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In vitro efficacy and storage stability of a talc-based *Trichoderma longibrachiatum* formulation against *Sclerotinia sclerotiorum*, the cause of stem and crown rot of alfalfa

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Research Article

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Abstract

Stem and crown rot caused by *Sclerotinia sclerotiorum* is a damaging disease of alfalfa (*Medicago sativa* L.), and biocontrol formulations that stay viable in storage are needed to reduce reliance on fungicides. This study developed a talc-based formulation of *Trichoderma longibrachiatum* (isolate FMB 0031) and assessed its in vitro efficacy against *S. sclerotiorum* (isolate FMB 0090) and its storage stability. The formulation was prepared by mixing a millet-grown *T. longibrachiatum* powder with talc, potato powder and carboxymethyl cellulose (CMC). It was tested at 1/100, 1/1000 and 1/10,000 dilutions in a dipping assay, with colony growth recorded after 3, 5 and 7 days. Viability was determined monthly for six months of storage at room temperature by serial dilution plating. All dilutions reduced pathogen growth significantly relative to the untreated control. Mean colony growth was 0.61, 0.70 and 0.77 cm at 1/100, 1/1000 and 1/10,000, respectively, against 3.71 cm in the control, equivalent to 83.6, 81.1 and 79.2% inhibition. The 1/100 dilution was the most effective at every observation. The viable count was 3.4×10^8 cfu g^{-1} at preparation and declined slowly over the first three months (87% of the initial count retained), then more quickly, reaching 1.5×10^8 cfu g^{-1} (44% of the initial count) after six months. The talc-based formulation therefore combined strong in vitro suppression of *S. sclerotiorum* with substantial viability over six months of storage. Evaluation in pot and field trials is required before the formulation can be recommended for alfalfa production.

Highlights

• Talc–CMC formulation of *Trichoderma longibrachiatum* from millet inoculum.

• All tested dilutions strongly suppressed *Sclerotinia sclerotiorum* in vitro.

• The 1/100 dilution gave the highest inhibition (83.6%) of the pathogen.

• Viable count remained at 1.5×10^8 cfu g^{-1} after six months.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial forage legume grown widely for its high protein content, multiple annual cuttings and capacity to fix atmospheric nitrogen (Russelle, 2001). In Pakistan it is widely grown as a winter fodder. Forage yield and stand life are reduced by several fungal diseases, among which stem and crown rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is particularly damaging in cool, humid conditions (Bolton et al., 2006).

S. sclerotiorum is a necrotrophic pathogen with a very broad host range. It survives between crops as melanised sclerotia that can persist in soil for several years and germinate either myceliogenically or carpogenically to produce airborne ascospores (Adams and Ayers, 1979; Abawi and Grogan, 1979; Purdy, 1979). Because the sclerotia are long-lived and the host range is wide, rotation and sanitation alone

seldom give adequate control, and fungicides add cost and carry environmental and resistance risks (Bolton et al., 2006). Biological control is therefore an important component of integrated management.

Species of *Trichoderma* are among the most widely used fungal biocontrol agents. They act through mycoparasitism, antibiosis, competition for nutrients and space, and induction of plant defences (Harman et al., 2004; Vinale et al., 2008; Verma et al., 2007). *Trichoderma longibrachiatum* is a member of section Longibrachiatum (Samuels, 2006). The field performance of any biocontrol fungus, however, depends on a formulation that keeps the propagules viable during storage and delivers them in a form that is easy to apply (Whipps, 2001; Woo et al., 2014). Talc-based powders are among the simplest and cheapest carriers and have been used successfully for fungal and bacterial agents (Vidhyasekaran and Muthamilan, 1995). Solid inocula grown on cereal grains provide a convenient and inexpensive source of propagules for such formulations (Papavizas et al., 1984).

