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FIRST MOLECULAR INSIGHT INTO *NIGROSPORA GUILINENSIS* INFECTING *ROSA INDICA* LEAVES IN PAKISTAN

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ABSTRACT

The rose (*Rosa indica*) has significant economic value due to its ornamental uses, as well as its medicinal and nutritional properties. Rose cultivation worldwide faces significant losses due to various fungal diseases, many of which have detrimental socio-economic impacts. Some of these include foliar diseases like leaf spot, which are very common in Pakistan. Determining the correct fungal pathogens causing the disease is critical to implementing effective disease control management. In the present investigation, a systematic field survey was conducted across multiple sites in Lahore, Punjab, during August to September in both 2020 and 2021. Leaf spot symptoms were seen with high prevalence on *Rosa indica* plants. Incidence, severity, and prevalence of disease were recorded. The symptomatic leaves were used to isolate and characterize pathogens based on their morphological characteristics. Molecular identification was done by amplifying rDNA regions with β-tubulin (Bt2a/Bt2b) and elongation factor (EF) gene-specific primers. The pathogen was characterized as *Nigrospora guilinensis* based on integrated morphological and molecular evidence**.** Phylogenetic analysis was performed using MEGA 6 software to determine the evolutionary relationship of the fungal isolate. Pathogenicity was verified by fulfilling Koch's postulates through artificial inoculation of *Rosa indica* seedlings under both in vitro and greenhouse conditions. The recurrence of characteristic symptoms, along with successful re-isolation of the identical fungal strain, confirmed the pathogenic role of *Nigrospora guilinensis***.** To the best of our knowledge, this study provides the first documented evidence of *N. guilinensis* as a foliar pathogen of *Rosa indica* in Pakistan.

Key Words: Identification, Genetic analysis, *Nigrospora guilinensis,* phylogenetic relationship, *Rosa indica*, Pathogenicity test.

INTRODUCTION

The rose carries important economic, medicinal, and therapeutic significance. It belongs to the family Rosaceae, and is one of the most ancient cultivated ornamental plants, with indications that it was domesticated more than 5,000 years ago (Leus *et al*., 2018; Ljubojević and Božanić Tanjga, 2025). The genus Rosa is widely spread throughout the northern hemisphere and comprises about 150 wild species and over 2,800 cultivated varieties (Singh *et al*., 2023). Rose plants, which have a shrubby growth habit, are highly variable in terms of size and flower color, from yellow and red to white. Due to their aesthetic appeal, roses have become a prominent feature in public parks, ornamental gardens, and residential landscapes worldwide, including in Pakistan (Rahnema *et al*., 2019). Their demand spans domestic markets, international trade, and ceremonial uses, making them integral to the floriculture sector.

In addition to their beauty, some of the petals of the Rosa species are used in traditional remedies for obesity, skin diseases like acne, and digestive problems (Takahashi, 2025). The cultivation of roses is primarily done to produce valuable products, including rose oil, rose water, rose atar, gulkand, and various perfumes (Masoodi *et al*., 2025). Rose hips are highly valued for their significant vitamin C content, which surpasses that of oranges. Additionally, they are valued for their anti-inflammatory, digestive, and anti-cancer properties (Anushree and Veena, 2023).

Floriculture has also been steadily growing in Pakistan with rising local and international demand. In 2013, approximately 17,000 acres were planted with flowering crops, of which roses held a substantial amount (Shetty *et al*., 2018). In the 2006–2007 period, roses were cultivated on approximately 8,000 acres in Pakistan, with the area under cultivation steadily increasing in subsequent years (Hashmi *et al*., 2024; Younas *et al*., n.d.). Pakistan has also established itself as a regular exporter of cut flowers to several international markets, including Dubai, Germany, and Egypt (Zeb *et al*., 2007; Rasheed *et al*., 2016).

Although they are economically and horticulturally important, rose plants face a broad spectrum of biotic stress factors, such as bacterial, viral, and fungal diseases, as well as insect-resistant pests (Rzyska *et al*., 2024). These

diseases and pest attacks can happen throughout the entire lifespan of the crop, active growth and pre-harvest periods, and post-harvest handling, transport, and storage. In particular, fungal diseases like dieback, stem blight, powdery mildew, *Alternaria* leaf spot, and black spot are predominantly overriding (Lavanya, 2025). Some of the other diseases caused by fungi are black mold, Botrytis gray mold, brown canker, cane blight, *Cercospora* leaf spot, stem and crown cankers, rust, Septoria leaf spot, Verticillium wilt, and anthracnose, most of which are widespread around the world (Sharma and Singh, 2002; Webster and Weber, 2007; Chase and Daughtrey, 2013).

