MOLECULAR INVESTIGATION OF ENDOPHYTIC BACTERIA ISOLATED FROM ROOT, STEM AND LEAVES OF *CITRUS LIMON*

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The German botanist *Heinrich Friedrich* described endophytes first in 1809. Endophytes are optional or obligatory symbiotic microorganisms, primarily bacterial and fungal species, which reside in healthy internal plant tissues and have not caused the plant any disease. (Schulz andBoyle., 2006). Endophytes are known to supply nutrients to plant by fixing atmospheric nitrogen and solubilizing ion which protect plant from infection by plant pathogens, increase in plant immune and have a role in removal of soil contaminants (Barac*et. Al.,* 2004)(Doty *et al*., 2009). An important area to explore significance of endophytes and its role in plant metabolism. From plant to plant and from species to species endophytic population was known to vary. Similar species of plant may also show different endophytic population occurring at different regions. occurrence of entophytes are affected by temporal and climatic changes (Dhanya Nair and Padmavathy., 2014). Scientists use the cultivation and non-cultivation method to research the plant-bacteria relationship.The cultivation method has some disadvantages as the bacteria can only be available for cultivation if they are metabolic and in vitro physiological needs can be produced (Nadkarni *et al*., 2009).Non-cultivation approach was used to amplify the 16S rRNA gene from the plant's metagenome sample using polymerase chain reaction (PCR). (EL-Ghareeb, D *et al*., 2012). Several studies have recorded the isolation of endophytic bacteria from different plant tissues, including roots, stems, leaves, flowers and seeds. (Rosenblueth andMartínez.,2006) (Compant *et al*., 2011). Bacterial endophytes play a major role in improving plant growth, increasing biotic tolerance and the development of secondary metabolites. (Hardoim,*et al*., 2015) (Khan.*et al*., 2016) (Hassan., 2017)

Lemon, *Citrus limon* (L.) Osbeck, is a small evergreen tree species native to South Asia in the flowering plant family Rutaceae. Lemon is a rich source of vitamin C which provides 64% of the daily

value in a reference amount of 100 g. There is a small proportion of other basic nutrients (Pennisto*net al*., 2008).

Recent progress in experimental techniques and methods such as the use of 16s rRNA engineering in molecular biology is gradually contributing to a better understanding of the nature of endogenous bacteria isolated from the environment. This concept paves opportunities to investigate the interactionsbetween endogenous bacteria and their host plants in-depth.

The present study aimed to study and identify endophytic bacteria present in the tissue of roots, stem and leaves of *Citrus limon* from Saudi Arabia and to evaluate these endophytes.The technique of 16s rRNA for partial sequencing is accomplished to identify the isolated strains.This research also focuses on obtaining a comprehensive picture of the endophytic bacterial population present in an arid habitat growing plant of *Citrus limon*.

MATERIALS AND METHODS

Location of the Study

Plant samples were collected from Saudi Arabia – Makkah Almokkarama city(21°26'N / 39°46'E). These study areas belong to a hot desert climate. Makkah Almokkarama retains its hot temperature in winter, temperature range from °C (64 °F) at night to 30°C (86 °F)during the day light. Summer temperatures are extremely hot, often being over 40 °C (104 °F) during the day light , dropping to 30 °C (°F) at night ("Mecca, Saudi Arabia - Climate data" 2017).

Collection of plant samples

For the present study healthy fresh flowering plants of *Citrus limon* plant species were carefully selected, removed and were collected in previously sterilized zip lock poly bagsfrom different cultivation area in Makkah.

Pre-treatment

Endophytes bacteria were isolated from root, stem and leaves of the healthy flowering *Citrus limon* plant. The isolation was carried out from the plant immediately after collection. The plant samples were washed under running tap water for 10-15 min. to remove adhering soil particles, air-dried and roots, stem and leaves were separated out. The separated plant roots, stem and leaves were weighed up to one gram on a weighing balance and then were rinsed three times with distilled water in the laminar air flow cabinet.

Surface sterilization

Surface sterilization is the initial and mandatory step for isolation of endophyte in order to kill all the surface microbes. It is usually accomplished by treatment of plant tissues with oxidant or general sterilizing agent for a period and then by 3-5 times sterile rinse. The most commonly used isolation procedures combine surface sterilization of the plant tissue, plating small sterilized segments onto nutrient agar and by the maceration

of the plant tissue and streaking onto nutrient agar, or vacuum or pressure extraction. Theoretically, the sterilizing agent should kill any microbe on the plant surface without affecting the host tissue and the endophytic microorganisms. Though, this is challenging to achieve because the conditions required to kill the any microbe on the surface may already be lethal for some endophytic microorganisms and in time the agent may penetrate the plant tissue.

