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In-vitro antagonistic potency of *Trichoderma* against root rot fungi and root-knot nematodes isolated from medicinal plants

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Abstract

Bioinoculants holds a great promise for eco-friendly and sustainable management of the phytopathogens that is considered one of the major biotic stress for crop production. In the current study, fifteen fungal endophytes isolated from leaves, stems, and roots of medicinal plants collected from different localities of Karachi University. Out of which five strains of bioinoculants viz., *Trichoderma harzianum*, *Trichoderma viridi*, *Trichoderma asperellum*, *Penicillium expansum* and *Penicillium chrysogenum* were evaluated in vitro against four important phytopathogens of agricultural crop plant roots viz., *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum* and *F. solani*. showing inhibition zones up to 16 mm. *T. harzianum*, *T. viridi*, and *P. expansum* displayed strong antifungal activity. Strong nematicidal effects on *Meloidogyne javanica* juveniles were observed with culture filtrates, where isolates SpS-TR3 exhibited the highest mortality (40.28%) within 24 hours, and SpS-TR2 and SpS-TR3 achieved 58.39% and 56.12% mortality after 48 hours. By 72 hours, SpS-TR3, AiL-PE1, and AvR-PE2 all isolates exhibited complete nematode mortality. Results suggested that these bioinoculants act as bio fertilizer and can be used alternative to synthetic pesticide which harm ecology of agricultural production.

Keywords: Endophytic fungi, Biocontrol agents, Root rot pathogens, Root-knot nematodes, Medicinal plants, Antifungal activity

Introduction

Endophytic fungi are microorganisms that reside within plant tissues for all or part of their life cycle, maintaining a mutualistic association without harming the host plant (Farhat et al., 2022). These fungi are integral to the plant micro-ecosystem, enhancing host physiology by producing hormones such as indole acetic acid, facilitating nutrient biosynthesis and acquisition, and secreting stress-related metabolites to shield plants from herbivores and pathogens, thereby boosting abiotic stress tolerance (Farhat et al., 2022). This study reviews the interactions between endophytic fungi and nematodes, with a focus on their agricultural impact.

Fungi and nematodes are among the most abundant soil organisms. Endophytic fungi, especially species within the Ascomycota division (e.g., *Penicillium*, *Alternaria*, *Trichoderma*, and *Aspergillus*), were isolated from medicinal plants. These fungi, residing within plant tissues, produce bioactive metabolites with significant pharmacological and agricultural applications. Advances in omics technologies have facilitated research into symbiotic

relationships between endophytic fungi and plants, driving the discovery of novel biomolecules with biotechnological potential for sustainable agriculture (Bogas et al., 2022).

Endophytic fungi inhabit the intercellular and intracellular spaces of plant tissues, producing antibiotics, enzymes, and other bioactive compounds that enhance competitive survival. These secondary metabolites offer plant protection against pathogens and promote plant growth (Farhat *et al.,* 2023). Endophytes have drawn significant research interest due to their vast chemical diversity and potential applications in medicine, agriculture, and environmental science, where they contribute to host photo-protection and photochemical efficiency.

Nematodes, particularly roundworms, are highly diverse and predominantly inhabit the top 15 cm of soil, where they play a role in the nitrogen cycle through mineralization. Unlike decomposer fungi, nematodes are parasitic or free-living organisms that feed on living matter, impacting nutrient cycles. Both fungi and nematodes co-exist in a variety of ecosystems, particularly around plant roots, where they interact in ways that are crucial to agriculture and forestry. Consequently, understanding fungal-nematode interactions is increasingly important for agricultural science.

Antagonistic relationships between fungi and nematodes are well-documented. Some nematodes, such as *Aphelenchus avenae* and *Aphelenchoides* spp., consume various fungal species and are known as fungivorous nematodes. Conversely, certain fungi, such as *Arthrobotrys oligospora*, prey on nematodes and their eggs, serving as biological control agents. These fungi are termed nematophagous due to their nematode-killing abilities.

