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Prospective oleaginous endophytic fungi isolated from biodiesel plants: An assessment of diversity and lipid content

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ABSTRACT

Seven biodiesel plants, namely*Jatropha curcas, Ricinus communis, Pongamia pinnata, Sapindus mukorossi, Mesua ferrea, Terminalia bellerica* and *Cascabela thevetia* were collected from oil fields of Assam for isolation of endophytic fungi. Besides morphological characterization, molecular identification of the endophytic isolates was done by sequencing the internal transcribed spacer regions (ITS). The sequences were submitted to NCBI GenBank while the phylogenetic tree was submitted to TreeBase for obtaining accession numbers. Diversity assessment of the total 27 fungal isolates obtained from 155 segments of different plants was carried out to assess the distribution patterns. Ten numbers of fungal isolates obtained from *Cascabela thevetia* were dominant when compared to other plants. There were high colonization rate in leaf part of plants when compared to other parts which was also indicated by one way ANOVA with a significant colonization of fungal isolates in leaf part of the plants. Density of *Colletotrichum* sp. was dominant with 26% relative frequency. Total lipid extraction of the fungal isolates was performed and application of one way ANOVA indicated a significant percentage of total lipid found in isolates SPSRJ27 and SPSRJL36 isolated from leaf part and isolate SPSRJL35 isolated from stem part of the plants. Further studies on growth optimization of lipid producing isolates may open up avenues for their use as oleaginous fungi.

Key words: Oleaginous, endophytic fungi, biodiesel plants, diversity.

INTRODUCTION

Endophytic fungi represent an important component of fungal biodiversity. These endophytes are known to affect plant community diversity and structure (Gonthier *et al*., 2006; Krings *et al*., 2007). High estimates of endophytic fungal species diversity have been studied recently from tropical and temperate forests (Santamaria and Diez, 2005; Sanchez Marquez *et al*., 2007). There are a significant proportion of prospective novel fungal genera of endophytic fungi inhabiting diverse niches and are in untold numbers (Smith *et al*., 2007). Though morphology is widely accepted but internal transcribed spacer (ITS) sequence data is helpful in identification of fungi in the absence of morphological data (Schoch *et al*., 2012; Hibbett and Taylor, 2013). Fungal endophytes and their host plants carry a range of relations, starting from mutualistic or symbiotic to antagonistic or slightly pathogenic (Arnold, 2007).Host-specificity, host-recurrence, host selectivity, or host-preference represents the relationships of endophytes with single or multiple plants (Zhou and Hyde, 2001; Cohen, 2006).

Production of a plethora of substances by endophytes has seen potential use to modern medicine, agriculture, and industry. Various natural products produced by endophytic fungi reveal incidence of exceptional structures and bioactivities (Tan and Zou, 2001; Zhang *et al*., 2006). One of the various products produced by endophytic fungi is the production of biofuel lipids which has attracted the attention of scientists. Microorganisms producing more than 20% of the total lipid content are termed as oleaginous (Ratledge, 1991), which has the potential to be further studied for biofuel generation. Considering the easy growth manipulation and cultivation of the fungal forms, the present study is aimed to screen oleaginous endophytic fungi associated with biodiesel plants and assess their diversity to find out the richness of such species in various parts of the biodiesel plants along with profiling of the production of lipid by the isolates under laboratory conditions.

MATERIALS AND METHODS

Collection and sampling site: Collection of biodiesel plants from oil fields of Dibrugarh and Tinsukia Districts of Assam were undertaken and the collected plant parts were transported aseptically in pre-sterilized sample collection bags to the laboratory for further processing.