Information on formulated *T. longibrachiatum* against *S. sclerotiorum* from alfalfa is limited. The objectives of this study were therefore (i) to prepare a talc-based formulation of *T. longibrachiatum* from millet-grown inoculum, (ii) to evaluate its in vitro efficacy against *S. sclerotiorum* at three dilutions over 3, 5 and 7 days, and (iii) to determine its viability during six months of storage.

2. Materials and methods

2.1. Fungal cultures

Sclerotinia sclerotiorum (isolate FMB 0090; GenBank accession MT995056) and *Trichoderma longibrachiatum* (isolate FMB 0031; GenBank accession MF767445) were obtained from the Fungal Molecular Biology (FMB) Laboratory culture collection, Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Cultures, stored as dried filter-paper stocks, were revived on potato dextrose agar (PDA) by single-hyphal-tip transfer. Spore suspensions of *T. longibrachiatum* were prepared by flooding 4–5-day-old colonies with 15 mL sterile distilled water and adjusting the concentration to approximately 10^5 spores mL^{-1} with a haemocytometer.

2.2. Mass multiplication of T. longibrachiatum

Pearl millet grains (100 g per flask) were washed, soaked in 2% sucrose solution and autoclaved at 121°C for 20 min in two flasks. Each flask was inoculated with 1 mL of the *T. longibrachiatum* spore suspension and incubated until the grains were fully covered with green sporulating growth (10 days). The colonised grains were removed, crushed to a fine powder and stored in sealed plastic bags at refrigeration temperature until use.

2.3. Preparation of the talc-based formulation

Potato powder was prepared by washing and slicing 1 kg of potato tubers, sun-drying the slices completely and grinding them. To prepare the carrier, 100 g of talc powder was mixed with 10 g of potato powder and 1% (w/w) carboxymethyl cellulose (CMC), and the mixture was sterilised by dry heat (160°C

for 1 h). After cooling, 5 g of the *T. longibrachiatum* powder was added aseptically, mixed thoroughly and the container was sealed with aluminium foil.

2.4. In vitro efficacy against S. sclerotiorum

The talc-based formulation was diluted in sterile distilled water to prepare three working concentrations. The 1/100 dilution (T1) was prepared by mixing 1 g of formulation with 99 mL of sterile distilled water. The 1/1000 dilution (T2) was prepared by adding 1 mL of T1 to 9 mL of sterile distilled water, and the 1/10,000 dilution (T3) by adding 1 mL of T2 to 9 mL of sterile distilled water (ten-fold serial dilutions). Mycelial discs (5 mm) were cut from the actively growing margin of *S. sclerotiorum* colonies, dipped in the respective dilution for 1 min and transferred to fresh PDA plates. Untreated discs served as the control (T4). The plates were sealed with Parafilm and incubated at 25 ± 2°C in the dark. Radial colony growth was measured at 3, 5 and 7 days after inoculation, and the percentage inhibition of radial growth (PIRG) relative to the control was calculated as PIRG (%) = [(C − T)/C] × 100 (Vincent, 1947), where C and T are the colony growth in the control and treatment, respectively. The experiment was carried out in a completely randomised design (CRD) with three replications per treatment.

2.5. Shelf life of the formulation

The formulation was packed in low-density polyethylene (LDPE) zip-lock bags and stored at room temperature (25 ± 3°C) under ambient conditions. At monthly intervals from 0 to 6 months, 1 g of formulation was suspended in 10 mL of sterile distilled water (10^{-1} dilution base), shaken vigorously and allowed to stand for 30 min. Ten-fold serial dilutions were then prepared up to 10^{-7}, and a 1 mL aliquot of the 10^{-7} dilution was spread on PDA plates. The plates were incubated at 28 ± 2°C for 7 days and the colonies of *T. longibrachiatum* were counted. Viable count was calculated from the mean number of colonies per plate as colony-forming units per gram (cfu g^{-1}) = (number of colonies × dilution factor)/volume plated (mL), where the dilution factor was 10^7 relative to 1 g of formulation. The change in viable count over time was described by regression of the mean count on storage period.