Of all these, approximately 20% of agricultural spoilage is caused by a particularly devastating leaf spot disease, causing huge losses of about 80% of the crop (Asim *et al*., 2019), plant vigor, and flower quality. Since roses are of economic significance and must be kept ornamental, it is important to identify and characterize the causal agents of leaf spot disease. Therefore, this study aimed to isolate, identify, and characterize the fungal pathogen(s) responsible for leaf spot symptoms in *Rosa indica* under local conditions, to support targeted disease management strategies.

MATERIALS AND METHODS

Analysis of Field Survey

A field survey was carried out during August and September of 2020 and 2021 to investigate the prevalence of leaf spot disease in rose plants. The survey sites included Manawan, Canal Road site, the University of the Punjab's main campus site, the University's Botanical Garden, and Jallo Park sites, all located in Lahore. Rose leaves exhibiting infection were identified by the presence of necrotic areas, lesions, and signs of wilting. Measurements and observations were made concerning lesion size, discoloration patterns, and the physical characteristics of the spots. Photographs were captured to document these symptoms for later analysis and verification. Statistical analysis of the field survey data was done to analyze the distribution of disease over the assigned areas of Lahore and to determine the degree and severity of the disease in plants by applying the given formulas. Infected leaves were gathered into sterilized polyethylene bags, brought to the laboratory for pathogen analysis, and placed in the refrigerator at 4°C until further processing for future research. The following formulas were applied:

Disease prevalence (%) = (Number of diseased fields / Total number of fields) × 100;
Disease incidence (D.I.) (%) = (Number of diseased plants in a field / Total number of plants in the field) × 100;
Disease severity (%) = (Affected area of plant leaves / Total leaf area) × 100.

Isolation of Fungal Pathogen

Fungal growth medium 2% Malt Extract Agar (MEA; pH 6.5) was made for the isolation and purification of the pathogen from the infected rose plants. Approximately 3–4 necrotic spots were excised from the infected leaf and trimmed into small fragments measuring about 3 mm and immersed in 1% sodium hypochlorite solution for 5 minutes to kill any pathogenic flora, and then washed several times with sterilized distilled water. Roughly 4–5 leaf fragments were aseptically placed onto Malt Extract Agar (MEA) media plates and placed in an incubator and maintained at 25 ± 2 °C. Daily, the plates were observed for any evidence of mycelial growth emanating from the periphery of the affected lesions. Mycelium derived from fungus growing on inoculated leaf fragments was sub-cultured onto MEA plates for purification at 25 ± 2 °C, and the resulting pure cultures were stored at 4 °C.

Pathogenic Identification

Morphological Characterization of Pathogen

Morphological observations were made on pure 4-7-day-old fungal cultures grown on MEA medium. Colony morphology was determined both macroscopically and with a naked eye by inspecting the front and reverse surfaces of the growth area. Microscopic features were determined for the identification of fungi, including the structure of mycelial growth. Both submerged and aerial septation of hyphae and characteristics of conidiophores, such as pigmentation, morphology, direction, and conidial density, were observed. Photographic documentation was used to capture both macroscopic and microscopic structures of the culture.

Genetic Characterization

The nucleon reagent method was used for fungal DNA extraction. The crushed cells were incubated at 37 °C for 30 min in 2 mL Nucleon reagent B (400 mM Tris pH 8, 120 mM EDTA, 150 mM NaCl, and 1% SDS) and 0.5 µL of RNase A (10 mg/mL). After incubation, 0.5 mL of 5 M sodium perchlorate was added to the cells and mixed thoroughly. Furthermore, 2 mL of ice-cold chloroform was also mixed. These cells were centrifuged at 4000 rpm for 5 min, and supernatant was carefully transferred into a new sterile tube. Addition of 2 mL of ice-cold 96% ethanol to

supernatant caused the DNA precipitation. Precipitated DNA was pelleted by centrifugation at 4000 rpm for 5 min. Supernatant was discarded and DNA pellet was washed with 70% ethanol and resuspended in 50 µL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8). Extracted DNA was incubated at 65 °C for 15 min to inhibit activity of DNase and stored at -20 °C. Agarose gel electrophoresis (1%) was carried out to check the quality and Length (bp) of isolated DNA. The samples were subjected to electrophoresis at 70 V for 45 minutes, and DNA bands were observed using UV transillumination.

