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Diversity and Molecular Characterization of Dominant *Bacillus amyloliquefaciens* (JNU-001) Endophytic Bacterial Strains Isolated from Native Neem Varieties of Sanganer Region of Rajasthan

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Abstract

Higher plants are ubiquitously colonized with bacterial endophytes that often lack readily detectable structures. In the present study seven novel endophytic bacterial species viz; *Bacillus amyloliquefaciens* (JNU-001), *Burkholderia denitrificans* (JNU-002), *Pseudomonas aeruginosa* (JNU-003), *Xanthomonas campestris* (JNU-004), *Azotobacter tropicalis* (JNU-005), *Acetobacter xylinum* (JNU-006) and *Azospirillum lipoferum* (JNU-007) has been recovered from native neem varieties at Sanganer areas namely Khonagorion, Muhana, Jagatpura, Goner, Watika, Kalwara and Bagru of Rajasthan state. In which *Bacillus amyloliquefaciens* was found dominant and associated endophytically in all the plants. Molecular characterization of the isolates was performed using 16S r-DNA restriction analysis. Similarity index in unweighted pair group method with arithmetic mean programme clustered the isolates according to their geographical distribution and confirmed the endophytic association between these novel bacterial strains and neem varieties.

Keywords: Endophytes; Nutrient agar; UPGMA analysis; NTSYS; ARDRA

Introduction

Microorganisms have long served mankind by virtue of the myriad of the enzymes and secondary metabolites that they produce. The diversity of microbial life is enormous and the niches in which microbes live are truly amazing, ranging from deep ocean sediments to the earth's thermal pools.

One specialized and unique biological niche that supports the growth of microbes is the intracellular space between cells of higher plants. It turns out that each plant supports a suite of microorganisms known as endophytes [1]. Many plants depend on a symbiotic relationship with nitrogen-fixing microorganisms for proper growth. These microorganisms do not cause overt symptoms on the plants in which they live. Further more, because so little work on these endophytes has been done, it is suspected that untold numbers of novel fungal and bacterial genera exist as plant associated microbes and their diversity might parallel that might have the higher plants.

According to the World Health Organization (WHO) about 70% of the human population uses medicinal plants in their primary health care and neem is one of the most important medicinal plants out of these. The recent studies suggest that endophytic microorganisms isolated from medicinal plants do produce the same metabolites as their hosts. Therefore, there is a great potential in exploring endophytes as a source of therapeutic agents.

Rajasthan is one of the twenty hot spots of global biodiversity with approximately 5,000 species of distinguished plants. Endophytes of neem are considered as a novel source of useful medicinal compounds [2]. The xerophytic conditions of the rajasthan accommodated a large number of endophytes and this area has not been explored to understand the role of these microbes in host plants. Therefore the present study was aimed to isolate and identify the bacterial endophytes from the native neem varieties [3].

Nitrogen-fixing microbes inhabit varieties of tissues and organelles in plants such as seeds, roots, stems and leaves [4]. Plants are benefited extensively by harbouring these endophytic microbes and confer enhanced resistance against pathogens [5-8] by introducing antibiotics and siderophores [9,10].

These endophytes from different environmental, geographical and physiological conditions may have resulted in allopatric polymorphism. The present study aims to characterise morphological, biochemical and plant growth promotory (PGP) properties of the isolates. The ribosomal RNA genes of bacteria, especially those for 16S and 23SrRNA, are excellent molecular markers for phylogenetic studies because of their functional constancy and their ubiquitous distribution [11].

Materials and Methods

Site description and Sample collection

Neem plants from the entire seven sites of sanganer region (Khonagorion, Muhana, Jagatpura, Goner, Watika, Kalwara and Bagru) especially those with an unusual biology and possessing novel strategies for survival were selected for the study. The plant samples were collected in sterilized polythene bags, during the months of July-November [3].

Isolation of bacterial endophytes

The roots, leaves and stems were used as explants for isolation of bacterial endophytes. All explants were surface-sterilized by dipping in

75% ethanol for 1 minute, 4% sodium hypochlorite for 5 minutes followed by rinsing three times in sterilized double distilled water. In each Petri dish (9 cm diameter), a total of four-five processed explants were evenly spaced onto the surface of Nutrient Agar (NA) media supplemented with 200 µg/ml griseofulvin. Cultures were incubated at 37°C and observation was recorded regularly. The emerging bacterial colonies appeared on the plates were carefully isolated, sub-cultured and pure culture were maintained [7,8].