Isolation: Isolation of endophytic fungi was performed according to the method reported by Hallmann *et al*.(2007) with minor modifications. The plant samples were washed thoroughly in running water before processing to eliminate attached dust and debris. Samples were then cut into 2 mm segments and were surface sterilized with 70% ethyl alcohol for 1 min, soaked in 2% sodium hypochlorite solution for 3 min, and rinsed with 70% ethyl alcohol for 1 min. They were finally rinsed with sterile distilled water and blot dried on sterile filter paper. The surface sterilised explants were inoculated into the Petri dishes containing water agar (WA) (Himedia, Mumbai, India) according to the method of Strobel *et al*. (1996) and kept in incubator for 7 to 15 days. Periodically the plates were checked for fungal growth. The fungi growing out from the plant explants were then subcultured in PDA (Potato Dextrose Agar) (Himedia, Mumbai, India) plates following the method of Nath *et al*. (2012).The cultures were deposited to MCC (Microbial Culture Collection), National Centre For Cell Science, (NCCS), Pune, India to obtain accession numbers.

Staining: Lactophenol cotton blue staining was performed for fungal mycelium and the slides were observed in light microscope under 100X. Microscopically, the endophytic fungal isolates were identified on the basis of their hyphal features, arrangement of spores and reproductive structures (Nagamani *et al*., 2006; Nath *et al*., 2014). Qualitative estimations of oil accumulated in microbial cells were done using Sudan black B staining technique (Evans *et al*., 1985).

Molecular identification

DNA isolation and molecular characterisation of the fungal isolates: The mycelia were grown in potato Dextrose Broth (PDB) at 25 ± 2°C for 3-5 days. These were then harvested and crushed in a sterilized pestle and mortar under liquid nitrogen. The genomic DNA was then obtained by using the HiPurA fungal DNA isolation kit (Himedia, India) as per the manufacturer's instructions. DNA samples were stored at 4°C for immediate use and at - 20°C for long-term storage.

PCR amplification and sequencing: To amplify the ITS region in rDNA, the universal primers ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' were used (White *et al*., 1990). The PCR reaction mixture composed of 10 µl fungal DNA, 5µl 10 × PCR buffer, 1 µl of 10 mM dNTP, 0.25µl Taq polymerase, 2 µl each of the forward and the reverse primers in a total reaction volume of 50 µl. PCR was executed in a Gene Amp 9700 Thermal Cycler (Applied Biosystems, USA) beginning with a denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 52°C for 30 sec, and 72°C for 1 min, with an ultimate extension step of 72°C for 10 min (Bhagobaty and Joshi, 2011). Gel electrophoresis with 1.5% agarose in 1X Tris-acetate-EDTA was used to analyze the amplified ITS with a marker ladder of 1kbp and ethidium bromide staining. The amplified ITS products were purified using QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Big dye ready reaction terminator cycle sequencing kit (Applied Biosystems, USA) was employed and sequenced in Applied Biosystems 3700 Genetic Analyzer.

Identification of endophytic fungi and phylogenetic evaluation: BLAST algorithm was used for analysis of obtained sequences and closely related phylogenetic sequences obtained from the National Centre of Biological Information (NCBI) database. MEGA 5.0 program with an alignment of sequences prepared using ClustalW software was used for phylogenetic relationship analysis (Tamura *et al*., 2011). Phylogenetic tree was constructed using Neighbour Joining algorithm with the Kimura 2-parameter (Kimura, 1980). The stability of relationships was checked by a bootstrap analysis with a resampling of 1,000 times. The sequences were deposited to the NCBI database and phylogenetic tree was submitted to TreeBase to obtain the accession numbers.

The presumptive identification of the endophytic fungal isolates was carried out with the help of standard monographs (De Gruyter, 2002; Samson *et al*., 2006; Webster and Weber, 2007; Huang *et al*., 2008; Maharachchikumbura *et al*., 2011; Udayanga *et al*., 2011; Bensch *et al*., 2012; Weir *et al*., 2012; Herrera *et al*., 2013; Nong *et al*., 2013; Mohamed K. Refai and AtefA Hassan, 2013; Mohamed Refai and El-Yazid, 2014 and Dou *et al*., 2017). Some other online databanks that were used for identification included CBS-KNAW collections, MycoBank, JGI (Joint Genome Institute) MycoCosm.

Colonization Rate: Colonization rate (CR) was calculated as the total number of segments/pieces colonized by endophytic fungi divided by the total number of segments/pieces incubated for the plant sample (Huang *et al*., 2008).

