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Identification and Biological Control of *Neopestalotiopsis chrysea* Causing Leaf Spot Disease in *Ocimum sanctum* L.

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Key words: Tulsi, Leaf spot disease, *Neopestalotiopsis chrysea*, Antagonistic fungi, Botanical extracts, Vegetative growth

Abstract

Neopestalotiopsis chrysea has emerged as an aggressive pathogen in nurseries and fields, causing concerns among medicinal plant growers. The purpose of the present study was to isolate, identify, and characterize the pathogenic fungus associated with leaf spot disease of tulsi (*Ocimum sanctum* L.), and to investigate as well as into the antagonistic effects of specific plant extracts and selected biocontrol agents, such as *Trichoderma* spp., as alternatives to conventional fungicides. *Neopestalotiopsis chrysea* causes leaf spot symptoms in *Ocimum sanctum* during its early and mature phases. The polymerase chain reaction (PCR) results showed an internal transcribed spacer (ITS) region of 557 bp in *N. chrysea* isolate JUF0129 and its GenBank accession number PX376321.1, and a BLAST search indicated a 100% sequence match with *N. chrysea* (PV866874.1). Pathogenicity testing confirmed the correlation between the fungus and disease symptoms in greenhouse environments. Mycelial growth of *N. chrysea* was highest on the potato dextrose agar (PDA) medium (55.33 mm), followed by the malt extract agar (MEA) medium, and lowest on the yeast extract agar (YEA) medium (31.11 mm). *N. chrysea* grew and developed mycelially best at 25°C and pH 7. Against the mycelial growth and development of *N. chrysea*, *Trichoderma atroviridae* exhibited the highest mycelial growth inhibition (68.94%), followed by *T. virens* (63.82%). The antifungal properties of *Andrographis paniculata* are more effective than those of *Lawsonia inermis*. *A. paniculata* exhibited the maximum inhibition (60.85%) at the highest concentration (30%), which was much more than that of *L. inermis*. Fungal growth is considerably inhibited as the treatment concentration increases. This study provides a valuable resource for future investigation and the development of effective environment-friendly disease management strategies against pathogenic fungi.

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Introduction

Tulsi (*Ocimum sanctum*), commonly known as holy basil, is a widely cultivated medicinal herb, appreciated for its therapeutic, fragrant, and religious significance, and belongs to the Lamiaceae family (Baliga et al. 2013). It is believed to have originated in north-central India, although its cultivation has grown across tropical and subtropical nations, including Nepal, Bangladesh, Sri Lanka, Myanmar, Thailand, some regions of Africa, and South America (Cohen 2014). It is a branched, erect herb, and a mature plant attains a height of roughly 75 to 90 cm. Its leaves are typically spherical and up to 5 cm long with a margin. *O, sanctum* grows best at temperatures ranging from 20 to 35°C, and it requires moderate rainfall of 70-100 cm per year (Kumar et al. 2013). In Bangladesh, tulsi is grown both as a household plant and in small commercial farms, particularly in the districts of Barishal, Khulna, Cumilla, and Rajshahi.

O. sanctum is sensitive to several biotic stresses, particularly fungal diseases that affect its leaves, stems, and roots. The fungus *Curvularia trifolii* and *Rhizoctonia solani* generate the leaf spot and leaf blight diseases, which inflict both quantitative and qualitative losses to this crop (Yadav et al. 2024). Leaf spot symptoms often start as small, water-soaked lesions on the leaves, which eventually grow, become necrotic, and frequently result in premature leaf drop (Patel and Hedawoo 2022). Fungal infections significantly reduce leaf production and quality, with severe infections causing yield losses of up to 40-60%. Khatun et al. (2024) examined leaf spots on tulsi, identifying *Alternaria* spp. as one of the causative organisms.