Identification of bacterial endophytes

The isolated bacterial endophytes were identified on the basis of morphological features like colony characterization, growth on different media, colour of colony size, shape and staining properties. Bacterial isolates appeared on semisolid nutrient agar medium and plates showed typical light or heavy orange yellow surface pellicle on the medium. Gram staining and morphological studies were performed on the isolates [5-8].

Frequency distribution and colonization rate

Data analysis was carried out on the basis of Colonization rate (%) of bacterial endophytes which was equal to the number of segments colonized by a single endophyte divided by the total number of segments observed X 100 [3].

Functional Characterization of the Bacterial Isolates

Antibiotic sensitivity test

One millilitre of actively growing bacterial cultures was pour plated in nutrient agar plates. Antibiotic discs (Himedia) of Ceftriaxone (Cf30; 30 lg/disc), Chloremphenicol (Cl30; 30 lg/disc), Tetracyline (T30; 30 lg/disc) and ampicillin (Ac5; 5 lg/disc), were placed at four corners of solidified plates. Plates were incubated for 2–3 days at 30 ± 2°C. Inhibition zones appeared after the incubation was noted for individual organisms and antibiotic(s).

Cellulase activity

Freshly growing bacterial culture(s) were spot inoculated on nutrient agar plates supplemented with 0.2% carboxy methyl cellulose (CMC), plates were incubated at 30°C for 3–5 days and were overlaid with Congo-red (1 lg/ml) solution for 15 min. After washing the plate surface with 1 M NaCl, clear zone around colony indicates cellulose production.

Gelatin hydrolysis

Nutrient gelatin medium was inoculated with a loopful of actively growing bacterial culture and incubated for 3 days at 30°C. Control tubes solidified when placed in ice whereas medium in inoculated tubes remained unsolidified, showing positive gelatin hydrolysis test.

Starch hydrolysis

Nutrient agar plates supplemented with 0.3% soluble starch were inoculated with actively growing bacterial culture(s) aseptically and incubated for 3 days at 30°C. When flooded with Gram's iodine, a clear yellow zone around the inoculation spots indicates starch hydrolysis.

Casein hydrolysis

Skim milk agar (Skim milk powder 100 g/l, agar 15 g/l) plates were spot inoculated with actively growing culture(s) aseptically and incubated for 3 days at 30°C. Clear zones around the inoculation spots indicate casein hydrolysis.

Catalase activity

Yeast extract tryptone broth tubes, inoculated with actively growing bacterial culture(s) were incubated for 3 days at 30°C. Catalase activity was observed by adding few drops of 3% H_2O_2 to the broth cultures, kept on the glass slides. Formation of oxygen bubbles confirms the positive result.

Production of H_2S

SIM agar [Peptone 30 g/l, Beef extract 3 g/l, ferrous ammonium sulphate 0.20 g/l, sodiumthiosulphate 0.025 g/l, agar 3 g/l (pH 6.0)] stabs were inoculated with actively growing culture(s). Blackening along the line of inoculation shows a positive test after 3–5 days at 30°C.

Molecular characterization of bacterial endophytes

Assessment of genetic diversity among seven endophytic bacterial isolates of *B. amyloliquefaciens* isolated from different ecological niche were carried out using PCR based 16S rDNA amplification. Amplified 16 S rDNA restriction analysis of the amplicons (ARDRA) offers a powerful tool for bacterial species identification. The 16S rDNA amplicons of the isolates and standards were restriction digested individualy with Alu I, Msp I, Hae III and Mnl II.

16S rDNA amplification of genomic DNA

Genomic DNA was isolated from all the bacterial isolates and was used as template for PCR. Primers used for the amplification of part of 16S rDNA were 16SF (5'- Ag A gTT TgA TCM Tgg CTC-3') and 16SR (5'- AAg gAg gTg WTCCAR CC-3') and were selected based on the previous reports of Chun and Goodfellow (1995). PCR was carried out in a 50 lL reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 lM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a MycyclerTM (Bio-Rad, USA) with the initial denaturation at 94°C for 3 min, cyclic denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 2 min with a final extension of 7min at 72°C. The PCR product was checked by agarose gel electrophoresis, purified and was further subjected to sequencing. The sequence data was checked by BLAST analysis [12].