Materials and Methods

Collection of plant samples for isolation of endophytic fungi

Samples from medicinal plants, including *Salvadora persica* L., *Abutilon indicum* (L.) Sweet, *Achyranthus aspera* L., *Euphorbia hirta* L., *Amaranthus viridi* L., *Calotropis procera* (Aiton) W.T.Aiton, and *Azadirachta indica* A.Juss., were collected from the University of Karachi. Samples were stored at low temperatures until use. The roots, stems, and leaves were washed with tap water, then cut into 1 mm small segments, and sterilized with 1% Sodium hypochlorite (NaOCl) for 3–4 minutes. The segments were cultured on Potato Dextrose Agar (PDA) plates and incubated for 5–7 days, during which fungal isolates displaying mycelium and spores were examined under a microscope.

Identification of fungal endophytes

Fungal cultures were stained with lactophenol cotton blue for identification and analyzed using bright-field and phase-contrast microscopy. Morphological traits, including growth patterns, hyphal structure, colony color, surface texture, aerial mycelium, and sporulation, were evaluated following standard identification guidelines following Barnett & Hunter (1998), Domsch et al., (1980), Dugan (2006), Ellis (1971), Gilman (1957).

Isolation of root-knot nematodes (*Meloidogyne* spp.,)

Root-knot nematode eggs were extracted from infected brinjal roots by washing, cutting, and immersing the roots in a $Ca(OCl)_2$ solution, followed by vigorous shaking for 5–10 minutes. The egg masses were then filtered through 40, 200, and 400 mesh sieves and the final filtrate

was placed in cavity blocks with distilled water at room temperature for 48 hours to allow juvenile hatching.

Isolation of root-rotting fungi from soil

A: Soil dilution technique for the isolation of *Fusarium* spp*:* Dilutions was prepared by adding 1g of soil sample mixed in 9 mL of 0.1 % agar suspension. From the final dilution, 0.1 percent agar suspension of 1 mL aliquot was poured on petri dishes containing Pentachloronitrobenzene (PCNB) media (Nash and Snyder, 1962) and the suspension was spread on the surface of agar by rotating the petridishes. For 5 days plates were incubated at 28°C and after *Fusarium* species were identified.

B: Baiting technique for the isolation of *Rhizoctonia solani:* Sterilized sorghum seeds used as bait were spread on a damp soil surface. Baits were removed after 24 hours, washed with tap water, and then transferred on to a potato dextrose agar (PDA). For radial growth and characterization of *R. solani* pH was maintained at 5.50 (Wilhelm, 1955). The % of seeds colonized was used to estimate the population of *R. solani* in the soil.

C: Wet sieving and dilution technique for the isolation of *Macrophomina phaseolina:* Method for the isolation of *Macrophomina phaseolina* by wet sieving was suggested by Sheikh and Ghaffar (1975). Twenty gram soil samples were sieved through a 100 (150- µm) mesh screen before being placed on a 300 (53- µm) mesh screen. The residue from the 53- µm screen was washed under tap water for one minute and then transferred into a beaker containing (0.5 % Ca $(OCl)_2$) and diluted up to 100 mL to make a 1:5 dilution. The suspension of sclerotia was put on a magnetic stirrer and 1 mL aliquot was evenly spread onto the surface of plates containing PDA supplemented with penicillin (100, 000 units/L) and streptomycin 1g/L). For five days the plates were incubated at 28 °C and were identified by the appearance of brown to black colonies.

Preparation of Culture Filtrates of Antagonistic Microorganisms

To prepare culture filtrates, 5 mm discs of fungal test cultures were inoculated in 250 mL flasks containing fungal broth and an equal volume of hexane, the resulting filtrates were then used for further testing.

Nematicidal Potential of Culture Filtrates

Culture filtrates (1 mL) were introduced to pit blocks that contained 15 second-stage juveniles of *Meloidogyne javanica*, using distilled water as a control. The blocks were maintained at a temperature range of 25–30°C, and the mortality of the nematodes was observed under a stereomicroscope after 24, 48, and 72 hours as compared to the control for each treatment at time 0.