Relative frequency: Relative frequency was calculated as the number of isolates of one species divided by the total number of isolates, and expressed as percentage (Huang *et al*., 2008).

Screening for of total lipid production: One gram each of the oven dried fungal biomass at 60°C was taken for total lipid extraction. The extraction was done according to the method of Bligh and Dyer (1959) using 1:2(v/v) Dichloromethane: Methanol with minor modifications.

Statistical analysis: All experiments were done in triplicate and data expressed as mean ± Standard Error of the Mean (SE). One-way ANOVA was performed to calculate significant differences in treatment means. Graph Pad Prism 4 was used for interpretation of the data. Mean separations were performed by Tukey's post hoc tests.

One-way ANOVA was also performed to compare the colonization of fungal endophytes from different parts of seven different plants using Student-Newman-Keulis (SNK) (Kumar and Hyde, 2004) test. Graph Pad Prism 4 was used for interpretation of the data.

RESULTS

Collection and sampling site: Different biodiesel plants, namely *Jatropha curcas, Ricinus communis* (N27°18.882, E095°17.287; El-201m), *Mesua ferrea* (N27°19.264, E095°16.635; El-136m) from Oil India Ltd. Duliajan, *Pongamia pinnata* (N27°19.260, E095°16.652; El-143m), *Cascabela thevetia* (N27°18.823,E095°17.283; El-205m) from Bhekulajan [Early production station (EPS)] Duliajan, *Sapindus mukorossi* (N27°18.914, E095°17.421; El-220m), *Terminalia bellerica* (N27°19.271, E095°16.659; El-158m) from Balijangaon, Duliajan were collected from oil fields of Assam (**Fig. 1**).

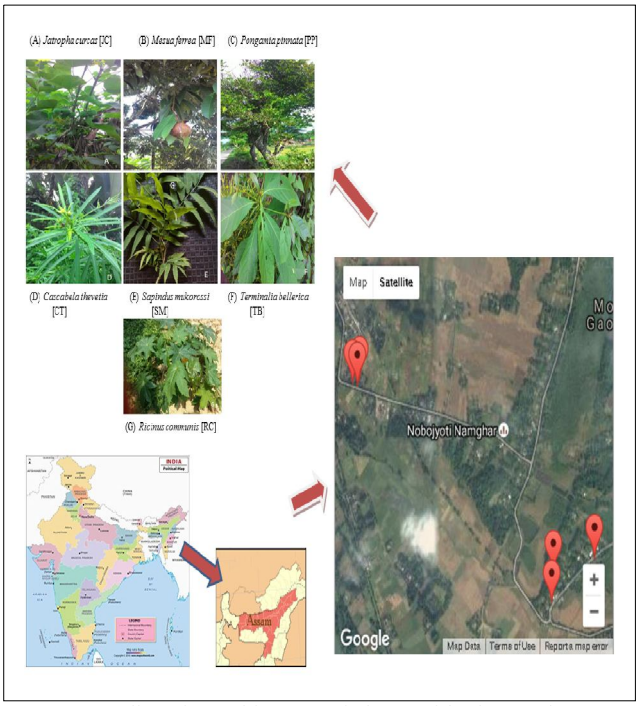

Fig. 1: Sampling site and images of plants with plant code.

Table 1(A): Number of endophytic fungi isolated from different biodiesel plants.

Host Plant	Bark	Leaf	Stem	Root	Seed	Petal	Total
Jatropha curcas(JC)	-	3	2	-	-	-	5
Ricinus communis(RC)	-	2	1	-	-	-	3
Pongamia pinnata(PP)	-	1	-	2	-	-	3
Sapindus mukorossi (SM)	1	-	1	-	1	-	3
Mesua ferrea(MF)	-	-	-	-	1	-	1
Cascabela thevetia (CT)	-	6	2	1		1	10
Terminalia bellerica (TB)	-	-	1	-	1	-	2

Table 1(B): Number of isolates obtained from different plant parts and colonization rate.