ARDRA – Amplified 16S rDNA restriction analysis of the amplicons

16S rDNA amplicons were digested separately with four restriction enzymes in 25 µl reaction volume containing, Amplicon 20 µl, Digestion buffer 2.5 µl (10X), Enzyme 0.1 µl and the rest being TDW (triple distilled water). Reaction mixtures containing Alu I, Msp I, Hae III and Mnl II each were incubated at 37°C for 3 hours.

Phylogenetic analysis

ARDRA profile of the isolates was analysed on 2.5% agarose gel. The specific band pattern was scored with binary characters

(1=presence and 0=absence, of bands) Amplification products were scored from the gel images as presence or absence of bands. Each band was treated as one marker. Homology of bands was based on thedistance of migration of amplified DNA fragments according to their molecular weights in the gel. The presence of band percentage of the total number of bands produced in fingerprinting profiles. Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method Analysis, a sub programme of online software http://genomes.urv.cat/ UPGMA/index.php?entrada=Example2 clustering method [13]. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the clones using computer program NTSYS pc version 2.02 [14].

Results

Isolation of bacterial endophytes from Neem

Based on the reported growth characteristics of seven suspected bacterial endophytes from different neem samples were recovered, purified and assigned the respective codes (Table 1). These were observed for their shape, size, pigmentation and margin along with the colony/cell morphology of individual isolates (Table 1). All the isolates were able to grow in semi-solid LGIP medium (pH 5.5), indicating the necessity of acidic environment for these cultures [15]. Heavy or moderate mucous secretion was observed for all the isolates, which might help to maintain optimum O_2 concentration without inhibiting nitrogenase activity and cell metabolism [16].

Characteristics	JNU-001	JNU-002	JNU-003	JNU-004	JNU-005	JNU-006	JNU-007
Location	Khonagorion	Muhana	Jagatpura	Goner	Watika	Kalwara	Bagru
Explant	Stem	Root	Stem	Root	Root	leaf	leaf
Colony morphology							
Configuration	Round	Round	Oval	Oval	Round	Round	Oval
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Pigmentation	Creamy	Light Creamy	Creamy	Creamy	White	Creamy	Creamy
Cell Morphology							
Gram's reaction	_	_	_	_	_	_	_
Shape	Long rods	Long rods	Short rods	Short rods	Short rods	Short rods	Short rods
Arrangement	Single	Single	Single	Single	Paired	Single	Single
Biochemical Characteristics							
H2S Production	+	+	+	+	+	+	+
Gelatin Hydrolysis	_	_	_	_	_	_	_
Casein Hydrolysis	_	_	_	_	+	+	_
Starch Hydrolysis	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Cellulase production	_	_	_	_	_	_	_
Urease production	+	_	_	_	_	+	_
Endospore formers	+	_	_	_	_	_	_

Table 1: Characteristic features of different isolates. (Positive test +, Negative test --).

Biochemical characterization of bacterial endophytes

In biochemical analysis of 7 (JNU 001-JNU 007) isolated bacterial endophytes, all the isolates formed acids by fermenting glucose out of which 5 were found positive with gas production. On contrary, only two isolates gave positive result for production of acid from lactose. All the isolates were hydrolysing starch and producing H_2S and none of the isolates showed any response towards gelatin hydrolysis and cellulase production (Table 1). Five isolates out of seven showed casein hydrolysis (Table 1). Catalase activity was shown by all the isolates. Hence these properties can be ascertained as an index of similarity or relatedness among the isolates and also signifies their agricultural importance (Figure 1 A-F).

Antibiotic susceptibility pattern of bacterial endophytes

Out of 7 isolates, 5 were sensitive to ampicillin, 1 was sensitive to chloremphenicol, and tetracycline respectively (Figure 1D).