Neopestalotiopsis chrysea is emerging as a destructive pathogen responsible for leaf spot symptoms. These illnesses not only impair photosynthetic efficiency and biomass growth but also adversely affect the production of bioactive compounds, consequently reducing medicinal quality (Chowdhury et al. 2021). Therefore, there is a compelling need to explore sustainable biocontrol strategies against these growing diseases, especially under both *in vitro* and *in vivo* situations. Plant pathogenic fungi cause various plant diseases on a variety of tropical and sub-tropical plant parts such as roots, fruits, seeds, storage tissues, stems, and vascular wilt. Moreover, isolates of *Fusarium* sp. can spread through soil and from infected plant debris (Rahman et al. 2024). The maximum mycelial growth, as well as the sporulation of *Alternaria* sp., were recorded at a temperature of 25°C and pH 6.5 on PDA medium (Sultana et al. 2020).

The genomic DNA containing the internal transcribed spacer (ITS) region is highly helpful for identification and evaluating the phylogenetic relationships, as shown by recent molecular phylogenetic research (Alam and Rahman 2020). The ITS region of rDNA is thought to vary between species and even between strains (Alam et al. 2010). There is a prodigious opportunity and need for conducting a comprehensive study on the molecular depiction of *N. chrysea*, which causes leaf spot disease of tulsi. Therefore, the present research work has been undertaken to isolate and identify the pathogenic fungus that causes leaf spot disease of *O. sanctum* using classical fungal taxonomy and

molecular techniques. Furthermore, the effects of cultural conditions, botanical extracts, and biological control on the isolated and identified fungus were investigated.

Materials and Methods

Leaf spot disease of *Ocimum sanctum* was collected from the Botanical garden, at the Department of Botany, Jahangirnagar University, and the farmhouse garden in Manikganj from November 2024 to March 2025. The collected diseased samples were sealed in sterile polyethylene bags to prevent secondary infection. The laboratory experiments were conducted in the Department of Botany, Jahangirnagar University (JU), Savar, Dhaka-1342, Bangladesh. Using conventional protocols and relevant literature, a pathogenic fungus was isolated and identified using the tissue planting methods based on the characteristics of the colony, mycelium, conidiophore, and conidia (Akter et al. 2022). The net house in the Botanical Garden of JU was used for *in vivo* pathogenicity testing.

For molecular identification, 10-day-old fresh culture mycelia of the chosen pathogenic fungus, *Neopestalotiopsis chrysea*, were collected from PDA culture media. White et al. (1990) reported that several fungal species employ the universal primers ITS1F (5′-TCCTTAGGTGAACCTGCGG-3″) and ITS4R (5′-TCCTCCGCTTATTGATAT GC-3″) for PCR amplification of rDNA. The PCR was performed using an Applied Biosystems 2720 Thermal Cycler PCR device. After 25-30 cycles, the best amplification of the intended products is frequently achieved. To improve the quality of the PCR results, we conducted 35 cycles. Samples were sent to First BASE Laboratories (SdnBhd, Malaysia) for sequencing following the PCR product's purification. The DNA sequences were confirmed using MEGA11 and Bioedit.

The BLAST program from the NCBI was used to assess the recovered sequence in more detail. Using their default settings, Mega Blast and nucleotide BLAST were chosen to compare the ITS sequence. The similarity percentage and BLAST lowest expected value were used to identify the species. After analysis, GenBank received the sequence. The sequence's accession number was found based on the submission results. Each fungal isolate's nucleotide sequences were retrieved from GenBank in order to construct the phylogenetic tree. Phylogenetic analysis was done using MEGA 11 software, and sequence alignment was done using the Clustal-W algorithm (Thompson et al. 1994). To undertake phylogenetic inference, the Maximum Parsimony (MP) approach was applied with bootstrap values of 1000 replicate runs (Cho et al. 2010, Lee et al. 2010).

For the mycelial growth and development of *N. chrysea*, six distinct culture media were utilized in this experiment: Potato Dextrose Agar (PDA), Richard Agar Medium (RAM), Yeast Extract Agar (YEA), Carboxymethyl Cellulose Agar (CMC), Czapek's Dox Agar (CDA), and maltose yeast extract agar (MEA). To determine a suitable temperature for the investigated fungus, experiments were conducted at 15, 20, 25, 30, and 35°C. Five distinct pH levels, viz. pH 5, 6, 7, 8 and 9 were adjusted to the PDA medium using standard methods (Sikder et al. 2024).

