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Molecular Characterization and Plant Growth Promotion Potential of Endophyte Isolated from Desert Plant

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ABSTRACT

The leaves of the *Leptadenia pyrotechnica* plant, native to the Bikaner region of Rajasthan, India, are the source of the bacterial endophyte *Bacillus subtilis* strain LP-B (Accession number: OP535977). To identify the bacterial species 16s-rDNA ribotyping was used followed by evaluation of the isolated bacterial strain for its plant growth promotion activities such as ability to fix nitrogen, produce IAA, HCN, siderophores, ammonia and so on. Furthermore, the endophyte's ability was analyzed to produce extracellular enzymes likewise amylase, chitinase, cellulase, protease and catalase. The isolated endophyte produced IAA27±0.375 µg/ml with 5 mg/ml tryptophan after 14 days of inoculation. The phylogenetic analysis exhibited that the isolated strain LP-B had ancestry and maximum nucleotide sequence similarity (99%) with *Bacillus subtilis* isolates of India and Iraq. The findings indicated that the isolated endophyte was a good candidate for plant growth promoting inoculants to help reduce chemical input in conventional agricultural practices and plant tolerance in stress conditions such as drought and heat. It is worthwhile to invest in the future use of these extracellular enzymes for medicinal and industrial purposes.

Key words: Endophyte, plant growth promotion activities, phylogenetic analysis, indole acetic acid

INTRODUCTION

As a result of climate change, desert plants are becoming increasingly vulnerable to the abiotic stressors that are characteristic of deserts. Desert endophytes have applications in other parts of the world (Adeleke *et al.,* 2022). It is likely that the lack of water and nutrients found in desert soils fosters the development of symbiotic relationships between plants and microbes, particularly in the rhizosphere and root endophytic compartments of plants (Fouda *et al.,* 2021; Byregowda *et al.,* 2022). Nevertheless, desert plants continue to be susceptible to diseases caused by pathogens, despite the fact that instances of these attacks occurring in the wild are rarely observed. Studies that have been published indicate that endophytes on desert plants contribute to host fitness in the same way that endophytes on plants in other habitats do. As a result of climate change, the frequency of extreme weather events that mimic desert conditions is expected to rise, which could make desert endophytes more beneficial for agricultural purposes than endophytes from other regions (Fadiji and Babalola, 2020; Zhang and White, 2021). In order to meet the ever-increasing need for food, this factor, together with the rise in the number of people living in dry areas, has necessitated the creation of plants that are more resistant to the adverse effects of abiotic factors and have the ability to produce high yields. It is well recognized that desert endophytes boost a host plant's ability to take up nitrogen and phosphate, as well as the plant's resistance to heat, water and salt stress, as well as its tolerance to biotic stress. They are typically generalists that may colonise and benefit a wide variety of hosts, which may be helpful for the production of agricultural products that are applicable to a wide range of environments (Shah *et al.,* 2021; Liu-Xu *et al.,* 2022). Desert endophytes stand out in comparison to traditional agrochemicals due to their capacity to deliver all the benefits described above while being kind to the environment (Pirttilä *et al.,* 2021). Many endophytes produced bioactive metabolites as antibacterial, antiviral, anticancer, antioxidant,

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anti-inflammatory, immunosuppressive and other chemicals, proving their long-term value as pharmaceutical bioactive substances (Wang *et al.,* 2022).

The Great Indian Desert, Thar Desert, encompasses over 0.32 million square kilometres of the earth's surface, constituting nearly 10% of India's total geographical area. The arid climate of the Thar Desert is characterized by low humidity, sandstorms and minimal rainfall, extremes in precipitation and high yearly temperature (Yadav and Meena, 2021). The Thar Desert occupies approximately 25,000 km the North-Western portion of Rajasthan. Jodhpur, Jaisalmer, Bikaner and Barmer districts constitute the arid and semi-arid region. *Leptadenia pyrotechnica* is distributed across the state of Rajasthan in arid habitats, particularly in desert regions. It is commonly known by the name of Khimp and belongs to family Apocynaceae. The plant *L. pyrotechnica* and its components have been utilized historically for a variety of applications. *L. pyrotechnica* fibre is used as expectorant and antihistamine. It is used as a traditional medicine in the majority of nations where it grows, and is used to cure a variety of serious disorders ranging from simple (e.g. stomach) to complex (e.g. hepatitis) types. It has also been extensively investigated pharmacologically. Relevant research demonstrated that this plant possessed numerous important pharmacological properties, including wound healing, anthelmintic, anticancer, antidiabetic, antioxidant, antimicrobial, cytotoxic properties anti-lipoxygenase, anti-atherosclerotic and hypolipidemic (Umaru *et al.,* 2018). Flavonoids, sterols, phenolic acids, cardiac glycosides, terpenes, pregnane glycosides, alkaloids, fatty acids, amino acids and sugars and hydrocarbons are reported to be abundant in *L. pyrotechnica* (El-Fitiany and Khasawneh, 2022). Alteration in gene expression and host plant physiology has been found to be the primary mechanism by which endophytes confer stress tolerance, according to a large body of research (Byregowda *et al.,* 2022). Characterization of endophytes acquired from Thar Desert plants and their application in crop plants is still in its early stages, however, considerable research has concentrated on the medical and industrial applications of desert medicinal plants.

