Supplementary Information

For

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

Kaja Borup Løvschall,[a,b] Sara T. R. Velasquez, [a,b] Beata Kowalska [c], Magdalena Ptaszek [c], Anna Jarecka [c], Magdalena Szczech,[c] Frederik R. Wurm [a,b*]

a) Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany.

b) Sustainable Polymer Chemistry (SPC), Department of Molecules and Materials, MESA+ Institute for Nanotechnology, Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede (Netherlands), f.r.wurm@utwente.nl

c) The National Institute of Horticultural Research, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

1. Materials

All chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise stated. Glycidyl trimethylammonium chloride (GTAC) was purchased from TCI Chemicals.

Trichoderma strains were obtained from the collection of the Department of Microbiology and Rhizosphere, The National Institute of Horticultural Research (Poland). These strains were originally isolated from soil or mushroom growing farms. All isolates from the collection (more than 100) were subjected to the tests for their antagonistic and plant-promoting performance in the laboratory and

greenhouse conditions – see Table 1 (Szczech et al. data not published). They were identified by DNA barcoding, based on the sequences of internal transcribed spacers 1 and 2 (ITS1 and 2) of the ribosomal RNA gene cluster and on the sequences of translation elongation factor 1 alpha (tef1), chitinase 18-5 (chi18-5), and RNA polymerase II subunit (rpb2) gene fragment [1].

Table S**1**- Characteristic of *Trichoderma* strains used in the experiments.

Strain	**classification**	***In vitro* antagonism against *Fusarium***	**volatiles**	**antibiotics**	**lytic enzymes**	**plant growth promotion**	**resistance induction in plants**
TRS14	*T. atroviride*	***	**	***	**	***	***
TRS75	*T. simmonsii*	**	*	*	***	**	nd
TRS123	*T. gamsii*	*	***	**	**	*	***

* - the number of stars indicates the intensity of the strain activity.

nd – not detected.

2. Equipment

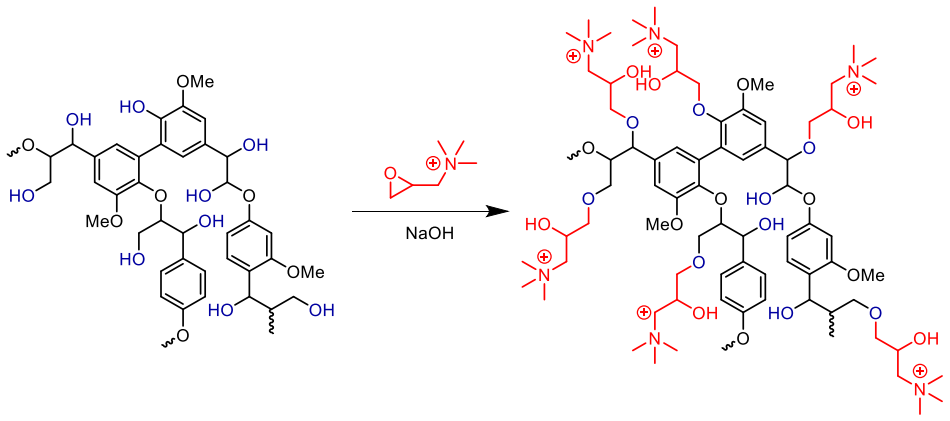
Nuclear magnetic resonance (NMR) was performed to characterize the lignin polymers used for layer-by-layer encapsulation of *Trichoderma* spores. The ^{1}H NMR measurements were performed on a Bruker Avance III™ HD 400 MHz using D_2O as solvent. The spectra were analysed using MestReNova from Mestrelab Research S. L. and referenced to the solvent signal. Fourier transform infrared (FTIR) spectroscopy was performed on a Bruker Alpha. Before each measurement, a baseline measurement was conducted. The samples were then applied as powders and could be measured in ATR mode. The measurements were performed with 64 scans from 4000 to 400 cm^{-1}.

Scanning electron microscopy (SEM) was performed on a JSM 7610 FPlus by JEOL. Zeta potential measurements were performed on Malvern Zetasizer Advanced Range Pro. Equipment used for

spore work was sterilized by autoclaving using a HMC Europe HG-50. Optical microscopy used for the quantification of *Trichoderma* spores was performed on Olympus BX60 with 20x magnification.

3. Preparation of lignin-based polyelectrolytes for LbL

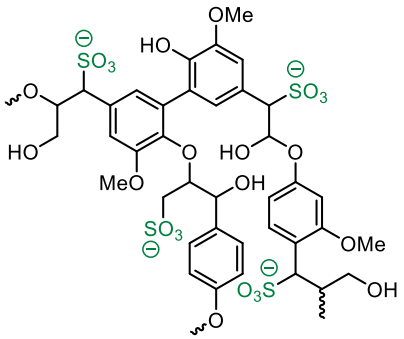
3.1. Synthesis of cationic lignin



Cationic lignin was synthesized using a modified literature protocol, which provided an improved yield[2]. Kraft lignin was determined to have 6.13 mmol of hydroxyl groups as described previously in our work[3]. Kraft lignin (15 g) was dissolved in 0.2 M sodium hydroxide (1 L) under stirring. Afterwards, GTAC (39 g) was added slowly to the solution and the mixture was stirred at 70 °C for an hour. The reaction mixture was allowed to cool down followed by neutralization with 10wt% sulfuric acid. The solution volume was reduced and afterwards subjected to dialysis against pure water using 1 kDa cut-off. The dialysis was performed for 48 hours with exchange of water 5 times. The final product was isolated by freeze-drying yielding the pure product (13.3 g).

