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Antagonistic effect of *Nigrospora oryzae* against pathogenic fungi in dual culture assays

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Abstract: This study aimed to evaluate the antagonistic activity of the endophytic fungus *N. oryzae* against four important plant pathogenic fungi, namely *Fusarium solani*, *F. oxysporum*, *Alternaria alternata*, and *Acremonium kiliense*, using *in vitro* dual culture assays. The antagonistic activity of *N. oryzae* was assessed using a dual culture method on Czapek Agar (CA) medium. Pure cultures were incubated at 25 °C for 72 h, after which 5-mm mycelial plugs were placed at a distance of 3 cm from the pathogenic fungi and incubated for an additional 72 h. Antagonistic activity was quantified by measuring colony radii (r_1 and r_2). Microscopic observations of the interaction zone were conducted using 1 × 1 cm² agar blocks stained with lactophenol cotton blue to identify antagonistic mechanisms. The results showed that *N. oryzae* inhibited the growth of all tested pathogens, with inhibition rates of 75.26% against *A. alternata*, 62.07% against *A. kiliense*, 43.63% against *F. solani*, and 33.26% against *F. oxysporum*. Macroscopic and microscopic observations indicated that the antagonistic interaction primarily involved competition for space and nutrients, as well as hyphal interactions resembling mycoparasitic behavior, including direct hyphal contact and hyphal coiling. These findings suggest that *N. oryzae* exhibits *in vitro* potential as a biological control agent against certain plant pathogenic fungi, particularly *Alternaria* and *Acremonium*. However, its antagonistic activity against *Fusarium* species was relatively lower, indicating that further studies are required to confirm the underlying mechanisms and to evaluate its effectiveness under more complex conditions.

Keywords: Biocontrol agent, dual culture assay, endophytic fungi, mycoparasitism

Abstrak: Penelitian ini bertujuan untuk mengevaluasi aktivitas antagonistik fungi endofit *N. oryzae* terhadap empat jamur patogen tanaman, yaitu *Fusarium solani*, *F. oxysporum*, *Alternaria alternata*, dan *Acremonium kiliense*, menggunakan uji kultur ganda secara *in vitro*. Aktivitas antagonistik *N. oryzae* diuji menggunakan metode *dual culture* pada media Czapek Agar (CA). Kultur murni diinkubasi pada suhu 25°C selama 72 jam, kemudian potongan miselium berdiameter 5 mm dipasangkan dengan patogen pada jarak 3 cm dan diinkubasi kembali selama 72 jam. Nilai antagonisme dihitung berdasarkan pengukuran jari-jari koloni (r_1 dan r_2). Pengamatan mikroskopis pada zona interaksi dilakukan dengan menyiapkan potongan agar berukuran 1 × 1 cm² yang diwarnai menggunakan lactophenol cotton blue untuk mengidentifikasi mekanisme antagonistik. Hasil penelitian menunjukkan bahwa *N. oryzae* mampu menghambat pertumbuhan keempat patogen dengan tingkat penghambatan yang bervariasi, yaitu 75,26% terhadap *A. alternata*, 62,07% terhadap *A. kiliense*, 43,63% terhadap *F. solani*, dan 33,26% terhadap *F. oxysporum*. Pengamatan makroskopis dan mikroskopis mengindikasikan bahwa interaksi antagonistik terutama melibatkan kompetisi ruang dan nutrisi serta interaksi hifa yang menyerupai perilaku mikoparasitisme, termasuk kontak langsung dan pelilitan hifa. Temuan ini menunjukkan bahwa *N. oryzae* secara *in vitro* memiliki potensi sebagai agen pengendali hayati terhadap beberapa jamur patogen tanaman, khususnya *Alternaria* dan *Acremonium*. Namun, efektivitasnya terhadap patogen *Fusarium* relatif lebih rendah, sehingga diperlukan kajian lanjutan untuk mengonfirmasi mekanisme antagonisme dan mengevaluasi aplikasinya dalam kondisi yang lebih kompleks.