For botanical extracts, fresh leaves of *Andrographis paniculata* and *Lawsonia inermis* were collected, washed, and processed to produce aqueous extracts using standard methods (Rahman et al. 2024). Following the integration of these botanical extracts into the PDA medium, three distinct concentrations by volume were attained (T1: 10%, T2: 20%, and T3: 30%). To assess the extracts' inhibitory efficacy against *N. chrysea*, the poisoned food technique following the standard method (Mondal et al. 2017).

To assess the efficacy of antagonistic fungi, *Trichoderma atroviridae* and *T. virens* have been used against *N. chrysea* with conventional techniques. Following that, the sample was incubated at 27 ± 2°C for seven days. The control plates containing the fungus being tested were also grown to facilitate comparisons. The experiment was repeated three times. After incubation, the radial mycelial growth of the experimental plates was measured. According to Alam et al. (2023), the percentages of Trichoderma's growth inhibition of the examined fungi were calculated.

The data on mycelial development and inhibition of the isolated fungus by culture media, temperature, pH, and treatment with environmentally friendly control measures were considered normal using one-way ANOVA with Duncan's post-hoc test in SPSS.

Results and Discussion

Infected leaf symptoms include distinct necrotic lesions. Leaf spots are circular or sub-circular dark brown dots with slightly swollen centers and darker borders. The circles vary in size and are scattered across the leaf lamina (Fig. 1A). Mycelial growth on a PDA plate is resilient and concentric. The colony is cottony or filamentous, mostly white, with abundant aerial mycelium that forms unique ring-like patterns from the center outward (Fig. 1B). The hyphae are narrow, branching, and visibly septate. The staining reveals the various cell compartments in the mycelial network (Fig. 1C). They are multicelled and normally have four cells separated by transverse septa. The median cells appear darker, olivaceous to brown, but the terminal cells remain lighter (Fig. 1D).

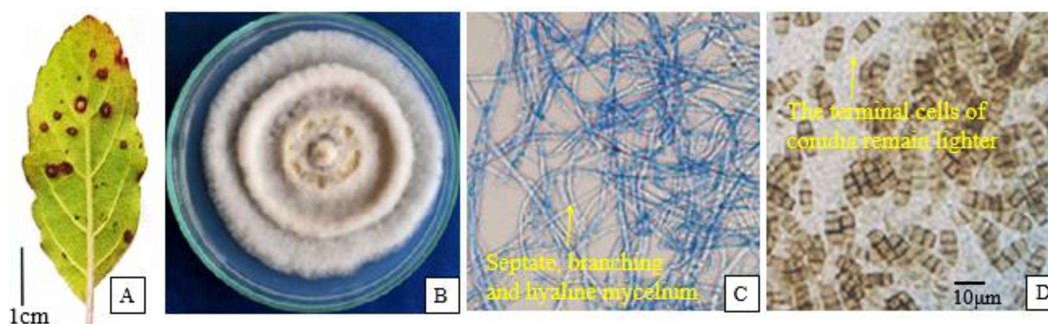

Fig. 1. Morphological characteristics of *Neopestalotiopsis chrysea* causes leaf spot disease in *Ocimum sanctum* L leaf: (A) symptoms of leaf spot disease, (B) mycelial growth, (C) septed mycelia, and (D) conidiophore and conidia of *N. chrysea*.

Similar leaf-spot symptoms on tulsi were reported by Mukherjee et al. (2025). According to Maharachchikumbura et al. (2014), *Neopestalotiopsis* species are typically characterized by white, cottony-to-fuzzy colonies that later develop gray-to-black pigmentation, and fusiform conidia with 3-5 septa, features that closely mirror those of our isolate. Similarly, Liu et al. (2019) reported that *N. chrysea* generates fast-growing colonies on PDA with light to dark pigmentation over time, along with septate, branching, hyaline mycelia, and ellipsoid to fusiform conidia that are pale brown and multi-septate. The findings revealed that *N. chrysea* is a filamentous fungus with septate hyphae and distinctive multicelled conidia that induce necrotic spots on tulsi leaves.