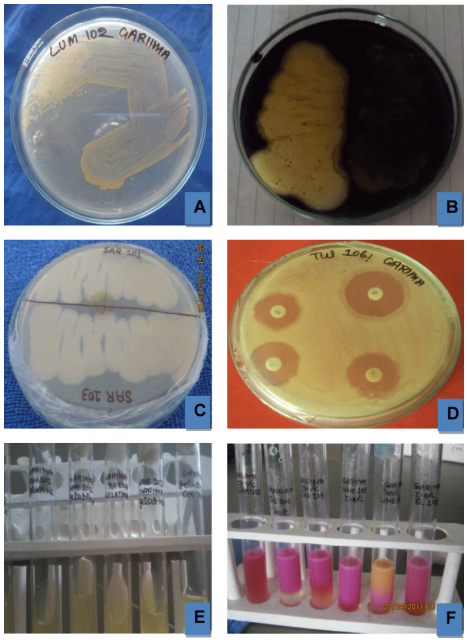

Figure 1: A - Pure culture of the endophytic bacterium B. *amyloliquefaciens* isolated from Neem; B - Starch hydrolysis by the *B. amyloliquefaciens*; C- Casein hydrolysis by the *B. amyloliquefaciens*; D - Antibiotic sensitivity assay by the *B. amyloliquefaciens*; E - Gelatin hydrolysis by the endophytic bacterial isolates ; F - Mannitol utilization by the endophytic bacterial isolates

Molecular characterization of the isolates using ARDRA

ARDRA and subsequent UPGMA analysis offers a powerful tool for bacterial species identification. 16S rDNA amplicons of the isolates and standards were restriction digested individually with Alu I, Msp I, Hae III and Mnl II. For any isolate, to belong to a given species, there must be at least 80–85% similarity based upon unweighted pair group method analysis and for strain level identification there must be 98-100% similarity required. Since, *Bacillus amyloliquefaciens* showed dominant endophytic association as compared to other endophytic bacterial communities (JNU 001-JNU 007). Therefore the seven *B. amyloliquefaciens* strains were further investigated for their genetic diversity in nature.

UPGMA cluster analysis resulted in two major clusters based upon the similarity index calculated using Jaccard coefficient from 16s r DNA amplification alienated 7 isolates (Figure 2). The first group is further divided into 2 subgroups (A & B). Sub group A has 3 isolates [11,16,17] whereas, subgroup B contain 2 isolates [4,6]. Group second contain only 2 isolates (Figure 2). This has confirmed that the *B. amyloliquefaciens* bacterial communities association with the neem varieties.

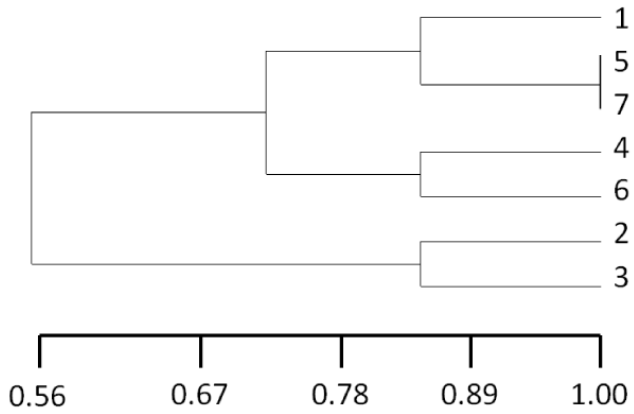

Figure 2: Dendrogram showing the UPGMA clustering of 7 isolates of *Bacillus amyloliquefaciens*.

Discussion

The bacillus species was previously reported to be capable of nitrogen fixation, Ration et al. [18] and Rana et al. [19] worked with these strains which indicated suitable environments for searching new inoculants for biotechnological purposes.

The present work explores the new strains of Bacillus amyloliquefaciens, (JNU001-JNU007) which are dominantly associated with the neem plants. These strains can fix the atmospheric nitrogen as they carry the nif H genes and results confirmed there endophytic diversity within these native neem varieties. Therefore these strains can be utilized for the exploitation of biotechnological purposes.

Conclusion

Endophytic bacterial communities from different habitats are indeed different: communities associated with Sanganer region of Rajasthan are poorly investigated group of microorganisms that represents an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agriculture and industrial arenas. The results from the study demonstrated the diverse community of endophytic bacteria associated with sanganer region of rajasthan. Among these endophytic bacterial isolates obtained, Bacillus amyloliquefaciens (JNU-001) was dominant and diversified in all the neem plants. Seven strains of Bacillus amyloliquefaciens (1-7) were molecularly investigated and shown endophytic diversity in all the neem plants of sanganer region. This assemblage must be plant growth promoting and interacting with these neem varieties in many ways. The mechanism through which endophytes exists and respond to their surrounding must be better understood in order to be more predictive about which higher plants to seek and spend time in isolating microfloral compounds. This may facilitate the product discovery processes. Certainly, one of the major problems facing the future of endophyte biology and natural-product discovery is the rapid diminishment of rainforests, which holds the greatest possible resource for acquiring novel microorganisms and their products.

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