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Diversity of leaf spot pathogen associated with vegetable from Chhatrapati Sambhajinagar, Maharashtra

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ABSTRACT

Vegetables are important constituents of Indian agriculture and nutritional security due to their short duration, high yield, nutritional richness, economic viability and ability to generate on-farm and off-farm employment. The fungal diseases are serious threats to vegetable crop production. The leaf spot infection can result in chlorosis, defoliation, and a decline in the photosynthetic capacity of plant, potentially reducing fruit yield by up to 100% loss. During this investigation the genetic diversity of leaf spot pathogen, the infected samples were collected from different localities of Chhatrapati Sambhajinagar in Kharif and Rabi season of 2023-2024. It was found that the five different leaf spot pathogens via; *Cladosporium cladosporioides*-1, *Cladosporium cladosporioides*-2, *Curvularia crepinii*, *Curvularia geniculata* and *Macrophomina pseudophaseolina* from different localities were shows the morphological as well as genetic diversity among each other. The genetic variability in the leaf spot pathogen isolates may be due to difference in geographical and environmental conditions. The ITS primer is used to analysis, it was found diversity among leaf spot pathogen isolates at molecular characterization. The phylogenetic analysis using ITS Primer showed the grouping of leaf spot pathogen isolates strictly according to their cultural characteristics and degree of pathogenicity and not the geographical origin.

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INTRODUCTION

India is a major global producer of vegetables, with an estimated production of 205.80 million tonnes in 2023-24, and is known for producing a wide variety of vegetables including onions, tomatoes, potatoes, and cauliflower. India is the second largest producer of vegetables in the world and accounts for 16% of global vegetable production (Tripathi *et al.*, 2019). India is rich in biodiversity of vegetables and it is primary/secondary centre of origin of many vegetables. Vegetables are important constituents of Indian agriculture and nutritional security due to their short duration, high yield, nutritional richness, economic viability and ability to generate on-farm and off-farm employment. Our country is blessed with diverse agro-climates with distinct seasons, making it possible to grow wide array of vegetables. Vegetables are vital sources of proteins, vitamins, minerals, dietary fibers, micronutrients, antioxidants and phytochemicals in our daily diet. Apart from nutrition, they also contain a wide array of potential Phyto-chemicals like anti-carcinogenic principles and anti-oxidants (e.g. flavonoids, glucosinolates and isothiocyanates). Vegetables, consumed either raw or cooked, act as major sources of vital nutrients required for the growth and development of the human body. In fact, vegetables are excellent sources of essential minerals for the human body, such as macronutrients, including, K, Ca, Mg, P, and S (Chakrabarty *et al.*, 2018; Sarker *et al.*, 2018a, b) and micronutrients, including Fe, Cu, Mn, Zn, Na, Mo, and B (Sarker *et al.*, 2015b, 2017), protein with essential amino acids (Sarker *et al.*, 2014), dietary fiber (Sarker *et al.*, 2018c), and vitamins (Sarker *et al.*, 2015a, 2016). Besides, the phytochemicals present in vegetables and other plants show high antioxidant activity, help in increasing immunity, and reducing age related health issues (Forni *et al.*, 2019). Phytochemicals, basically, are the non-nutrient bioactive compounds found in plants as the products of secondary metabolism (da Silva *et al.*, 2016) which constitute their defence system (Sarker & Oba, 2018a, b).

In recent years, the varieties and frequency of vegetable diseases have continued to increase worldwide with the increase of the vegetable planting area and species. Particularly, due to