The weighed samples were soaked in distilled water and drained. The samples were then surface-sterilized by dipping in 70% ethanol for 1 minute, then (2 %sodium hypochlorite for 5 minutes 3 times then treated with 70% ethanol for 30 sec. followed by rinsing five times in sterilized distilled water. The surface sterilized samples were blot-dried using sterile filter paper. The samples are treated with the same method by different concentration of sodium hypochlorite (5 and 10%) (Anjum and Chandra., 2015).

Test for effectiveness of surface sterilization

Only after complete surface sterilization of the plant tissue is confirmed, then the isolated microorganisms are said to be endophytes. Validation of the surface sterilization procedure was done by imprinting the surface sterilized plant tissue onto nutrient media (Pleban *et al*., 1995) (Schulz *et al*., 1998), culturing aliquots of water from the last rinsing onto nutrient media (McInroy and Kloepper,1994), dipping the surface sterilized explants into nutrient broth (Anjum and Chandra., 2015). Explants were incubated as control of the tested sample.

Media for isolating endophytic bacteria

The choice of the growth medium is crucial as its directly affects the number and type of endophytic microorganisms that can be isolated from the different tissues. Nutrient agar media were used for the isolation of endophytic bacteria. Since there is no component in nutrient agar which can suppress the growth of endophytic fungi, so the media used for the isolation of endophytic bacteria were supplemented with an antifungal agent, nystatin at a concentration of 30 µg/mL for each to suppress fungal growth.

Isolation, purification, and subculture of endophytic bacteria

The samples of leaves, stem and roots were cut into small pieces and macerated separately in phosphate buffer of pH 7.2 in laminar air flow cabinet with a sterile pestle and mortar. Tissue extract were then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-5}, 10^{-6}, and 10^{-7})were prepared from this extract. Inoculations of 0.1ml of the aliquot was added toNutrient Agar medium. The inoculations were done in triplicates separately for both roots, stem and leaf tissues extract.These plates were then incubated at 28±2°C in order to recover the maximum possible colonies of bacterial endophytes. Observations were taken after 48 to 72 hrs (S. Suhandono *et al*., 2016).

After 72hrs from the bacterial cultures, morphologically different bacterial colonies were selected and are repeatedly streaked in order to achieve bacterial isolates. All selected isolates were subculture in nutrient agar slants and finally, all the purified endophytes were maintained at 4°C till further used.

liminary characterization of endophytic bacteria

Phenotypic characteristics such as microscopic features, gram reaction, endospore staining, were determined by using standard procedures.

olation of genomic DNA

The isolation of genomic DNA from the selected isolates was conducted usingWizard® Genomic DNA Purification Kit – Promega. For genomic DNA isolation, the isolates were separately inoculated into 10 mL of Luria Bertani broth and incubated at 30°C in an orbital shaking incubator. After incubation, the cells were harvested by centrifuging the culture at 8000×g for 10 min at 4°C. To the pellet, 750 µL of 1X buffer was added and was re-suspended by vortexing, after which, 5 µL of RNase was added to the mixture followed by incubation at 65°C for 10 min with intermittent mixing.To this preparation, 1 mL of lysis buffer was added, followed by inverting the tubes 10 to 12 times. The tubes were then incubated at 65ºC for 15 min and again centrifuged at 10000×g for 5 min. The clear lysate collected was loaded on to spin column in 2 mL collection tube (600 µL each time). This was then centrifuged at 10000×g for 1 min at room temperature, the content of lection tube was then discarded and the spin column was placed back in collection tube. After that 500 µL of 1X wash buffer was added to the column and centrifuged at 10000×g for 1 min at room temperature. Then the content of the collection tube was discarded and the spin column was placed back in the collection tube. The empty column was then centrifuged at 10000×g for 3 min at room temperature. The spin column was then placed in a fresh 1.5 mL vial and 50 µL of warm elution buffer already kept at 65°C was added into the spin column. The vial along with the spin columnkept at 65°C and temperature and the eluted DNA was labeled and stored at -20°C.3.3.5.2.Agarose gel electrophoresis for this, 0.8% (w/v) Agarose gel was prepared in 0.5X TBE (Tris-Cl 40 mM (pH 8.3), boric acid 45 mM, EDTA 1 mM) and melted by stirring until the mixture became clear. The solution was then allowed to cool upto 60°C and 0.2 µg/mL (final concentration) of ethidium bromide was added. The solution was gently mixed and poured carefully without forming air bubbles into the casting tray, with comb in place. After solidification, the comb was removed and the solidified gel was placed in an electrophoresis tank containing 0.5X TBE. DNA samples were then loaded to the wells by mixing it with 5X gel loading dye (Glycerol 60%, Tris-HCl (pH 7.6) 10 mM, EDTA 60 mM, Bromophenol Blue 0.03%, Xylene Cyanol FF 0.03%) and the gel was run at 60 V. The gel was then visualized

