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In vitro Evaluation of Three Unexploited Fungi for Their Biocontrol Potential against Selected Phytopathogenic Fungi

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ABSTRACT

Background: The increasing incidence of fungal phytopathogens poses a serious threat to the balance of our ecosystem. While chemical fungicides are commonly used for disease control, their excessive application leads to environmental contamination, the development of resistant pathogen strains and the disruption of beneficial soil microbiota. This has intensified the search for eco-friendly and sustainable biocontrol alternatives.

Methods: In the present study, phytopathogenic fungi *Penicillium citrinum*, *Bipolaris sorokiniana* and *Alternaria alternata* were isolated from diseased *Morus alba* and *Melia azedarach*. Simultaneously, rare and underexplored non-pathogenic fungi, *Purpureocillium sodanum*, *Talaromyces pinophilus* and *Aspergillus ochraceopetaliformis* were isolated from the rhizospheric soil of healthy *Morus alba* trees growing near the Bhakhra riverbank. Fungal identification was conducted based on colony morphology, microscopic features and molecular analysis using 18S rRNA sequencing. The antagonistic potential of these non-pathogenic isolates was evaluated in vitro using dual culture assays against the phytopathogenic fungi.

Result: Dual culture assays revealed significant antagonistic effects, with inhibition percentages ranging from 33.3% to 85.7%. Among the isolates, *P. sodanum* showed the highest inhibition (85.7%) against *A. alternata*, surpassing the commonly reported efficacy of standard *Trichoderma* strains. Enzymatic assays further indicated the production of hydrolytic enzymes, suggesting multiple mechanisms of pathogen suppression. This study presents *Purpureocillium sodanum* as a highly effective in vitro antagonist with superior inhibition compared to standard *Trichoderma* strains. These findings support its potential as a novel, eco-friendly biocontrol agent and warrant further investigation for broader agricultural applications.

Key words: Antagonistic fungi, Dual culture assay, Pathogenic fungi, Rare fungal species, Sustainable agriculture.

INTRODUCTION

Fungal pathogens remain a persistent threat in agriculture and forestry, contributing to significant losses in crop productivity, tree health and ecological stability. Among these pathogens, *Penicillium citrinum*, *Alternaria alternata* and *Bipolaris sorokiniana* are also considered important due to their association with various plant diseases observed during the study. *P. citrinum* causes post-harvest decay in fruits and cereals, often leading to mycotoxin contamination and market rejection (Coutinho *et al*., 2020). *A. alternata*, a necrotrophic fungus, induces leaf spots, blights and fruit rots in various trees and crops (Fagodiya *et al*., 2024). *B. sorokiniana* is a major constraint which triggers root rot, crown rot and leaf blight in grains, ultimately reducing yield (Al-Sadi, 2021).

To manage such diseases, chemical fungicides are commonly employed. However, their widespread and repetitive use has led to critical concerns, including the emergence of resistant fungal strains (Valarmathi, 2018). In addition to resistance, these fungicides contribute to soil and water pollution and negatively affect beneficial microbial populations essential for soil health. Specific compounds such as tebuconazole, propiconazole and kresoxim methyl have been linked to toxicological effects ranging from neurotoxicity and reproductive harm to carcinogenic risks (Li *et al*., 2022; Tabassum *et al*., 2016; Kanungo and Dewhurst, 2019). Over time, these issues undermine the sustainability and effectiveness of chemical-based disease management (Zubrod *et al*., 2019).

In light of these challenges, attention has shifted toward environmentally sound solutions, most notably, biological control using antagonistic fungi. *Trichoderma* spp. are among the most studied biocontrol agents, valued for their ability to outcompete pathogens, parasitise fungal hyphae and produce a wide array of antifungal metabolites (Mukherjee *et al*., 2022). However, despite their prominence, *Trichoderma*-based biocontrol faces notable limitations, including inconsistent field performance due to environmental variability and limited activity against certain pathogens (Pertot *et al*., 2016).