the changes of global climate and farming system, vegetable diseases have exhibited the characteristics of wide distribution, high disaster frequency and strong suddenness. The spread of diseases has seriously affected the quality and yield of vegetables, not only causing economic losses of billions of dollars each year, but also bringing potential threats to food safety. Therefore, strengthening the disease control measures has become the key to ensuring high yield and high quality of vegetable products. Diseases and their insect vectors are the major threats to vegetable production in the world. Achieving a 'zero hunger' world by 2030 depends on doubling of agricultural productivity and reduction of crop losses due to plant diseases. The Foliar fungal diseases are serious threats to vegetable food security. Various disease recognition techniques are required for the rapid detection of leaf diseases in the early phases of plant growth and fungal infection (Singh & Misra, 2017). The current approach for leaf disease recognition is based on the identification and detection of fungal pathogens by comparison with databases from national or regional biological information systems. However, this approach is not fully reliable. Over the past two decades, leaf diseases have been investigated using visible array images. Nevertheless, the methodology to date is imperfect and tends to rely upon idealized disease models and scenarios (Barbedo, 2016). Although the focus here is the correct identification of foliar fungal diseases, environmental speciation may render this task difficult and challenging. The tropical climate, with its high humidity, exacerbates these challenges by creating ideal conditions for fungal pathogens. Many of these pathogens have short latent periods, leading to rapid spread and secondary infections (Liang *et al.*, 2022). Local farmers have faced substantial setbacks, as traditional pesticide applications, often formulated without precise knowledge of pathogen behaviour, have proven ineffective. These difficulties highlight the need for a more targeted and scientifically informed approach to pest and disease control. A promising solution lies in implementing site-specific pest and disease management strategies. However, effective control systems require correct identification of the specific pathogenic microorganisms involved (Srivastava *et al.*, 2020). Molecular-based identification methods, particularly DNA sequencing, provide the precision needed to identify the exact species responsible for infections, thereby significantly enhancing the effectiveness of disease control measures (Nizamani *et al.*, 2023).

Leaf spot symptoms include blackish-brown acervuli and concentric ring on leaves, which release fungal conidia, spreading the infection (Back *et al.*, 2015). This infection can result in chlorosis, defoliation, and a decline in the photosynthetic capacity of plant, potentially reducing fruit yield by up to 100% if left unmanaged (Sastrahidayat & Nirwanto, 2016). Leaf samples collected from different vegetable crop field of Chhatrapati Sambhajinagar, were analysed through DNA extraction, PCR amplification, and sequencing to identify the specific fungal species responsible. The fungal pathogen *Cladosporium cladosporioides*, *Curvularia crepinii*, *Curvularia geniculata* and *Macrophomina pseudophaseolina* attacking the living leaves and causing leaf spot disease on affecting plant growth and development. Early detection and proper identification of the causative agent to the specific level are essential to adopt the most effective management strategies, including cultural practices, fungicides, biocontrol, and resistant plant varieties. This study represents the first effort to apply molecular techniques for leaf spot pathogen identification in vegetable cultivation, aiming to establish a foundation for more effective and targeted disease management strategies. The leaf spot pathogen was analysed morphologically as well as by busing ITS primer. The highly distributed and most destructive pathogen of causing leaf spot disease is genetically much diversified and the phylogenetic analysis showed the grouping of isolates. The ITS primer was found genetic diversity and genetic relationship of leaf spot pathogen. The cluster analysis using ITS primer showed the grouping of pathogen isolates strictly according to their cultural characteristics and degree of pathogenicity and not the geographical origin. This information will be helpful for pathologist and plant breeder to design effective resistance breeding programmers in vegetable crop taking into account the diversity in leaf spot pathogen.

MATERIAL AND METHODS

The present work was carried out at the Government College of Arts and Science, Chhatrapati Sambhajinagar, during the year of 2023-2024. The details of field and laboratory procedure followed during this research work are described in this chapter.

Collection of Samples

The leaf spot affected samples were collected from different vegetable growing crop field of Chhatrapati Sambhajinagar. The leaf spot affected sample are collected in early morning in a sterilized paper bag or zip lock polythene bag, each samples locality takes the GPS camera photograph and polythene bag was labeled properly by in the locality of collection, date and time (Figure 1). Then the collected sample was taken to the laboratory and these samples were preserved at 4 °C in refrigerator and used for further studies.

