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Antibacterial Potential of Fungal Endophytes Isolated from *Carica papaya*

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ABSTRACT

Background: Drug resistant bacteria related health problems are major concern globally and search for newer and most effective antibacterial agents is the urgent need to combat with these challenges. The study was undertaken with the aim of isolating and identifying endophytic fungi associated with *Carica papaya* and assessing their potential as antibacterial agents.

Methods: *Carica papaya* plants were collected from different locations and endophytic fungi were isolated and characterized phenotypically and genotypically by microscopy, colonial characteristics and ITS gene sequencing, respectively. Antibacterial activity of endophyte was assessed against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella* Typhimurium, *Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*, using the agar plate diffusion assay method with Ciprofloxacin as a positive control.

Result: Thirty four (34) fungal endophytes of two genera *Fusarium* and *Penicillium* were recovered from 60 samples of *Carica papaya* and they showed the antibacterial activity against *Staphylococcus aureus*. Thus, the endophytic fungi have the potential to be used as an antibacterial agent.

Key words: Antibacterial agents, Carica papaya, Endophyte, Fungi, Fusarium, Penicillium.

INTRODUCTION

Drug resistant bacteria related health problems are major concern in the modern world and search for newer and most effective antibacterial agents are the urgent need tocombat with these challenges (Singh *et al.,* 2017). There has been a tremendous increase in interestin screening endophytes for their antimicrobial activities.

Endophytes are the bacterial and fungal microorganisms that live inside the living plant tissues for at least part of their life without causing any apparent disease symptoms in the host (Christina *et al.,* 2013). They are ubiquitous, colonize in all plants and recognized as one of the most chemically promoting groups of microorganisms in terms of diversity and pharmaceutical potential (Thaslimmunisha *et al.,* 2016). The plantendophyte interaction is a mutual relationship (Ginting *et al.,* 2013), with plants offering residence, nutrients and protection to the endophytes; whilst endophytes provide several benefits to improve growth and health of their plant hosts (Pereira *et al.,* 2016, Morare *et al.,* 2018). Metabolites produced by endophytes have shown various biological activities such as anti-microbial, anti-cancer, anti-oxidant and anti-diabetic (Nair and Padmavathy, 2014, Morare *et al.,* 2018). Endophytic fungi are a good source of natural products that have been observed to kill a wide variety of harmful disease causing agents (Sandhu *et al.,* 2014). Medicinal plants and their fungal endophytic communities produce similar pharmaceutical products (Prabukumar *et al.,* 2015).

Papaya (*Carica papaya*) is a fruit crop widely grown in tropical and subtropical environments. Traditionally, the plant is used for the treatment of a wide range of ailments like wounds, ulcers, dyspepsia, diarrhea, bleeding, haemorrhoids, whooping cough, dysentery, skin diseases (Saeed *et al.,* 2014, Krishnan *et al.,* 2012). Lot of information on the nutritive importance and antimicrobial properties of papaya fruit are available. However, the endophytes associated with papaya plant are yet to be elucidated and functionally characterized. Papaya, a sugar crop, possessing many saccharides like glucose, fructose and sucrose, harbours numerous endophytes and and their study can provide important clues about the characteristic medicinal features (Krishnan *et al.,* 2012). Many of the recent researches are pointing towards identifying novel natural pharmaceutically active compounds from various plant sources. The fungal endophytes are also helpful in controlling antimicrobial resistance. Any breakthrough in this direction will be a significant achievement in fight against bacterial diseases of human and animals. To best of our knowledge very scanty database is available with respect to characterization of

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endophytic bacteria from papaya plants. Therefore, the present study was undertaken with the aim of isolating and identifying endophytic fungi associated with *Carica papaya* and assessing their potential as antibacterial agents.

MATERIALS AND METHODS

The experiment was conducted in the year 2018-19 in the Department of Veterinary Microbiology, College of Veterinary Science and A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India.

Ethical approval

The research work was conducted as approved by the College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, India.

