(Original Research article)

Studies on symptomatology, Morphological and molecular characterisiation of *Erysiphe Cichoracearum* causing powdery mildew of okra

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ABSTRACT

Okra (*Abelmoschus esculentus* (L.) Moench) is a globally important annual vegetable belongs to family malvaceae, it is most broadly distributed vegetable all over the world. Among the fungal diseases affecting okra crop, powdery mildew caused by *Erysiphe cichoracearum* DC. is the most important disease causing considerable yield losses. The disease was characterized by the appearance of small white powdery specks on upper surface of the lower leaves which enlarged and coalesced forming bigger patches. Later on the disease spread to the upper leaves, coalescing of the white powdery specks and enlargement in their size ultimately resulted in complete coating of white fungal mass on the leaves. The morphological studies of the pathogen in the laboratory revealed, that the mycelium is septate, ectophytic, hyaline and adjacent to the mycelium, basal septum of the conidiophores was seen. The conidiophores were erect, on which conidia were produced either singly or in short chains. The conidia were found cylindrical or barrel in shape, hyaline, non-septate and measured 75.23 µm x 40.65 µm. Molecular characterisation of the native isolate resulted in the DNA amplicon at the region 531 bp. Further, DNA sequence was obtained for ITS Rdna and was deposited in NCBI gene bank. Accession number 'MW774352' was obtained and it was confirmed that the causal pathogen of okra powdery mildew of Raichur region is *Erysiphe cichoracearum*

KEY WORDS: *Erysiphe cichoracearum, conidiophores, conidia, DNA amplicon.*

1. INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench) is a globally important annual vegetable belongs to family malvaceae, it is most broadly distributed vegetable all over the world. In the world it is cultivated in tropical, sub-tropical and warm temperate regions. The crop is usually grown both under irrigated and rainfed conditions. In India, okra occupies a prominent position because of the easy cultivation, dependable yield and adaptability to the varying weather conditions and it is one of the home garden vegetable. The native of okra is considered to be Africa and India is taken into consideration as the secondary centre of diversity.

Among the fungal diseases affecting okra crop, powdery mildew caused by *Erysiphe cichoracearum* DC. is the most important disease causing considerable yield losses. The disease initiates as white minute powdery patches first on the upper surface of leaf and lower older leaves and then spreads to younger ones. Grayish white powdery coating is visible on severely affected leaves. Leaves finally show necrosis resulting in withering, drying and defoliation. Powdery mildew affects plants at all the growth stages and may result in yield losses up to 17 to 86.6 per cent (Sridhar and Sinha, 1989)(1).

Powdery mildew occurs in severe form at a particular stage of the crop covering large host surface. Sometime, crop gets infected at an early stage, if the inoculum potential coincides with favorable environmental conditions. In Karnataka, serious outbreak of powdery mildew on okra was observed around Bangalore since 1970 (Sokhi and Sohi, 1976) (2) and also during last few years the disease is occurring in an endemic form in most areas of Northern Karnataka. Hence, an attempt was made to study symptoms caused by pathogen under filed conditions, collect diseased leaf samples and study morphology of pathology, further molecular characterization was also done and sequence was deposited to NCBI gene bank.

2. MATERIALS AND METHODS

Symptomatology

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To study the symptom development on okra, the seedlings of susceptible variety Arka Anamika were raised in pots under glasshouse conditions. Severely infected powdery mildew leaves were collected from field. Then the white powdery mass containing conidia of *Erysiphe cichoracearum* DC. were dusted on to the fully expanded leaves of Arka Anamika. The inoculated seedlings were placed under shade. Observations on symptom development were recorded at regular intervals after initiation of infection.

Morphology of the pathogen

To study the morphological features of *E. cichoracearum,* powdery mildew infected okra leaves of Arka Anamika were brought to the laboratory. The white powdery mildew mass having conidial growth was dislodged in to sterile water using camel hair brush. The spore suspension was placed on cavity slides and the observations were recorded for shape, size of the conidia, mycelium, conidiophores and germination of conidia under compound microscope at 10X and 40X magnification.

Molecular characterization of the pathogen

The protocol for DNA extraction protocol, primers used and PCR amplification were followed as per Chen *et al*. (2008) (3) with minor modifications. Total genomic DNA was extracted by scraping the mycelium from the powdery mildew infected leaves, collected from the susceptible cultivar Arka Anamika following the standard CTAB method with minor modifications.

Procedure: The conidia were collected from the fresh powdery mildew infected okra leaves (Arka Anamika) with the help of camel brush. The DNA extraction method was standardized and certain steps were optimized to produce good concentration of DNA.

