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Full Length Research Paper

Molecular phylogeny and genetic diversity studies of some potent endophytic fungi isolated from medicinal plants (*Calotropis procera* and *Catharanthus roseus*) using 18S rRNA and RAPD analysis

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Molecular identification of four endophytic fungi (*Penicillium singorense, Curvularia geniculata, Aspergillus neoflavipes and Alternaria alternata*) from two important medicinal plants, *Calotropis procera* (L.) R.Br. and *Catharanthus roseus* (L.) G.Don. was carried out by 18SrRNA sequencing. Genetic diversity using two RAPD primer viz. OPB-07 and OPC-06 showed good result with high polymorphism between the four strains. The sample DRC1 Accession No. MG322179 indicated strong homology with *Penicillium singorense*, strain DI16-118 (Accession No. LT558940), sample DRC2 Accession No. MG322180 with *Curvularia geniculata* strain F1 (Accession No. KX022497), sample DRC3 Accession No. MG322181 with *Aspergillus neoflavipes* strain AJR1 ribosomal gene (Accession No. KX218387), sample DRC4 Accession No. MH021686 with *A. alternata* strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene and ITS 2 complete sequence (Accession No. KY676196). Phylogenetic tree showing relatedness of sample DRC1 Accession No. MG322179 with 19 strains of *Penicillium* sp, DRC2 Accession No. MG322180 with 34 strains of *Curvularia* sp, DRC3 Accession No. MG322181 with 24 strains of *Aspergillus* sp and DRC4 Accession No. MH021686 with 16 strains of *Alternaria* sp of diverse origin. The present investigation thus gave an insight for the detection and genetic diversity study among the fungal endophytes isolated from two important Indian medicinal plants (C. *procera* and *C. roseus*) and it would probably the first report of its own kind from West Bengal, India.

Key words: *Calotropis procera*, *Catharanthus roseus*, National Center for Biotechnology Information (NCBI), homology search, endophytic fungi, DNA marker, Random amplification of polymorphic DNA (RAPD) analysis, 18SrRNA sequencing.

INTRODUCTION

Medicinal plants are the reservoir of biopharmaceutical compound traditionally used for the treatment of human ailment. Deforestation or constant use of medicinal plants makes them threatened or endangered (Chinedu, 2017). Conservation of medicinally important plant through sustainable practices is one of the methods for their availability. Another alternative approach for the production of secondary metabolites or biopharmaceutical is the search for fungal endophytes having medicinal importance. The long association of fungi with plants in

mutuality's mode probably makes fungi rich sources for bioactive compounds (Pimentel, 2011).

The living organism is made up of DNA, RNA and protein, similar organism has the similar type of these molecules. Molecular phylogeny generally made with such data to infer a "relationship tree" that shows the probable evolution of various organisms. After the discovery of Sanger sequencing in 1977 it became possible to isolate and identify these molecular structures (Sanger and Coulson, 1975; Sanger et al., 1977). Studies on the diversity of fungal endophytes are expected to yield potential sources of natural products and bioactive compounds for medicinal, agricultural and industrial uses, such as new antibiotics as well as novel biocontrol agents (Molina et al., 2012). Molecular identification and phylogeny of an organism generally accomplished by the help of hereditary molecular differences, mainly in DNA sequences. DNA sequencing is therefore specific identification of an individual than conventional culture-based methods: identification is refined by including internal transcribed spacer (ITS) sequence analyses with phenotypic characterization. Nuclear-encoded ribosomal DNA (rDNA) gene has been the gene of interest to analyze phylogenetic relationships and resolve taxonomic problems in different taxonomic levels (Cai et al., 2005; Jeewon et al., 2004). The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) have become a more popular marker for systematic and phylogenetic studies of closely related species of animals, plants and fungi (Von der Schulenburg et al., 2001), recently phylogenetic and taxonomic analysis in 100 endophytic fungi from *Chenopodium quinoa* has been carried out (Gonzalez-Teuber et al., 2017). Endophytic fungi often possess 'cryptic' morphological characters (Ganley et al., 2004). The cryptic morphological identity exhibited by fungal endophytes can be resolved with the help of molecular genetic marker-based identification (Bhagobaty and Joshi, 2011).

Two common medicinal plant, namely *Calotropis procera* and *Catharanthus roseus* commonly available in tropical area, mainly West Bengal has potential to provide various active ingredients of pharmaceutical importance. In the natural product research, secondary compounds leads to search an alternative way to obtain already existing active ingredients and new active phyoto-chemical from the mutulaistic symbionts mainly fungi, has gained momentum rapidly (Carvalho et al., 2016).

In the present investigation, an attempt has been made to identify endophytic fungi from *C. procera* (L.) R.Br. and *C. roseus* (L.) G.Don., the two important medicinal plant and phylogenetic classification with the collective patterns generated by partial 18S rDNA sequencing and random amplification of polymorphic DNA (RAPD).

MATERIALS AND METHODS

Genomic DNA isolation

Genomic DNA extraction from pure culture isolated from *C. procera* and *C. roseus* of *Alternaria* sp (Sample No.1), *Penicillium* sp (Sample No. 3), *Curvularia* sp (Sample No. 4) and *Aspergillus* sp (Sample No. 5) was performed following the standard protocol (Gontia- Mishra et al., 2013) with little modification. In brief fungal mycelium (800 mg of each) were taken from pure and fresh fungal culture, crushed with (3.2 ml) extraction buffer (0.1 M Tris HCl pH 8,10 mm EDTA pH 8, (2.5M) NaCl, 3.5% CTAB,150 µl of 20 mg/ ml of proteinase K) with sterilized fine sand in preatuoclaved mortar pestle. The mixture was transferred to 10 ml centrifuge tube (Tarsons) vortexes vigorously, then incubated in a water bath at 65°C for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform- isoamyl alcohol (25:24:1) was added mix thoroughly, followed by centrifugation at 10.000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of chloroform-isoamyl alcohol (24:1) was added, mix thoroughly followed by centrifugation at previous condition. Supernatant was then collected, equal volumes of chilled isopropanol added, allowed to precipitate at - 20°C freezers for overnight. Samples were the again centrifuge at 13.000 rpm for 15 min to form the DNA pellet. Supernatant was discarded; DNA pellet washed thoroughly with 1 ml of absolute ethanol by centrifugation at 500 rpm for 5 min. DNA pellet was dried under Laminar Air Flow, dissolved in 200 µl of sterile high performance liquid chromatography (HPLC) grade water. The genomic DNA was treated with RNase A according to manufacturer's instruction to remove RNA and the purified DNA was quantified spectrophotometrically (NanoDrop 8000 spectrophotometer) to know their concentration and quality.

Amplification of 18S rRNA gene with ITS 1 and ITS 4 primer

Polymerase chain reaction (PCR) amplification of the 18S rRNA gene of four fungal isolates was performed using the respective genomic DNA with the primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCGCTTATTGATATGC-3' (White et al., 1990). PCR was performed in 25 µl reaction volume containing 2.5µl of 10X Taq DNA polymerase, 2.0 µl of 2.0 mM dNTP mix, 15 pmol of each primer ITS1 and ITS4, 50 ng template DNA and 1.5U of Taq polymerase (Promega) using the following program: initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53.5°C for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min on a thermal cycler (ABI Veriti Thermal Cycler).

18 SrRNA detection and sequencing of amplicon

The amplicon generated were resolved in 1.5% agarose gel with ethidium bromide containing a (100 bp) DNA ladder. The applicon was the enzymaticaly purified followed by EDTA-Ethanol purification method. The bi-directional DNA sequencing reaction of PCR amplicon was carried out with 18S sequencing primers (1F CTGGTGCCAGCAGCCGCGGYAA, 4R CKRAGGGCATYACWGACCTGTTAT) using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The PCR condition

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was 94°C 3 min, 94°C 30 s, 48°C 30 s, 72°C 1 min, 72°C 7 min, 4°C forever, the total cycles were 30.

Submission of 18Sr RNA gene sequence in the NCBI database and construction of phylogenetic tree

The partial 18S rRNA gene sequence of selected fungal antagonists was submitted to NCBI GenBank for obtaining Accessions. Parsimony Methods were conducted in MEGA 5 software. The evolutionary history was inferred by using the Maximum Likelihood (Tamura et al., 2004). Evolutionary Genetics Analysis was performed using Maximum Likelihood, Evolutionary Distance (Tamura et al., 2011). The bootstrap value was calculated and shown in the dendrogram.

RAPD polymerase chain reaction

PCR was carried out in a final reaction volume of 25 µl in ABI Veriti Thermal Cycler with three RAPD primers: OPB-07 5'- GGTGACGCAG -3' (10 nts.) OPC-06 5'- GAACGGACTC-3' (10 nts.) and OPC-07 5'- GTCCCGACGA-3' (Gonita-Mishra et al., 2013). Composition of reaction mixture for PCR is : Nuclease free water to make-up volume for 25 µl, DNA 50 ng, Primer (10 pmole) 1.0 µl, 2X PCR Master Mix 10 µl, total Volume 25 µl. PCR tubes containing the mixture were tapped gently and spin briefly. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 40 cycles for the primers used is: initial denaturation 94°C, 4 min; denaturation 94°C, 1 min; annealing 37°C, 1 min; extention 72°C 2 min and final extension 72°C, 5 min.

Visualization of PCR product

To confirm the targeted PCR amplification, 10 µl of PCR product from each tube was mixed with 2 µl of 6X gel loading dye and electrophoreses on 2.0% agarose gel containing ethidium bromide and co run with 100 bp DNA ladders, at the constant 5 V/cm for 45 min in 1X TAE buffer. The amplified products were visualized under UV light and documented by a gel documentation system (BIORAD).

Data analysis and construction of dendrograms from RAPD profile

Clear and distinct bands amplified by RAPD primers were scored for the presence (1) and absence (0) of the corresponding band through OPC-06 and OPB-07 primers, in the gDNA of the sample. The data were entered into the binary matrix and subsequently analyzed. Using FreeTree Software, cluster analysis was performed by agglomerative technique using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean, replication no. 3) method. Relationships between the samples were graphically represented in the form of dendrograms.

RESULTS

Qualitative and quantitative estimation of genomic DNA

Nanodrop reading of genomic DNA of four samples under study viz: 1,3,4 and 5 showed high yield and good quality 847.4 ng/ µl (*Alternaria* sp*)*, 469.8 ng/ µl (*Penicillium* sp*)*, 811.3 ng/ µl *Curvularia* sp and 240.3 mg/µl *(Aspergillus* sp) respectively.

Amplification of 18S rRNA using ITS1 and ITS 4 primer

Amplification of the rDNA regions of four sample under study with ITS1 and ITS 4 oligonucleotide primers yielded amplicon of approximately 900 bp as estimated by 1.5% agarose gel electrophoresis using known standard DNA ladder.

