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Research Article

# Pathogenicity, Molecular Identification, and Integrated Disease Management of *Nigrospora oryzae* on Rice

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## ABSTRACT

The opportunistic fungal pathogen *Nigrospora oryzae* poses a growing threat to global rice production, especially given its enhanced virulence in co-infections with other pathogens. This study aimed to characterize the pathogen and identify effective integrated disease management strategies. Isolates from Indonesia were identified using morphological and molecular markers, including the ITS and ACT genes, which confirmed a strong genetic link to a rice-native strain from China, ruling out a recent host jump from oil palm. Pathogenicity assays demonstrated the fungus's opportunistic nature, as its virulence and disease progression were significantly heightened when co-inoculated with *Curvularia lunata. In vitro* fungicide tests revealed a high sensitivity to methyl thiophanate ($EC_{50}$ ~250 ppm ) but a lower sensitivity to azoxystrobin. Furthermore, silicon application effectively suppressed the disease, highlighting that some rice varieties responded better than others; the IPB 3S variety experienced a 30% reduction in disease incidence compared to 16% in Situ Bagendit. These findings establish a comprehensive framework for integrated disease management, combining varietal selection and silicon application with targeted chemical control to combat this emerging threat.

## 1. Introduction

Rice (*Oryza sativa*) production, an essential factor of global food security, faces persistent constraints, primarily due to plant diseases. Fungi constitute the largest and most significant group of causal agents, responsible for extensive yield losses. The fungal disease landscape is dynamic, encompassing long-recognized established pathogens and increasingly concerning emerging fungal species (Pedrozo *et al.* 2025). The emergence of these novel threats is driven by factors such as the introduction of foreign races to a new region, the evolution of pathogens to overcome existing host resistance, and the development of fungicide resistance. Furthermore, global climate change, with its associated increases in temperature, humidity, and elevated $CO_2$, acts as a powerful selective pressure, favoring the virulence and genetic adaptation of these pathogens (Roussin-Léveillée *et al.* 2024). The evolving nature of this threat requires continuous monitoring to secure global rice productivity.

*Nigrospora oryzae*, a widespread Ascomycete fungus, has traditionally been categorized as a weak or opportunistic pathogen in rice. Unlike primary invaders, its invasion is often dependent on pre-existing wounds or lesions caused by primary infections or insect damage, utilizing these sites as penetration points. This opportunistic lifestyle contrasts with the endophytic and often beneficial roles played by other *Nigrospora* species (Wiyono *et al.* 2020; Widodo *et al.* 2022; Sodhi and Saxena 2023). However, recent reports documenting significant associated 25% yield losses and its notable saprophytic capacity, which causes research challenges

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through frequent contamination of primary fungal isolations (Wicaksono *et al.* 2017; Liu *et al.* 2021), suggest a potential shift in its virulence status. This potential is heightened by its extremely broad host range, which spans rice, other cereals, and economically crucial tropical crops like oil palm.

Despite the increasing evidence of its pathogenic potential and its high risk as a global threat, there is a critical lack of detailed pathological information regarding *N. oryzae* in Indonesia. Existing studies are limited in scope, sometimes leading to confusion between its symptomatology and that of major pathogens like *Pyricularia oryzae*. The absence of comprehensive data on its virulence and infection risk slows the development of effective integrated disease management strategies, although work on antagonistic bacteria and plant extracts has been reported (Suriani *et al.* 2022). Therefore, the objectives of this study are to thoroughly characterize the symptomatology, verify the identification using morphological and molecular markers, confirm the origins, and determine the virulence of *N. oryzae* on its primary host through controlled single inoculation and co-inoculation trials with established pathogens. Furthermore, the study aims to evaluate the efficacy of novel control tactics, including the application of silica to enhance the host plant's physical barrier and the use of key fungicides, in order to establish a comprehensive integrated disease management approach against this emerging threat to rice productivity.

## 2. Materials and Methods

### 2.1. Pathogen Isolation and Morphological Identification

Fungal pathogens were isolated from symptomatic rice leaves collected from a rice cultivation area within an oil palm nursery field. A direct plating method was employed for the isolation process. The collected leaves were washed thoroughly under running tap water to remove surface debris, then allowed to air dry. Subsequently, small, approximately 5 × 5 mm leaf pieces were cut from the lesion margins to include both healthy and diseased tissue. For surface disinfection, the pieces were immersed in 70% ethanol for one minute, followed by three times of one-minute rinses in sterile distilled water (Ramalashmi *et al.* 2017). The sterile pieces were air-dried on sterile filter paper before being aseptically placed onto petri dishes containing potato dextrose agar (PDA) (HiMedia, IN). The plates were incubated at 25±3°C under a 12/12-hour dark/light photoperiod until fungal colonies began to grow. Individual colonies were then sub-cultured to new PDA plates to obtain pure isolates. Initial identification of the isolates was performed by examining their macroscopic and microscopic characteristics. Macroscopic features recorded included colony colour, texture, and growth rate. For microscopic analysis, a portion of the colony was mounted on a slide with a drop of sterile water to observe the morphology of key structures such as mycelia, conidia, and conidiophores under a light microscope.