Collection of plant materials

A total of 60 samples consisting 20 samples each from leaves, roots and shoots tip of *Carica papaya* were collected from four different locations of Jabalpur (Table 1) and immediately transported to Laboratory of Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Jabalpur, India.

Isolation and identification of endophytic fungi

The disinfection of roots, leaves and shoots tip of *Carica papaya* plants and isolation of endophytic fungi from the collected samples was done according to Mahajan *et al.* (2014) with some modifications. The isolated fungal endophytes were identified on the basis of morphological features like colony characteristics, growth of fungi, colour of colony (front and reverse) on SDA plates (Promputtha *et al.,* 2005) and their microscopic appearance like shape of conidia. The microscopic identification of fungal endophytes was carried out by lactophenol cotton blue staining and visualization under high power lens using light microscope.

Molecular identification of endophytic fungi and phylogenetic analysis

Isolation of DNA was performed as per the protocol described by Gul *et al.* (2017). DNA was dissolved in nuclease free water and preserved in -20ºC until further use. Molecular characterization of endophytic fungi was performed using universal primers (ITS1: 5'- TCCGTAGG TGAACCTGCGG -3' and ITS4: 5'- TCCTCCGCTTATTGAT ATGC -3') of internal transcribed spacer ribosomal DNA (ITS rDNA) sequences (Sushma *et al.,* 2018). The PCR products were tested for amplification of gene by agarose gel electrophoresis using 1% agarose gel. The amplified DNA products from endophytic fungi was sent for sequencing at Triyat Genomics, Nagpur, India. Endophytic fungi were identified by comparing similarity between ITS rDNA sequences with sequences available in (NCBI) GenBank database using BLAST tool.

Antibacterial activity of endophytic fungi

The agar plate diffusion assay method as described by Nwakanma *et al*. (2016) The modified agar plug method described by Devaraju and Satish (2011) was used to evaluate screen the antibacterial activity of the fungal endophytes with some modifications against six bacteria *viz. Escherichia coli* (ATCC25922), *Klebsiella pneumonia* (ATCC700603), *Salmonella* Typhimurium (ATCC13311), *Bacillus cereus* (ATCC11778), *Staphylococcus aureus* (ATCC6538), *Streptococcus pyogenes* (ATCC12386). Briefly the Muller-Hinton agar (MHA) was inoculated with 100µl of the bacterial suspension and spread uniformly. The mycelial discs (6 mm) of each endophytic fungal isolate (15 days old) grown on sabouraud dextrose agar (SDA) were obtained from actively growing margins using a sterile cork borer and placed on the surface of the MHA medium previously seeded with test organisms. The plates were sealed using para film and incubated at 37°C for 24hours. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition in millimeters and compared with ciprofloxacin as positive and distilled water as negative control.

Determination of minimum inhibitory concentration (MIC) for endophytic fungi

Minimum inhibitory concentration (MIC) was determined using broth dilution method as per the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2006). The fungal plates were incubated at 25-28°C for 42 hours and were visually observed for the presence or absence of turbidity. The minimum concentration of the compound showing no turbidity was recorded as MIC.

RESULTS AND DISCUSSION

Fungi are among the most important groups of eukaryotic organisms they are well known for producing many novel metabolites which are directly used as drugs or function as lead structures for synthetic modifications. The advent of endophytes has shifted the focus of drug discovery from plants to microorganisms.

Isolation of fungal endophytes

In present study the disinfection of *Carica papaya* leaves, roots and shoot tipsurface was adequate as all control plates did not have any microbial growth. While, a total of 34

Table 1: Sample collection from *Carica papaya* plants.