To broaden the biocontrol collection, there is an need to explore lesser-known fungal genera for their potential antagonistic activity against plant pathogens. In this context,

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Purpureocillium sodanum, *Talaromyces pinophilus* and *Aspergillus ochraceopetaliformis* have emerged as promising candidates. *Purpureocillium* species are known for their dual role in suppressing pathogens and promoting plant growth through induced systemic resistance and improved nutrient uptake (Elsherbiny *et al*., 2021; Lan *et al*., 2017; Rigobelo *et al*., 2024). *Talaromyces* spp. have shown notable antifungal activity (Oiuphisittraiwat *et al*., 2024). While many *Aspergillus* species are established biocontrol agents (Ngo *et al*., 2021; Attia *et al*., 2022), *A. ochraceopetaliformis* is a rare, non-pathogenic species with unexplored biocontrol potential. This study aims to evaluate the in vitro antagonistic potential of these three underexplored fungi against the above phytopathogens. The findings will contribute to identifying new, sustainable biocontrol agents capable of mitigating plant diseases while reducing dependence on harmful chemical fungicides.

MATERIALS AND METHODS

Isolation and purification of pathogenic and Non-pathogenic fungal strains

Pathogenic fungal isolates were obtained from plant samples exhibiting visible signs of fungal infection, collected from various locations across Bathinda District, Punjab (India). *Penicillium citrinum* was isolated from infected fruits of *Morus alba* showing signs of fruit rot, *Alternaria alternata* from leaves of *Melia azedarach* exhibiting leaf spot disease and *Bipolaris sorokiniana* from *Morus alba* leaves showing symptoms of leaf spot. The infected plant tissues, including symptomatic leaves and fruits, were surface sterilised using 1% sodium hypochlorite for one minute, followed by three rinses with sterile distilled water to eliminate surface contaminants. Small sections of the sterilised tissue were aseptically transferred to Potato Dextrose Agar (PDA) plates supplemented with ciprofloxacin (1 g/L) to inhibit bacterial growth. The plates were incubated at 28±1°C for 5–7 days to promote fungal growth.

Among the non-pathogenic isolates, *P. sodanum*, *A. ochraceopetaliformis* and *T. pinophilus* were obtained from soil samples collected along the Bhakhra riverbank in Bathinda District. For the isolation of non-pathogenic fungi, soil samples were collected from the zones having healthy-looking trees. Serial dilutions (10^3 to 10^6) of soil samples were prepared using sterile distilled water and aliquots were spread onto PDA plates. The plates were incubated at 28±1°C for 5-7 days (Li *et al*., 2024; Aniekwu *et al*., 2014). Fungal colonies were identified based on their colony morphology and microscopic characteristics.

All experimental procedures, including fungal isolation and preliminary identification, were conducted in the Botany Laboratory at Akal University, Talwandi Sabo, Punjab, during the period from August 2023 to June 2024.

Purification of pathogenic and non-pathogenic fungi

Fungal colonies exhibiting distinct morphologies were subcultured onto fresh PDA plates until pure cultures were obtained. Sub-culturing was performed every 3-5 days to ensure the isolation of single fungal species.

Preliminary identification of pathogenic and non-pathogenic fungi

The pure fungal isolates were characterized based on colony morphology, including colour, texture and growth pattern, followed by microscopic examination using Lacto-Phenol Cotton Blue (LPCB) staining (Gilman, 1957).

Selection and justification of antagonistic fungal isolates

Based on a review of existing literature, the selected antagonistic fungal isolates *P. sodanum*, *T. pinophilus* and *A. ochraceopetaliformis* were identified as non-pathogenic under standard environmental conditions. These fungi have not been reported to cause diseases in plants or humans and have been previously described for their beneficial roles in biological control. Their selection in this study was guided by documented evidence of their antifungal properties and ecological safety, making them suitable candidates for *in vitro* antagonistic assays against phytopatho- genic fungi (Rigobelo *et al*., 2024; Abbas *et al*., 2025; Mohamed *et al*., 2025).

Molecular identification

Pure fungal cultures of both pathogenic and non-pathogenic strains were sent to Barcode Biosciences, Bangalore, for molecular identification through 18S rRNA sequencing. The resulting sequences were analysed and compared with those in the NCBI GenBank database to confirm species identification.