on a UV transilluminator for detecting the presence of DNA in the eluted solution.

Molecular identification

Molecular identification of the selected isolates was accomplished using 16s rRNA approach, the genomic DNA isolated was used as the template for PCR amplification of 16S rRNA gene, using primers 16SF (5′ – GAG TTT GAT CCT GGC TCAG – 3′) and 16SR (5′– GAT ATT ACC GCGGCGCCT G – 3′) according to(Chun and Goodfellow., 1995). PCR was carried out in a 50 µL reaction volume containing 50 ng of genomic DNA, 20 Pico-moles of each primer (both forward and reverse), 1 X EmeraldAmp GT PCR Master Mix (Takara Bio Inc.). PCR was carried out for 35 cycles in a Mycycler™ (Bio-Rad, USA) with the initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 2.5 min with a final extension of 7 min at 72°C. The PCR product formation was confirmed by agarose gel electrophoresis and the product was further purified for its use as the template for sequencing PCR, using Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequence data further subjected to BLAST analysis (Dewedar *et al.,* 2018). The phylogenetic analysis of the 16S rDNA sequence was conducted by MEGA X using neighbor-joining method with 1,000 bootstrap replicates (Osman *et al.*, 2013). (B. Jasim *et al.,* 2014).

Data processingSequencing

Process was made at Macrogen Inc., Korea. Sequencing results were compared with existing sequences using-Basic Local Alignment Search Tool program on National Center forBiotechnology Information site (www.ncbi.nlm.nih.gov) to obtainthe homology. Sequences obtained from Basic Local AlignmentSearch Tool results and then analyzed using MEGA X software to determine the level of kinship. Construction of phylogenetic trees was created using character-based parameter models, Neighbor-Joining method. The bootstrap method with 1000 replication was used to evaluate phylogenetic trees.Similarity value of each isolate was calculated manually using ascale that is produced by the software (Abouseadaa*et al*., 2015).

RESULTS AND DISCUSSION

Surface sterilization

To check the efficiency of different concentration of sodium hypochlorite as effective sterilizing agents and survival percentage of treated explant and to see the effect of sodium hypochlorite on explant treated individually. At 2% of sodium hypochlorite a high percentage of the explants were found contaminated while at 10% of sodium hypochlorite damaged the explant and plant died. While at 5% of sodium hypochlorite the percentage of survival explants was high and no contamination appear on explant when it subculture on agar media.

Surface sterilization effectiveness

Three methods of sterility check are used. The surface sterilized plant tissue are imprintedonto nutrient media, Aliquots of water from the last rinsing onto nutrient media are cultured and dipping the surface sterilized explants into the nutrient broth. All the three methods gave comparable results. Under optimal condition, no microbial growth occurred on the control medium,so the surface sterilization is considered complete and isolated bacteria are considered as endophytes.The endophytic bacterial communities of healthy looking roots, stems, and leaves of *Citrus limon* plant was assessed in surface disinfested plant parts upon cultivation in nutrient agar medium. The result of effectiveness of the surface sterilization protocol was determined. Each sample showed no microbial growth on nutrient medium after incubation at 30°C for 15 days, indicating that the epiphytic microbes were completely removed by this surface sterilization procedures. To ensure the sterility of the plant surface is the major key to succeed in isolating and studying endophytes (Hallmann *et al*., 1997). The diversity of isolated endophytic bacteria was also largely dependent on the isolation methods (Das *et al*., 2007).

Isolation and purification

The isolation of endophytic bacteria and different colonies was done by nutrient agar media and have been recovered after culture plate. Twenty two endophytic bacteria have been isolated in pure form from *C.limon* plant used in this study. Thirteen bacterial endophytes from root, six from stem and 3 from leaves.

