Tobacco Research 48(2): 66-70, 2022

ISOLATION AND MOLECULAR IDENTIFICATION OF DAMPING-OFF DISEASE CAUSATIVE PATHOGEN (*PYTHIUM* SP.) IN TOBACCO NURSERIES USING ITS SEQUENCE CHARACTERIZATION

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(Recieved on 19th Oct., 2022 and accepted on 14th Nov., 2022)

Damping-off disease is caused by several species of fungi found under various weather conditions in nurseries throughout the world. It is a serious problem in tobacco nurseries, both in heavy clay and light sandy soils. Different classes of fungi viz. *Rhizoctonia Fusarium, Pythium* etc. can cause and have similar symptoms of Damping-off disease in various crops. In order to identify the damping-off disease causative agent at molecular level, samples were collected from damping-off infected tobacco nursery fields of Central Tobacco Research Institute (CTRI), Rajahmundry and its Research stations viz., Jeelugumilli, Dinhata and Hunsur. A total of 16 pathogen isolates were collected from the infected soils and cultured on PDA medium. Genomic DNA was isolated from all these pathogen isolates and purified. Intact genomic DNA of all the isolates were analysed with ITS (Internally Transcribed Sequence) specific primers using polymerse chain reaction. The PCR amplicons of ITS region comprising ITS1, ITS2 and 5.8S regions of all the isolates were purified and sequenced. The sequences obtained were annotated using NCBI BLAST and compared with the Gen Bank submitted sequences. The results revealed that *Pythium* spp are the predominant causative agent of damping-off disease in tobacco nurseries and *P. aphanideramtum*. is the most prevalent species. These findings will aid in designing the appropriate control measures against the specific pathogen (*Pythium*) to protect the tobacco nurseries from damping-off disease.

INTRODUCTION

Tobacco, the golden leaf in India is cultivated in more than seven states in a total area of 0.45 million ha, producing around 760 million kg annually. FCV, *bidi, hookah*, chewing, cigar-wrapper, *cheroot, burley, oriental*, HDBRG, *lanka, pikka, natu* etc., are the major tobacco types grown in the country, with FCV and burley tobacco being the main exportable types. As the tobacco seed is very small in size (<1 mm dia) it is sown in nursery and the grownup seedlings (55-60 days) will be planted in main field. Generally tobacco nurseries are grown on sandy or sandy loam soils. Damping-off is a serious problem in tobacco nurseries both in heavy clay soils and light sandy soils (Fajola et al, 1974). It occurs in two stages, i.e. the pre-emergence and the post-emergence phases of seed germination. In the pre-emergence the phase, the seedlings are killed just before they emerge from the soil. The young radical and the plumule are killed leading to complete rotting of the seedlings. In the post emergence period, the effected seedlings show shriveling and brown discoloration of the stems near the soil subsequently causing rotting of the seedlings. At this stage, the disease quickly spread over the whole seedbed causing near total loss of nursery in a very short span of 48 hours. Damping off is caused by several soil borne fungi and predominantly by *Pythium* and *Rhizoctonia* species. As the causative agents belongs to different classes, different methods of control are required for the effective management of the disease. Identification of the dominant fungal pathogen causing the disease will aid in choosing the right control measures to protect the tobacco nurseries. Hence, it is propose to isolate and identify the predominant pathogen causing damping off in tobacco nurseries. Apart from the conventional methods of identification of fungal pathogens, the emerging molecular tools became handy in identification of the pathogens. The nuclear ribosomal internal transcribed spacer (ITS)

Key Words: ITS, Phythium, Damping off, Nursery and Pathogen

region is the most commonly chosen genetic marker (Schoch et al., 2012) for the molecular identification of fungi in environmental sequencing and molecular ecology studies. Hence in the present study, an attempt was made to identify the damping off causative organisms through ITS sequence characterization.

MATERIAL AND METHODS

In order to identify and ascertain the damping-off disease causative pathogens, a total of 16 samples were collected from tobacco nurseries of ICAR-CTRI Rajahmundry and its research stations located at Jeelugumilli, Hunsur and Dinhata from three different states i.e Andhra Pradesh, Karnataka and Tamilnadu. The infected soil samples were incubated in a desiccator with small pieces of ridge gourd at congenial temperature for 3-4 days. The grown up hyphal filaments were used to develop monoculture in a PDA (Potato Dextrose Agar) medium at 25^{0}C for three days and were subculture at regular intervals for studying the growth parameters.

Genomic DNA extraction

The DNA extraction was carried out by modified CTAB method as described (Doyle and Doyle 1987). The cultured fungal hyphae were grinded manually in 0.5 ml CTAB (Cetyl Trimethyl Ammonium Bromide) extracting buffer and homogenized with plastic pestle. The samples after addition of proteinase-K were incubated in water bath at 65-70^{o}C for one hour and extracted with of chloroform and iso-amyl alcohol (24:1) and precipitated with chilled isopropnal. The precipitated DNA was washed twice with 70% ethanol, dried and resuspended in TE buffer. Quality of the isolated DNA was checked on 0.8% agarose gel and quantified using Nano Drop Spectrophotometer (ND-1000) (M/s Thermo Scientific make). The stock DNA was diluted, to make a working solution of 30 ng/µl for PCR analysis.

