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A novel chitosan biopolymer based *Trichoderma* delivery system: Storage stability, persistence and bio efficacy against seed and soil borne diseases of oilseed crops

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ABSTRACT

Management of seed and soil borne fungal plant pathogens using fungal species belonging to the genus *Trichoderma* is gaining importance. Seed coating with powder based formulations of *Trichoderma* is most widely adopted by the researchers and farmers as well. Delivery system that leads to good adherence of fungal propagules on seed surface, minimizing the wastage of active ingredient, sustained and timely release during treatment process is very important for effective season long protection. Chitosan-PEG (Polyethylene glycol) (Cts-PEG) blend containing *Trichoderma harzianum* (Th4d) (Cts-PEG-Th) spores is developed and its storage stability, persistence in soil and bio efficacy against seed and soil borne pathogens of groundnut and safflower crops is studied. The blend was stable without much changes in pH throughout the storage period. Persistence studies conducted for 3 months revealed that Cts-PEG-Th amended soil, *Trichoderma* got released from polymer film slowly and reached a maximum of log 8 CFUs by 30 days and there after started declining to retain log 6 CFUs at 90 days. In shelf life study, the chitosan blend was able to maintain *Trichoderma* counts of log 10.0 and log 10.2 over a period of 6 months at storage temperatures of 30 °C and 4 °C, respectively and the antagonistic activity unaffected against three plant pathogens viz. *Macrophomina phaseolina*, *Fusarium oxysporum*f. sp.*ricini* and *Aspergillus niger* over a period of 6 months of storage. Bio efficacy testing in germination towels and green house pot studies revealed the effectiveness of seed treatment with Cts-PEG-Th blend significantly increasing the germination and seedling vigour and reducing the diseases in groundnut (peanut) and safflower.

1. Introduction

Biotic stresses caused by organisms like bacteria, fungi, viruses, parasites, weeds and insects can inflict yield losses in crops. Among all, fungi have devastating effects on yield of crops, hence there is a need to control such damages otherwise, this could lead to economic losses. Use of chemical protectants against variety of biotic stresses has been the main weapon for decades but, using eco-friendly alternatives like use of bio control agents is gaining importance worldwide in order to minimize hazardous effects of chemicals on environment, humans and animals. Many species of *Trichoderma* have been exploited as potent bio control agents (Mastouri et al., 2010; Ahanger et al., 2014), bio stimulants (Fernando et al., 2018; López-bucio et al., 2015), endophytic root colonizers and for plant growth promotion (Singh et al., 2010; Singh et al., 2014). *Trichoderma* also known to produce volatile and non-volatile secondary metabolite which affects the growth of other fungi. Due to their antagonistic activity against wide range of fungi, *Trichoderma* can be employed in management of array of diseases caused on root, stem and leaves of crop plants (Reddy et al., 2014; Woo et al., 2014).

Delivery of *Trichoderma* to the target point in the form of formulations with suitable carriers is adopted by many researchers and industries for commercial use. This kind of approach results in reducing the wastage of bio control agent, proper adherence to treated surfaces and tenable biological control activity (Swaminathan et al., 2015). Sustained and timely release of bio control agent also can be achieved with formulated products for efficient functioning. Inert carriers like talc, bentonite clay, kaolin for powder based formulations and vegetable oils and mineral oils for liquid based formulations are generally used (Prasad & Rangeshwaran, 2000). Natural polymer like chitosan which is antimicrobial in nature used as plant defense inducers, growth promoter and in management of plant pathogens can be exploited as

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better carrier for delivery systems of bio control agents (Raphaël & Meimandipour, 2017; Rakesh et al., 2017a,b; Mohamed et al., 2016).

Different methods of application of *Trichoderma* include seed treatment, soil amendment, and foliar spraying etc. Seed treatment helps the seed in combating soil ecological impacts and protects from seed and soil borne pathogens, early emergence and helps in establishment of healthy plants (Callan et al., 1997). Chitosan was reported as an antifungal compound against post-harvest horticultural diseases in horticultural crops caused by fungal pathogens *viz.*, *Fusarium sulphureum* (Yong-Cai et al., 2009), *Alternaria alternata*, *Aspergillus niger* and *Rhizopus oryzae* (Zhang et al., 2003; Sebti et al., 2005;Bhaskara Reddy et al., 1998). In an approach, combination of *Trichoderma harzianum* and chitosan were used for the control of sapstain fungi (Chittenden & Singh 2009). Chitin/chitosan supplemented formulations proved to be effective against several phytopathogens besides significantly increasing the plant growth (Manjula & Podile, 2005). Chitin supplemented peat formulation of *Bacillus subtilis* AF1 was very effective in improving growth of groundnut and pigeon pea (Manjula & Podile 2001; Manjula & Podile 2005). Using combination of chitosan in combination with *Trichoderma* for seed coating can lead to a better disease management apart from enhancement in plant vigour (Rakesh et al., 2017a,b). Apart from antimicrobial nature, the film forming nature of chitosan can also give a physical protection to seed. The permeable property of film allows optimum vapour and gas exchange for healthy germination resulting in better plant stand. Though film formation on seed surfaces by chitosan, protecting seed and bio agents from stresses reported (Fernandez-Saiz et al., 2009), our experience shows formation of a non-uniform brittle layer rather than formation of a continuous film coat on seeds which was overcome by a combination of a plasticizer and cross linker (Chandrika et al. 2019). For effective use of any formulation, the physical, chemical and biological stability during storage period and conditions of storage plays a vital role (John & Jeeva, 2014). The viability of potent strain in formulation and a suitable delivery system are important factors for effective management of plant diseases.

The objective of this study was to evaluate the storage stability of the chitosan-PEG (Cts-PEG) blend containing *Trichoderma harzianum* (Th4d) (Cts-PEG-Th) spores, its persistence and bio efficacy against seed- and soil-borne pathogens of groundnut and safflower crops in growth chamber and greenhouse conditions.