Sequencing of 18SrRNA gene

The four 18SrRNA amplicon subjected to sequencing namely Sample No.1 (*Alternaria alternata* DRC 4), Sample No. 3 (*Penicillium* sp DRC1), Sample No.4 (*Curvularia* sp DRC2), Sample No. 5 (*Aspergillus* sp DRC 3) gave a good sequence reading of standard fragment size. The sequences used for the final homology search, phylogenetic analysis were done after manual counting trimming and corresponded to the ITS 1 and 2 complete regions, 5' portion of the 18S gene, 5.8 S complete sequences and the 3' end of the 28S gene.

GenBank accession followed by homology searching

The FASTA format of four (sample DRC1, DRC2, DRC3, DRC4) 18 SrRNA sequence after submitting in NCBI database, the GenBank Accession were obtained (DRC1 MG322179, DRC2 MG322180, DRC3 KX218387, DRC4 MH021686) respectively. Sample 3 that is, DRC1 Accession No. MG322179 showed strong homology with *Penicillium singorense*, strain DI16-118, sample 4 that is, DRC2 Accession No. MG322180 showed strong homology with *Curvularia geniculata* strain F1, sample 5 that is, DRC3 Accession No. MG322181 showed strong homology with *Aspergillus neoflavipes* strain AJR1 ribosomal gene, sample 1 that is, DRC4 Accession No. MH021686 showed strong homology with *Alternaria alternata* strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene and ITS 2 complete sequence Accession No. KY676196 (Table 1). The phylogenetic analysis of these four isolated and identified strain reveals their relatedness with respective species (Figures 1, 2, 3 and 4).

RAPD analysis

Among the three RAPD primer (OPB- 07, OPC-06 and OPC-07) tested to identify genetic diversity good

Table 1. Homology and annotation of 4 GenBank Accession of isolated endophytic fungi strains.

Strain code	NCBI Accession No	Homology	Length (bp)	Annotation	Max score	Total score	E-value	Identical (%)
DRC1	MG322179	LT558940	599	*Penicillium singorense* , strain DI 16-118 genomic DNA contains ITS1, 5.8SrRNA gene, ITS2, 28SrRNA gene	1033	1033	0.0	99
DRC2	MG322180	KX022497	597	*Curvularia geniculata* strain F1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene& ITS 2 complete sequence.	1055	1055	0.0	99
DRC3	MG322181	KX218387	896	*neoflavipes* strain AJR1 18S rRNA gene, partial sequence.	1594	1594	0.0	99
DRC 4	MH021686	KY676196	574	*Alternaria alternata* strain AE1 18SrRNA gene, partial sequence, ITS1, 5.8S rRNA gene & ITS 2 complete sequence.	1061	1061	0.0	100

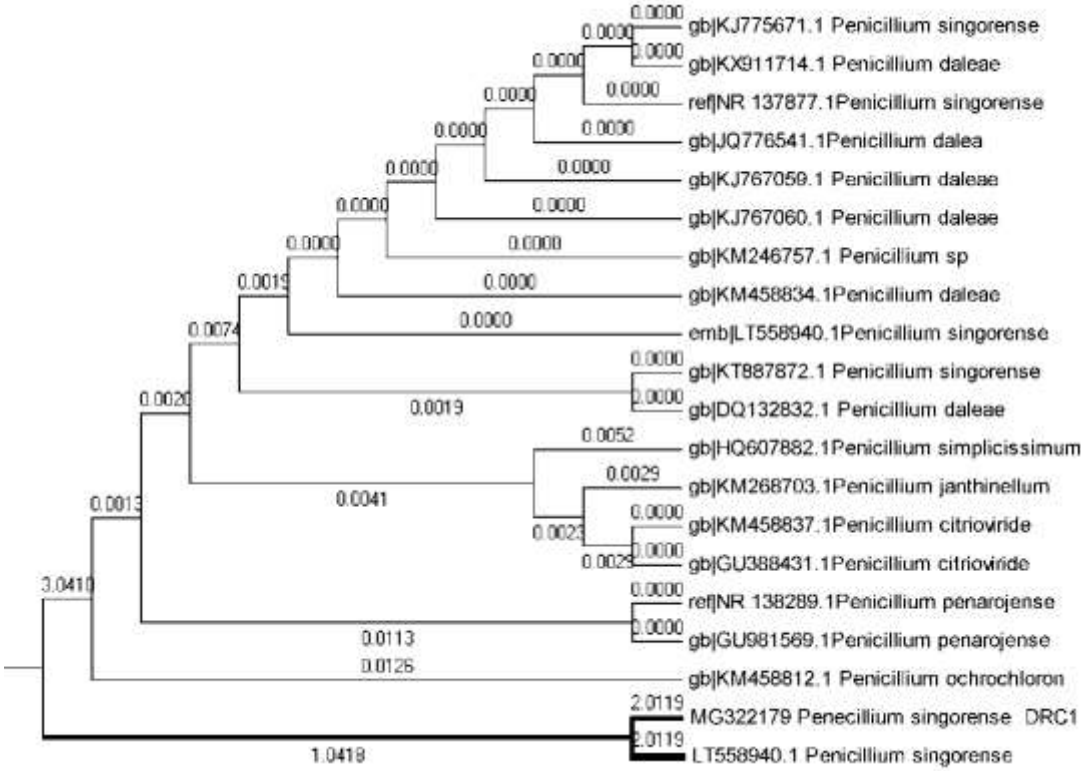

Figure 1. Phylogenetic tree showing relatedness of DRC1 Accession No. MG322179 with 19 strains of *Penicillium* sp. of diverse origin showing highest similarity with *Penicillium singorense*, strain DI16-118 (Accession No. LT558940).

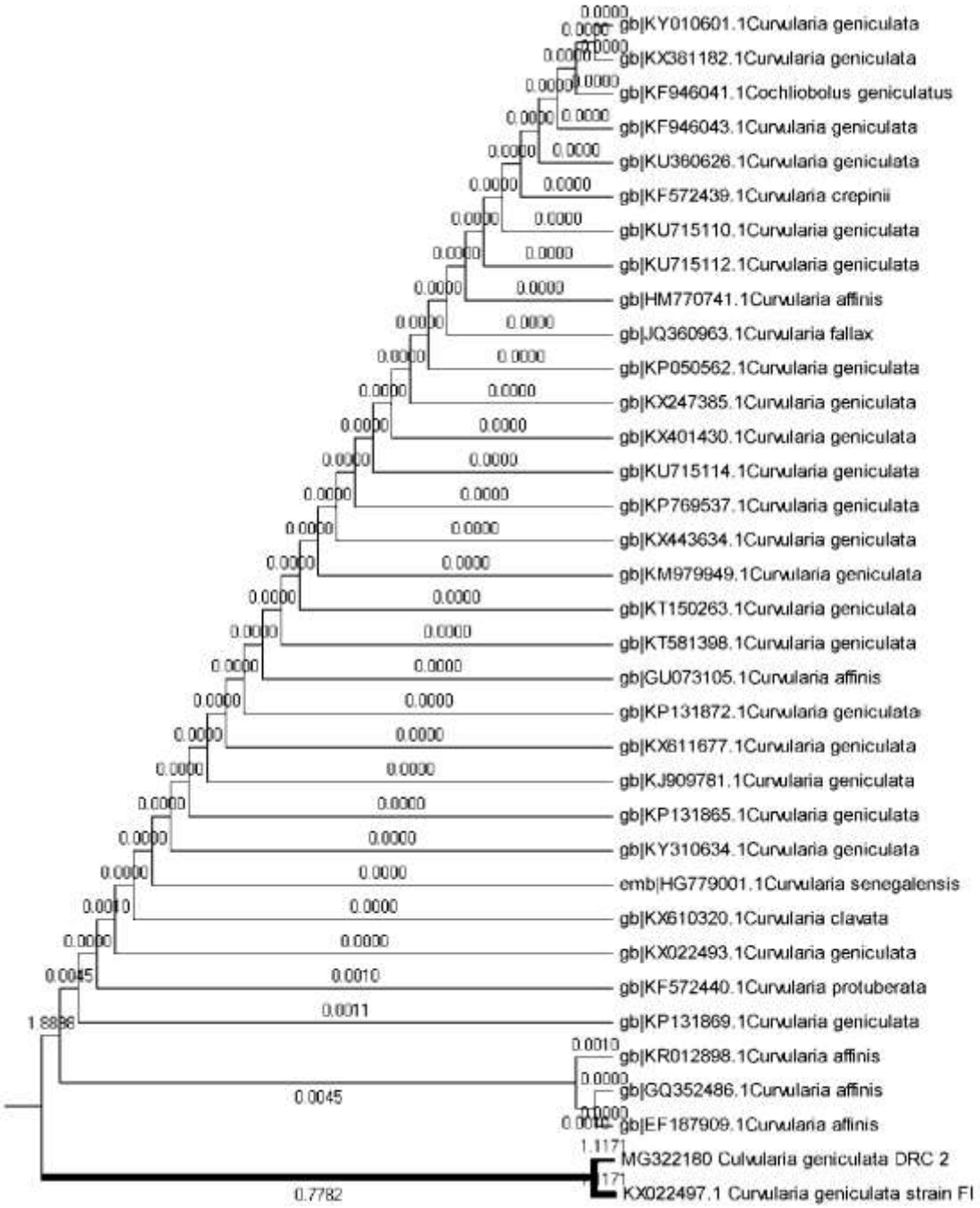

Figure 2. Phylogenetic tree showing relatedness of DRC2 Accession No. MG322180 with 34 strains of *Curvularia* sp of diverse origin showing highest similarity with *Curvularia geniculata* strain F1 (Accession No. KX022497).

amplification was responded by two primers namely OPB-07 and OPC-06. The RAPD profile of four samples showed polymorphism and genetic diversity among them with fragment size of approximately 150 to 1.5 kb (Figure 5). The highest amplification was recorded with OPB-07 primer. RAPD marker has also been used (Gontia-Mishra et al., 2013) to amplify the genomic DNA of several species of *Aspergillus* and showed similar result.