Fungicidal Potential of Culture Filtrates Using Disc Diffusion Method

Culture filtrates of endophytic fungi in concentrations 20µL, 40µL, and 60µL were applied to sterilized filter paper discs, which were then positioned on CDA (CZapek Dox Agar) plates surrounding a central disc that contained root-rot fungi. A disc without sterilized broth is used as a control, while a disc loaded with pesticide (carbendazim 1%) served as a positive control. The plates were incubated at 30°C for 5 to 7 days, after which the inhibition zone between the fungal growth and the discs loaded with filtrate was measured.

Statistical Analysis

Analysis of variance (ANOVA) encompasses minimum significant difference (LSD), Duncan multiplier, mean, and standard deviation, were analyzed using IBM SPSS statistics 21 in accordance to experimental design as described by Gomez and Gomez 1984.

Results

Isolation of Endophytic Antagonistic Fungi from Medicinal Plants

In this study antagnistic fungi including *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma viride*, and *Penicillium expansum* were predominantly isolated from the roots and stems of *Salvadora persica*. *Trichoderma asperellum* was also isolated from the roots of *Abutilon indicum*, while *Penicillium expansum* and *T. asperellum* were recovered from the leaves of *Calotropis procera*. However, *Penicillium chrysogenum* was predominant from the roots of *Amaranthus viridi* and *Salvadora persica*.

Evaluation of Antagonistic Fungi for In Vitro Biocidal Activity Against Pathogenic Fungi and Nematodes

In vitro testing was conducted to assess the effectiveness of antagonistic fungal strains against pathogenic fungi and root-knot nematodes. The formation of zones of inhibition by disc diffusion method indicated that all tested fungi effectively suppressed root rot pathogens. *Penicillium expansum* (AiL-PE1) showed the highest inhibitory effect against all tested root-rotting fungi, including *F. solani*, *F. oxysporum*, *M. phaseolina*, and *R. solani*, with maximum inhibition zones ranging from 6.66 mm to 7.33 mm at the highest concentration (60 µL/disc). Among the *Trichoderma* species, *Trichoderma viride* (SpR-TR2) exhibited greater inhibition as compared to *T. asperellum* (SpS-TR3) and *T. harzianum* (SpR-TR1), with inhibition zones reaching up to 6.66 mm for *F. solani* and 6.33 mm for *M. phaseolina*. *T. asperellum* showed moderate inhibition, with maximum zones ranging from 4.33 mm to 6.00 mm, while *T. harzianum* displayed the least inhibition, with zones not exceeding 5.00 mm. Overall, *Penicillium expansum* emerged as the most effective antagonist, followed by *T. viride*, *T. asperellum*, and *T. harzianum* (Table 3).

However, all the antagonistic filtrates including SpR-TR1, SpR-TR2, SpSTR3, AiL-PE1, and AvR-PE2 exhibited substantial inhibition against *M. phaseolina*, with inhibition zones ranging from 9.5 to 16 mm. Occasional overlapping of pathogenic growth with antagonistic cultural disc was also observed.