### 2.2. Molecular Identification using ITS and ACT Genes

Fungal colonies grown on PDA were subcultured into potato dextrose broth (PDB) (HiMedia, IN) to promote mycelial growth. A 5 mm mycelial plugs from 7-d-old colony were transferred from a pure culture into tubes containing 500 µL PDB, which were then incubated on an orbital shaker for 3 days. Genomic DNA was subsequently isolated from the mycelia using the Abd-ElSalaam *et al.* (2003) method. The isolated DNA was then used as a template for PCR amplification using two primer sets. The primers for the ITS region were ITS1F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4R (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR program was as follows: pre-denaturation at 94°C for 5 minutes; followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 50 seconds, and extension at 68°C for 1 minute; with a final extension at 68°C for 7 minutes.

Because this pathogen has a wide host range, genetic relationships among the host-origin isolates were determined using the Actin (ACT) gene. For the ACT gene, the primers were ACT512F (5'-ATG TGC AAG GGT TTC GC-3') and ACT783R (5'-TAC GAG TCC TTC CCC AT-3'). The program was similar but with a different annealing temperature: annealing at 60°C for 30 seconds, and extension at 68°C for 1 minute; with a final extension at 68°C for 7 minutes. The resulting PCR amplicons were visualized by gel electrophoresis using a 2% agarose gel (Simply GeneDirex, US) in TAE1x (HiMedia, IN). Electrophoresis was performed at 100 volts for 30 minutes, and DNA bands were visualized with GelRed (Biotium, US).

DNA sequencing was performed by Apical Scientific (formerly 1st BASE) via PT Genetika Science. Sequence editing and consensus construction were performed using BioEdit, and the resulting contig was analyzed via NCBI BLAST to identify sequence similarity and taxonomic affiliation. Phylogenetic analysis was conducted in MEGA X, where a Neighbor-Joining tree was inferred with 1,000 bootstrap replicates to assess node support.

### 2.3. Pathogenicity Assay and Co-Inoculation Test

A pathogenicity assay was established using leaf samples from rice and oil palm, following a completely randomized design (CRD) with five replications per treatment. To facilitate a co-inoculation treatment, a Curvularia lunata isolate, previously obtained from rice and known to be pathogenic to oil palm (Susanto and Prasetyo 2013; Priwiratama *et al.* 2024), was selected. The *N. oryzae* and *C. lunata* isolates used were originally collected from infected rice leaves in Riau, Indonesia. The *C. lunata* isolate sequence was submitted to GenBank (accession number PV698394.1).

The experiment comprised four treatments: P1 (rice inoculated with *N. oryzae*), P2 (rice co-inoculated with *N. oryzae* and *C. lunata*), P3 (oil palm inoculated with *N. oryzae*), and P4 (oil palm co-inoculated with *N. oryzae* and *C. lunata*). The primary goal of including the co-inoculation treatments (P2 and P4) was to confirm the proposed role of *N. oryzae* as a weak or opportunistic pathogen by comparing disease progression against the single-pathogen treatments (P1 and P3).

The pathogenicity assay followed the methodology described by Petrović *et al.* (2023). Leaf samples were first washed, air-dried, and placed on moistened tissue in sterile containers. The leaves were then surface-sterilized using an alcohol-soaked cotton swab. Wounding was performed by pricking the leaves with a sterile needle before a 5-mm agar plug containing $2.3 \times 10^5$ conidia $mL^{-1}$ (*N. oryzae*) and $3.3 \times 10^5$ conidia $mL^{-1}$ (*C. lunata*) fungal mycelium at 7-day-old was placed on the inoculation site. For co-inoculations (P2 and P4), a separation distance of 2 cm was maintained between the two fungal plugs. All plugs were covered with sterile-water-injected tape to maintain the high humidity necessary for conidial germination. The tape was removed after 24 hours. Disease severity was subsequently quantified by measuring the percentage of infected leaf area by visual scale on days 1, 3, 5, and 7 after inoculation. The single-pathogen treatments (P1, P3) served as the baselines for evaluating the effects of co-inoculation.