PCR Amplification for DNA Sequence Analysis

PCR amplification targeting the partial coding regions of the β-tubulin (Bt2a: 5′-GGTAACCAAATCGGTGCTGCTTTC-3′, Bt2b: 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) and elongation factor (EF1: 5′-ATGGGTAAGGA(A/G) GACAAGAC-3′, EF2: 5′-GGA(G/A) GTACCAGT(G/C) ATCATGTT-3′) genes was performed using fungal genomic DNA as the template. The primer was used at a concentration of 1 µL from a 10 pmoL/µL stock for both the forward and reverse primers. A commercially available 2X Amp Master TMTaq DNA polymerase (GeneAll Biotechnology Co., LTD) was used for polymerase chain reaction (PCR) amplification in a 30 µL reaction volume. The thermocycling procedure included a 5-minute initial denaturation at 94 °C, 35 amplification cycles that included 30 seconds of denaturation at 94 °C, 30 seconds of primer annealing at 60 °C, and 10 minutes of strand extension at 72 °C. The amplified DNA sequences were subjected to analysis using the Nucleotide Basic Local Alignment Search Tool (BLAST). To identify fungal species taxonomically, sequence alignment results were compared to homologous sequences from the GenBank repository.

Pathogenicity Confirmation Test

In vitro Method

A spore suspension containing 5×10^5 spores/mL was prepared in sterilized NaCl solution using a haemocytometer and further used as inoculum. Then the sterilized petri plates were lined with 2 filter papers in each. Around 2 mL of double-distilled water moistened the filter paper. Leaves detached from healthy plants were arranged in Petri plates with the petiole ends resting on damp absorbent paper. Afterward, a sterile micropipette was used to put 2 mL of spore suspension (5×10^5 spores/mL) on the leaf surface. The plates were incubated at 25 ± 2 °C and observed regularly for the emergence of disease symptoms. After the onset of disease, re-isolation of the pathogen from the infected leaves was carried out to fulfil Koch's pathogenicity postulates.

In vivo Method

Washed earthen pots were filled with sterilized soil at the rate of 1 kg per pot. The grafted rose stems were sown into earthen pots, watered properly, and placed into a growth room at 30 ± 2 °C. The pathogenicity test was confirmed by inoculating 15 ml of suspension of spores (containing 5×10^5 spores/mL) into stem internodes and nodes with the assistance of an aseptic syringe and also by spraying fungal spore suspension over the soil surface. The control received distilled water in the same quantity. Plants were maintained under polythene bag covers for 48 h to maintain sufficient moisture for spore germination and the development of disease. After that, the plants were kept in shade under optimum temperature, i.e., 26 °C, and watered properly. Symptoms of *Rosa indica* disease began within 2-3 days, and pathogenicity was assessed using a disease rating scale and visual assessment of symptoms in *Rosa indica* seedlings.

RESULTS

Surveying and Diagnostic Assessment of Diseased Plant Material

A survey of *Rosa indica* fields was conducted between August and September 2020 to collect samples of infected rose leaves. The survey sites selected included the fields at Manawan, Canal Road, the Botanical Garden of the University of the Punjab, and Jallo Park in Lahore. During the field investigation, distinct symptoms such as dark brown to black leaf spots, lesions, and numerous necrotic patches were observed (Fig. 1). The lesion diameters ranged from 2 to 3 mm, with approximately 50–60% of the leaf surface area exhibiting infection. The extent of infection was quantified to determine disease prevalence, incidence, and severity.

Fig. 1. Survey of *Rosa indica* fields of Lahore; (A): Diseased Rose plant, (B): Foliar disease symptoms.

Disease Prevalence and Incidence

The field data reflected extensive disease presence in every selected location. The highest prevalence was recorded in Manawan, i.e., 72.33%, followed by Jallo Park and Canal Road, where 70.4% and 67.66% disease prevalence were noted, respectively, with insignificant differences. While the lowest disease prevalence (51.0%) was observed in the botanical garden (Fig. 2). Survey analysis revealed maximum disease incidence (45%) in Jallo Park. While the disease incidence was insignificantly lower in the rose growing area of Canal Road and Manawan, i.e., 42.3% and 37.66%, respectively. The botanical garden exhibited the fewest records of disease incidence (Fig. 3).