In this study, the endophytic *Bacillus subtilis* strain LP-B (Accession number: OP535977) was isolated from a *Leptadenia pyrotechnica* plant growing in the Bikaner district of Rajasthan under unfavourable conditions. Extracellular enzyme production (amylase, chitinase, cellulase, protease and catalase), nitrogen fixation, IAA, HCN, siderophore and ammonia generation and P-solubilization were evaluated as plant growth promoting (PGP) activities of the bacterial strain. The isolated endophytes are promising candidates for plant growth-promoting inoculants to reduce chemical input in conventional agriculture and plant tolerance to drought and heat.

MATERIALS AND METHODS

In the month of January, the whole plant *Leptadenia pyrotechnica* (Khimp) was obtained from Bikaner district, Rajasthan. The surface sterilization was performed using sterile distilled water for 1 min, 70% ethanol for 1 min, 2.5% sodium hypochlorite for 4 min, 70% ethanol for 30 sec, and a final series of three sterile distilled water rinses in three separate containers. Final rinse by water approximately 100 µl was inoculated on nutrient agar to check success of the surface sterilization (Khalil *et al.,* 2021). The sterilized plant parts were cut into small segments and were placed on nutrient agar plates and kept for incubation at 37°C in incubator. The endophytic isolate was designated LP-B (LP-B *Leptadenia pyrotechnica* Bikaner). Subsequently, pure culture of endophyte was obtained by subculturing.

The identification of bacteria was based on the 16S rRNA sequencing analysis of the gene. The genomic DNA of isolate was extracted using a modified version of Miller method. Individual colonies were removed from an agar plate using an inoculating loop and suspended in 50 ml of sterile deionized water. The cell suspension was placed in a water bath heated to 97°C for 10 min. The cell lysate was centrifuged (15,000×g for 10 min), and the DNA-containing supernatant was recovered. Using a spectrophotometer, the DNA concentration was measured by measuring its absorbance at 260 nm in the UV spectrum. A partial 16S rDNA fragment was amplified using PCR and the universal bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'

GGTTACCTTGTTACGACTT-3'). The PCR master mix containing 1X PCR buffer, 0.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase, 0.25 mM dNTP, 0.5 M of each primer, and roughly 5 ng of bacterial genomic DNA were used for amplification. The PCR cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for one min, with a final extension at 72°C for 10 min. The amplified product was analyzed by 0.8% agarose electrophoresis and followed by sequencing using Sanger sequencing method employing forward and reverse primer. The 16S rRNA sequence was then compared to the GenBank database using a BLAST nucleotide search conducted by the NCBI. A multiple sequence alignment was constructed on approximately 1.5 kb of 16S rRNA gene fragment using the Clustal X 1.8 software (*http://www.clustal.org/clustal2*), and a phylogenetic tree was constructed using the Maximum Likelihood method in the MEGA v6.1 software (*www.mega-software.net*), with confidence assessed by bootstrap analysis (1000 repeats) (ALKahtani *et al.,* 2020; Verma *et al.,* 2020).

Indole-3-acetic acid (IAA) production was evaluated by cultivating the isolates for 24 h in nutrient broth in a 37°C incubator with shaking. After that, the 100 µl of fresh broth culture were transferred to a test tube containing 4 ml of nutritional broth and 1 ml of tryptophan and incubated at 37°C for four days in a shaking incubator and followed by centrifugation at 5000 rpm for 15 min. 1 ml of supernatant was added to 2 ml of Salkowski's reagent. Salkowski's reagent was composed of ferric chloride (FeCl3), sulfuric acid and distilled water at a concentration of 0.5 M. The media was then incubated for 25 to 30 min at room temperature. Orange colouration indicated positive IAA production by bacterial species (Gang *et al.,* 2019).