Sterilization of Samples

To minimize contamination from non-pathogenic surface microbes, the infected leaf sample were surface sterilized with 0.1% Mercuric Chloride ($HgCl_2$) for 2 minutes, followed by 2-3 times rinses with sterilized double distilled water to remove traces of $HgCl_2$ Mercuric Chloride ($HgCl_2$) is a widely used disinfectant in Mycology and Plant Pathology, effectively eliminating surface pathogens while preserving the integrity of internal fungal structures. The repeated rinsing with sterile water ensures the removal of any residual bleach, which could otherwise inhibit fungal growth during subsequent culturing (Lee *et al.*, 2011).

Leaf Spot Pathogen Isolation and Purification

Cut the Small sections (3-5 mm) of the sterilized leaf spot sample then placed on to Potato Dextrose Agar Media (PDA) containing streptomycin (0.12 gm/L) and incubated at 30 °C for 4-5 days in the alternate dark and light condition for one week.

Figure 1: Collection of leaf spot sample from different region of Chhatrapati Sambhajinagar

The dark environment inhibits the growth of photosynthetic organisms and aids in isolating fungal colonies. Potato Dextrose Agar Media (PDA) is basic medium, supports fungal growth while minimizing the proliferation of bacterial contaminants. Once fungal colonies appeared, they were purified by subculturing on to PDA, a nutrient-rich medium designed to promote fungal growth and facilitate the isolation of distinct fungal species (Sastrahidayat & Nirwanto, 2016). All the isolates pathogen was purified by single spore isolation method (Hansen, 1926) and sufficient care was taken to maintain the purity of the isolates throughout the study.

Selection of Media for Isolation

Potato Dextrose Agar (PDA) (Rangaswami, 1972): Peeled Potato - 200 g, Dextrose - 20 g, Agar-Agar - 20 g, Streptomycin - 0.2 g, Distilled Water (D/W) - 1000 mL, pH - 5.5 to 5.6.

Procedure

Take 200 gm of potatoes for 1 L of PDA media preparation. Wash the potato to remove dirt and peel off the skin and dry them. Add the pieces to 300 mL of distilled water and boil for 20-25 minutes on a hot plate, after boiling collect the extract through the muslin cloth. Weigh the ingredients separately with respect to the volume of the media. Dissolve the compound in the beaker using stirrer or glass rod. Transfer the potato infusion or potato extract into conical flask and mix ingredients well by shaking the conical flask. After that mix the Agar-Agar powder separately into warm distilled water by continuous shaking of glass rod and add into potato extract. Then close the mouth of the flask with a cotton plug seal it further with a newspaper and rubber band. Autoclave for 20-25 minute at 15 psi/121 °C on liquid cycle, after autoclave cooled the media about 45 to 60 °C then adjust the PH of the media to 5.5-5.6 using 0.1 N HCI and 0.1 NaOH and mix well. Then add antibiotics and mix well, pour into sterilize Peri-plate and solidify them.

Microscopic Identification Leaf Spot Pathogen

After the growth of pathogen on the petri plates, the macroscopic observation was done by external mycelial feature, texture, front and reverse colony color, growth rate and microscopic characteristics are arrangement of conidia. Macroscopic and microscopic features of fungi are helpful in the correct identification of fungi. The identification of fungi was done by using the various research paper, monographs and other literature such as The Illustration of Fungi (Mukadam, 2006), Dematiaceous Hyphomycetes (Ellis, 1971).

Morphological Characterization

To study morphology of macroconidia and microconidia 7-9 day old culture of each isolate grown on PDA medium was stained with 0.1% Lactophenol cotton blue on slide and observed under compound microscope.

Slide Preparation

On a clean slide, a drop of lactophenol cotton blue was taken & then the culture from the edge of the colony was placed over it followed by a cover slip. Examine the Fungal structure under a microscope.

Growth Pattern and Morphological Characteristics of Leaf Spot Pathogen

In order to study growth pattern and morphological characteristics of leaf spot pathogenic fungi which are isolated from different infected vegetable leaf sample, were grown on solid Potato Dextrose Agar (PDA) medium. These isolates where incubated room temperature at 30 °C for 7-9 days on Potato Dextrose Agar (PDA) medium and the result were in the form of front and reverse mycelia colony colour, colony pattern and colony diameter. The study of Morphology of conidia and conidiophore after 7-9 days old culture of each isolate grown on PDA medium was stained with 0.1% lactophenol cotton blue on slide and observed under compound microscope.

