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Molecular Characterization and Plant Growth Promotion Potential of *Paenibacillus Dendritiformis* Endophyte Isolated from *Tecomella Undulata* (Roheda)

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Abstract

In this study, we have isolated a bacterial endophyte *Paenibacillus dendritiformis* strain RAE13 (Accession number: OR259131) from the leaves of *Tecomella undulata* (Roheda) plant. The identification of bacterial species was carried out using 16s-rDNA ribotyping. Subsequently, the isolated bacterial strain was gauged for its potential to endorse plant growth through various mechanisms such as nitrogen fixation, IAA production, HCN synthesis, siderophore generation, and ammonia production. Furthermore, the evaluation focused on the endophyte's capacity for producing extracellular enzymes, including cellulase, chitinase, protease, amylase, and catalase. The endophyte exhibited to synthesize an average of 18±0.375 µg/ml of indole-3-acetic acid (IAA) after being subjected to a concentration of 5 mg/ml of tryptophan over a 14-day incubation period. The endophytic isolate RAE 13 produced an average of 42.4±0.004 µg/ml of Gibberellin, solubilized phosphate in the range of 70.2 µg/ml to 135.5 µg/ml, and produced an average of 45.5 µg/ml of ammonia. The phylogenetic analysis unveiled that the isolated strain RAE13 had a common ancestor and had a maximum nucleotide sequence similarity of 98.30% with Paenibacillus sp isolates of Uttar Pradesh, India. To diminish the consumption of chemicals in conventional farming, the results indicated that the isolated endophyte had great potential as a plant growth-stimulating inoculant. Henceforward, utilization of these extracellular enzymes for medical and industrial applications will be highly beneficial. Additionally, it could enhance plant tolerance to challenging environmental circumstances including drought and high temperatures.

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Introduction

Increasing agricultural yield is necessary to nurture the mounting population in emerging nations and frequently depends on applying chemical fertilisers. Enduring utilisation of these fertilisers has been demonstrated to diminish the bacterial population in soil and can lead to adverse environmental impacts, including phosphorus and nitrogen leakage into groundwater, as well as elevated soil and groundwater contamination[1] Effective microorganisms that mobilize nutrients reduce chemical fertiliser use and improve sustainable agriculture. Plant growth-promoting bacteria that establish symbiotic relationships with their host plants improve plant productivity and health under many environmental conditions.[2-3] In contrast to pesticides, biofertilizers that contain endophytes are superior since they can perform all of the aforementioned functions.[4] Desert plants are increasingly susceptible to abiotic stresses prevalent in deserts due to climate change.[5] There is probably less water and nutrients in desert soils, which helps plants and bacteria form symbiotic relationships.[6]. This is especially true in the rhizosphere and root endophytic parts of plants.[7-8] Research indicates that desert plant endophytes play a similar role in plant health as those found in other habitats. Desert endophytes help a host plant to uptake more nitrogen and phosphate and are more resistant to heat, water, and salt stresses.[9] This could make desert endophytes better for farming than endophytes from other areas.[10-11] Low humidity, sandstorms, and little to no rain are all signs of the Thar Desert of Rajasthan.[12] *Tecomella undulata* can be found all over Rajasthan, mostly in dry places like deserts. It comes from the Bignoniacea family and is often called Roheda.[13] *Tecomella undulata* is recognised in both traditional and folk medicinal practices for its significant therapeutic potential. The plant possesses a broad array of medicinal properties, consisting of hepatoprotective, anti-inflammatory, antimicrobial, anticancer, antifungal, anti-termite, immunomodulatory, analgesic, cytotoxic, anti-bacterial, and anti-obesity activity.[13] In this study, we have isolated a bacterial endophyte from the *Tecomella undulata* plant and evaluated its plant growth promotion activities. In addition, we have quantitively estimated the IAA production capacity of the isolated endophyte. Furthermore, we have evaluated the extracellular enzyme production activity of the isolate.