Dendrograms from RAPD profile

Dendrogram constructed from two RAPD profile depicted in Figures 6 and 7 reveals their relationship. In both the tree there are two groups showing the close relationship with sample number 1 (*Alternaria* sp.) and three (*Penicillium* sp.) and other with sample number 4 (*Curvularia* sp.) and 5 (*Aspergillus* sp.). Both the groups

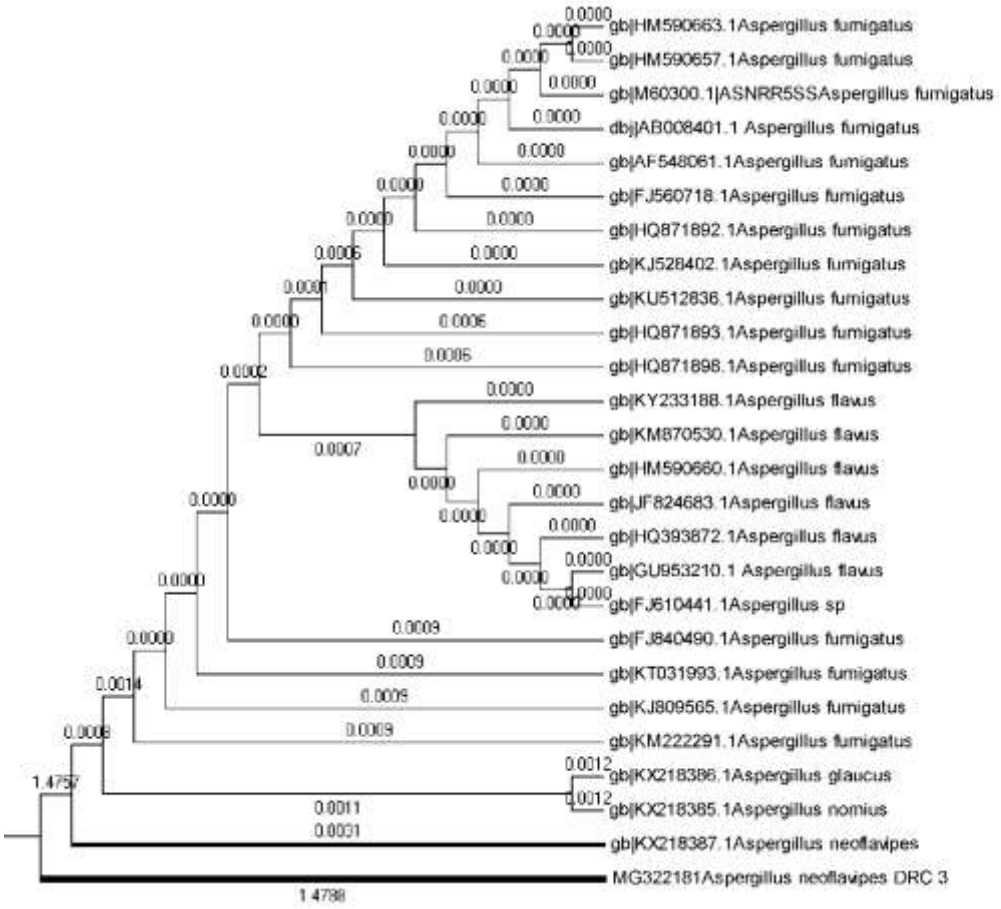

Figure 3. Phylogenetic tree showing relatedness of DRC3 Accession No. MG322181 with 24 strains of *Aspergillus* sp. of diverse origin showing highest similarity with *Aspergillus neoflavipes* strain AJR1 ribosomal gene (Accession No. KX218387).

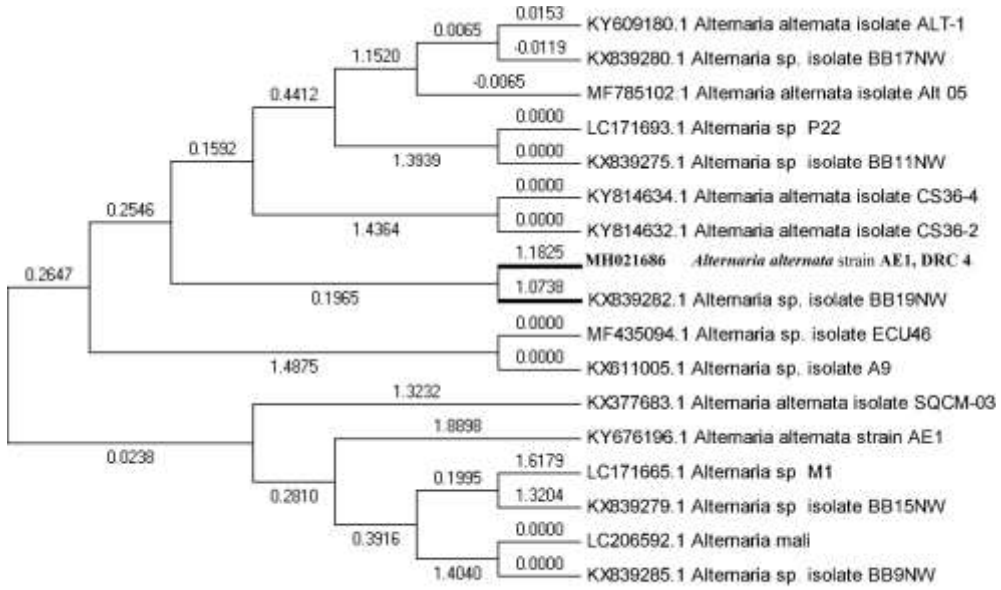

Figure 4. Phylogenetic tree showing relatedness of DRC4 Accession No. MH021686 with 16 strains of Alternaria sp of diverse origin showing highest similarity with *Alternaria alternata* strain AE1 18SrRNA gene (Accession No. KY676196).

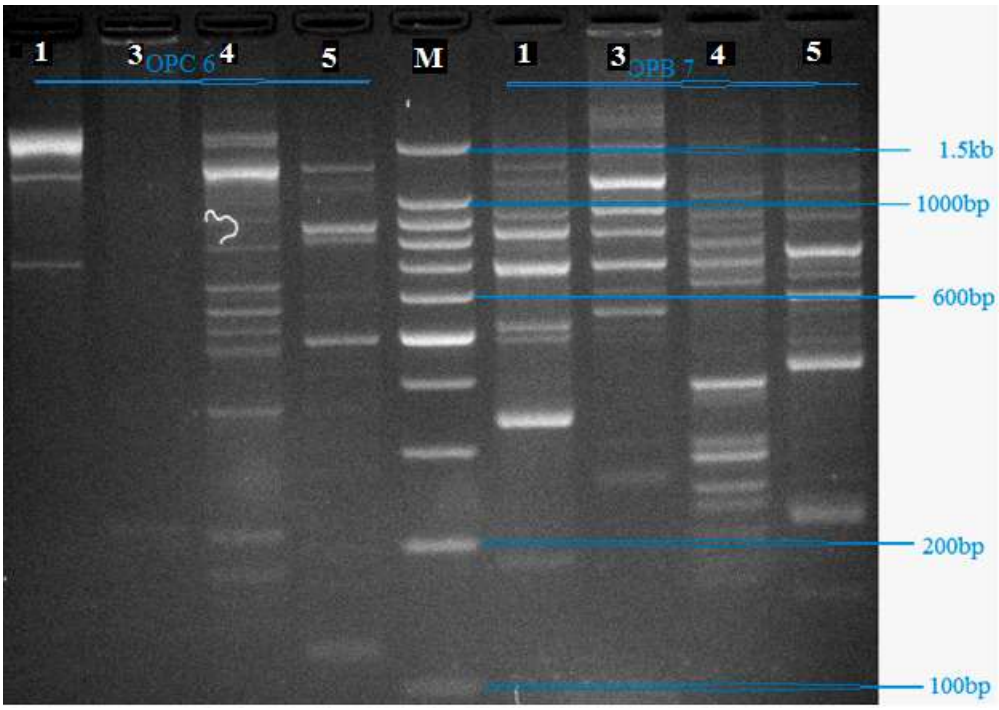

Figure 5. 2% Agarose gel electrophoresis showing gel image of 1, 3, 4, 5 g DNA with primer OPC-06 and OPB-07.Lane M: 100bp DNA ladder.

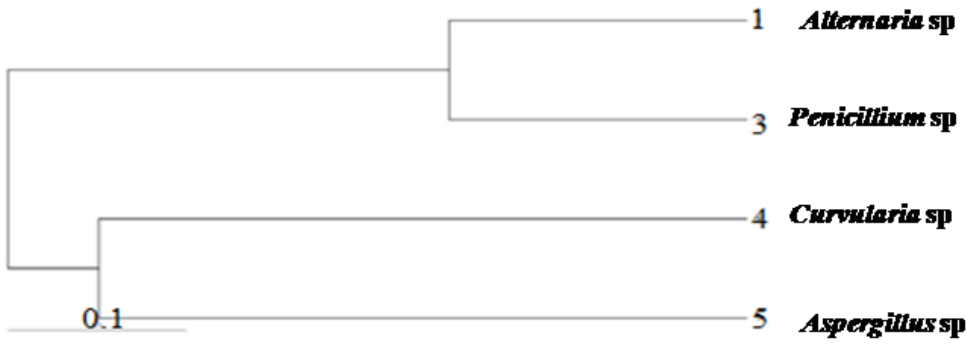

Figure 6. Total homology search using RAPD Primer OPB-07 of the four DNA samples (Sample No. 1 *Alternaria* sp. sample No. 3 *Penicillium* sp. sample No. 4 *Curvularia* sp. and sample No. 5 *Aspergillus* sp.); cluster analysis was performed by agglomerative technique using the unweighted pair group method with arithmetic mean (UPGMA) method showing relationships between the samples.

in other hand distantly related in the case of both primers (Figures 6 and 7).

Total homology searching between the four fungal isolates namely *Penicillium* sp, *Curvularia* sp, *Aspergillus* sp. and *Alternaria* sp. revealed that *Alternaria* sp., *Penicillium* sp. and *Curvularia* sp., *Aspergillus* sp. are also closely related to each other. The molecular analyses proved that these four fungal isolates to the diverse genera.

DISCUSSION

After thorough investigation for the presence of fungal endophytes from two important Indian medicinal plants (*C. procera* and *C. roseus*), four pure fungal isolates of *Penicillium* sp., *Curvularia* sp., *Aspergillus* sp. and *Alternaria* sp. has been identified by using molecular tools and techniques. From the perusal of literature it has been revealed that the four isaolated fungi have great

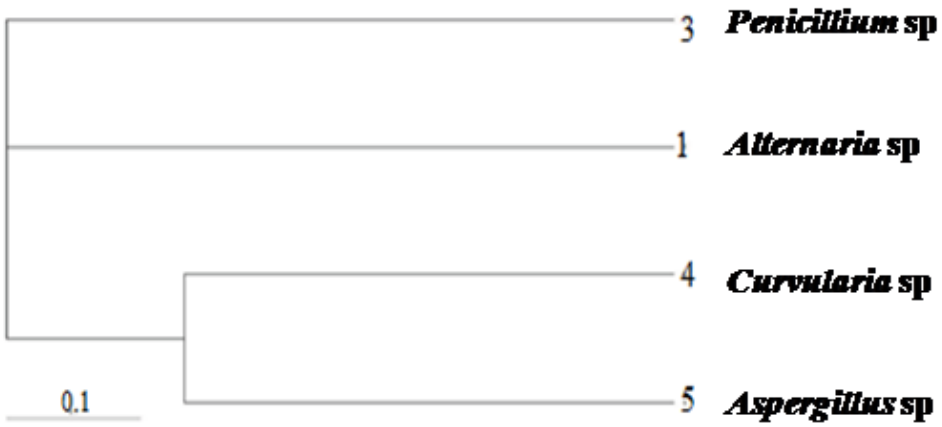

Figure 7. Total homology search using RAPD Primer OPC-06 of the four DNA samples (sample No. 1 *Alternaria* sp., sample No. 3 *Penicillium* sp. sample No. 4 *Curvularia* sp. and sample No. 5 *Aspergillus* sp.); cluster analysis was performed by agglomerative technique using the unweighted pair group method with arithmetic mean (UPGMA) method showing relationships between the samples.

medicinal value, as they produce several bioactive compounds of pharmaceutical importance. *Penicillium singorense* is a species of the genus *Penicillium* which was isolated from house dust (Visagie et al., 2014). *Curvularia* is a hyphomycete fungus which is a facultative saprophyte of many plant species and common in soil. Most *Curvularia* are found in tropical to temperate regions. However literature reveals that *Curvularia affinis*, *Curvularia fallax*, and *Curvularia senegalensis* are synonymous to C. *geniculata.* The presence of the mycotoxins, fumifungin, fumiquinazoline A/B and D, fumitremorgin B, gliotoxin, sphingofungins, subroutines, and verruculogen in *Aspergillus fumigatus* a close relative of A. n*eoflavipes* has been reported (Tamiya et al., 2015). The presence of isochromenone, from an endophytic fungus *Aspergillus fumigatus* isolated from *Bacopa monnieri* plant and proof its antioxidant and antitubercular activity has been investigated (Thakur et al., 2015). The methanol extract of *A. fumigatus* inhibits the growth of the virulent strain of *Mycobacterium tuberculosis* HRV with minimum inhibitory concentration 500 µg/ml.

