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Endophytic Fungal Diversity in *Eclipta prostrata* (L.) L through Illumina MISEQ Platform

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ABSTRACT
Plant- microbe interaction is a common and inevitable phenomenon that has been studied in detail by many researchers. Some of these relationships are beneficial while others are harmful. Among the beneficial interactions, endophytic association of a bacteria or fungi with plants are of great interest. These beneficial plant- microbe interactions are said to affect plant health, resistance and stress tolerance level in a positive way. Some of them have influenced in their metabolic pathways too. The present study is an attempt to understand the endophytic fungal diversity in the leaves, stem and roots of Eclipta prostrata (L.) L through metagenomic approach using illumina MiSeq platform. Metagenomic diversity analysis were based on the sequencing of ITS region of endophytic fungi. OTU analysis at different taxonomic level clearly catalogues two phyla viz. Ascomycota and Basidiomycota in all the three samples. OTU heatmap analysis elucidated the most prevalent species in the sample viz. Vishniacozyma, Wallemia, Cladosporium, Verticillium, Pichia and Acremonium with a prevalence of 1.0 at a minimum threshold of 0.010%. Comparative analysis revealed 164 fungal endophytes that were common to both leaf, stem and root while 174 genera were exclusive to leaf and stem and 80 were unique to the root sample. This diversity analysis has helped in identifying the endophytic fungi in E. prostrata and would form a base for further investigation on their role in metabolic pathways or in contributing to ethnomedicinal properties.
Keywords: *Metagenome, Illumina Miseq, OTU, diversity analysis, relative abundance*

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INTRODUCTION

Plants can harbour microbiota both in its rhizosphere and the phyllosphere ecosystems [1]. They can be archeae, fungi, bacteria, viruses or oomycetes [2, 3] and the interactions can be either pathogenic or beneficial [4]. Mutualism, symbiosis, commensalism or neutralism are some of the beneficial interactions . Out of these, endophytes are an important group of microorganisms that execute a mutualistic beneficial interaction in plants which can include both fungi and bacteria. They enter the plant through rhizosphereic soil and colonise its internal tissues [5] without showing any harmful effects on plant health [6,7]. Studies demonstrated that these endophytes developed certain strategies that influence the production of metabolites in plants [8]. The recent research conducted in endophyte biology exposed that almost all plants are symbiotic with mycorrhizal fungi and/or fungal endophytes [9]. These associations could be traced back to the time of plant origin and can be regarded as a key to bring plant evolution on land . Endophytes reside within the plant tissue and can grow and then sporulate within them without producing any harm to the host [10,11,12]. Based on evolutionary relations, taxonomy, plant hosts, and ecological purposes there are two types of fungal endophytes viz.; the clavicipitaceous endophytes (C-endophytes) and the nonclavicipitaceous endophytes (NC-endophytes). The former commonly infects grasses and the latter are found from tissues of ferns, conifers, non- vascular plants and angiosperms [13]. Metagenomics is a culture independent way of understanding microbial populations which opened a new dimension of ecosystem analysis. It encompasses sampling genome sequences of a group of micro-organisms occupying a common environment [14]. Over the past decade next generation sequencing (NGS) is extensively used in metagenomics because of its high speed, low cost and technical advancements [15] that increased the number of metagenomic projects dramatically [16]. The first metagenomic study using NGS technique was based on pyrosequencing to explore the microbial sample in Soudan Mine, USA, that used a systemic approach by integrating biology, chemistry, bioinformatics

which lead to a milestone in advancing modern microbial ecology. Earlier, molecular taxonomy of fungal classification was much more difficult [17,18,19,20] because of the presence of intermediate forms, the instability of morphological characters and the phenotypic overlay among various taxa [21]. *Eclipta prostrata* (L.) L, commonly called as *Bhringaraj* in India, is a plant of great ethnomedical significance. This plant belongs to Asteraceae family can be seen commonly in tropical and subtropical regions [22]. A number of compounds like Wedelolactone, Ecliptalbine, Demethylwedelolactone, Eclalbatin, Dasyscyphin C and Ecliptine are reported from *E. prostrtata* [23] that contributed hepatoprotective, hair growth promoting, antidiabetic, analgesic, anti- inflammatory, neurological, antimicrobial, antioxidant, antimalarial, cardiovascular, anticancer, antiulcer, immunomodulatory and antiepileptic effects [24]. The endophytic microflora and its metadiversity remain an underexplored area in *E. prostrtata*. The present work is an attempt to analyse the diversity of endophytic fungi in the leaves, stem and roots of *Eclipta prostrata* (L.) L using metagenomic approach through illumina Miseq platform.