Materials & Methods

In May, the leaves of the plant *Tecomella undulata* (Roheda) were collected from the Bikaner district, Rajasthan. The leaves were surface sterilized using the following steps: immersed in sterile distilled water for one minute, followed by 70% ethanol for one minute, then 2.5% sodium hypochlorite for four minutes. After that, it was treated with 70% ethanol for 30 sec. Finally, the leaves were doused three times with sterile double distilled water in separate containers.[14] The last rinsed water served as a control and was inoculated onto the nutrient agar plates to ensure the absence of any microorganisms present on the leaf surface.[15] The sterilised leaves were subsequently cut into small pieces, which were then placed on nutrient agar plates and incubated at 37°C. A pure culture of endophytes was then obtained through subculturing. Furthermore, the total bacterial DNA was extracted through the use of the phenol-chloroform extraction method. This was then followed by PCR amplification of the 16S rDNA fragment of the isolated endophyte using universal bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 0.8% agarose electrophoresis was used for amplified fragment separation. The amplified products were sequenced using the Sanger sequencing method. After obtaining the sequence of the 16s rDNA fragment, the sequence was deposited to the NCBI Genbank database, followed by alignment and phylogenetic analysis. The NCBI-BLAST tool was used for the alignment analysis. For the phylogenetic analysis, we used MEGA v11. software (www.megasoftware.net) and used the Maximum Likelihood algorithm with 1000 bootstraps.[16-17]

For Plant growth promotion activities, the freshly grown culture bacterial-isolated RAE-13 was used. We assessed the production of indole-3-acetic acid (IAA) by culturing the isolates in nutrient broth for 24 hours at 37°C with agitation. Subsequently, 100 µl of fresh broth culture was then put into a test tube with 4 ml of nutrient broth and 1 ml of tryptophan. The tube was then put in an incubator with shaking (120 rpm) at 37°C for four days and then centrifuged at 5000 rpm for 15 minutes. Two ml of Salkowski's reagent were mixed with one ml of supernatant, followed by incubation at room temp for 25 to 30 minutes. The presence of orange colouration showed that the bacteria were producing IAA.[18] We used varying concentrations of tryptophan

to quantitively evaluate the IAA production by endophytic bacteria at various time intervals ranging from 2 to 14 days. The quantitative IAA assay mandated the inoculation of endophytic bacterial isolates into nutrient broth containing 5 mg ml-1 tryptophan, which was determined to be the optimal concentration for IAA production through qualitative analysis, followed by incubation at 33 ± 2°C for 14 days. Every 2 days, from day 2 to day 14, approximately 4 ml of the inoculated culture was collected and subjected to centrifugation at 3000 rpm for 30 minutes. The following ingredients were added to 1 mL of supernatant: 2 mL of Salkowski's reagent and one drop of orthophosphoric acid. A UV-visible spectrophotometer was used to measure the intensity of the pink colour's development at a wavelength of 530 nm. The amount of indole-3-acetic acid (IAA) formed was assessed through a reference IAA graph to pinpoint the ideal days for IAA synthesis.[7] To ascertain the isolates' ability to produce the catalase enzyme, the catalase test was conducted. The catalase enzyme catalysed the conversion of H_2O_2 into H_2O and oxygen. First, in a controlled environment, a small amount of isolate was smeared on glass slides. Then, a few drops of H_2O_2 were added. The formation of air bubbles leads to positive results of the catalase test.

The bacterial culture was moved from agar plates to nutrient broth and left to grow overnight at 37°C in a shaking incubator to perform the HCN production test. A bacterial strain that lives on surfaces was introduced to nutrient agar that has been supplemented with glycine. 0.5% picric acid and 2% sodium carbonate (Na2CO3) were added to a solution containing the chopped Whatman filter paper No. 1. Incubation was carried out at 37°C after the filter paper was placed onto the agar plate. When the hue changed from yellow to orange, it meant that hydrogen cyanide (HCN) was being produced.[19] Then, 5 mL of bacterial culture was transferred to a test tube, and the strips of filter paper were dehydrated. To prevent the escape of any potentially volatile compounds, the tubes were sealed with cotton. The tubes were positioned in an incubator and kept at 28±2 °C for 3–5 days. When the incubation time was up, we transferred the strips to fresh tubes with 10 mL of distilled water and mixed them well with a vortex. Then we observed that the colour changed. The samples underwent evaluation for their optical density at a wavelength of 515 nm. A standard curve was established utilising potassium cyanide at concentrations between 10 and 100 µg/mL.[20]

A nitrogenase test was performed to assess the ability to have nitrogen fixation activity in isolated endophytes. A bacterial strain was introduced to Jensen's Nitrogen-Fixing bacteria medium and incubated at 37°C for 4 days. The positive nitrogenase test demonstrated the existence of bacterial growth.[19]