Kata kunci: Agen biokontrol, uji kultur ganda, jamur endofit, mikoparasitisme

INTRODUCTION

Diseases caused by fungi are among the major obstacles to plant health and biological productivity, as fungal pathogens disrupt cellular integrity, interfere with physiological processes, and alter host–microbe interactions (Hajji-Hedfi et al., 2025; Jayathissa et al., 2025). Plant pathogenic fungi employ diverse infection strategies, including necrotrophy, vascular colonization, and latent endophytism, which contribute to their persistence and adaptability across different host species and environmental conditions. Understanding the biological characteristics and interaction mechanisms of these fungi is therefore essential for exploring alternative approaches to disease regulation beyond chemical control.

Among the most biologically significant plant-pathogenic fungi are members of the genera *Alternaria*, *Fusarium*, and *Acremonium*. Species of *Alternaria* are cosmopolitan necrotrophic pathogens capable of infecting more than 400 plant species. Their pathogenicity is closely associated with host tissue necrosis and the production of secondary metabolites, such as alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA), which function as phytotoxins and contribute to disease severity (Fernandes et al., 2023; Ramires et al., 2018). The ability of *Alternaria* species to colonize a wide range of hosts and survive under variable environmental conditions facilitates their ecological success and persistence.

Species of *Fusarium* represent another major group of plant pathogens, particularly as soil-borne fungi that invade root and vascular tissues. *Fusarium solani* and *F. oxysporum* are well known for causing wilt, root rot, and damping-off diseases through mechanisms involving xylem colonization and toxin production (Sampaio et al., 2021). In addition to direct pathogenic effects, many *Fusarium* species produce mycotoxins, including trichothecenes, fumonisins, and zearalenone, which further complicate host–pathogen interactions and enhance their ecological fitness (Colombo et al., 2019). Their persistence in soil and tolerance to chemical treatments underscore the biological limitations of conventional disease control strategies (Chhabra et al., 2024).

Although *Acremonium* spp. are frequently reported as endophytic fungi, they have also been documented as opportunistic pathogens in several plant species. This dual lifestyle enables *Acremonium* to colonize plant tissues asymptomatically and subsequently shift toward pathogenic behavior when hosts experience physiological stress (Aroge et al., 2025). The biological plasticity of *Alternaria*, *Fusarium*, and *Acremonium* highlights the complexity of fungal–plant interactions and the need for control strategies that consider microbial interactions at the biological level.

Biological control has gained attention as an approach to regulate plant pathogenic fungi through antagonism, competition, and mycoparasitism. To date, most biocontrol research has predominantly focused on well-established antagonistic microorganisms, particularly species of *Trichoderma*, owing to their consistent antagonistic performance and well-characterized mechanisms of action (Ramzan et al., 2023; Srivastava et al., 2026). While

these organisms have been extensively studied, the emphasis on a limited number of model biocontrol taxa has resulted in comparatively less attention being given to other fungi with potential antagonistic capabilities.

N. oryzae is a filamentous fungus commonly isolated as an endophyte or saprophyte from a wide range of plant hosts (Dutta et al., 2023). Previous studies have reported that *N. oryzae* produces diverse secondary metabolites with antibacterial and antifungal activities, suggesting its involvement in microbial interaction networks (Li et al., 2014; Liao et al., 2024; Sharma et al., 2025; Thanabalasingam et al., 2015). However, systematic evaluations of its antagonistic activity against multiple plant pathogenic fungi remain limited. Although *N. oryzae* has been described as weakly pathogenic under certain conditions (Zheng et al., 2024), its ecological behavior appears to be strongly context-dependent, emphasizing the importance of controlled *in vitro* studies to characterize its interactions with other fungi.

At present, comparative data examining the antagonistic behavior of *N. oryzae* against pathogens with distinct infection strategies, such as necrotrophic (*Alternaria*), vascular (*Fusarium*), and opportunistic (*Acremonium*) fungi, are still scarce. Consequently, the biological mechanisms underlying its antagonistic interactions and its inhibitory capacity across different pathogenic genera remain insufficiently understood. Addressing this knowledge gap is essential for expanding current understanding of fungal antagonism beyond well-established biocontrol taxa. Therefore, this study aims to evaluate the antagonistic activity of *N. oryzae* against *Alternaria*, *Fusarium*, and *Acremonium* species using *in vitro* dual culture assays, with particular emphasis on growth inhibition patterns and interaction mechanisms. The findings are expected to contribute biological insights into the antagonistic behavior of *N. oryzae* and to clarify its potential role within ungal interaction systems.