^{1}H-NMR (400 MHz, D_2O) δ 6.88 (bs, 1H), 4.24 – 2.28 (m, 5H).

3.2. Purification of lignosulfonate

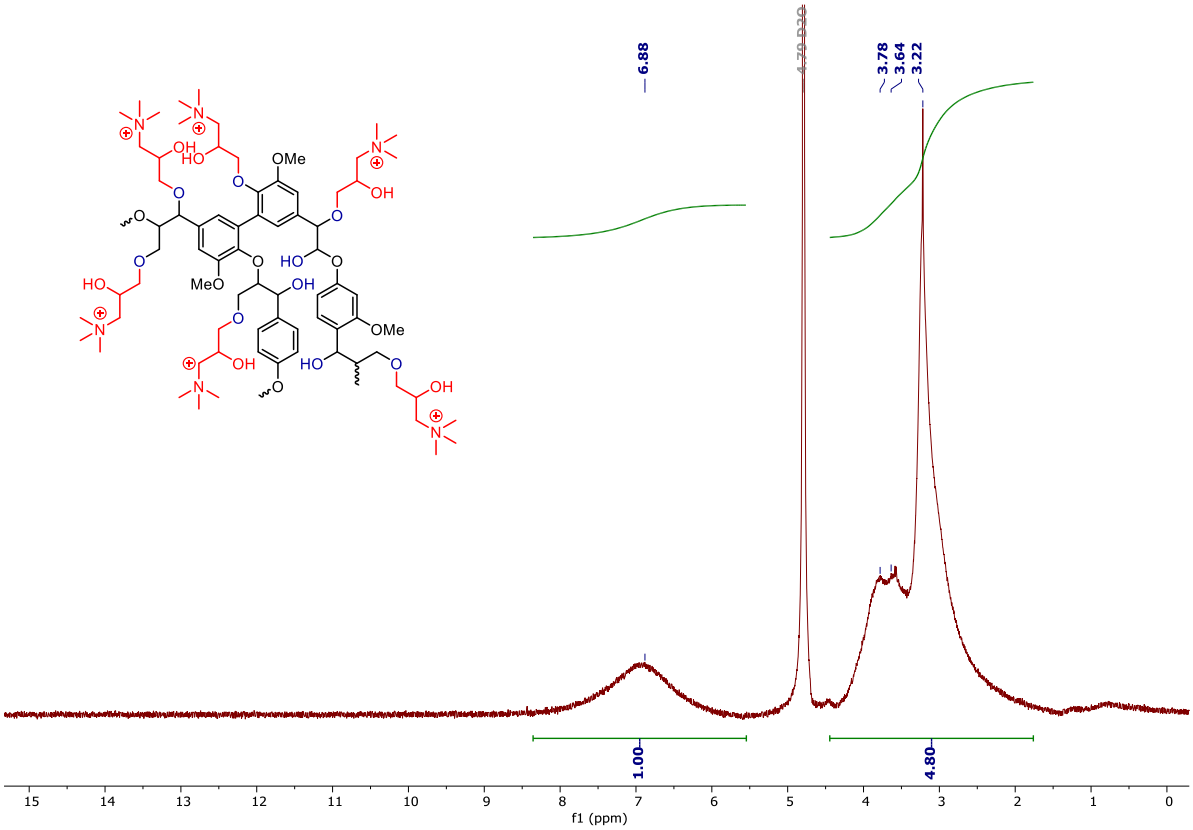


Lignosulfonate was purified by dialysis before use to remove minor impurities. Lignosulfonate (10 g) was dissolved in pure water (150 mL) and added to dialysis bags with a cut-off of 1 kDa. The dialysis ran in pure water for 24 hours and the water was exchanged 3 times. The pure lignosulfonate was isolated by lyophilisation (7.1 g).

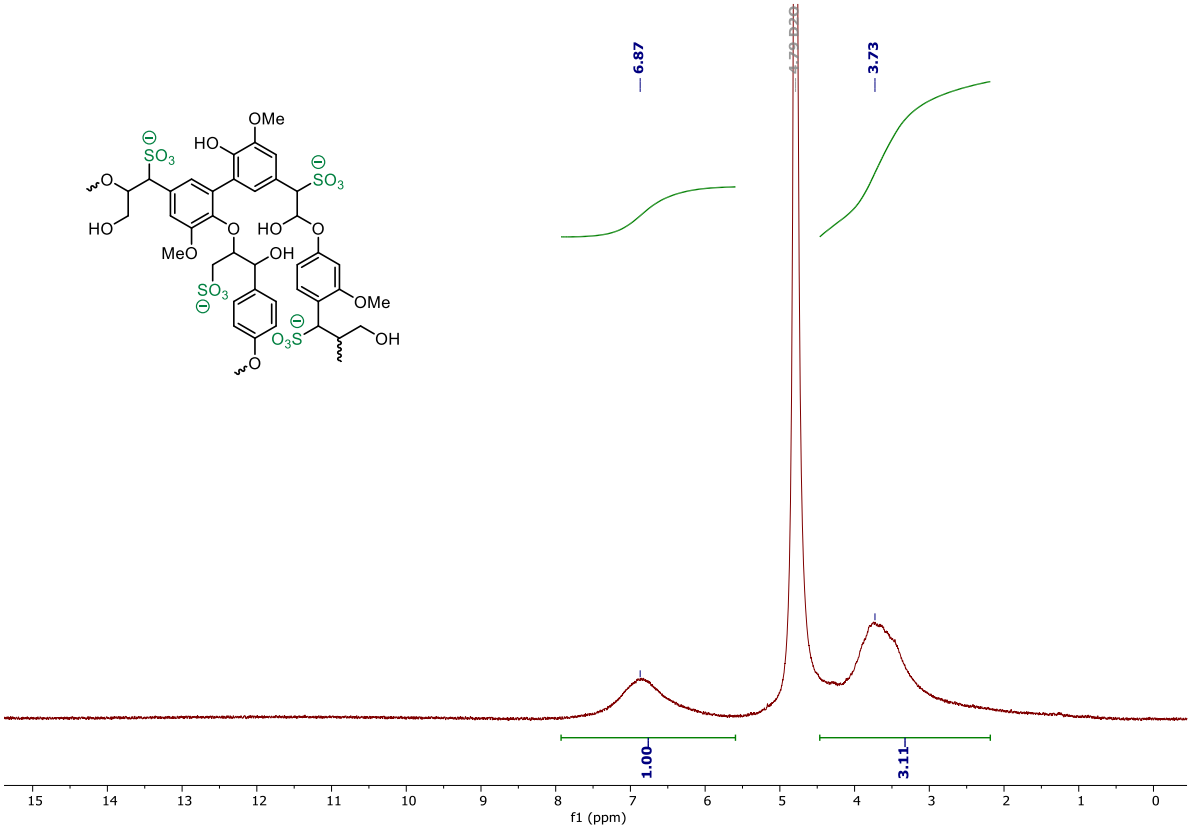
^{1}H-NMR (400 MHz, D_2O) δ 6.87 (bs, 1H), 3.73 (bs, 3H).