The endophyte isolate was evaluated for its ability to quantitatively synthesise the phytohormone gibberellin. The bacterial culture was introduced into NB media supplemented with 1mM of L-tryptophan and incubated at 37°C for 24 hours under conditions of 150 rpm. The culture underwent centrifugation at a velocity of 10,000 revolutions per minute for 5 minutes. The supernatant, free of cells, was collected and used for the quantification of gibberellic acid. The measurement of gibberellin production was performed utilising the Folin-Ciocalteu reagent. 1 mL of bacterial cell extract was introduced into the test tube, subsequently accompanied by the addition of 1 mL of Folin-Ciocalteu reagent and 1 mL of concentrated hydrochloric acid to the test tubes. The solution underwent heating in a water bath for 5 minutes and was subsequently permitted to cool to room temperature. The spectrophotometer measured the greenish-blue colour at a wavelength of 760 nm. The experiment utilised gibberellic acid (GA3) at concentrations varying from 10 to 100 mg/mL.[21-22]

A bacterial strain was introduced into a nutritious broth for the ammonia synthesis test, followed by a 24-hour incubation at 37°C in a shaking incubator. A total of 100 µl of freshly prepared broth culture was introduced into a test tube that contained 5 ml of peptone media. The solution was subsequently transferred to a shaking incubator and maintained at a temperature of 37°C for four days. A peptone medium was maintained without any bacterial inoculation to serve as a control. After incubation, 0.5 ml of Nessler's reagent was added to each test tube. The presence of a yellow to orange colour indicates a positive ammonia test result. Furthermore, the concentration of ammonia was quantified through spectrophotometric analysis at a wavelength of 600 nm. A standard curve was established within the concentration range of 10-100 µg/mL.[23]

The isolate was assessed for its qualitative ability to dissolve calcium phosphate utilising Pikovskaya agar. The isolates were inoculated onto Pikovskaya agar plates and incubated at a temperature of 28±2°C for 7 days. The presence of a halo zone indicated the ability to dissolve phosphate. The measurement of phosphate release was conducted utilising the chlorostannous reduced molybdophosphoric acid blue method. A 1 mL sample of bacterial culture was added to 100 mL of sterile Pikovskaya broth in an Erlenmeyer flask. Following this, the flask was placed in an incubator maintained at a precise temperature of 28±2°C for 11 days. Throughout this period, the flask underwent vigorous agitation at a frequency of 120 revolutions per minute. The uninfected broth served as a control. The complete experiment was duplicated. On the 3rd, 5th, 7th, and 10th days, 10 mL of broth was extracted from each sample to assess the concentration of soluble phosphorus and observe pH variations. The cultures were subjected to centrifugation at a speed of 10,000 revolutions per minute for 15 minutes. A total of 100 µL of the liquid left after centrifugation was introduced into a flask with 10 mL of chloromolybdic reagent while stirring, followed by additional dilution with 40 mL of distilled water. Subsequently, 5 drops of chlorostannous acid reagent were introduced around the periphery of the flask and thoroughly mixed. The final volume was adjusted to 50 mL by the addition of distilled water. The resultant blue colour was quantified using spectrophotometry at 660 nm, with a reference blank sample as the baseline for comparison. A standard curve was established within the concentration range of 10-50 µg/mL.[20]

To assess siderophore production, the approach proposed by Schwyn and Neilands was used. A new culture was added to Chrome azurole S agar plates, and then they were placed in an incubator at 37°C. The siderophore test appeared to have been successful when a yellow zone appeared.[19]

The extracellular enzymatic activities were evaluated by introducing endophytic bacteria into a mineral salt agar medium (MSA). Following the incubation of the inoculation plates at 37°C for 48 hours, specific reagents were employed to examine the development of halos surrounding the bacterial growth. The production of amylase was measured by cultivating a strain of endophytic bacteria on an MSA medium, which contained 1% (weight/volume) soluble starch. The inoculation plate was then treated with a 1% iodine solution after the incubation period had ended. To measure the amylolytic activity, the diameter (in millimetres) of the see-through areas surrounding the bacterial growth was measured.[7] Endophytic bacteria were introduced onto an MSA medium containing 1% colloidal chitin produced from commercially available chitin, and the chitinase activity was then evaluated. The evaluation of bacterial chitinase production was conducted by examining the clear zone indicative of chitin degradation.[7,3] Using an MSA medium supplemented with 1% (w/v) carboxymethylcellulose, the endophytic bacterial strains' cellulase enzyme production capacity was investigated. Following treatment with Logule's iodine solution, a distinct inhibition zone on the agar plates indicated cellulase activity after the incubation time.[7]