	Bark	Leaf	Stem	Root	Seed	Petal	Total
Incubated plant segments	25	35	35	25	25	10	155
Number of endophytic fungal isolates	1	12	7	3	3	1	27
Colonization Rate (%)	4	34.3	20	12	12	10	17.42

Isolation and staining: A total of 27 endophytic fungi from 155 plant segments/pieces were isolated (**Table 1 A-B; Fig. 2**) and lactophenol cotton blue staining performed for fungal mycelium which showed microscopically, the presence of septate or aseptate hyphal structures as well as the presence or absence of spores (**Fig. 3A**). The Sudan black B staining intensity revealed the lipid producing isolates which were further studied for lipid quantification (**Table 2; Fig. 3B**).The accession numbers obtained for few isolates on deposition of

Table 2: Intracellular Lipid concentration of different fungi.

Identity	Lipid staining intensity
SPSRJ 2	+++
SPSRJ4	++
SPSRJ5	++
SPSRJ6	++
SPSRJ7	+
SPSRJ8	++
SPSRJ9	++
SPSRJ10	++
SPSRJ11	+
SPSRJ12	++
SPSRJ13	+
SPSRJ14	++
SPSRJ15	++
SPSRJ16	+
SPSRJ17	+++
SPSRJ21	++
SPSRJ22	+
SPSRJ23	++
SPSRJ26	+
SPSRJ27	++++
SPSRJ30	++
SPSRJL33	++
SPSRJL35	++++
SPSRJL36	++++
SPSRJ5A	++
SPSRJ6A	+++
SPSRJ7A	+++

+: indicates very low amount of lipid,
++: indicates low amount of lipid,
+++: indicates considerable amount of lipid,
++++: indicates very high amount of lipid.

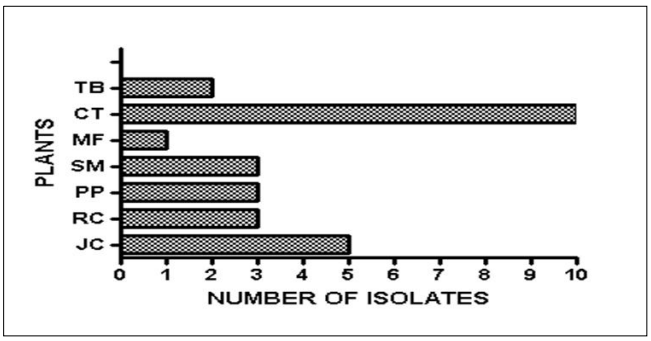

Fig. 2: Number of endophytic fungal isolates obtained from different plants.

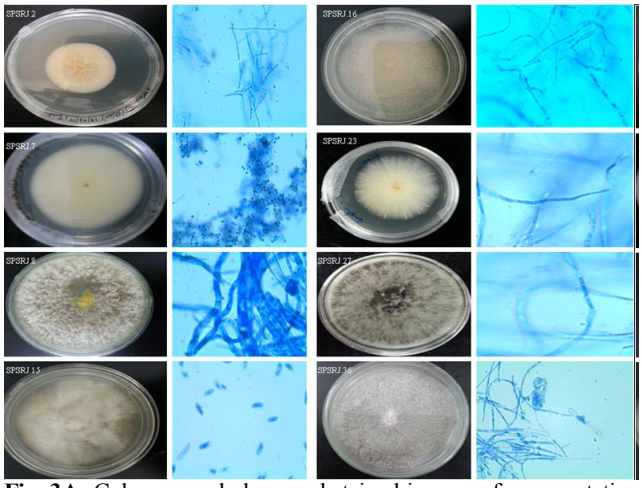

Fig. 3A: Colony morphology and stained images of representative isolates.