DNA Extraction

Fungal DNA extraction was extracted by using DNA extraction method (Abd-Elsalam *et al.*, 2003). The leaf spot pathogenic Fungi were cultured in 50 mL of Potato Dextrose Broth (PDB) for 72 hours on a contentious shaker to promote optimal growth and

biomass production of fungi. After centrifugation at 10,000 rpm, the fungal mycelia were collected and washed with Tris-EDTA (TE) buffer to stabilize the DNA and remove contaminants, then ground to release cellular contents. The extraction buffer contained Tris-HCl to maintain a stable pH, NaCl to stabilize DNA and disrupt protein interactions, EDTA to chelate divalent cations and inhibit DNases, and SDS, a detergent that lyses cells and dissolves membranes. Sodium acetate was added to precipitate proteins and other contaminants, and the supernatant containing the DNA was transferred to a new tube for further processing. DNA precipitation was achieved using isopropanol, which causes the DNA to aggregate and form a visible pellet upon centrifugation. The 70% ethanol is used to wash and to remove salts and other impurities that could interfere with subsequent PCR amplification. Finally, the DNA pellet was air-dried and resuspended in Tris-EDTA (TE) buffer for storage at -20 °C, ensuring the integrity of DNA and protection from degradation.

Amplification of Internal Transcribed Spacer (ITS)

The internal transcribed spacer (ITS) region of fungal rDNA was amplified using Hi-PCR® REDy Master Mix. The ITS region is highly variable among fungal species and is widely used as a genetic marker for species identification. Primer pairs ITS1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse) (5'-TCCTCCGCTTATTGATATGC-3') were selected because they target conserved regions flanking the ITS region, enabling amplification across a broad range of fungal species (White *et al.*, 1990).

DNA Sequencing

PCR products were processed for cleanup to remove unincorporated nucleotide and residual primers using exonuclease-I and shrimp alkaline phosphatase enzyme (1unit/µL) followed by cycle sequencing reaction using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). For ITS amplicon sequencing same PCR primers were used. The thermal cycler conditions were an initial denaturation of 2 min at 96 °C and 35 cycles of 30 sec at 96 °C, 15 sec at 55 °C, and 4 min at 60 °C. The Cycle sequencing is followed by sequencing cleanup by ethanol precipitation followed by dissolving template in HiDi formamide and bidirectionally sequenced in ABI 3730 genetic analyser.

Sequence Alignment and Assembly

PCR products were then processed for direct bi-directionally sequencing using ABI PRISM 3730 × 1 Genetic Analyzer (Applied Biosystems, USA). The resulting DNA sequences were aligned using CLUSTALW embedded in MEGA 11 (Tamura *et al.*, 2021), manually trimmed and edited to obtain complete sequences. The confirmation of species depends on the sequence similarity score. Homology searches were carried out using the BLAST program against the NCBI GenBank database. Phylogenic tree was constructed using MEGA 11 with all positions containing gaps and missing data were included for analysis.

RESULT AND DISCUSSION

Isolation of Fungal Pathogens

During this investigation isolates different five leaf spot pathogen, were obtained from different location of Chhatrapati Sambhajinagar (Khultabad, Paithan, Vaijapur, Phulambari, Kannad), primarily responsible for causing leaf spot symptoms. Potato Dextrose Agar (PDA) medium is used to isolation of fungal pathogen. These fungal pathogens are responsible for cause the leaf spot diseases. To infect the leaf, pathogens must first break down cellulose, which is predominantly found in plant tissue (Shirsath *et al.*, 2018). Potato Dextrose Agar (PDA) medium contains only distilled water, dextrose and cellulose fiber from the agar, providing an ideal carbon source for cellulose-degrading fungi, including those capable of causing leaf spot symptoms (Cheng *et al.*, 2021). The purification was carried out using Potato Dextrose Agar (PDA) medium, where the fungal colonies were inoculated and incubated at 30 °C for 5-7 days in the alternate dark and light condition for one week until purified isolates were obtained.