The proteolytic activity of the endophytic isolates was evaluated by inoculating them on an MSA medium supplemented with 1% (w/v) gelatin. The gelatin hydrolysis presented itself as a clear zone lacking bacterial colonies following the application of acidic mercuric chloride on the plates, acting as a definitive indicator.[7]

Results and Discussion

In the present study, bacterial endophytes were extracted from *Tecomella undulata* (Roheda) leaves that were collected in the Bikaner district of Rajasthan, India. With the use of morphological, physiological, and molecular criteria, the bacterial endophyte was identified. We used sequence analysis to verify that the bacterial endophyte strain's 16S rRNA gene fragments were indeed identified. Through 16S rRNA gene amplification and sequencing, the endophytic bacterial strain was identified as *Paenibacillus dendritiformis* strain RAE13 (Accession number: OR259131). After analysing the identified bacterial species against the GenBank 16S rRNA-related sequence, a similarity of 98.30% was found using the BLAST (Basic Local Alignment Search Tool). Based on the maximum likelihood (ML) phylogenetic tree of matched genomes, the *Tecomella undulata* endophyte isolates RAE13 were found to have a tight ancestral relationship with other isolates from India (Fig. 1).

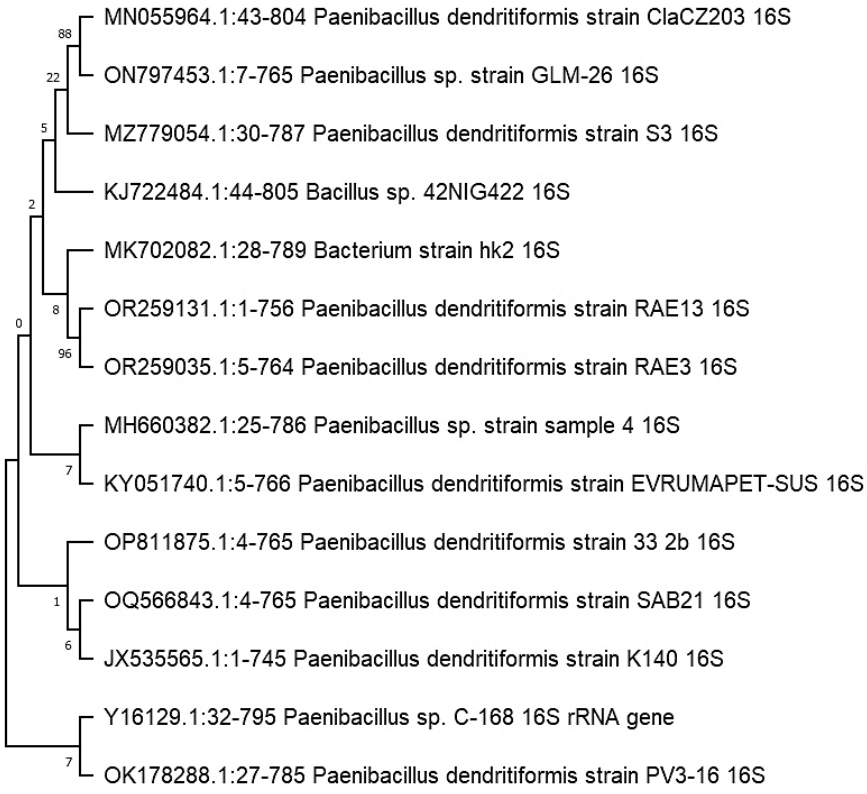

Fig.1: The Maximum Likelihood Phylogenetic tree of isolated endophyte *Paenibacillus dendritiformis* strain RAE13 showing close ancestry with other Indian isolates of *Paenibacillus* sp.

The study involved the isolation of a strain of *Paenibacillus dendritiformis*, namely the RAE13 strain (Accession number: OR259131), from a *Tecomella undulata* (Roheda) plant. This plant was found to be thriving in a challenging environment in the Bikaner area of Rajasthan. The bacterial strain displayed various activities that promote plant growth, including the secretion of extracellular enzymes such as protease, chitinase, cellulase, and catalase. Additionally, it exhibited nitrogen fixation, production of ammonia, IAA, HCN, siderophores, and solubilization of phosphorus (Table 1).

