Journal of Crop and Weed, 16(1): 180-185 (2020)

ISSN- O : 2349 9400 ; P : 0974 6315

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Molecular identification of fungi associated with mulberry root rot disease in Eastern and North Eastern India

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Received : 25.02.2019 ; Revised : 22.04.2020 ; Accepted : 22.05.2020

DOI : 10.22271/09746315.2020.v16.i1.1291

ABSTRACT

Root rot of mulberry is a devastating disease which completely kills the whole plants and renders plantation unfit for mulberry cultivation. Five fungal cultures were isolated on PDA from root samples infected with root rot disease from Assam, Arunachal Pradesh, Mizoram and West Bengal. Pathogenicity of isolates was confirmed by modified root inoculation method. Four isolates were characterized by white and cottony colonies, turned into bluish brown producing hyaline, thick walled, slightly curved short and bent falcate macroconidia and oval/cylindrical, hyaline, smooth microconidia belonging to Fusarium spp. All the isolates produced abundant terminal or intercalary, smooth, globose to subglobose chlamydospores. A fungal isolate with chocolate brown colony producing medianly septate, dark brown, thick walled ellipsoidal conidia with truncate base and longitudinal striations was identified as Lasiodiplodia sp. Total genomic DNA was isolated from the fungal isolates and ITS regions were amplified using universal primers ITS1 and ITS4. BLAST analysis revealed that isolates from Karjora and Pipulkhola (West Bengal), shared maximum nucleotide identity (98.7% and 99.8%) with Fusarium solani F5 (MG711899) and F. solani Fs2 (KC156594), respectively. Similarly, the isolate from Sille (Arunachal Pradesh) and Jorhat (Assam) exhibited maximum nucleotide similarity (98.09% and 98.67%) with F. solani OSHSL-5.4 (KR017036) and F. solani AV1 (MH517359), respectively. Isolate from Kolasib (Mizoram) was 99.03% homologous to Lasiodiplodia theobromae Bl16 (MK813947).

Keywords: Molecular identification, mulberry, phylogeny and root rot

Mulberry (*Morus* spp.) is a quick growing, perennial plant widely cultivated for its nutritious leaves essential for the production of most valued silkworm cocoon.Sericulture provides livelihood for rural people in 26 different states of Indian subcontinent. In the tropical zone, Karnataka, Andhra Pradesh and Tamil Nadu, contributes about 90 per cent of Sericultural activities, whereas West Bengal, Himachal Pradesh and the North Eastern statesleads in the sub-tropical zone. In West Bengal major contribution is from Malda, Mushidabad, Birbhum and Nadia districts (Datta, 2019).

Pest and disease infestation is one of the major hindrances for successful sericulture in India. Being highly nutritious, mulberry is prone to the attack of fungi, bacteria, viruses, mycoplasmas and nematodes. In the recent years incidence of root rot has been reported from different districts of West Bengal (Dutta *et al*., 2017).The root rot is asoil borne disease, characterized by quick withering of leaves and subsequently rotting of roots and mortality of the plants are also observed. During initial stages the symptoms are observed on few plants in isolated patches that act as inoculum source further spreading disease to healthy plants within a short time. Once the pathogenmoves to the cortex of the root, cortex slowly dries and disintegrates leading to the colonization by secondary invaders finally causing decay of the roots. Sudden death of the plants, irrespective of age has been also reported due to root rot disease (Munshi *et al*., 2009).

Unlike foliar diseases, soil borne diseases are difficult to manage and often management measures are less effective, since pathogen perpetuates in the soil. If not managed initially, root rot disease can spread gradually leading to 100 per cent damage and gardens have to be abandoned. It is essential to develop management measures for the root rot disease of mulberry to prevent such unacceptable loses. To develop any successful disease management practice diagnosis of the disease and proper identification of the causal agent is essential.Considering this, present investigation was carried out to isolate and identify fungal pathogens associated with the root rot of mulberry.

MATERIALS AND METHODS

Location

The currentstudy was carried out at Central Sericultural Research and Training Institute (CSRTI), Berhampore, West Bengal (Latitude: 24°5’28.01"N and Longitude: 88°15’56.37"E) during January-September, 2019.

Isolation of fungal pathogen(s)

The infected mulberry root samples were sourced from the farmers’fields of Karjora and Pipulkhola (West Bengal), Jorhat (Assam), Sille (Arunachal Pradesh) and Kolasib (Mizoram). The portion of the infected root was removed from the plant, cut into smallpieces (3-5 mm).