The PCR amplification targeting the ITS region of *N. chrysea* yielded an amplicon of approximately 557 bp (Fig. 2). Amplification of the ITS region using universal primers (ITS1 and ITS4) produced a distinct band of approximately 560 bp, which is consistent with the expected size range for the fungal family of sporocadoceae (Billah et al. 2021).

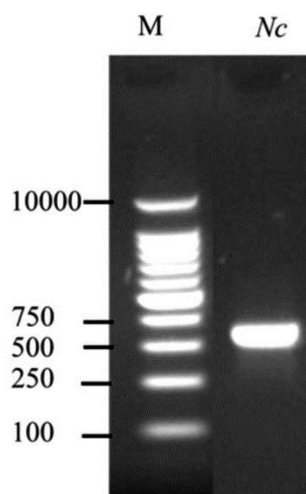

Fig. 2. PCR products of the ITS region of *Neopestalotiopsis chrysea*. Lane M, molecular marker (1 kb DNA ladder); Lane *Nc*, *N. chrysea*.

The phylogenetic tree based on the nucleotide sequence of the ITS region comprises 24 taxa of representative strains of Sporocadaceae, including our studied taxa (PX376321.1), *Neopestalotiopsis chrysea* (JUF0129), and *Alternaria alternata* (NW017306268.1) as an outgroup (Fig. 3). A phylogenetic tree was generated by the neighbor-joining (NJ) method utilizing the ITS sequence. In this tree, Clade-I consists of eleven taxa under the genus *Neopestalotiopsis* with a 99% bootstrap value. Clade-II consists of five taxa under the species *N. rosae* with a 99% bootstrap value. Clade-IV had five taxa under *N. clavispora* with 99% bootstrap value. *Alternaria alternata* functioned as an outgroup in the evolutionary tree. Our examined fungus, *N. chrysea* (JUF0129), and its GenBank accession (PX376321.1) showed a 100% sequence match with *N. chrysea* (PV866874.1), which belongs to the Sporocadaceae family.

The ITS sequence analysis and BLAST findings suggest that the isolated fungus is *Neopestalotiopsis chrysea*, displaying strong nucleotide similarity with other *Neopestalotiopsis* strains (>98%). This is similar with recent findings, where ITS sequencing consistently identified species within the *Neopestalotiopsis* complex (Maharachchikumbura et al. 2014, Liu et al. 2019). The high identity values discovered in BLAST demonstrate low sequence divergence among *N. chrysea* isolates from varied hosts, suggesting a conserved ITS region for this species, as also observed by Xu et al. (2020).