MATERIAL AND METHODS

Plant Sample Collection and Sterilization

Healthy and flowering plant samples of *E. prostrata* were collected from three sites (Ernakulam 10◦0'12.1" N 76◦17'30.8"E) with same soil conditions, pooled and used for DNA extraction after surface sterilization. The collected plants were washed in soap solution followed by running tap water for 30 min. Plants were then immersed in 0.1 % mercuric chloride for 5 minutes followed by a quick dip in 70% ethanol. It was followed by immersion in distilled water thrice, 5 min for each wash. The final wash with distilled water was poured into a sterile nutrient agar plate that served as a control to ensure that the sample is devoid of any external contaminants.

DNA Isolation

DNA isolation was carried out using Himedia Higenome Kit Method.

Experimental Process And Sequencing

DNA samples were quantified using a Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). 50-100ng DNA was used to generate amplicons using a panel of primers designed.

Amplicon Generation

Oligonucleotide primers were designed to anneal to relatively conserved sequences spanning fungal ITS regions. ITS2 region was amplified using forward primer containing sequence "GTGAATCATCGARTC" and reverse primer containing sequence "TCCTCCGCTTATTGAT". Besides the ITS target-specific sequences, the primers also contain adaptor sequences allowing uniform amplification of the library with high complexity ready for downstream NGS sequencing on Illumina Miseq platform. All PCR reactions were carried out in 50µL reactions with 25µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.4µM of forward and reverse primers, and about 20 ng template DNA. PCR amplification was initiated by denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s followed by finally extension at 72°C for 5 min.

PCR Product quantification

The PCR products were electrophoresed on 2% agarose gel for detection. Samples with one bright main band between 400-450bp were chosen for further experiments.

PCR Product Purification

PCR products were purified by bead based purification method [25].

Library preparation and sequencing

Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 4.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2x300/250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) embedded in the MiSeq instrument.

Analysis Workflow

At first, adapters and low quality data were filtered out from the original data. Then the chimera sequences were removed to obtain the effective sequences for cluster analysis. Each cluster was called an OTU (Operational Taxonomic Unit). The taxonomy analysis of the representative sequence of each OTU was then performed to obtain species distribution information. UPGMA clustering tree can be constructed based on Unifrac distance to illustrate the differences in community structure between different samples or groups.

Sequencing data quality optimization

Sequencing errors such as point mutations might occur in high-throughput sequencing, and it's common that bases toward the end of the sequence reads have lower than average quality. In order to obtain higher quality and more accurate bioinformatics analysis results, it is necessary to optimize the raw data of the sequencing to obtain higher quality and more accurate bioinformatics analysis results.

Analysis software: Trimmomatic (0.39), vsearch (v2.14.1)

Data Analysis

The QIIME [26] data analysis package was used for ITS rRNA data analysis. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on joined sequences was performed and sequence which did not fulfill the following criteria were discarded: sequence length = 20. Then the sequences were compared with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed. The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH（1.9.6） against the UNITE ITS database (https://unite.ut.ee/) pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. The RDP classifier uses the UNITE ITS database which has taxonomic categories predicted to the species level. Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree from beta diversity distance matrix was builded.

RESULT

Sequencing Data Quality Optimization

The data obtained after high- throughput sequencing was further analyzed using Trimmomatic (0.39), vsearch (v2.14.1) softwares to obtain higher quality and more accurate bioinformatics analysis results. The sequence data has been deposited at NCBI under Sequence Read Archieve database (accession numbers PRJNA773862, PRJNA774103).