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The pieces were surface sterilized with 4% sodium hypochlorite (five minutes), 70% Ethanol(two minutes), washed twice in sterile distilled water, dried on sterile tissue paper and plated on Potato Dextrose Agar (PDA). After inoculation Petri plates were incubated at ambient conditions and observed daily for fungal growth. Developing fungal mycelia were inoculated on to fresh PDA plates and the isolates were maintained on PDA at 4°C. Sub-culturing was done at 21 days interval.

Morphological Characteristics

Morphological characteristics of the isolates were studied once the cultures were grown on PDA for ten days. Slides were prepared with Lactophenol cotton blue and examined under compound microscope to record the morphology. Characteristics like colour of the colony, morphology of conidia and chlamydosporeswere studied.

Pathogenicity Studies (Inoculation by root dip method)

To prove the pathogenicity, conidial suspension was prepared from 7-14 days old fungal cultures. The mycelial mat was suspended in sterilized water (500 ml for each sample) collected in a container. The suspension was mixed thoroughly for equal distribution of conidia and with the help of haemocytometer, final count was adjusted to 10^6 ml^{-1}. Six month old saplings of mulberry variety S-1635 grown in nursery bed were carefully removed and roots were washed with running tap water. The roots of the saplings were slightly injured by rubbing with sand paper and dipped inconidial suspension overnight. After inoculation the mulberry saplings were replanted into the pot filled with sterile soil. The mulberry saplingswere maintained in a glasshouse without irrigation for two days. After two days, irrigation was carried out regularly. Forty five days post inoculation,disease incidence was scored by the presence or absence of symptoms of root rot.

Isolation of genomic DNA and amplification of ITS region

The total genomic DNA of root rot pathogen isolateswere extracted using CetylTrimethyl Ammonium Bromide (CTAB) method. Universal primers of ITS rRNA gene (Forward primer-ITS1: 5'TCCGTAGGTGAACCTGCGG3' Reverse primer - ITS4:5'TCCTCCGCTTATTGATATGC3') were used for amplifying genomic DNA of root rot pathogen isolates using DNA thermal cycler (Eppendorf Nexus gradient master cycler). PCR reaction mixture (25ìl) consisted of primers (1ìl each of forward and reverse), 2.5ìl template DNA, 8ìl DNase-free Water and 12.5ìl Master mix (Himedia). The PCR mixture was subjectedto one cycle of initial denaturation at 95ÚC (5 min) followed by 35 cycles of denaturation at 95ÚC (30 sec), annealing at 55ÚC (60sec), extension at 72ÚC (60sec) and a final extension at 72ÚC (6 min). PCR amplicons were stored at 4ÚC till further use.

Agarose gel electrophoresis

Amplification by polymerase chain reaction was confirmed by performing agarose gel electrophoresis [2% (w/v) in TAE buffer at 15 V cm^{1}] by loading 5 ìl each of PCR productand DNA ladder (Himedia-100 bp DNA Ladder, MBT130).Ethidiumbromide (0.5 ìg ml^{-1})was used to stain the gel, visualized in gel documentation system (GelDoc EZ imager of Biorad).

Sequencing

Amplified PCR products were directly sequenced employing Sanger sequencing (ABI 3730xl 96 capillary system using Big Dye Terminator v3.1 kit) through outsourcing (M/s. Xcelris Labs, Gujarat, India).

BLAST search

The ITS sequences (approx. 600bp length) weresubmitted to NCBI-BLAST search analysis (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) to determine the nearest phylogenetic neighbours.

Phylogenetic analysis

The CLUSTAL W algorithm (MEGA 6.0) was utilized for sequence alignments and MEGA 6.0 software was used for phylogenetic analysis. Distances were computed using Kimura correction in a pair wise deletion manner. Neighbour-joining (NJ) method was used to construct the dendrogram(s). Percentage support values were achieved using bootstrap procedure (1000 replications).