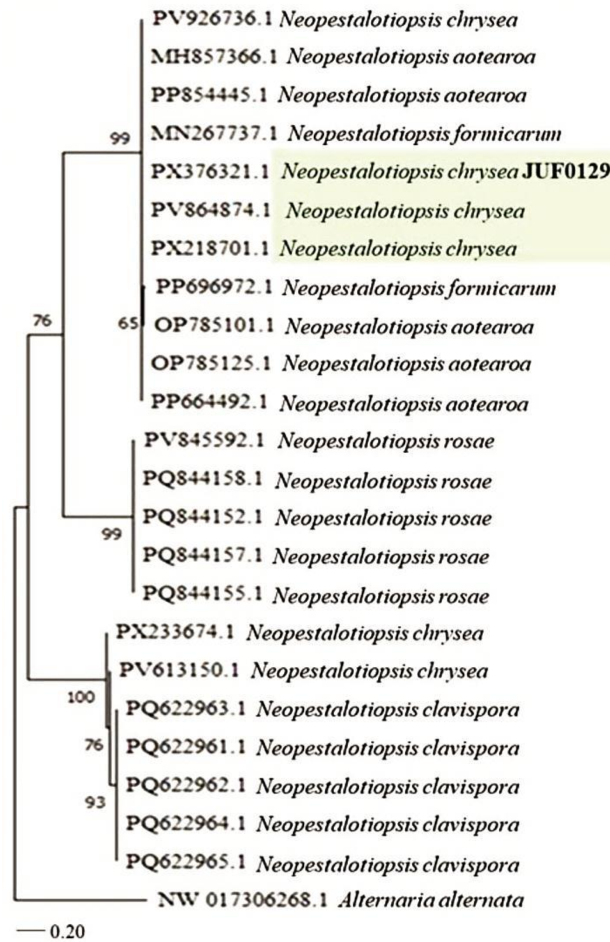

Fig. 3. Neighbor-joining tree showing the phylogenetic relationship among the selected fungal isolates, including our studied taxa (PX376321.1) with 1000 bootstraps. *Alternaria alternata* was used as an outgroup in the tree.

The development of necrotic lesions in the leaf within 7-9 days after inoculation is consistent with prior results on pathogenic fungi producing symptoms within a brief latent time under favorable humidity and temperature (Dean et al. 2012). Re-isolation of

N. chrysea from diseased tissues on PDA confirmed their consistency in pathogenicity. Similar re-isolation approaches were undertaken by Maharachchikumbura et al. (2014) in fungal disease confirmation.

Six distinct culture media, PDA, RAM, YEA, CMC, CDA, and maltose yeast extract agar (MEA) were employed to assess the optimal conditions for the growth and development of *N. chrysea*. The findings indicated that the PDA medium facilitated the most significant mycelial growth, reaching 55.33 mm, followed by the MEA medium at 44.50 mm and the RAM medium at 40.78 mm. Conversely, the YEA medium resulted in the least growth, measuring only 31.11 mm (Fig. 4A). These findings are consistent with Sultana et al. (2022), who showed that PDA promotes strong vegetative growth in *N. chrysea*, causing leaf spot disease of strawberry plants in Bangladesh. PDA is a typical fungus culture medium prepared from potato infusion and dextrose that serves as an optimal carbon source for *N. chrysea* development (Rahman et al. 2025).

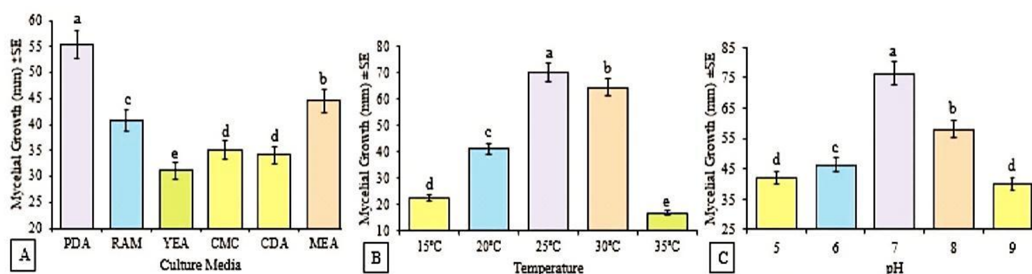

Fig. 4. Effects of culture media: (A) temperature, (B) pH and, (C) on the mycelial growth of *Neopestalotiopsis chrysea* at 7dpi. Data represent the mean value ± standard error of three replicates. Significant differences are presented as letters on the top of the bar and letters with the same value do not differ significantly at 5% level.

The present study indicated that the radial mycelial growth of *N. chrysea* increased steadily until reaching 25°C, after which it began to decline at 30°C. The species achieved its peak growth of 70.22 mm at 25°C, followed by a growth measurement of 64.44 mm at 30°C, while the lowest mycelial growth recorded was 16.88 mm at 35°C (Fig. 4B). Ahmmed et al. (2022) found that *Pseudopestalotiopsis thae*, which causes Aloe vera leaf spot disease, exhibited maximal radial development at 25°C, with an important decrease in growth at 15°C and 35°C, which was quite comparable to our results. Therefore, temperature is a major environmental element impacting the mycelial development and sporulation of *N. chrysea*. According to Sultana et al. (2021), the ideal temperature for fumonisin synthesis by *Pseudopestalotiopsis* species is between 25 and 30°C under high humidity conditions.