Identification of Fungal Pathogens

The purified leaf spot pathogen isolates were then subjected to DNA extraction and sequencing. DNA sequences were identified through an Internal Transcribed Spacer (ITS) primer and BLAST search on the NCBI database. Based on the molecular identification, the five main isolates were classified into four species, as shown in Table 1 with their macroscopic appearance presented in Figure 2. The identification of *Cladosporium cladosporioides*-1, *Cladosporium cladosporioides*-2, *Curvularia crepinii*, *Curvularia geniculata* and *Macrophomina pseudophaseolina* as the major pathogens responsible for leaf spots in vegetable crop cultivars in Chhatrapati Sambhajinagar region. These findings enhance our understanding of the pathogen diversity associated with leaf spot disease and can aid in developing effective pest and disease management strategies.

Morphology of Leaf Spot Pathogen

In general, all leaf spot pathogen, the aerial mycelium had a cottony, fluffy appearance, was initially grey but gradually turned brown or black as it aged, and becoming dark brown or black in some areas but in RG1 showing grey olive and in RG4 mycelium shows greyish black colour, RG5 show whitish brown colour similarly, the RG6 show blackish brown colour and RG7 shows grey black mycelium. The different leaf spot pathogen shown difference in colony diameter. The highest growth was observed in RG6 (3.3 cm) but RG5 shows lowest growth (2.7 cm). All the leaf spot pathogen are difference between mycelia colour, texture of mycelia, growing pattern of mycelia, colony diameter as well as it also differences between its conidia and conidiophore structure as shown in Table 2 (Figure 2).

Table 1: Molecular identification of leaf spot pathogen isolated from infected vegetable leaf

Sample Codes	Name of Fungi	Isolate Host Name	Location	Accession No.	Identity
RG1	*Cladosporium cladosporioides-1*	Tomato	Khultabad	KJ596320.1	99.79%
RG4	*Cladosporium cladosporioides-2*	Spinach	Paithan	HQ315845.1	100.00%
RG5	*Curvularia crepinii*	Cabbage	Vaijapur	KF572439.1	99.42%
RG6	*Curvularia geniculata*	Brinjal	Phulambari	KX401430.1	99.61%
RG7	*Macrophomina pseudophaseolina*	Cowpea	Kannad	ON797334.1	97.67%

Table 2: Morphological and cultural characteristics of leaf spot pathogen

Sample Codes	Front colony Colour	Reverse Colony Colour	Colony Diameter (Cm)	Type of Mycelium
RG1	Grey Olive	Black Velvety	2.8	Irregular, Scattered, Compact Aerial Mycelia
RG4	Greyish Black	Olive Black	3.0	Rounded, Cottony, Compact Aerial Mycelia
RG5	Whitish Brown	Dark Brown	2.7	Rounded, Cottony, Fluffy Aerial Mycelia
RG6	Blackish Brown	Dark Brown	3.3	Cottony, Fluffy, Compact Aerial Mycelia
RG7	Grey Black	Dark Black	3.1	Rounded Cottony, Fluffy Aerial Mycelia

