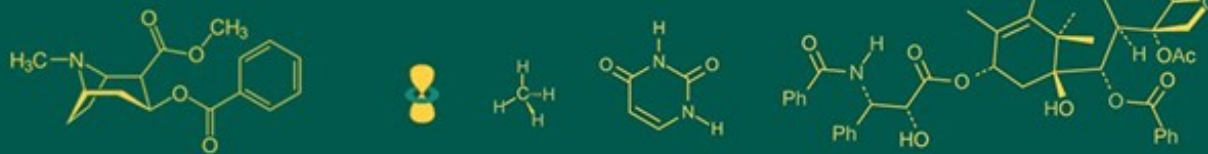


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To study in vitro antagonist activity against *Fusarium oxysporum* f.sp. *ciceri*

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ABSTRACT

This study establishes a reliable and efficient protocol for evaluating the in vitro antagonistic activity of Endophytic fungi against *Fusarium oxysporum* f.sp. *ciceri* (Foc), the causal agent of *Fusarium wilt* in chickpea (*Cicer arietinum* L.). Seven Endophytic isolates recovered from healthy chickpea roots were *Aspergillus niger*, *A. flavus*, *Trichoderma asperellum*, *T. harzianum*, *T. hamatum*, *Metarhizium anisopliae*, and *Penicillium chrysogenum* were screened against Foc using the dual culture technique on Potato Dextrose Agar. Antagonism was assessed by measuring radial growth of the pathogen in dual culture against an uninoculated control, and percent inhibition of mycelial growth was calculated using Vincent's formula. Plates were incubated at 28 ± 2 °C and monitored until the pathogen fully colonized the control. *Aspergillus Niger* showed the highest antagonistic activity, inhibiting Foc mycelial growth by 88.02%, followed by *Metarhizium Anisophille* 81.03% inhibition. *Penicillium* recorded the lowest inhibitory effect 53.36%. Statistical analysis (ANOVA, CRD) confirmed significant differences among isolates in their inhibitory potential ($p < 0.05$). The study confirms that *Aspergillus Niger*, *Metarhizium Anisophille*, *Trichoderma Asperellum* possess strong biocontrol potential against Foc under in vitro conditions. This optimized screening protocol is applicable for advanced studies in field-level biocontrol validation, formulation development, and sustainable management of *Fusarium wilt* in chickpea.

Keywords: Biocontrol, Chickpea (*Cicer arietinum* L.), Dual culture technique, Endophytic fungi, *Fusarium oxysporum* f.sp. *ciceri*, In vitro antagonism, Mycelial inhibition

1. Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceri* (Foc), continues to be the single most destructive biotic constraint on chickpea production, with the pathogen's persistence as soil-borne chlamydospores and its capacity to generate new virulent races steadily eroding the durability of host resistance (Haware and Nene, 1982; Jiménez-Díaz *et al.*, 1989). While chemical fungicides and resistant cultivars remain the mainstay of disease management, their limitations including fungicide resistance, residue accumulation, and the breakdown of race-specific resistance have driven sustained interest in biological alternatives (Trapero-Casas and Jiménez-Díaz, 1985).

Among biocontrol agents evaluated against Foc, species of *Trichoderma* have received the most extensive attention, owing to their multiple modes of antagonism mycoparasitism, antibiosis, and competition for space and nutrients (de Cara-García *et al.*, 2021) [1]. Dennis *et al.* (1971) [2] were among the first to characterize the antibiotic basis of *Trichoderma* antagonism, and this mechanistic framework has since been extended to numerous *Fusarium* pathosystems. Dubey *et al.* (2007) [3] demonstrated that *Trichoderma* species could be integrated into chickpea wilt management programmes with measurable gains in seed germination and grain yield, while comparative *in vitro* screenings have repeatedly shown wide variation in antagonistic efficacy among *Trichoderma* isolates and species against Foc, with inhibition of mycelial growth ranging from below 40% to above 85% depending on isolate origin and species (Kumari *et al.*, 2023; Kumari *et al.*, Legume Research). More recent work has confirmed this variability under both native and consortium-based applications, with combined *Trichoderma* inoculations often outperforming single-isolate treatments in suppressing Foc mycelial growth and reducing wilt incidence in pot and field trials (Chohan *et al.*, 2024) [4]. Beyond *Trichoderma*, other components of the chickpea-associated mycobiota including *Aspergillus* and *Penicillium* species have also been reported as Endophytic antagonists of soil-borne pathogens, although their biocontrol potential against Foc specifically remains far less characterized than that of *Trichoderma* (Rajamanikyam *et al.*, 2017). The standard method for screening such antagonistic potential remains the dual culture (confrontation) technique on PDA, in which radial growth of the pathogen in the presence of a candidate antagonist is compared against an uninoculated control, and percent inhibition is calculated using the formula of Vincent (1947) [5]. The nature of the interaction overgrowth, mycoparasitism, or the formation of