Places	Leaves	Roots	Shoots tip	Total
Department of Botany, Jawaharlal Nehru KrishiVishwaVidyalaya, Jabalpur	05	05	05	15
State Forest Research Institute, Jabalpur	05	05	05	15
Tropical Forest Research Institute, Jabalpur	05	05	05	15
College of Veterinary Science and Animal Husbandry, Jabalpur	05	05	05	15
Total	**20**	**20**	**20**	**60**

endophytic fungi were recovered from the *Carica papaya* leaves (Table 2), roots (Table 3) and shoot tips (Table 4). Isolates of one group of endophytic fungi had sickle shaped conidia and formed dark brown colored colony from front view and yellowish colour at reverse view of petridish. Isolates of second group formed filamentous velvety green colony from front view and white colour colony at reverse view of petridish and microscopically unbranching chain of round conidia were observed. Phenotypically first isolate was characterized as *Fusarium* species whereas second isolate was characterized as *Penicillium* species. Mahajan *et al.* (2014) conducted a study to isolate and characterize endophytes from plants on kings B media. During the study fungal species were isolated and characterized on the basis of various morphological and biochemical assays. The variation in morphological characteristics of fungi may be due to variation in host of endophytic fungi and the endophytic fungi which was isolated. Since the papaya plants used in this study appeared healthy and *Fusarium spp* and *Penicillium spp* are known to be pathogens, it appears that these fungal isolates are avirulent, hypovirulent, or virulent but in a latent phase, causing no harm to papaya plant. Sushma *et al.* (2018) screened four plants with medicinal properties *Carica papaya*, *Phyllanthus amarus*, *Tinospora cordifolia* and *Azadiracta indica* for the isolation of endophytic fungi. The leaves of these plants revealed two, four, six and three isolates of endophytic fungi belonging to the family Hypomycetes, Ascomycetes, Dothideomycetes, Coleomycetes, respectively. The roots, leaves and shoots tip of *Carica papaya* was chosen for isolation of fungal endophytes. However, only two fungal endophytes were isolated in this study, providing limited diversity of fungal endophytes as compared to other similar studies (Tenguria and Khan, 2015, Jariwala *et al.,* 2018). This may be due to the temperature, rainfall and atmospheric humidity (Selvanathan *et al.,* 2011), plant used, age, seasonal collection the isolation method (Jasim *et al.*, 2014) media composition (Costa *et al.,* 2002, Kumar *et al.,* 2015) and culture conditions used (Kumar *et al.,* 2015). Huang *et al.* (2007) indicated that isolation method whereby plant material is cut intosmall pieces and placed on growth medium yield highly numerous endophytes.

Molecular characterization and phylogenetic analysis

The amplified PCR products of the bacterial isolates were run on agarose gel electrophoresis. The amplified DNA fragments with 700bp were observed (Fig 1). Endophytic fungi were identified by comparing similarity between ITS gene sequences with sequences available In NCBI GenBank database using BLAST tool. Based on ITS sequence

Table 2: Morphological characteristic of endophytic fungi isolated from leaves of *Carica papaya* in Sabouraud dextrose agar.

Isolates	Macroscopic view		Microscopic	Fungal type
	Front	Reverse		
VFL1	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
VFL3	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
VFL4	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
VFL5	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFL6	Filamentous velvety green	White	Conidia round, unbranch chains	*Penicillium spp*
TFL7	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFL8	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
TFL10	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
JFL11	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
JFL12	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFL13	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
JFL15	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*

Table 3: Morphological characteristic of endophytic fungi isolated from roots of *Carica papaya* plant in Sabouraud dextrose agar.

Isolates	Macroscopic view		Microscopic	Fungal type
	Front	Reverse		
VFR1	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
VFR2	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
VFR5	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
TFR6	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFR8	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFR9	Filamentous velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
TFR10	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFR11	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFR12	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFR13	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*

analysis 20 endophytic fungiisolates were classified as *Fusarium*species (Fig 2) and 14 isolates were classified as *Penicillium* species.