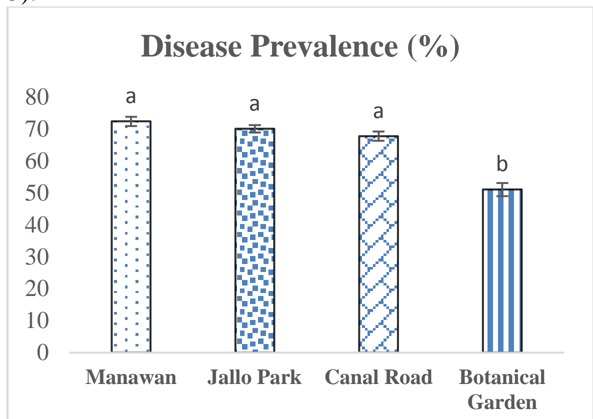

Fig. 2. Prevalence of the disease in distinct areas of Lahore.

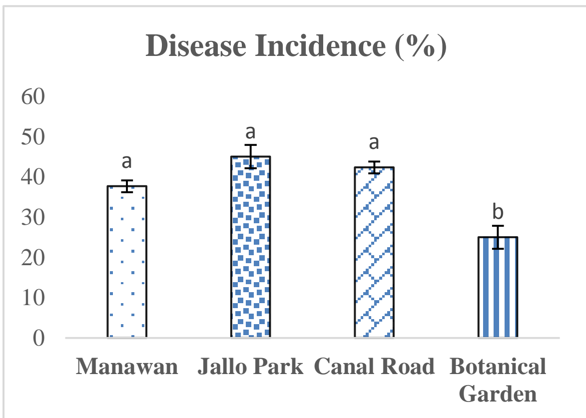

Fig. 3. Incidence of disease across different areas of Lahore.

Vertical bars represent standard errors of the means (n=3), and different letters indicate significant differences by ANOVA ($p \leq 0.05$) using the LSD test in Statistix 8.1.

Disease Severity

In case of disease severity, the rose field of the Botanical Garden was found to be at the highest risk. All data records of survey analysis revealed that though the prevalence of disease was least in Botanical Garden rose plants but the severity of disease was maximum here, i.e., 55%. In Manawan, the canal road, roses boundary lane, and Jallo park had the least disease severity, with a range of 40 – 45% was calculated with insignificant differences (Fig. 4).

Characterization of Isolated Pathogen

The pathogen that was isolated from the infected samples was characterized initially by its morphological characteristics and subsequently by its nucleotide sequence. The morphological characterization included assessing the macroscopic characteristics and then microscopically observing the fungal isolate. Thereafter, the fungus was confirmed genotypically using sequence alignment with specific primers.

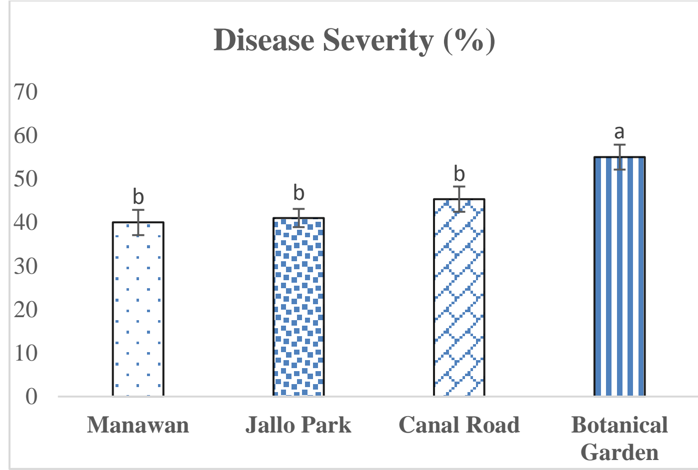

Fig. 4. Disease severity of different regions of Lahore.

Vertical bars represent standard errors of the means (n=3), and different letters indicate significant differences by ANOVA ($p \leq 0.05$) using the LSD test in Statistix 8.1.