The new colonies were scraped from the leaf surface with a small brush and ground in a Pestle and Mortar with nine ml of CTAB extraction buffer and mixed gently by inversion. Tubes were incubated for 60-90 minutes, with occasional inversion at 65 ^{0}C. The samples were allowed to cool by keeping the tubes in water at room temperature.

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Five ml of chloroform and isoamyl alcohol (24:1) was added, the tubes were rocked gently to mix the content for five min. The samples were subjected to spinning in a centrifuge for 15 min at 6500 rpm at room temperature. The aqueous layer was transferred to a fresh tube and 25 μl RNase A (20 mg ml^{-1}) was added.

The samples were mixed gently by inversion and incubated for 30 min. at room temperature. Six ml of isopropanol was added to each tube and mixed gently by inversion until a white fluffy DNA precipitate appeared. The contents were centrifuged at 6500 rpm for 15 min to pellet the DNA. After 2-3 min, eight ml of cold wash buffer was added and incubated for 20 min at room temperature.

The tubes were centrifuged to pellet the DNA at 6500 rpm for 15 min. The supernatant was discarded and eight ml of cold 70 per cent ethanol was added to the tube containing the DNA pellet. One ml of elution buffer was added and mixed gently to dissolve the pellet and kept at 4 ^{0}C overnight.

The DNA solution appeared to be turbid after standing overnight at 4^{0} C and the samples were heated to 65 ^{0}C for 10 min, inverting the tube every 3 min. Insoluble material was removed by centrifugation at 6500 rpm for 15 min and the clear supernatant containing DNA was transferred to a fresh 1.5 ml tube discarding the pellet.

Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analysed by running 2 μl of each sample mixed with 2 μl of 10X loading dye in one per cent agarose gel. The DNA from the native isolate produced clear sharp bands in one per cent agarose gel indicating the good quality of DNA. The DNA was quantified by comparing with the 100 bp size marker (Genei, Bangalore) and by using biophotometer.

Polymerase chain reaction

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of a copy of 18S, 5.8S and 28S like rDNA and its spacer like internal transcribed spacers (ITS) and intergenic spacers (IGS). The rDNA

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has been employed to analyze evolutionary events because it is highly conserved, where as ITS rDNA is more variable. Hence, it has been used to investigate species level relationships. The ITS region was amplified with the universal primers ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTAT TGATATGC-3') given by White *et al.* (1990) (4). Amplification was performed in 50 µl of reaction mixture containing 50 Pico mol of 1 µl of ITS 1, ITS 4 primers, 0.25 µl *Taq* DNA polymerase, 1 µl (10 mM) of dNTP, 5 µl of 10X PCR buffer, 1 µl (40 µg) of template DNA and PCR water 39.75 µl and subjected to PCR in a thermal cycler. The PCR conditions followed were, initial denaturation at 94 ^{0}C for 4 min, denaturation at 94 ^{0}C for 1 min, annealing at temperature 55 ^{0}C for 1min, its extension at 72 ^{0}C for 1.5 min and final extension at 72 ^{0}C for 5 min**.**

Agarose gel electrophoresis

Separation of amplified products by agarose gel electrophoresis

Requirements

- Electrophoretic unit, gel casting, gel comb, power pack and UV-trans illuminator
- Agarose 1 per cent
- Bromophenol blue
- Ethidium bromide (0.5 µg ml^{-1})
- 50X TAE (stock): Tris-free base-60.5 g
- Glacial acetic acid - 14.25 ml
- 0.5 M EDTA - 25 ml
- Made up the volume to 250 ml, PH - 8.0
- Working solution (1X TAE): 20 ml of 50X TAE was made up to 1,000 ml by using distilled water (Appendix I)

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Procedure

- One gram of agarose was weighed and added to a 250 ml conical flask containing 100 ml of 1X TAE buffer.
- The agarose was melted by heating the solution in an oven and the solution was stirred to ensure even mixing and complete dissolution of agarose.
- The solution was cooled to about 50 ^{0}C.
- Two to three drops of ethidium bromide (0.5 µg ml^{-1}) was added.
- The solution was mixed and poured into the gel casting platform after inserting the comb in the trough.
- While pouring, sufficient care was taken for not allowing the air bubbles to trap in the gel.
- The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1X TAE) so as to cover the wells completely.
- The amplified products (20 µl) to be analyzed were carefully loaded into the sample wells, after adding bromo phenol blue with the help of micropipette.
- Electrophoresis was carried out at 60 volts, until the tracking dye migrated to the end of the gel.
- Ethidium bromide stained DNA bands were viewed under gel doc unit and photographed for documentation.

