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Bioactivities of Collet Otrichumgloeosporioides-An Endophyte of Justicia adhatoda.

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ABSTRACT

This study focused on the isolation of the endophytic fungi from *Justicia adhatoda* which has ability to be used in bioremediation of leachate. Two endophytic fungi were recorded from this medicinal plant. In this investigation both of them were tested for their bioremediation ability to degrade leachate. They showed ability to grow on 50% and 100% leachate incorporated with Malt Extract Agar. The radial growth was measured every 48hours for a period of nine days after incubation at 30^0Cand growth rates were determined. Among them one endophyte showed active positive result. We identified this fungus using morphological characteristics as well as on the basis of CgInt primer(5'–GACCCTCCCGGCCTCCCGCC–3') and universal primer ITS4(Internal Transcribed Spacer4)(5'–TCCTCCGCTTATTGATATTGC–3'). The identified fungus, *Colletotrichumgloeosporioides* was tested for its ability to produce extracellular enzymes such as amylase, protease and lipase by the qualitative assays and showed positive results.

Key words: endophytic fungi, leachate, bioremediation, *Colletotrichumgloeosporioides*

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INTRODUCTION

Endophytes are microorganisms, which inhabit in healthy living tissues for all or part of their cycle without causing apparent harmful symptoms to the host [1]. They have attracted great attention in the past few decades due to its ability to produce novel secondary metabolites for medical, agricultural and industrial use. They are also considered as an outstanding source of bioactive compounds due to its ability to occupy any plants at any environments [2]. Endophytic fungi from medicinal plants could be a rich source of functional metabolites [3].

Landfill is one of the main methods for disposing of municipal and industrial solid waste. The degrading of the organic fraction of the waste in the landfill in combination with the percolation of rain water produces a polluted liquid called leachate. Leachate emitted by the landfill is likely to contain various types of pollutants. [4].

The following study was designed and carried out to achieve objectives, (1) isolation of endophytic fungi from a medicinal plant, *Justicia adhatoda*, (2)to find out Percentage of density of colonization (colonization frequency) and Percentage of Endophytic Infection Rate (3)to detect the bioremediation activity of the endophyteson leachate, (4) to identify these endophytesmorphologically and molecularly and (5)to assay the extracellular enzymes like amylase, protease and lipase production by the endophytes.

MATERIAL AND METHODS

Collection of Host Plant Material

Healthy, disease free leaves of mature *Justicia adhatoda* plants were collected from laterite soils of Central Kerala, India. The specimen was authenticated by Dr. V T Antony, Head, Department of Botany, St. Berchmans College, Changanacherry and a voucher specimen was deposited at Regional Herbarium Kerala with field no. RHK 6301 and accession no. 7564.

Isolation of Endophytes

Isolation of endophytes was done after removing epiphytes from leaf surface using water and sterilants. Leaves were washed in running tap water, and then rinsed in 70% ethanol for 1 min, 0.1% mercuric chloride for 3 min. A final wash was given with sterile distilled water for four times. Leaves were cut into small pieces about 5-10cm in length (tip region, distal region with or without midrib, basal and petiole segment were taken). Then the leaf pieces were blotted on sterile blotting paper [5].

Inoculation and Incubation

The leaves were placed on Sabouraud's dextrose agar (SDA, Hi Media Laboratory Ltd)plates supplemented with streptomycin sulphate (300mg/L).Imprints of the surface

sterilized leaf pieces were taken on SDA and Potato dextrose agar (PDA, Hi Media Laboratory Ltd)plates to check the efficiency of surface sterilization. The plates were sealed and incubated at 27^{o} C and they were observed every day for fungal growth.

Identification of fungal endophytes

Fungal endophytes were identified according to their macroscopic and microscopic characteristics.Colonization frequency ofendophytic fungal isolates and Percentage of Endophytic Infection Rate (EIR) [1] were analyzed.

Colonization Frequency

$$CF\ (\%) = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total number of segments analyzed}} \times 100$$

Endophytic Infection Rate (EIR %)

$$EIR\ (\%) = \frac{\text{Number of infected segments}}{\text{Total number of segments screened}} \times 100$$

Bioremediation effect of endophytes on leachate

Raw leachate was collected from the landfills areas in a sterile bottle. A piece of SDA with endophytic fungal culture was then placed on the centre of each Malt Extract Agar(MEA, Hi Media Laboratory Ltd)plate which contains 50% and 100% leachate. After inoculation plates were incubated at 25^{o} C for obtaining fungal growth rate data for each of the fungal isolate. Then the colony radius from the edge of the SDA piece was measured every 48 hours upto nine days to get the fungal growth rate for each fungal isolate in the different treatments. For each isolate three replicates trials were conducted [4].