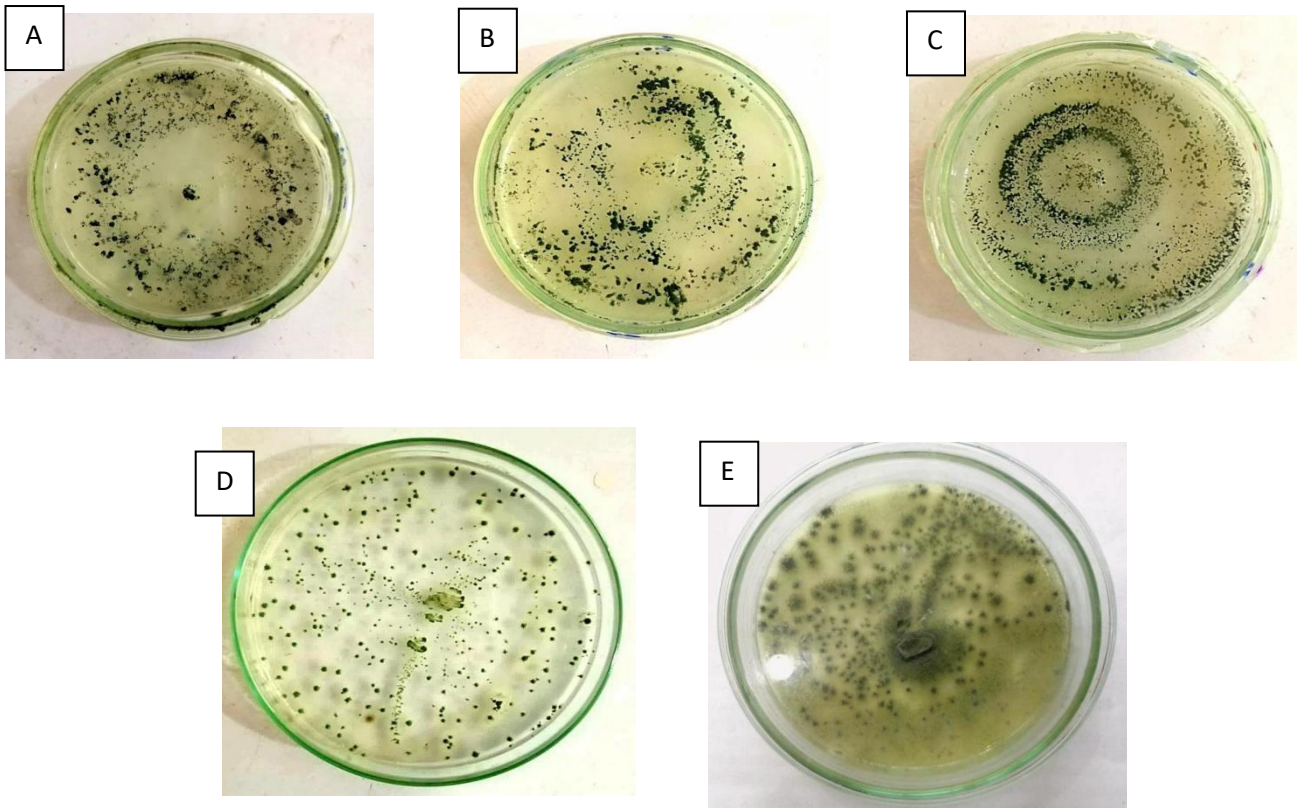

Fig 1: Pure cultures of antagonistic microbes maintaned on PDA: A= SpR-TR1, B= SpR-TR2, C= SpR-TR3, D= AiL-PE1 and E= AvR-PE2

Table 1: List of fungal species isolated from medicinal plant at University of Karachi.

S.no	Plants names	Plant Part	Name of fungi	Localities
01	*Salvadora persica*	Roots	*Trichoderma harzianum* *Alternaria solani* *Penicillium expansum*	Department of Zoology, UoK.
02	*Salvadora persica*	Stems	*Trichoderma viridi* *Alternaria solani* *Trichoderma asperellum*	Department Of Physics, Uok.
03	*Abutilon indicum*	Roots	*Rhizoctonia solani* *Aspergillus flavus* *Aspergillus niger*	Department of Pharmacy, UoK.
04	*Salvadora persica*	Roots	*Trichoderma viridi* *Penicillium chrysogenum* *Trichoderma harzianum*	M.H.Library Admin block, UoK.
05	*Achyranthus aspera*	Roots	*Rhizoctonia solani* *Fusarium solani* *Trichoderma viridi*	Department of Botany, UoK.
06	*Euphorbia hirta*	Roots	*Fusarium solani* *Rhizoctonia solani* *Rhizopus spp*	Department of Chemistry, UoK.
07	*Amaranthus viridi*	Roots	*Fusarium oxysporum* *Rhizopus spp* *Macrophomina phaseolina* *Penicillium chrysogenum*	Department of Zoology, UoK.
08	*Calotropis procera*	Roots	*Aspergillus niger* *Rhizoctonia solani* *Fusarium oxysporum*	Department Of Physics, Uok.
09	*Azadirachta indica*	Leaves	*Penicillium chrysogenum* *Rhizopus spp* *Aspergillus niger*	Department of Botany, UoK.
10	*Salvadora persica*	Stems	*Penicillium chrysogenum* *Trichoderma viridi* *Aspergillus spp*	Department of Pharmacy, UoK.
11	*Salvadora persica*	Stems	*Alternaria solani* *Trichoderma harzianum* *Trichoderma viridi* *Penicillium expansum*	Department Of Physics, Uok.