2.6. Statistical analysis

Colony growth data were analysed by analysis of variance for a CRD with treatment and observation day as factors, using Statistix 10 (Analytical Software, Tallahassee, FL, USA). Treatment means were compared by Fisher's least significant difference (LSD) test at $P \leq 0.05$.

3. Results

3.1. Efficacy of the formulation against S. sclerotiorum

All three dilutions of the *T. longibrachiatum* formulation significantly reduced colony growth of *S. sclerotiorum* compared with the untreated control at each observation (Table 1, Fig. 1). Treatment, observation day and their interaction were all significant. Averaged over the three observation days,

colony growth was 0.61 cm at 1/100, 0.70 cm at 1/1000 and 0.77 cm at 1/10,000, against 3.71 cm in the control.

The 1/100 dilution was the most effective at every observation. At 7 days, growth was 0.68 cm at 1/100, 0.80 cm at 1/1000 and 0.88 cm at 1/10,000, compared with 4.60 cm in the control. The 1/1000 and 1/10,000 dilutions did not differ at 3 days, and the 1/100 and 1/1000 dilutions did not differ at 5 days; the three dilutions were all different from one another at 7 days (Table 1). Growth in treated plates increased only slightly with time, whereas growth in the control increased by about 70% between 3 and 7 days.

Percentage inhibition ranged from 76.7 to 85.2% across dilutions and days (Table 2). Mean inhibition over the three observations was 83.6% at 1/100, 81.1% at 1/1000 and 79.2% at 1/10,000. Inhibition at the 1/100 dilution increased from 81.5% at 3 days to 85.2% at 7 days.

Table 1
Colony growth (cm) of *Sclerotinia sclerotiorum* after treatment with different dilutions of the talc-based *Trichoderma longibrachiatum* formulation.

Treatment	3 days	5 days	7 days	Mean
Formulation 1/100 (T1)	0.50 c	0.65 c	0.68 d	0.61
Formulation 1/1000 (T2)	0.60 b	0.70 c	0.80 c	0.70
Formulation 1/10,000 (T3)	0.63 b	0.81 b	0.88 b	0.77
Untreated control (T4)	2.70 a	3.83 a	4.60 a	3.71

Values are means of three replications. Within a column, means followed by the same letter do not differ significantly (Fisher's LSD, $P \leq 0.05$; LSD = 0.072 for treatment × day means).

Table 2
Percentage inhibition of radial growth (PIRG) of *S. sclerotiorum* by the formulation.

Treatment	3 days	5 days	7 days	Mean
Formulation 1/100 (T1)	81.5	83.1	85.2	83.6
Formulation 1/1000 (T2)	77.8	81.7	82.6	81.1
Formulation 1/10,000 (T3)	76.7	79.0	80.9	79.2

PIRG was calculated from treatment means relative to the control mean for the same day; the "Mean" column is calculated from the three-day mean growth.

3.2. Shelf life of the formulation

The viable count of the formulation was 3.4×10^8 cfu g^{-1} at preparation (Table 3, Fig. 2). It declined only slowly during the first three months, falling to 3.3×10^8 cfu g^{-1} after one month and 2.96×10^8 cfu g^{-1} (87% of the initial count) after three months. The rate of decline then increased: the count was 2.6×10^8 cfu g^{-1} at four months, 2.1×10^8 cfu g^{-1} at five months and 1.5×10^8 cfu g^{-1} at six months, i.e. 44% of the initial count, a reduction of 0.35 log units. The decline followed a curvilinear pattern that was described well by a quadratic regression ($\text{cfu} = 337.6 + 0.79t - 5.29t^2$, where cfu is in units of 10^6 g^{-1} and t is storage time in months; $R^2 = 0.997$).

Table 3
Viable count of the talc-based *T. longibrachiatum* formulation during storage.