Statistical Analysis

Data were expressed as mean± SD for three replicates. Statistical analysis of biodegradation activity of isolated endophytes were done by ANOVA using statistical package, INSTAT and mean were compared by Turkey- Kramer multiple comparison test.

Analysis of enzymatic activity of the isolated endophytes

The fungal endophytes were analyzed for amylase, lipase and protease enzyme activities. Endophytes were inoculated on media supplemented with appropriate substrates and incubated for 3-5 days at room temperature and the results were determined by clear zone formed around the fungal colony [1].

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Molecular characterization of the Endophyte

The DNA from the *Colletotrichum gloeosporioides* isolates was subjected to PCR reaction with the specific primers. The CgInt primer(5'–GACCCTCCCGGCCTCCCGCC–3') and universal Primer ITS4 (5'–TCCTCCGCTTATTGATATTGC–3') were used and the amplification reaction was performed [6]. Genetic similarity was calculated using the Jaccard coefficient. Matrix and genetic clustering analyses wereperformed using the UPGMA method and the NTSYS-pc software program at Scigenome, Cochin, India.

RESULT AND DISCUSSION

Isolation of endophyte

Twentyendophytic fungal isolates were obtained from the medicinal plant, *Justicia adhatoda.* They were identified on the basis of fungal morphology and cultural characteristics as *Colletotrichumgloeosporioides* and *Aspergillusniger* (Figure: 1).

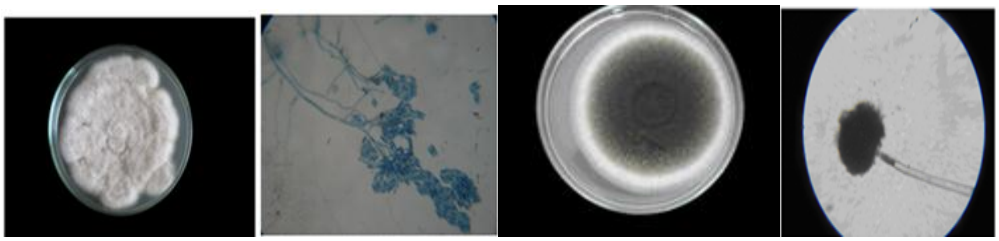

Figure 1: Macroscopic and microscopic view of *Colletotrichumgloeosporioides*and *Aspergillusniger*.

Bioremediation effect on leachate

Among the two endophytic fungi studied only *Colletotrichumgloeosporioides* showed the most prominent growth on MEA incorporated with 50% and 100% leachate (Table: 1). The other isolate showed degradation activity only in MEA with 50% leachate (Table:1). The growth rate of *C. gloeosporioides* on 50 % leachte was 10.83mm and on 100% leachate was 4.87 mm (Figure: 2). Whereas the growth rate of *A. niger* on 50 % leachate was 10.37 mm and no growth on MEA with 100% leachate (Figure:3). Growth rate was compared with growth on SDA. Statistical analysis was done by ANOVA INSTAT and the results obtained are statistically significant.

Species of *Colletotrichum*was very frequently isolated as endophytesand they have also been considered as almost exclusive endophytes, showing their ability to adapt to an endophyticmode of life [1]. The percentage of colonization frequency of *Aspergillusniger* in *Justicia adhatoda* was found to be the highest, ie about 12 % (Table: 2).

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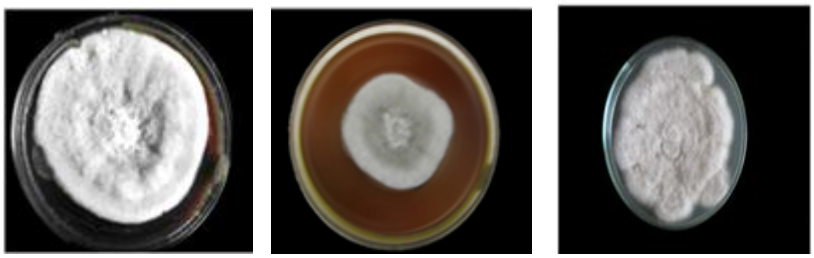