3.3. ^{1}H NMR spectra

3.3.1. ^{1}H NMR spectrum of cationic lignin

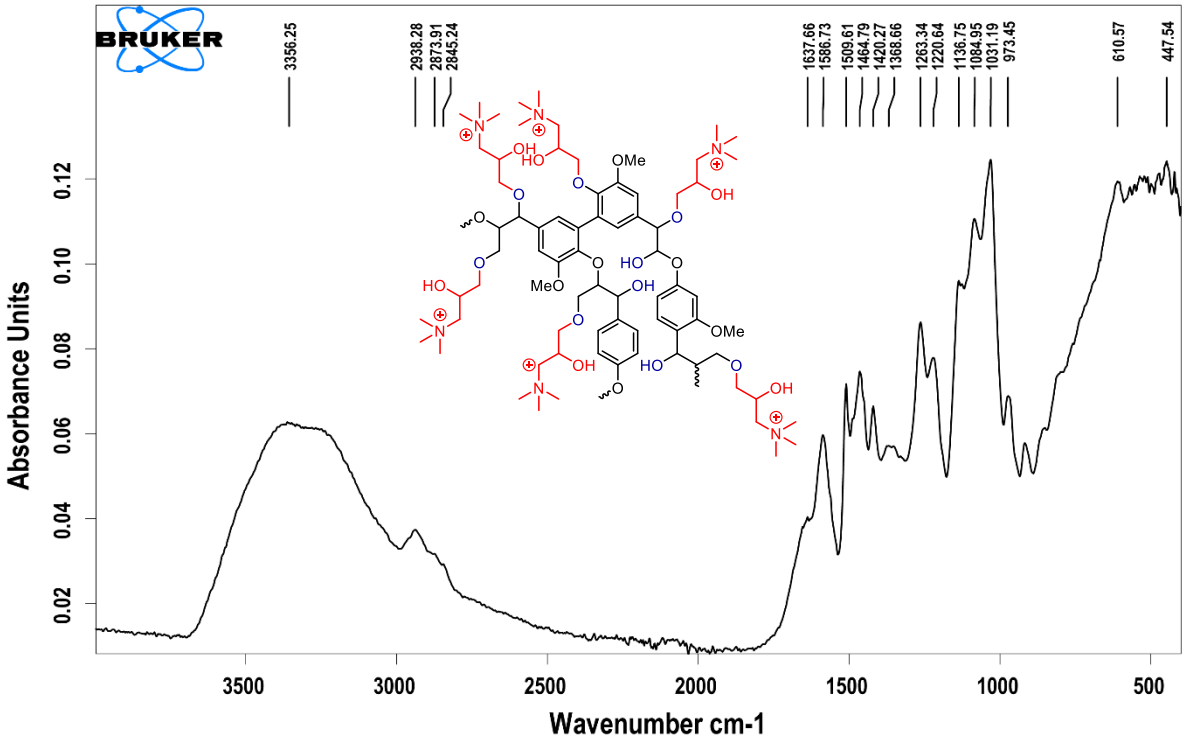


3.3.2. ^{1}H NMR spectrum of purified lignosulfonate

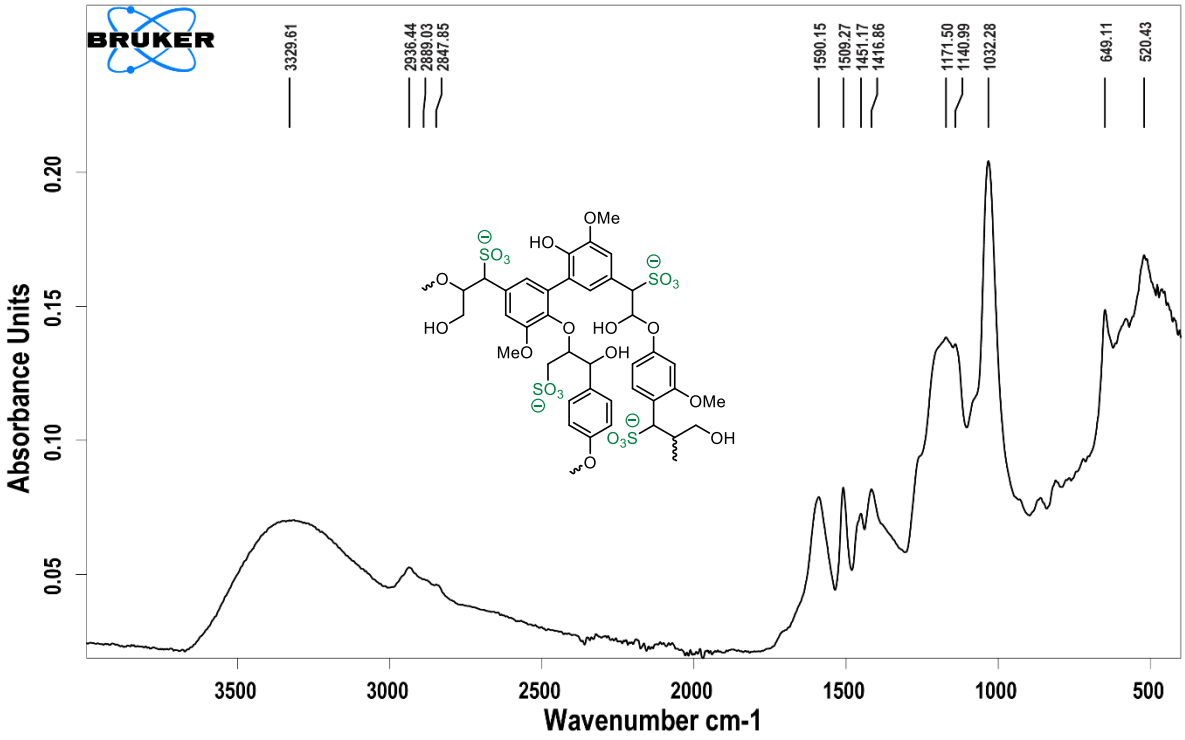


3.4. FTIR spectra

3.4.1. FTIR spectrum of cationic lignin



3.2.2. IR spectrum of purified lignosulfonate



4. *Trichoderma* experiments

4.1. Preparation of YMG medium

Yeast extract (2 g), malt extract (5 g), and glucose (5 g) were added to a 1 L Duran® glass bottle with a magnetic stirring bar. MilliQ water (1 L) was added to the bottle and the mixture was stirred for 5 minutes until fully dissolved. Next, agar (18 g) was added to the bottle yielding a suspension. The pH of the mixture was adjusted to 5.5 by addition of concentrated HCl. The resulting suspension was autoclaved at 121 °C for 40 minutes and cooled to 50°C. After autoclaving, the solution was stirred for 1 minutes followed by pouring into sterile petri dishes in a flow bench. The petri dishes were left to cool at room temperature overnight and afterwards placed in the fridge at 4 °C for storage. Petri dishes with YMG medium were used for all experiments.

4.2. Culturing of *Trichoderma* spores

A fragment of *Trichoderma* spores (either from strain TRS14, TRS75, or TRS123) was added to a petri dish with YMG medium and incubated for 7-10 days at room temperature being exposed to light minimum 30 minutes every day. Upon full spore coverage of the plate, the spores could be harvested. 10 mL of saline solution (0.85% NaCl in pure water) was added to the agar plate and the spores and mycelium were gently detached from the surface using a bacterial spreader. The suspension was filtered through a Miracloth® filter to isolate the spores from the mycelium. Freshly harvested spores were used directly for experiments.

4.3. Preservation of *Trichoderma* spores