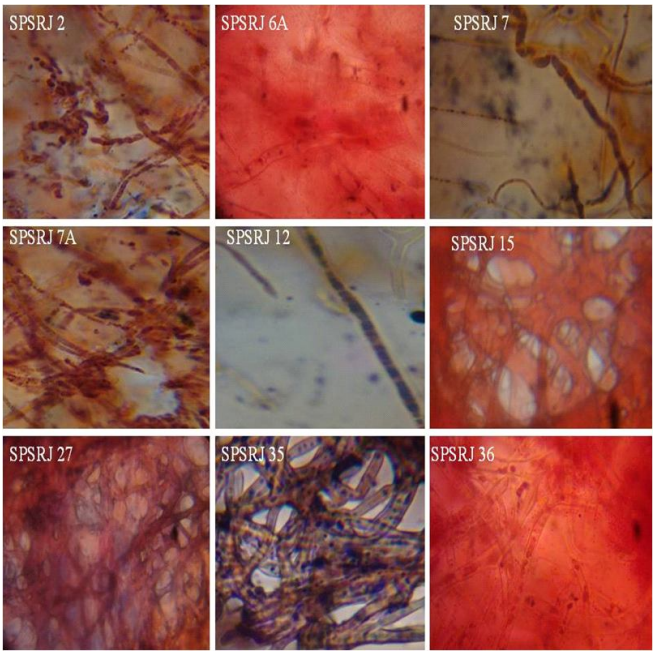

Fig. 3B: Microscopic structures of Sudan black B stained hyphal structures of representative fungal isolates.

Table 3: Relative frequency of endophytic fungi with monographs/databanks details used for identification.

Fungal taxa	Bark	Leaf	Stem	Root	Seed	Petal	Total	Relative Frequency (%)	Monographs/Databanks used for identification
Xylariales sp.	-		1	-	-	-	1	3.70	Huang *et al*., 2008, JGI, MycoCosm
Xylariaceae sp.	-	1	-	-	-	-	1	3.70	Nong *et al*., 2013, Mycobank
Phoma sp.	1	1	-	-	-	-	2	7.41	De Gruyter, 2002; Mohamed Refai and El-Yazid, 2014
Diaporthe sp.	-	1	1	-	1	1	4	14.82	Gomes *et al*., 2013, CBS
Ilyonectria sp.	-	-	-	1	-	-	1	3.70	Mycobank
Colletotrichum sp.	-	5	2	-	-	-	7	25.93	Weir *et al*., 2012, CBS
Phomopsis sp.	-	1	1	1	-	-	3	11.12	Udayanga, *et al.,*2011; CBS
Aspergillus sp.	-	1	-	-	1	-	2	7.41	Samson *et al.,* 2006, Mohamed K. Refai and Atef A Hassan, 2013
Fusarium sp.	-	-	-	1	-	-	1	3.70	Webster and Weber, 2007; Mohamed K. Refai and Atef A Hassan, 2013
Pseudocosmospora sp.	-	-	-	-	1	-	1	3.70	Herrera *et al*., 2013; Mycobank
Pestaliotopsis sp.	-	-	1	-	-	-	1	3.70	Maharachchikumbura *et al*., 2011
Lasiodiplodia sp.	-	1	-	-	-	-	1	3.70	Mohamed Refai andEl-Yazid, 2014; Dou *et al.,* 2017
Cladosporium sp.	-	1	1	-	-	-	2	7.41	Bensch *et al.,* 2012; Mohamed Refai and El-Yazid, 2014

pure culture to MCC, Pune, India of which few were provided with the accession numbers/reference numbers and the rest are in the process of being alloted the deposition numbers (**Table 4**).

Table 4: Accession numbers of endophytic fungi isolated from biodiesel plants.