Dual culture assay

To assess the antagonistic potential of the fungal isolates, dual culture assays were performed. Potato Dextrose Agar (PDA) plates were inoculated with a 5 mm diameter agar disc of the pathogenic fungus on one side of the plate and a 5 mm diameter agar disc of the antagonistic fungus on the opposite side, ensuring a 3 cm distance between the two. The plates were incubated at 28°C for 5-7 days. The inhibition zone between the two fungal colonies was measured in millimetres. Control plates contained only pathogenic fungi, incubated under the same conditions. The diameter of inhibition zones was recorded as an indicator of antagonistic activity (Talapatra *et al*., 2017).

Statistical analysis

All experiments were conducted in triplicates and the data were expressed as the mean±standard de*via*tion. The inhibition of pathogen growth by the antagonistic fungi was measured by comparing the radial growth of the pathogen (R_2 Pathogen without antagonist) and (R_1 Pathogen with antagonist). The inhibition percentage was calculated using the following formula:

$$\text{Inhibition percentage} \quad \text{Inhibition percentage} = \frac{R2 - R1}{R2} \times 100$$

RESULTS AND DISCUSSEN

Isolation and identification of pathogenic and antagonistic fungi Three pathogenic fungal species were successfully

isolated from Trees in Bathinda, Punjab (India), which were preliminarily identified based on their morphological characteristics, such as colony appearance, spore formation and microscopic features (Fig 1). The species names of some fungi were confirmed through molecular identification using 18S rRNA sequencing from Barcode Biosciences, Bangalore.

The isolates of Antagonistic fungal strains were subcultured to obtain pure isolates and their preliminary characteri- zation was performed using microscopic observations and colony Morphological characteristics (Fig 2). The species of antagonistic fungi were further identified through molecular methods, specifically 18S rRNA sequencing.

Antagonistic activity of non-pathogenic fungi

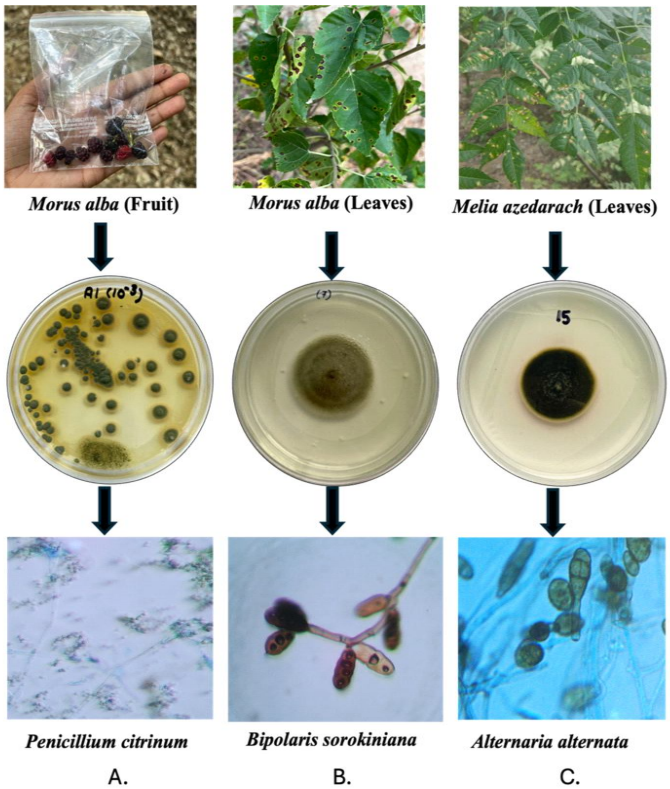
The antagonistic activity of non-pathogenic fungi against the tested pathogenic fungi was evaluated and exhibited varying levels of inhibition against the *tested pathogens* based on the inhibition zone and percentage.

Among the tested endophytic fungi, *P. sodanum* demonstrated the highest inhibition against *A. alternata*, with an inhibition zone of 3.0±0.1 mm (85.7%). This was followed by its activity against *B. sorokiniana* (3.7±0.1 mm, 77.1%) and *P. citrinum* (2.2 ± 0.2 mm, 55.8%).

T. pinophilus exhibited moderate inhibition against *A. alternata* (2.7 ± 0.1 mm, 77.1%) but showed relatively lower inhibitory effects on *P. citrinum* (1.8±0.1 mm, 45.8%) and *B. sorokiniana* (1.6±0.2 mm, 33.3%). *A. ochraceopetaliformis* displayed a balanced inhibitory effect on all tested pathogens, showing the highest inhibition against *B. sorokiniana* (3.0±0.1 mm, 62.5%), followed by *P. citrinum* (2.0±0.2 mm, 50.8%) and *A. alternata* (2.2±0.1 mm, 61.9%) (Table 1).

