Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Comparative in vitro antagonism of *Trichoderma longibrachiatum*, *T. harzianum* and *T. asperellum* against *Sclerotinia sclerotiorum*, the cause of stem and crown rot of alfalfa

Muhammad Ans

muhammadans430@outlook.com

University of Agriculture Faisalabad

Research Article

Keywords: Antagonism, Biological control, Dual culture, Sclerotinia sclerotiorum, Trichoderma

Posted Date: November 3rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11579274/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Abstract

Stem and crown rot caused by *Sclerotinia sclerotiorum* is a serious disease of alfalfa (*Medicago sativa* L.), and effective antagonists are needed for its biological management. This study compared the in vitro antagonism of three *Trichoderma* isolates, *T. longibrachiatum* (FMB 0031), *T. harzianum* (FMB 0012) and *T. asperellum* (FMB 0032), against *S. sclerotiorum* (FMB 0090) in a dual-culture assay. Pathogen colony growth toward the antagonist was recorded after 3, 5 and 7 days in a completely randomised design with three replications, and percentage inhibition of radial growth (PIRG) was calculated relative to the untreated control. All three antagonists significantly reduced pathogen growth at every observation. Averaged over the three observations, pathogen growth was 0.48 cm with *T. longibrachiatum*, 0.56 cm with *T. harzianum* and 0.71 cm with *T. asperellum*, against 3.33 cm in the control. At 7 days, PIRG was 86.5, 85.1 and 78.7%, respectively. *T. longibrachiatum* and *T. harzianum* did not differ significantly, and both suppressed the pathogen more than *T. asperellum* at 7 days. Inhibition by *T. asperellum* rose from 73.0% at 3 days to 81.6% at 5 days, then declined, whereas inhibition by the other two isolates was maintained. *T. longibrachiatum* and *T. harzianum* are therefore the more promising candidates for formulation and further testing against stem and crown rot of alfalfa. Confirmation in pot and field studies, and study of the mechanisms involved, is still required.

Highlights

• Three *Trichoderma* species were compared against *S. sclerotiorum* in dual culture.

• All antagonists cut pathogen growth by 73–87% over 3–7 days.

• *T. longibrachiatum* and *T. harzianum* gave the strongest suppression.

• *T. asperellum* was weaker and lost some inhibition by day 7.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial forage legume valued for its protein content, repeated cuttings and nitrogen fixation (Russelle, 2001). In Pakistan it is a major winter fodder, and its production is limited by several diseases. Stem and crown rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is among the most damaging in cool, humid conditions (Bolton et al., 2006).

S. sclerotiorum has a very wide host range and survives in soil as sclerotia for several years (Adams and Ayers, 1979; Purdy, 1979). These features limit the value of rotation and sanitation, and repeated fungicide use raises cost, environmental and resistance concerns (Bolton et al., 2006). Biological control with antagonistic fungi is a promising component of integrated management.

Species of *Trichoderma* are the most widely used fungal biocontrol agents. They suppress pathogens through mycoparasitism, antibiosis, competition and induction of plant resistance (Harman et al., 2004; Vinale et al., 2008; Verma et al., 2007). Their efficacy differs markedly between species and between

isolates of the same species (Harman, 2006), so candidate antagonists must be compared directly against the target pathogen before formulation. The dual-culture technique is a standard first screen, as it exposes the pathogen to competition, diffusible metabolites and hyphal interaction in a single assay (Dennis and Webster, 1971).

The objective of this study was to compare the in vitro antagonism of three *Trichoderma* isolates (*T. longibrachiatum*, *T. harzianum* and *T. asperellum*) against *S. sclerotiorum* from alfalfa over 3, 5 and 7 days, in order to identify the most promising antagonist for further development.

2. Materials and methods

2.1. Fungal isolates

The isolates used were *S. sclerotiorum* FMB 0090 (GenBank accession MT995056), *T. asperellum* FMB 0032 (MF767444), *T. harzianum* FMB 0012 (MG029259) and *T. longibrachiatum* FMB 0031 (MF767445), all obtained from the Fungal Molecular Biology (FMB) Laboratory culture collection, Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Cultures held as dried filter-paper stocks were revived on potato dextrose agar (PDA) by single-hyphal-tip transfer. The isolates were identified on cultural and morphological characters (colony colour and texture, hyphae, conidiophores and conidia for *Trichoderma*; mycelium and sclerotia for *S. sclerotiorum*) and are held with GenBank accessions as listed.

2.2. Dual-culture assay

Antagonism was evaluated by the dual-culture technique (Dennis and Webster, 1971). A 6-mm agar disc from a 4-day-old culture of each antagonist was placed on one side of a PDA plate, and a 6-mm disc of *S. sclerotiorum* was placed opposite at a distance of 60 mm. Control plates carried the pathogen alone. The plates were incubated at 25 ± 2°C in the dark. Radial growth of the pathogen toward the antagonist was measured at 3, 5 and 7 days along the line of approach. Percentage inhibition of radial growth (PIRG) was calculated as "PIRG (%)"=[(C-T)/C]×100 (Vincent, 1947), where C is pathogen growth in the control and T is pathogen growth in the presence of the antagonist. The treatments were T1, *T. longibrachiatum*; T2, *T. harzianum*; T3, *T. asperellum*; and T4, untreated control. The experiment was laid out in a completely randomized design (CRD) with three replications.