Storage (months)	Mean colonies per plate (10^{-7})	Viable count ($\times 10^6$ cfu g^{-1})	$\log_{10}$ cfu g^{-1}	Of initial (%)
0	34.0	340	8.53	100.0
1	33.3	333	8.52	97.9
2	31.0	310	8.49	91.2
3	29.6	296	8.47	87.1
4	26.0	260	8.41	76.5
5	21.0	210	8.32	61.8
6	15.0	150	8.18	44.1

Counts were made on PDA at the 10^{-7} dilution (1 mL plated). Percentages are relative to the count at month 0.

4. Discussion

The talc-based formulation of *T. longibrachiatum* strongly suppressed *S. sclerotiorum* in vitro, with 79–84% inhibition of colony growth across the dilutions tested. Inhibition was greatest at the highest concentration (1/100) and declined with dilution, which is consistent with a dose-dependent response, although the difference between the 1/100 and 1/10,000 dilutions was small in absolute terms (about 4 percentage points of mean inhibition). The fact that even the 1/10,000 dilution retained about 79% inhibition suggests that the formulation delivered a high density of active propagules, as expected from a preparation containing about 10^8 cfu g^{-1}, but this was not tested directly and the mechanism of suppression was not examined here. Antagonism by *Trichoderma* is generally attributed to mycoparasitism, antibiosis and competition (Harman et al., 2004; Vinale et al., 2008), and these mechanisms would need to be confirmed for this isolate by microscopy or enzyme and metabolite assays.

Viability is a key requirement for a practical biocontrol product, because the usefulness of a formulation depends on the number of live propagules delivered at the time of application (Whipps, 2001; Woo et al., 2014). In this study the count at room temperature (25 ± 3°C) remained within 13% of its initial value for three months and was still 1.5×10^8 cfu g^{-1} after six months. The accelerating loss after month three suggests that storage beyond about three to four months would shorten the useful life of the product; the effect of other storage temperatures, moisture content and packaging on this trend should be examined, because these factors strongly influence the survival of talc-based formulations (Vidhyasekaran and Muthamilan, 1995). The inclusion of CMC as an adhesive and potato powder as a nutrient base probably contributed to propagule survival, but their individual contributions were not tested.

Several limitations should be noted. First, all efficacy data are from in vitro plate assays, which indicate antagonistic potential but do not necessarily predict disease suppression on plants or in soil. Second, a carrier-only control (talc, potato powder and CMC without *Trichoderma*) was not included, so the contribution of the carrier ingredients to the observed inhibition cannot be separated from that of the fungus. Third, viability was determined from mean plate counts without replicate-level variance, so the decline over time is described but not tested statistically. Pot trials on alfalfa and field evaluation, with carrier-only and fungicide comparators, are required before the formulation can be recommended.

5. Conclusions

A talc-based formulation of *T. longibrachiatum* (isolate FMB 0031) reduced the in vitro growth of *S. sclerotiorum* by 79–84%, with the 1/100 dilution most effective, and retained 44% of its initial viable count (1.5×10^8 cfu g^{-1}) after six months of storage. These results support further evaluation of the formulation as a component of integrated management of stem and crown rot of alfalfa, beginning with pot experiments including a carrier-only control, followed by field trials and studies of storage conditions.

Declarations

CRediT authorship contribution statement

Muhammad Ans: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Resources, Writing – original draft, Writing – review & editing, Project administration. Imran ul Haq: Supervision. Amjad Abbas: Supervision. Siddra Ijaz: Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data supporting this study are available from the corresponding author on reasonable request.