Population size of bacterial endophytes

The results clearly showed that roots had more number of bacterial endophytes than either by stem or leaves, whereas stem had more number of bacterial endophytes than leaves (13 bacterial endophytes for root, six for stem and 3 for leaves).

Preliminary characterization of endophytic bacteria

Morphological characterization, isolated endophytic bacteria exhibited the diverse colony, different shapes, color, margins and textures including round to irregular colonies, off white, yellow colonies with regular or wavy margins (Jalgaonwala *et al.,* 2010). The endophytic bacterial community isolated from *C.limon included Rhizobium sp., B. megaterium, and Pseudomonas sp. B. cereus , B. firmus, B. licheniformis, B. axaarquiensis, B. fiamentosus, B. endophyticus, Geobacillius stearothermophilus, B. mojavensis, B. albus, B. thurigensis, B .proteolyticus*, has been previously characterized as endophytes (Cho *et al.,* 2007) while *B. pumilus, Bacillus subtilis, B. megaterium, Pseudomonas sp*. were isolated as an endophyte from the root of Plant *Chlorophytum-borivilianumSafedmusli* (Panchal and Ingle. 2011). *B. licheniformis* has been identified in *Jacaranda decurrens* plant (Carrim*et al*., 2006), *Bacillus firmus* (Osman *et al*., 2015a). Some species of *Bacillus*

firmus are very alkaline-tolerant and may grow in environments with pH as high as *Bacillus firmus* was evaluated against the root-knot nematode *Meloidogyne incognita* in a laboratory, greenhouse and under field conditions on tomato plants (Metasebia Terefe *et al*., 2009). *B. megaterium* possesses plant growth promoting activity including biocontrol ability against plant pathogens (Gunajit Goswami *et al*., 2018). Rhizobium forms a symbiotic relationship with certain plants such as legumes, fixing nitrogen from the air into ammonia, which acts as a natural fertilizer for the plants.

Extraction and Quantitative Detection of Total DNA from Samples

The total DNA and DNA concentration of isolates extracted from the 22 samples were detected by agarose gel electrophoresis. The results showed that the DNA bands were clear and complete, which met the requirements of the follow-up test.

Molecular characterization of a bacterial endophyte

Molecular identification of the isolates was done by sequencing part of the 16S rRNA gene. The amplification of the 16S rRNA was confirmed by agarose gel electrophoresis.The PCR product was eluted from the gel and sequenced. The sequence data of the 16S rRNA was subjected toBLAST analysis. As 16S rRNA gene sequence provide accurate grouping of organism even at subspecies level (Jill and Clarridge,. 2004).

Neighbors joining phylogenetic tree (Fig. 1) were generated using sequence data from gene bank for strains that showed high percentage of similarities with our strains(Osman *et al*., 2015b).

Sequencing and analyses of DNA

The identification of the endophyte isolate strain 1 and 2 by the 16S rRNA gene partial sequencing were performed at Macrogen Inc., Korea using the universal primers. The partial16s rRNA gene of selective isolate of each tissue extract was sequenced and presented in FASTA format. Finally16S rRNA sequence of the endophyte isolate was compared with that of other bacterial sequence by way of BLAST(http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). To check the relationship and similarity with the endophytic isolate, the result was compared with the sequence of Gene Bank based on partial 16S rRNA gene sequence.

Evolutionary relationships of taxa

the Neighbor-Joining method was used to inference ethe evolutionary history (Saitou and Nei .,1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Neighbor-Joining method (Tamura *et al*., 2004) and are in the units of the number of base substitu-

tions per site. This analysis involved 88 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

SUMMARY

There is an mutual benefit interaction between plants and endophytic bacteria. Plant provides food for bacteria and bacteria defend the plant from pathogen, aiding in the synthesis of phytohormone and nitrogen fixation, leading to an increase in the absorption of minerals and known as plant-growing bacteria. Endophytic bacterial isolates were isolated from roots, stem and leaves of lemon (*Citrus limon*). A total number of 22 bacterial endophytes were isolated. Roots have a greater number of bacterial endophytes than either stem or leaves. The most effective solution for the surface sterilization was 5% sodium hypochlorite and 70% ethanol rather than (2% - 5% - 10%) sodium hypochlorite and 70% ethanol.16s rRNA Sequence analysis performedby using the algorithms BLAST (National Center for Biotechnology Information For identification. Multiple sequence alignment methods were conducted using a freely available alignment program, MEGA X. 16s rRNA gene sequence similarity was used for bacterial identifications. phylogenetic trees evaluate was done by the bootstrap method with 1000 replication. Neighbors joining phylogenetic tree were created usinggene banksequence data for strains showing high percentage of similarities with our strains .