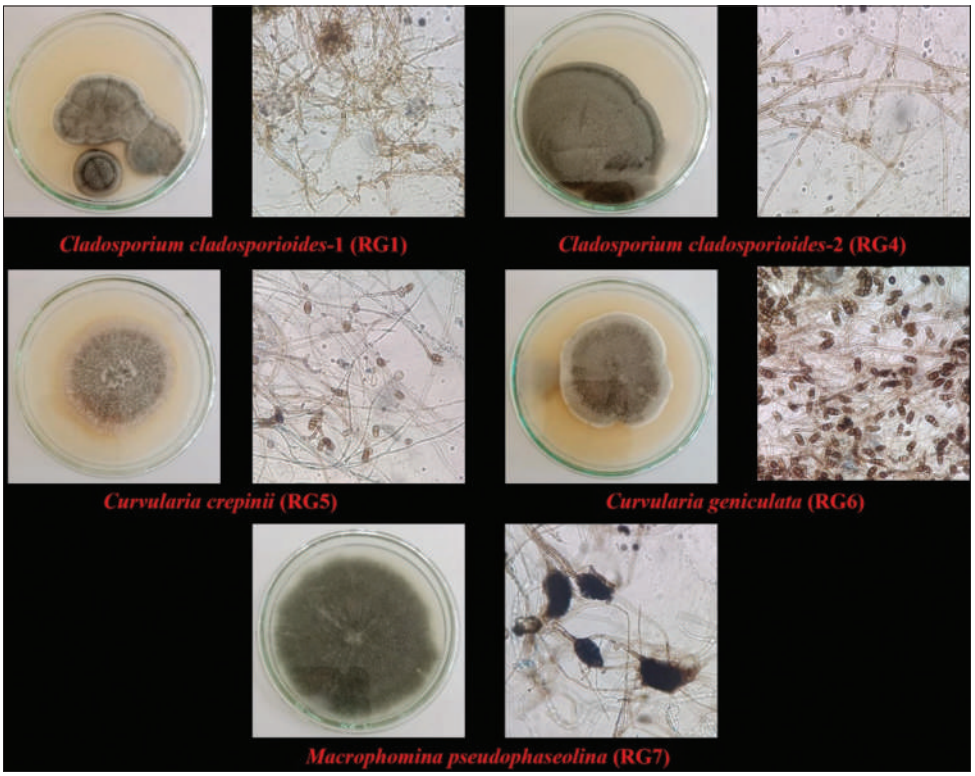

Figure 2: Pure culture and microscopic photograph of different vegetable leaf spot pathogen

Genetic Diversity Analysis of Leaf Spot Pathogen using ITS Primer

The Genome analysis of leaf spot pathogen isolates by ITS primer, in this study has provided evidence that this Pathogenic fungus varies genetically. This variability should be taken into consideration in different crop. In order to determine the extent of genetic variation of this economically important fungus and relationship with cultural and pathogenic traits, the DNA fingerprint was generated through ITS primer, based on the DNA sequencing data for each ITS primer for five leaf spot pathogens. The five leaf spot pathogen were differentiated on the basis of their ITS primer sequence pattern (Tables 3 - 6). All pathogen is indicated existence of genetic variation and high level of genetic variability in the study suggests among selected five isolates (RG1, RG4, RG5, RG6, RG7).

Genetic Diversity Analysis among Five Leaf Spot Pathogen Based on Phylogenetic Tree Generated using ITS Primer

The phylogenetic tree was constructed on the basis of their variants forms the entire data set of primers assisted identification and analysis homology of each gene sequence of five different leaf spot pathogen. The phylogenetic analysis shows the evolutionary history, diversification and relationship between different strains (Figure 3). The phylogenetic analysis studies can help to identify the specific leaf spot