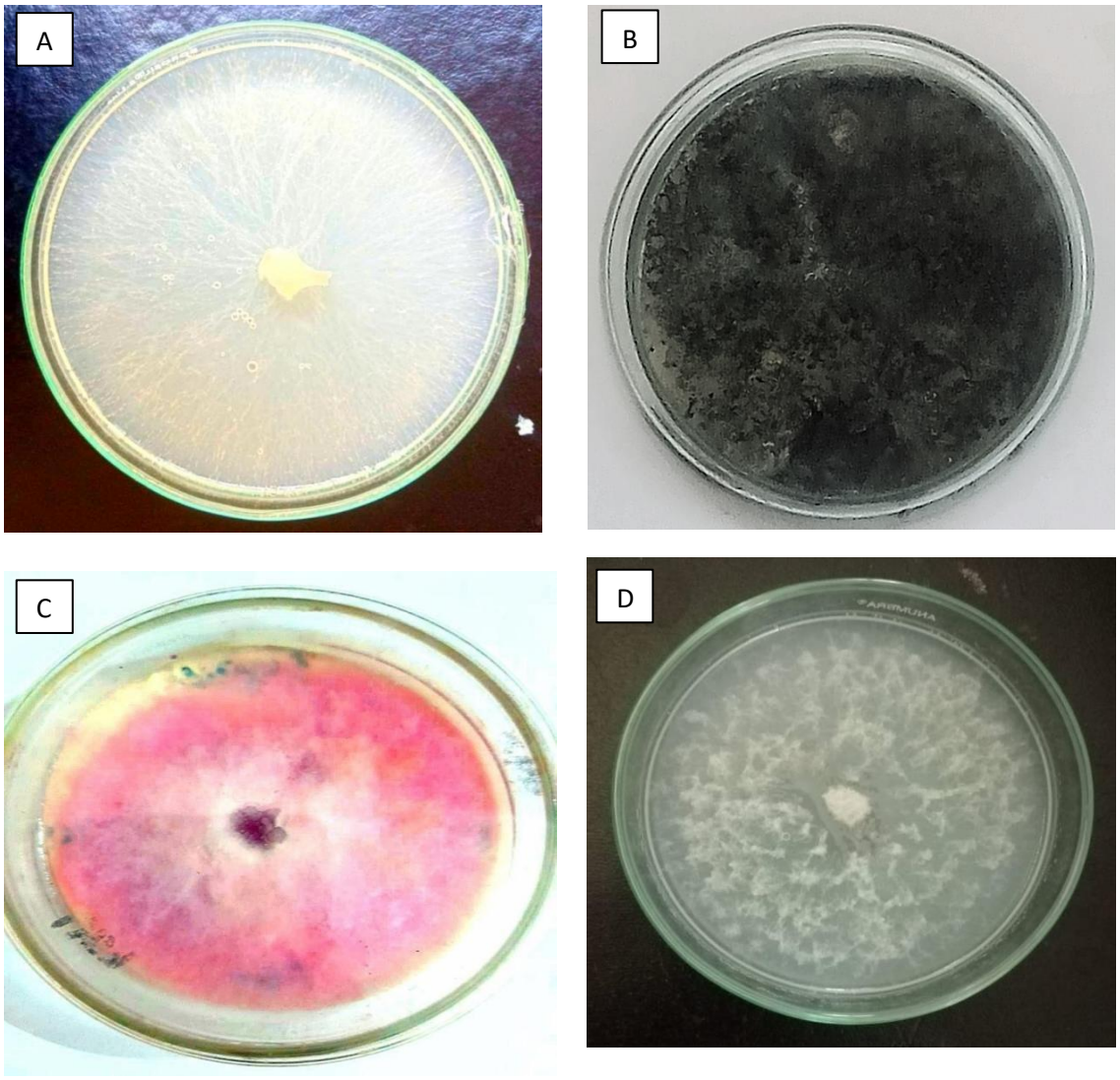

Fig 2: Pure cultures of root rotting fungi maintaned on PDA: A-*Rhizoctonia solani*, B-*Macrophomina phaseolina*, C-*Fusarium oxysporum*, D-*Fusarium solani*

Table 2 Effect of Cell Culture Filtrates from Antagonistic Fungal Strains on Juvenile Mortality of *Meloidogyne incognita* Over 24, 48, and 72 Hours.