Table 1. Preliminary Data Analysis- Raw Data Statistics

Sample	Raw reads	Raw bases	Raw data (w)	Q20 (%)	Q30 (%)	GC (%)
1LSITS_1	100179	30153879	64.5	95.4	87.9	49
1LSITS_2	115235	34685735	64.5	87.8	75.2	50
2RITS_1	139714	42053914	89.9	92.5	84.6	48
2RITS_2	139714	42053914	89.9	85.2	73.1	48

Data Analysis

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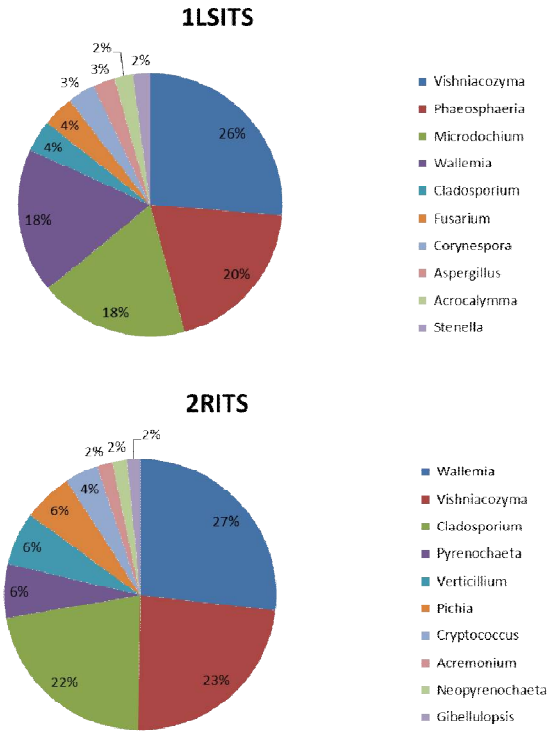

Fig 1. Pie chart showing relative abundance of genus in 1LSITS and 2RITS

Genus abundance Heat- map

The abundance distribution of most dominant genera among all samples were analysed using Plot by R software and is displayed in the genus abundance heatmap.

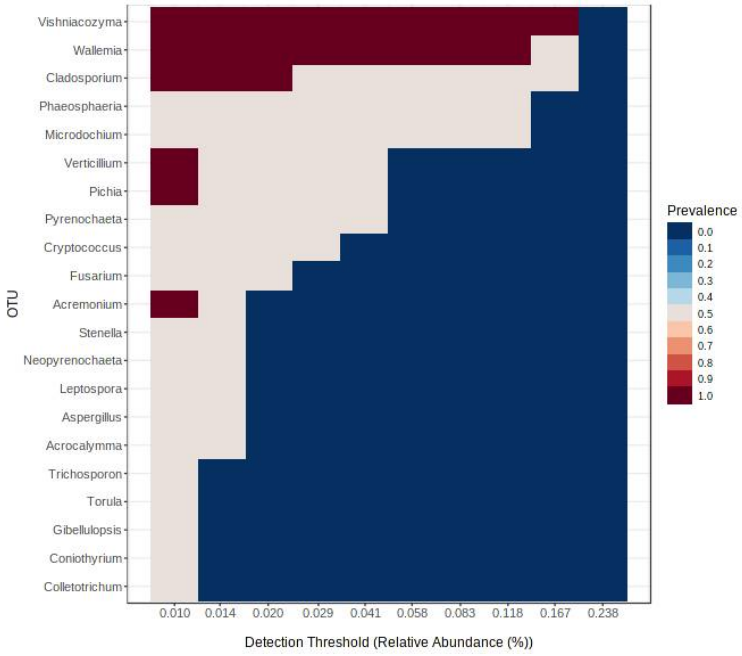

Fig 2. OTU Heatmap

Venn diagram

A Venn diagram (aka Euler diagram) is a method of visualizing the amount of overlap between two (or three) lists of data, using circles to signify the size of each circle and positioning the circles such that the area of overlap represents the amount of list overlap.

