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Isolation and Characterization of Pathogen causing Black Leaf Spot in Rose

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ABSTRACT: Black leaf spot, a common disease found in roses, is a major threat to rose production in India. *Diplocarpon rosae* is believed to be the major pathogen associated with this disease. There have been some controversial reports regarding its etiology. In a study conducted in the Department of Plant Pathology, College of Agriculture, Vellayani revealed that the causal organism responsible for black spot of rose in the southern part of Kerala state, *i.e.,* Thiruvananthapuram and Quilon district, was *Colletotrichum gleosporioides.* The present study focused on the isolation, cultural, morphological, and molecular characterization of the causal organism. Diseased samples were collected, isolated and Koch's postulates were proved. Morphological and molecular characterization of the pathogen was done. The results confirmed the presence of *Colletotrichum gleosporioides,* showing 99.7% similarity.

Keywords: Rose, *Colletotrichum gleosporioides*, black leaf spot, morphology, molecular.

INTRODUCTION

The diverse nature of India makes it a homely place for the establishment of a variety of crops, including ornamentals. Floriculture, as an integral part of horticulture plays apivotal role in the socio-economic aspects of the country. Rose as the 'Queen of flowers' has always been a piece of aesthetic and a symbol of love, even from the origin of mankind. Rose (*Rosa indica* L.) is a commercially cultivated ornamental crop belonging to the family Rosaceae. According to Gajraj *et al*. (2022) the rose holds the top rank in the global flower trade. It is grown for a variety of purposes, such as cut flowers, loose flowers, garden flowers, potted plants, essential oil extraction etc. Angel *et al.* (2024) stated that India accounts for over 45 per cent of the global area dedicated to cut rose cultivation (2024). However, rose cultivation is severely affected by many diseases. The loss of valuable potted flowers due to plant pathogens has been estimated to reach 100%, and plant diseases represent a significant bottleneck in flower production and distribution (Haque *et al.*, 2024). Black spot, a fungal disease on rose casts a long shadow over rose cultivation worldwide. It not only reduces the aesthetic appeal of these valuable flowers but also poses a significant threat to their health and productivity, thus claiming to be the most widespread and destructive disease of roses (Sinclair *et al*., 1987).

Diplocarpon rosae is the major fungal pathogen associated with this foliar disease (Walker *et al*., 1995). In addition to this, many other fungal pathogens are also involved in the black spot, *viz*., *Colletotrichum* sp., *Penicillium* sp., and *Rhizoctonia* sp. (Mohd Asmadi *et al.*, 2020). *Colletotrichum gloeosporioides* is the fungal organism that was observed to produce black spot on rose during this study.

The fungus comprises *C. gloeosporioides* as anamorph, and *Glomerella cingulata* as sexual (perfect) teleomorph state. *G. cingulata* occurs in a broad range of host species, producing acervuli within the host tissue during the asexual (mitotic) phase of their life cycle. The teleomorph state has the ability to cause serious disease (Cannon *et al*., 2012). The penetration into host tissues generally relies on the formation of specialized infection structures known as appressoria. These appressoria allow the fungus to penetrate the host cuticle and epidermal cell wall directly by a narrow penetration peg that emerges from the base of the appressorium (Ajaykumar, 2014).

Colletotrichum species have a general life cycle in which they overwinter as mycelia or sclerotia in various plant remnants, producing acervuli, which then create primary conidia. Conidia that propagate by rain allow infections to progress into lesions, which in turn give rise to acervuli that generate conidia for subsequent infection cycles, as the asexual conidia are known to be the important stage for disease development (Salotti *et al*., 2023).

Symptoms of infection mainly include round spots with a black and feathery edge on the front side of the leaves (Blechert and Debener 2005). In severe conditions, spots appear to be converged, and yellow halos may encircle these spots. Leaflets or leaves may get severely chlorotic as a result of an infection. Yellowed leaves wither early, especially on cultivars that are susceptible. Middle and upper leaves typically become infected following the lower leaves. The quantity and quality of leaves and blooms, as well as the length and size of the

stem, are all decreased by excessive defoliation. If the disease is not controlled, it can adversely affect the plant's health, resulting in reduced flowering and eventually potential plant breakup, which makes them more susceptible to other pathogens. It is therefore essential to have systematic study of the pathogens inciting black spot as well as to develop suitable management practices to control the disease.

MATERIALS AND METHODS

Collection of samples. Samples were collected from the plants that showed the highest disease severity based on the typical symptoms, including black circular spots with perforated edges appearing on the upper leaf surface (Le *et al.*, 2022).