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Figures

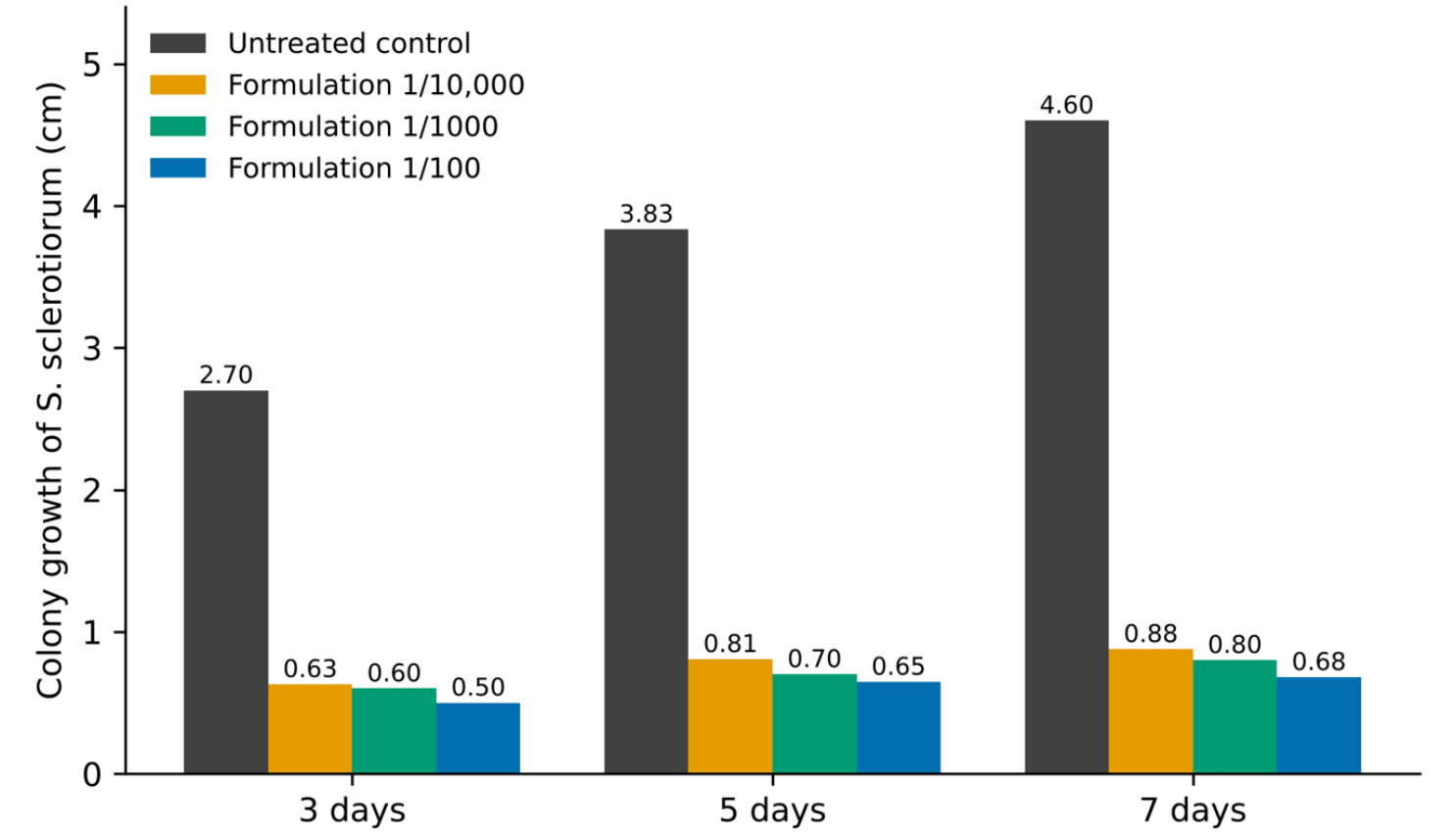


Figure 1

Colony growth of *S. sclerotiorum*at 3, 5 and 7 days after treatment with dilutions of the *T. longibrachiatum* formulation and in the untreated control. Bars are treatment means (*n* = 3).