Catalase test was performed to determine whether the isolates were capable of producing the catalase enzyme. The catalase enzyme converted H_2O_2 to H_2O and oxygen. Initially, a smear of isolate was generated in laminar air flow on glass slides, followed by the addition of a few drops of H_2O_2.

For the HCN production test, bacterial culture was harvested from agar plates, inoculated into nutrient broth, and incubated overnight at 37°C in a shaking incubator. An endophytic bacterial strain was distributed on nutritional agar containing glycine. The Whatman filter paper was cut and dipped in a solution containing 0.5% picric acid and 2% sodium carbonate (Na_2CO_3). The filter paper was then placed on the agar plate and incubated at 37°C. The transition from yellow to orange in colour signified the formation of HCN (Kumar *et al.,* 2020).

Nitrogenase test was conducted to investigate the presence of nitrogen fixation. A bacterial isolate was streaked onto Jensen's Nitrogen-Fixing bacteria medium and cultured for four days at 37°C. The presence of bacterial growth indicated a positive nitrogenase test (Kumar *et al.,* 2020).

For the ammonia synthesis test, nutritional broth was infected with a bacterial strain, which was then cultured for 24 h at 37°C in a shaking incubator. 100 µl of freshly produced broth culture was transferred to a test tube containing 5 ml of peptone media and incubated for four days at 37°C in a shaking incubator. As a control, peptone medium was maintained without bacterial inoculation. 0.5 ml of Nessler's reagent was added to each test tube following incubation. The appearance of a yellow to orange colour indicated a positive ammonia test result.

Schwyn and Neilands' approach was utilized to evaluate siderophore production. Plates of chrome azurole S agar injected with fresh culture and incubated at 37°C. A yellow zone formation indicated a positive siderophore test (Kumar *et al.,* 2020).

For screening the extracellular enzymatic activities; the endophytic bacteria were used to inoculate a mineral salt agar medium (MSA; comprising g/l: $NaNO_3$, 5; KH_2PO_4, 1; KH_2HPO_4, 2; $MgSO_4.7H_2O$, 0.5; KCl, 0.1; $CaCl_2$, 0.01; $FeSO_4.7H_2O$, 0.02; agar, 15; distilled H_2O, 1L) supplied with particular substrates. The inoculation plates were then incubated at 37°C for 48 h before being flooded with particular reagents to visualise halos around the bacterial growth (Fouda *et al.,* 2021).

To identify amylase production, an endophytic isolate was grown on MSA containing 1% (w/v) soluble starch. After the incubation time, 1% iodine was flooded onto the inoculated plate. Amylolytic activity was determined by measuring the size (in millimetres) of clear zones surrounding bacterial growth (Fouda *et al.,* 2021).

Chitinase activity was determined following

the inoculation of endophytic bacteria on MSA supplemented with 1% colloidal chitin made from commercial chitin. By assessing the clear zone, which showed chitin breakdown, the ability of bacteria to create chitinase was evaluated (Fouda *et al.,* 2021).

Endophytic bacteria's ability to produce cellulase enzyme was investigated on MSA containing 1% (w/v) carboxymethylcellulose. After the incubation period, cellulase activity was observed as a clear zone on the agar plates after being flooded with Logule's iodine solution (Fouda *et al.,* 2021).

Endophytic isolates' proteolytic activity was evaluated by inoculating them on MSA containing 1% (w/v) gelatin. After flooding the plates with acidic mercuric chloride, gelatin hydrolysis was visible as a clear zone surrounding the bacterial colonies as a signboard (Fouda *et al.,* 2021).

The bacterial strain was inoculated in nutrient broth (NB) media supplemented with 1.0, 2.0, and 5.0 mg/ml of L-tryptophan or without L-tryptophan and cultured at 35°C for 14 days in a shaking incubator to determine the IAA production potential of the identified isolated bacterial endophyte (150 rpm). 5 ml of culture was aspirated from the fermentation broth and centrifuged at 6000 rpm for 30 min at 4°C; 1 ml of the supernatant was combined with 1 drop of orthophosphoric acid and 2 ml of Salkowski's reagent (300 ml of concentrated sulphuric acid, 500 ml distilled water, and 15 ml of 0.5 M $FeCl_3$) and incubated in the dark for 30 min. At 530 nm, optical density was measured with a Spectrophotometer (ALKahtani *et al.,* 2020; Fouda *et al.*, 2021). The production of IAA was approximated by using a conventional IAA.