Figure 2: Growth rate of *C. gloeosporioides*on 50 %, 100% leachate on MEA & growth on SDA

Figure 3: Growth rate of *A. niger* on 50 % leachate on MEA & growth on SDA

Table 1: Bioremediation activity of*C.gloeosporioides*and *A.niger* on leachate

No. of Days	Growth of Endophyton on 50% leachate (mm)		Growth of Endophyte on 100% leachate (mm)		Growth of Endophyte on SDA (mm)	
	C.gloeosporioides	*A.niger*	*C.gloeosporioides*	*A.niger*	*C.gloeosporioides*	*A.niger*
1.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	No growth obtained	0.00 ± 0.00	0.00 ± 0.00
2.	13. 66 ± 1.52	19.33 ± 1.15	13.33 ± 0.57		30.00± 1.00	29.00±1.00
3.	34.66 ± 0.57	37.00 ± 1.00	19.66 ± 0.57		51.66±1.52	45.66±0.57
4.	50.00 ± 1.00	55.66 ± 0.57	23.00 ± 1.00		70.00±0.57	59.00±1.00
5.	70.00 ± 1.00	71.33 ± 0.57	26.33 ± 1.52		81.33±1.15	74.00±1.00
6.	74.00 ± 1.00	75.00 ± 1.00	31.00 ± 1.00		94.00±0.57	79.66±0.57
7.	76.66 ± 0.57	77.00 ± 1.00	34.00 ± 1.00		94.00±0.57	79.66±0.57
8.	78.00 ± 1.00	80.33 ± 0.57	37.00 ± 1.00		94.00±0.57	79.66±0.57
9.	80.00 ± 0.00	83.00 ± 1.00	39. 00 ± 1.00		94.00±0.57	79.66±0.57

Table 2: Colonization frequency and enzymatic activity of endophytic fungi isolated from *J.adhatoda*

Sl.No	Name of the endophytic fungi	Percentage of Colonization Frequency (%)	Enzymatic activity of endophytic fungi		
			Amylase	Lipase	Protease
1	*Colletotrichumgloeosporioides*	8	+	+	+
2	*Aspergillusniger*	12	-	-	-

Enzyme Assay

In the present investigation endophytic fungal isolates were screened for the presence of extracellular enzymes such as amylase, lipase and protease. Among the two endophytic fungi

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Colletotrichumgloeosporioides showed positive zones for amylase, lipase and protease activity (Figure: 4, Table:2). *C.gloeosporioides* was found to be the best of producing alkaline lipase and also hydrolase wide range of oils [7]. The amylase of fungal origin was more stable than bacterial amylase enzyme [1].

Amylase Lipase Protease

Figure 4: Enzyme activity of *Colletotrichumgloeosporioides*

Molecular characterization of*Colletotrichumgloeosporioides*

All the isolates amplified with the CgInt and ITS4 primers, confirming that the isolates pertained to *Colletotrichumgloeosporioides*(Figure: 5).

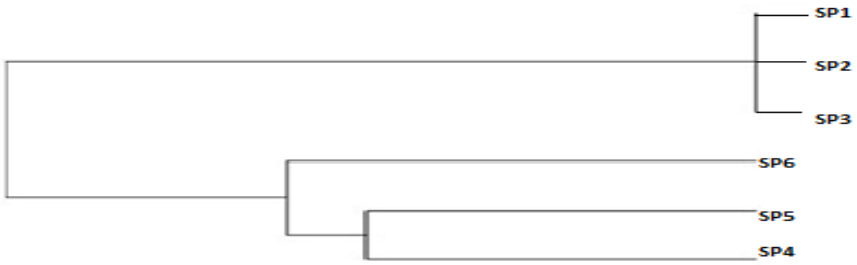

Figure 5: Dendrogram of *Colletotrichumgloeosporioides* isolates using the UPGMA method and the NTSYS-pc software program.

CONCLUSION

- The endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host.
- In the present study two different genera were isolated from *Justicia adhatoda*with 20%endophytic infection rate.
- During the study, a screening method for the selection of potential fungi which has the ability to be used in bioremediation of leachate was carried out and was found that *Colletotrichumgloeosporioides* can grow on MEA incorporated with 50% and 100% leachate, therefore have the most potential to be used in leachate bioremediation.

- Screening of endophytic fungi for enzymes will be useful for the development of new technologies in the industrial purpose. Among the isolated endophytes during the study, *C. gloeosporioides*exhibit a wide range of enzyme activity.

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