Antibacterial activity of endophytic fungi

Among 34 isolated endophytic fungi only 20 isolates belonged to *Fusarium* species showed antibacterial activity (Table 5). Isolates of endophytic fungi *Fusarium* species were effective against *Staphylococcus aureus*. None of the endophytic fungal isolates showed antibacterial activity against *Escherichia coli, Streptococcus pyogenes*, *Salmonella* Typhimurium and *Klebsiella pneumoniae*. Present study results were similar with the findings of Eze *et al.* (2019). They investigated the antibacterial activity of secondary metabolites of endophytic fungi isolated from leaves of *Carica papaya*. In the antimicrobial assay, the extracts PPL-LE2 displayed mild antibacterial activity against both Gram negative and Gram positive test bacteria. PPL-LAC extract showed mild activity only against *Staphylococcus aureus*. Likewise, Phongpaichit *et al.* (2006) screened the antimicrobial activity in endophytic fungi of five Garcinia plants. Isolates displayed antimicrobial activity against at least one pathogenic microorganism, such as *Staphylococcus aureus*, a clinical isolate of methicillin-resistant *Staphylococcusaureus*, *Candida albicans* and *Cryptococcus neoformans*. Similarly, Romasi *et al.* (2011) reported that the extracts of papaya leaves could inhibit the growth of pathogenic bacteria. Their findings strongly support that the endophyte from papaya can be rich source with antimicrobial properties.

In the present study *Carica papaya* plant fungal endophyte showed antibacterial activity against *Staphylococcus aureus* suggests that metabolites of endophytic fungi might had diffused in the culture medium and suppressed the growth of pathogenic bacteria. The

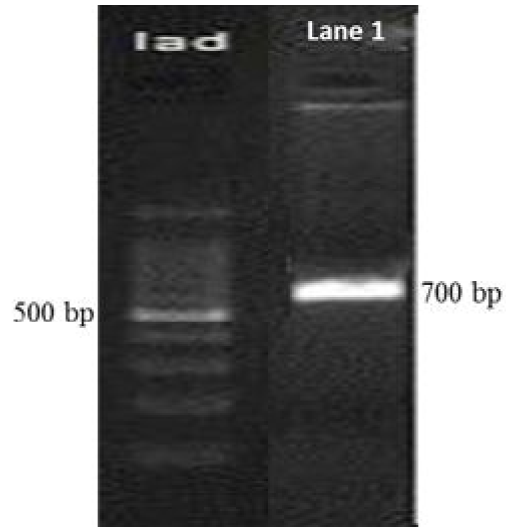

Fig 1: Agarose gel electrophoresis of ITS gene PCR product of endophytic fungi.
Lad: 100 bp ladder, **Lane 1:** 700 bp PCR product.

Table 4: Morphological characteristic of endophytic fungi isolated from shoots tip *Carica papaya* in Sabouraud dextrose agar.

Isolates	Macroscopic view		microscopic	Fungal type
	Front	Reverse		
VFS1	Filamentous, velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
VFS4	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
VFS5	Filamentous, velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
TFS6	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFS7	Filamentous, velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
TFS8	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
TFS10	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFS11	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFS12	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFS13	Dark brown	Yellow	Macroconidia sickle shaped	*Fusarium spp*
JFS14	Filamentous, velvety green	White	Conidia round, unbranching chains	*Penicillium spp*
JFS15	Filamentous, velvety green	White	Conidia round, unbranching chains	*Penicillium spp*

Table 5: *In vitro* antibacterial activity of endophytic fungi isolated from *Carica papaya* plant.

Bacteria	Leaves	Roots	Shoots tip	Total
Staphylococcus aureus	06	07	07	20
Streptococcus pyogenes	00	00	00	00
Bacillus cereus	00	00	00	00
Salmonella Typhimurium	00	00	00	00
Klebsiella pneumonia	00	00	00	00
E. coli	00	00	00	00
	Total	**06**	**07**	**07 20**

Table 6: Minimum inhibitory concentration (MIC) of endophytic fungi against pathogenic bacteria.