RESULTS AND DISCUSSION

In this study, a bacterial endophyte was isolated from leaves of *Leptadenia pyrotechnica* collected from the Bikaner area of Rajasthan, India (Fig. 1). The bacterial endophyte was identified using established morphological physiological, and molecular criteria. Sequence analysis was done to confirm the identification of bacterial endophyte strain 16S rRNA (16S ribosomal RNA) gene fragments. On the basis of amplification and sequencing of the 16S rRNA gene, strain LP-B of endophytic bacteria was identified as a *B. subtilis* species. The

Fig. 1. *Leptadenia pyrotechnica* plant growing in the Bikaner district of Rajasthan.

detected bacterial species were compared to the 16S rRNA-related sequence in GenBank, and the BLAST (Basic Local Alignment Search Tool) analysis revealed a 99% degree of similarity. The alignment analysis revealed that the *B. subtilis* strain LP-B (OP535977) had a maximum sequence identity (98.32%) with *B. subtilis* strain CAS-916 (MG641153) of India. The maximum likelihood (ML) phylogenetic tree of aligned sequences exhibited the close ancestry of the *Leptadenia* endophyte isolate LP-B with other geographical isolates of India and Iraq (Fig. 2.).

LP-B, a bacterial endophyte, was quantitatively evaluated for its ability to create IAA after 14 days in the absence and presence of 1.0, 2.0 and 5.0 mg/ml tryptophan as an IAA precursor in the NA medium. The bacterial endophyte demonstrated the ability to synthesise IAA with or without tryptophan. LP-B was shown to produce IAA 27±0.375 µg/ml with 5 mg/ml tryptophan after 14 days of inoculation.

In this study, the endophytic *B. subtilis* strain LP-B (Accession number: OP535977) was isolated from a *L. pyrotechnica* plant growing in the Bikaner district of Rajasthan under unfavourable conditions. Extracellular enzyme production (amylase, chitinase, cellulase, protease and catalase), nitrogen fixation, IAA,

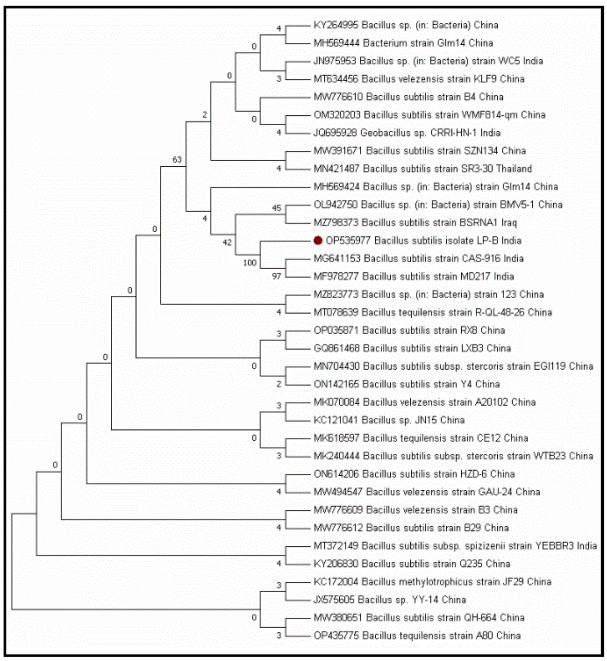

Fig. 2. The maximum likelihood (ML) phylogenetic tree of aligned sequences (16s-rDNA) of *Bacillus subtilis* exhibited the close ancestry of the Leptadenia endophyte isolate LP-B with other geographical isolates of India and Iraq.