Trichoderma was preserved in the -80°C freezer for long-time storage. To protect the spores, a spores suspension was mixed with sterile glycerol (0.7 mL spores suspension + 0.3 mL of glycerol) before it was placed at -80°C. To revive the spores after freezing, one drop of the suspension was placed on the surface of a plate with fresh YMG medium and incubated and harvested as described in 4.1.

4.4. Spores quantification by Neubauer counting chamber

A Neubauer counting chamber was used to estimate the spore concentration. The spore suspensions were diluted to around 250,000-2,500,000 spores/mL and 10 µL was added to a Neubauer counting chamber. Using an optical microscope, the number of spores in the four big squares of the counting grid were counted. The concentration of spores in the original sample (C_{sp}) was determined by the equation below.

$$C_{sp} = \frac{N * 10{,}000}{N_{sq}} * d$$

N is the number of counted spores, N_{sq} is the number of big counting squares that were counted, and d is the dilution factor from the original spore suspension. C_{sp} was determined in spores/mL.

4.5. Germination test

Each germination test was performed in the same way and always performed in triplicates. A suspension containing 5,000 spores/mL was prepared and from that suspension, 10 µL was taken and added to a plate with fresh YMG medium yielding 50 spores/plate. The spores were distributed all over the plate using a sterile bacterial spreader. After 48-72 hours of incubation at room temperature, it was possible to observed the mycelium by eye and the number of germinating spores could be determined. The germination (G) was calculated from the equation below.

$$G = \frac{N_G}{N_t} * 100\%$$

N_G is the number of germinating spores, and N_t is the total number of spores added to the plate.

The Gaussian error propagation was used to determine the germination error by combining the standard deviation of the germination and the error connected to the Neubauer counting method, which was assumed to be 0.3[3, 4].