Plant code	Fungal isolates	Identified endophytic fungi	NCBI Accession No.	MCC Accession/ Reference number No.
JC	SPSRJ 2	*Xylariales* sp.	KX951470	D_NOV_16_001
	SPSRJ4	*Phoma labilis*	KX951471	Yet to be alloted
	SPSRJ22	*Colletotrichum gloeosporioides*	MF595899	Yet to be alloted
	SPSRJL35	*Pestaliotopsis microspora*	MF595902	Yet to be alloted
	SPSRJL36	*Phomopsis* sp.	MF595903	Yet to be alloted
RC	SPSRJ5A	*Cladosporium cladosporioides*	MF143554	Yet to be alloted
	SPSRJ6A	*Xylariaceae* sp.	MF143555	Yet to be alloted
	SPSRJ7A	*Cladosporium tenuissimum*	MF143556	Yet to be alloted
PP	SPSRJ7	*Ilyonectria radicicola*	KX951474	D_NOV_16_006
	SPSRJ15	*Fusarium solani*	KX951482	Yet to be alloted
SM	SPSRJ5	*Phoma exigua*	KX951472	Yet to be alloted
	SPSRJ6	*Diaporthe* sp.	KX951473	MCC 1290
	SPSRJ27	*Lasiodiplodia exigua*	MF143558	Yet to be alloted
	SPSRJ30	*Diaporthe phaseolorum*	MF143560	Yet to be alloted
MF	SPSRJ16	*Pseudocosmospora vilior*	KX951483	Yet to be alloted
CT	SPSRJ8	*Diaporthe* sp.	KX951475	MCC 1289
	SPSRJ9	*Colletotrichum gloeosporoides*	KX951476	Yet to be alloted
	SPSRJ10	*Colletotrichum gloeosporoides*	KX951477	Yet to be alloted
	SPSRJ11	*Colletotrichum gloeosporoides*	KX951478	D_NOV_16_004
	SPSRJ12	*Phomopsis* sp.	KX951479	Yet to be alloted
	SPSRJ13	*Phomopsis* sp.	KX951480	D_NOV_16_003
	SPSRJ14	*Colletotrichum gloeosporoides*	KX951481	D_NOV_16_002
	SPSRJ17	*Diaporthe* sp.	MF595897	Yet to be alloted
	SPSRJ23	*Aspergillus niger*	MF595900	Yet to be alloted
	SPSRJ26	*Colletotrichum gloeosporoides*	MF143557	Yet to be alloted
TB	SPSRJ21	*Aspergillus niger*	MF595898	Yet to be alloted
	SPSRJ33	*Colletotrichum siamense*	MF595901	Yet to be alloted

Molecular identification: The amplified rDNA-ITS region of the fungal isolates after sequencing were associated to different genera (**Table 3**) .The sequence data were then submitted to the NCBI GenBank with accession numbers (**Table 4**). Phylogenetic tree submitted to TreeBase with submission ID 2165 (in progress http://purl.org/phylo/treebase/phylows/study/TB2:S21675). Phylogenetic trees of endophytic fungi isolated from different plant samples of biodiesel plants were constructed using neighbor- joining method and aligned to different genera (**Fig. 4 A & B**).

Details of monographs and databank used for the study used are presented in **Table 3**.

Diversity assessment: Colonization rate was found to be dominant in the leaf (**Table 1; Fig. 5 A & B**). *Colletotrichum* sp. found to be dominant as indicated by the relative frequency (**Table 2; Fig. 6**).

Lipid content analysis by extraction of total lipids: The total lipid extraction for the isolates revealed three positive isolates having more than 20% of total lipid of their dry biomass (**Fig. 7**).

Statistical analysis: One-way analysis of variance (ANOVA) showed significant differences in means with total lipid content on the tested parameters. Total lipid content of the fungal isolates were found to be significant ($p<0.0001$; $r^2= 0.9959$).

The overall colonization of fungal isolates in the leaves of different plants was found to be significantly higher than those in the bark, roots, petals and seeds ($p<0.001$) with the exception of stem where the significance was noticed at $p<0.01$. The one-way ANOVA test reveals significant differences ($p<0.0001$) in the mean values among the total number of isolates per tissue type. This indicates that there are differences in the total number of isolates and their means from each tissue type used for the isolation of the prospective fungal endophytes.