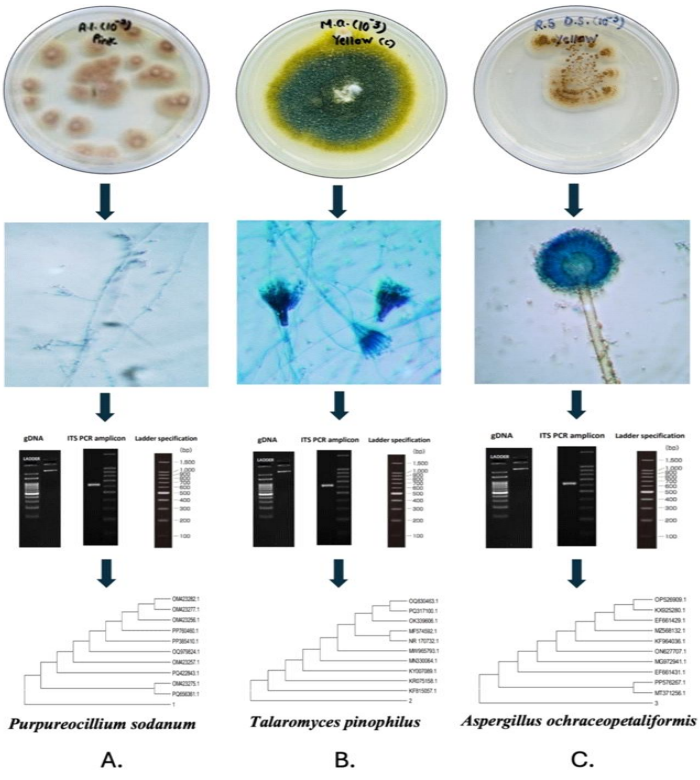
Table 1: Inhibition zone (mm) and inhibition percentage (%) of *P. sodanum*, *T. pinophilus* and *A. ochraceopetaliformis* against the pathogenic fungi *P. citrinum*, *A. alternata* and *B. sorokiniana* in dual culture assays.

Endophytic fungi	*P. citrinum*		*A. alternata*		*B. sorokiniana*	
	Inhibition zone	Inhibition %	Inhibition zone	Inhibition %	Inhibition zone	Inhibition %
P. sodanum	2.2±0.2	55.8	3±0.1	85.7	3.7±0.1	77.1
T. pinophilus	1.8±0.1	45.8	2.7±0.1	77.1	1.6±0.2	33.3
A. ochraceopetaliformis	2.0+0.2	50.8	2.2±0.1	61.9	3+0.1	62.5



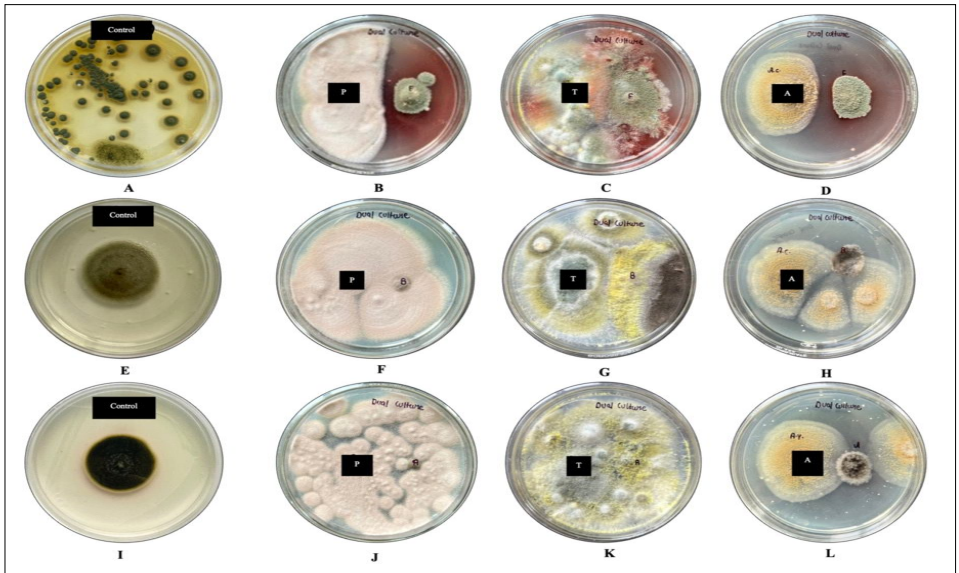
(A) *Penicillium citrinum*, (B) *Bipolaris sorokiniana* and (C) *Alternaria alternata* showing: infected Tree parts (top), Pure cultures and conidiophore structure under 40X magnification.