Treatment	Mortality %		
	After 24 hours of treatment	**After 48 hours of treatment**	**After 72 hours of treatment**
Control	0	0	0
SpR-TR1 (*T. harzianum*)	28.27	49	95.23
SpR-TR2 (*T. viride*)	33.33	58.39	96
SpS-TR3 (*T. asperellum*)	40.28	56.12	100
AiL-PE1 (*Penicillium expansum*)	39.28	54.67	100
AvR-PE2 (*P. expansum*)	19.04	33.33	100

Strong nematicidal effects on *M. javanica* juveniles were observed with culture filtrates, where *Trichoderma asperellum* (SpS-TR3) exhibited the highest mortality (40.28%) after 24 hours,, followed closely by *Penicillium expansum* (AiL-PE1) at 39.28%. *Trichoderma harzianum* (SpR-TR1) and *T. viride* (SpR-TR2) showed moderate mortalities of 28.27% and 33.33%, respectively, while AvR-PE2 showed the lowest mortality (19.04%) (Table 2).

By 48 hours, mortality increased significantly for all treatments, with *T. viride* (58.39%) and *T. asperellum* (56.12%) performing slightly better than *Penicillium expansum* (54.67%) and *T. harzianum* (49%). AvR-PE2 remained the least effective at 33.33%.

After 72 hours, complete mortality (100%) was observed for *T. asperellum*, *Penicillium expansum*, and AvR-PE2, indicating their full effectiveness. *T. viride* (96%) and *T. harzianum* (95.23%) also showed near-complete mortality. Overall, all fungal strains were highly effective by 72 hours, with variations in their speed of action.

Table 3 In Vitro Effects of Cell Free Culture Filtrates from Antagonistic Microbial Strains Against *Fusarium oxysporum*, *Fusarium solani*, *Macrophomina phaseolina*, and *Rhizoctonia solani.*

In-vitro antagonistic potency of Trichoderma against root rot fungi and root-knot nematodes isolated from medicinal plants

Treatment	*Fusarium solani*	*Fusarium oxysporum*	*Macrophomina phaseolina*	*Rhizoctonia solani*
		Zone of Inhibition (mm)		
		***Trichoderma harzianum* (SpR-TR1)**		
-ve control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+ve control	6.66 ± 0.05	5.00 ± 0.64	8.66 ± 0.85	6.66 ± 0.68
20µL/disc	4.66 ± 0.05	2.66 ± 0.51	4.33 ± 0.52	3.66 ± 0.08
40µL/disc	6.66 ± 0.05	4.66 ± 0.52	10.66 ± 0.51	9.33 ± 0.52
60µL/disc	14.00 ± 0.01	12.66 ± 0.52	13.00 ± 0.01	13.00 ± 0.01
		***Trichoderma viridi* (SpR-TR2)**		
-ve control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+ve control	4.00 ± 0.58	6.66 ± 0.68	11.33 ± 0.04	1.66 ± 0.05
20µL/disc	3.33 ± 0.05	4.33 ± 0.05	4.66 ± 0.51	3.33 ± 0.05
40µL/disc	4.66 ± 0.50	6.33 ± 0.05	10.33 ± 0.16	10.00 ± 0.64
60µL/disc	10.00 ± 0.07	9.66 ± 0.50	12.66 ± 0.04	12.00 ± 0.64
		***Trichoderma asperellum* (SpS-TR3)**		
-ve control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+ve control	8.33 ± 0.96	6.00 ± 0.58	10.66 ± 0.05	3.00 ± 0.03
20µL/disc	3.33 ± 0.05	4.66 ± 0.51	4.00 ± 0.05	4.66 ± 0.05
40µL/disc	9.33 ± 0.72	6.66 ± 0.78	6.33 ± 0.51	7.33 ± 0.21
60µL/disc	10.33 ± 0.68	8.33 ± 0.37	15.00 ± 0.03	12.33 ± 0.16
		***Penicillium expansum* (Ail-PE1)**		
-ve control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+ve control	7.00 ± 0.35	2.66 ± 0.51	7.66 ± 0.16	4.33 ± 0.85
20µl/disc	2.66 ± 0.51	6.66 ± 0.05	1.66 ± 0.88	2.66 ± 0.51
40µl/disc	8.33 ± 0.05	4.66 ± 0.51	3.33 ± 0.51	5.33 ± 0.50
60µl/disc	8.33 ± 0.02	4.33 ± 0.85	7.66 ± 0.65	7.33 ± 0.93
		***Penicillium chrysogenum* (AvR-PE2)**		
-ve control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+ve control	11.00 ± 0.04	5.00 ± 0.07	3.00 ± 0.06	3.33 ± 0.05
20µl/disc	17.33 ± 0.31	4.00 ± 0.60	4.66 ± 0.50	5.33 ± 0.08
40µl/disc	6.66 ± 0.57	6.33 ± 0.51	5.00 ± 0.35	5.33 ± 0.05
60µl/disc	6.66 ± 0.05	7.33 ± 0.05	7.66 ± 0.65	8.33 ± 0.15