Assessment of disease severity. The pathogen was isolated from the infected rose leaflets collected from the rose garden of the College of Agriculture, Vellayani. Black spot disease on each plant was scored (Gachomo, 2005) (Table 1) based on the symbolic representation (Yasin *et al.,* 2016) (Fig. 1) and disease severity in percentage (Palmer *et al*., 1966) was calculated among 5 plants confined in an area of 1×1 sq m using the following formula:

$$\text{Disease index} = \frac{\Sigma (v \times n)}{N \times Z} \times 100$$

Where,
v = disease score
n = the number of infected plants/leaves observed in each category
N = total number of plants/leaves
Z = maximum possible disease score

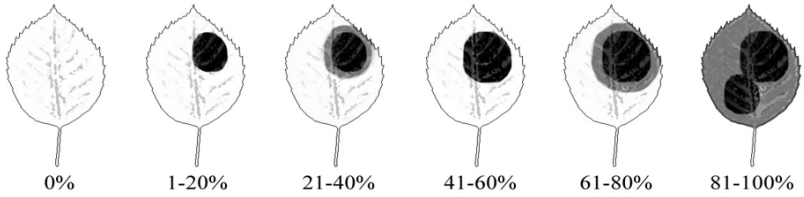

Fig. 1. Symbolic representation of disease severity scoring in percentage.

Table 1: Disease severity scale with symptom description

Class/scale	Disease severity	Symptoms description
1	0%	Leaflet with no black spot symptoms
2	1-20%	Leaflet with black spots having size less than 5 mm. No yellowing of leaflet and no premature defoliation
3	21-40%	Leaflet with black spots having less than 5 mm size along with yellowing and/or defoliation symptoms
4	41-60%	Leaflet with black spots having 5-10 mm size with no yellowing and/or defoliation symptoms
5	61-80%	Leaflets with black spots of 5-10 mm along with yellowing and/or defoliation symptoms
6	81-100%	Leaflets with black spots of more than 10 mm along with yellowing and/or defoliation symptoms

Isolation of pathogen. The black spot infected samples were isolated using standard procedure. After the sterilization of diseased leaves in 0.01% $HgCl_2$ solution and sterile distilled water, it is transferred to potato dextrose agar (PDA) media, followed by incubation at room temperature for 5 to 7 days (Chandel *et al*., 2017). Pure culture was prepared (Fig. 2) and utilized for microscopic observation of the fungus.

Pathogenicity test. Pathogenicity test was conducted on detached rose leaves by inoculating pathogen by pin prick method (Udhayakumar *et al*., 2019). The pathogen was reisolated from the infected leaf tissues on PDA medium, and Koch's postulates were confirmed. Microscopic characteristics of the pathogen were examined and found to be similar to the tested pathogen.

Morphological characterization of pathogen. The morphology of conidia and appressoria was studied using the slide culture method. The fungal isolate obtained from this study was identified based on both macroscopic and microscopic features, including pattern and colour of cultural growth. Using a light microscope, spores, hyphae, and other related structures were examined.

Molecular characterization of pathogen. DNA Barcoding was done using universal primers of ITS 1 and 4. For this, about 100 mg of the tissue/mycelium is homogenized using liquid nitrogen and the powdered. Tissue is transferred to a microcentrifuge tube. After adding 400 microlitres of buffer PL1, vortex for a minute. Ten microliters of RNase A solution are added and inverted. The homogenate is incubated at 65°C for 10 minutes. After getting transferred to a Nucleospin filter, the lysate is centrifuged for two minutes at 11000 x g. The filter will be disposed once the liquid flow through it, has been collected. Four hundred and fifty microliters of buffer PCs are added and mixed well. The liquid gets disposed when the solution is moved to a Nucleospin Plant II column and centrifuged for one minute. The column is filled with 400 microlitres of buffer PW1, centrifuged for one minute at 11000 x g, and the supernatent is discarded. The flow-through liquid is subsequently disposed after 700 µl of PW2 is added and centrifuged at 11000 × g. Lastly, 200 µl of PW2 is added, and the silica membrane is dried for two

minutes by centrifuging at 11000 × g. The DNA is then eluted from the column by centrifuging it for one minute at 11000 x g and stored at 4°C. Agarose gel electrophoresis technique facilitated the quality assessment of the isolated DNA. 5µl of DNA was combined with 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0). 0.5X Tris-Borate-EDTA (TBE) buffer containing 0.5 µg of ethidium bromide per millilitre was used to prepare a 0.8% agarose gel, onto which samples were loaded. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei), and the image was captured under UV light using the Gel documentation system (Bio-Rad).