PCR amplification and sequencing

The internal transcribed spacer (ITS) region of fungal isolates were amplified, using the universal primers ITS1 (TCCGTTGGTG AACCAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) (White et al. 1990) using Taq DNA polymerase (ILS). The final volume of each PCR reaction mixture (sample) was 20 il containing; 2 il 10 PCR buffer, 0.5 il of each primer (10 pmol), 2 il dNTPs (2 mM), 3 il template DNA (30 ng/il), 1 unit Taq polymerase. PCR amplification was performed using the following conditions: Initial denaturation at 94 C for 3 min followed by 35 cycles each consisting of final denaturation at 94 C for 30 s, annealing temperature at 55 C for 30 s, initial extension for 1 min, and final extension at 72 C for 5 min. PCR-amplified products were separated on a 1% agarose gel and visualized with ethidium bromide under UV illumination, and images were captured using gel documentation system (BioVis). The PCR amplicons were sequenced using Sanger's method. The sequences analysis was carried out using NCBI tools.

RESULTS AND DISCUSSION

Different classes of fungi can cause and have similar symptoms of Damping-off disease in various crops. Hence in order to find out the predominant pathogen involved in manifestation of Damping-off disease in tobacco nurseries, causative agent was isolated from infected tobacco nursery fields of ICAR-CTRI Rajahmundry and Research stations at Jeelugumilli, Hunsur and

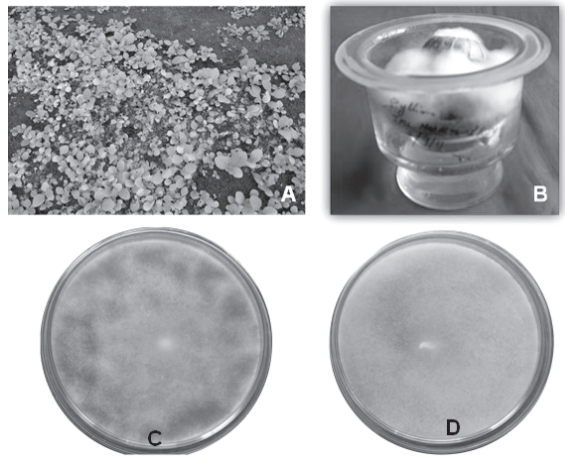

Fig.1: Isolation of Damping off Causative fungus from Tobacco nurseries. (A) Damping off infected Tobacco nursery, (B) Isolation of fungi from infected soil with ridge gourd, (C) and (D) Pure culture of fungi on PDA medium

Dinhata. The monoculture of each isolate was developed and growth patterns were observed on PDA medium (Fig.1). All the isolates exhibited white, cottony growth without any significant morphological differences among the isolates.

Molecular characterization of damping-off disease pathogen

A total of 16 damping off pathogen isolates were collected from different regions, in order to identify the pathogen at molecular level the respective monocultures were used for ITS sequence characterization. Genomic DNA was isolated from all the cultured pathogen isolates, purified and analysed by agarose gel electrophoresis and quantified by spectrophotometry. Pure and intact genomic DNA of all the isolates were analyzed with ITS (Internally Transcribed Sequence) specific primers in a polymerse chain reaction. ITS1 and ITS4 primers amplified the entire ITS rDNA region (ITS-1, 5·8S, ITS-2) and parts of the 18S small and 28S large subunit rDNA with amplified product of ~800 bp in all the isolates (Fig.2). The rDNA region consists of multiple copies arranged in tandem repeats of 18S small subunit, the 5.8S, and the 28S large subunit genes separated by internal transcribed spacer regions (ITS1 and ITS2) (Bruns et al., 1991; Liew et al., 1998). Generally, molecular identification of plant pathogenic fungi is carriedout by PCR amplification of ITS region followed by either restriction analysis (Durán et al., 2010) or direct sequencing and BLAST search in GenBank or other databases (White et al., 1990). Hence, the amplified product of ITS region from all the isolates were purified and sequenced. The sequences obtained were analyzed using NCBI BLAST. The results from the BLAST hits revealed that (Table 1) that all the pathogen isolates belong to *Pythium* species only. Further, 13 of the isolates have shown more than 90 % similarity to *Pythium aphnidermatum* with HN4-0 and HN2-0 isolates in the NCBI data. Only three isolates each from CTRI Rajahmundry, Jeelugumilli of Andhra Pradesh and one from CTRI RS Dinhata, West Bengal has shown similarity of more than 75% with *P. myriotylum* species. Sequenced ITS regions of four *Pythium* isolates from different regions were submitted to the NCBI (Table2).