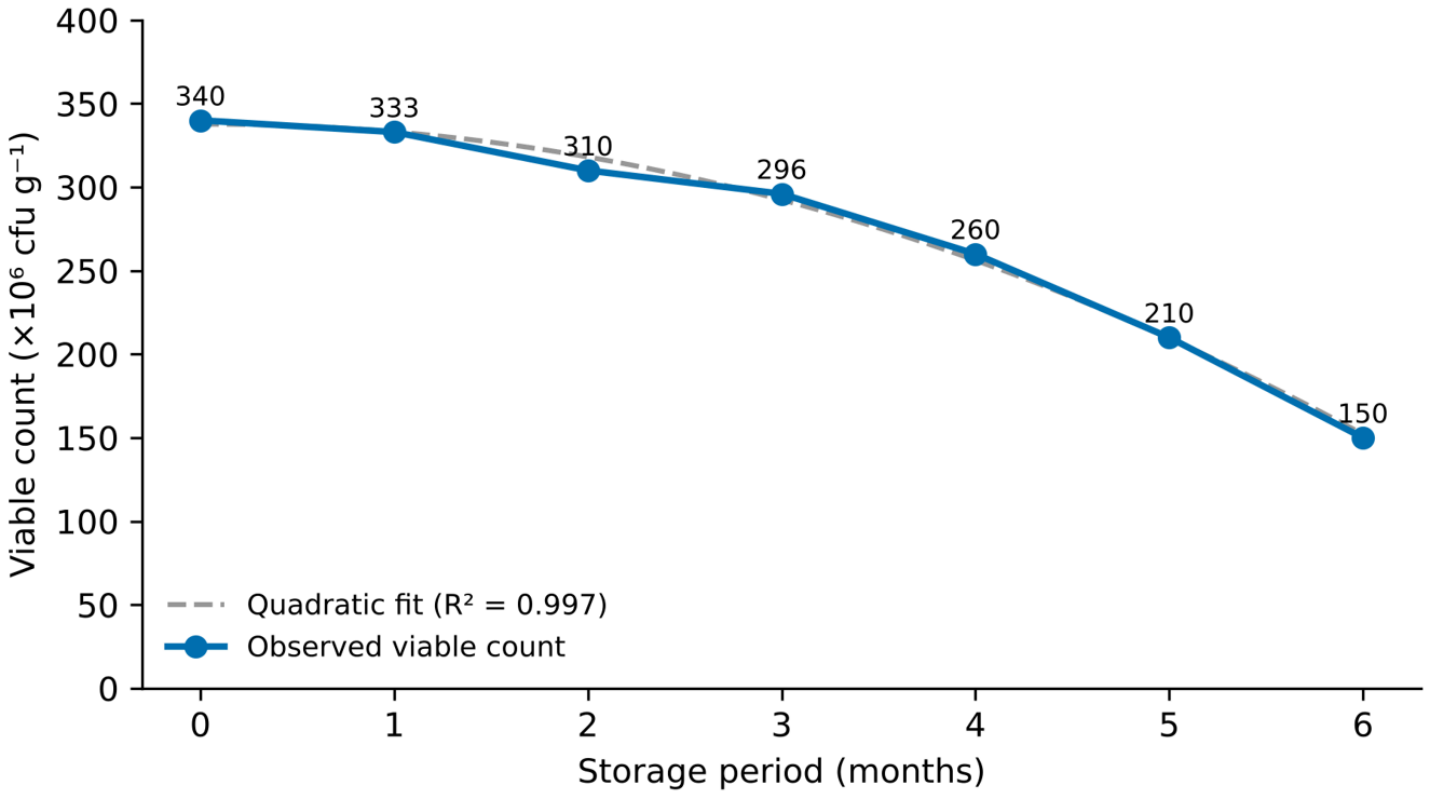


Figure 2

Change in viable count of the talc-based *T. longibrachiatum* formulation during six months of storage. The dashed line is a quadratic regression fitted to the means.