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Isolation and Characterisation of Endophytic Nitrogen Fixing Bacteria from Roots of *Cymbopogon* Species of Barak Valley, Assam

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Plant constitute vast and diverse niche for endophytic microbes. Endophytic bacteria reside within plants without causing disease symptoms. During the association the invading bacteria benefit the acquired host with a marked increase in plant growth, vigor and yield. The diversity of endophytic microbes to a large extent remains unexplored. In this study fresh roots of Cymbopogon species were used for the isolation of endophytic diazotrophic bacteria using standard methods. Cymbopogon species are widely used all over the India as an aromatic and medicinal plant. The most frequently endophytic diazotrophs isolated from Cymbopogon species are *Klebsiella pneumoniae, Bacillus pumilus, Pseudomonas fluorescence and Burkholderia cenocepacia*. Determining genetic relationship between the species or genera is very important for genetic improvement and phylogenetic studies. In silico study was conducted to understand the major evolutionary relationships among the endophytic bacteria of *Cymbopogon* species using nucleotide sequence of 16s ribosomal RNA with other bacteria obtained from GenBank.

Key words: Endophyte, Diazotroph, 16S rRNA, Diversity, In silico.

The Barak Valley of Assam is very rich in plant diversity, as a wide range of climate influences it. The genus *Cymbopogon* belongs to the grass family, Poaceae which is a very large cosmopolitan family. *Cymbopogon* comprises about 55 species, native to warm temperate and tropical regions of the world. *C. nardus* or citronella is used as a tropical application for rheumatism. The cultivation of citronella has potential to rehabilitate Jhum lands cultivation and for sustaining the livelihoods of the local people in Arunachal Pradesh. Citronella oil is a raw material for production of geraniol, citronellal, hydroxyl citronellal and similar high value perfumery bases. Hydroxy citronella has a sweet flowery odour and is being extensively used in manufacture of high value perfumes. The citronella is also used as – anti-infectious, antiseptic, antibacterial, antidepressant, antispasmodic, anti-inflammatory, deodorant, diaphoretic fungicidal, insect repellent (mosquito), stomachache, excessive perspiration, rheumatism and arthritic pains etc. The essential oil obtained from Cymbopogon citratus are geranial (40.9 %), neral (29.7 %), myrcene (11.3 %), linalool (1.7 %) and geranylacetate (1.6 %)[1].

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects[2]. Plants benefit extensively by harbouring these endophytic microbes and confer enhanced resistance to various pathogens[3] by producing

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antibiotics [4] and also produces unusual secondary metabolites of plant importance. Diazotrophic endophytic bacteria such as *Azoarcus* spp., *Herbaspirillum* spp. and *Acetobacter diazotrophicus* can infect the interior tissue of graminaceous plants without causing any pathogenic symptoms but do not survive in soil [5]. Objective of this study was to investigate the diversity of endophytic diazotrophs of *Cymbopogon* sp. and also to determine the genetic relationship between the different endophytic microbial species.

MATERIALS AND METHODS

Study site

The study was conducted in three districts of Barak Valley, Assam (lies approximately between $24^0$15' to $25^0$9'N latitude and $92^0$16' and $93^0$15'E longitude). Samples were collected randomly from 3 locations of Karimganj, 5 locations of Cachar and 3 locations of Hailakandi during summer, rainy and winter season of 2009 (February to December).

Isolation of endophytic diazotrophic bacteria

For isolation of endophytes, small root pieces were cut from the citronella plants and placed in sterile polyethene bags, labelled, transported in ice box to the laboratory and placed in a refrigerator at 4^0C. All samples were processed within 24h of collection. The roots were washed thoroughly in running tap water and air dried. Roots were cut into 1-2 cm long sections, rinsed with 70% ethanol for 30 seconds and then sterilized with 0.1% $HgCl_2$ for 3 minutes [6]. Root samples were rinsed three times in sterile distilled water and dried on sterile blotters under laminar airflow to ensure complete drying. Samples were placed in nitrogen free media. In each petri-dish 10-12 root segments were placed. The plates were incubated at 30 ± 2^0C for 48-72 hours. Pure cultures were obtained with further subculturing. Endophytic isolates were identified on the basis of culture charecteristics, morphology and biochemical charecteristics. Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al*., (2003)[7].

$$\text{Colonization frequency (\%)} = \frac{\text{Number of segments colonized by an endophyte}}{\text{Total no. of segments analysed}} \times 100$$

Genotypic charecterisation of endophytic bacteria

In the present study six endophytic bacterial strains (AU_SW3_M, AU_SC5_M, AU_SW2_M, AU_SW4_M, AU_SW5_M, SMEND03) were taken for 16s ribosomal RNA analysis. Sequencing of the gene was performed using universal primer. Sequences were examined and edited with the BioEdit Sequence Alignment Editor. The Basic Local Alignment Tool (BLAST) at the National Center for Biotechnology Information was used to search for similar known sequences. These sequences were aligned with Clustal X2. Phylogenetic and molecular evolutionary analysis with MEGA version 4.1. Neighbor joining consensus trees were obtained with the Kimura two-parameter substitution model and bootstrap test.