Table 1: Plant Growth Promotion activities of bacterial endophyte *Paenibacillus dendritiformis* strain RAE13 isolated from *Tecomella undulata* (Roheda) plant of Thar Desert of Rajasthan, India

Strain	Plant Growth Promotion Activity	Extracellular Enzymatic Activity
Paenibacillus dendritiformis strain RAE13 (Accession number: OR259131)	Nitrogen Fixation, IAA, Siderophore production, Ammonia production, Phosphate solubilisation, Gibberellin production	Protease, Chitinase, Cellulase, Catalase

The production of ammonia by the PGPR has an indirect impact on the growth and development of plants. The nitrogenous components of peptones that are produced by the PGPR decompose into

ammonia, which is then released into the soil and utilised by plants as a source of nutrients.[24] HCN synthesis is linked to bioremediation and serves as a biocontrol for promoting growth and inhibiting antagonistic activities.[25] Gibberellins are phytohormones that act as plant growth regulators and have a significant impact on seed germination and stem elongation.[26]

The quantitative assessment was conducted to determine the capacity of the bacterial endophyte *Paenibacillus dendritiformis* strain RAE13 to manufacture indole-3-acetic acid (IAA) after 14 days on the NA medium, both with and without 1.0, 2.0, and 5.0 mg/ml tryptophan as a precursor for IAA. The bacterial endophyte demonstrated the capacity to synthesise indole-3-acetic acid (IAA) regardless of whether tryptophan was present or not. RAE13 was found to produce 18±0.375 µg/ml of indole-3-acetic acid (IAA) after 14 days of inoculation, using 5 mg/ml of tryptophan (Table 2). An average of 42.4±0.004 µg/ml of Gibberellin was generated by the endophytic isolate RAE 13. Additionally, 70.2 µg/ml to 135.5 µg/ml of solubilized phosphate was produced by the same strain, and an average of 45.5 µg/ml of ammonia was produced by the same isolate (Table 2).

Table 2: Quantitative estimation of Plant Growth Promotion activities of bacterial endophyte *Paenibacillus dendritiformis* strain RAE13 isolated from *Tecomella undulata* (Roheda) plant of Thar Desert of Rajasthan, India

Strain	IAA (µg/ml)	Ammonia production (µg/ml)	Phosphate solubilisation (µg/ml)	Gibberellin production (µg/ml)
Paenibacillus dendritiformis strain RAE13 (Accession number: OR259131)	18±0.375 µg/ml	45.5 µg/ml	Day 3: 70.5 µg/ml Day 5: 92.5 µg/ml Day 7: 135.5 µg/ml	42.4 µg/ml

The ability of cellulolytic bacteria to enter plant tissues and establish symbiotic relationships with their hosts is well-known. The endophytes produce extracellular hydrolytic enzymes, which indirectly promote plant growth while improving disease resistance.[27-28] Endophytes are categorised as amylase bio-producers due to their ability to manufacture amylolytic enzymes.[16] Similarly, bacterial endophytes that were obtained from mangrove trees[28] exhibited amylase production. The various enzymatic activities of the isolated endophyte demonstrate their potential for use in industrial and agricultural applications, as well as their capacity to facilitate various biochemical processes.[29] Endophytic Bacillus was obtained from two mangrove species in Brazil through isolation. The isolates demonstrated extracellular enzymatic activity for amylase, esterase, lipase, protease, and endoglucanase, rendering them well-suited for utilisation in industrial settings.[28] Moreover, these enzymes facilitate the infiltration of endophytes into plant tissues, establishing a mutually beneficial association with their host plant. This association safeguards the host plant from illnesses by degrading the cell wall of the pathogen.[30] Endophytes produce hydrolytic enzymes that can contribute to plant growth by breaking down the cell walls of phytopathogens.[7] The catalase enzyme serves as the primary defensive mechanism for microorganisms. They neutralise harmful free radicals caused by biological and environmental stressors, hence enhancing plant growth. Chitinase activity in the fungal cell wall can boost plant growth by suppressing phytopathogenic fungi through the hydrolysis of chitin.[31] This approach could elucidate how these endophytic bacteria demonstrate effectiveness against phytopathogenic fungi under controlled laboratory settings. Comparably, it has been identified that the extracellular enzymatic activity of endophytic bacteria improved the plant's ability to contribute to systemic resistance.[7] The activity of hydrolytic enzymes that are produced by these endophytic bacteria has the potential to enhance the breakdown of proteins and polysaccharides, offering advantages in various industrial applications.