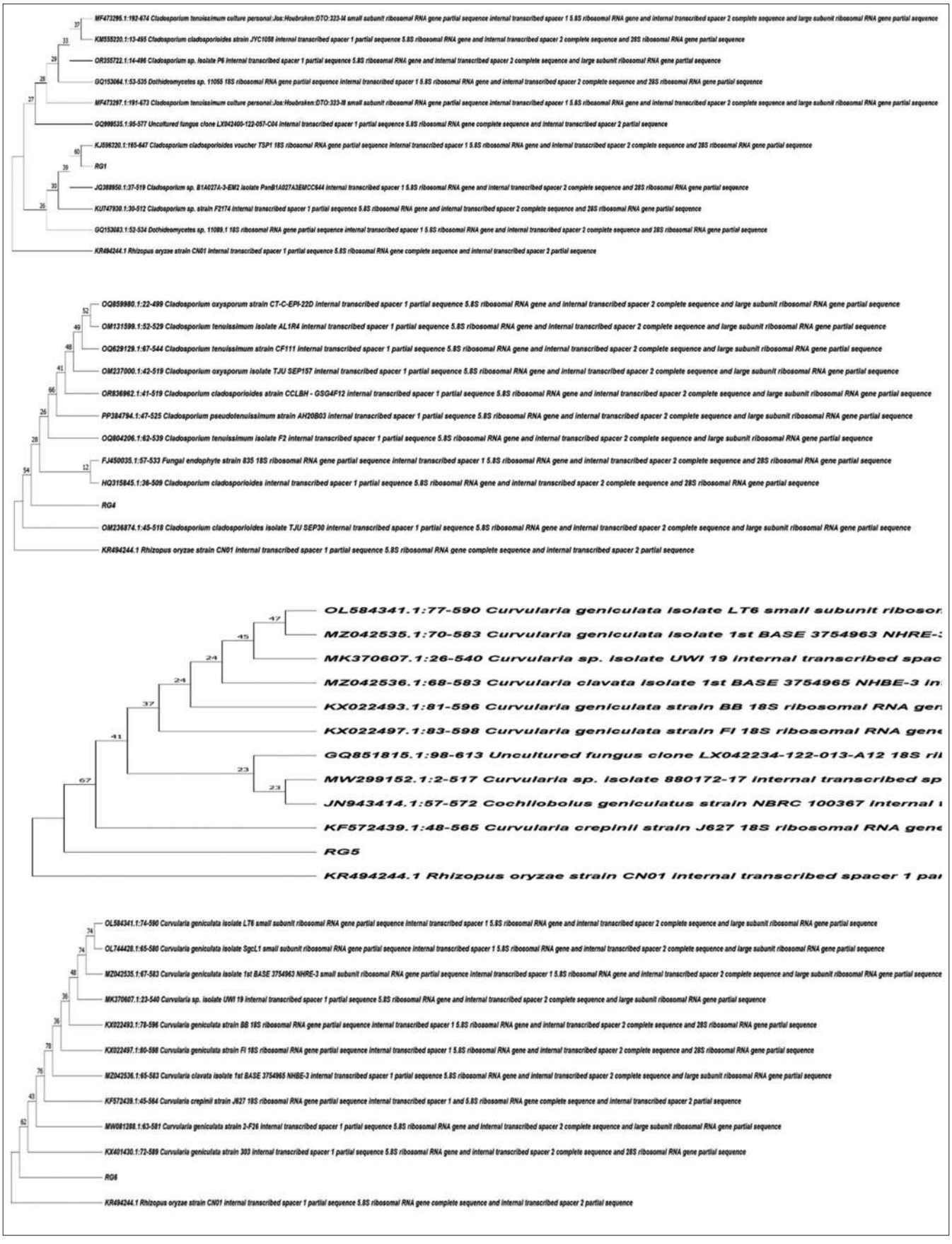

Figure 3: Phylogenetic tree demonstrating the relationship of different leaf spot pathogen of vegetable crop

