. Rasool, African Journal of Biotechnology 2011, 10.

[23] F. Zhang, Y. Huo, A. B. Cobb, G. Luo, J. Zhou, G. Yang, G. W. T. Wilson, Y. Zhang, Front Microbiol 2018, 9, 848; A. H. a. P. Dwivedi, J Pharmacogn Phytochem 2018, 7, 758.

[24] M. T. a. A. R. Sanjeev Kumar, African Journal of Agricultural Research 2014, 9, 3838; A. Puyam, Journal of Applied and Natural Science 2016, 8, 1100.

[25] Y. Martinez, J. Ribera, F. W. M. R. Schwarze, K. De France, Appl. Microbiol. Biotechnol. 2023, 107, 5595.

[26] M. Gigli, G. Fellet, L. Pilotto, M. Sgarzi, L. Marchiol, C. Crestini, Front Plant Sci 2022, 13, 976410.

[27] A. Boarino, H. Wang, F. Olgiati, F. Artusio, M. Özkan, S. Bertella, N. Razza, V. Cagno, J. S. Luterbacher, H.-A. Klok, F. Stellacci, ACS Sustainable Chemistry & Engineering 2022, 10, 14001.

[28] P. H. C. d. Lima, D. R. Antunes, M. M. d. L. Forini, M. d. S. Pontes, B. D. Mattos, R. Grillo, Frontiers in Nanotechnology 2021, 3.

[29] S. Peil, S. J. Beckers, J. Fischer, F. R. Wurm, Mater Today Bio 2020, 7, 100061.

[30] M. C. García, J. A. Díez, A. Vallejo, L. García, M. C. Cartagena, Industrial & Engineering Chemistry Research 1996, 35, 245.

[31] M. Stanisz, Ł. Klapiszewski, M. N. Collins, T. Jesionowski, Materials Today Chemistry 2022, 26.

[32] F. Kong, K. Parhiala, S. Wang, P. Fatehi, European Polymer Journal 2015, 67, 335.

[33] H. Lange, F. Rulli, C. Crestini, ACS Sustainable Chemistry & Engineering 2016, 4, 5167.

[34] E. Zussman, Polymers for Advanced Technologies 2011, 22, 366.

[35] H. Sadeghifar, A. Ragauskas, Polymers 2020, 12, 1134.

[36] N. G. Reed, Public Health Reports 2010, 125, 15.

[37] M. Faria, I. Martins, D. A. Souza, G. M. Mascarin, R. B. Lopes, Biol Control 2017, 107, 87.

[38] Y. Martínez, M. Heeb, T. Kalač, Z. Gholam, F. W. M. R. Schwarze, G. Nyström, K. De France, Appl. Microbiol. Biotechnol. 2023, 107, 1465.

[39] W. Herrera, O. Valbuena, D. Pavone-Maniscalco, Egyptian Journal of Biological Pest Control 2020, 30; B. Swathi, A. K. Patibanda, J. K. Prasadji, P. V. Krishnayya, M. L. Ahamed, International Journal of Current Microbiology and Applied Sciences 2020, 9, 301.

[40] X. Yao, H. Guo, K. Zhang, M. Zhao, J. Ruan, J. Chen, Frontiers in Microbiology 2023, 14.

[41] A. Sharma, A. Srivastava, A. K. Shukla, K. Srivastava, A. K. Srivastava, A. K. Saxena, in *Phytobiomes: Current Insights and Future Vistas*, (Eds: M. K. Solanki, P. L. Kashyap, B. Kumari), Springer Singapore, Singapore 2020, 225.

[42] A. E. Sehim, O. A. Hewedy, K. A. Altammar, M. S. Alhumaidi, R. Y. Abd Elghaffar, Frontiers in Microbiology 2023, 14.

Graphical Abstract