Fig 1: Morphological identification of pathogenic fungi.



(A) *Purpureocillium sodanum*, (B) *Talaromyces pinophilus and* (C) *Aspergillus ochraceopetaliformis* showing sequential identification steps: pure culture, conidiophore structure under 40X magnification, 18s rRNA sequence and phylogenetic tree.

Fig 2: Morphological and molecular identification of antagonistic fungi.



(A) Control *P. citrinum (B) P. citrinum* with *P. sodanum*; *(C) P. citrinum* with *T. pinophilus*; *(D) P. citrinum* with *A. ochraceopetaliformis*; *(E)* Control *B. sorokiniana*; *(F) B. sorokiniana* with *P. sodanum*; *(G) B. sorokiniana* with *T. pinophilus*; *(H) B. sorokiniana* with *A. ochraceopetaliformis*; *(I)* Control plate of *A. alternata*; *(J) A. alternata* with *P. sodanum*; *(K) A. alternata* with *T. pinophilus*; *(L) A. alternata* with *A. ochraceopetaliformis*.

Fig 3: Dual culture assay of pathogenic fungi with antagonistic fungi.

Overall, *P. sodanum* was the most compelling antagonist, particularly against *A. alternata*, while *T. pinophilus* exhibited the lowest inhibition against *B. sorokiniana*. These results highlight the potential of endophytic fungi as biocontrol agents against pathogenic fungi (Fig 3).

When these findings are compared with previous studies, the superior performance of *P. sodanum* becomes evident. *Trichoderma* spp., which are widely studied and commonly used as biocontrol agents, have been reported to inhibit *A. alternata* by 60-70% (Pandey, 2010) and *B. sorokiniana* by 60-73.7% (Singh *et al*., 2018). In contrast, *P. sodanum* exhibited higher inhibition percentages (85.7% and 77.1%, respectively) in the present study, indicating its strong antagonistic potential. Additionally, no reports were found on the antagonistic activity of Trichoderma against *P. citrinum*, which emphasises the novelty of exploring *P. sodanum* and related genera for biocontrol applications. The enhanced antagonism observed may be attributed to mechanisms such as competition for space and nutrients, secretion of antifungal metabolites, and interference with pathogen growth and development.

CONCLUSION

The present study highlights the significant in vitro biocontrol potential of *Purpureocillium sodanum*, *Talaromyces pinophilus*, and *Aspergillus ochraceopetaliformis* against key phytopathogenic fungi. Dual culture assays revealed inhibition rates ranging from 33.3% to 85.7%, reflecting varying levels of antagonistic activity. Among the tested isolates, *P. sodanum* showed the strongest inhibition, with 85.7% against *A. alternata* and 77.1% against *B. sorokiniana*, positioning it as a promising biocontrol candidate. In contrast, *T. pinophilus* and *A. ochraceopetaliformis* exhibited moderate antagonistic activity and may require further investigation against a broader range of pathogens to fully assess their potential.

The observed antagonism likely results from multiple mechanisms, including nutrient and space competition, secretion of antifungal compounds, and interference with pathogen development. While these in vitro findings are encouraging, further research is essential to validate the biocontrol potential of these fungi under real-world conditions. Future studies should include greenhouse and field trials, along with investigations into the biochemical and molecular mechanisms underlying their antagonism, such as secondary metabolite production and induction of host plant defences. Overall, this study provides important preliminary evidence, especially underscoring *P. sodanum* as a potent, eco-friendly biocontrol agent worthy of further development and testing.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis and decision to publish, or preparation of the manuscript.

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