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an inhibition zone is further assessed using the antagonism rating scale of Bell *et al.* (1982) [6], which remains the most widely applied qualitative index in fungal biocontrol screening.

Given that the Endophytic isolates recovered from healthy chickpea roots in the present study include genera with established or plausible biocontrol activity particularly *Trichoderma asperellum*, *T. harzianum*, and *T. hamatum* evaluating their *in vitro* antagonistic potential against Foc represents a logical extension of the isolation and characterization work already completed. This assessment was therefore undertaken to determine which of the seven recovered endophytes possess measurable antagonistic activity against Foc, and to characterize the nature of that antagonism, providing a screened shortlist of candidates for future glasshouse and field-level biocontrol validation.

2. Materials and methods

2.1 Test pathogen and antagonists

The virulent isolate of *Fusarium oxysporum* f.sp. *ciceri* (Foc), purified and maintained, served as the test pathogen. The seven Endophytic fungal isolates recovered and identified as *Aspergillus niger*, *A. flavus*, *Trichoderma asperellum*, *T. harzianum*, *T. hamatum*, *Metarhizium anisopliae*, and *Penicillium chrysogenum* served as the test antagonists. Both pathogen and antagonist cultures were maintained on PDA slants at 8 °C and sub-cultured onto fresh PDA plates 5-7 days prior to the assay to ensure actively growing mycelium.

2.2 In vitro screening by dual culture technique

The antagonistic potential of each Endophytic isolate against Foc was evaluated *in vitro* by the dual culture (paired-plate/confrontation) technique on PDA, following the method of Dennis *et al.* (1971) [2] as widely adapted in subsequent biocontrol screening studies (Dubey *et al.*, 2007) [3]. A 5 mm mycelial disc, cut from the actively growing margin of a 5-7 day old culture using a sterile cork borer, was placed on one side of a 9 cm Petri plate containing 20 ml of PDA, approximately 1 cm from the periphery. A corresponding 5 mm disc of the test antagonist was placed on the opposite side of the same plate,

equidistant from the centre. Plates inoculated with Foc alone, without an antagonist, served as the control. All plates were incubated at 28 ± 2 °C in a BOD incubator and observed daily.

Each treatment (seven antagonists + control) was replicated three times and arranged in a Completely Randomized Design (CRD).

2.3 Measurement of radial growth and percent inhibition

Radial growth of the Foc colony was measured in two directions toward the antagonist and toward the plate periphery using a millimeter scale/digital Vernier caliper, once the pathogen had completely covered the control plate (usually 6-7 days post-inoculation). Percent inhibition of mycelial growth was calculated using the formula of Vincent (1947) [5], as adopted in numerous subsequent dual culture antagonism studies (Dubey *et al.*, 2007; Kumari *et al.*, 2023) [3, 7]:

$$I(\%) = \frac{C - T}{C} \times 100$$

Where,

I = percent inhibition over control,

C = radial growth of Foc in the control plate (mm), and

T = radial growth of Foc in the direction of the antagonist in dual culture (mm).

2.4 Categorization of antagonistic interaction

The nature and intensity of each antagonistic interaction was further classified using the rating scale of Bell *et al.* (1982) [6], a five-class index widely used to score the extent of antagonist overgrowth over the pathogen colony Class 1: antagonist overgrows the pathogen completely; Class 2: antagonist overgrows at least two-thirds of the plate; Class 3: antagonist and pathogen colonize approximately equal areas; Class 4: pathogen overgrows at least two-thirds of the plate; Class 5: pathogen overgrows the antagonist completely). Plates were also observed under a stereo-binocular microscope for visual evidence of mycoparasitism, hyphal coiling, or a distinct zone of inhibition at the interaction interface.