ITS rDNA Sequencing:

The PCR product (50 µl) was sent to Chromus Biotech Laboratory, Bangalore for direct sequencing. DNA sequence for the internal transcribed spacer region (ITS) of the 28S rDNA was obtained. After collecting the sequence from Chromus biotech lab, the

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sequence obtained in this study was submitted to the NCBI (National Centre for Bioinformatics) Gen Bank to check the identity of the isolate and previously published sequences used for phylogenetic analysis.

3. RESULTS AND DISCUSSION

Symptomatology

The disease appeared on leaves during flowering to fruiting stage in the month of September to January. The symptoms of the powdery mildew were observed both on stem and leaves. The initial symptoms observed were small, white, powdery specks on upper surface of the lower leaves which enlarged and coalesced forming bigger patches. Later on the disease spread to the upper leaves. Coalescing of the white powdery specks and enlargement in their size ultimately resulted in complete coating of white fungal mass (consisting mycelium, conidiophores and conidia) on the leaves. In the severe and later stages senescence and defoliation of leaves was noticed (Plate 1 and Plate 2).

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1. Small white powdery specks noticed on lower leaves

2. Specks enlargement

3. Powdery growth on petiole

4. Severely infected leaf

5. Defoliation

Plate 1: Manifestation of powdery mildew symptoms on okra

Plate 2: Pathogenicity test for *Erysiphe cichoracearum*

Morphology of *E. cichoracearum*

The morphology of the fungus at 10X magnification revealed, that the fungus had superficial mycelium, which is septate, ectophytic, hyaline and adjacent to the mycelium, basal septum of the conidiophores was seen (Plate 5a and 5b). The conidiophores were erect, on which conidia were produced either singly or in short chains. The conidia were found cylindrical or barrel in shape, hyaline, non-septate and measured 75.23 μm x 40.65 μm and are in agreement with earlier workers *viz*., Sharma (2016)(5) and Sushma (2019)(6).

Molecular characterization of the pathogen

The molecular detection methods for identification of the pathogen based on ITS sequences were found to be more accurate over microscopic observations (Takamatsu and Kano 2001) (7). To confirm the pathogen more reliably and accurately, molecular characterization of the native isolate was carried out by extracting the DNA from the powdery mildew samples followed by PCR amplification of ITS region of ribosomal DNA product of the pathogen by using powdery mildew-specific ITS primer pair ITS1 and ITS4 which resulted in the amplified product of size 531 bp DNA fragment length against 100 bp DNA ladder. The amplified PCR product was sequenced.

Isolation of Genomic DNA

Genomic DNA of the fungus was isolated by CTAB (Cetyl trimethyl ammonium bromide) method. The DNA so obtained was observed by running on one per cent agarose gel electrophoresis.

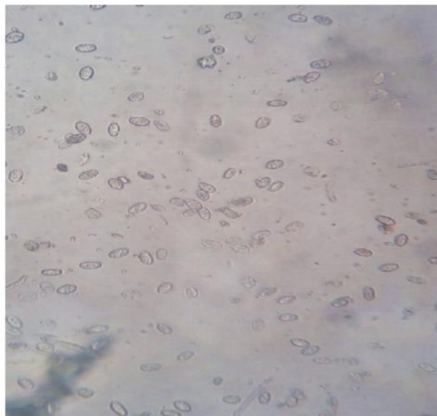
Amplification of ITS1 and ITS4 region

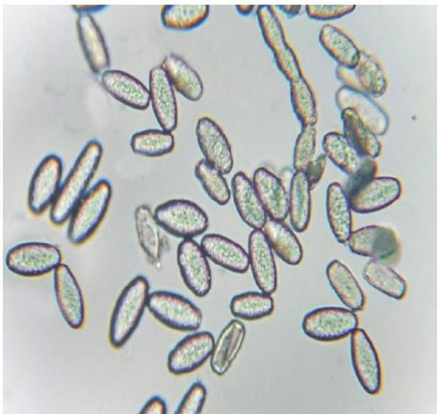
The full length ITS rDNA region was amplified with ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’)