HCN, siderophore and ammonia generation, and P-solubilization were classified as plant growth promoting (PGP) activities of the bacterial strain. It is known that cellulolytic activity allows bacteria to penetrate plant tissues and form symbiotic relationships with their host plants. Endophyte-produced extracellular hydrolytic enzymes contribute indirectly to plant growth promotion and disease resistance (Castro *et al.,* 2014). Based on their amylolytic activity, endophytes can be characterized as amylase bio-producers (ALKahtani *et al.,* 2020). Similarly, bacterial endophytes isolated from mangrove plants possessed amylase-related activities (Castro *et al.,* 2014). The numerous enzymatic activities of the isolated endophyte demonstrated their capacity to catalyse many biochemical reactions as well as their potential for agricultural and industrial uses. Similarly, they isolated endophytic Bacillus from two Brazilian mangrove species; the isolates exhibited extracellular amylase, esterase, lipase, protease and endoglucanase activity and therefore suitable for industrial uses (Castro *et al.,* 2014). Furthermore, these enzymes could enable endophytes to penetrate plant tissues and establish a symbiotic connection with their host plant, in addition to defending the host from infections by degrading the pathogen cell wall (Hassan, 2017). Endophyte-secreted hydrolytic enzymes may enhance plant growth by hydrolysing the cell walls of phytopathogens. Catalase enzymes are the first line of defence for microorganisms. They get rid of harmful free radicals caused by environmental and biological stress, which in turn help plants grow.

Chitinase activities could benefit plant growth via biocontrol of phytopathogenic fungi by hydrolysis of chitin in the fungal cell wall (de Almeida Lopes *et al.*,2016). This process could explain the effectiveness of these endophytic bacteria against phytopathogenic fungi *in vitro*. Similarly, Fouda *et al.* (2021) revealed that the extracellular enzymatic activity of endophytic bacteria enhanced the plant's generation of systemic resistance. The hydrolytic enzyme activity of these endophytic bacteria could speed up the breakdown of protein and polysaccharides, which could be useful in industrial settings.

Endophytic microorganisms that generate IAA play a crucial role in mutualistic interactions between the host plant and endophytes and thus govern plant growth. Indole-3-acetic acid (IAA) is a phytohormone that plants and other microbes can create. In addition to promoting plant growth, this hormone also contributes to the interaction between plants and microbes. In this study, the endophytic bacterial strain was capable of producing IAA in the absence and presence of tryptophan, the precursor for IAA synthesis. In microorganisms and higher plants, L-tryptophan is a physiological precursor for auxin synthesis. Ammonia generation and phosphate solubilization are two other strategies employed by endophytic bacteria to boost plant growth (Afzal *et al.,* 2016). Endophytic bacteria that create beneficial metabolites for the plant, including ammonia, can improve the fresh weight of the inoculated plant and prolong the root and shoots of the plant (Passari *et al.*, 2016). Intriguingly, the bacterial species examined in this study were capable of creating ammonia. The bacterial endophytes could produce ammonia from chains of amino acids via proteolytic activity where ammonia is the result of the hydrolysis of amide nitrogen, or by the initial proteolytic breakdown of the molecule followed by deamination, as reported for Corynebacterium.

Ammonia is produced by microorganisms through the hydrolysis of urea into ammonia and carbon dioxide. Ammonia satisfies the plant's nitrogen requirements and reduces pathogen colonisation (Rodrigues *et al.,* 2016). Phosphorus is the third most important nutrient required for plant growth. However, phosphate solubilization is necessary to transform insoluble forms into those that plants can use (Fadiji and Babalola, 2020). Similarly, N-fixing endophytic bacterial species are regarded as an environmentally beneficial method for increasing the N content and, thus, enhancing plant growth. Siderophores are small molecular compounds that are capable of chelating iron and making it available for plant use while depriving pathogens of iron. Siderophores are produced by endophytic bacteria and contribute to plant growth promotion because they are able to make iron available for plant use while depriving pathogens of iron. Some of the siderophores known to be produced by endophytes have biocontrol properties, including hydroxymate, phenolate, and/or catecholate forms (Fadiji and Babalola, 2020). A desert ecosystem is the most challenging site for plant growth because of environmental challenges like water deficiency and high temperatures. Microorganisms called endophytes colonise the intracellular spaces inside of plant cells. Despite these setbacks, endophytic bacteria are effective biocontrol agents and can boost plant growth and yield. Natural substances produced by endophytes with potential medical and agricultural uses are yet another way in which these microorganisms contribute to the health of their host plants. The isolated endophytes from this study can be used as a biofertilizer for crops such as wheat, maize and legume plants that have low growth and production due to abiotic and biotic stress. The metagenomics metabolomics and metabolic profiling to discover endophyte and plant biosynthetic pathways, protein-protein interaction maps, and endophytic nanoparticle research will assist in defining how desert endophytes can be exploited in agriculture, environment, medicine and industry for human welfare.

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