METHOD

Testing the antagonism of *N. oryzae* against several species of pathogenic fungi

Pure cultures of *N. oryzae*, *A. alternata*, *A. kiliense*, *F. solani*, and *F. oxysporum* were obtained from laboratory-maintained fungal collections. All isolates were previously identified based on macroscopic colony morphology and microscopic characteristics following standard mycological identification keys (Samson et al., 2004; Samson et al., 2014). Prior to experimentation, each isolate was subcultured to ensure culture purity and active growth.

Fungal culture plugs (5 mm in diameter) were aseptically excised using a sterile cork borer and inoculated in paired combinations on the surface of Czapek Agar (CA) medium, consisting of *N. oryzae* and each pathogenic species. The distance between the inoculation points was set at 3 cm. Czapek Agar was selected as a chemically defined medium to reduce the influence of complex nutrients and to facilitate clearer observation of antagonistic interactions between fungal colonies. The plates were incubated at 25 °C for an additional 72 hours. After incubation, antagonistic interactions were assessed by measuring radial growth of the pathogenic fungi. The percentage of growth inhibition was calculated

according to the formula described by Pukalski et al., (2024), where r_1 represents the radius of the pathogenic fungal colony growing away from *N. oryzae*, and r_2 represents the radius of the colony growing toward *N. oryzae*.

$$\text{Antagonism} = \frac{r1-r2}{r1} \times 100\% \quad (1)$$

Antagonistic activity was evaluated using a qualitative comparative approach by comparing inhibition percentages and visual interaction patterns among the tested pathogenic species.

Macroscopic and microscopic observation of the antagonism mechanism between *N. oryzae* and pathogenic fungi

Macroscopic observations focused on colony morphology, growth patterns, and interaction zones between *N. oryzae* and the pathogenic fungi. For microscopic examination, agar sections (approximately 1 × 1 cm^2) were aseptically excised from the interaction zones using a sterile scalpel. The sections were placed on glass slides, stained with lactophenol cotton blue, and covered with cover glass. The prepared slides were then examined under a light microscope to identify morphological interactions and antagonistic mechanisms between *N. oryzae* and the pathogenic fungi, such as hyphal contact, coiling, and growth inhibition at the interaction interface.

RESULTS AND DISCUSSION

Antagonistic activity of *N. oryzae* against four pathogenic fungi

Dual culture assays revealed that *N. oryzae* exhibited antagonistic activity against all four tested plant pathogenic fungi, ie: *A. alternata*, *A. kiliense*, *F. solani*, and *F. oxysporum*. Both qualitative observations of colony interactions (Figure 1) and quantitative growth inhibition measurements (Table 1) demonstrated clear interspecific differences in susceptibility. These findings indicate that the antagonistic behavior of *N. oryzae* is pathogen-specific and influenced by biological and physiological characteristics of the interacting fungi.

The highest growth inhibition was observed against *A. alternata* (75.26%), followed by *A. kiliense* (62.07%). In contrast, *F. solani* and *F. oxysporum* showed lower inhibition values of 43.63% and 33.26%, respectively. This pattern suggests that *N. oryzae* is more effective against necrotrophic and opportunistic pathogens than against vascular or soil-borne fungi with stronger stress tolerance mechanisms. Similar species-dependent antagonistic responses have been reported in previous dual-culture studies involving endophytic fungi (Hiremani et al., 2020; Meng et al., 2023).

Table 1. Antagonistic activity of *N. oryzae* against four pathogenic fungi

Antaginistic Fungi	Pathogenic Fungi	Antagonistic Activity (%)
N. oryzae	*F. solani*	43.63
N. oryzae	*F. oxysporum*	33.26
N. oryzae	*A. alternata*	75.26
N. oryzae	*A. Kiliense*	62.07