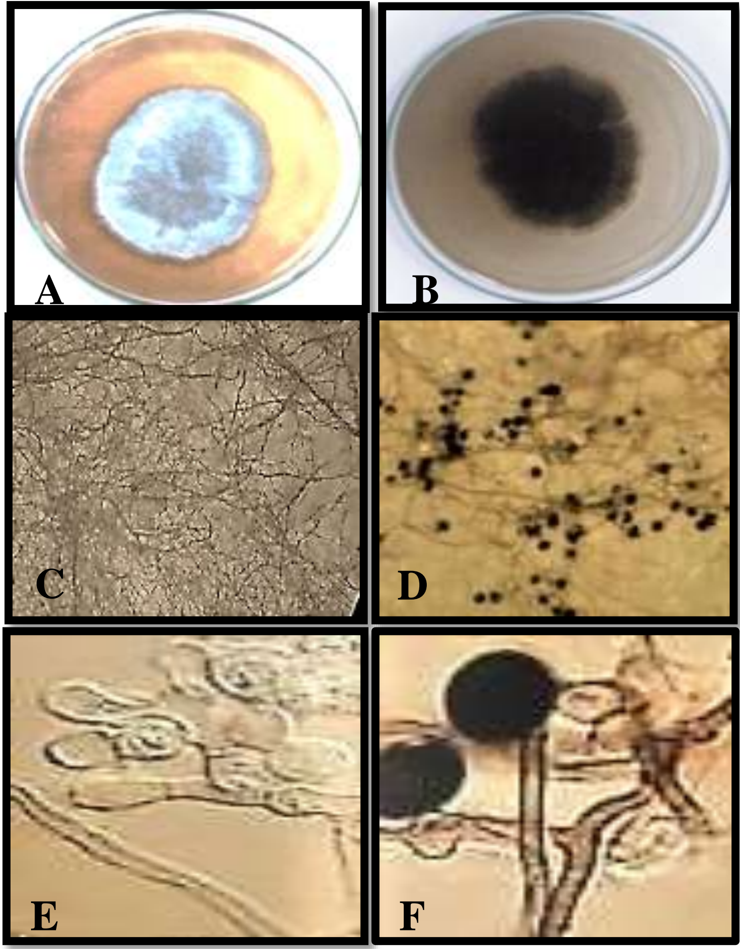

Fig. 5. *Nigrospora guilinensis* (A): Colony morphology from the front end (B) reverse side on Malt Extract Agar (MEA); (C): Branching structure visualized using a stereoscope and (D-F): Microscopic images showing mycelia and conidiogenous cells at 10X and 40X magnification, and conidia at 100X magnification, respectively.

Macroscopic/ Microscopic Features

The fungal isolate exhibited slow growth, producing white colonies after incubation on Malt Extract Agar (MEA) at an incubation temperature of 25-27 °C. The colonies completely covered the surface of the Petri plate in 10-12 days. At first, the colony presented a white color on the top side that progressed to a greyish black color as it matured. The backside of the Petri plate showed entirely black mycelial growth. (Fig. 5A, B). The fungal mycelium, which consisted of septate hyphae, produced one black conidiogenous cell. The conidiogenous cell was globose and swollen in shape, and it was located at the apex of the conidiophore. The conidiophores were elongate, branched, and clustered. They were around 10-12 µm, and transparent to whitish. The conidiogenous cell was approximately 12 × 14 µm and contained conidia. The conidia were black, single-celled, and spherical. Based on its morphological characters, the fungus was identified as *Nigrospora guilinensis*. (Fig. 5C-F).

Genetic Characterization

Genetic characterization of the fungal pathogen *N. guilinensis* was concluded by the isolation of DNA. For further nucleotide sequencing, the isolated DNA was used in PCR amplifications by elongation factor (EF) and β-tubulin primers. PCR amplification showed that all β-tubulin products from the pathogenic fungi produced bands on an agarose gel between 300-400 base pairs in length, while elongation factor (EF) gene sequences were between 200-300 base pairs. Nucleotide sequences were further analyzed by BLAST using the National Center for Biotechnology Information (NCBI) website. The identification of fungal species was confirmed based on Blast sequences (90-100%). Alignment of the β-tubulin sequence of *N. guilinensis* revealed a 99.46% similarity with the sequence of *N. guilinensis* (KY019608.1), while the sequence KY019459.1 exhibited 99.45% homology with the rDNA of *N. guilinensis* (Fig. 6). When comparing the translation elongation factor (EF) gene of *N. guilinensis*, it was found to be 98.77% similar to KY019404.1 and 97.53% similar to KY019292.1, producing a 549 bp PCR product (Fig. 7).