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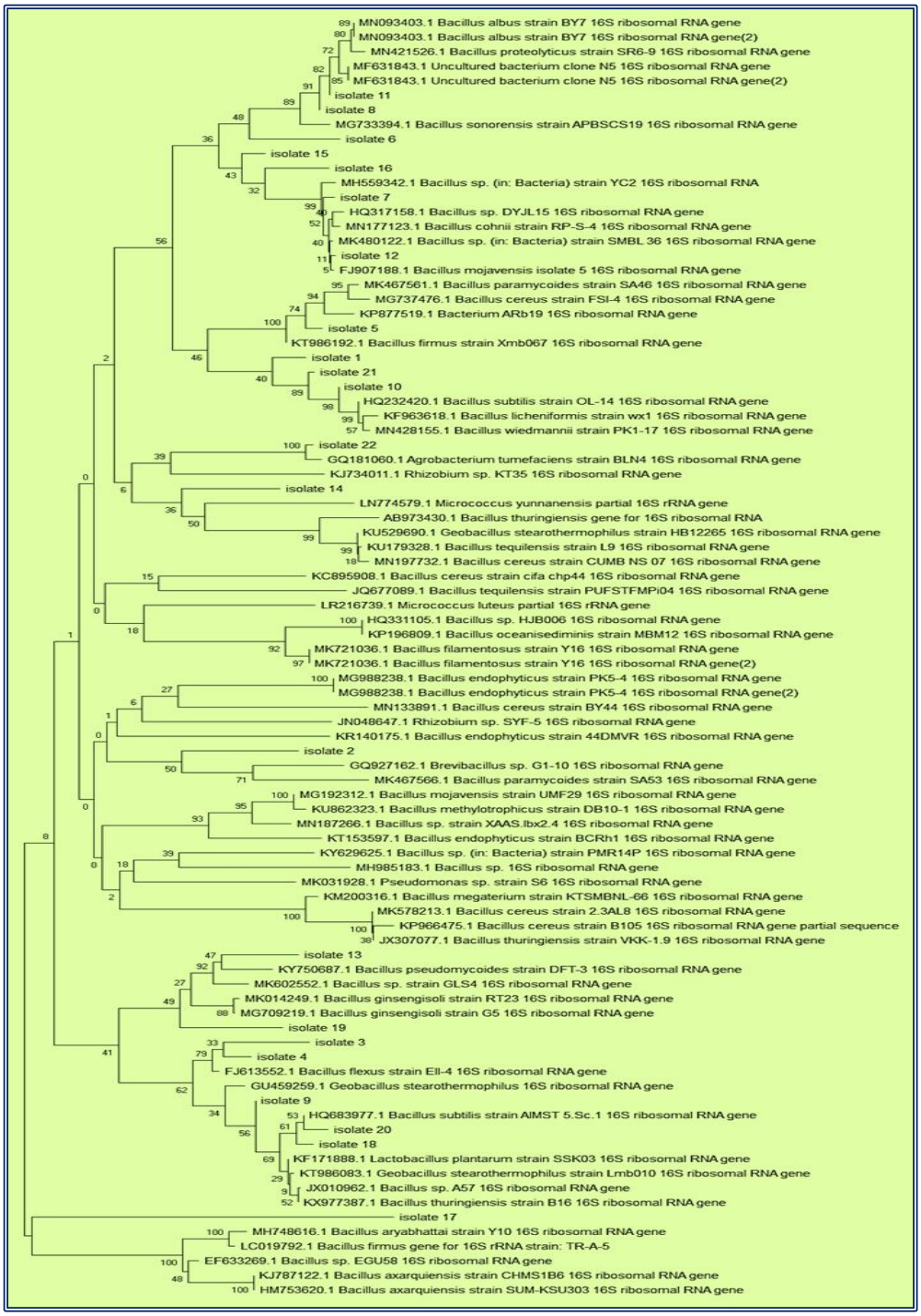

Fig. (1): Phylogenetic analysis of16S rDNA sequences of the bacterial isolates (Isolate 1– isolate 22) from *Citrus limon* along with the sequences from NCBI. The analysis was conducted with MEGA X using neighbor-joining method.