### 2.4. *In-Vitro* Fungicide Sensitivity Test

The sensitivity of *N. oryzae* to fungicides was evaluated using the fungicide-amended PDA, as described by Boonsang *et al.* (2014). This test used two fungicides with the active ingredients azoxystrobin and thiophanate methyl at five concentrations: 0 ppm (control), 250 ppm, 500 ppm, 750 ppm, and 1,000 ppm. Each treatment was replicated three times. Stock solutions of each fungicide were prepared according to the manufacturers' recommendations. A 5 mm diameter agar plug from a 7-day-old *N. oryzae* culture was inoculated onto the center of each plate. The plates were incubated at 25-28°C for 8 days, or until the colony in the control treatment reached the full diameter of the plate. Colony diameter was measured daily, and the relative inhibition rate (RIR) was calculated for each treatment using the following formula:

$$\text{RIR}\ (\%) = \frac{\begin{matrix}\text{Colony diameter} \\ \text{on control}\end{matrix} - \begin{matrix}\text{Colony diameter} \\ \text{on treatment}\end{matrix}}{\text{Colony diameter on control}} \times 100\%$$

### 2.5. Efficacy of Silicon Application on Disease Incidence

The efficacy of silicon in controlling *N. oryzae* infection was evaluated in a factorial completely randomized design. The experiment included two factors: two rice varieties (IPB 3S and Situ Bagendit) and two treatments (control and silica application), with three replications for each combination. Before planting, all seeds were treated with hot water at 50°C for 20 minutes to eliminate other potential seed-borne pathogens. After this, all seeds were inoculated by submerging them in a suspension of *N. oryzae* for 24 hours. The seeds were then planted in trays containing sterilized soil.

For the silica treatment, 2 g of silica (commercial $SiO_2$ 99.8%) per kg of soil were thoroughly mixed into the growing medium. The control trays received only sterilized soil without silica. Each tray had 20 planting holes. The percentage of germination was observed at 1 and 2 weeks after sowing (WAS). At 3 WAS, observations for disease symptoms began and were recorded. Disease incidence was calculated using the formula from Cooke (2006): $I\ (\%) = n/N \times 100$ with I: disease incidence (%), n: number of infected plants, and N: total number of plants observed. Re-isolation of the pathogen was performed from symptomatic plants in all treatments to confirm that *N. oryzae* indeed caused the observed symptoms.

### 2.6. Data Analysis

Data obtained were analyzed using ANOVA and furtherly tested by Tukey test at $\alpha$ 0.05. For the fungicide treatment, the value of effective concentration at 50% level ($EC_{50}$) was determined by Probit analysis using Polo Plus Le Ora software at 95% significance level.

## 3. Results

### 3.1. Isolation and Identification *Nigrospora oryzae* From Rice Leaves

The symptom of *Nigrospora oryzae* infection on rice is a distinct leaf spot that initially presents as a whitish-yellow lesion encircled by a light brown necrotic border. While the initial symptoms of this pathogen can be difficult to distinguish from those of rice blast caused by *Pyricularia oryzae*, the diseases are readily separable in their advanced stages. Specifically, *N. oryzae* infection produces lesions that are relatively oval and frequently located on the leaf margin, contrasting sharply with the characteristic diamond-shaped or rhomboidal lesions caused by *P. oryzae*. Furthermore, the early symptoms of blast consistently feature a straw-gray spot with a distinct central accumulation of grayish spores (conidia), a visual trait absent in *N. oryzae* lesions. Colonies of *N. oryzae* are white for up to 2 days post-inoculation (DPI) before turning light gray and developing a cottony texture due to profuse aerial hyphae growth; the fungus also exhibits a rapid growth rate of approximately 13 mm per day. Microscopically, the isolate is characterized by septate hyphae and distinctive lunate (semicircular) conidiophores that bear terminal, black, and spherical (globose) conidia, all features summarized in Figure 1.

The identity of *N. oryzae* was confirmed through molecular characterization targeting the internal transcribed spacer (ITS) region of the ribosomal DNA. Amplification using the ITS1 and ITS4 primer pair yielded a product of approximately 500 base pairs (bp), as verified in Figure 2A. Subsequent nucleotide homology analysis demonstrated that the test isolate exhibits over 99% sequence homology with previously registered N. oryzae isolates in GenBank. This sequence data has been deposited of the isolate in GenBank under the accession number PV698395.1.

Based on sequence analysis compared to GenBank data, the closest related isolates to the *N. oryzae* isolate from this study showed relatively broad sequence diversity (Figure 3). Some highly homologous isolates originated from diverse hosts, including rice, bamboo, and apple. Given the potential for differences in host specificity between *N. oryzae* isolates from rice and other hosts, and the inherent time required for