PCR Amplification. The reaction mixture used in the PCR analysis experiment included one microliter of DNA sample, four microliters of distilled water, five microliters of 2X Amplitaq Mix, and 0.25 microliters of each of the forward and reverse primers. Primers known as ITS-1F (forward) and ITS-4R (reverse) were specifically created to target the Internal Transcribed Spacer (ITS) region of the DNA. The PCR reaction was subjected to 40 cycles of distinct temperatures, known as thermal cycling, which included initial denaturation at 95°C for two minutes, denaturation at 95°C for thirty seconds, primer annealing at 50°C for forty seconds, and the final extension step was carried out at 60°C for four minutes, after which it was held at 4°C. The amplified DNA fragments underwent size separation on a 1.2% agarose gel (gel electrophoresis), ethidium bromide staining, and UV visualisation. The size of the amplified product was estimated using a size marker (DNA ladder). In order to avoid interacting with further DNA sequencing, ExoSAP-IT enzyme was used to remove any residual primers and nucleotides from the PCR result after amplification. Then, using a Big-Dye Terminator v3.1 kit, the purified PCR product was sequenced in a thermal cycler. Before determining the DNA sequence, the sequencing reaction product was purified using an ethanol-containing solution to eliminate undesirable chemical constituents. The purified DNA was then placed onto a DNA sequencer for analysis to ascertain the actual DNA sequence.

Phylogenetic Analysis. The program Basic Local Alignment Search Tool-Nucleotide, or BLASTn server, was used to edit and align the ITS sequences (White *et al*., 1990) and the similarity between strains of 18S rRNA gene sequences was calculated using Clustal X Windows (Hall, 1999; Thompson *et al*., 1997). The phylogenetic tree was constructed in Mega XI software (Kumar *et al*., 2018).

RESULT AND DISCUSSION

Collection of diseased samples. Symptoms with varied sizes ranging from 2mm to 10mm were collected for the study (Fig. 2) where a few spots were seen along with yellowing, and they were coalesced to form bigger necrotic spots which covered the overall part of the leaflet. Leaves with 0% to 81-100% disease severity were obtained and classified on each scale ranging from 1 to 6 (Fig. 3). Leaflets in plant 1 showed a highest disease severity of 22.6% (Table 2). Therefore, diseased samples for isolation were taken from plant 1.

Fig. 2. General symptoms of black leaf spot of rose.

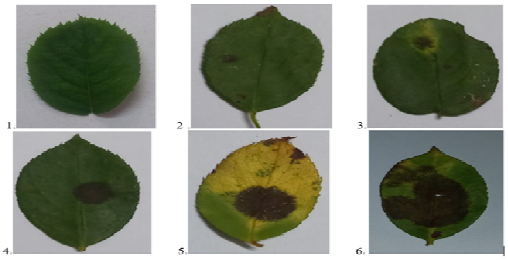

Fig. 3. Collection and scoring of infected leaves from 1 to 6.

Table 2: Comparison of disease severity of plants in selected area.

Class/scale	No. of leaflets in Plant 1	No. of leaflets in Plant 2	No. of leaflets in Plant 3	No. of leaflets in Plant 4	No. of leaflets in Plant 5
1	183	179	111	162	140
2	2	6	1	2	3
3	5	4	2	1	2
4	3	3	1	2	2
5	7	4	4	1	0
6	5	2	2	0	0
Disease severity (%)	22.6	20.79	21.34	18.05	18.1

Isolation and identification of pathogen. From the infected samples collected, the fungal pathogen *Colletotrichum gloeosporioides* was obtained. Circular white colonies (Malvi *et al*., 2023) with dark green pigmentation at the center (Fig. 4) with a cloudy growth pattern were observed after 5 to 7 days of incubation at room temperature, and radial growth of the mycelium was noted (Table 3) showing a growth of 4 cm on the 7th day of incubation. From slide culture technique, it was observed that the conidia (Fig. 5) and brown colored appressoria (Fig. 6) spread over the cover slip's underside after a few days. Thao *et al.* (2024) pointed out that the typical characteristics of *Colletotrichum gleosporioides* species complex is its conidia having cylindrical shape with round ends. They were elongated and slightly curved, resembling a slender barrel with a size range from 10.90µm to 12.28µm and hyphal length between septa ranging from 15.92µm to 23.38µm.

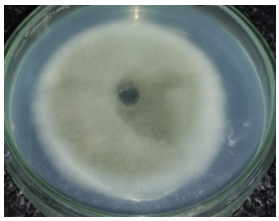

Fig. 4. Pure culture of *Colletotrichum gloeosporioides.*

Table 3: Radial growth of mycelium on different days during incubation.