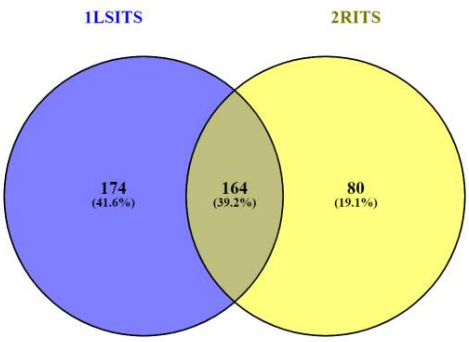

Fig 3 . Venn Diagram of samples; 1LSITS and 2RITS

DISCUSSION

OTU and Taxonomic Composition Analysis

The distribution of top most abundant classifications of endophytic fungus in the sample at different taxonomic levels were analyzed using Qiime software. OTU analysis at different taxonomic level clearly catalogues two phyla viz. *Ascomycota* and *Basidiomycota* in the leaf stem sample. *Basidiobolomycetes, Dothideomycetes, Eurotiomycetes, Mortierellomycetes, Tremellomycetes* and *Wallemiomycetes* were the recognized classes. Out of these classes, 7 orders such as *Capnodiales, Eurotiales, Pleosporales, Pleosporales, Tremellales, Wallemiales* and *Xylariales* were distinguished. Most abundant families were viz. *Bulleribasidiaceae, Mycosphaerellaceae, Phaeosphaeriaceae, Trichocomaceae and Wallemiaceae. Vishniacozyma, phaeosphaeria, Microdochium, Wallemia, Cladosporium, Fusarium, Corynespora, Aspergillus, Acrocalymma, Stenella* shows the direction of Genus in its decreasing relative abundance from leaf stem sample.

Ascomycota and *Basidiomycota* are the most important phyla obtained from root sample. The most abundant classes were viz. *Basidiobolomycetes, Dothideomycetes, Mortierellomycetes, Tremellomycetes* and *Wallemiomycetes*. OTU analysis at different taxonomic level clearly catalogues two phyla viz. Ascomycota and Basidiomycota. In these classes, three orders such as *Tremellales, Cystofilobasidiales* and *Wallemiales* were identified. Among these, three families viz. *Tremellaceae, Wallemiaceae* and *Trichosporonaceae* were represented predominantly whereas *Rhynchogastremataceae, Sirobasidiaceae, Carcinomycetaceae, Cystofiobasidiaceae* were less represented. *Wallemia* was the most abundantly seen endophytic genus while *Vishniacozyma, Cladosporium, Pyrenochaeta, Verticillium, Pichia, Cryptococcus, Acremonium, Neopyrenochaeta* and *Gibellulopsis* showed decreasing relative abundance in the root sample.

Only a few studies have been conducted in plants for investigating the biodiversity of fungal endophytes using illumina Miseq platform. A total of 249 fungal endophytes were isolated from *Eclipta prostrata* in our study. Both these samples represented two phyla viz. Ascomycota and Basidiomycota as the predominant one which was in agreement with a previous study of metagenomic fungal diversity analysis in strawberry plants [27]. Screening of endophytic fungal associates from *Eclipta prostrata* was done by and they reported the presence of *Aspergillus niger, Cladosporium oxysporum, Fusarium moniliformae and Phoma sp.* as the culturable fungal endophytes [28]. This study coincides very much with our results were we found *Acremonium, Acrocalymma, , Aspergillus, , Cladosporium, Corynespora, , Cryptococcus, , Fusarium, Gibellulopsis, Microdochium, Neopyrenochaeta , Phaeosphaeria, Pichia, Pyrenochaeta, Stenella , Verticillium, Vishniacozyma, Wallemia* as the most abundant ones.

According to some studies, the abundance of endophytes were found more in the roots than the stems and leaves [29]. However, in this study leaf-stem as well as the root sample showed almost a wide range of endophytic composition with the leaf stem sample contributing much more unique orders and families to the group. Relative abundance (Fig 1) shows that *Vishniacozyma, Wallemia* and *Cladosporium* are three genera that are commonly found in greater richness in both the samples. The relative abundance of *Vishniacozyma, Wallemia* and *Cladosporium* in the leaf stem sample is 26%, 18%, 4% and the belowground root sample is 23%, 27% and 22% respectively. All most all the isolated fungal genera were found to have bioactive properties. Cladosporium is described to have antioxidant, antimicrobial [30] and phyto beneficial properties [31], *Phaeosphaeria* is a GA producing fungus [32], *Microdochium* is reported to have bioactive isocoumarins [33], *Wallemia* is a halotolerant fungus [34], *Fusarium* and *Aspergillus* is reported to have antioxidant characters [35,36] and *Acremonium* is the one with antibiotic property [37].