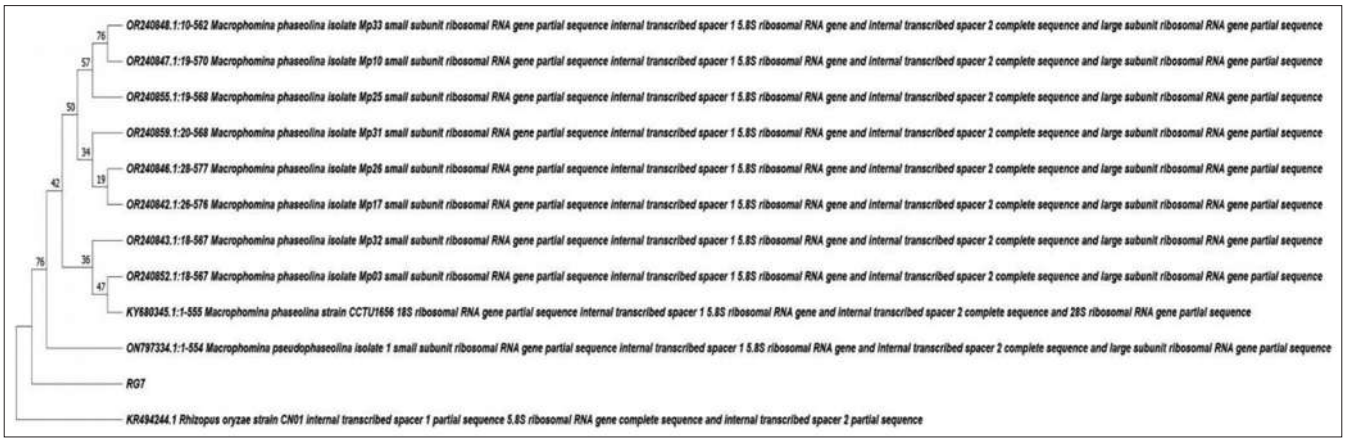

Figure 3: *(Continued)*

Table 3: Details of ITS polymerase chain reaction composition

S. No.	Component	Component Volume (µL)
1	Hi-PCR® REDy Master Mix	12.5
2	ITS1 primer	1.5
3	ITS4 Primer	1.5
4	Template DNA	3.0
5	Nuclease-Free Water	6.5
6	Total reaction volume	25

Table 4: Primers used for ITS gene amplification and its sequencing

S. No.	Name of Primer	Oligo Name	Sequence (5`à3`)
1	ITS1 Primer	ITS Forward	5'TCCGTAGGTGAACCTGCGG3'
2	ITS4 Primer	ITS Reverse	5'TCCTCCGCTTATTGATATGC3'

Table 5: ITS thermal cycling conditions used for amplification

S. No.	Steps	Temperature (°C)	Duration	Cycle
1	Initial Denaturation	95	3 Min	35 Cycles
2	Denaturation	95	45 Sec	
3	Annealing	55	59 Sec	
4	Primer Extension	72	59 Min	
5	Final Extension	72	10 Min	
6	Hold	4	-	-

Table 6: ITS primer used in the DNA sequencing of leaf spot pathogen isolates

S. No.	Sample Code	Name of Fungi	Primer	Sequence (5`à3`)
1	RG1	*Cladosporium cladosporioides-1*	ITS	5'TTGTCCGACTCTGTTGCCT3'
2	RG4	*Cladosporium cladosporioides-2*	ITS	5'TCCGACTCTGTTGCCTCCG3'
3	RG5	*Curvularia crepinii*	ITS	5'GAGTATTTTATTACCCTTGT3'
4	RG6	*Curvularia geniculata*	ITS	5'CTGGAGTATTTTATTACCCT3'
5	RG7	*Macrophomina pseudophaseolina*	ITS	5'GGAGGATCGGGACGGAGCC3'

pathogen cause the disease in vegetable crop and other host. The phylogenetic analysis is often combined and verify the accuracy of morphological and pathogenicity to provide a more comprehensive understanding of the pathogen. The phylogenetic tree shows genetic variation of these isolates of leaf spot pathogen and aiding in understanding their evolutionary relationship with cultural and pathogenic traits.

CONCLUSION

The leaf spot disease has become major concern in vegetable production. The four different fungal species were found to primarily infect vegetable crop on Chhatrapati Sambhajinagar, specifically in the five districts (Khultabad, Paithan, Vaijapur, Phulambari, Kannad). These five fungi were *Cladosporium cladosporioides*-1, *Cladosporium cladosporioides*-2 *Curvularia crepinii*, *Curvularia geniculata* and *Macrophomina pseudophaseolina*. This study provides molecular identification and characterization of the major pathogens responsible for Leaf spot disease in vegetable crop cultivars on Chhatrapati Sambhajinagar. The leaf spot pathogen is showing the high level genetic and morphological variability due to difference in geographical and environmental conditions. The ITS primer was used to find out diversity among different isolates of leaf spot at the molecular level characterization. The genetic high-level variability also recorded in five different leaf spot pathogens. The genetic variation of these isolates and relationship with cultural and pathogenic traits.

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