2. Materials and Methods

2.1. Preparation of Cts-PEG-Th blend

Detailed synthesis method of chitosan-PEG (Cts-PEG) blend containing *Trichoderma harzianum* (Th4d) (Cts-PEG-Th) spores is as per previous published work (Chandrika et al., 2019). Briefly the Cts and PEG (1:0.33% w/v) was prepared by dissolving in distilled water under acidic conditions (0.1% glacial acetic acid). Glycerol (Gly of 0.66% w/v) plasticizer was added to the above liquid and was kept on magnetic stirrer overnight at 60–80 °C and 300 rpm. The blended system was utilized for *Trichoderma* dry conidial spores impregnation @ 1% w/v (10^{10} CFUs).

2.2. Entrapment efficiency and loading capacity

The loading capacity (LC) and entrapment efficiency (EE) of Cts-PEG-Th was determined by the method described by Lotfipour et al. (2012). The colony forming units (CFUs) of *Trichoderma* was determined and extracted from the Cts-PEG blend by serial dilution method and pour plate technique onto TSM -*Trichoderma* Selective Medium (Elad et al., 1981). Serial dilution was done after complete dissolving of Cts-PEG-Th (1% w/v) in autoclaved distill water by vortexing for 30 minutes. The process was done in triplicates. The EE and LC (%) was calculated by the following equation.

Table 1
Properties of soil used for persistence studies.

pH	Moderately alkaline
Sand (%)	73.75
Silt (%)	6.75
Clay (%)	19.50
Type of soil	Sandy loam
Field capacity(FC)	26.5 %
Permanent wilting point (PWP)	12.3%
Organic carbon	0.5 %

$$\text{Entrapment efficiency (EE)} = (\text{Log10 N}/\text{Log10 No}) \times 100$$

Where N is the number of entrapped viable fungal spores and No displays the free viable fungal spores before entrapment.

$$\text{Loading capacity LC (\%)} = \log 10\ \text{N}/\text{amount of carrier used}$$

2.3. Survival and persistence studies of T. harzianum in Cts-PEG-Th blend

To study the persistence of *T. harzianum* (Th4d spores) in soil after entrapment in Cts-PEG films (0.1 % - 0.1 g film / 100 g soil) and without entrapped spores (0.05% - 0.05 g dry conidial spores / 100 g soil) the film was properly mixed with sterile soil and packed in a closed HDPE container. Natural field soil used in the study was collected from farm of ICAR-Indian Institute of Oilseeds Research, Hyderabad and autoclaved to remove any unwanted microbes (Table 1). The containers were placed at room temperature for 90 days. One gram of soil was drawn at intervals of 0, 15, 30, 60 and 90 days for enumeration of *Trichoderma* spores in that given conditions. The samples were serially diluted and plated on *Trichoderma* selective medium and number of colony forming units on medium were counted and represented as CFU/gm. of soil. The fungal colony counts obtained were the average of 3 replications. Pure dry *Trichoderma* (Th4d) conidial spores without carrier and untreated soil served as controls in the study.

2.4. Storage stability of Cts-PEG-Th blend

Viability of *Trichoderma* spores in Cts-PEG blend stored in HDPE bottles at 2 different temperatures viz., 4 °C and 27 ± 2° was studied at intervals of 30 days for 6 months. Sample (1 ml) was taken for serial dilution followed by plating on *Trichoderma* selective medium (TSM) and incubated at 25 ± 2 °C. Experiment was carried out in a completely randamosized design (CRD) with 3 replications for each dilution used. Colony forming unit (CFU) was calculated by using the formula.

$$CFU = \frac{Avg.\ number\ of\ colonies \times Dilution\ factor}{weight\ of\ the\ sample \times Aliquote\ taken}$$

Properties like pH were evaluated at intervals of 30 days for 6 months. The container with blend was properly shaken to confirm uniform dispersion of spores in liquid and pH was recorded. Five replicates were maintained for each experiment.

2.5. Antagonistic activity of Trichoderma in Cts-PEG-Th blend

Antagonistic ability of *Trichoderma* in Cts-PEG blend was evaluated at interval of 30 days for 6 months by growing *Trichoderma* from Cts-PEG-Th blend on agar medium and actively growing culture is used for testing antagonistic activity. The antagonistic activity of *Trichoderma* was tested against plant pathogens like *Macrophomina phaseolina*, *Fusarium oxysporum* and *Aspergillus niger* by dual culture technique (Skidmore & Dickinson, 1976) where a disc of *Trichoderma* and test pathogen are paced in a potato dextrose agar plates at opposite periphery of same petriplate. After incubation at 25 °C for 7 days, radial growth of the pathogens in control plate and plate containing *Trichoderma* was recorded and percentage inhibition (PIGR) was calculated by

using the formula developed by Skidmore and Dickinson (1976)

$$PIGR(\%) = \frac{R1 - R2}{R1} \times 100$$

2.6. Bio efficacy of Cts-PEG-Th blend

2.6.1. Seed material and pathogens

Safflower (*var*. A1) and groundnut (*var*. kadiri-6) which were used in the study was obtained from Seed Unit of ICAR-Indian Institute of Oilseeds Research (IIOR), Hyderabad, India. Pathogens used in the study include *Macrophomina phaseolina* and *Aspergillus niger* and cultures were obtained from culture collection of pathology laboratory, ICAR-IIOR.

2.6.2. Seed coating with Cts-PEG-Th blend

Ten millilitres of Cts-PEG-Th blend after dilution with water was used for treating 1 kg seed. Required amount of liquid was poured on the seeds and uniform coating was achieved by manual mixing and dried in shade. Commercial synthetic seed coating polymer under trade name of super bond (M/s Reliable Corporation, Chennai, India) was procured from market and used as control. Ten ml of commercial synthetic polymer in 100 ml of water was used after adding with dry *Trichoderma* conidial spores as it was added to the blend @ 1% w/v (10^{10} CFUs) for treating 1 kg of seed.