4.6. Toxicity test

To access if the polymer solutions had any toxicity to the *Trichoderma* strains, a toxicity experiment was conducted. Fresh *Trichoderma* spores (TRS123, TRS14, and TRS75 in separate experiments) were grown and harvested as described in section 4.2. Samples of 500,000 spores/mL were prepared in either pure water (control), 0.2wt% cationic lignin, or 0.2wt% lignosulfonate and left for 2 hours. Afterwards, the samples were subjected to a germination test as described in section 4.5. The results of the germination test for all three strains are shown **Figure S1**. Independent on the treatment, the germination is similar indicating no toxicity of the polymer solutions.

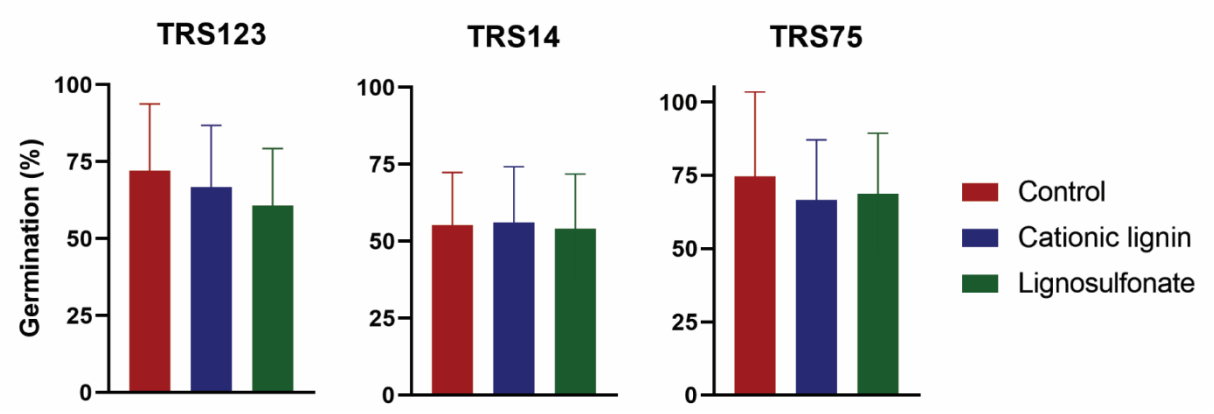


Figure S1: Germination test of the three Trichoderma strains (TRS123, TRS14, and TRS75) after treatment with either no treatment (red), 0.2wt% cationic lignin (blue), or 0.2wt% lignosulfonate (green) for 2 hours.

4.7. Layer-by-layer encapsulation *Trichoderma* spores

The layer-by-layer encapsulation was performed in a similar way as described previously[3] and all three strains were treated in the same manner. A suspension of *Trichoderma* spores (1 mL) with a concentration of 50,000,000 to 500,000,000 spores/mL was prepared in a sterile 1.5 mL reaction tube. The spore suspension was centrifuged, the supernatant was removed and resuspended in 1 mL of sterile water by vortexing for 15 seconds. This washing cycle was repeated one more time, but the spores were only resuspended in 500 µL of pure water. After resuspension, 500 µL of the polycation solution (0.2 wt% of cationic lignin in sterile water) was added to the reaction tube and vortexed for 15 seconds. The spore suspension was left for 10-15 minutes to ensure absorption of the polycation

to the negatively charged surface of the spores. Afterwards, the suspension was centrifuged and the supernatant was removed. The spores were once again suspended in 500 µL sterile water followed by addition of 500 µL of the polyanion solution (0.2 wt% of lignosulfonate in sterile water). The spores were vortexed for 15 seconds and left for 10-15 minutes for the polyanions to absorb. The suspension was centrifuged and the supernatant removed. The process was repeated until reaching the wanted number of layers and after the addition of each layer, the zeta potential of the sample was measured to ensure the successful addition of the polyions. When all the polymer layers were added, the spores were washed 3 times in sterile water to remove any leftover polymer. SEM samples were prepared right away and afterwards the layered spores were stored in 0.85% saline solution in the fridge. A control sample without layers was always prepared the same except that sterile water was added to the samples instead of the polyions.

4.8. Zeta potential measurements

The zeta potential measurements were used to ensure that the polyions were successfully added to the surface of the spores. Therefore, the zeta potential was measured after the addition of each layer. The spores without any layers were also measured. The optimal concentration of the spore suspension for measuring zeta potential was 10,000,000 spores/mL in pure water. The measurements were performed on Malvern Zetasizer Advanced Range Pro and were performed in triplicates. The result of the measurements are shown in **Figure S2** and **Figure 1C**.

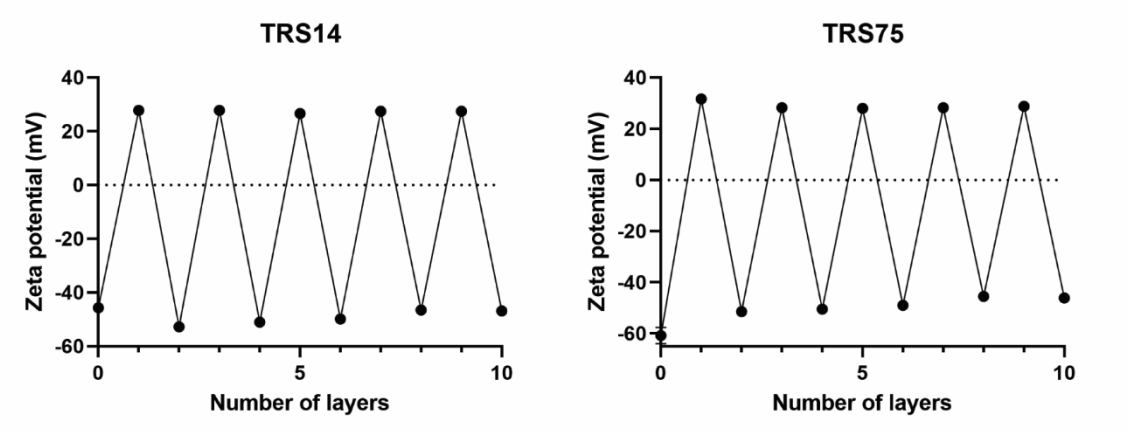


Figure S2: Zeta potential (mV) of Trichoderma strains TRS14 and TRS75 with 0-10 layers of lignin polymer (results of TRS123 can be found in Figure 1C in main text).