DISCUSSION

Micro organisms use less space to grow and produce bioactive compounds of interest (Lang *et al*., 2001) useful to medicine, agriculture, and industry (Tan and Zou, 2001). So, in the present study, seven biodiesel plants growing under three different geographical locations were selected to explore the oleaginous endophytic fungi. *Cascabela thevetia* was found to hold more number of isolates with higher diversity of endophytic fungi. Colonization rate of endophytic fungi from medicinal plants is reported to range from 36.7% to 100% (Huang *et al*., 2008) and in this study 27 isolates from 155 plant segments were recovered with a

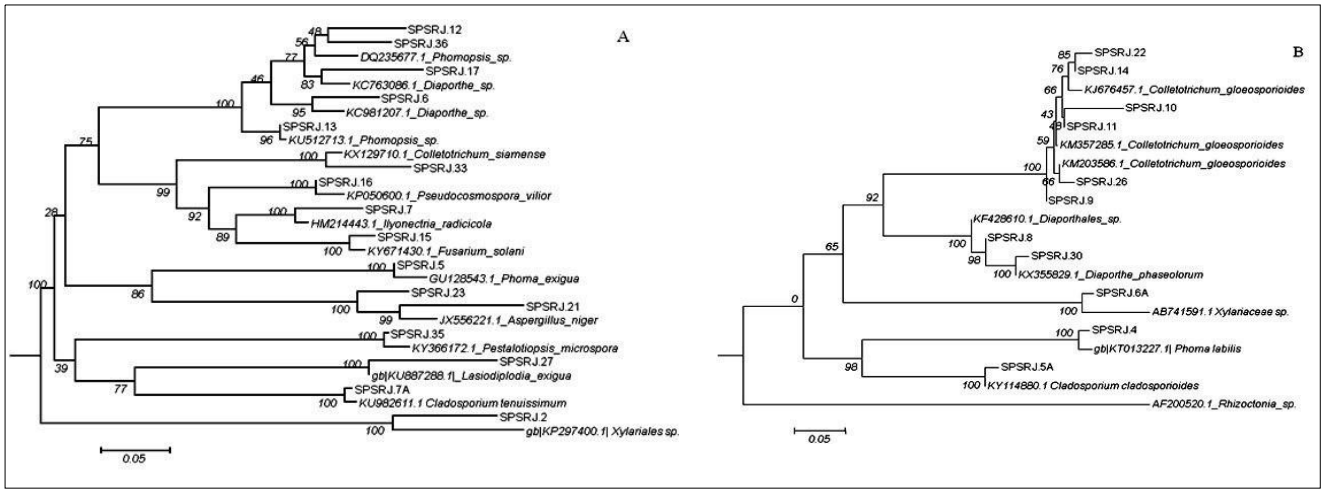

Fig. 4 (A-B): Evolutionary positions of the endophytic fungal isolates with other concurrent fungal species based on internal transcribed spacer (ITS) sequence match.