Nucleotide sequence accession numbers

The sequences obtained in this study were deposited in GenBank.

RESULTS

The physico-chemical analysis of soil from the three district is given in Table 1. The pH value ranged from 5- 6.8. Soil was found to contain very low concentration of organic carbon, nitrogen, phosphorus and potassium. During the study period rainfall was 1025mm, temperature range (15-35^0C) and relative humidity (>75%). Maximum rainfall occured between May to August.

Table 1. Physico-chemical parameters of soil of three districts of South Assam

Sampling site	pH	Organic carbon (%)	Moisture (%)	Nitrogen (%)	Phosphorous (kg/hectare)	Potassium (kg/hectare)
(Karimganjdistrict)	5.6±0.57	1.06±0.15	40	0.55±0.03	47.25±3.4	94.08±0.01
(Hailakandi district)	6.4±0.40	0.63±0.04	70	0.55±0.02	48.47±2.3	101±1.5
(Cachar district)	5.7±0.73	0.4±0.05	60	0.44±0.01	44.62±1.7	97.53±0.59

Table 2. Frequency of endophytic bacteria isolated from roots of citronella plant.

Endophytic bacteria	Frequency of colonization (%)		
	Cachar district	Karimganj district	Hailakandi district
Pseudomonas sp.	40	23	18
Klebsiella pneumoniae	23	52.75	—
Burkholderia sp.	12.5	6.76	13
Bacillus pumilus.	19.5	14.7	28
Bacillus cereus	13	32	20
Azospirillum sp.	5	1.79	39

Table 3. Genetic characteristics of endophytic diazotrophs isolated from Cymbopogon sp.

Bacterial strain	Plant tissue	Accession number	Hit in NCBI database	Max indent (%)
AU_SW2_M	root	JN210574	*Pseudomonas fluorescenes*	98
AU_SW3_M	root	JN375551	*Bacillus cereus*	95
AU_SC5_M	root	JN375552	*Klebsiella pneumoniae*	98
AU_SW4_M	root	JN210575	*Bacillus pumilus*	99
AU_SW5_M	root	JN210576	*Burkholderia cenocepacia*	99
SMEND3	root	JF838291	*Klebsiella pneumoniae 342*	95

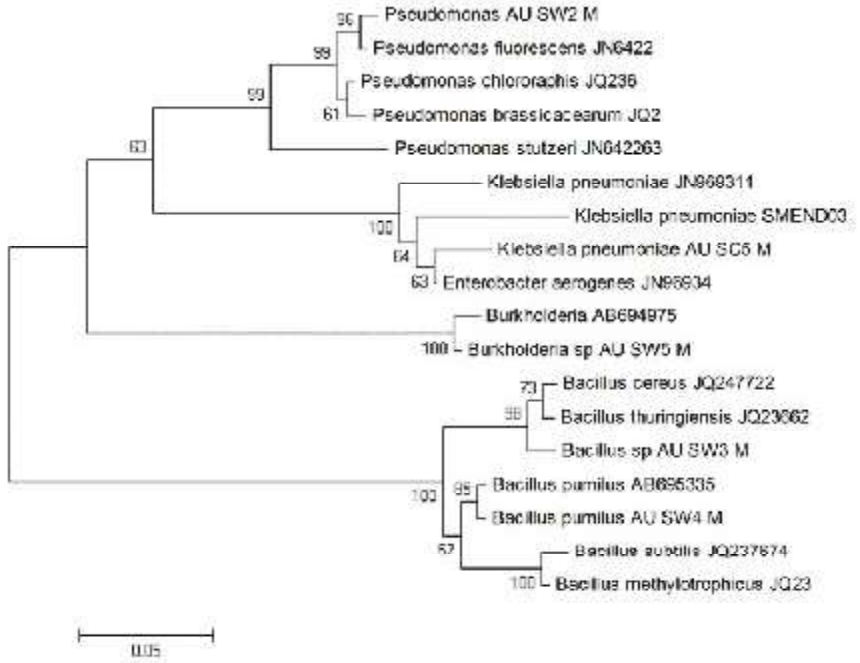

Fig. 1. Neighbour-joining tree of 16SrRNA gene sequences from endophytic bacterial isolates of Cymbopogon sp with 16s rRNA of other bacteria obtained from genebank. The Kimura two-parameter substitution model was used and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown