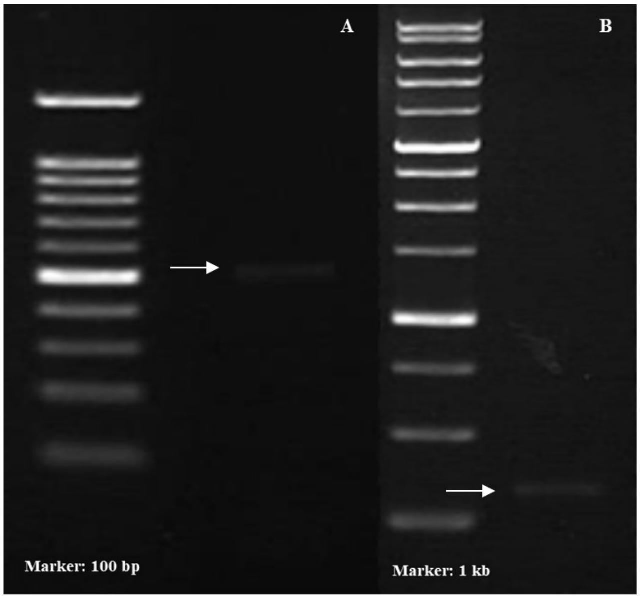

Figure 2. Electrophoresis visualization of *Nigrospora oryzae* isolates. (A) Amplified DNA shows band at ±500 bp for ITS locus amplification, (B) positive result at ±300 bp for ACT locus

Figure 1. Nigrospora leaf spot on rice leaves by *Nigrospora oryzae*. (A-B), Symptoms at initial infection appeared as a white spot surrounded by a light-brown edge and turned longer after 5 days. (C), colony appearance on potato dextrose agar medium 7 days after subculturing stage. (D), septate hyphae, conidiophore (hyaline) and conidia (dark, rounded structure) (C). Bar: 10 µm

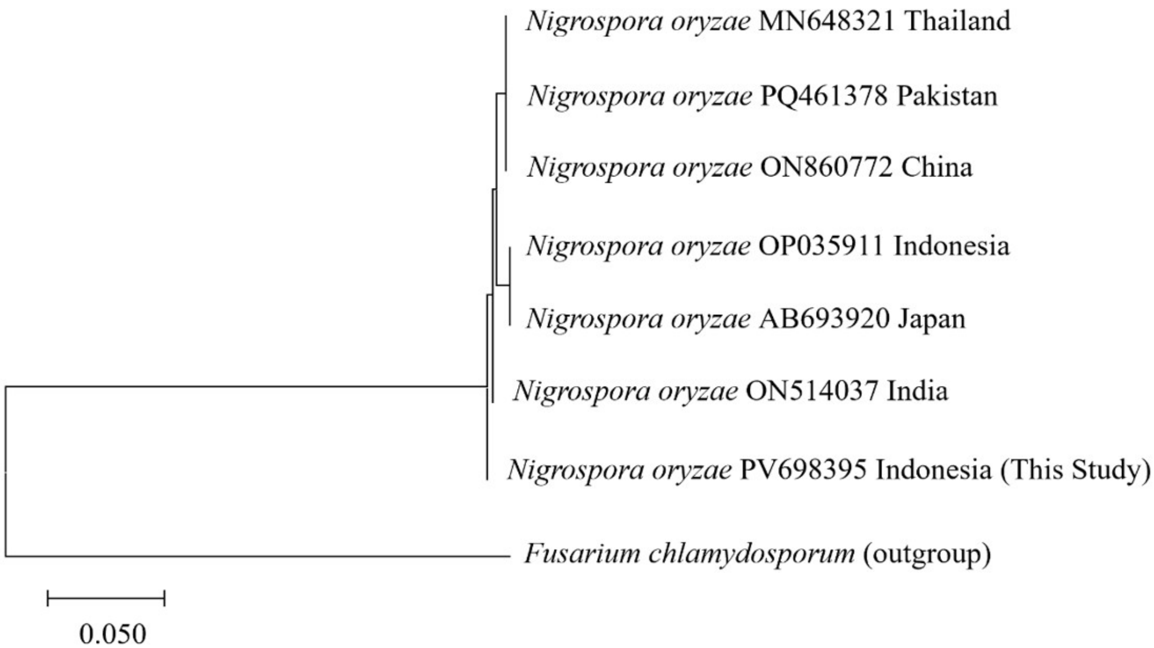

Figure 3. Dendrogram of *Nigrospora oryzae* isolates from this study compared to other *N. oryzae* isolates on the GenBank using neighbour-joining tree on MEGA X

host adaptation, the precise identity of the *N. oryzae* isolate concerning its original host required further confirmation.

To resolve the question of host origin, Actin gene amplification was performed, yielding a DNA band of approximately 300 base pairs (bp) (Figure 2B). Nucleotide homology analysis of this gene region demonstrated a 100% of homology with a *N. oryzae* isolate causing rice leaf spot disease from China (accession number CP096802.1), which has undergone whole genome sequencing. This finding strongly suggests that the rice-origin isolate in this research is native to rice.