2.6.2.1. Screening in germination towels. Seeds of safflower were first treated with the micro sclerotia suspension (conc. 10^3 /ml) of *Macrophomina phaseolina* cultured on potato dextrose agar (PDA) for a period of 10-days. Groundnut seeds were treated with spore suspension of *Aspergillus niger* containing 10^4 spores /ml.The treated seeds were air dried for a period of 5 hr. Seeds thus treated with different pathogens separately were used for seed coating with the treatments to be evaluated. The experiment was carried out in a complete randomized design (CRD) containing 8 treatments (Table 5) with 5 replications of each treatment for each crop. Twenty seeds were placed on wet germination towels then rolled as a bundle and placed in growth chamber at 25 °C, 70% humidity and 10 hr. of light. Moisture was maintained in germination towels by spraying sterile water at regular intervals. After 10 days, parameters like germination percentage, root length, shoot length, plant dry weight were recorded. Seedling vigour was calculated by multiplying germination percentage and seedling length (sum of root and shoot length) and the disease incidence was calculated by formula, disease incidence = No. of plants with root rot symptoms/ Total no. of plants.

2.6.2.2. Greenhouse experiment

2.6.2.2.1. Preparation of pathogen and soil infestation. Sorghum grains were washed thoroughly, soaked in water for 3 hr and autoclaved in autoclavable polythene bags. Sterilized sorghum grains in bags were aseptically inoculated with 5 mm discs of pathogen culture from 7-day-old culture plate separately and incubated for 7 days at 25 °C or till the sorghum grains were completely covered with the pathogen mycelium. Plants raised in soil in pots without pathogen served as control. Pathogens grown on sorghum grains was properly mixed with sterile soil at concentration of 10 g of sorghum grain inoculum of pathogen/ kg soil and 3 kg of soil was filled in each pot.

2.6.2.2.2. Evaluation of Cts-PEG-Th blend against soil borne pathogens. A green house pot study was conducted to evaluate the bio control efficacy of Cts-PEG-Th against Macrophomina root rot of safflower and Aspergillus collar rot of groundnut. The treatments include Cts-PEG-Th based blend, Cts-PEG alone (without Th4d), Th4d alone, synthetic seed polymer, synthetic seed polymer + Th4d and fungicide (Table 6). Groundnut and safflower seeds were coated separately. Ten seeds treated with Cts-PEG-Th blend were sown in each pot and irrigated regularly to maintain sufficient moisture. Five replications of each treatment were maintained. Untreated seed sown in pots without pathogen served as control. Plant stand and disease incidence was recorded at regular intervals for a duration of 50 days.

2.7. Statistical analysis

Data was subjected to analysis of variance (ANOVA) and data in per cent were arcsine transformed before analysis using SPSS software 16.0 version. The treatment means were compared by Tukey's HSD test critical difference (p = 0.05).

3. Results and discussions

3.1. Entrapment and loading

The ability of the carrier to entrap the bio control agent is the determining factor for development of a bio control agents based delivery system. Study exhibited almost 87 % of the entrapment efficiency of *T. harzianum* spores, indicative of better spore entrapping ability which could be attributed to biocompatibility and excellent tolerability of the chitosan. The loading capacity of *T. harzianum* spores expressed as the number of spores per 1 g of dry carrier is 1×10^{10} CFUs. This can be explained in terms of network properties of Cts-PEG blend system which makes it suitable to impregnate sufficient number of spores. Haghshenas et al. (2015), Marín et al. (2016), Vinceković et al. (2017) reported entraping the biological control agents in different biopolymeric matrix for enhanced survival and efficacy in disease control.

3.2. Survival and persistence studies of T. harzianum

The study monitored persistence of number of spores of *Trichoderma* in Cts-PEG film after application to sterile soil in the absence of plants over 3 months period (Table 2). Pure conidial powder of *Trichoderma* added to sterile soil and sterile soil alone served as a control. The samples was drawn from various treatments at different periods are 0, 15, 30, 60 and 90 days after application (DAA) to soil. The initial colony

Table 2
Persistence of Th4d in Cts-PEG-Th blended film in soil.

Duration	Persistence of *Trichoderma* spores				
	1 day	7th day	15th day	30th day	60th day	90th day
T_1-Control (untreated)	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
T_2- *Trichoderma* Th4d spores	6.3^{a}	6.5^{a}	6.8^{a}	6.0^{b}	5.2^{c}	4.0^{d}
T_3-Chitosan + *Trichoderma* Th4d film	1.2^{e}	3.8^{d}	7.5^{b}	8.0^{a}	7.1^{b}	6.0^{c}
SEm ±	0.01	0.03	0.1	0.0	0.01	0.04
C.D (p = 0.05)	0.2	0.1	0.4	0.0	0.05	0.1
C.V (%)	2.6	3.2	3.9	0.02	0.7	2.5

The values (Log CFU's) indicated are means of 3 replications (average no. of colonies x dilution). Mean values within a row followed by the same letter at different time intervals are not significantly different by Tukey's HSD (P ≤ 0.05).

forming units (CFU) (1 DAA) in pure conidial powder of *Trichoderma* treated soil are about 6.0 log CFUs per gram of soil. Similarly the Cts-PEG-Th blend has 1.2 log CFUs/ gm. released into soil after on 1 DAA as *Trichoderma* will be slowly released from chitosan film into soil and showed 7.5 log CFUs/ gm. of soil on 15 DAA. The soil treated with *Trichoderma* spores alone (T_2) and Cts-PEG-Th films (T_3) at 1, 7, 15, 30, 60 and 90 days in soil along with absolute control was given in Table 2. In soil treated with pure *Trichoderma* spores alone the population declined gradually and in case of Cts-PEG-Th film applied soil there was a slight increase in population of *Trichoderma* (log 8.0) till 30 days and started declining thereafter. According to Baker and Cook (1974), bio control agent must establish and proliferate in the area of application for obtaining effective results. In present study *Trichoderma* remained in higher numbers though there was a slow decline in population which is anticipated in the absence of plant root and exudates the association which is known to support proliferation of rhizosphere microflora. Presence of plant roots favours the growth and proliferation of rhizosphere competent *Trichoderma*, which they colonize readily (Harman and Kubicek, 1998). In soil applied with Cts-PEG film containing *Trichoderma*, there was a slight increase in *Trichoderma* population due to chitosan film which will serve as nutrient source to *Trichoderma* upon degradation by hydrolytic enzymes secreted by the biological control agent. A decline in the CFUs was observed over a period of 3 months in all the treatments which could be due to the disintegration, degradation and lysis of spores, which is in agreement with findings of Lewis and Papavizas (1984). The persistence of viable propagules of *Trichoderma* in good numbers in soil when applied as Cts-PEG-Th film may benefit crops during significant part of their growth, offering protection against soil borne pathogens.