Isolates	Fungal type	Zone of inhibition (mm)	MIC (mg/ml)
		Staphylococcus aureus	
VFL1	*Fusarium spp*	17 mm	64
VFL3	*Fusarium spp*	12 mm	128
VFL4	*Fusarium spp*	10 mm	256
VFL5	*Fusarium spp*	14 mm	64
TFL7	*Fusarium spp*	13 mm	128
JFL12	*Fusarium spp*	12 mm	128
VFR1	*Penicillium spp*	11 mm	128
VFR5	*Penicillium spp*	16 mm	64
TFR6	*Fusarium spp*	19 mm	64
TFR9	*Penicillium spp*	13 mm	128
TFR10	*Fusarium spp*	10 mm	256
JFR11	*Fusarium spp*	12 mm	128
JFR12	*Fusarium spp*	15 mm	64
VFS4	*Fusarium spp*	11 mm	128
TFS6	*Fusarium spp*	13 mm	128
TFS8	*Fusarium spp*	17 mm	64
TFS10	*Fusarium spp*	12 mm	128
JFS11	*Fusarium spp*	13 mm	128
JFS12	*Fusarium spp*	15 mm	64
JFS13	*Fusarium spp*	10 mm	256

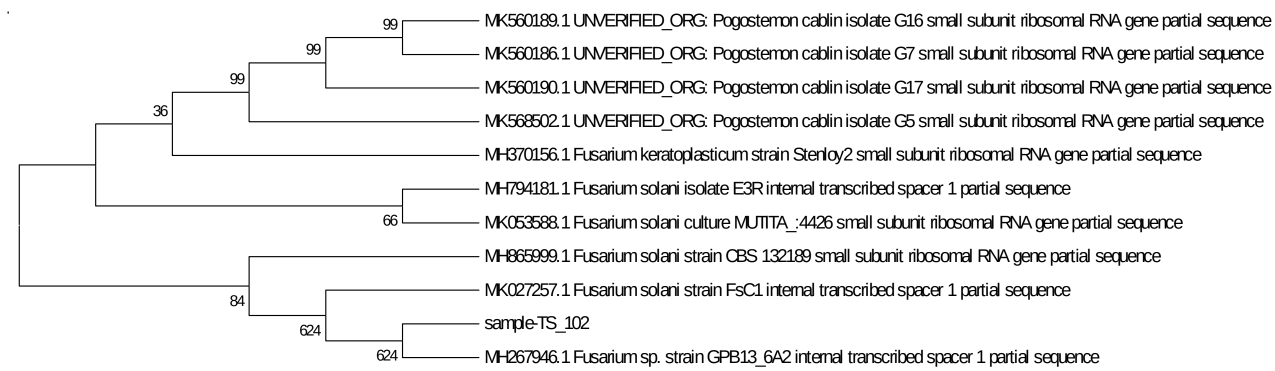

Fig 2: Construction of phylogenetic tree of isolated endophytic fungi.

bioactive compound could easily move into the bacterial cell membrane via the general bacterial porins, which might be responsible for several metabolic functions of the cell or they may form various pores in the outer cell membrane of bacteria, thus resulting in the leakage of internal substances to the outside, causing cell lysis and death (Islam *et al.,* 2018).

The endophytic fungi which showed antibacterial activity, their minimum inhibitory concentration (MIC) was determined. The antibacterial activity of endophytic fungi *Fusariumspp.*and *Penicillium spp*. was observed against *Staphylococcusaureus* and their minimum inhibitory concentration (MIC) ranged between64 to 128 mg/ml (Table 6).

CONCLUSION

In the present study, two endophytic fungi *Fusarium spp* and *Penicillium spp* were isolated from the leaves, roots and shoots tip of *Carica papaya* plant. Molecular characterization based on ITS1 and ITS4 gene sequence analysis, the endophytic fungus present was confirmed as *Fusarium spp* and *Penicillium spp*. Endophytic fungi *Fusarium spp* and *Penicillium spp*. possess the antibacterial activity against *Staphylococcus aureus*. Thus, study suggests that endophytic fungi isolated from leaves, roots and shoots tip of *Carica papaya* have the potential to be used as antibacterial agents.

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