Fungal endophytes are reported to secrete a diverse group of biomolecules extracellularly which are capable of reducing metal salts at a rapid scale under optimized conditions. One such endophyte is isolated from healthy leaves of *Andrographis paniculata* and employed for rapid synthesis of silver nanoparticles. The synthesized nanoparticles were evaluated for bactericidal activity against significant human pathogens (Azmath et al., 2016). The presence of paclitaxel from newly described endophytic fungus *Pestalotiopsis microspora*, isolated from the pine needles of *T. wallichiana* has been reported (Strobel et al., 1996). This study indicates that a large scale production of the natural product paclitaxel can be made at a lower cost just by mass production of the fungus in culture. Paclitaxel can be obtained from Yew (*Taxus* sp.), but such obtaining requires destruction of trees. Chemical synthesis of paclitaxel by cell and tissue culture from the Taxus sp are cost effective and production is not up to the mark (Guenard et al., 1993). Recently, paclitaxel as well as other toxins have been detected in shells and leaves of *Corylus avellana* and also in leaves of *Ocimum basilicum* (Ottaggio et al., 2008; Gangadevi and Muthumary 2017). It is well known that the tissues of living plant residue the endophytic fungi which possess analogical ability to perform a biosynthesis of secondary metabolites as the host plant. The ability of the endophytic fungus of Hazel plant *Penicillium aurantiogriseum* NRRL 62431 to independently synthesize paclitaxel was detected by liquid chromatography-mass spectrometry and proton nuclear magnetic resonance. The presence of Taxol from *Phoma* sp isolated from *Calotropis gigantea* has been detected (Hemamalini et al., 2015). The genome of *Penicillium aurantiogriseum* NRRL 62431 was sequenced and gene candidates that may be involved in paclitaxel biosynthesis were identified by comparison with the 13 known paclitaxel biosynthetic genes in *Taxus* (Yang et al., 2014). Two new metabolites, from a soil fungus, *Curvularia affinis* strain HS-FG-196. pyrenocine J (1) and pyrenochaetic acid D (2), together with two known metabolites, pyrenocine A (3) and pyrenochaetic acid A (4) has reported (Zhang et al., 2012). Pyrenocine J showed cytotoxic activity against the human hepatic cancer cell line HepG2 with an IC (50) value of 28.5 µg/ml. The presence of different mycotoxins, gliotoxin, sphingofungins, subroutines, and verruculogen in *Aspergillus fumigatus* a close relative of *A. neoflavipes* has also been reported (Tamiya et al., 2015) which are of

great commercial value. The capacity of producing Vinblastin, an anticancerous drug from *Alternaria* sp isolated from *C. roseus* was reported (Guo et al., 1998). The paclitaxel a highly valued anticancerous compound was also produced by *A. alternata* TPF6 isolated from *Taxus chinensis* var. *mairei* (Tian et al., 2006).

The present study highlights the detection and genetic diversity among the fungal endophytes isolated from two important Indian medicinal plants. The endophytic fungi are expected to be a potential source of many natural bioactive products including paclitaxel and other taxanes. The search for a bioactive compound from fungal endophytes gaining momentum, thus the identification of fungal endophytes would be of great importance to the discovery of the newly active compound. The present study would probably the first report of its own kind from West Bengal, India.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Detection of β-tubulin gene from benomyl sensitive isolates of *Colletotrichum gloeosporioides* causing anthracnose disease in mango

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Twenty six isolates of *Colletotrichum gloeosporioides* from anthracnose infected mango fruits were isolated from different places of Tamil Nadu, India and these isolates were identified as *C. gloeosporioides* by Internal transcribed spacer (ITS) and species specific (CgInt) primers. The sensitivity of *C. gloeosporioides* isolates to benomyl fungicide were evaluated at five different concentrations *viz.,* 0.5, 1, 2, 5, 10 mg/l through poisoned food technique and were expressed very less to highly sensitiveness to the fungicide. All the twenty six isolates were able to grow at the lowest concentration of 0.5 mg/l benomyl amended medium and the average mycelial growth was 70.70 mm at even days after inoculation. At the highest concentration of 10 mg/l benomyl, only two isolates viz., MCG 7 and 16 were able to grow with the mycelial diameter of 12.00 and 18.00 mm, respectively and 100% inhibition was found in the remaining isolates. In addition, benzimidazole sensitive and resistance β - tubulin gene sequences of TUB 1 and TUB2 were amplified from the benomyl sensitive isolates of *C. gloeosporioides*. The results indicated the differential resistance or sensitivity to benomyl fungicide against *C. gloeosporioides* and thereby allowed to identify the variability and diversity of the isolates on regional basis.

Key words: Mango*, C. gloeosporioides*, benomyl sensitive, β - tubulin gene.

INTRODUCTION

Many diseases are affecting mango, of which anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Sacc is the most devastating disease and major constraint in production and export of mango. Several fungicides have been used to manage the mango anthracnose as pre- and post-harvest sprays, including benzimidazoles, triazoles, strobilurins, acid amides, dithiocarbamates and heterocyclic compounds (Fei and Wang, 2004). Hot benomyl dips have been reported for effective control of anthracnose in harvested mango fruit (Kim et al., 2007). Benomyl has been used in agriculture for approximately 30 years and numerous cases of

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resistance have been reported, including *Colletotrichum* spp. on various crops (Whiteside, 1980; Picinini, 1994; Hewitt, 1998). Benzimidazoles are specific inhibitors of microtubule assembly that act by binding to their heterodimeric subunit (Davidse, 1986). Several studies have demonstrated that benzimidazole resistance is associated with point mutations in codon 198 or 200 of the β-tubulin gene in various fungi, including *Venturia inaequalis*, *Botrytis cinerea*, *Monilinia fructicola*, *C. gloeosporioides* and *Mycosphaerella fijiensis* (Ma et al., 2003; Peres et al., 2004; Chung et al., 2006; Kim et al., 2007). Other point mutations in the β-tubulin gene (Jung et al., 1992; Cooley and Caten, 1993) as well as in the putative leucine zipper protein CaBEN1 (Nakaune and Nakano, 2007) have also been associated with resistance to benzimidazoles. Maymon et al. (2006) reported that sequence analyses of the β-tubulin genes, *TUB1* and *TUB2*, of five sensitive and five resistant representative isolates of *C. gloeosporioides* from *Limonium* spp. revealed that the benomyl resistant isolates had an alanine substitute instead of a glutamic acid at position 198 in *TUB2*. Benzimidazole resistant isolates of *C. gloeosporioides* from different crops have been identified, but the molecular characteristics of these resistant isolates are still not well defined (Pei, 1981; Tsai et al., 2006). The molecular identification and monitoring of benzimidazole resistant fungi has been recognized as a useful tool in the study of fungicide resistance (Ma et al., 2003; Canas-Gutierrez et al., 2006). Housekeeping genes including the nuclear β-tubulin genes, which show higher DNA sequence variability, are more extensively used to develop diagnostics for fungi, (Fraaije et al., 2001; Mostert et al., 2006; Aroca et al., 2008). Partial β-tubulin sequences from *C. gloeosporioides* isolates of mango were amplified (Albertini et al., 1999; Yarden and Katan, 1993) as a molecular tool for the identification of benzimidazole-resistant isolates of *C. gloeosporioides*. Sanders et al. (2000) evaluated 158 isolates of *C. gloeosporioides* and reported, 17.7% were resistant to benomyl with 8.5% highly resistant due to mutations in the β - tubulin gene using this information, *C. gloeosporioides* isolates from mango fruits collected in India were evaluated for benomyl sensitivity and mutations in the β - tubulin gene in the present study.

MATERIALS AND METHODS

C. gloeosporioides from mango

Twenty six isolates of *C. gloeosporioides* collected from different districts of Tamil Nadu, India were evaluated in the study (Table 1). Their pathogenicity, variability in virulence was previously reported by Archana et al. (2014).

Sensitivity of *C. gloeosporioides* to benzimidazole fungicide – benomyl

Each isolate was cultured on Potato Dextrose Agar (PDA) medium in petri plates at 28±2°C for three days, after that mycelial disc (3 mm diameter) were cut from colony margins and a single disc was transferred to the centre of Petri plate containing PDA medium amended with 0.5, 1, 2, 5 and 10 mg/ml of the active ingredient of benomyl fungicide, which was added to PDA medium after autoclaving. Test cultures were incubated in dark at 28±2°C and then mycelial growth was measured 11 days after inoculation (Chung et al., 2010) and control plate was maintained by growing the isolates in the Petri plate containing PDA medium alone.

PCR based detection and diagnosis of *C. gloeosporioides* isolates using internal transcribed spacer (ITS) and CgInt primers

Genomic DNA for *C. gloeosporioides* isolates were extracted from the mycelial mat by Cetyl trimethyl ammonium bromide (CTAB) method as described by Knapp and Chandlee (1996). The ITS1-5.8S-ITS2 region of ribosomal DNA was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') oligonucleotide primers. The amplification was performed in 50 µl reaction containing 1.5 units of *Taq* DNA polymerase (Qiagen, Germany), 1x polymerase chain reaction (PCR) buffer, 200 µM each dNTP, 0.2 µM each primer and 100 ng of template DNA followed by the thermal cycler reaction of 30 cycles (0.5 min at 94°C, 0.5 min at 56°C and 2 min at 72°C) with the final step at 72°C for 7 min (Tosa et al., 2004).