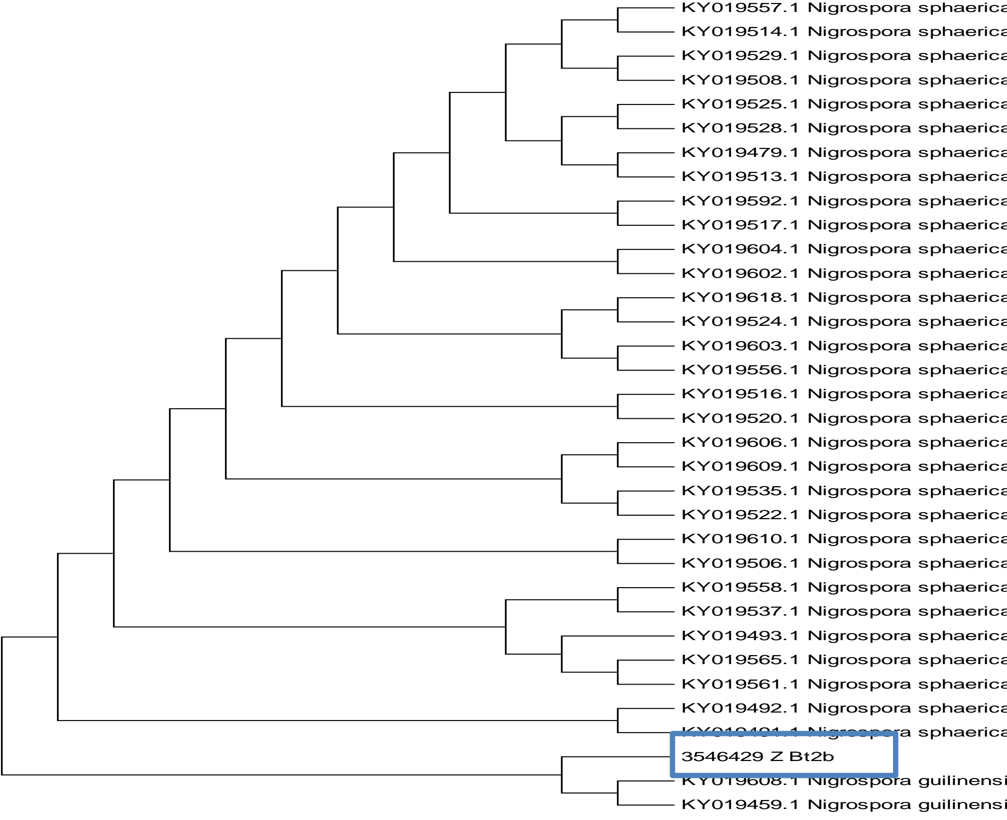

Fig. 6. Evolutionary relationships of the β-tubulin region of *N. guilinensis* rDNA were analyzed by constructing a phylogenetic tree using the maximum likelihood method, implemented in MEGA 6 (Tamura *et al*., 2013) with the Jukes-Cantor substitution model (Jukes and Cantor, 1969).