3.3. Storage stability of Cts-PEG-Th blend

The population count (CFU) was found to be high in the Cts-PEG-Th blend for 6 months period (Table 3). The viability of Th4d spores stored at two temperatures i.e. at 30 °C and 4 °C has not come down significantly. At 4 °C, Cts-PEG-Th blend retained more viable propagules (10 log CFU) compared to blend stored at 30 °C which has retained 9.5 log CFU after a storage period of 6 months. Maintaining the viability of a bio control agent in a carrier material throughout storage period is very essential factor for its efficient functioning after application in the field. Decline in viable colonies of *Trichoderma* from 10.7 log CFU to 9.5 log CFU at room temperature is very minimal and signifies the efficiency of Cts-PEG-Th blend in supporting viability of the bio agent. Many studies reported the decline of CFU over a period of time which can be attributed to desiccation, disintegration and depleting nutrients and other storage and environmental factors (Prasad and Rangeshwaran, 2000). The optimization of carrier to be used in formulation is critical to deliver extended shelf life of the antagonist (Rodham et al., 1999). The success of the bio formulation depends not only on the microbial load and its survival mechanism adopted by it but also on compatible carriers used in development of formulation, where as non-compatible carriers can adversely affect the efficacy of bio control agent apart from formulations storage stability. In present study biopolymer chitosan is used as an efficient carrier source as well as a nutrient source to bio agent which was evident from slight increase in viable counts of bio agent for few days as well as an antimicrobial agent upon its disintegration in soils. Similar observations were made by Chittenden and Singh (2009) who have reported that the chitosan used stimulated *T. harzianum* growth. Improvement in bio control potential and plant growth promoting efficacy of *Bacillus subtilis* AF1 in formulations supplemented with chitin has been reported (Manjula et al., 2004).

The minimal pH change from 4.3 to 4.9 of the Cts-PEG-Th blend (Table 3) was observed over the of period of 6 months resulting in the stability of the of product and maintaining viability of *Trichoderma* spores.

Table 3
Storage stability of *Trichoderma harzianum* Th4d in Cts-PEG-Th blend.

Duration	Log CFUs		pH of formulation
	30 ± 2°C	4 ± 2°C	
0 months	10.7^{a}	10.7^{a}	4.3
1 month	10.5^{b}	10.6^{b}	4.5
2 months	10.3^{c}	10.6^{c}	4.5
3 months	10.1^{d}	10.5^{d}	4.7
4 months	10.0^{e}	10.3^{de}	4.7
5 months	9.8^{f}	10.1^{ef}	4.8
6 months	9.5^{g}	10.0^{f}	4.9
SEm ±	0.51	0.33	
C.D.(p = 0.05)	1.6	1.09	
C.V.(%)	1.13	0.62	

Spore viability (Log CFU's) of Chitosan based formulation of Th4d stored at room temperature (30 ± 2 °C) and at 4 °C in HDPE bottles. The values indicated are means of 3 replications (average no. of colonies x dilution). Mean values within a column followed by the same letter at different time interval are not significantly different by Tukey's HSD ($P \leq 0.05$).

3.4. Antagonistic activity of Trichoderma in Cts-PEG-Th blend

Retention of intended biological activity of stored bio formulation throughout storage period is essential for its optimum performance during field usage. A study was conducted to assess the antagonist activity of *Trichoderma* retrieved from different storage periods. *Trichoderma* Th4d was cultured from Cts-PEG-Th blend stored at room temperature at different storage periods and used for testing against pathogens. A significant mycelial growth inhibition (PIRG %) of three plant pathogens *viz. Macrophomina phaseolina, Fusarium oxysporum* f. sp. *ricini* and *Aspergillus niger* was observed over a period of 6 months of storage (Table 4, Fig. 1). The inhibitory potential of the *Trichoderma* did not get affected significantly due to entrapment in Cts-PEG polymer blend.

3.5. Bio efficacy of Cts-PEG-Th blend

3.5.1. In vitro screening

Cts-PEG-Th based seed treatment in groundnut resulted in 100 % germination of seeds whereas untreated check recorded a germination of 90 % and pathogen treated seeds have shown a germination of 66.6% (Table 5). Significantly low collar rot disease incidence (23.3%) compared to pathogen check (59.6%) was also recorded in the same treatment (Fig. 2). Among different treatments, fungicide treatment resulted in very low disease incidence of 13.3%. All the treatments have shown significantly high vigour index compared to pathogen check. Cts-PEG-Th seed treatment resulted in highest vigour index (3280) whereas *Trichoderma* Th4d alone treatment resulted in a vigour index of 3106. In safflower, significant increase in germination (96%) was observed with Cts-PEG-Th seed treatment over fungicide (86.6%), *Trichoderma* (90%) treatments and pathogen check (83.0%). There was a significant increase in vigour index (2037), root length (11.5 cm) and shoot length (9.4 cm) in Cts-PEG-Th treatment followed by fungicide treatment (Fig. 2). The root rot incidence was significantly low in Cts-PEG-Th treatment (3.3%) and fungicide treatment (6.6%) compared to all other treatments. In a similar study by Chittenden and Singh (2009), chitosan in combination with *T. harzianum* was found effective in controlling sapstain than chitosan or *T. harzianum* alone. Seed treatment with Cts-PEG-Th blend in both crops resulted in reduction in disease incidence indicating the bio efficacy of the blend and it was superior in terms of crop vigour enhancement and disease control in both the crops compared to synthetic seed coating polymer.

Table 4
Mean PIRG values of *Trichoderma* Th4d from Cts-PEG-Th blend against plant pathogens by dual culture method.

