Journal of Advances and Scholarly Researches in Allied Education
Vol. 16, Issue No. 6.1, May-2019 (Special Issue), ISSN 2230-7540

Study on the Molecular Characterization and Diversity of Fungi Causing Root Rot Disease in Mulberry MORUS SPP

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ABSTRACT

The study reports the findings of survey undertaken on root rot in mulberry, a serious disease caused by fungi viz., Botryodiplodia theobromae (black rot), Macrophomina phaseolina (charcoal rot) and Fusarium spp. (dry rot) in important sericulture areas of South India. A total of 95 isolates of fungi associated with root rot were recovered from different mulberry gardens. These isolates were subjected to in-depth characterization by cultural and morphological parameters and molecular markers (RAPDs and SSRs). B. theobromae isolates showed variability in growth of mycelia, sclerotial shape and abundance and conidial size whereas, M. phaseolina exhibited variability in pycnidial abundance and conidial size. Fusarium isolates comprising of two species – F. oxysporum and F. solani revealed variability in hypha, macroconidia, microconidia and septation. Among the virulent isolates, one each of B. theobromae (BT-2) and F. solani (FS-25) recorded 100% root rot of host root system. But, in case of M. phaseolina, four isolates from Tigulahusahalli (MP-1), Peddavaram (MP-5), Parigi (MP-7) and Vellivalasa (MP-10) recorded complete root rot infection. Majority (virulent and moderately virulent) of the isolates of M. phaseolina (91.6%) showed infectivity compared to B. theobrome (80%) and Fusarium (72%). Stepwise MRA could identify markers associated with root rot infection in Fusarium spp. (30), M. phaseolina (19) and B. theobromae (5). Among the phenotypic markers, only mycelia growth was found to be associated with rotting of roots in case of M. phaseolina. The microconidial width and pigmentation of reverse colony of Fusarium spp. were found to be associated with root rot (%) but, only microconidial width showed positive correlation. The grouping of isolates based on genetic analysis did not reveal correlation either with geographical distribution or pathogenicity, possibly because of the rapid fungal spread due to anthropogenic activities, thus preventing population isolation and stratification. Genetic variability and diversity in the root rot associated fungi is bewildering and may contribute to the evolution of new strains with more virulence.

Keywords – *Genetic Diversity, Mulberry, Somatic Mutation.*

Article submitted at :
National Conference on Advances in Multidisciplinary Research (NCAMR-2019)
Organized by : Kurukshetra Institute of Professional Studies, Kurukshetra, Haryana

INTRODUCTION

Mulberry (Morus spp.) is a perennial, heterozygous, deciduous tree belonging to the family Moraceae. Its foliage is the exclusive source of food for the domesticated silkworm Bombyx mori (Linnaeus, 2018) for the production of elegant and lustrous silk – the Queeen of textiles. Indian sericulture largely depends on mulberry silk production as a sustainable and economically profitable venture. Sericulture is a highly remunerative enterprise for rural mass and contributes significantly to the Indian foreign exchange earnings to the tune of USD 312.12 million (CSB, 2017). Silk productivity is directly proportional to the quantum and quality of leaves utilized in rearing of high yielding bivoltine silkworm races (Ghosh et al., 2016).

Mulberry leaf production is constrained by several biotic and abiotic factors. Among the biotic factors, root rot is a devastating disease threatening the sustainability of mulberry cultivation in South India. Root rot disease of mulberry has been a major problem for mulberry cultivation in countries like China, Thailand and Japan (Aoki, 2-016). In India, the damage caused by this disease has been of less significance till now. However, a recent study has revealed its prevalence in all the sericulture areas of South India (Yadav et al., 2018). The disease results in severe yield loss due to wilting, defoliation, and death of plants. The disease is observed both in nurseries as well as established gardens. The problem has attained epidemic proportions due to the involvement of more than one pathogen (microbial consortium) with a potentiality to kill the plants and even wipe out entire plantation (Ganeshamoorthi et al., 2014). The disease can spread rapidly with devastating effect, rendering the land unsuitable for further cultivation. Many factors like mechanical injury to the plant, poor soil nutrition, higher temperature etc. are known to contribute to the manifestation and spread of the disease (Chowdary, 2016). The fungal pathogens which cause root rot disease in mulberry are soilborne. The unfavourable conditions in the soil like higher temperature, low organic matter and available moisture play a significant role in activation of the fungal pathogen and multiplication. Its epidemiological requirements may vary greatly for invading the host and development of visible symptoms. The prevalence of the fungi causing root rot infestation in the soil makes it difficult to control the disease through conventional plant protection measures.