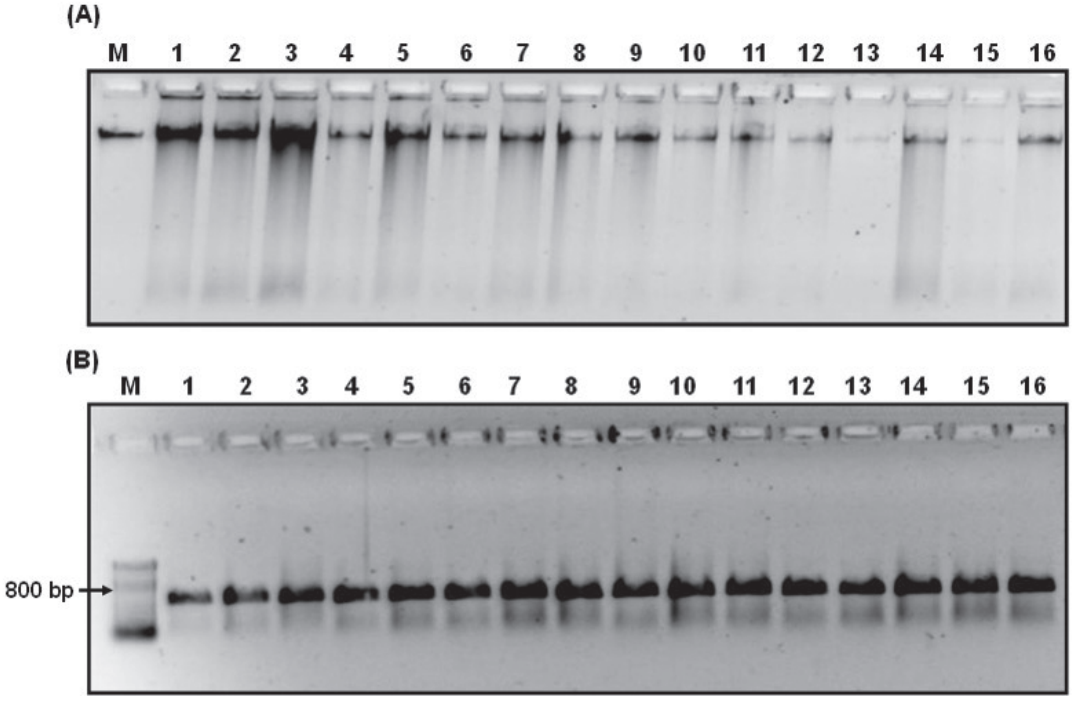

Fig. 2 : (A) Genomic DNA extraction from 16 pathogen isolates from different tobacco nurseries, (B) PCR amplification of ITS region from 16 pathogen isolates

Table 1: List of ITS sequenced fungal isolates along with percent similarity hits from the NCBI database.

Name	Similarity	Percentage (%)
	CTRI Rajahmundry (Andhra Pradesh)	
Pa-1	*Pythium aphanidermatum* isolate HN2-0	96
Pa-3	*Pythium aphanidermatum* isolate DC16	98
Pa- 4	*Pythium aphanidermatum* isolate HN 4-0	98
Pa-5	*Pythium myriotylum*	79
	CTRI RS Jeelugumilli (Andhra Pradesh)	
Pa-7	*Pythium myriotylum* 18S ribosomal RNA gene, partial sequence; internal	89
Pa-9	*Pythium aphanidermatum* isolate HN2-0	99
Pa-10	*Pythium aphanidermatum* isolate HN2-0	98
Pa-11	P*ythium aphanidermatum* isolate HN4-0 18S ribosomal RNA gene, partial	81
	CTRI RS Hunsur (Karanataka)	
Pa-13	*Pythium aphanidermatum* isolate HN4-0	89
Pa-14	*Pythium aphanidermatum* isolate HN4-0	96
Pa-15	*Pythium aphanidermatum* isolate HN4-0	98
Pa-16	*Pythium aphanidermatum* isolate HN2-0	99
	CTRI RS Dinhata (West Bengal)	
Pa-17	*Pythium aphanidermatum* isolate HN2-0	96
Pa-18	*Pythium aphanidermatum* isolate HN4-0	98
Pa-19	*Pythium myriotylum* isolate PCTu237 18S ribosomal RNA gene	87
Pa-20	*Pythium aphanidermatum* isolate HN1298 18S ribosomal RNA gene	83

Table 2: List of Pyhtium isolates with ITS sequence regions submitted to NCBI along with the Accession Number assigned by Gen Bank.

Name of the isolate	Organism	Gen Bank Accession No.
PA1	*Pythium aphanidermatum*	JX473000
PA14	*Pythium aphanidermatum*	KF425540
PA16	*Pythium aphanidermatum*	KF425541
PA18	*Pythium aphanidermatum*	KF425542

Although *Rhizoctonia solani* and some *Fusarium* spp. have been associated with damping-off (Lida et al. , 1983; Abbasi et al. , 2004), Pythium spp. have been shown to be the most frequent cause of damping-off disease. The present investigation through ITS sequence characterization of Damping-off infected samples revealed that *Pythium* spp are the predominant causative agent of damping-off disease in tobacco nurseries and *P. aphanideramtum* is the most prevalent species. These findings will aid in designing the appropriate control measures against the specific pathogen (*Pythium*) to protect the tobacco nurseries from damping-off disease.

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