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Research Article

Rhizobacterial Diversity of Umorok in Juvenile, Flowering, and Fruiting Stages

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Abstract

Rhizosphere soil of Umorok, *Capsicum chinense*, was collected from juvenile, flowering, and fruiting stages to study the distinct bacteria and plant development associations. Soil samples were collected from three growth stages, serially diluted, and retrieved the pure cultures. Genomic DNA was isolated from the bacteria and amplified the 16S rRNA partial gene sequence for sequencing information. Bacteria were characterized based on the sequencing data by searching in nucleotide database. Phylogenetic tree was constructed to interpret the evolutionary relationship and diversity of the rhizobacteria. Several rhizobacteria were molecular characterized, and 127 novel bacterial strains were identified. Bacterial grouping revealed alphaproteobacteria, betaproteobacteria, gammaproteobacteria, actinobacteria, and firmicutes. Among the phyla, gammaproteobacteria were dominantly present, and majorly Pseudomonas was found. Stage specific dominant bacterial analysis of Umorok confirmed that juvenile stage was associated with 40% of Agrobacterium and Rhizobium, flowering stage was associated with 50% of Pseudomonas and Burkholderia, and fruiting stage was associated with 50% of Burkholderia. Betaproteobacteria were found to be absent in the juvenile stage. Phylogenetic tree was constructed from the representative bacterial strains of each phyla using maximum likelihood method. Phylogenetic tree provided five distinct bacterial phyla and two clusters of gram positive and negative bacteria. The results of this study provided the comprehensive and specific rhizobacterial diversity in three growth stages of the Umorok. During the plant development, rhizobacterial community was changed. The stage specific diversity facilitates the rhizobacterial application in plant growth and health improvement.

INTRODUCTION

Plants access not only nutrients from the soil through roots but also form a symbiotic association with plant growth promoting rhizobacteria [1,2]. The distinct rhizosphere bacterial populations facilitate the plant growth and inhibit pathogenic fungi and bacteria from the surrounding region. Because plants selectively recruit the rhizobacteria [3], various developmental stages and ecosystems constitute specific bacteria [4,5]. Plants associate with a unique set of diverse rhizobacteria during the juvenile; flowering; and fruiting stages. Molecular evolutionary pattern of the rhizobacteria signifies the microbial signature; adaptation; and diversity during the growth stages [6]. Diverse rhizobacteria and plant root interactions accelerate the growth and yield traits and competitively inhibit the undesirable bacteria from the rhizosphere.

Umorok; *Capsicum chinense*; is an indigenous chilli variety of Northeast India and contains high capsaicin and pungency [7]. Effective HPLC methods were developed to measure the capsaicinoids [8], impact of environment and the genotypic variants on the capsaicinoid production in hot pepper hybrids was studied [9], and chemical profile and components of fruits were enlisted [10,11]. Genetic parameters of fruit traits [12], diseases [13-15]; and sustainable irrigation methods that are suitable for *Capsicum chinense* were described [16]. However; plant growth promoting rhizobacterial diversity of Umorok is yet to be studied.

Rhizobacteria of rice; maize; and chickpea were explored and used as plant growth promoting bacteria to improve the root and shoot system; and commercially important traits [17,18]. Additionally; rhizobacteria were used as an antagonist; biofertilizer; and biocontrol agent to inhibit the diseases and insect pests and to promote the plant growth [19-21]. Comprehensive understanding of rhizobacterial diversity of the Umorok enables to identify critical microbes and potential applications.

MATERIALS AND METHODS

Isolation of rhizobacteria

Umorok seeds were collected from the local farmer in Imphal; Manipur; India to interpret the associated rhizobacterial diversity. Seeds were sterilized with 70% ethanol and 4% hypochlorite; sprouted on 1% agar; and transferred to unsterilized soil in the pots. Umorok growth was divided into juvenile; flowering; and fruiting stages to collect the stage specific rhizosphere associated bacteria. Rhizoplane separates the ectorhizosphere or surrounding soil from the plant root system. Since the study focuses on rhizobacterial diversity in the

ectorhizosphere; unattached and unassociated soil was removed manually. Rhizosphere soil of three growth stages was collected in sterile water; briefly vortexed; and sonicated to suspend the bacteria. The turbid water with rhizobacteria containing soil was centrifuged for 5 min at 8000x g. The bacteria were serially diluted from the 1 g of soil (10^{-5}) and plated on Luria Bertani Agar; King's B Medium; Pikovskaya's Agar; Tryptic Soy Agar; Brain Heart Infusion Agar; and Rojo Congo Medium. Bacteria were incubated at 30°C for 2-3 days to facilitate the growth; morphologically distinct colonies were subcultured to obtain the pure cultures.

Molecular characterization of bacteria

Rhizobacterial pure colonies were cultured overnight in liquid media; and standard lysozyme method was used to isolate the genomic DNA from the bacteria [22]. For molecular characterization of rhizobacteria; 16S rRNA gene was amplified using the reported 63F and 1387R primers [23]. The PCR product was sequenced using Sanger DNA sequencing method and searched in the National Center for Biotechnology Information (NCBI) nucleotide database using Basic Local Alignment Search Tool (BLAST). Novel bacterial strains were submitted to the NCBI – Nucleotide database.