Pathogens	Growth inhibition (%)								
	0 month	1 month	2 month	3 month	4 month	5 month	6 month	SEm ±	C.D. (p = 0.05)	C.V (%)
Macrophomina phaseolina	78.4^{e}	80.5^{d}	81.4^{cd}	82.3^{bc}	83.6 ^{ab}	83.7^{ab}	84.9^{a}	0.5	2.4	1.7
Fusarium oxysporum f.sp. *ricini*	81.6^{e}	82.7^{de}	83.7^{cd}	84.5^{bc}	85.1^{bc}	85.4^{ab}	86.7^{a}	0.4	2.0	1.4
Aspergillus niger	73.1^{c}	73.2^{c}	74.0^{bc}	74.5^{bc}	75.1^{b}	77.1^{a}	78.6^{a}	0.5	2.2	1.6

Mean values within a row followed by the same letter for each pathogen are not significantly different by Tukey's HSD ($P \leq 0.05$).

Table 5
Evaluation of Cts-PEG-Th blend against Aspergillus collar rot in Groundnut (*var.* kadiri-9) and Macrophomina root rot in safflower (*var.* A1) in germination towel tests.

Treatments	Groundnut						Safflower					
	Germination (%)	Shoot length (cm)	Root Length (cm)	Vigour index	Dry Wt. (gms)	Disease Incidence (%)	Germination (%)	Shoot length (cm)	Root Length (cm)	Vigour index	Dry Wt. (gms)	Disease Incidence (%)
Untreated Control	90.0 (74.6)^{ab}	7.3 ^{ab}	22.6 ^{a}	2654 ^{a}	3.0 ^{a}	0.0 (0.9)^{f}	96.0 (78.7) ^{ab}	3.8 ^{c}	6.7 ^{b}	1008^{d}	0.22^{d}	0.0 (0.9)^{f}
Pathogen check	66.6 (50.7)^{c}	2.6 ^{c}	11.2^{b}	1170 ^{b}	1.8 ^{c}	59.6 (68.8) ^{a}	83.0 (65.6)^{e}	2.2 ^{c}	3.7 ^{c}	490^{h}	0.12^{e}	73.3 (59.0) ^{a}
Trichoderma (Th4d)	93.3 (80.5)^{ab}	8.9 ^{a}	24.5 ^{a}	3106 ^{a}	2.8 ^{ab}	30.0 (33.0)^{cd}	90.0 (71.5)^{cd}	5.9 ^{b}	7.2 ^{b}	1098^{c}	0.33 ^{ab}	16.0 (22.5) ^{c}
Cts-PEG	86.6 (72.4) ^{ab}	6.2 ^{ab}	22.4 ^{a}	2884 ^{a}	2.9 ^{a}	50.0 (45.0) ^{bc}	97.0 (72.5)^{cd}	3.2 ^{c}	6.6 ^{b}	910^{e}	0.31 ^{bc}	32.6 (34.8) ^{b}
Cts-PEG-Th (Th4d)	100.0 (89.0)^{a}	8.1 ^{ab}	24.7 ^{a}	3280 ^{a}	3.4 ^{a}	23.3 (28.0)^{de}	96.0 (80.1) ^{a}	9.4 ^{a}	11.5 ^{a}	2037^{a}	0.36 ^{a}	3.3 (6.7)^{ef}
Carboxin + Thiram 2.5 g/kg	93.3 (77.4) ^{ab}	5.4 ^{bc}	24.8 ^{a}	3043 ^{a}	2.9 ^{a}	13.3 (18.0)^{e}	86.6 (78.5)^{bc}	8.7 ^{a}	11.2 ^{a}	1910^{b}	0.3 ^{bc}	6.6 (12.5)^{de}
Commercial polymer	83.3 (66.1)^{bc}	4.7 ^{bc}	20.1 ^{a}	2217^{ab}	2.2 ^{ab}	53.3 (46.9) ^{b}	93.0 (68.6)^{de}	2.8 ^{c}	6.1 ^{b}	810^{g}	0.28^{c}	35.0 (36.1) ^{b}
Commercial polymer + *Trichoderma* (Th4d)	83.3 (69.7)^{bc}	5.1 ^{bc}	22.6 ^{a}	2509 ^{a}	2.9 ^{a}	20.0 (26.0)^{de}	96.0 (75.0)^{a}	2.4 ^{c}	7.1 ^{b}	893^{h}	0.33 ^{ab}	18.0 (24.4)^{cd}
SEm ±	5.3	0.9	2.2		0.2	6.7	1.5	0.7	0.7		0.01	3.4
C.D (p = 0.05)	16.2	2.8	6.6		0.6	13.5	4.2	2.0	2.2		0.03	9.9
C.V (%)	10.9	17.6	11.6		13.0	13.4	3.2	24.4	17.0		7.8	14.0

*Values in parentheses indicate angular transformed values of germination percentage
$P < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

Table 6
Green house evaluation of Cts-PEG-Th against Aspergillus collar rot in Groundnut (*var.* Kadiri-9) and Macrophomina root rot in safflower (*var.* A1).

Treatments	Groundnut		Safflower	
	Germination (%)	Disease incidence (%)	Germination (%)	Disease incidence (%)
Untreated Control	96.6 (83.2) ^{a}	0.0 (0.9)^{c}	86.6 (68.8)^{bc}	0.0 (0.9)^{d}
Pathogen check	66.6 (54.7) ^{b}	90.0 (74.6)^{b}	46.6 (43.0)^{d}	63.3 (52.7)^{a}
Trichoderma (Th4d) WP @10 g/kg	96.6 (83.2) ^{a}	0.0 (0.9)^{c}	93.3 (77.4)^{ab}	16.6 (23.8)^{b}
Cts-PEG	96.6 (83.2) ^{a}	24.4 (29.5)^{d}	90.0 (71.5)^{bc}	20.0 (26.5)^{b}
Cts-PEG-Th (Th4d)	100.0 (89.0) ^{a}	0.0 (0.9)^{c}	96.6 (83.2)^{a}	6.6 (12.5)^{c}
Carboxin + Thiram 2.5 g/kg	96.6 (83.2) ^{a}	13.3 (13.6)^{c}	83.3 (66.1)^{c}	23.3 (28.7)^{b}
Commercial polymer	96.6 (83.2) ^{a}	43.3 (40.8.)^{e}	83.3.0 (66.1)^{c}	53.3 (47.0)^{a}
Commercial polymer + *Trichoderma* (Th4d)	96.6 (83.2) ^{a}	18.0 (23.4)^{f}	90.0 (71.5)^{bc}	26.6 (30.9)^{b}
SEm ±	5.4	6.1	3.6	3.3
C.D (p = 0.05)	15.5	17.7	10.2	9.6
C.V (%)	11.5	16.5	8.6	19.8