4.9. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on a JSM 7610 FPlus by JEOL. The samples were prepared by taking 10 µL of a spore suspension of around 20,000,000 spores/mL in pure water and add it to a silicon wafer. The samples were dried in a vacuum oven overnight. The results of the SEM measurements can be found in **Figure S3** and **Figure1E-F**.

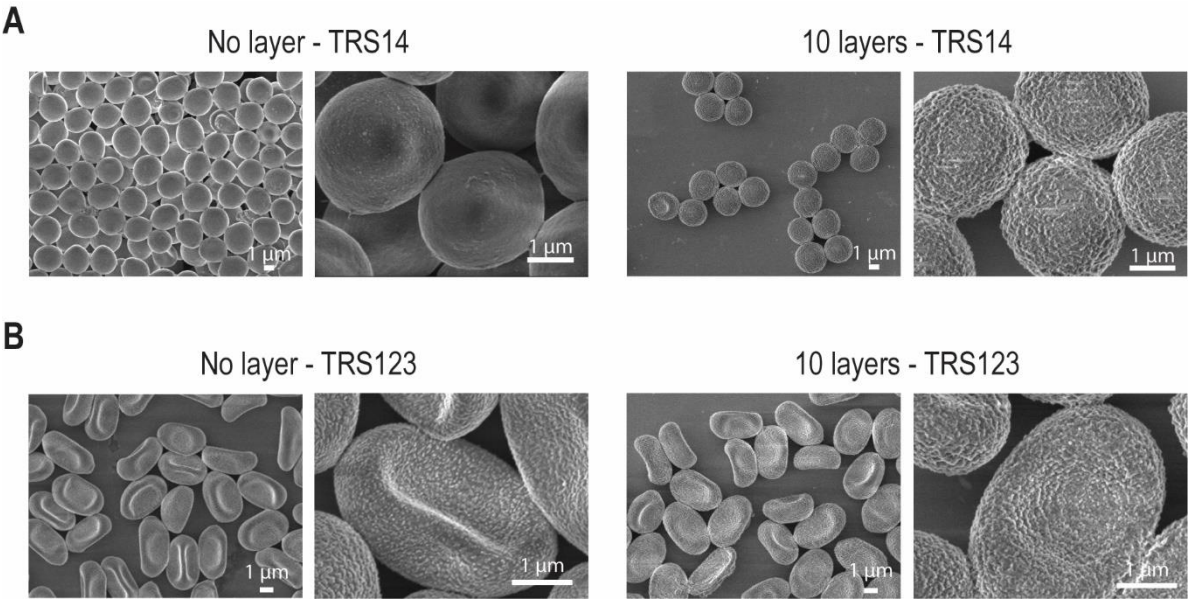


Figure S3: Scanning electron microscopy of A) TRS14 and B) TRS123 with and without polymer layers.

4.10. Stability of encapsulated *Trichoderma* spores to physical stimuli

All the stability studies were performed in a similar manner but varied in the type of treatment the spores were exposed to. The specifications of each treatment can be found in section **4.10.1-4.10.4**. In a standard experiment samples of *Trichoderma* spores without polymer layers and with 10 polymer layers in pure water were used. The number of spores in both samples were quantified as described in **4.5** and the samples were diluted to contain 5,000 spores/mL. The samples were divided into two where one was used as a none-treated control while the other sample was treated. After treatment the samples were subjected to a germination test and the none-treated controls (both with and without layers) were set to 100% viable and the treated samples were compared directly to them to determine the viability after treatment. Because the control and treated sample originated from the same sample, it was possible to exclude the counting error. The germination test was performed in triplicates (n=3) and the experiment was performed minimum 3 times on different days (N≥3).

4.10.1. Heating

The samples were prepared in closed reaction tubes and were stored in the oven at 50°C for 2 hours. Afterwards, the samples were allowed to cool to room temperature before the viability was determined as described in **4.10**.

4.10.2. Freezing

The samples were prepared in closed reaction tubes and were stored in the freezer at -20°C for 2 hours. After treatment, the samples were allowed to heat to room temperature before the viability was determined as described in **4.10**.

4.10.3. UVB light treatment

Instead of only dividing each samples (with and without layers) into two, the samples were divided into five to be able to treat them five different way. 1) no treatment, 2-5) treatment with different energies of UVB light. The different energies were 2) 0.10 J/cm^2, 3) 0.25 J/cm^2, 4) 0.50 J/cm^2, and 5)

0.75 J/cm^2. The samples were prepared in a transparent 96-well plate and one treatment was performed at a time. The UVB light treatment was performed using a UVP Crosslinkers CL-1000 from Analytik Jena at 302 nm and 8 W. The viability after treatment was accessed by germination test as described in **4.10**. P-values for the statistical difference between spores with no layers and spores with 10 layers in connection to **Figure 2C** with treatment 2-5 were: 2) P = 0.0411 (*), 3) P = 0.0178 (*), 4) P = 0.0200 (*), 5) P = 0.0362 (*).