PCR amplification using the oligonucleotide primers ITS 4 (TCCTCCGCTTATTGATATGC) and CgInt (GGCCTCCCGCCTCCGGGCGG) were used for species identification. PCR reaction were as described above with amplification as follows: denaturation at 95°C for 5 min, 30 cycles of 30 s. at 95°C, 30 s at 48°C and 90 s at 72°C and a final extension of 10 min at 72°C (Maymon et al., 2006) in an Eppendorf gradient thermal cycler.

Analysis of benomyl resistance of *C. gloeosporioides* isolates using partial sequence of β-tubulin gene

PCR amplification of β-tubulin gene (*TUB2* and *TUB2B*) fragments were carried out using generic primer GENC (5′-GAGGAATTCCCAGACCGTATGATG- 3′) and TUB2 (5′-GACATCCTTCATAGCG-3′) to identify the benomyl resistant isolates (Koenraadt et al., 1992) and primer GENC and TUB2B (5′-GAC(A/G)TCCTTCAT(A/G)GCGA-3′) to identify benomyl sensitive isolates (Panaccione and Hanau, 1990). *TUB1*β-tubulin gene was amplified using the primer GENC (5′GAGGAATTCCCAGACCGTATGATG-3′) and TUB 1C (5′-TCAATCTGCTTGGTCGACACCTT-3′) to identify the resistant isolates.

Reactions prepared as above and PCR reactions were performed with 60 s of denaturation at 94°C; followed by 35 cycles consisting of 30 sec at 94°C, 30 sec at 50°C and 60 sec at 72°C; and a final extension period of 10 min at 72°C.

Agarose gel electrophoresis

Amplification products for the above 5 evaluations were separated in 1.2% (w/v) agarose gel in1x TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 85 V and the gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The sizes of the PCR products were determined by comparing with standard 100 bp or 1 kb molecular marker (Bangalore Genei Pvt. Ltd., Bangalore, India).

Table 1. Characterization of *C. gloeosporioides* isolates based on cultural characters in PDA medium.

Isolates	Variety	Place of collection	Colony colour	Pigmentation	Condia (µm)	
					Length	Width
MCG 1	Neelum	Rajapalayam	Dull White	-	11.34^{a}	6.23^{m}
MCG 2	Mallika	Krishnagiri	White	-	15.55^{kl}	5.30^{hi}
MCG 3	Himayuddin	Dharmapuri	white	-	13.33^{fg}	4.30^{b}
MCG 4	Raspuri	Krishnagiri	White	-	16.28^{m}	5.77^{jk}
MCG 5	Mulgoa	Palani	Blackish white	Black	11.76^{ab}	6.01^{l}
MCG 6	Nadusalai	Coimbatore	Dull white	-	12.45^{d}	5.87^{kl}
MCG 7	Himayuddin	Periyakulam	White	-	13.99^{h}	4.50^{cd}
MCG 8	Banganapalli	Tenkasi	Blackish white	Black	15.05^{j}	5.20^{h}
MCG 9	Mulgoa	Paiyur	Blackish white	Black	12.78^{de}	6.30^{m}
MCG 10	Alphonso	Tirunelveli	Blackish white	Black	11.87^{bc}	5.77^{jk}
MCG 11	Alphonso	Paiyur	Blackish white	Black	16.33^{m}	4.30^{b}
MCG 12	Kalapad	Nagercoil	Dull white	-	15.34^{jk}	5.80^{jk}
MCG 13	Senthuram	Trichy	White	-	13.74^{gh}	4.87^{fg}
MCG 14	Mulgoa	Erode	Blackish white	Black	16.01^{lm}	4.70^{ef}
MCG 15	Alphonso	Thiruvallur	White	-	12.75^{de}	5.20^{h}
MCG 16	Neelum	Theni	White	-	11.93^{bc}	4.34^{bc}
MCG 17	Neelum	Thanjavur	Blackish white	Black	12.56^{d}	4.60^{de}
MCG 18	Banganapalli	Kanyakumari	Black	Dark black	13.78^{gh}	5.46^{i}
MCG 19	Senthuram	Namakkal	Purple	-	15.98^{lm}	5.67^{j}
MCG 20	Neelum	Salem	Salmond orange	-	15.02^{j}	4.77^{ef}
MCG 21	Banglora	Dharmapuri	Yellowish white	Yellow	12.34^{cd}	4.10^{a}
MCG 22	Karuneelum	Paiyur	White	-	13.32^{fg}	4.80^{f}
MCG 23	Banglora	Nagapattinam	Blackish White	Black	14.48^{i}	4.30^{b}
MCG 24	Neelum	Madurai	white	-	15.32^{jk}	5.01^{g}
MCG 25	Neelum	Kalakad	Dull white	-	11.56^{ab}	4.72^{ef}
MCG 26	Mulgoa	Kayathar	White	-	13.08^{ef}	4.87^{fg}

Mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by DMRT.

Statistical analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, Philippines (Gomez and Gomez, 1984). Data were subjected to analysis of variance (ANOVA) at two significant levels ($P< 0.05$ and $P< 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Molecular detection of *C. gloeosporioides* isolates using ITS and CgInt primers

PCR amplification using universal primers of ITS 1 and ITS 4 produced the expected 560 bp fragment, confirming that 26 isolates belonged to the genus *Colletotrichum* (Figure 1). PCR amplification with *Cg*Int and ITS 4 species specific primers produced the expected 450 bp amplicon confirming the isolates were *C. gloeosporioides* (Figure 2).

Screening of *C. gloeosporioides* against benzimidazole fungicide – benomyl

The sensitivity of *C. gloeosporioides* isolates to the fungicide benomyl was evaluated based on the method of poisoned food technique at five different concentrations viz., 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l (Figure 3). The growth of mycelia on the benomyl amended PDA medium was directly correlated to the sensitiveness of the isolates benomyl. Based on this, twenty six *C. gloeosporioides* isolates were designated as susceptible, moderately resistant and resistant to the fungicide benomyl. At the lowest concentration of 0.5 mg/l benomyl amended medium, all the twenty six isolates were able to grow and the diameter of the mycelium was measured eleven days after inoculation. The isolate MCG 21 produced the least growth of 27.23 mm followed by MCG 22(33.24 mm) and

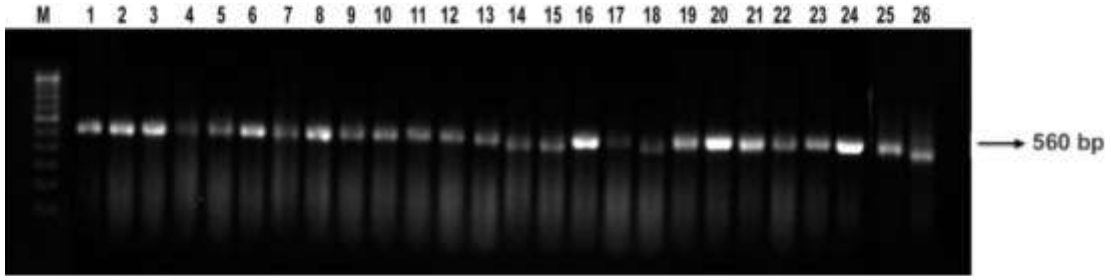

Figure 1. PCR amplification of ITS fragments of *Colletotrichum* isolates from Mango fruits infected by anthracnose disease, (M – Marker (100 bp); 1 – 26: MCG 1 – MCG 26 isolates of *Colletotrichum).*

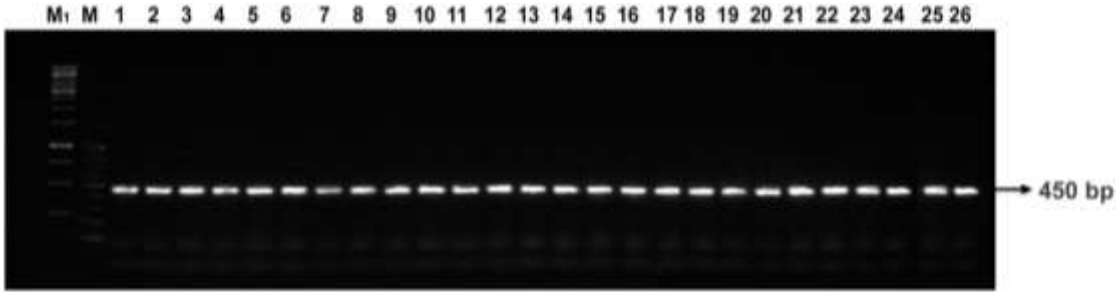

Figure 2. PCR amplification of *Colletotrichum* isolates using specific primer (CgInt + ITS 4), **(**M1 – Marker (1 kb); M – Marker (100 bp); 1 – 26: MCG 1 – MCG 26 isolates of mango anthracnose).

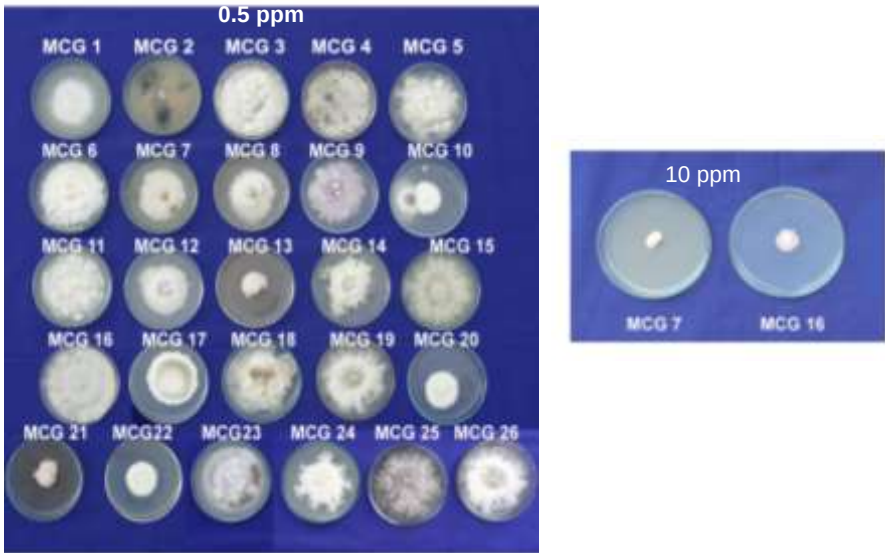

Figure 3. Sensitivity of *C. gloeosporioides* isolates to benomyl fungicides (Poisoned food method using PDA medium amended with different concentration of benomyl to evaluate the sensitivity of *C. gloeosporioides*).

MCG 13 (33.72 mm) which were significantly different from other isolates. The mycelial growth of 90 mm was observed in the isolates of MCG 3, 16, 23 and 26 at 0.5 mg/l concentration (Table 2). At the concentration of 1 mg/l of benomyl, the percent inhibition over control was higher in the isolate MCG 21 accounting 73.16%, which was on par with the isolates MCG 10 (71.60%) and MCG 22 (71.21%). Least inhibition of 3.06% was observed in

Table 2. Sensitivity of *C. gloeosporioides* isolates from mango to benomyl fungicide.