Various forms of disease – dry, black, charcoal, violet and white root rots have been reported in mulberry from different countries across the world. Among them dry, black and charcoal root rots are reported in India (Philip et al., 2015; Radhakrishnan et al., 2015; Chowdary, 2016). Charcoal root rot is most commonly found in the southern states of Karnataka, Andhra Pradesh, Tamil Nadu and Telangana, and these four states account for about 80% of silk production in the country. Once the mulberry plants are vulnerable to infection, the fungus dominates inside the roots multiplying the hyphae rapidly in the cortical tissues and extending up to pith. It enters the xylem vessels and causes death of plants (Radhakrishnan et al., 2015; Sukumar and Padma, 2019; Sharma and Gupta, 2015).

The fungi causing root rot diseases in mulberry are wide spread and pathogenic to many other crop plants (Ma et al., 2019; Bahar and Shahab, 2016). These fungi are also the essential components of the terrestrial eco-system as decomposers, symbionts and pathogens (Mueller et al., 2017). Studies have indicated the existence of variability in cultural and morphological characteristics of fungi associated with the root rot diseases in many crop plants (Khalil et al., 2016; Stojsin et al., 2017). Several studies have been undertaken to characterize the genetic and

pathogenic variability in M. phaseolina. Molecular techniques such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR) have contributed to a better understanding of the genetic and pathogenic variability within the populations of these pathogens (Fuhlbohm, 2017; Su et al., 2018; Jimenez, 2016). Cultural and morphological markers have been widely used to characterize these fungi, and to assess the genetic variability. However, these markers are limited by number and fail to make an accurate prediction due to environmental effect. In contrast, DNA based markers are plenty, and used along with phenotypic markers to assess the genetic variability, diversity, population structure, gene flow etc. in many fungal pathogens such as Botryosphaeria spp. and their anamorphs (Zhou et al., 2018; Barnes et al., 2015; Slippers et al., 2015).

Development of improved cultivar/genotype having resistance to root rot pathogens through breeding programmes is a long lasting and effective approach to avoid crop loss, and to improve the sustainability of sericulture as a venture. Towards this objective it is very important to estimate the genetic variability and diversity of the pathogens in order to ensure the development of durable resistant mulberry. It is also essential to assess the genetic variability within and between pathogen populations and study the interaction between the pathogens and host plant before initiating the breeding programmes.

OBJECTIVE OF THE STUDY

1. To study the Collection and isolation of fungi causing root rot disease in mulberry from hotspot areas of South India.

2. To study the Characterization of pathogenic isolates based on cultural, morphological and molecular markers, and estimation of genetic diversity

MATERIALS AND METHODS

Survey of root rots disease in South India

Survey was undertaken in major sericultural regions of South India during 2015-2017 based on the information provided by the extension workers and reports of different Regional Sericultural Research Stations (RSRS) and Research Extension Centres (REC) under CSRTI, Mysuru. This included important mulberry cultivation areas covering 18 districts in the different states of Andhra Pradesh, Karnataka, Tamil Nadu and Telangana.

Collection of infected root samples and rhizosphere soil

Root and soil samples from rhizhosphere were collected from healthy as well as infected mulberry gardens. The implements used for sample collection were disinfected each time with 70% ethanol. The root samples were sealed in sterile polypropylene bags after collection. Soil samples (~200 g) from 15−30 cm below the surface were also collected.

Isolation and identification of fungal isolates

Root rot associated fungal organisms were isolated from the roots by the tissue segment method on Potato Dextrose Agar (PDA) medium (Trigiano et al., 2018). The roots were cut into small pieces of 5-7 mm length. These bits were surface sterilized with 0.1% mercuric chloride solution for one minute, rinsed thoroughly with autoclaved distilled water and transferred onto PDA plates supplemented with streptomycin sulphate (50 mg/l). Inoculated plates were incubated at 28 ± 2°C for 3−7 days. The isolates were pure cultured by hyphal tip method (Rangaswami and Mahadevan, 1999) and the purified cultures of were maintained on PDA slants for use in further studies. The fungi were isolated from the soil samples by dilution plate technique (Waksman, 2017). Lactophenol blue staining used for microscopic observations of the fungal isolates for identification based on mycelia color, fruiting body and conidia. The isolates were stored on PDA in plates sealed with Parafilm for pathogenicity test. Subsequent subculturing was also done on PDA slants for further experimental wor

Pathogenicity test on mulberry cutting

Pathogenicity tests were conducted using Koch's Postulate to confirm the Fusarium species as the causal agent of root rot of mulberry. The plants used were 4 months old and showed no disease symptoms.