4.10.4. UVC light treatment

The samples were prepared in a transparent 96-well plate and were exposed to UVC light for 3 minutes. UV sterilizer PHY-6UVS-1 was used for the experiment producing 6 W at 235 nm wavelength. After the treatment, the viability of the samples was established by a germination test as described in **4.10**.

4.11. Stability of encapsulated *Trichoderma* spores to long-time storage

The *Trichoderma* strain TRS14 was encapsulated by layer-by-layer as described in section **4.7**. Samples of spores either with no lignin layers (naked spores) or with 10 layers of lignin were prepared in 0.85% saline solution and stored in the dark at room temperature for up to 9 months. After storage the stability of the spores was determined by a germination test, which was performed as described in **4.5**. The results of the germination test after 2 months are shown in **Figure S4** and after 9 months in **Figure 2E** in the main text. The germination test after 9 months was performed in triplicates (n=3) and reproduced three time on different days (N=3).

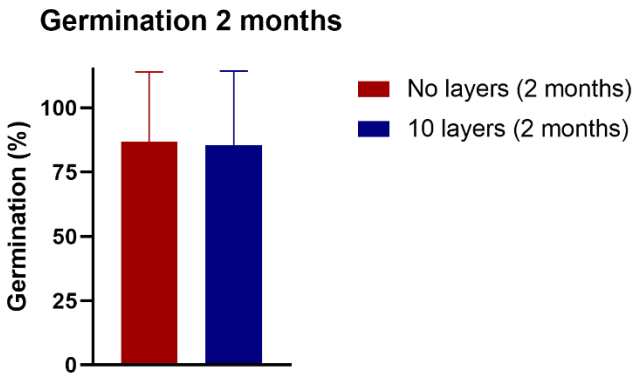


Figure S4: Germination test of TRS14 either without (red) or with (blue) lignin polymer encapsulation after 2 months of storage in saline solution at room temperature.

4.12. Examination of encapsulated *Trichoderma* spores in greenhouse tests with tomato plants

4.12.1. Inoculation of growing medium

The EasyGrowing Klasmann TS1 peat growing medium (PH 5.8) was used for greenhouse experiments with encapsulated *Trichoderma* spores. Two weeks before use, the growing medium was infested with the strain of *Fusarium oxysporum* f.sp. *lycopersici* FOL (the causal agent of tomato fusarium wilt) and inoculated with the spores of selected *Trichoderma* fungi. To prepare inoculum of *Fusarium*, the oat flakes in glass Petri plates (90 mm) were sterilized twice in an autoclave at 120 ^{o}C, and inoculated with 5 mm mycelial disc of 7 days old FOL, cultured on PDA medium. After two weeks of incubation at 24 ^{o}C the content of the plates was homogenized in sterilized water, in a dose of one plate in 150 ml of water. The slurry was mixed with peat substrate in a ratio one plate per 1 L of the substrate. The mixture was stored in a plastic bag at 18 – 22 ^{o}C for 10 – 14 days, and then used for infestation of TS1 growing medium at a ratio 1 : 9 vol. Immediately after infestation of TS1 growing medium with *Fusarium*, the medium was divided into separate containers and inoculated with encapsulated or fresh, not capsulated spores of *Trichoderma* strains TRS14, TRS75 and TRS123. All spores were suspended in 0.85% saline solution. The inoculum concentration was 10^4 spores g^{-1} of TS1 medium. Two kg of inoculated medium was prepared for each treatment. The medium not inoculated with

Fusarium and *Trichoderma* (C), and medium infested only with *Fusarium* (F) were prepared as controls.

4.12.2. Greenhouse experiments

All batches of growing medium were incubated in the containers for additional two weeks at 18 – 22 ^{o}C. After incubation with *Fusarium* and *Trichoderma*, the growing media from each treatment were divided into 10 plastic pots and planted with the transplants of tomato cv Remiz F_1. Immediately after transplanting to the pots, each transplant height was measured and plants were marked with consecutive numbers. This measurement was then used to determine the effect of *Trichoderma* inoculation on plants increment (cm) according to equation $IN = H_F – H_B$ (IN plant increment, H_F final height of tomato plants; H_B initial height of the seedling).

The plants were kept in a greenhouse until clear symptoms of fusarium wilt develop. Then the plants were cut at the stem base and their height and fresh weight were measured. The stem base of each plant was then cut lengthwise to determine the degree of fusarium wilt infection of the vascular system. The infection was estimated according to the 0 – 4 index, where: 0 – no fusarium wilt symptoms, 1 – browning of 25% vessels; 2 - browning of 50% vessels; 3 - browning of 75% vessels; 4 - browning of 100% vessels. Tomato roots were carefully extracted from the growing medium and used for microbial analyse to determine rhizosphere colonization by *Trichoderma* and *Fusarium*. The experiment was repeated three times at the same conditions.

4.12.3. Microbial analyses

In each experiment, microbiological analyses were carried out on: (i) the samples of growing substrates contaminated with FOL and inoculated with Trichoderma strains, after a two-week incubation, before tomato transplanting; (ii) the rhizosphere of tomatoes grown in these media, at the end of the experiment. Ten gram of the substrate or the roots carefully shaken from the loosely

adhering substrate, were suspended in 100 ml of 0.85% water saline solution, and shaken for 30 min using rotary shaker. Next, from the obtained suspensions, the decimal dilutions were prepared with the same diluent, and used for standard serial dilution plating method, to determine Trichoderma and Fusarium numbers. The quantity of *Trichoderma* spp. was evaluated on peptone-rose bengal agar and Fusarium spp. on Komada's medium[5]. The colonies of Trichoderma spp. were counted after 7 days and Fusarium after 14 days of incubation at 25 °C. The results were expressed as colony-forming units per gram of dry weight of the substrate or gram of the roots. Each treatment was performed in triplicate.