## 3.2. Pathogenicity Evaluation of *Nigrospora oryzae* using Single and Co-Inoculation

Inoculation with *N. oryzae* demonstrated variability in symptom appearance, incubation period, and disease progression (lesion area expansion) across the different host and inoculation treatments. Specifically, single inoculation of *N. oryzae* on rice leaves resulted in symptoms beginning at 3 DPI. Conversely, on oil palm leaves, symptoms only manifested from *N. oryzae* when the leaves were co-inoculated with *C. lunata*, although in this co-inoculation scenario, the incubation period for *N. oryzae* was notably shorter, at 2 days (Table 1). Interestingly, the pathogenicity assay revealed that the symptom appearance of *N. oryzae* consistently preceded that of *Curvularia* sp., particularly in the co-inoculation treatments (Figure 4).

Table 1. Incubation period of *Nigrospora oryzae* pathogenicity assays with co-inoculation with *Curvularia lunata*

| Treatment | Incubation period (days post inoculation)$^{a}$ |
|---|---|
| P1 rice *N. oryzae* only | 3.0±0.0$^{b}$ |
| P2 rice *N. oryzae* + *C. lunata* | 3.0±0.0$^{b}$ |
| P3 oil palm *N. oryzae* only | - |
| P4 oil palm *N. oryzae* + *C. lunata* | 2.0±0.4$^{a}$ |

[a] Means followed by same letter is not significantly different by Tukey test at α 0.05

Both pathogenic fungi (*N. oryzae* and *C. lunata*) were capable of infecting both rice and oil palm leaves. Disease severity was consistently higher in treatments where *N. oryzae* was co-inoculated with *C. lunata* on both rice and oil palm, compared to single-inoculation treatments. This data on lesion area strongly indicates that the *N. oryzae* fungus grows and develops more rapidly when an infection by another pathogen is present. Separately, the lesion area for each fungus was measured and terminated once the two lesions met to prevent measurement bias. Interestingly, in the co-inoculation treatments, the symptom appearance of *N. oryzae* on rice coincided with the first symptoms of *C. lunata*, whereas *N. oryzae* infection on oil palm appeared earlier than the first *C. lunata* symptoms, showing a rapid disease progress (Figure 5).

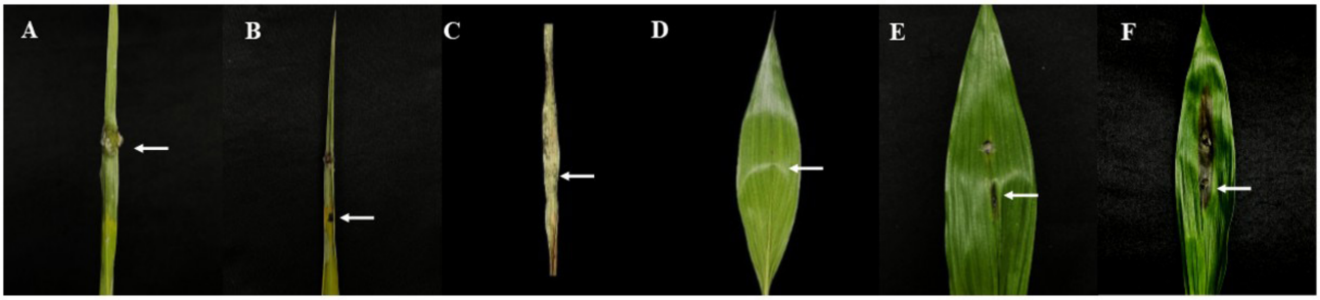

Figure 4. Development of symptoms resulting from *Nigrospora oryzae* inoculation on rice (A-C) and oil palm (D-F). (A) *N. oryzae* without *Curvularia lunata*, (B) *N. oryzae* co-inoculated with *C. lunata* at 3 days post-inoculation (DPI), (C) *N. oryzae* co-inoculated with *C. lunata* at 7 DPI, (D) *N. oryzae* without *C. lunata*, (E) *N. oryzae* co-inoculated with *C. lunata* at 3 DPI, (F) *N. oryzae* co-inoculated with *C. lunata* at 7 DPI