The Fusarium isolates used were the representative of isolates, which was successfully isolated and identified from root of mulberry. To obtain inoculum, Fusarium spp. was grown on pieces of mulberry twig that cut into 0.5 mm and autoclave twice. The plugs of Fusarium spp. were added into the pieces and incubated at 28 ºC for 14 days.

Mulberry cutting cultivar Noi were washed and surface sterilized before grown in 10 cm diameter plastic glasses using sterile peat moss and Fusarium spp. inoculum (107 cfu/ml) were placed on the bottom before planting. The experiment was conducted in a completely Randomized Block Design (RCBD) with five replicates for each Fusarium species and control. Disease incidence was observed.

Pathogenicity test on mulberry cutting

Thirty days after inoculation, disease incidences were observed. All eleven of Fusarium species were pathogenic on mulberry cutting. F. solani shows the highest infection with a disease incidences of 90.0%, significantly from others species. There were no significant difference of recovery from root let between F. solani, F. anthophilum and F. dlamini showed that 94 – 96% recovery. There were no significant difference of recovery from lateral root between F. solani, F. phaseoli, F. anthophilum, F. dlamini, F. dimerum and F. beomiforme showed that 80 – 94% recovery. There were no significant difference of recovery from basal between F. solani, F. phaseoli, F. culmorum, F. anthophilum, F. dlamini and F. beomiforme showed that 36-48.02% recovery.

THE DISEASE

Mulberry crop is affected by many pests and diseases. But, some diseases are highly infectious and cause serious damage to the plants. Among them, root rot is a devastating disease

threatening the sustainability and profitability of sericulture. The disease is considered more serious because of its potentiality to kill the plants completely (Govindaiah et al., 2015). The disease is most pronounced when plants are stressed by adverse environmental conditions such as drought and higher temperature (Khan, 2017). Application of weedicides during cultivation predisposes to root rot disease in many agriculturally important crops (Levesque and Rahe,2017 Harikrishnan and Yang, 2018). The symptoms of root rot are first visible on shoots as sudden withering and defoliation of leaves followed by the death of the affected plants. The disease initially appears in isolated patches and then quickly spread to the surrounding areas, leads to severe crop loss within a short period of time. The disease spreads through the irrigation water, soil and implements, to nearby plants both in same row and adjacent rows where root zones overlap.

PATHOGENS

Dry root rot is caused by Fusarium solani and F. oxysporum (Philip et al., 2015; Sharma et al., 2018). Rhizoctonia bataicola (anamorph of Macrophomina phaeseolina) extensively cause charcoal root rot disease in South India and is reported to be the most virulent pathogen (Chowdary, 2006; Marimuthu et al., 2015) responsible for root rot in mulberry. The other fungus, viz, Botryodiplodia theobromae (syn = Lasidiplodia theobromae) cause black root rot in mulberry (Sukumar and Padma, 2016; Sharma et al., 2017).

CULTURAL AND MORPHOLOGICAL VARIABILITY

B. theobromae showed considerable morphological variability because of widespread occurrence and is composed of a number of cryptic species (Punithalingam, 2017). Pavlic et al. (2017) found that isolates have typical conidialsize collected from different countries viz., USA, South America, South Africa and Asia. Based on these morphological characteristics and DNA-based phylogenies, isolates were distinguished in to new species L. gonubiensis. Similarly, Burgess et al. (2019) reported three novel species of Lasidiplodia based on morphological characters (septation of the paraphyses, size of the spores, thickness of spore walls, colour of pycnidia) and ITS and EF1-a nucleotide sequences.

Punithalingam, (2016) has reported physiological aspects like effects of light, pH, temperature, carbon and nitrogen sources and vitamin requirements in B. theobromae. Mycelial growth and sclerotia formation results in inhibition of sporulation when subjected to optimum photoperiods i.e. 16 hours daily (Perera and Lago, 2016). Sclerotia produces conidia, which results in wilting of host. During discharge the conidia would be in single cell state later it become two-celled state and produces cellulolytic, pectic enzymes (Punithalingam, 2019). Phenotypic characters like mycelial growth (fluffy or depressed), colony colour (cottony white, black), pycnidia abundance were used for characterization of the fungus but, molecular markers are known to provide a great potential for diversity assessment (Shah et al., 2018).