The mycelial growth and development of *N. chrysea* exhibited an upward trend until reaching a pH of 7, beyond which it declined. At this optimal pH of 7, *N. chrysea* demonstrated the most significant mycelial expansion, measuring 76.33 mm. In contrast,

under basic conditions at pH 9, the fungus experienced its least growth, recorded at 40.11 mm (Fig. 4C). In the present study, the fungal isolates exhibited optimal growth under slightly basic to neutral conditions, particularly within the pH range of 7.0 to 8.0. Colony diameter, pigmentation, and sporulation were most prominent at these pH levels. Sultana et al. (2021) found that *N. chrysea* isolated from strawberry displayed optimal radial growth at a pH around 7.0, with a notable decline in biomass production at both pH 5.0 and pH 9.0. These results support the conclusion that *N. chrysea* favors mildly basic environments, which are often prevalent in natural substrates like decaying plant matter or infected crops. This finding revealed that pH not only influences mycelial growth but also regulates secondary metabolism in fungi (Alam et al. 2007).

The results of the three different *A. paniculata* and *L. inermis* concentrations against *N. chrysea* mycelial growth inhibition have been presented in Fig. 5A. The maximum inhibition is observed in T3, when *L. inermis* reaches 55.05%, and *A. paniculata* reaches about 60.85%. The proportion of mycelial growth inhibition increases significantly while the treatment concentration increases from T1 to T3. Results suggested that *A. paniculata* is a better botanical inhibitor than *L. inermis*, especially at higher concentrations. The percent mycelial growth inhibition of *N. chrysea* at 7 dpi due to the effect of antagonistic fungi has been presented in Fig. 5B. *T. atroviridae* showed the maximum mycelial growth inhibition (68.94%) against the mycelial growth and development of *N. chrysea*, followed by *T. virens* (63.82%). In contrast to *T. virens*, *T. atroviride* is a more effective biocontrol agent for decreasing the growth of the target pathogen.

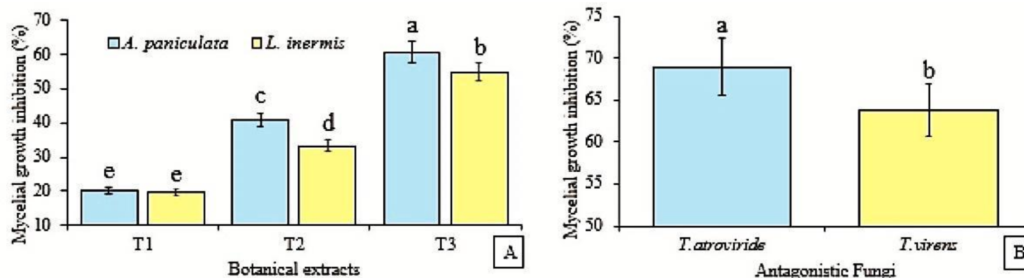

Fig. 5. Percent mycelial growth inhibition of *Neopestalotiopsis chrysea* at 7 dpi. (A) 10% (T1), 20% (T2), 30% (T3) extracts of *A. paniculata* and *L. inermis*, (B) *T. atroviride* and *T. virens*. Data represent the mean value ± standard error of three replicates. Significant differences are presented as letters at the top of the bars, and letters with the same value do not differ significantly at the 5% level.

Similar observations were obtained by Khatun et al. (2025), where certain plant extracts displayed the highest antifungal activity at lower dosages, probably due to improved solubility and bioavailability of active metabolites. Botanical extracts showed a typical dose-dependent response, with 30% concentration exhibiting the highest inhibition, which aligns with previous studies where higher concentrations effectively suppressed the pathogenic fungi (Ahmmed et al. 2021). The present findings coincide with recent research indicating that *T. virens* and *T. atroviride* are effective against various

plant diseases, including *Fusarium oxysporum, Rhizoctonia solani,* and *Alternaria alternata* (Kakvan et al. 2013, Naraghi et al. 2013). Tulsi cultivation is seriously threatened by *N. chrysea*, although its growth can be efficiently inhibited by applying extracts of *A. paniculata* and selected *Trichoderma* species as a biological control.

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