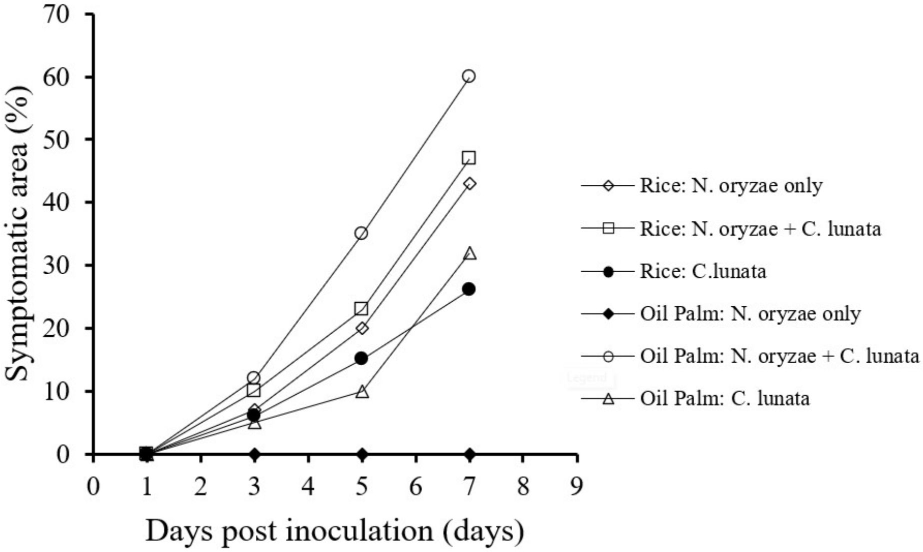

Figure 5. Development of symptomatic area of *Nigrospora oryzae* both in single and co- inoculation methods compared to that of *Curvularia lunata* alone

This evidence confirms that *N. oryzae* functions as a secondary pathogen or opportunist. Secondary pathogens can escalate the severity of existing symptoms and disease, even though they are not the primary causal agent. *N. oryzae* likely exploits the host plant's weakened condition and associates with the primary pathogen, perhaps, as the rapid progression suggests, by being triggered by a chemical compound or metabolite produced by *C. lunata* that enables it to grow and develop more aggressively than the primary causal agent. The *N. oryzae* symptoms on rice were characterized by leaf chlorosis accompanied by dark brown leaf spots, while *C. lunata* produced blackish spots. On oil palm, *N. oryzae* caused dark brown to blackish, spreading lesions, whereas *C. lunata* exhibited its characteristic symptom: spots with a distinct black border and a grayish-white center. The symptoms produced by both pathogens in this assay were consistent with those observed in the field, confirming that both are capable of infecting rice and oil palm plants.

### 3.3. *In Vitro* Sensitivity Assays to Fungicide

An *in vitro* sensitivity test was conducted using two fungicides with broad-spectrum control: azoxystrobin and methyl thiophanate. The results revealed significant differences in the ability of the fungicides to suppress *N. oryzae*. Growth of *N. oryzae* was still observed at the highest tested concentration of azoxystrobin (1,000 ppm), with the fungus maintaining a nearly normal radial growth pattern despite substantial inhibition (Figure 6). Conversely, methyl thiophanate achieved maximal inhibition of *N. oryzae* growth at a much lower concentration of 250 ppm. Probit analysis confirmed this differential sensitivity, showing the concentration required for 50% inhibition ($EC_{50}$) was approximately 400 ppm for azoxystrobin but only 250 ppm for methyl thiophanate (Table 2). This data indicates that *N. oryzae* is highly sensitive to methyl thiophanate, making this fungicide a promising component for an integrated disease management strategy aimed at suppressing nigrospora leaf spot disease.

Table 2. Effective concentration 50% ($EC_{50}$) of azoxystrobin and thiophanate methyl in *Nigrospora oryzae* inhibition at 8 days post-inoculation

| Fungicide's active ingredients | $EC_{50}$ (ppm) | $EC_{90}$ (ppm) |
|---|---|---|
| Azoxystrobin | 388 (298–425) | 1,081 (886–1,451) |
| Tiophanate methyl | 249 (233–338) | 678 (504–812) |