Isolates	Diameter of mycelial growth (mm) in benomyl amended PDA medium and in control plate					
	0.5 mg/l	1 mg/l	2 mg/l	5 mg/l	10 mg/l	Control
MCG 1	60.12^{e}	60.13^{e}	45.14^{l}	21.00^{jk}	0.00^{a}	90.00^{a}
MCG 2	62.34^{ef}	65.45^{fgh}	43.56^{k}	20.32^{ijk}	0.00^{a}	90.00^{a}
MCG 3	90.00^{m}	90.00^{o}	60.33^{p}	25.09^{m}	0.00^{a}	90.00^{a}
MCG 4	88.56^{lm}	87.25^{n}	55.55^{n}	22.12^{l}	0.00^{a}	90.00^{a}
MCG 5	77.34^{k}	67.56^{hij}	31.57^{e}	16.90^{fg}	0.00^{a}	90.00^{a}
MCG 6	88.10^{lm}	66.67^{ghi}	39.14^{hi}	19.32^{h}	0.00^{a}	90.00^{a}
MCG 7	63.00^{fg}	35.13^{c}	28.18^{d}	20.04^{hi}	12.00^{b}	90.00^{a}
MCG 8	65.03^{gh}	68.23^{ij}	47.62^{m}	17.31^{g}	0.00^{a}	90.00^{a}
MCG 9	74.12^{j}	69.33^{j}	54.34^{n}	21.16^{k}	0.00^{a}	90.00^{a}
MCG 10	45.45^{c}	25.56^{a}	18.54^{b}	10.05^{b}	0.00^{a}	90.00^{a}
MCG 11	88.26^{lm}	77.10^{kl}	37.16^{g}	13.45^{e}	0.00^{a}	90.00^{a}
MCG 12	63.16^{fg}	64.11^{f}	33.88^{f}	11.01^{c}	0.00^{a}	90.00^{a}
MCG 13	33.72^{b}	30.31^{b}	17.17^{b}	9.32^{b}	0.00^{a}	90.00^{a}
MCG 14	75.41^{jk}	65.09^{fg}	38.37^{gh}	20.19^{hij}	0.00^{a}	90.00^{a}
MCG 15	88.23^{lm}	79.87^{m}	40.37^{i}	21.17^{k}	0.00^{a}	90.00^{a}
MCG 16	90.00^{m}	85.44^{n}	41.82^{j}	30.41^{n}	18.00^{b}	90.00^{a}
MCG 17	68.00^{i}	55.33^{d}	28.17^{d}	12.36^{d}	0.00^{a}	90.00^{a}
MCG 18	87.08^{l}	75.23^{k}	37.77^{gh}	16.23^{f}	0.00^{a}	90.00^{a}
MCG 19	88.09^{lm}	76.12^{kl}	55.24^{n}	21.15^{k}	0.00^{a}	90.00^{a}
MCG 20	48.14 ^{d}	78.35^{lm}	57.31^{o}	20.00^{hi}	0.00^{a}	90.00^{a}
MCG 21	27.23^{a}	24.16^{a}	13.15^{a}	8.33^{a}	0.00^{a}	90.00^{a}
MCG 22	33.24^{b}	25.91^{a}	17.16^{b}	10.13^{b}	0.00^{a}	90.00^{a}
MCG 23	90.00^{m}	87.18^{n}	61.42^{p}	31.43^{o}	0.00^{a}	90.00^{a}
MCG 24	65.80^{hi}	54.53^{d}	26.34^{c}	14.04^{e}	0.00^{a}	90.00^{a}
MCG 25	87.73^{lm}	76.52^{kl}	31.35^{e}	13.22^{e}	0.00^{a}	90.00^{a}
MCG 26	90.00^{m}	85.14^{n}	43.36^{k}	22.16^{l}	0.00^{a}	90.00^{a}

*Mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by DMRT;

the isolate MCG 4. In addition, at the concentration of 2 mg/l the percent inhibition of mycelial growth was ranged from 31.76 to 85.39% and at the concentration of 5 mg/l it ranges from 65.08 to 90.74%. At the highest concentration of 10 mg/l, only two isolates viz., MCG 7 and MCG 16 were able to grow in benomyl poisoned petriplate with the mycelial diameter of 12.00 and 18.00 mm possessing 86.67 and 80.00% inhibition over control, respectively (Figure 4) among 26 isolates. By comparing the mycelial growth of *C. gloeosporioides* isolates at five different concentrations tested, it was concluded that the isolate MCG 21 was considered sensitive; MCG 7 and MCG 16 were resistant to benomyl fungicide.

PCR amplification of β-tubulin gene from *C. gloeosporioides*

In all the twenty six isolates of *C. gloeosporioides,* partial β – tubulin gene sequences of TUB 2 and TUB 1 were amplified by the appropriate primers from genomic DNA (Figure 5).

The results from PCR amplification, showed that the primer pairs of GENC and TUB2 amplified the resistant allele of TUB 2, (563bp fragment) amplified in ten isolates (MCG 3, MCG 7, MCG 8, MCG 11, MCG 12, MCG 13, MCG 16, MCG 19, MCG 20 and MCG 21) (Figure 5a). The sensitive allele of TUB 2 (563bp fragment) was amplified from nine isolates (MCG 2, MCG 3, MCG 6, MCG 8, MCG 19, MCG 20, MCG 21, MCG 24 and MCG 25) (Figure 5b). In addition, another β-tubulin resistant allele of TUB 1 (506bp fragment) was amplified by the primer pair of GENC and TUB1C from four isolates (MCG 19, MCG 20, MCG 24 and MCG 25) (Figure 5c). Results from this study have confirmed most of the isolates used as benzimidazole-resistant, which were collected from anthracnose infected mango fruits of different places.

Nucleotide sequence accession number

The sequence obtained in this study was assigned

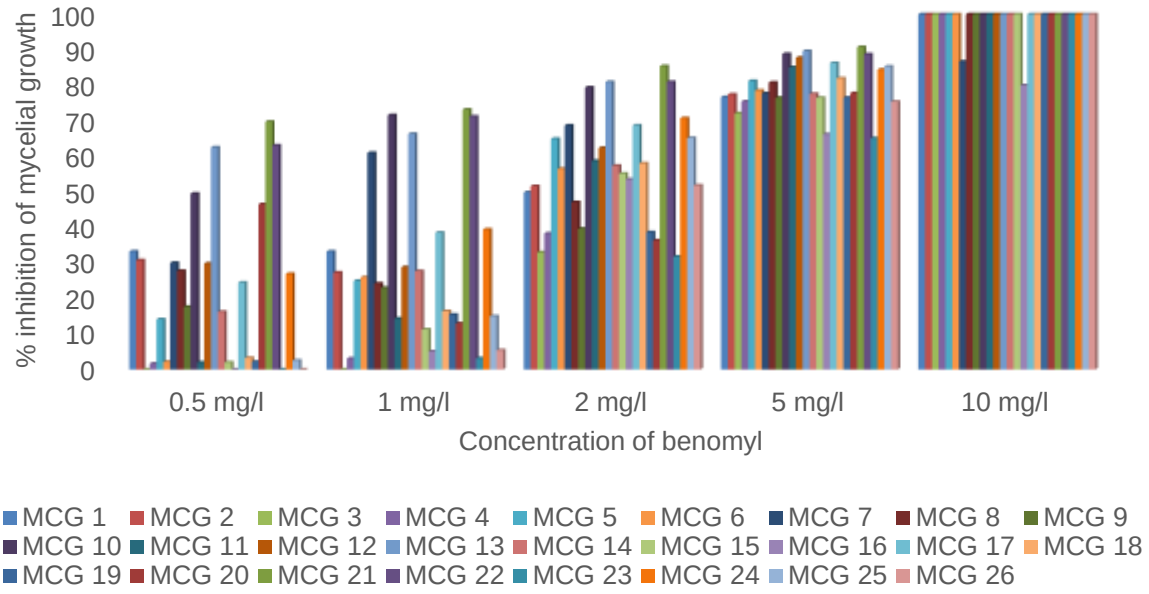

Figure 4. Efficiency of benomyl fungicide against *C. gloeosporioides* isolates *in vitro* analysis.

GenBank accession number of β-tubulin resistance gene isolates MCG 11-KJ470629.1 and MCG 16-KJ462468.1

DISCUSSION

The fungus *C. gloeosporioides* is known to be highly variable in nature. All twenty six isolates of the present study were identified as *C. gloeosporioides* based on the symptoms on host, substrate, conidial size and shape, shape of appressoria, growth rate in culture, colour of cultures as reported by von Arx (1970) and Sutton (1980). The traditional method of relying on cultural and morphological grounds for identification of *C. gloeosporioides*is variable rarely standardized and tends to mislead, since it is subjective in nature. Molecular genetic studies have provided useful data for clarifying the systematics of the genus *Colletotrichum* (Martinez et al., 2002). The polymerase chain reaction (Mullis and Faloona, 1987) offers the opportunity to characterize fungal symbionts by amplification of specific sequences and provide very accurate quantitative data required for control and quarantine decisions. Hence, the present study was resorted into exploitation of ITS and *Cg*Int derived primers for identification. The ITS region contains two variable non-coding regions that are nested within the rDNA repeat between the highly conserved small subunit, 5.8S and large subunit rRNA genes (Gardes and Bruns, 1993). The internal transcribed spacer regions (ITS1 and ITS4) within the nuclear ribosomal gene clusters are attractive loci of PCR-based detection assays since they are readily accessible using universal primers (White et al., 1990). In the present study, ITS 1 and ITS 4 primers amplified a fragment of 560 bp corresponding to the region of 18S rDNA sequence in all the twenty six isolates and further confirmed to the group of *Colletotrichum.* The results confirmed the findings of Kamle et al. (2013) in amplification of ITS region of *C. gloeosporioides* from mango yielded 560 bp amplicon. Further, identification of ITS regions of nuclear rDNA has been good targets for differentiation and phylogenetic analysis of fungi (Dunne et al., 2002). In the present research, a sensitive PCR-based species-specific designed primer pair of *Cg*Int and ITS 4 was used with the aim to detect *C. gloeosporioides* of mango anthracnose pathogen from the infected fruit tissue, which amplified the DNA at 450 bp in all isolates. These results were supported by the earlier findings for the taxonomic identification of *Colletotrichum* species which have been used to distinguish between *Colletotrichum acutatum*, *C. gloeosporioides* and *Colletotrichum fragariae* (Forster and Adaskaveg, 1999; Urena-Padilla et al., 2002). Similarly, Sakinah et al. (2013) reported that, 38 *Colletotrichum* isolates from banana were identified as *C. gloeosporioides* (35) and *Colletotrichum musae* (3) through the amplification of ITS regions and β-tubulin gene using ITS 4 and 5 and Bt2a and Bt2b primers, respectively. Fungicide resistance is a key factor in limiting the efficacy and disease control strategy. The resistance may also be an important aid to our understanding, at a molecular level, of the fungicidal mechanism of action. In the present study, the sensitivity of *C. gloeosporioides* isolates to benomyl, a benzimidazole fungicide was determined, based on the mycelial growth at five different concentrations viz., 0.5, 1, 2, 5 and 10 mg/l. The twenty six isolates were