RESULTS AND DISCUSSION

Symptoms of root rot

In farmers' fields, root rot appeared in isolated patches. The disease first appeared on couple of plants, from which it spread to adjacent plants, leaving large number of plants affected within a short period. Infected plants were characteristically stunted with chlorotic leaves that defoliated prematurely. The disease damaged the fine feeder roots followed by rapid decay of main root system leaving the stump dead. In advanced stages, the affected plants exhibited yellowing and dropping of the leaves. Discolouration and rotting of root were also observed (Fig. 1),while severely affected plants lost the hold in the soil and could be easily uprooted. The bark of the roots comes out easily emanatingfoul smell.These symptoms of root rot were similar to the observations of Govindaiah *et al*. (2005) and Sutthisa *et al.* (2010).

Fig. 1 : Discolouration of root due to root rot

Fig. 3: Symptoms of root rot on mulberry 45 days after inoculation

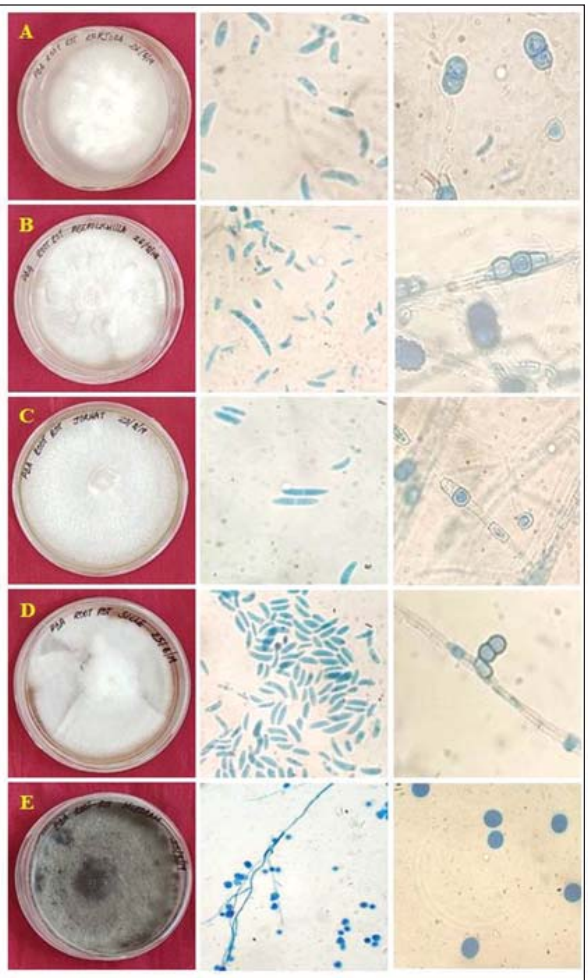

Fig. 2: Colony morphology, conidia and chlamydospores of Karjora (A), Pipulkhola (B), Jorhat (C) and Sille (D) isolates. Colony morphology and conidia (40x and 100x) of Kolasib isolate (E)

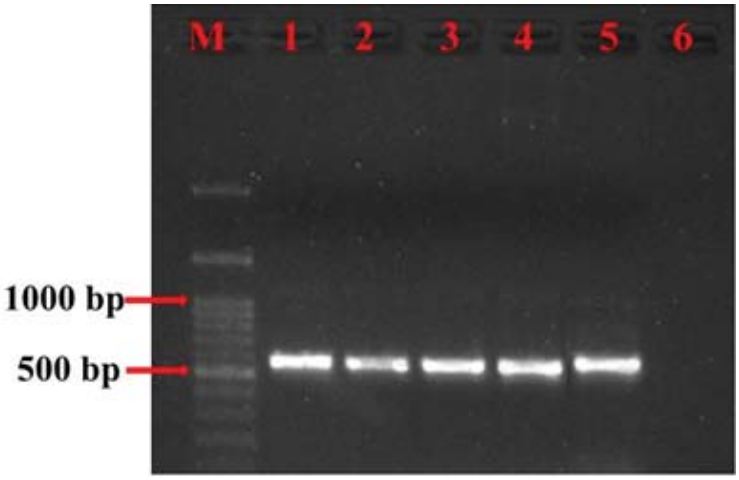

Fig. 4: PCR amplicons of ITS regions of different isolates

M-DNA ladder, 1-Karjora, 2-Pipulkhola, 3-Jorhat, 4-Sille, 5-Kolasib&6- Water control