No. of days	Radial growth of mycelium (cm)
Day 1	0.6
Day 3	1.8
Day 5	3.3
Day 7	4

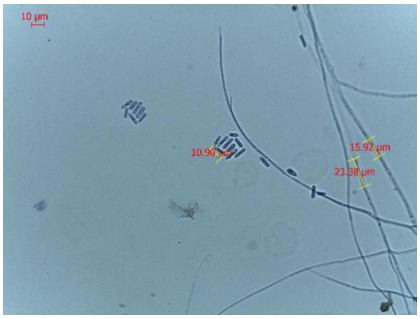

Fig. 5. Spores of *Colletotrichum gloeosporioides.*

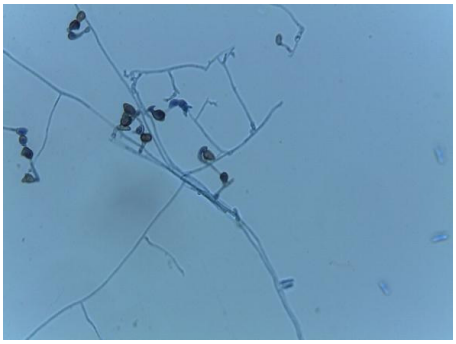

Fig. 6. Presence of appressoria.

Pathogenicity test. Koch's postulates were satisfied by constant re-isolation of the same pathogen from inoculated leaves (Guarnaccia *et al*., 2021). A pathogenicity test on detached rose leaves showed that after 4 days of inoculation, typical symptoms developed on all leaflets inoculated with *Colletotrichum gloeosporioides,* whereas the untreated control leaves did not exhibit any symptoms (Fig. 7).

Fig. 7. Pathogenicity test on rose leaves.

Molecular characterization and phylogenetic analysis. The ITS regions and 5.8S gene area of 18S rDNA were amplified with the primers ITS 1 and ITS 4 and got amplified with 500 base pairs, which has confirmed it as *C. gloeosporioides* (Fig. 8). The amplified 18S-rDNA(ITS1F:TCCGTAGGTGAACCTGCGG and ITS4R:TCCTCCGCTTATTGATATGC) region was purified individually and sequenced by sangar dideoxy sequencing in NCBI. The sequence of ITS regions has shown 99.7% sequence homology with GenBank sequences with BLASTn analysis. A phylogenetic tree was generated from the 18s rDNA sequence of *Colletotrichum gloeosporioides* named Isolate KAU (Fig. 9) using Neighbour likelihood analysis in MEGA11.

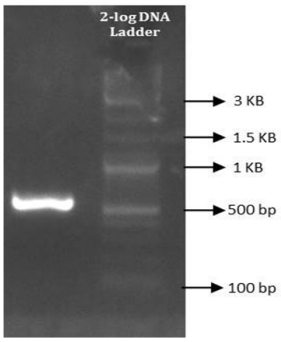

Fig. 8. ITS fragment amplification of *Colletotrichum gloeosporioides* isolate using universal primers ITS 1 and ITS 4.

Fig. 9. Phylogenic tree generated from 18s rDNA sequence of *Colletotrichum gloeosporioides* (Isolate KAU) using Neighbour likelihood analysis in MEGA11.

CONCLUSIONS

In this study, rose leaves affected by black leaf spot were harvested from plants exhibiting the most severe disease symptoms. Subsequently, a standardized isolation protocol was employed, leading to the growth of *Colletotrichum gloeosporioides* after an incubation period of 5 to 7 days. Koch's postulates were confirmed, and both cultural, morphological, and molecular characterizations of the pathogen were conducted, yielding results that corroborate the presence of *Colletotrichum gloeosporioides.*

Some results from the previous studies on the causal organism of black spot were found to be *Diplocarpon rosae* (Gachomo *et al*., 2006) which was different from the present study. Li *et al*. (2023) found the pathogenic fungi of rose black spot was *Alternaria* sp. on PDA plates, and they also reported the presence of new fungi, *Gnomoniopsis rosae*, which belongs to the phylum Ascomycota, order Diaporthales, family Gnomoniaceae, and genus Gnomoniopsis, for the black spot of rose. In another study done by Mohd Asmadi *et al*. (2020), four fungal isolates have been successfully isolated from rose black leaf spot namely, *Rhizoctonia* sp. (one isolate), *Colletotrichum* sp. (two isolates) and *Penicillium* sp. (one isolate). So, it is crucial to accurately identify fungal infections in order to plan an effective strategy for managing the black spot disease in rose production.

FUTURE SCOPE

Further mycological studies that causes black leaf spot on roses, have great potential in various domains. Research on pathogenicity will improve our comprehension of virulence mechanisms and host-pathogen interactions whereas molecular strategies, like DNA barcoding, can improve species identification and taxonomic clarity. Recognition of genetic markers for resistance will aid in breeding initiatives that aims at generating resistant rose cultivars, thereby lowering the requirement for chemical fungicides.

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Conflict of Interest. None.

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