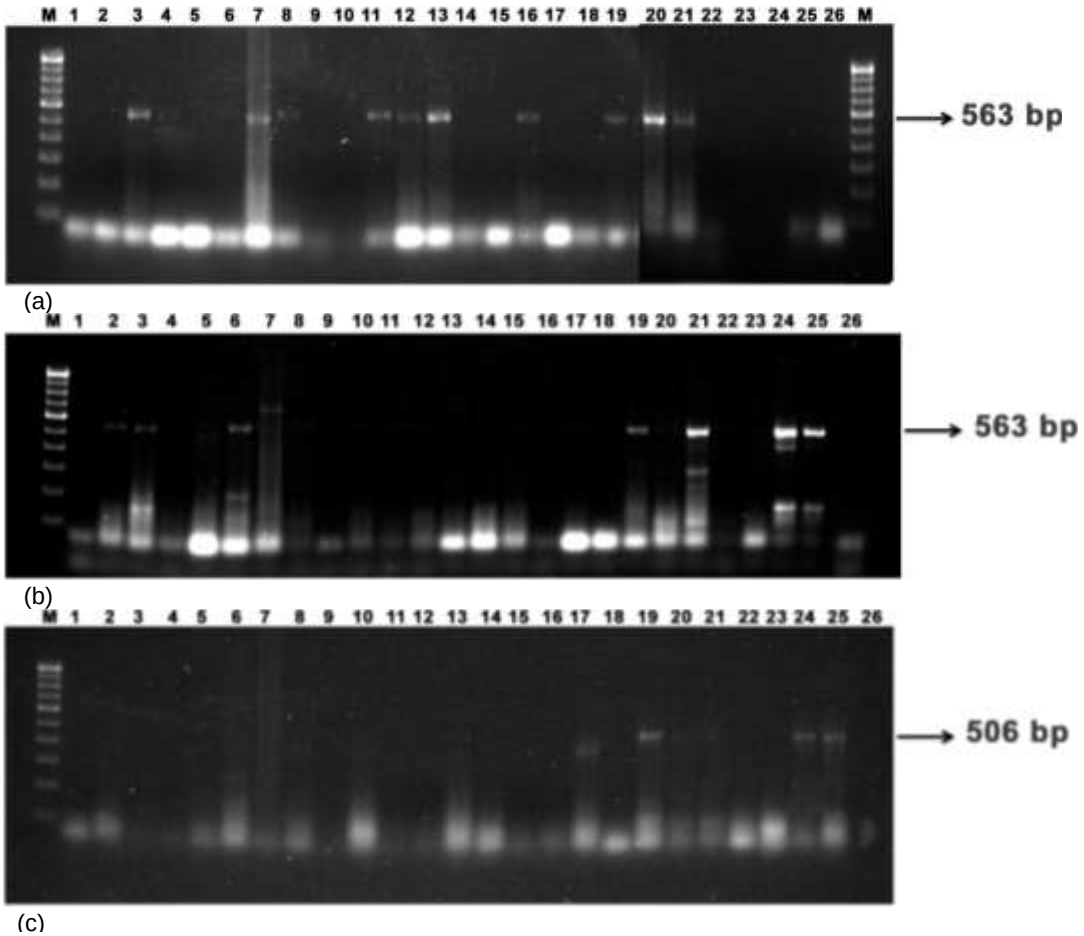

Figure 5. PCR amplification of partial β-tubulin sequence from benomyl resistant and sensitive isolates of *C. gloeosporioides,* (a. β-tubulin gene (*TUB2*) - b*enomyl resistant gene; b. β-tubulin gene (TUB2) - benomyl sensitive gene; c.*β-tubulin gene*TUB1* – benomyl resistant gene).

categorized as three viz., susceptible, moderately resistant and resistant, since significant variation was observed among the isolates in respect to sensitivity. In aqueous solution benomyl is rapidly hydrolysed to methyl benzimidazole – 2 – carbamate and this is probably the active fungi toxicant against pathogen. At the highest concentration of 10 mg/l benomyl, only isolates MCG 7 and MCG 16 were able to grow eleven days after inoculation, which confirmed their sensitiveness of the remaining isolates to benomyl. This might be due to benomyl does not inhibit the spore germination and conidial production but acts primarily by slowing the mycelial growth before or after infection (Peres et al., 2002).

Based on this, isolate MCG 21 was considered as sensitive MCG 5 as moderately resistant and MCG 16 as resistant to benomyl fungicide. This varied reaction obtained in the present study are similar to the findings of Chung et al. (2010), which demonstrated the sensitivity of thirty one *C. gloeosporioides* isolates from mango and strawberry to benzimidazole fungicides viz., benomyl, carbendazim and thiabendazole. In that, they reported that seventeen isolates of *C. gloeosporioides* were grouped as sensitive, two isolates were moderately resistant, nine isolates were resistant and three isolates were classified as highly resistant based on the colony diameter on PDA medium amended with benomyl at 10, 100 and 500 mg/ml concentration. It confirms the earlier reports of Sariah (1989), where the growth of sensitive isolates of *Colletotrichum capsici* were completely inhibited at 2.5 µg/ml benomyl while the resistant isolates grew on agar containing 1000 µg/ml fungicide. Peres et al. (2004) reported that mycelial growth of sensitive isolates was completely inhibited at 1.0 µg/ml of benomyl, whereas resistant isolates grew even at 10 µg/ml of benomyl. Similarly, Joshi et al. (2013) reported that, out of 30 isolates of *C*. *gloeosporioides*, isolate Cg 42 from mandarin orange was found to be insensitive to Benomyl with mean IC50 value of 318.85 µg/ml; while Cg 41 and Cg 43 was highly sensitive to Benomyl. In addition

Nalumpang et al. (2010) reported the sensitivity of *C. gloeosporioides* isolates from mango to carbendazim fungicide at various concentrations of 0.1, 1, 10, 100, 500 and 1,000 mg/l. They found that, among 59 isolates of *C. gloeosporioides*, 49 isolates were highly resistant (HR) phenotypes, 4 isolates were sensitive (S) phenotypes, none showed weakly resistance (WR) and moderately resistance (MR) phenotypes to carbendazim.

Benzimidazole fungicides act by inhibition of tubulin biosynthesis (Davidse, 1973), due to the mutations of β-tubulin gene which has been related to specific amino acid substitutions at several distinct regions within the ß-tubulin molecule (Fujimura et al., 1992). All the twenty six isolates of the current study were used to identify the presence of resistance and susceptible β-tubulin genes. Most often benzimidazole tolerance is due to mutations in the β -tubulin gene which reduce benzimidazole binding (Cooley and Caten, 1993; Reijo et al., 1994). These mutations can be used to rapidly identify tolerant strains with nucleic acid-based methods (Luck and Gillings, 1995).

Further, rapid identification of tolerant strains can aid in determining the fungicide resistance management policies. In the present research, partial β - tubulin gene sequences were amplified by the appropriate primers to the DNA fragment for TUB 2 and TUB 1 gene. Isolates MCG 3, MCG 7, MCG 8, MCG 11, MCG 12, MCG 13, MCG 16, MCG 19, MCG 20, MCG 21 had showed amplification product at 563 bp for benomyl resistant gene while the isolates of MCG 2, MCG 3, MCG 6, MCG 19, MCG 21, MCG 24, MCG 25 produced amplification for the primers of β-tubulin sensitive gene. In addition, alternative gene TUB 1 β-tubulin was amplified from four isolates viz., MCG 19, MCG 20, MCG 24 and MCG 25 at 506 bp. Peres et al. (2004) found that eight isolates of *C. gloeosporioides* were amplified by the portion of β – tubulin gene, with primer TB2R and TB2L.

Resistance to benzimidazole fungicides has been reported in many filamentous fungi, whereby the fungicide is unable to bind to a mutated β-tubulin protein containing an altered amino acid sequence (Davidse and Flach, 1977). Several studies have demonstrated that sequence analysis of the β-tubulin gene of benomyl-resistant fungi revealed that, resistance almost always is due to point mutations in codon 198 or 200 of the β-tubulin gene. Amino acid substitutions at these two codon positions result in different benomyl-resistance phenotypes (Albertini et al., 1999; Yarden and Katan, 1993).

Conclusion

The study reveals that a rapid benomyl tolerance screening could be necessary for examining the potential of different fungicide resistance. Pathogen *C. gloeosporioides* responsible for anthracnose in mango is composed of both benomyl-resistant and sensitive populations that are genetically distinct, which might be due to a single β-tubulin gene existing within the fungal genome; however, in others, an additional gene may be present that is divergent in sequence.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sero-prevalence of hepatitis E virus (HEV) genotype 3 in goats from Sokoto Metropolis, Nigeria

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Hepatitis E virus (HEV) is a cause for public health concern in many developing countries where sanitation conditions are poor. Increasing attention has been focused on the zoonotic nature of HEV. Goats, along with other species of small ruminants have been reported to be susceptible to HEV infection. This study was designed to determine the infection rate of HEV3 by detecting antibodies against capsid proteins of HEV genotype3 in goats from Sokoto using indirect enzyme linked immunosorbent assay (ELISA). An overall infection rate of HEV3 27.84% (49/176) was recorded in the study. Goats, one year and above, had a prevalence of 32.56% (42/129) while those below the age of one year showed a prevalence of 14.89% (7/47). Their was a statistical significant association beween age and HEV3 infection (P-value<0.05). However, the infection was not significantly associated with breed and sex (P-value>0.05). It was concluded that goats from Sokoto are exposed to HEV3 infection. Goats older than one year appeared to be more exposed. It is suggested to study other domestic animals to determine the magnitude of HEV infection as well as carry out detailed molecular characterization of the virus to evaluate the epidemiological pattern of the virus infection in all domestic animals in the area of this study.

Key words: Hepatitis E virus 3 (HEV3), enzyme linked immunosorbent assay, sero-prevalence, goats, Sokoto metropolis, Nigeria.

INTRODUCTION

Hepatitis E is a disease caused by infection with hepatitis E virus (HEV), an RNA virus that exists in both enveloped and non-enveloped forms and was first recognized in the early 1980s. The virus is a member of the Hepeviridae family and the genus *Orthohepevirus*. At least 4 mammalian genotypes (1 to 4) belonging to a single serotype are recognized by Perez et al. (2015). Genotypes 1 and 2 are found only in humans, whereas genotypes 3 and 4 have also been found in several mammalian species. The virus is relatively stable in the environment and is sensitive to heat, chlorination, and ultraviolet light (Perez et al., 2015).The viral genome contains three non-overlapping open reading frames (ORF 1-3) (Di-martino et al., 2017). Open reading frame

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1 codes for the non-structural protein like helicase, ORF2 codes for the viral capsid protein which is the target of neutralizing antibodies against HEV. ORF3 codes for the multifunctional phosphoproteins that modulate cellular activities.