2.3. Statistical analysis

Growth data were analysed as a factorial CRD (treatment × observation day) using Statistix 10 (Analytical Software, Tallahassee, FL, USA). Means were separated by Fisher's least significant difference (LSD) test at $P \leq 0.05$.

3. Results

3.1. Pathogen growth in dual culture

All three *Trichoderma* isolates significantly reduced the growth of *S. sclerotiorum* compared with the untreated control at each observation (Table 1, Fig. 1a). Treatment, observation day and their interaction were significant. Pathogen growth in the control increased from 2.12 cm at 3 days to 4.12 cm at 7 days, whereas growth in the presence of an antagonist increased only slightly over the same period (for example, from 0.39 to 0.56 cm with *T. longibrachiatum*).

Averaged over the three days, pathogen growth was lowest with *T. longibrachiatum* (0.48 cm), followed by *T. harzianum* (0.56 cm) and *T. asperellum* (0.71 cm); the control mean was 3.33 cm. *T. longibrachiatum* and *T. harzianum* did not differ significantly, and both were significantly better than *T. asperellum*. At 3 days the three antagonists did not differ from one another. By 7 days, *T. asperellum* allowed significantly more pathogen growth (0.88 cm) than *T. harzianum* (0.61 cm) or *T. longibrachiatum* (0.56 cm).

3.2. Percentage inhibition

PIRG at 7 days was 86.5% for *T. longibrachiatum*, 85.1% for *T. harzianum* and 78.7% for *T. asperellum* (Table 2, Fig. 1b). Mean PIRG over the three observations was 85.5, 83.1 and 78.6%, respectively. Inhibition by *T. longibrachiatum* and *T. harzianum* increased between 3 and 5 days (from 81.6 to 86.7% and from 76.9 to 84.3%) and was then maintained. Inhibition by *T. asperellum* increased from 73.0% at 3 days to 81.6% at 5 days but fell to 78.7% at 7 days, because pathogen growth in these plates increased more between days 5 and 7 (0.69 to 0.88 cm) than in plates with the other antagonists.

Table 1
Growth (cm) of *Sclerotinia sclerotiorum* toward different *Trichoderma* antagonists in dual culture.

Treatment	3 days	5 days	7 days	Mean
T. longibrachiatum (T1)	0.39 b	0.50 c	0.56 c	0.48 c
T. harzianum (T2)	0.49 b	0.59 bc	0.61 c	0.56 c
T. asperellum (T3)	0.57 b	0.69 b	0.88 b	0.71 b
Untreated control (T4)	2.12 a	3.75 a	4.12 a	3.33 a

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 = 0.19 for treatment × day means and 0.11 for treatment means).

Table 2
Percentage inhibition of radial growth (PIRG) of *S. sclerotiorum* by *Trichoderma* antagonists.

Treatment	3 days	5 days	7 days	Mean
T. longibrachiatum (T1)	81.6	86.7	86.5	85.5
T. harzianum (T2)	76.9	84.3	85.1	83.1
T. asperellum (T3)	73.0	81.6	78.7	78.6

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

4. Discussion

All three *Trichoderma* isolates suppressed *S. sclerotiorum* strongly in dual culture, reducing its growth by 73–87% relative to the control. *T. longibrachiatum* and *T. harzianum* were the most effective, and *T. asperellum* was less effective by 7 days. The differences among the antagonists were small in absolute terms (a range of about 0.3 cm in pathogen growth at 7 days) but consistent across observations, which supports the ranking. Differences in antagonism among *Trichoderma* species and isolates are well known and are attributed to differences in growth rate, secondary metabolite production and hydrolytic enzyme activity (Harman et al., 2004; Vinale et al., 2008), although these traits were not measured here.

The time course is also informative. The two stronger antagonists reached their highest inhibition by 5 days and maintained it, whereas inhibition by *T. asperellum* declined between 5 and 7 days as pathogen growth resumed. A suppression that is not sustained is a disadvantage in a pathogen such as *S. sclerotiorum*, which can regrow and form sclerotia once the antagonist’s early advantage is lost. A longer observation period with measurement of sclerotial production would be needed to confirm whether the inhibition by each isolate is sustained.

Several limitations apply. The assay is in vitro, on a rich medium and at a single incubation condition, and dual-culture results do not always predict performance on plants or in soil (Whipps, 2001). Only one isolate per species was tested, so the differences should be read as differences between isolates rather than between species. Mechanisms (mycoparasitism, antibiosis, competition) were not examined, and the number of replications was small. The isolates were identified by morphology with GenBank accessions held in the culture collection; the molecular identification should be reported in full in any later study. Nevertheless, the results justify selecting *T. longibrachiatum* and *T. harzianum* for formulation and for pot and field evaluation against stem and crown rot of alfalfa.