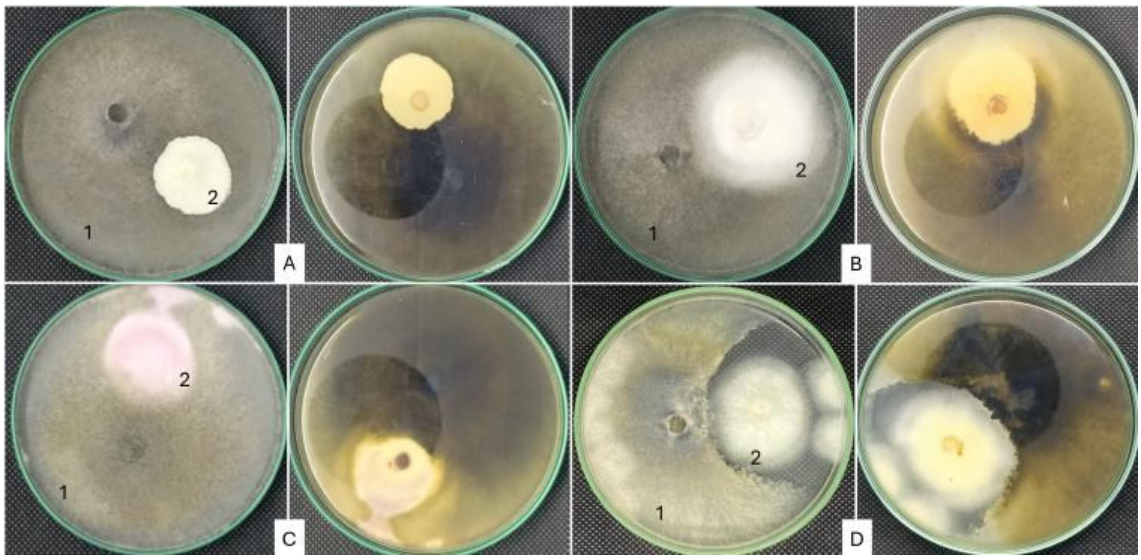

Figure 1. Antagonistic activity of *N. oryzae* against four pathogenic fungi in dual-culture assays on Czapek Agar

(A) Interaction between *N. oryzae (1)* and *Alternaria alternata (2)*

(B) Interaction between *N. oryzae (1)* and *Acremonium kiliense (2)*

(C) Interaction between *N. oryzae (1)* and *Fusarium solani (2)*

(D) Interaction between *N. oryzae (1)* and *Fusarium oxysporum (2)*

Biological basis of differential antagonistic responses

The pronounced susceptibility of *A. alternata* may be attributed to its hyphal morphology, cell wall composition, and metabolic sensitivity. *Alternaria* species typically possess relatively thin-walled hyphae and rely heavily on secondary metabolites during host colonization, which may render them more vulnerable to antagonistic interference under in vitro conditions (Fernandes et al., 2023; Ravat et al., 2017). Moreover, endophytic fungi, including *Nigrospora* spp., are known to produce diffusible antifungal metabolites that can effectively suppress the growth of *Alternaria* species (Ahmed & Zakaria, 2025). These characteristics likely explain the consistently high inhibition values observed in this study.

In contrast, *F. oxysporum* exhibited the lowest level of inhibition, indicating a higher tolerance to biological stress. This tolerance may be linked to its robust physiological defense mechanisms, including thickened cell walls, efficient stress-response pathways, and rapid hyphal regeneration (Chhabra et al., 2024). Additionally, *Fusarium* species are capable of producing their own secondary metabolites and mycotoxins, which may confer competitive advantages during fungal–fungal interactions (Colombo et al., 2019). These traits likely

enable *F. oxysporum* to partially withstand antagonistic pressure from *N. oryzae*, resulting in reduced inhibition.

The intermediate inhibition observed for *F. solani* and *A. kiliense* suggests a transitional level of susceptibility. *Acremonium* spp. are recognized for their ecological plasticity, functioning as endophytes under certain conditions and as opportunistic pathogens under others (Aroge et al., 2025). This dual lifestyle may allow *A. kiliense* to tolerate moderate antagonistic pressure while remaining susceptible to spatial restriction and metabolite-mediated inhibition. Similarly, *F. solani* exhibits moderate physiological resilience, which may account for its intermediate response in dual culture assays.

Interpretation of interaction mechanisms and microscopic observations

Macroscopic observations indicated that antagonistic interactions were primarily characterized by spatial competition, directional colony growth, and restricted pathogen expansion. Microscopic examination revealed close hyphal contact between *N. oryzae* and the pathogenic fungi (Figure 2). However, the available light microscopy images do not provide sufficient evidence to conclusively demonstrate true mycoparasitism. Critical hallmarks of mycoparasitismsuch as host cell wall penetration, hyphal lysis, or internal structural degradation were not unequivocally observed.