Rhizobacterial diversity analysis

Sequencing result of partial 16S rRNA gene was used to deduce the evolutionary relationship of Umorok rhizosphere bacteria. Representative DNA sequences of diverse phyla from the sequencing result were used to construct the phylogenetic tree. Maximum likelihood method was used to construct the molecular phylogenetic tree following the reported method [24], in the MEGA platform [25], and the diversity was interpreted from the clusters of the tree.

RESULTS

Rhizosphere soil was collected from three growth stages of the chilli plant (Figure 1A) and serial diluted to obtain the pure bacterial cultures. As many as 300 pure bacterial cultures were isolated to extract the genomic DNA to amplify the 16S rRNA gene. Amplified product was sequenced and searched the sequence in NCBI nucleotide database; and bacteria were characterized based on the sequencing result. We identified 127 novel bacterial strains and submitted to the NCBI nucleotide database; accession numbers: KY038202 – KY038328. Juvenile flowering and fruiting stages were associated with 221 different varieties of bacteria (Figure 1B). Intermediate flowering stage was associated with highest number (77) of rhizobacteria; while juvenile (75) and fruiting stage (69) contained less bacteria. More number of rhizobacteria was observed in flowering stage followed by juvenile and fruiting stage. If the individual share of the bacterial genus was concerned in the growth stages; 40% of the juvenile plant bacteria were Agrobacterium and Rhizobium; 50% of the flowering plant bacteria were Pseudomonas and Burkholderia; and 50% of the fruiting plant bacteria were Burkholderia.

We grouped the isolated bacteria into phyla to identify the number of unique set of bacterial phyla that were associated with the Umorok. Grouping revealed five bacterial phyla: alphaproteobacteria; betaproteobacteria; gammaproteobacteria; actinobacteria; and firmicutes; however; the number of phyla was found to be different in each growth stage (Figure 2). More number of gammaproteobacteria was found in all the three growth stages whereas firmicutes were seen less. Interestingly; betaproteobacteria were completely absent in juvenile stage but present in other two stages. Results also confirmed the severalbetaproteobacteria and firmicutes in flowering and fruiting stages respectively. Unlike rhizobacterial number which is more in flowering stage; rhizobacterial diversity; number of distinct rhizobacteria; were found more in juvenile stage. Although diversity appeared different in three growth stages; number looks seemingly close (69-77) in all the three plant stages. Nevertheless; bacterial colony count from each stage unveiled the high bacterial density (load) in fruiting stage followed by juvenile and flowering stages.

Molecular phylogenetic tree was constructed from the amplified product of partial 16S rRNA gene. Despite we isolated about 300 microorganisms from the Umorok rhizosphere soil; phylogenetic tree was constructed from the representative phyla to show the sensible rhizobacterial diversity. Because DNA sequences of a specific genus form single cluster; only the single bacterial genus was opted instead of multiple species and strains of the same kind. Maximum likelihood method was used to construct molecular phylogenetic tree to infer the rhizobacterial diversity. Bacteria formed five distinct clusters: alphaproteobacteria; betaproteobacteria; gammaproteobacteria; actinobacteria; and firmicutes (Figure 3). It was evident from the phylogenetic tree that 16S rRNA gene sequence information has the capacity to distinguish gram positive and negative bacteria. Phylogenetic tree was constructed for all novel bacterial strains that we isolated from the Umorok rhizosphere. Noticeably; we identified two large clusters that formed the five bacterial phyla in each clade. 16S rDNA based molecular characterization of Umorok rhizobacteria substantially produced bacterial diversity with adequate evolutionary relationship.

DISCUSSION

Root system of juvenile; flowering; and fruiting stages penetrate into distinct layers of the soil to uptake the nutrients and water. Rhizobacteria is expected to vary in each growth stage of Umorok like other plant species [26]. Growth specific and diverse rhizobacteria associated with plant root system anticipated to affect the plant growth and health [27]. Rhizobacteria were isolated from the juvenile; flowering; and fruiting stages of the Umorok in the soil of Northeast Indian Territory. Wide number of rhizobacteria were observed in juvenile stage; but increased diversity; distinct bacterial species and strains; was associated with fruiting stage. Although these findings are detailed and incomparable; earlier growth stage of maize and flowering stage of wheat were individually studied for the dominant and diverse rhizobacteria in the similar fashion [28,29]. While genus Pseudomonas; gammaproteobacteria; was predominantly present across the stages in Umorok; Juvenile; flowering; fruiting stages were specifically dominated with Agrobacterium and Rhizobium; Pseudomonas and Burkholderia; and Burkholderia respectively. Pseudomonas is one of the dominant soil microflora of Northeast India [30], hence; its presence in Umorok rhizosphere is an obvious attribute. Members of Pseudomonas in