Table 1. Instruments and Equipment Used

Instrument/Equipment	Purpose
Laminar Air Flow Cabinet	Aseptic inoculation and plating
Autoclave	Sterilization of media and glassware (121 °C, 15 psi)
BOD Incubator	Incubation of dual culture plates (28 ± 2 °C)
Stereo-binocular Microscope	Observation of colony interaction and mycoparasitism
Compound Microscope	Hyphal/conidial-level examination at interaction zone
Digital Vernier Caliper / mm scale	Measurement of radial mycelial growth
Cork borer (5 mm)	Cutting uniform mycelial discs
Digital camera	Photo-documentation of dual culture plates

2.5 Statistical analysis

Data on radial growth and percent inhibition were subjected to analysis of variance (ANOVA) appropriate to a Completely Randomized Design, using [OPSTAT / SPSS version XX / R software specify whichever you actually used]. Treatment means were compared using Critical Difference (CD) at 5% probability ($p < 0.05$).

3. Results and discussion

3.1 Effect of endophytic fungal isolates on the mycelial growth of *Fusarium oxysporum* f. sp. *ciceri*

The *in vitro* antagonistic activity of seven Endophytic fungal isolates against *Fusarium oxysporum* f. sp. *ciceri* (Foc) was evaluated using the dual culture technique on Potato Dextrose Agar (PDA). The results revealed considerable variation among the tested fungal isolates in their ability to inhibit the mycelial growth of the pathogen. Among

all the isolates tested, *Aspergillus niger* exhibited the highest antagonistic activity, resulting in 88.02% inhibition of the mycelial growth of Foc. This was followed by *Metarhizium anisopliae*, which recorded 81.03% inhibition. The results indicated that these fungal isolates possess strong antagonistic potential against the chickpea wilt pathogen under *in vitro* conditions.

The other isolates, namely *Aspergillus flavus*, *Trichoderma asperellum*, *T. harzianum*, *T. hamatum*, and *Penicillium chrysogenum*, also exhibited inhibitory effects against Foc. However, their antagonistic activity was comparatively lower than that of *A. niger* and *M. anisopliae*. *Penicillium chrysogenum* showed the lowest inhibitory effect, recording 53.36% inhibition of the pathogen's mycelial growth. Thus, the percentage inhibition among the tested Endophytic fungal isolates ranged from 53.36% to 88.02%, demonstrating considerable variation in their antagonistic potential.

The differences in inhibition among fungal isolates may be attributed to variation in their ability to compete with the pathogen for nutrients

and space and to produce inhibitory metabolites. Previous studies have also demonstrated considerable variation among fungal antagonists in their ability to suppress plant pathogenic fungi. Bell *et al.* (1982) [6] reported that the antagonistic activity of *Trichoderma* isolates varied depending on the interacting pathogen and antagonist combination. Similarly, Dubey *et al.* (2007) [3] demonstrated the potential of different *Trichoderma* species and isolates for suppressing *F. oxysporum* f. sp. *ciceri* and managing chickpea wilt.

The superior performance of *A. niger* in the present study indicates its potential as a promising fungal antagonist against Foc. Similarly, the high inhibition recorded by *M. anisopliae* suggests that this isolate may also possess significant antagonistic activity against the pathogen. However, as the present results were obtained under in vitro conditions, further investigations under greenhouse and field conditions are necessary before confirming their effectiveness for biological control of *Fusarium* wilt.

The present findings support previous research demonstrating that fungal biological control agents can suppress the growth of *F. oxysporum* and contribute to the management of chickpea wilt. Recent work has also shown that *Trichoderma* isolates can reduce the mycelial growth of Foc in dual culture and improve chickpea performance under pathogen-infested conditions.

3.2 Comparative antagonistic potential of endophytic fungi against *Fusarium oxysporum* f. sp. *ciceri*

The comparative evaluation of the seven Endophytic fungal isolates demonstrated significant differences in their antagonistic potential against *Fusarium oxysporum* f. sp. *ciceri*. Among the tested isolates, *Aspergillus niger* was identified as the most effective antagonist, followed by *Metarhizium anisopliae*. These results indicate that both fungal isolates have the potential to suppress the growth of Foc effectively under laboratory conditions.