5. Conclusions

In dual culture with *S. sclerotiorum* from alfalfa, *T. longibrachiatum* (FMB 0031) and *T. harzianum* (FMB 0012) gave the strongest and most sustained inhibition (86.5% and 85.1% at 7 days), whereas *T.*

asperellum (FMB 0032) was less effective (78.7%) and lost some of its early inhibition by day 7. These two isolates are the better candidates for formulation and for testing in pot and field experiments, together with studies of the mechanisms of antagonism.

Declarations

Credit authorship contribution statement

Muhammad Ans: Conceptualization, Investigation, Formal analysis, Resources, Writing – review & editing. – original draft.

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.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors thank the Fungal Molecular Biology Laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad, for providing the fungal isolates and laboratory facilities. This paper is based on the MSc (Hons) thesis of the first author.

Data availability

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

References

1. Adams PB, Ayers WA (1979) Ecology of *Sclerotinia* species. Phytopathology 69:896–899

2. Bolton MD, Thomma BPHJ, Nelson BD (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7, 1–16
3. Dennis C, Webster J (1971) Antagonistic properties of species-groups of *Trichoderma* III. Hyphal interaction. Trans Br Mycol Soc 57:363–369
4. Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96:190–194
5. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species — opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56
6. Purdy LH (1979) *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 69:875–880
7. Russelle MP (2001) Alfalfa Am Sci 89:252–259
8. Verma M, Brar SK, Tyagi RD, Surampalli RY, Valéro JR (2007) Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. Biochem Eng J 37:1–20
9. Vincent JM (1947) Distortion of fungal hyphae in the presence of certain inhibitors. Nature 159:850
10. Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*–plant–pathogen interactions. Soil Biol Biochem 40:1–10
11. Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Figures

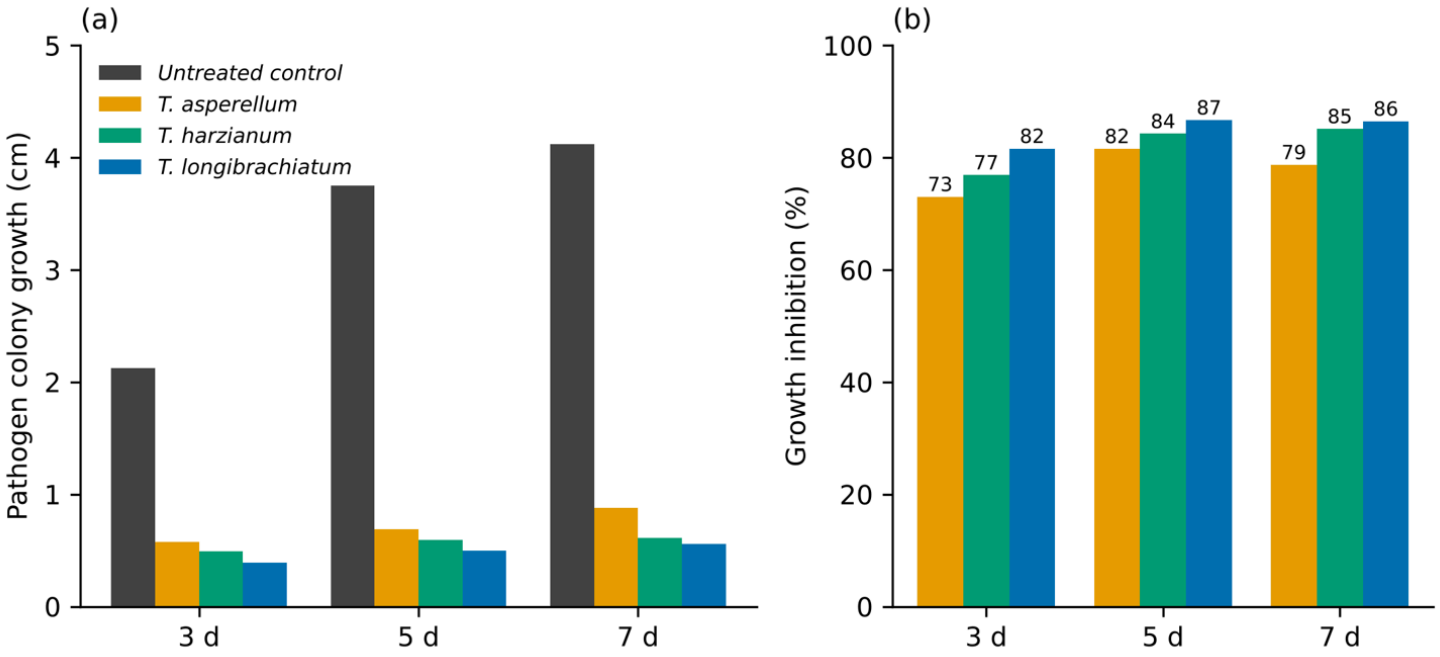


Figure 1

(a) Growth of *S. sclerotiorum*in dual culture with three *Trichoderma* species and in the untreated control, and (b) percentage inhibition of pathogen growth at 3, 5 and 7 days. Bars are treatment means (*n* = 3).