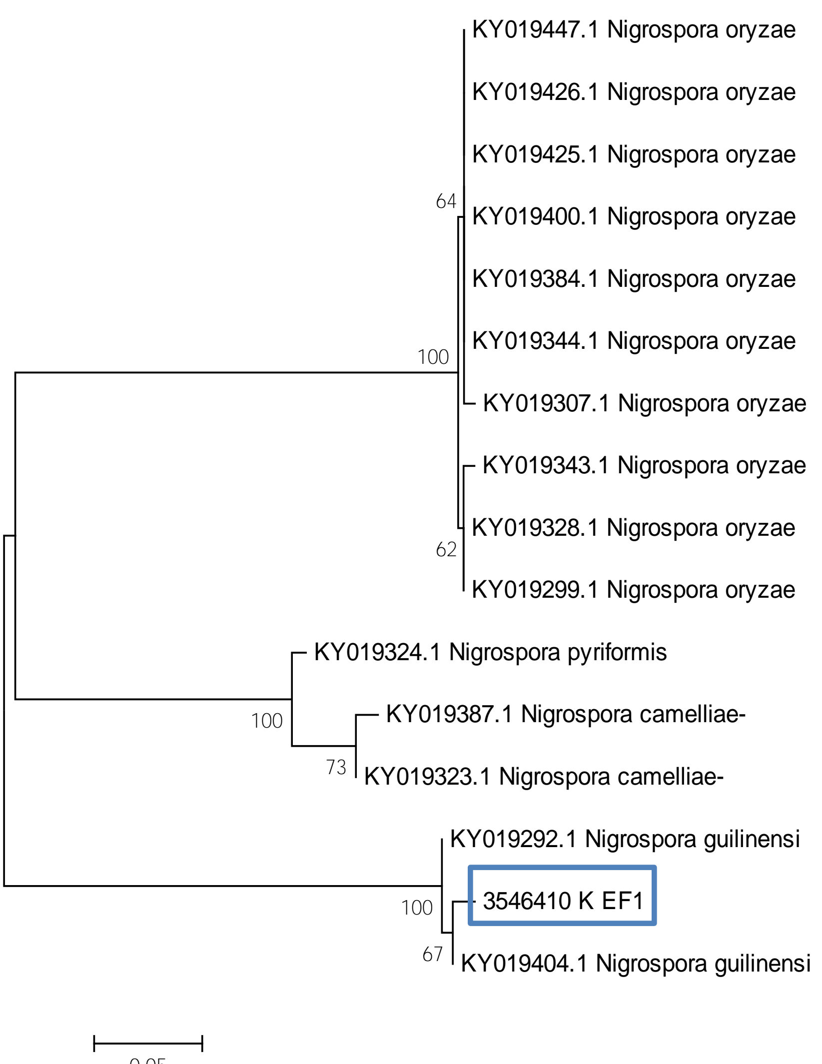

Fig. 7. Evolutionary analysis of the translation elongation factor (EF) region of *N. guilinensis* rDNA was performed by generating a phylogenetic tree using the maximum likelihood method in MEGA 6 (Tamura *et al*., 2013) with the Jukes-Cantor substitution model (Jukes and Cantor, 1969)**.**

Pathogenicity Analysis

Detached Leaf Method

Disease severity was determined based on a disease rating scale that was constructed following the appearance of symptoms within 5-15 days. After application of the inoculum of fungal species, symptoms started to appear on the leaves in the petri plates. Initial symptoms of yellowing appeared on the leaves 48 hours post-inoculation, progressing to complete leaf necrosis within 15 days under the detached leaf assay. *Nigrospora guilinensis* induced black lesions localized on the leaf midrib, followed by chlorosis. Symptom development extended from the midrib toward the petiole, culminating in leaf blight at the terminal stage (Fig. 8). Disease progression during the first week post-inoculation was relatively slow; however, a rapid increase in severity was observed thereafter, with approximately 95% of the leaf area exhibiting infection at fifteen days (Fig. 9).

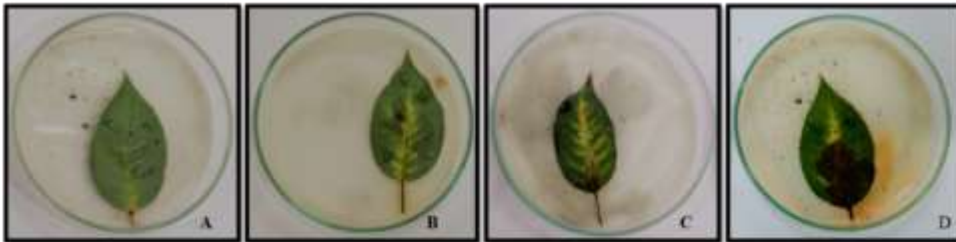

Fig. 8. Disease symptoms of *Rosa indica* caused by *Nigrospora guilinensis*.

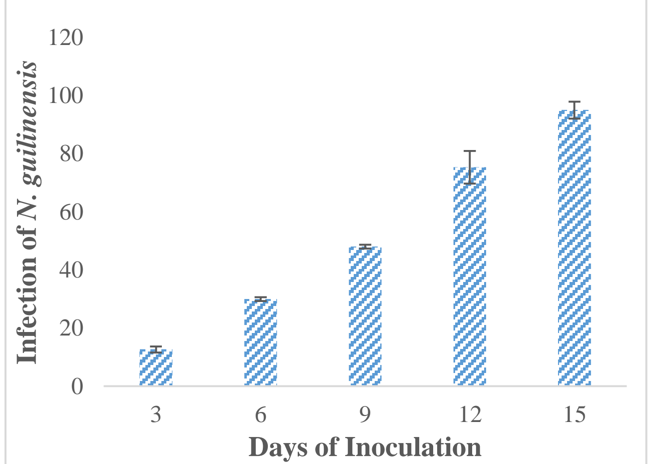

Fig. 9. Progression of disease caused by *Nigrospora guilinensis* on *Rosa indica* leaves, assessed via detached leaf assay.

Standard errors of three replicates are shown as vertical bars. Means with distinct letters differ significantly by ANOVA and LSD test ($p \leq 0.05$) via Statistix 8.1.