Survey of root rot disease in South India

Survey was undertaken in major sericultural regions of South India during 2017-2019 based on the information provided by the extension workers and reports of different Regional Sericultural Research Stations (RSRS) and Research Extension Centres (REC) under CSRTI, Mysuru. This

included important mulberry cultivation areas covering 18 districts in the different states of Andhra Pradesh, Karnataka, Tamil Nadu and Telangana.

Fungal genomic DNA extraction

Genomic DNA was extracted from pure cultures of all the fungal isolates grown on potato dextrose broth. The mycelial mat was harvested and thoroughly washed with sterilized distilled water. DNA was isolated using HiPurA Fungal Genomic DNA Extraction Kit (HiMedia, India) by following manufacturer's instructions. The genomic DNA was quantified on 1% agarose gel as per Sambrook and Russel (2017), and uniformly diluted to10 ng/µl for use in PCR.

RAPD–PCR AMPLIFICATION

PCR amplifications were carried out as per the protocol of Williams et al. (2017) with minor modification, in 20 µl reaction volumes containing 1X PCR Buffer, 2 mM $MgCl_2$, 0.1 mM of each dNTPs, 0.2 µM primer (Operon Technologies, USA), 0.5 U of GeNei Taq DNA polymerase (Merck, India) and 20 ng template DNA. The reactions were thermocycled on DNA Engine PTC-200 (MJ Research, USA) using the following profile: initial denaturation at 93°C for 2 min, followed by 40 cycles of 93°C for 1 min, 35°C for 1 min, 72°C for 2 min, and a final 7 min extension at 72°C. The amplified DNA fragments were separated on 1.5% agarose gel in 1X TAE (Sambrook and Russel, 2001), and documented using GeneGenius gel documentation system (Syngene, UK). λ DNA/EcoRI+HindIII marker (Fermentas, Lithuania) was used as a size standard.

SSR MARKER AMPLIFICATION

Fifty-seven SSR primers (Bogale et al., 2015; Baird et al., 2019; Mwang'ombe et al., 2018; Bahar and Shahab et al., 2017; Datta and Lal 2018) were used in the study. All primers were screened and PCR conditions were optimized using a subset of fungal isolates. The PCR amplifications was carried out in 10 µl reaction volume containing 10 ng template DNA, 1X PCR buffer, 2 mM $MgCl_2$, 100 µM of each dNTPs, optimized concentration of each primer and 0.5 U GeNei Taq DNA polymerase (Merck, India) on GeneAmp PCR System 9700 (Applied Biosystems, USA) programmed to the following cycling profile: initial denaturation at 94°C for 5 min, followed by optimized number of cycles of 94°C for 30 sec denaturation, primer specific annealing temperature for 30 sec and 72°C for 1 min extension, followed by the final extension step at 72°C for 8 min. The amplified DNA fragments were electrophoresed on 8% non-denaturing polyacrylamide gels (Sambrook and Russel, 2018). The SSR alleles were visualized by silver staining (Sanguinetti et al., 2016) and gels were documented using CanoScan FB1210U flatbed Scanner (Cannon, China). Allele sizes were estimated by comparing the bands generated with the pBR322 DNA-MspI digest ladder (New England BioLabs, USA).

CONCLUSION

Undoubtedly, mulberry root rot is the most devastating disease of mulberry with huge negative impact on its cultivation in terms of productivity and economics of the crop. Genetic variability and diversity in the root rot associated fungi is bewildering and contribute to the evolution of new strains with more virulence. Taking into account of complexities and role predisposing factors of the disease, consideration should be on ensuring the health of the soil. A healthy soil

supports vigorous plant growth. A healthy soil with abundant beneficial microflora will control population of pathogenic fungi and their invasion of host plant. The grouping based on genetic analysis did not reveal correlation with geographical distribution as well as virulence of isolates, possibly due to rapid fungal spread by anthropogenic activities, thus preventing population isolation and stratification. Modern day plant breeding provides an alternative strategy to combat the disease with development of cultivars by harnessing resistant genes/alleles from the crop gene pool. Genetic variability and diversity estimates of the pathogenic population can be used for screening for resistance in the mulberry. Screening of germplasm using multiple strains of pathogens will enhance possibilities of locating diverse resistant genes. Pyramiding of these genes will aid in development durable resistant mulberry cultivars. The molecular markers associated with virulence can be used to identify potential areas of infection and subsequently necessary control measures can be taken up on war footing to prevent the occurrence of this serious disease. Development of mulberry cultivar with durable resistance will ultimately help sericulture development in the South Indian sericulture zone and towards sustainable cultivation technology. In addition to contributing to the understanding of the diseases caused by the pathogen and improving crop productivity, these results will be useful for developing integrated strategies for disease management and breeding programs.

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