*Values in parentheses indicate angular transformed values of germination percentage. $P < 0.05$ ANOVA.
Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

3.5.2. Green house experiment

Cts-PEG-Th blend, *Trichoderma* Th4d WP alone, fungicide, synthetic polymer and synthetic polymer along with *Trichoderma* treatments were evaluated under greenhouse conditions as seed treatment against diseases of safflower and groundnut. Significant increase in germination and decrease in disease incidence in both crops was observed with all treatments over pathogen check (Table 6). In groundnut, Cts-PEG-Th treatment showed improvement in seed germination over other treatments though the differences in germination among treatments were not significant. No root rot disease incidence was observed in Cts-PEG-Th and *Trichoderma* Th4d alone treatments whereas pathogen check recorded a disease incidence of 90 % (Fig. 3). In safflower, a significant increase in germination with Cts-PEG-Th seed treatment was observed compared to rest of the treatments. Very low germination (46.6%) and also high incidence of root rot (63.3%) was observed in pathogen check (Fig. 3). Seed coating with Cts-PEG-Th in safflower resulted in significantly low disease incidence (6.6%) followed by treatments with *Trichoderma* Th4d alone (16.6%) and Cts-PEG alone (20.0%). Cts-PEG-Th blend was significantly superior to synthetic polymer seed coat in reduction of disease incidence in both the crops (groundnut and

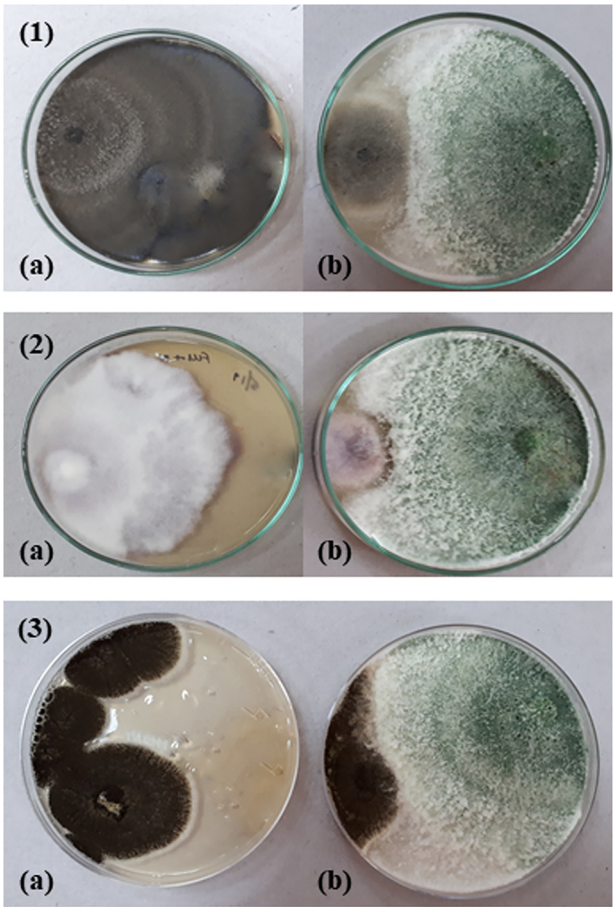

Fig. 1. Antagonistic activity of *Trichoderma* from Cts-PEG blends after 6 months storage at room temperature in dual culture plates. (a) Control (b) dual culture (pathogen + *Trichoderma*) (1) *Macrophomina phaseolina* (2) *Fusariumoxysporum* f. sp..*ricini* (3) *Aspergillus niger*.

safflower). In a similar study though chitosan powder was used in combination with *Trichoderma* but not as a liquid polymer seed coating in tomato, Nitu et al. (2016) have obtained low seedling mortality by soil borne pathogens. In a greenhouse trial conducted by El-Mohamedy et al. (2014), combined treatments with *T.harzianum* and chitosan (0.5 and 1.0 g/l) resulted in significantly low Fusarium crown and root rot incidence over control in tomato.

4. Conclusion

A chitosan biopolymer based *Trichoderma* (Cts-PEG-Th) liquid blend was developed which showed efficient entrapment and loading capacity of spores in the polymer matrix. The blend was stable without much changes in pH throughout the storage period. Persistence studies conducted for 3 months revealed that Cts-PEG-Th amended soil retained log 6.0 CFUs/ gm. of *Trichoderma* Th4d viable counts which is essential for effective disease management. The chitosan blend was able to maintain the viability of *Trichoderma* Th4d spores i.e. 10.0 and 10.2 log CFU over a period of 6 months at two different temperatures respectively and the antagonistic activity unaffected against 3 different plant pathogens. Bio efficacy testing in germination towel tests and green house studies proved the effectiveness of combination of the biopolymer chitosan and *Trichoderma* as a seed coating agent by significantly increasing the germination and seedling vigour and inhibiting the diseases in groundnut and safflower.

CRediT authorship contribution statement

Prasad R.D.: Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Methodology, Writing - review & editing. **Chandrika K.S.V.P:** Conceptualization, Data curation, Formal analysis, Methodology, Writing - review & editing. **Varsha Godbole:** Writing - original draft.