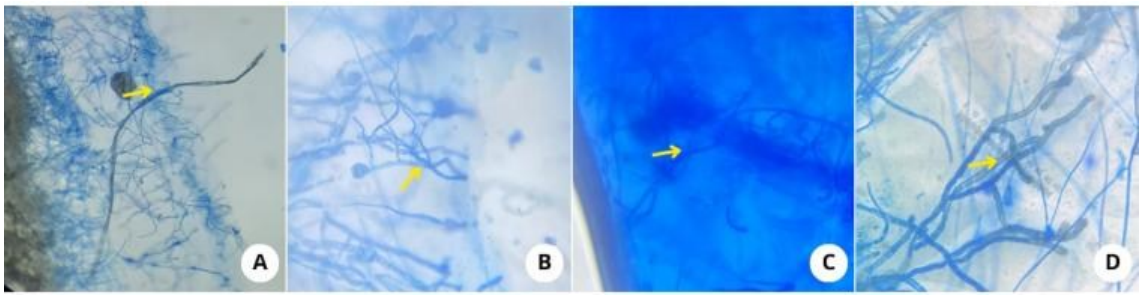

Figure 2. Microscopic observations of hyphal interactions between *N. oryzae* and pathogenic fungi
(A) Hyphal contact between *N. oryzae (yellow arrow)* and *Fusarium solani*.
(B) Directional hyphal growth of *N. oryzae (yellow arrow)* toward *Fusarium oxysporum*.
(C) Close hyphal proximity between *N. oryzae (yellow arrow)* and *Alternaria alternata*.
(D) Spatial dominance of *N. oryzae (yellow arrow)* over *Acremonium kiliense*.

Accordingly, the interactions observed in this study are more appropriately interpreted as contact-mediated antagonism and competitive interference, rather than definitive mycoparasitism. Similar caution has been emphasized in previous studies, which highlight the necessity of ultrastructural analyses (SEM/TEM), specific cell wall staining, or enzymatic assays to substantiate mycoparasitic claims (Harman et al., 2004; Mukherjee et al., 2022). Nevertheless, the close proximity of hyphae and directional growth patterns observed suggest the possible involvement of antibiosis or metabolite-mediated inhibition. *N. oryzae* has been reported to produce a range of bioactive secondary metabolites with antifungal properties, which may contribute to growth suppression of competing fungi (Landum et al.,

2016; Mustofa et al., 2025; Mustofa & Hastuti, 2024). Differences in inhibition outcomes among pathogens likely reflect variation in their physiological sensitivity to these compounds, as well as differences in growth rates and stress-response capacities.

Implications for biological control

The strong antagonistic activity of *N. oryzae* against *A. alternata* highlights its potential relevance in managing diseases caused by *Alternaria* species. Conversely, the lower inhibition observed against *Fusarium* spp. underscores the need for integrated approaches, such as combining multiple biological agents or optimizing formulation strategies. Overall, these findings support the view that *N. oryzae* may serve as a complementary component in biologically based plant disease management, particularly against *Alternaria* and *Acremonium*, while acknowledging its limitations against more resilient pathogens such as *Fusarium oxysporum*.

CONCLUSION

Based on the results of this study, *N. oryzae* exhibited variable antagonistic activity against four plant pathogenic fungi, namely *F. solani*, *F. oxysporum*, *A. alternata*, and *A. kiliense*. The highest level of growth inhibition was observed against *A. alternata*, followed by *A. kiliense*, while *F. solani* and *F. oxysporum* showed lower levels of inhibition. These differences indicate that the antagonistic effectiveness of *N. oryzae* is strongly influenced by the physiological characteristics and stress tolerance of the target pathogens. The antagonistic interactions observed in this study were primarily characterized by spatial competition and nutrient limitation, as evidenced by restricted colony expansion and directional growth patterns in dual culture assays. However, the interaction mechanisms should be interpreted cautiously, as the evidence obtained from light microscopy does not conclusively demonstrate mycoparasitism or other specialized antagonistic processes. The findings of this study demonstrate that *N. oryzae* possesses *in vitro* antagonistic potential against certain plant pathogenic fungi, particularly *A. alternata* and *A. kiliense*. These results provide preliminary biological insights into fungal interactions involving *N. oryzae* and highlight its relevance for further investigation. Additional studies involving *in planta* experiments, formulation development, biosafety evaluation, and assessment under greenhouse or field conditions are required before its applicability in plant disease management can be fully evaluated.

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