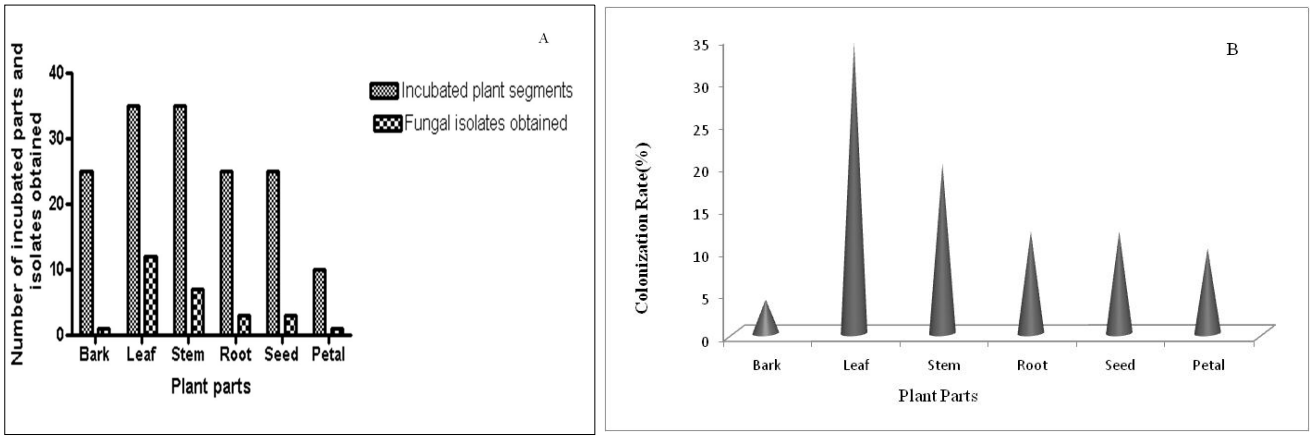

Fig. 5: (A) Fungal isolates obtained from incubated plant parts; **(B)** Colonization rate (%) of plant parts.

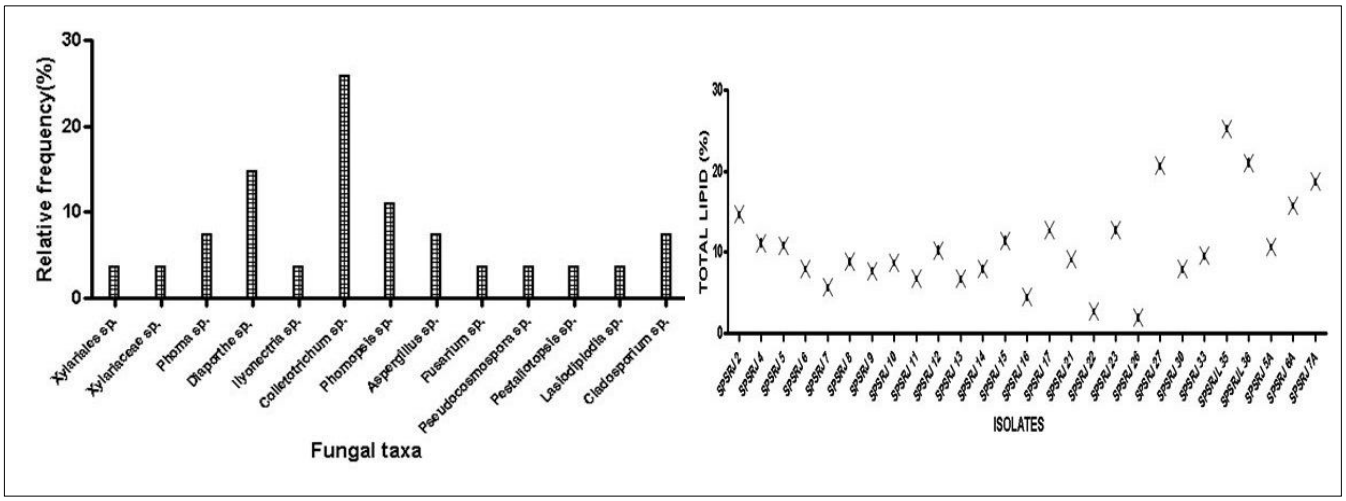

Fig. 6: Relative frequency of fungal taxa.

Fig. 7: Total lipid (%) obtained from the cultured biomass of fungi.

colonization rate of 17.42% in all the plants. Distribution of endophytic fungi was found to be high in leaf followed by stem part of the plants, which was also reported by Costa *et al*. (2012) that leaves of *Laguncularia racemosa* hosted the highest number of CFU and taxa in two seasons. Phylogenetic and diversity assessment of the endophytic fungi showed the isolates belonging to 13 genera, with *Colletotrichum* being the dominant genus which is found to be the most frequent group of endophytic fungi (Photita *et al*., 2005). Three isolates out of twenty seven were found to have more than 20% of total lipid of their dry biomass. Two of them were isolated from leaf part of the plant providing evidence on the occurrence of oleaginous endophytic fungi in the leaf part. This study provides baseline information about colonization of endophytic fungi mostly on multifoliate parts of biodiesel plants. Further study on growth optimization of the isolated fungi would open scope for exploring the oleaginous isolates to be used as minifactories for production of biofuels.

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