Clinical features of hepatitis E are indistinguishable from acute hepatitis caused by other hepatotropic viruses. The incubation period ranges from 15 to 60 days, with a mean of 40 days. Hepatitis E virus infected persons exhibit a wide clinical spectrum, ranging from asymptomatic infection through acute hepatitis to fulminant hepatitis (Kamar et al., 2008). Certain population sub-groups are at a higher risk for severe disease following HEV infection. This includes pregnant women, persons with pre-existing liver disease and persons with immunosuppression (Teshale et al., 2010).

Several studies have shown that pigs, sheep and goats amongst others can serve as reservoir hosts for HEV infection all over the world (Perez et al., 2015; Di Martino et al., 2016; Long et al., 2017). Antibodies against HEV genotype 3 has been detected in domestic animals all over the world. In pigs from Brazil (Santos et al., 2009), in sheep and goats from Egypt (El-Tras et al., 2013), in cows from China (Huang et al., 2016), in goats from the United States (Sanford et al., 2013), and sheep from China (Wu et al., 2015).

Recent studies have shown that there is evidence of HEV infection in Nigeria. Alkali et al. (2015), studied the prevalence of HEV genotype 3 in pigs from Zaria, Kaduna State, Nigeria and recorded a prevalence of 24.4%. In another study carried out by Junaidu et al. (2014) in domestic animals (pigs, goats and sheep) from Jos, Plateau State a prevalence rate of 24.1% was recorded. In Cross-River State, Nigeria, a sero-prevalence rate of 7.7% was recorded in children by Ekanem et al. (2015).

The evidence of non-A and non-B hepatitis in humans is of increasing concern (Goens et al., 2004). Very little information to the best of the authors knowlege is available on the sero-prevalence of HEV3 infection in the study area.

Determining the existence of HEV3 infection in goats which is a major source of meat in the study area, will provide baseline information for further studies. The findings from this study will hopefully assist policy makers and veterinary health authorities in devising effective and applicable control measures in the study area.

This study was designed to determine the infection rate of HEV genotype 3 in goats and to determine the association of this infection with sex, age and breed.

MATERIALS AND METHODS

Epidemiological study was conducted from Jauary 2017 to March 2017 in Sokoto metropolis area in the Sokoto State as shown in Figure 1. Systematic random sampling technique was used in this study.

Samples were drawn from each fifth animal with the first animal also being chosen at random. According to Ngere et al. (1984), there are three breeds of goat in Nigeria: Sahel (Sh), Red Sokoto (RS), and West African Dwarf (WAD) of which two of these breeds were sampled in this study. Blood samples were collected from goats brought to the State Veterinary Clinic, Aliyu Jodi Road and from backyard farms and within the metropolis. After proper restrain, 5 ml of blood was collected from the jugular vein of each animal sampled using a 5 ml syringe and transferred into a sterile plain vacutainer test tube and transported in an ice pack to Usumanu Danfodiyo University, City Campus Central Research Laboratory, Sokoto. The samples were kept on the bench for 60 min to allow for coagulation and seperated with a centrifuge at 3000 rpm for 10 min. The supernatants (sera) were carefully decanted into a 1 ml serum vial and preserved at -20°C until used.

Indirect enzyme linked immunosorbent assay (ELISA) kit from Innovative Diagnostic Vet© Montpellier, France was used to detect anti-HEV genotype 3 capsid protein antibodies in serum and plasma of goats and other species. Samples and controls were deposited in duplicate. One hundred and ninety microliters of sample dilution buffer was added to each well. Ten microliter of negative control was added to wells A1, A2, B1 and B2. Ten microliters of positive control to wells C1, C2, D1 and D2. Ten microliters of each sample was transferred to the remaining wells. Micro-plates were incubated for 45 min at room temperature (26°C). Wells were emptied by inversion and three cycles of manual washing was carried out. One hundred microliters of multispecies conjugate (1X) was added to each well. The plates were incubated for about 30 min at room temperature (26°C). Micro-wells were emptied and each well was washed with 300 µl of wash solution. One hundred microliters of substrate solution was added to each well, incubated for 15 min at room temperature (26°C). The reaction was stopped by adding 100 µl of stop solution (H_2SO_4) to each well. The absorbance of each sample was measured using a 96 wells spectrophotometer at a wavelength of 450 nm. The test was validated as recommended by the manufacturer and percentage sample to positive ratio (S/P) was calculated. Samples presenting S/P ratio of greater than 70% was considered positive, between 60 and 70% was considered doubtful and less than 60% was considered negative.

Sample size determination

The sample size was determined using a previous prevalence 10.5% (Junaidu et al., 2014) at 95% confidence interval, with desired precision of 5%, using the formula (Thrutfield, 2005):

$$n = z^2 x\ p^{exp}(1-p^{exp}) / d^2$$

where n = sample size, z = score for a given interval which is 1.96 (SE) at 95% confidence interval, p^{exp} = prevalence at 10.5%, and d^2 = precision at 0.05 (Junaidu et al., 2012).

$$n = (1.96)^2 \times 0.094 \times (1\text{-}0.094) / (0.05)^2$$

$$n = 0.26711 / 0.0025 = 106.84$$

Thus, n = 106.84 Sample size was adjusted to 176 to improve the chance of detection.

Data analysis

The results were recorded uing microsoft excel, presented in a table format, and Chi square test was used to test for association between sero-prevalence and epidemiological variables such as

Figure 1. Sokoto metropolis on the map is located at latitude 13/0514 (133'5.040"N) and longitude 5,2314 (513'53.004"E).
Source: http://www.yolasite.com.

Table 1. Sex distribution of HEV genotype3 infection in goats in Sokoto metropolis.

Sex	Tested number	Positive number (%)	Negative numbr (%)	$\chi 2$	p-value
Male	96	31 (32.29)	65 (67.71)		
Female	80	18 (22.5)	62 (77.5)	1.62	0.202
Total	176	49 (27.84)	127 (72.16)		

health status, breed, age and sex at 95% CI. The analysis was done using SPSS version 21.0 considering probability value <0.05 as significant.

RESULTS

According to the ELISA results, the infection rate of HEV genotype 3 in goats was 27.84% (49/176; Table 1).

The sex distribution of HEV genotype 3 antibodies in goats showed that the males have a higher infection rate 2.29% (31/96) than females 22.5% (18/80) as shown in Table 1. Chi square analysis showed no statistical significant association between HEV3 infection in goats and sex (p-value > 0.05).

The relation between age and HEV3 showed that goats, one year or above have a higher infection rate of 32.56% (42/129), than the younger goats 14.89% (7/47) as shown in Table 2. Chi square analysis shows statistical significant association between HEV3 infection and age in goats (p-value< 0.05).

The results in Table 3 show that Red Sokoto goats had a higher number of positivity of 33.33% (27/81) than Sahel goats 28.4% (22/95). Chi square analysis did not show any statistical significant association between HEV

Table 2. Age distribution of HEV genotype3 infection in goats in Sokoto metropolis.

Age (year)	Tested number	Positive number (%)	Negative numbr (%)	$\chi 2$	p-value
≥1	129	42 (32.56)	87 (67.44)		
<1	47	7 (14.89)	40 (85.11)	4.51	0.0337
Total	176	49 (27.84)	127 (72.16)		

Table 3. Frequency of HEV genotype3 antibodies in Red Sokoto goats and Sahel goats in Sokoto metropolis.

Breed	Tested number	Positive Number (%)	Negative numbr (%)	$\chi 2$	p-value
Red Sokoto	81	27 (33.33)	56 (66.67)		
Sahel	95	22 (28.4)	73 (71.6)	1.78	0.1827
Total	176	49 (27.84)	129 (72.16)		

infection and Breed (p-value >0.05).

DISCUSSION

HEV is a cause for public health concern in many developing countries where sanitation conditions are poor and waterborne epidemics are frequent. Both epidemic and sporadic forms of HEV infections exist in developing and under-developed countries. Pigs are the principal reservoir of HEV. However, goats have also been reported to be highly seropositive for anti-HEV IgG (Perez et al., 2015).

Here, the concentration was particularly on the food habits of the local inhabitants of Sokoto State, of whom the majority are Hausa-Fulani and the continuous contact of the inhabitants within the study area with goats. Goat meats and other small ruminants are common in their diet.

In this study, the infection rate of HEV3 was 28.7% and this percentage corroborates with results of several studies done in China such as Geng et al. (2014) and Zhang et al. (2010) who found 28.2 and 24% infection rate, respectively and lower than that reported in United States 16% (Sanford et al., 2013) and in Egypt 9.4% (El-Tras et al., 2013). A higher prevalence of 37.2% has been recorded in Plateau State, Nigeria (Junaid et al., 2014).

The findings in this study showed that male goats had a higher infection rate 31 (32.9%) and female had a lower number of positive samples of 18 (22.5%) and not statistically significant. This validates the findings of Sarah et al. (2013) who found no significant association between HEV infection and sex in pigs. However, our results are not in accordance with the findings of Alkali et al. (2015) who observed a significant statistical association between sex and HEV infection (p<0.05) in pigs.

Nevertheless, in order to fully comprehend the role of sex in the epidemiology of the disease, further investigations involving a larger population of goats are needed.

Regarding the age, the results of the present study show that goats, one year or older have a higher infection rate 32.56% (42/129), than younger goats 14.89% (7/47). Chi square analysis shows statistical significant association between HEV infection and age in goats (p-value<0.05). The outcome may be because sero-prevalence may increase with age as a consequence of repeated contact with the virus. The comparison made between breeds of goats showed that Red Sokoto goats had a higher prevalence of 33.33% (27/81) while Sahel goats had lower prevalence of 28.4% (22/95). Chi square analysis did not show any statistical significant association between HEV infection and breed (p-value >0.05). The difference observed between the breed groups in sheep and goats might be due to the sampling ratio. HEV genotype 3 infection is influenced by sex and sero-prevalence increases with age.

Goats may act as source of exposure for HEV infection in the study area, thus veterinarians and other individuals with extensive contact with goats may be at risk of being infected with HEV. Breed had no consequence on the prevalence of HEV infection. This outcome simply implies that all the breed tested in this study are susceptible to HEV3 infection.

The results show the need for the use of a more sensitive and specific technique like PCR to clarify the distinct pattern of HEV3 distribution and the extent of cross-species transmission. Better information to the public by veterinary health authorities will be required to help prevent current and future endemics. Good hygiene practices among people with extensive contact with goats might help to prevent future occurrence of the infection.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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