4.13. Statistical analysis

All experiments with statistics were performed in triplicates (n=3) and repeated minimum three times on individual days (N=3). For statistical analysis the sample sizes of N=3 or N=4 were used and the statistical significance was calculated from a t-test or a two-way ANOVA analysis using GraphPad Prism. The results were assumed statistically significant (*) with $P < 0.05$. Generally, $P > 0.05$ (ns), $0.01 < P < 0.05$ (*), and $0.001 < P < 0.01$ (**).

For greenhouse experiments and microbial analyses the statistical significance was calculated using Duncan test with $P < 0.05$, and the calculations were carried out in the statistical package STATISTICA 13 (Dell Inc.).

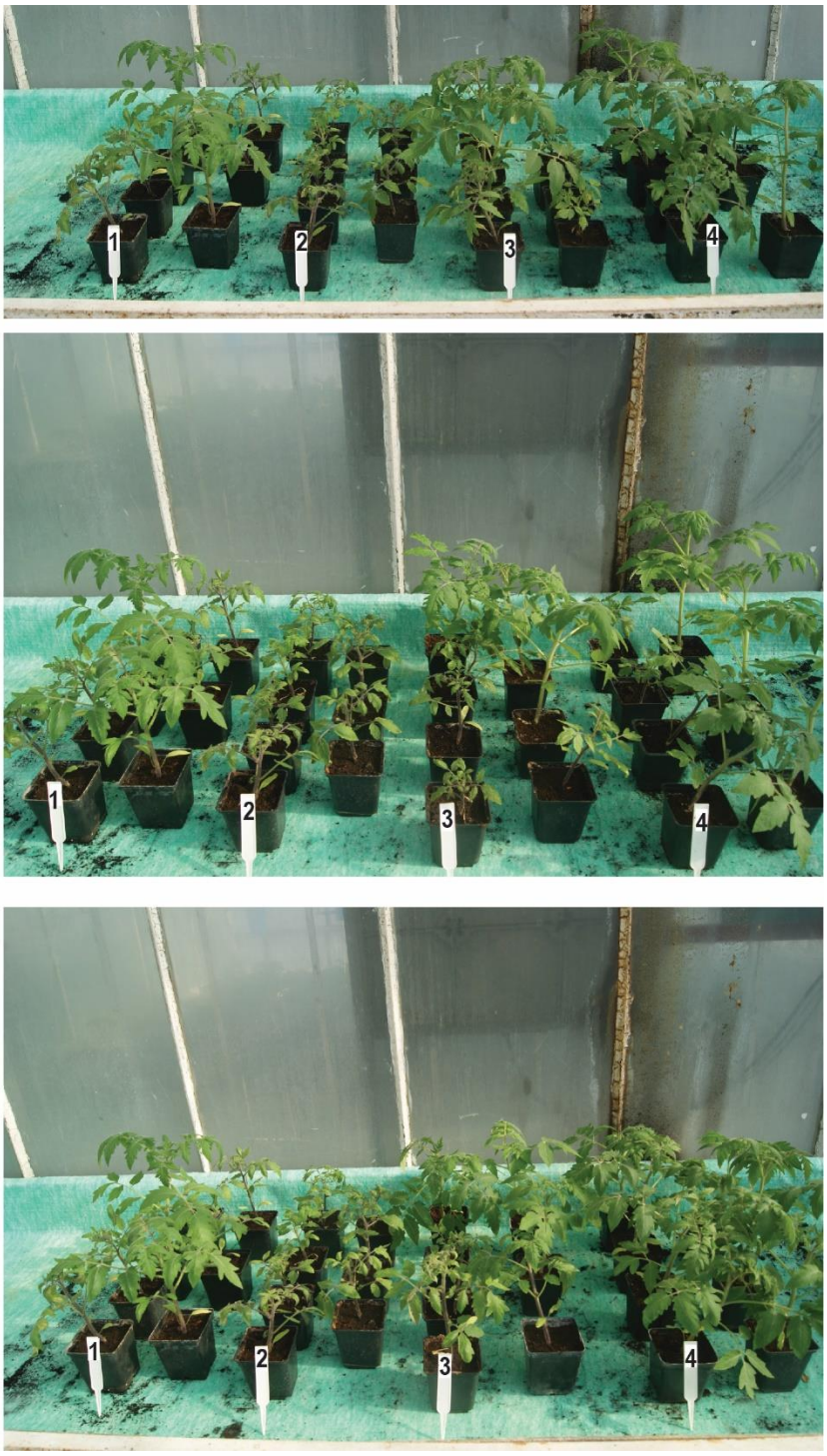


Figure S5: Photographs of the plant experiments showing the tomato plants after incubation with the pathogen and the spores: 1 – control without Fusarium; 2 – Fusarium; 3 – spores and substrate with Fusarium; 5 – encapsulated spores and substrate with Fusarium. A) Strain TRS14; B) Strain TRS75; C) Strain TRS123.

Additional References

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2. Kong, F., et al., *Preparation of cationic softwood kraft lignin and its application in dye removal.* European Polymer Journal, 2015. **67**: p. 335-345.
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4. Freund, M. and B. Carol, *FACTORS AFFECTING HAEMOCYTOMETER COUNTS OF SPERM CONCENTRATION IN HUMAN SEMEN.* Reproduction, 1964. **8**(2): p. 149-155.
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