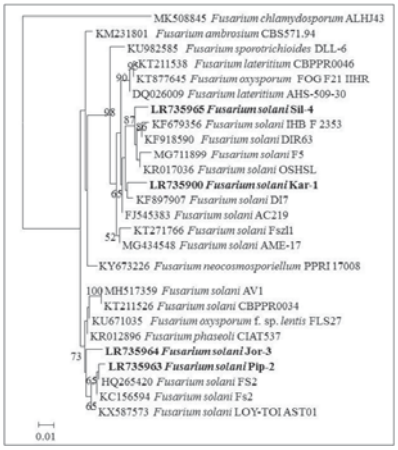

Fig.5: Neighbour Joining (NJ) Phylogenetic tree based on ITS sequences indicating phylogenetic relationship of *Fusarium*isolates with their closest phylogenetic neighbours. Numbers at the nodes denote bootstrap percentages. Gene Bank accession numbers for ITS gene sequences are given in parentheses. Bar 0.01 substitutions per nucleotide position.

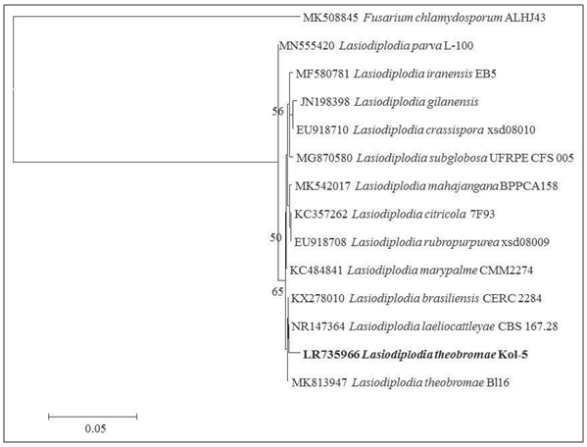

Fig.6: Neighbour Joining (NJ) Phylogenetic tree based on ITS gene sequences indicating phylogenetic relationship of *Lasiodiplodia* isolate with their closest phylogenetic neighbours. Numbers at the nodes denote bootstrap percentages. Bar 0.05 substitutions per nucleotide position.

Isolation and study of morphological characteristics

Five fungal isolates were cultured from root rot samplesobtained from five locationsof Eastern and North Eastern India (Fig. 2). Initially the fungi were identified based on their morphological characteristics (Barnett, 1972). Based on morphological characteristics, four isolates with white and cottony colonies which later turned bluish brown producing hyaline, thick walled, subcylindrical slightly curved short and bent falcate macroconidia with predominantly 3 septa and oval or cylindrical, hyaline, smooth microconidia were identified to be *Fusarium* spp. These isolates also produced abundant terminal or intercalary, smooth, globose to subglobose chlamydospores. Isolate from Kolasib (Mizoram) with chocolate brown colony, producing medianly septate, dark brown, thick walled ellipsoidal conidia with truncate base and longitudinal striations from apex to base was determined to be *Lasiodiplodia* sp. Isolates were designated as Kar-1, Pip-2, Jor-3, Sil-4 and Kol-5 based on the respective location of the root samples from where they were isolated.

Pathogenicity test

Healthy mulberry saplings of variety S-1635 were inoculated with conidial suspension. Roots were observed 45 days after inoculation with conidial suspension. Typical symptoms of root rot were observed (Fig. 3) and the pathogen(s) were isolated back from the infected roots, establishing pathogenicity.

Molecular identification of fungi

Total genomic DNA was isolated from different isolates and ITS regions were amplified. PCR amplification was confirmed by gel electrophoresis and expected ~600 bp band was observed (Fig. 4). Subsequently PCR products were sequenced. BLAST analysis revealed that Karjora isolate was 98.7% homologous to *Fusarium solani* isolate F5 (MG711899) isolated from Agarwood plant *(India), while it also showed 99.05% nucleotide identity with F. solani strain IHB F 2353 (KF679356), strain 54 (JX897000) and strain TGLS-1 (JN937588) isolated from Aquilariaa gallocha* (India), *Elaeisguineensis* (India) *and Lycopersicum esculentum* (South Korea) respectively. Root rot pathogen isolate from Pipulkhola showed 99.8% nucleotide similarity with *F. solani* isolate Fs2 (KC156594) and *F. solani* isolate FS2 (HQ265420) isolated from wilted roots of guava (India). It also showed 99.4 % nucleotide similarity with *F. solani* strain LOY-TOI (KX587573) isolated from *Plumbago zeylanica* (India) and 99.8% identity with *Fusarium* sp. 1RIFA (KF624785) reported from USA.