The initial value indicates the mean value and ± standard deviation.

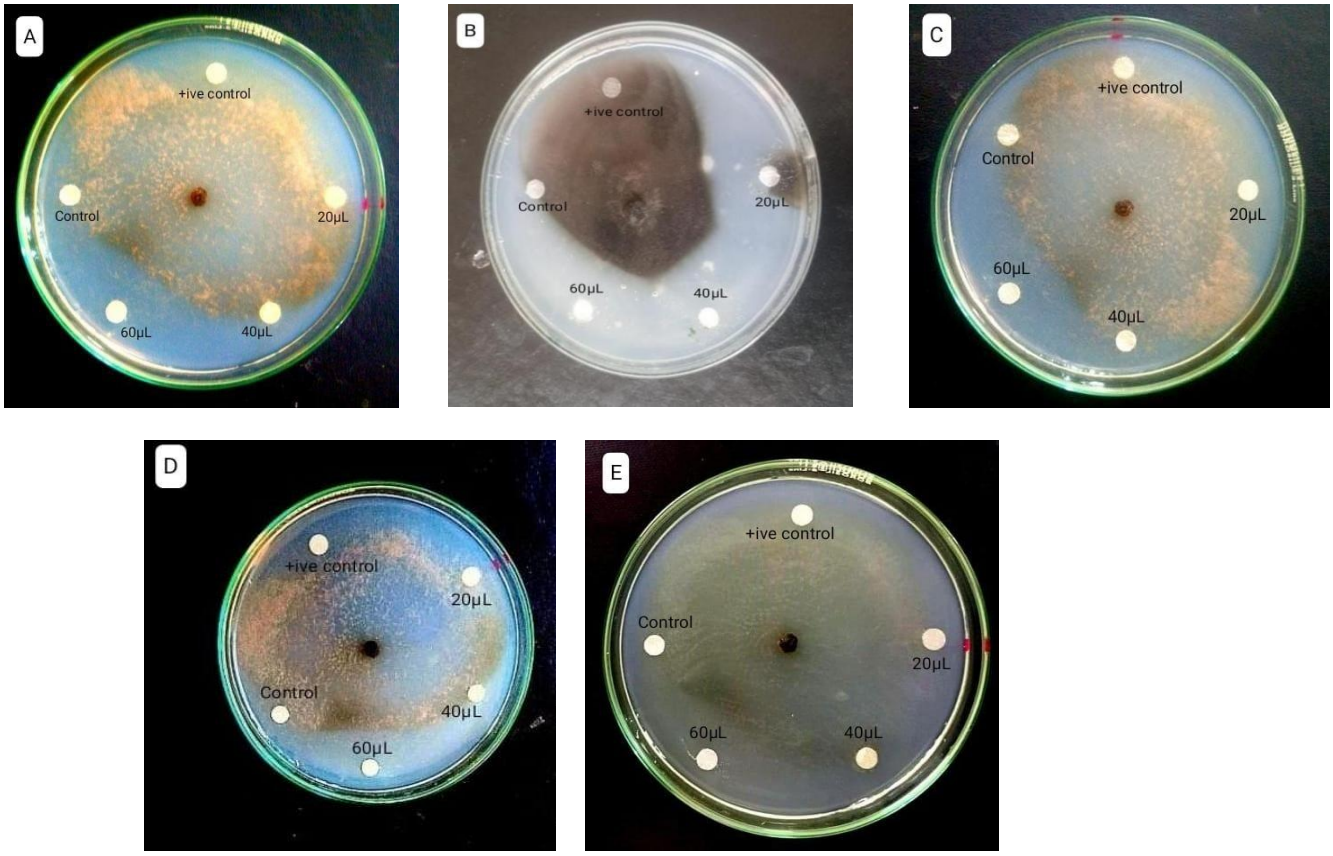

Fig 3: Inhibition of *M. phaseolina* by Culture filtrates from A= SpR-TR1, B= SpR-TR2, C= SpR-TR3, D= AiL-PE1 and E= AvR-PE2

Discussion:

The findings of this study underscore the significant potential of endophytic antagonistic fungi as biocontrol agents against pathogenic fungi and nematodes. The observed antimicrobial effects of cell-free filtrates from antagonistic fungal strains, including *Trichoderma harzianum*, *T. asperellum*, *T. viride*, *Penicillium expansum*, and *Penicillium chrysogenum*, suggest that biologically active compounds in these filtrates play a vital role in suppressing pathogenic microorganisms. This aligns with Rehan et al. (2024), who reported similar inhibitory effects by microbial cell-free filtrates. The application of these filtrates as eco-friendly alternatives to chemical pesticides offers a promising strategy for sustainable agriculture, consistent with the findings of Tariq et al. (2008).

In vitro evaluation revealed that *Penicillium expansum* exhibited the highest inhibitory effect against root-rotting pathogens, including *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina*, and *Rhizoctonia solani*. Its inhibition zones (6.66–7.33 mm at 60 µL/disc) were superior to those of other tested fungi, demonstrating its broad-spectrum antifungal activity. Among the *Trichoderma* species, *T. viride* showed greater effectiveness compared to *T. asperellum* and *T. harzianum*, with maximum inhibition zones of 6.66 mm for *F. solani* and 6.33 mm for *M. phaseolina*. These findings corroborate previous studies emphasizing the role of *Trichoderma* strains as potent biocontrol agents for managing soil-borne pathogens (Ashrafizadeh *et al*., 2005).