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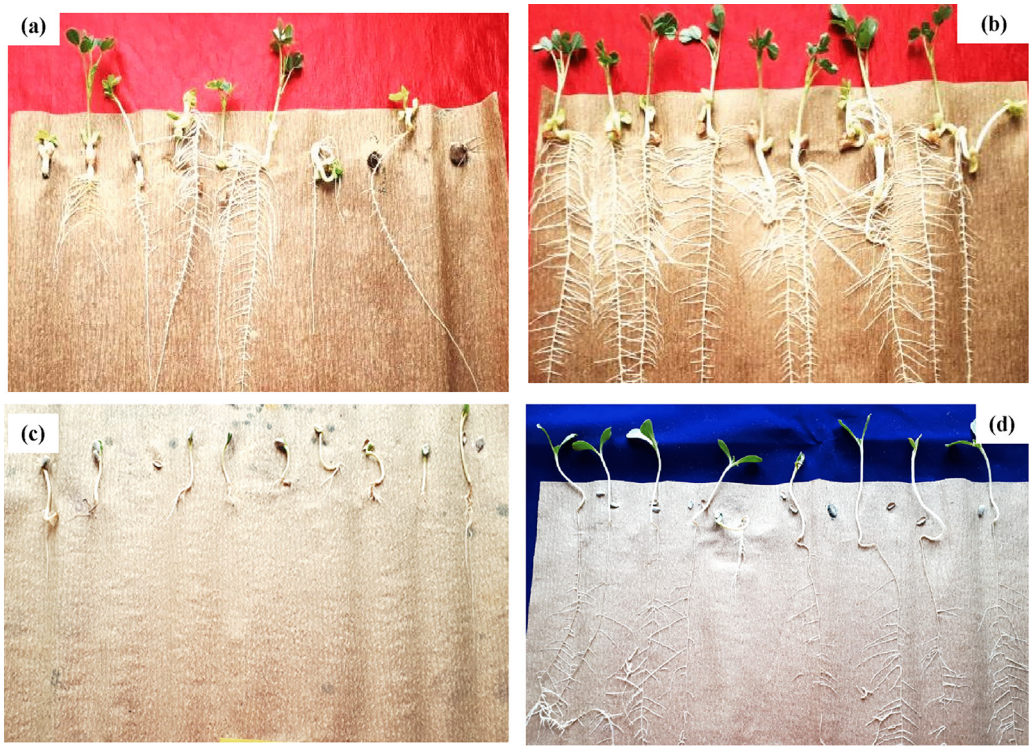

Fig. 2. Effect of chitosan + *Trichoderma* blend seed coating on Aspergillus collar rot incidence in groundnut (var. Kadiri-9) and Macrophomina root rot incidence in safflower (var. A1) under laboratory conditions. (a) Pathogen check- groundnut (b) chitosan + *Trichoderma* blend seed coating on pathogen infected seed of groundnut (c) pathogen check -safflower and (d) chitosan + *Trichoderma* blend seed coating on pathogen infected seed of safflower.

Fig. 3. Effect of chitosan + *Trichoderma* blend seed coating on Macrophomina root rot incidence in safflower (var. A1) and Aspergillus collar rot incidence in Groundnut (var. kadiri-9) under glass house conditions. (a) chitosan + *Trichoderma* blend seed coating on pathogen infected seed of safflower (b) pathogen check of safflower (c) chitosan + *Trichoderma* blend seed coating on pathogen infected seed of groundnut and (d) pathogen check of groundnut.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126487.

References

Ahanger, R.A., Bhatand, H.A., Dar, N.A., 2014. Bio control agents and their mechanism in plant disease management. Sciencia Acta Xaveriana 5 (1), 47–58.

Baker, K.F., Cook, R.J., 1974. In: Freeman, W.H. (Ed.), Biological Control of Plant Pathogens, pp. 433 San Francisco.

Bhaskara Reddy, B.M.V., Ait Barka, E., Castaigne, F., Arul, J., 1998. Effect of chitosan on growth and toxin production by *Alternaria alternata* f. sp. *lycopersici*. Biocon. Sci. Techn. 8, 33–43.

Callan, N.W., Mathre, D.E., Miller, J.B., Vavrina, C.S., 1997. Biological seed treatments: Factors involved in efficacy. Hortsci. 32 (2), 179–183.

Chandrika, K.S.V.P., Prasad, R.D., Godbole, V., 2019. Development of chitosan-PEG blended films using *Trichoderma*: Enhancement of antimicrobial activity and seed quality. Inter. J. Biol. Macromole. 126, 282–290.

Chittenden, C., Singh, T., 2009. In vitro evaluation of combination of *Trichoderma harzianum* and chitosan for the control of sapstain fungi. Biol. Con. 50, 262–266.

Elad, Y., Chet, I., Henis, Y., 1981. A selective medium for improving quantitative isolation of Trichoderma spp. from soil. Phytoparasitica 9, 59–67.

El-Mohamedy, R.S.R., Abdel-Kareem, F., Jabnoun-Khiareddine, H., DaamiRemadi, M., 2014. Chitosan and *Trichoderma harzianum* as fungicide alternatives for controlling *Fusarium* crown and root rot of tomato. Tunisian J. Plant Prot. 9, 31–43.

Fernandez-Saiz, P., Lagaron, J.M., Ocio, M.J., 2009. Optimization of the film- forming and storage conditions of chitosan as an antimicrobial agents. J. Agri. Food Chem. 57, 3298–3307. https://doi.org/10.1021/jf8037709.

Fernando, D., Milagrosa, S., Francisco, C., Urbano, D.S., 2018. Biostimulant activity of *Trichoderma saturnisporum* in melon (*Cucumis melo*). HortSci. 53 (6), 810–815. https://doi.org/10.21273/HORTSCI13006-18.

Harman, G.E., Kubicek, C.P., 1998. Trichoderma and Gliocladium, Vol. 2, Enzymes, Biological Control and Commercial Applications. Taylor & Francis, London, pp. 393.

Haghshenas, B., Abdullah, N., Nami, Y., Radiah, D., Rosli, R., Yari Khosroushahi, A., 2015. Microencapsulation of probiotic bacteria *Lactobacillus plantarum* 15HN using alginate-psyllium-fenugreek polymeric blends. J. Appl. Microbiol. 118, 1048–1057. https://doi.org/10.1111/jam.12762.

John, N.S., Jeeva, M.L., 2014. Efficacy of cassava by-products as carrier materials of *Trichoderma harzianum*, a biocontrol agent against *Sclerotium rolfsii* causing collar rot in elephant foot yam. J. Root Crops. 40 (1), 1–6.