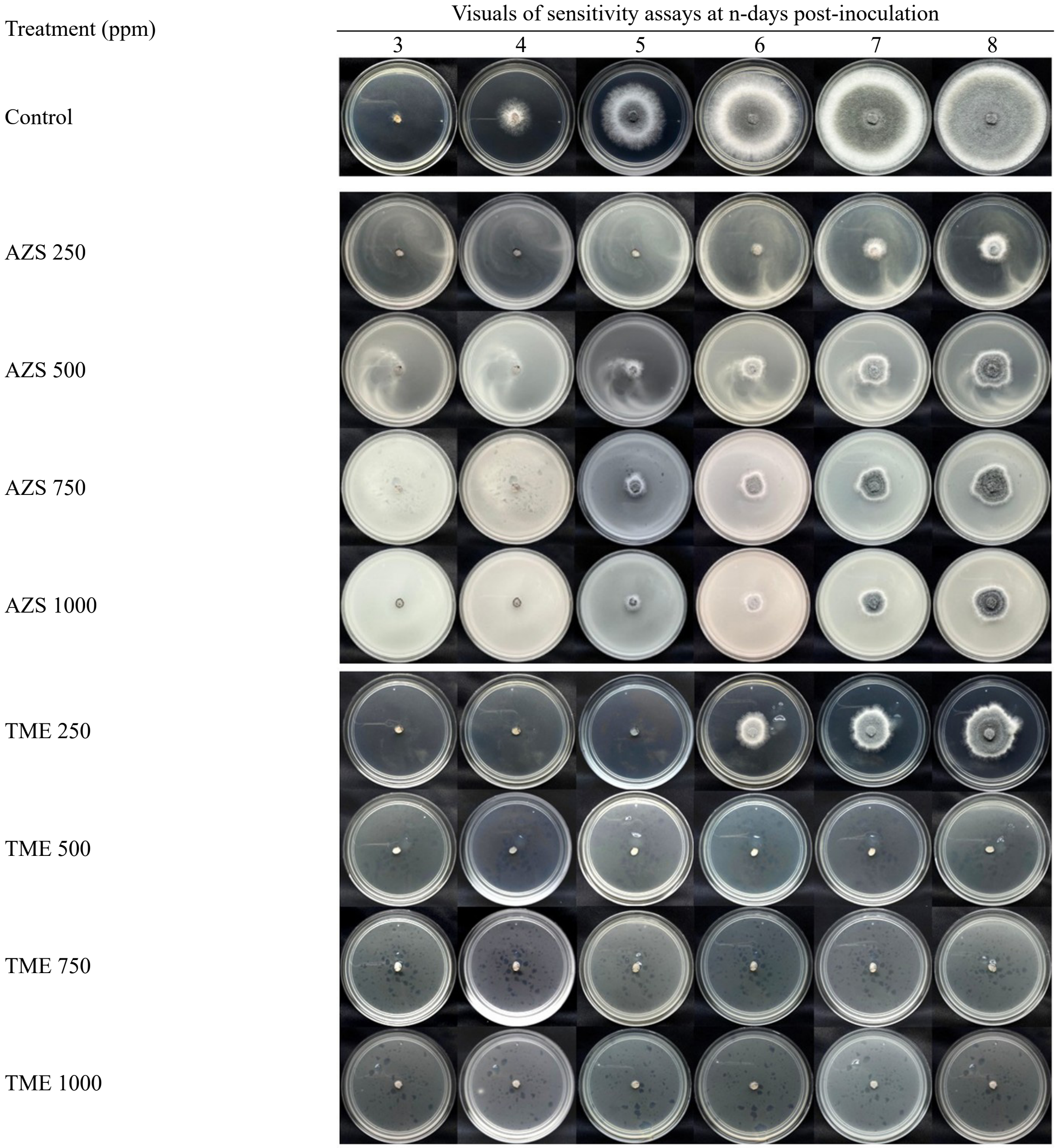

Figure 6. Visualization of *Nigrospora oryzae* growth on fungicide-amended potato dextrose agar at 3 to 8 days post-inoculation

## 3.4. Silicon Application to Disease Incidence

Following seed inoculation with the *N. oryzae* spore suspension, the first symptoms appeared at 2 weeks post-inoculation, peaking at 3 weeks post-inoculation. The symptoms were characterized by brownish spots on the leaves (Figure 7A). Compared to each control, the silica treatment reduced disease incidence, with a varied host response observed among the different rice varieties used (Figure 7B).

The percentage of disease incidence in the silica treatment was lower than in the control, confirming that silica application effectively promotes pathogen suppression. The suppressive effect of silica showed a distinct varietal response: it reached a maximum of 30% reduction in disease incidence for the IPB 3S variety, versus a 16% reduction for the Situ Bagendit variety. Because *N. oryzae* is a seed-borne pathogen, using silica is essential as an early defense against infection. Furthermore, choosing the right variety partners well with silica to improve its performance. This was clear in the results: IPB 3S had less initial infection and responded better to silica than Situ Bagendit. These findings confirm that combining plant genetics with farming practices provides the most reliable disease control.

## 4. Discussion

*Nigrospora oryzae* has been globally documented as a causal agent of leaf spot across diverse hosts, yet its specific role in rice pathosystems has remained under-characterized. In this study, morphological analysis proved to be a reliable primary diagnostic tool; the oval lesions observed on leaf margins provide a clear field distinction from the diamond-shaped lesions typical of rice blast caused by *Pyricularia oryzae*. Molecular validation using the Actin gene confirmed the isolate's identity, showing high sequence homology with rice-origin strains from China. This genetic relatedness suggests that the pathogen is a native rice inhabitant rather than the result of a recent host jump from oil palm (Abass and Mohammed 2014), a finding that helps improve disease control and biosecurity.