Isolate from Jorhat (Assam) exhibited maximum nucleotide identity of 98.67 % with *Fusarium solani* AV1 (MH517359) and *F. solani* CBPPR0034 (KT211526) while Silleisolate (Arunachal Pradesh) showed maximum nucleotide identity of 98.09 and 97.57 % with *F. solani* OSHSL-5.4 (KR017036) and *F. solani* DIR63 (KF918590). Isolate from Kolasib

(Mizoram) showed 99.03 % similarity with *Lasiodiplodia the obromae*(Syn. *Botryodiplodiatheo bromae*)Bl16 (MK813947) and 98.84 % similarity with *L. theobromae* BPPCA262 (MK530069).

The results of molecular identification are in agreementwith morphological observations andestablished the identity of the fungal isolates. The phylogenetic analysis (Fig. 5) of the ITS sequences of the *Fusarium*isolates in the present study and their closest phylogenetic neighbours indicate clustering with *F. solani*. Similarly isolate from Kolasib clustered with its nearest phylogenetic neighbor *Lasiodiplodia theobromae* (Fig. 6).

Based on macroscopic characteristics and microscopic examination, identification of fungi like Fusarium is often very tough or unreliable because of variability among isolates and overlapping characters between species. Similarly identification of *Lasiodiplodiatheobromae* using morphological and physiological characteristics is tedious as these characteristics are unstable and often influenced by environmental factors (Shah *et al*., 2010).In comparison molecular classification is quick and is also helpful in differentiating morphologically similar species (Gautam and Bhadauria, 2012). PCR applications and sequencing of ITS region have been increasingly used for identification and classification of fungi (Druzhinina *et al*., 2005; Hathout *et al*., 2014 and El-Neekety *et al*., 2016).Geib *et al.* (2012) amplified and sequenced the ITS region for identification of *Fusarium* strains. Abd-Elsalam *et al*. (2004) employed ITS region to identify *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum*, *F. moniliforme* and *F. solani*. Similarly, analysis of ITS sequences has been employed successfully to identify and study the genus *Fusarium* isolated from gladiolus (Córdova-Albores *et al*., 2014) cotton (Gonzalez-Soto, 2015),*Vasconcelleaheil bornii*(Robles *et al*., 2016) and different grain samples (Hussain *et al*., 2018).Recently, morphologicalcharacterisation and ITS sequences have been successfully used for identification of *Lasiodiplodiatheo bromae* (Munirah *et al*., 2017 and da Rosa *et al*., 2018).

In the present study, based on the cultural, morphological and molecular characterization,fungi associated with root rot disease of mulberry in different locations of Eastern and North Eastern India were identified to be *Fusarium solani* and *Lasiodiplodia theobromae*.Similarly *F. solani*f.sp.*mori* was reported to be associated with the roots of affected mulberry trees in North-Eastern Thailand (Sanoamuang *et al*., 1986). There are reports of association of various fungi *viz*., *F. solani*, *F.oxysporum*,*Rhizoctoniabataticola*, *R. solani*and *Botryodiplodiatheo bromae* with mulberry root rot (Xie *et al*., 2014, Gani *et al*., 2017 and Pratheesh Kumar *et al*., 2017). Of late Dutta *et al*. (2017) also reported that *F. solani*ca used root rot mulberry in West Bengal. More than 280 plant species have been found to be affected by *L. theobrombae* (Sutton, 1980, Khanzada *et al*., 2006 and Domsch *et al*., 2007). *L. The obrombae* has been reported to cause root rot of different plants includingAustralian bottle trees-*Brachychitonpopulneus* (Sandlin, 1992), Cassava (Onyenka *et al*., 2005) and *Jatrophacurcas* (Prajapati *et al*., 2014).

Based on the cultural, morphological and molecular characteristics, fungi associated with root rot of mulberry in different localities of Eastern and North Eastern India were identified to be *Fusarium solani* and *Lasiodiplodia theobromae*.

ACKNOWLEDGEMENT

The authors are grateful to Sri. Debashish Chakravarty, Scientist, CSR&TI, Berhampore for his continuous motivation and support.The help and support extended by scientists of Regional Sericultural Research Satiation, Jorhat and Research Extension Centre, Sille as well as Director of Sericulture, Aizwal, Mizoram, in collecting the root rot samples is also acknowledged.

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