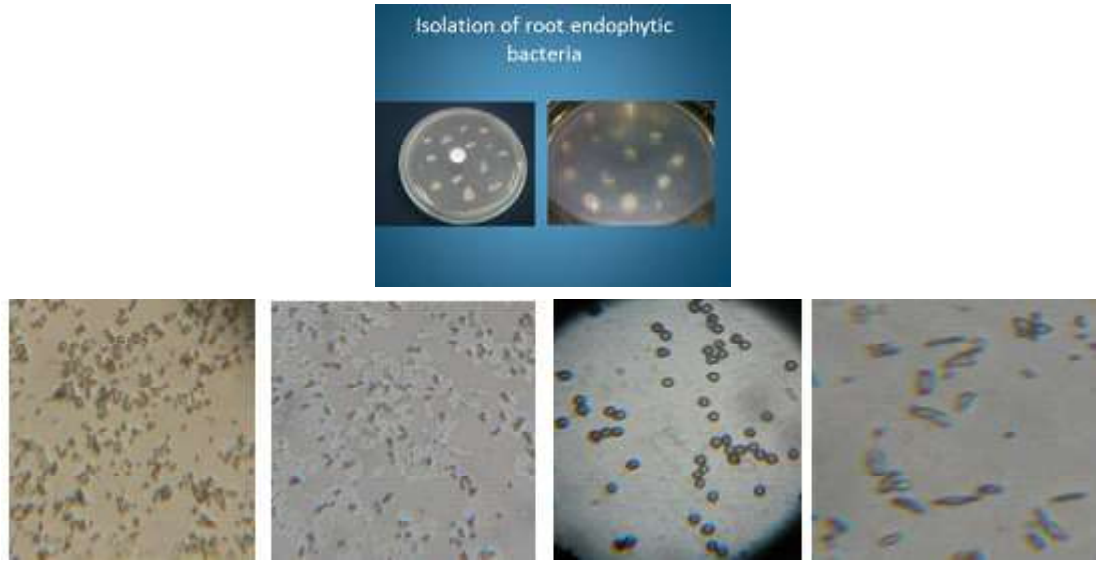

Fig. 2.

Eight hundred root segments from 25 plants of *Cymbopogon sp*. were processed for the isolation of endophytic diazotrophs. A total of five different diazotrophic bacteria belonging to six species were isolated. In Cachar district, highest population was found of *pseudomonas fluorescence* (40%) where as *Klebsiella pneumoniae* (52.75%) and *Azospirillum* sp.(39%) were higher in Karimganj district and Hailakandi district respectively.

DISCUSSION

Endophytes were abundant and diverse in the roots of *Cymbopogon* species in different environmental conditions.The endophytic nitrogen fixing bacterial community associated with *Cymbopogon species* harboured multiple genera with potential for plant growth promotion and disease control. The above endophytic diazotrophs have also been reported from grasses [8]. Danise *et al*., 2008 [9] isolated the genera such as *Klebsiella, Bacillus, Pseudomonas* etc. from different crop plants most of which are also recovered in the present study. The 16s rRNA gene sequences of the *Cymbopogon* isolates were compared to other corresponding sequences of bacteria from different host and locations obtained from Genebank database. A phylogenetic tree was generated based on 16s rRNA and depicted the phylogenetic divergence of the species in the tree. The phylogenetic tree showed four clades. 16s rRNA gene phylogenetic analysis suggested that the endophytic *Pseudomonas* strain isolated from *Cymbopogon* was closely related to *Pseudomonas fluorescence* type strain. *The Klebsiella* strain (SMEND3 and AUSC5M) showed 100 bootstraping value with *Klebsiella pneumoniae* strain. *Bacillus* strain (AUSW4M) isolated from *Cymbopogon* sp. closely related to *Bacillus pumilus* with bootstrap value 85. According to the 16srRNA based phylogenetic tree, it can be interpreted that *Bacillus* was the most ancient species whereas *Pseudomonas sp*. was the most recent one.

CONCLUSION

From the present study it can be concluded that the Cymbopogon nardus or citronella plant forms an diverse niche for endophytic microbes.These beneficial endophytic diazotrophic bacteria can be utalized as potential PGPRs.

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