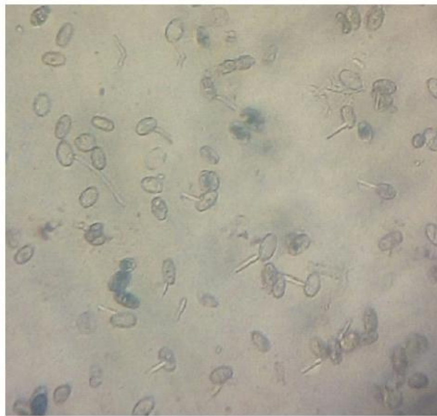
a) Conidia and conidiophore at 10X

b) Conidia and conidiophore at 40X

Plate 5a: Microphotographs of conidia and conidiophore of *Erysiphe cichoracearum*


a) Conidia at 10X


b) Conidia at 40X


c) Germinated conidia at 10X

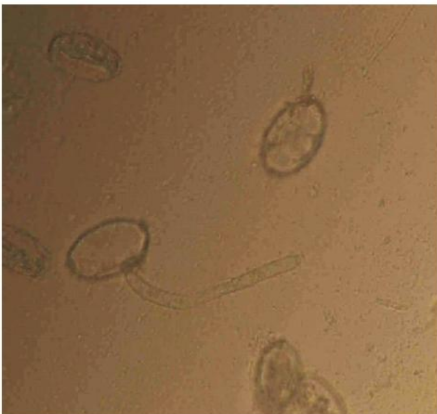

d) Germinated conidia at 40X

Plate 5b: Microphotographs of conidia and germinated conidia of *Erysiphe cichoracearum*

primers and the amplicon was observed at the region 531 bp (Plate 6). The amplified products were checked on one per cent agarose gel electrophoresis.

Sequence of *Erysiphe cichoracearum* ITS rDNA

DNA sequencing: The DNA sequence was obtained for ITS rDNA. The sequence of the isolate is given below.

AGTCATTCACAAGAGCGTAGAGACTCGGCCCGGGCGGTGTCCACGCGTGGTCGA
GTCGACCCTGCCACCCGTGTTGACTTTATCTGTTGCTGTGGCGGGCCAGGTGCCT
GGCGCGACCGGCTTCGTGCTGGCTCGTGTCCGCCAAAAGACCCAACCTAACTCGT
GTTGTCGTGTAGTCTGAGGAAGAAATATTGAATTGTTAAAACTATTCAACAACGGAT
CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTGAGTGAATCATCGAATCTTGTGAACGCACATTGCGCACCCTTGGTATT
CCGAGGGGCATGCCTGTTCGAGCGTCGTCACACCCCCTCAAGCCGCGCCGTGTG
TGTGGCTTGGTGTTGGGGCTCGTCCGTCGGAGCGGCCCTTAAAGACAGTGGCGG
TGCCGTTGTGGTCTCTACGCGTAGTACGATTCTCGCGACAGAGCGAGCAGCGGCG
GCTTGCCAATCAATCCTGTTCAGGTTAAAGGTTGAACCTCGAATCAGGTAGGGATA
CCACGCTGAACTTGAAGCAGTAATCAGATG

The sequence obtained was deposited in NCBI gene bank. Accession number 'MW774352' was obtained and it was confirmed that the causal pathogen of okra powdery mildew of Raichur region is *Erysiphe cichoracearum.*

Earlier Channaveeresh and Kulkarni (2017) (8) worked on the molecular identification based on rDNA-ITS sequence of *Erysiphe polygoni* causing powdery mildew in black gram. The results, indicated that the Dharwad isolate is having more than 96 per cent homology with reported *Erysiphe polygoni* isolates in NCBI gene bank from different geographical locations such as Mexico (97 %), Berkley (97 % and 96 %) and Iran (96 %). Their results confirmed *Erysiphe polygoni* as the causal organism for black gram powdery mildew in Dharwad region. Parameshwar (2017) (9) conducted study on the molecular identification based on rDNA-ITS sequence of *Erysiphe cichoracearum* causing powdery mildew in cucumber. The results of NCBI BLAST

analysis confirmed that, hits available in NCBI accessions were showing maximum of 96 per cent sequence similarity with ITS1 and 5.8S-ITS2 region of *Erysiphe cichoracearum*. Therefore, it was