Pot Trials

A pathogenicity test was also conducted in the field to confirm the efficacy level of the isolated fungal pathogen by spraying one-month-old *R. indica* plants. An 8-day-old mature fungal culture was utilized to prepare a 2 mL spore suspension, with a concentration of 5×10^5 spores per milliliter, for pathogenicity confirmation. The initial symptoms of the disease were demonstrated 15 days following inoculation. The symptoms were consistent with the pathogen and began with a gradual disease progression, where initially the leaf tips were black. *Nigrospora guilinensis* infection first produced water-soaking of the leaf surface, progressing to chlorosis at tips and margins that formed a brown blighted area. In the later stages of infection, the entire plant desiccated, with black lesions formed. (Table 1). The infected areas of *R. indica* plants were recorded in pot studies to assess the level of disease severity associated with *N. guilinensis*. The pathogen resulted in significant disease severity with a value of 85%.

Table 1. Pictorial representation of developed disease symptoms by *Nigrospora guilinensis*.

Key Scale	**Disease Symptoms**		**Disease Severity**
0	Healthy appearance		**0**

1	Dark spots on the leaf's lower surface		**20**
2	Yellow discoloration of the leaf		**40**
3	Leaf yellowing with brown spots		**60**
4	Slight leaf deformation and noticeable black lesions		**80**
5	Extensive black necrotic lesions with leaf abscission		**100**

DISCUSSION

Rosa indica is important horticulturally and has nutritional and medicinal value. *Rosa Indica* is significantly grown in Pakistan for ornamental, medicinal, and cosmetic purposes and is a commonly traded flower worldwide (Fayaz *et al*., 2024; Takahashi, 2025). Rose gardens are often negatively impacted by one of the most devastating foliar diseases, leaf spot, which significantly reduces their landscape value (Rudska *et al*., 2024). Leaf spot is a disease caused by several infections with pathogenic fungi, bacteria, and viruses (Kakade *et al*., 2006; Chase and Daughtrey, 2013). *Alternaria* and *Fusarium* species are among the most common fungal pathogens identified with leaf spot disease. The increasing incidence of this disease has resulted in significant economic loss to rose growers. A range of different adapted disease management strategies have been tested; the results have not been consistent. Thus, research continues to identify the most effective, sustainable approach (Agrios, 1988; Liu *et al*., 2021). In order to manage leaf spot disease from fungal pathogens, the first step is to isolate and identify the causal agents (Shafique *et al*., 2019). Fungal identification was traditionally done by conventionally and reliably identifying morphological features of fungi. However, advancements in molecular techniques, more precise identification can now be achieved, whereby phylogenetic relationships can be obtained by analyzing ribosomal DNA sequences (Mirhendi *et al*., 2007). Moreover, the use of mitochondrial small subunit rDNA sequencing, an alternative molecular method of classification and identification is also has been used to classify fungi (Kretzer *et al*., 1996).

In this research, *Nigrospora guilinensis* was the isolated and identified as the pathogen responsible for the leaf spot disease of *Rosa indica* based on morphological observations and rapid identification by using rDNA sequencing

with β-tubulin and elongation factor (EF) gene primers. The nucleotide sequences were later utilized with bioinformatics tools from two databases, NCBI and EBI. The nucleotide sequence alignment of the β-tubulin gene showed 99.46% similarity with N. guilinensis (KY019608.1) through BLAST, while the EF gene showed 98.77% similarity to *N. guilinensis* (KY019404.1). The use of morphological characterization of leaf spots and rDNA sequencing was previously done by Akhtar *et al*. (2016) to isolate *Phyllotactic aristolochiicola* from leaf spots on *Sonchus oleraceus*. Likewise, morphological characters supported with ITS region rDNA spacer sequencing for *Alternaria ochroleuca* identified it as the leaf spot pathogen of the money plant (Shafique *et al*., 2017).

Pathogenicity of *N. guilinensis* on *Rosa indica* seedlings was confirmed through Koch's postulates, employing detached leaf assays and pot trials. The fungus induced characteristic symptoms, characterized by yellowing of the foliage, lesion formation, and dark brown to reddish spots, progressively covering as much as 85% of the leaf surface. This finding aligns with previous research by Conner (2002), who demonstrated the pathogenic effect of *Cladosporium carygenum* on pecan leaves via detached leaf methods. Similarly, Shafique *et al*. (2015) documented the pathogenicity of multiple *Fusarium oxysporum* strains on various chili varieties, noting that strain B exhibited the earliest and most severe symptoms within 7 days, marking it as particularly aggressive.

Conclusion

The study concludes that fungal isolations and identifications are very helpful in the fungal biotechnology area and play an efficient role in the recommendation of authentic control measures for the pathogen.

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