The antagonistic activity was evaluated by comparing the radial growth of Foc in dual culture with its growth in the control treatment. The percentage inhibition of mycelial growth was calculated using the formula proposed by Vincent (1947) [5]:

$$I(\%) = \frac{C - T}{C} \times 100$$

where C represents the radial growth of the pathogen in the control and T represents the radial growth of the pathogen in the presence of the antagonist. The dual culture method and growth inhibition approach provide a useful preliminary screening technique for identifying potential fungal biocontrol agents. Vincent's original work remains the source commonly cited for this inhibition formula.

The variation in antagonistic activity among the isolates indicates that their biological control potential is species- and isolate-dependent. In fungal antagonism studies, the interaction between the antagonist and pathogen may involve competition for nutrients and space, production of inhibitory compounds, and direct antagonistic interactions. The qualitative assessment of fungal interactions may also be performed using the antagonism scale developed by Bell *et al.* (1982) [6], which evaluates the relative colonization and overgrowth of the interacting fungi.

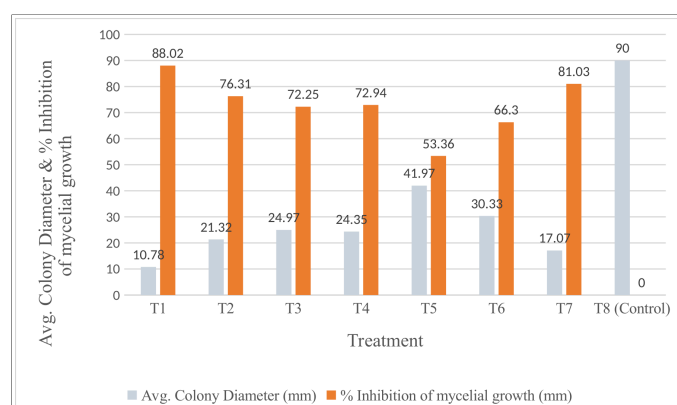
The statistical analysis of the present experiment, conducted using ANOVA under a Completely Randomized Design (CRD), confirmed significant differences among the fungal isolates in their ability to inhibit Foc. Overall, the results demonstrated that *Aspergillus niger* and *Metarhizium anisopliae* were the most promising antagonistic isolates, while the remaining Endophytic fungi also exhibited varying levels of inhibitory activity.

Based on the present findings, the effective fungal isolates can be considered promising candidates for further studies on the biological management of *Fusarium wilt* of chickpea. Their efficacy should subsequently be evaluated under controlled greenhouse and field conditions to determine their ability to reduce disease incidence and sever-

ity and to assess their potential for development as biological control agents.



Figure 1. *in vitro* antagonist activity against *Fusarium oxysporum* f.sp. *ciceri*



4. Conclusion

The present study demonstrated the potential of Endophytic fungi isolated from healthy chickpea roots as antagonistic agents against *Fusarium oxysporum* f. sp. *ciceri* (Foc), the causal organism of *Fusarium wilt* of chickpea. The in vitro evaluation using the dual culture technique revealed considerable variation in antagonistic activity among the seven fungal isolates tested.

Among all the isolates, *Aspergillus niger* exhibited the highest inhibitory activity, with 88.02% inhibition of the mycelial growth of Foc, followed by *Metarhizium anisopliae*, which showed 81.03% inhibition. In contrast, *Penicillium chrysogenum* recorded the lowest inhibition of 53.36%. The remaining isolates also demonstrated varying levels of antagonistic activity against the pathogen.

The results indicate that *Aspergillus niger* and *Metarhizium anisopliae* are promising fungal antagonists for the biological management of *Fusarium wilt* of chickpea under in vitro conditions. The significant differences observed among the fungal isolates further indicate that antagonistic potential is dependent on the individual isolate and its interaction with the pathogen.

Overall, the study confirms that Endophytic fungi obtained from healthy chickpea plants may serve as potential sources of biological control agents against *Fusarium oxysporum* f. sp. *ciceri*. However, since the present investigation was limited to laboratory-based in vitro evaluation, further studies under greenhouse and field conditions are required to validate the effectiveness of the most promising isolates. Future investigations may also focus on their formulation and application for the sustainable management of *Fusarium wilt* in chickpea.

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