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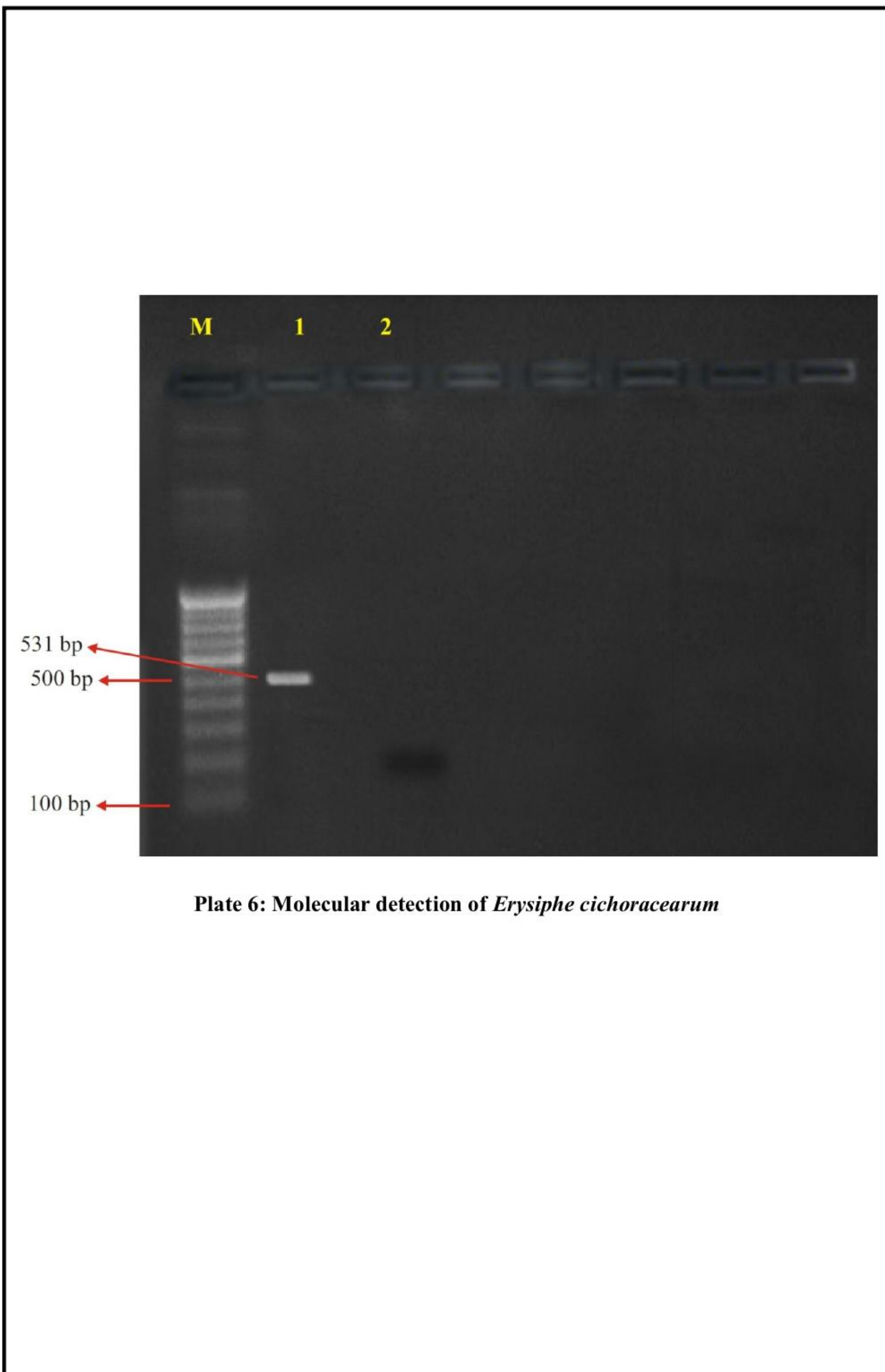

Plate 6: Molecular detection of *Erysiphe cichoracearum*

confirmed that the causal pathogen of cucumber powdery mildew of Dharwad region was *E. cichoracearum.*

4. CONCLUSION

The disease was characterized by the appearance of small white powdery specks on upper surface of the lower leaves which enlarged and coalesced forming bigger patches. Later on the disease spread to the upper leaves, coalescing of the white powdery specks and enlargement in their size ultimately resulted in complete coating of white fungal mass on the leaves. In severe and later stages senescence and defoliation of leaves was noticed. The morphological studies of the pathogen in the laboratory revealed, that the mycelium is septate, ectophytic, hyaline and adjacent to the mycelium, basal septum of the conidiophores was seen. The conidiophores were erect, on which conidia produced either singly or in short chains. The conidia were cylindrical or barrel shape, hyaline, non-septate and measured 75.23 μm x 40.65 μm. Molecular characterisation of the native isolate resulted in the DNA amplicon at the region 531 bp and hence it was confirmed at molecular level that the causal agent of okra powdery mildew is *Erysiphe cichoracearum.*

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