Additionally, all antagonistic filtrates demonstrated significant inhibition of *M. phaseolina*, with inhibition zones ranging from 9.5 to 16 mm, highlighting their strong antifungal potential. The occasional overlapping of pathogenic growth with the antagonistic fungal disc suggests variability in the diffusibility of active metabolites, a phenomenon noted in other studies on biocontrol efficacy (Tariq *et al*., 2008).

The nematicidal activity of these endophytic fungi against *Meloidogyne incognita* further reinforces their potential as biocontrol agents. For instance, cell-free culture filtrates of *Trichoderma asperellum* (SpS-TR3) and *Penicillium expansum* (AiL-PE1) resulted in complete juvenile mortality after 72 hours, demonstrating their efficacy in managing root-knot nematodes. This aligns with Farhat *et al.* (2022), who highlighted the potential of fungal endophytes in producing novel bioactive metabolites with nematicidal properties.

These findings emphasize the dual role of antagonistic fungi in sustainable agriculture: improving soil health by suppressing root-rot pathogens and nematodes, and reducing the dependence on chemical inputs. Furthermore, the production of secondary metabolites by these fungi holds promise for therapeutic applications in medicine and agrochemicals. This study's focus on indigenous *Trichoderma* strains and *Penicillium* species from diverse localities demonstrates the potential of locally adapted fungal strains in biocontrol, paving the way for the development of fungal-based products for agricultural and pharmaceutical use.

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