Core microbiome analysis based on relative abundance (Fig 2) showed sample prevalence of 21 fungal genera at minimum detection threshold of 0.010% and a maximum of 0.238%. Plotting detection

threshold against OTU gives the most prevalent species in the sample which are *Vishniacozyma, Wallemia, Cladosporium*, *Verticillium, Pichia and Acremonium* with a prevalence of 1.0 at minimum threshold of 0.010%. *Phaeosphaeria, Microdochium, Pyrenochaeta, Cryptococcus, Fusarium, Stenella, Neopyrenocheata, Leptospora, Aspergillus, Acrocalymma, Trichosporon, Torula, Gibellulopsis, Coniothyrium* and *Colleotrichum* are the least prevalent species with a prevalence of 0.0 at minimum threshold 0.010%. similar results were observed were Core microbiome investigation exhibited that 17 genera showed a minimum detection threshold of 0.001% . Here, the core microbiome characteristic were presented by Clostridium, Desulfomicrobium, Advenella, Tindallia, Parabacteroides, Sedimentibacter, Desulfuromonas, Pseudomonas, Eubacterium, Tissierella, Trichococcus, Azospira, Thauera, Erysipelothrix, Alkaliphilus, Delftia, and Azoarcus at a minimum detection threshold of 0.001% (38).

In comparative analysis (Fig 3), Out of the total endophytes sequenced, 164 elements were common to both 1LSITS and 2RITS (39.2%) whereas 174 elements (41.6%) were unique to 1LSITS and 80 elements (19.1%) to 2RITS sample. A core microbiome of 164 fungal genera is common to leaf, stem and root habitat. There ar 174 unique fungal genera present in leaf and stem and 80 unique fungal genera in root. In another study of endophytic fungal diversity analysis, root and the leaf sample shared about 0.9% of the total endophytic the fungal genera in the plant. 6.8% unique fungal elements were present in the leaf whereas root constituted about 0.5% unique elements of the total endophytic fungi. The unique and shared mycobiome may have vital roles. This mycobiome could have been also present in rhizosphere soil if facultative endophytes are taken into account [39].

In the present study, endophytic fungal biodiversity analysis through illumina Miseq platform has helped in proper identification of a large number of fungal members to generic level. Many such biodiversity studies using NGS techniques have been done in bacterial endophytes related to medicinal plants [40] but not much with fungal endophytes. Culture dependent method of isolation is more common however, is delicate as it is greatly influenced by surface sterilisation techniques, incubation settings, isolation protocol etc [41] and only <1% of the total endophytes can be isolated through this technique [42] as some endophytes grow slowly and may be outcompeted by other fast expressing members on artificial nutrient media. To ignore all these potential constraints, molecular analysis techniques can be employed to trace out all the endophytic members and to further elucidate some specific candidates that would have significant role properties of the plant *Eclipta prostrata.*

CONCLUSION

Our results shows that *Eclipta prostrata* harbours a large number of endophytic fungi in the leaf, stem and roots which would help in the further characterisation of prominent fungal species and to trace the role of endophytes in plant growth promotion, stress tolerance and involvement in metabolic pathways. OTU analysis at taxonomic level clearly catalogues two different phyla viz. Ascomycota and Basidiomycota as the prominent ones. Subsequent findings point 17 generas viz. *Acremonium, Acrocalymma, , Aspergillus, , Cladosporium, Corynespora, , Cryptococcus, , Fusarium, Gibellulopsis, Microdochium, Neopyrenochaeta , Phaeosphaeria, Pichia, Pyrenochaeta, Stenella , Verticillium, Vishniacozyma* and *Wallemia* as the most prominent ones. Knowledge in both the culture dependent and independent endophytic species can open new vistas to unreveal the mystery behind the plants ethnomedicinal properties.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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