The pathogenicity assays further clarify the ecological niche of *N. oryzae*, confirming its status as an opportunistic or secondary pathogen. While the fungus lacked the primary virulence factors necessary to infect oil palm independently, its incubation period was shortened and disease progression was significantly accelerated when in association with *Curvularia lunata*. This fungal synergy is likely mediated by the

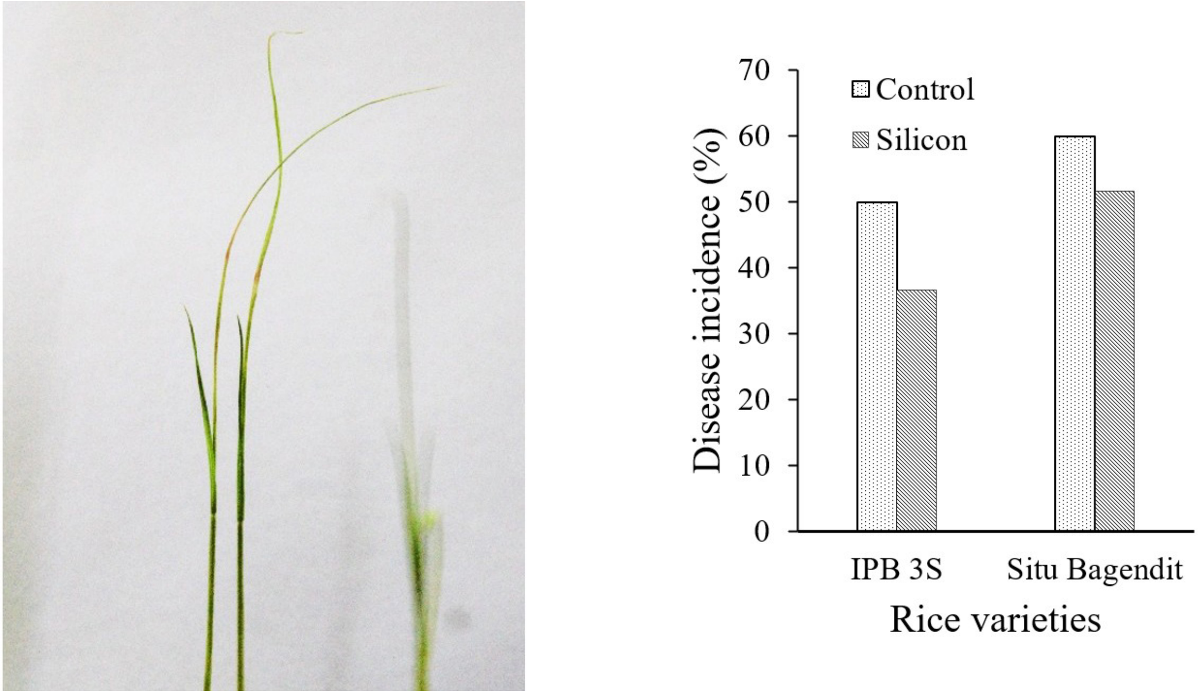

Figure 7. (A) The symptoms of leaf spot caused by *Nigrospora oryzae* infection on 3-week-old rice seedlings, (B) decrease of the disease incidence on two rice varieties upon silica treatment

production of specific phytotoxins by *C. lunata*, such as methyl 5-(hydroxymethyl)furan-2-carboxylate (M5HF2C) or (R)-(-)-mellein, which compromise host cell membrane integrity and suppress local defense responses (Mehta *et al.* 2022). Such a relationship highlights a critical aspect of pathosystem ecology: in a field setting, the impact of *N. oryzae* is likely underestimated, as its pathogenicity is largely driven by primary pathogens that compromise host defenses and facilitate secondary infection.

Regarding integrated disease management, our trials identified both chemical and cultural strategies to mitigate infection. *In vitro* assays revealed high sensitivity to thiophanate methyl ($EC_{50}$ approximately 250 ppm), which interferes with fungal mitosis, whereas the higher $EC_{50}$ for azoxystrobin (approx. 400 ppm) suggests a potentially reduced sensitivity or the early development of tolerance within the population. Beyond chemical control, silicon (Si) application proved effective, though its success varied by rice variety. The wetland cultivar (IPB 3S) achieved a greater reduction in disease incidence than the dryland variety (Situ Bagendit), likely stemming from superior Si-uptake efficiency and epidermal deposition. These results align with Vu *et al.* (2022), illustrating how Si functions as both a physical barrier and a biochemical signal. Furthermore, such Si-mediated defense is well-documented for enhancing resistance against major pathogens like *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani* (Song *et al.* 2016; Bahtiar *et al.* 2021).

In summary, this study changes how we view *N. oryzae*, showing it is a real threat rather than a harmless fungus, and offers practical ways to control it. While this study provides a broad overview, future research should use comparative genomics to explore why the fungus prefers certain hosts and use metabolomic profiling to identify the exact chemical signals driving the *N. oryzae–C. lunata* complex. These findings provide a complete framework for managing emerging leaf spot diseases through a combination of targeted fungicide applications and the use of silicon-enhanced host resistance.

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