Lewis, J.A., Papavizas, G.C., 1984. Chlamydospore formation by *Trichoderma* spp.in natural substrates. Canadian J. Microbio 30, 1–7.

López-Bucio, J., Pelagio-Flores, R., Herrera-Estrella, A., 2015. *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. Scientia Horti. 196, 109–123. https://doi.org/10.1016/j.scienta.2015.08.043.

Lotfipour, F., Mirzaeei, S., Maghsoodi, M., 2012. Preparation and characterization of alginate and psyllium beads containing *Lactobacillus acidophilus*. The Scientific World J. 8. https://doi.org/10.1100/2012/680108.

Mastouri, F., Bjorkman, T., Harman, G.E., 2010. Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. Phytopath. 100 (11), 1213–1221.

Manjula, K., Podile, A.R., 2001. Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. Canadian J. Microbio 47 (7), 618–625.

Mohamed, E.I., Badawy, Rabea, E.I., Taktak, N.E.M., El-Nouby, M.A.M., 2016. The antibacterial activity of chitosan products blended with monoterpenes and their biofilms against plant pathogenic bacteria. Scientifica 10. https://doi.org/10.1155/2016/1796256.

Manjula, K., Podile, A.R., 2005. Increase in seedling emergence and dryweight of pigeon pea in the field with chitin-supplemented formulations of *Bacillus subtilis* AF 1. World J. Microbio. Biotech. 21, 1057–1062.

Manjula, K., Kishore, G.K., Podile, A.R., 2004. Whole cells of *Bacillus subtilis* AF 1 proved more effective than cell-free and chitinase-based formulations in biological control of citrus fruit rot and groundnut rust. Canadian J. Microbio. 50, 737–744.

Marín, A., Cháfer, L.M., Atarés, A., Chiralt, R., Torres, J., Usall, T.N., 2016. Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA- 1 for control of *Botrytis cinerea* on grapes. Biol. Control. 96, 108–119.

Nitu, N.J., Masum, M.M.I., Jannat, R., Sultana, S., Bhuiyan, M.K.A., 2016. Application of chitosan and *Trichoderma* against soil-borne pathogens and their effect on yield of tomato (*Solanum lycopersicum* L.). Int. J. Biosci. 9 (1), 10–24.

Prasad, R.D., Rangeshwaran, R., 2000. Shelf life and bioefficacy of *Trichoderma harzianum* formulated in various carrier materials. Plant Dis. Res. 15 (1), 38–42.

Rakesh, P., Prasad, R.D., Devi, G.U., Bhat, B.N., 2017a. Effect of biopolymers and synthetic seed coating polymers on castor and groundnut seed. Int. J. Pure Appl. Biosci. 5 (4), 2043–2048.

Rakesh, P., Prasad, R.D., Devi, G.U., Bhat, B.N., 2017b. *In vitro* assessment of seedling

growth and management of castor wilt and groundnut collar rot through the combination of seed coat polymers, fungicides and bioagents. Bull. Env., Pharma. Life Sci. 6, 147–153.

Raphaël, K.J., Meimandipour, A., 2017. Antimicrobial activity of chitosan film forming solution enriched with essential oils ; an in vitro assay. Genetic Eng. Biotech. 15 (2), 111–119. https://doi.org/10.15171/ijb.1360.

Reddy, B.N., Saritha, K.V., Hindumathi, A., 2014. In vitro screening for antagonistic potential of seven species of *Trichoderma* against different plant pathogenic fungi. Res. J. Bio. 36, 29–36.

Rodham, D.K., Wang, Y., Cantwell, J.B., Winn, P.D., Foundling, J., 1999. Formulating microbial bio control agents. Pesti. Sci. 55, 340–342.

Skidmore, A.M., Dickinson, C.H., 1976. Colony interactions and hyphal interference between *Septoria Nodorum* and phylloplane fungi. Transac. British Myco. Society. 66, 57–64.

Sebti, I., Martial-Gros, A., Carnet-Pantiez, A., Grelier, S., Coma, V., 2005. Chitosan polymer as bioactive coating and film against *Aspergillus niger* contamination. J. Food Sci. 70 (2), 100–104.

Singh, S.P., Singh, H.B., Singh, D., Rakshit, A., 2014. *Trichoderma* -mediated enhancement of nutrient uptake and reduction in incidence of *Rhizoctonia solani* in tomato. Egyptian J. Bio. 16, 29–38. https://doi.org/10.4314/ejb.v16i1.4.

Singh, V., Singh, P.N., Yadav, R.L., Awasthi, S.K., Joshi, B.B., Singh, R.K., Lal, R.J., Duttamajumder, S.K., 2010. Increasing the efficacy of *Trichoderma harzianum* for nutrient uptake and control of red rot in sugarcane. J. Horti. Forestry. 2 (4), 66–71.

Swaminathan, J., Van, K.C., Henderson, H.V., Jackson, T.A., Wilson, M.J., 2015. Formulations for delivering *Trichoderma atroviridae* spores as seed coatings: effects of temperature and relative humidity on storage stability. J. Appli. Microbio. 120, 425–431. https://doi.org/10.1111/jam.13006.

Vinceković, M., Jurić, S., Pintarić, S.T., Viskić, M., Jalšenjak, N., Bujan, M., Đermić, E., Žutić, I., Fabek, S., 2017. Release of *Trichoderma viride* spores from microcapsules simultaneously loaded with chemical and biological agents. Agricult. Conspectus Scientific. 82 (4), 395–401.

Woo, S.L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., Pascale, A., Lanzuise, S., Manganiello, G., Lorito, M., 2014. *Trichoderma* -based products and their widespread use in agriculture. Open Myco. J. 8, 71–126.

Yong-Cai, L., Xiao-Juan, S., Yang, B., Yong-Hong, G.E., Yi, W., 2009. Antifungal activity of chitosan on *Fusarium sulphureum* in relation to dry rot of potato tuber. Agri. Sci. China 8 (5), 597–604.

Zhang, M., Tan, T.W., Yuan, H.Z., Rui, C.H., 2003. Insecticidal and fungicidal activities of chitosan and oligo-chitosan. J. Bioact. Compat. Poly. 18, 391–400.