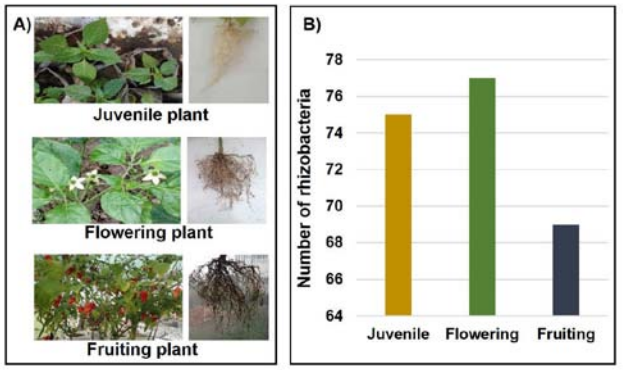

Figure 1 Three different plant growth stages of Umorok were associated with distinct number of rhizobacteria: A) The root system of juvenile; flowering; and fruiting stages appeared in different lengths and mature stages; B) Number of distinct rhizobacteria that were recovered from three growth stages – Juvenile; flowering; and fruiting stages contained 75; 77; and 69 rhizobacterial varieties.

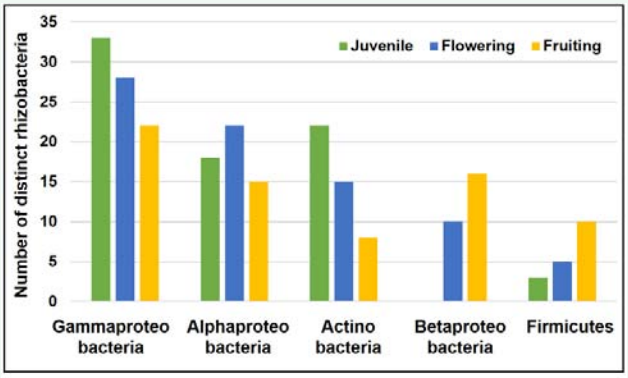

Figure 2 Rhizobacterial diversity of Umorok plant displayed five distinct bacterial phyla. Gammaproteobacteria were found more in all the three growth stages; particularly in juvenile stag; while betaproteobacteria were absent in juvenile stage. Betaproteobacteria and firmicutes were predominantly associated with flowering and fruiting stages.

the rhizosphere is an indicative feature of the plant health and protects from fungal infections [31]. Firmicutes were abundant in fruiting stage and these results are seemingly consistent with the existing evidence that plant changes rhizomicrobiome according to development [32]. We identified wide range of bacterial species and strains at the Umorok ectorhizosphere based on the 16S rRNA partial gene sequence; the sequencing data was used to construct the phylogenetic tree. Rhizobacteria were clustered into five distinct phyla as well as two groups of gram positive and gram negative bacteria. These results supported the rhizobacterial diversity and evolutionary relationship similar to the reported results [24]. In this study; we presented comprehensive evidence of Umorok associated rhizobacteria; identified rhizobacterial diversity based on 16S rRNA gene sequence; interpreted dominant bacterial species in general and plant growth specific microbes in particular; and established evolutionary relationship to support the diversity.

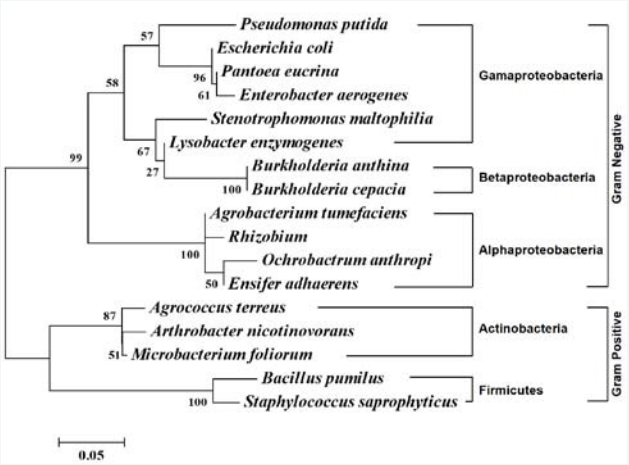

Figure 3 Rhizobacterial diversity of Umorok was inferred from the molecular phylogenetic analysis. Maximum likelihood method with Kimura 2-parameter model was used to construct the scaled phylogenetic tree. Gaps and missing data was partially ignored to analyze 17 nucleotide sequences.

CONCLUSION

Umorok; *Capsicum chinense*; is an indigenous chilli variety of Northeast India. We investigated complete rhizobacterial profile and diversity based on 16S rRNA gene sequencing; identified 127 novel rhizobacterial strains; and submitted to NCBI nucleotide database. The complete picture of the rhizosphere bacteria not only facilitates the insights into the microbial diversity associated with various growth stages of the plant but also provides the typical soil flora of the territory. Gammaproteobacteria Pseudomonas was dominated in juvenile; flowering; and fruiting stages of the plant. We clearly identified the stage specific predominant rhizobacteria and reported the bacterial load and diversity in each growth stage. Stage specific bacterial association with plant revealed the complete absence of betaproteobacteria; which signifies that plant changes the bacterial diversity during different developmental stages. Molecular phylogeny of rhizobacteria established and supported the evolutionary relationship and diversity. Findings of this study facilitate the rhizobacterial formulation to improve the plant growth and health in the further studies.

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