Endophytic bacteria are essential in regulating plant growth by synthesising IAA, which is vital for the mutualistic relationships between the endophytes and their host plants. The phytohormone indole-3-acetic acid (IAA) can be synthesised by a wide range of microorganisms as well as plants. This hormone is essential for plant growth and for developing an association between plants and microorganisms. The endophytic bacterial strain employed in this study exhibited the ability to synthesise indole-3-acetic acid (IAA) regardless of the presence of tryptophan, which is an essential precursor for IAA production. L-tryptophan serves as a biological precursor for the formation of auxin in both microorganisms and higher plants. Endophytic bacteria employ two additional mechanisms to enhance plant growth: phosphate solubilization and ammonia synthesis[32-33] found that endophytic bacteria can enhance the growth of the infected plant by increasing its fresh weight and promoting the development of its roots and shoots. This is achieved through the production of ammonia and other beneficial metabolites that support the plant's growth. It is noteworthy that the bacterial species examined in this study can synthesize ammonia. Bacterial endophytes can synthesize ammonia through proteolytic activity, by hydrolyzing amide nitrogen in chains of amino acids. Alternatively, as previously noted for Corynebacterium, the molecule can undergo proteolysis followed by deamination. A substantial ancestral link between strain RAE[13] and other Indian isolates was revealed by the maximum likelihood (ML) phylogenetic tree of aligned sequences (16s-rDNA) for *Paenibacillus dendritiformis*. Microorganisms hydrolyze urea to produce ammonia. Ammonia fulfils the nitrogen requirements of the plant while also reducing vulnerability to pathogen intrusion.[34] Phosphorus is the third most essential nutrient prerequisite for plant growth. To convert insoluble forms into forms that plants can effectively utilise, the process of phosphate solubilization is necessary.[10] Similarly, it is believed that endophytic bacterial species capable of nitrogen fixation offer an ecologically sound approach to enhance nitrogen levels and stimulate plant development. Siderophores, which are small molecules, can chelate iron. This process allows plants to get iron while simultaneously depriving pathogens of their iron source. Siderophores, which are formed by endophytic bacteria, facilitate the availability of iron for plants while preventing pathogens from accessing it. This, in turn, promotes plant growth and serves as a biocontrol agent employing specific siderophores, such as hydroxymate, phenolate, and/or catecholate forms.[10] The challenging conditions of a desert ecosystem, characterised by high temperatures and limited water availability, provide significant obstacles to plant growth. Endophytes are microorganisms that reside within the cells of plants and inhabit their interior areas. Despite these disadvantages, endophytic bacteria can enhance plant productivity and growth, making them valuable for controlling pests and diseases. These bacteria also contribute to the well-being of their host plants by producing natural chemicals that could be utilised in agriculture and medicine.[35] The endophytes identified from this work can be used as a biofertilizer to improve the growth and productivity of crops such as wheat, maize, and legume plants, which are affected by abiotic and biotic stress. The multi-faceted plant growth-promoting capabilities of P. dendritiformis RAE13 demonstrate significant potential for addressing specific agricultural challenges in nutrient-depleted soils. The strain's substantial phosphate solubilization capacity (70.2-135.5 µg/ml) is particularly relevant for agricultural systems in calcareous soils, where phosphorus availability is a limiting factor for crop productivity. This trait could be especially beneficial for cereal crops, which require significant phosphorus uptake during grain-filling stages.[36] The strain's extensive enzymatic profile, including cellulase, chitinase, and protease production, suggests potential applications in enhancing nutrient cycling efficiency in organic farming systems. For commercial application, these characteristics can be utilized through several approaches: (i) development of crop-specific inoculant formulations optimized for different soil types,[37] (ii) integration into existing organic farming protocols as a supplement to conventional fertilizer strategies,[38] (iii) creation of drought-resistant biofertilizer preparations, particularly valuable for rainfed agriculture in arid regions,[39] and (iv) development of novel seed coating technologies for improved germination and establishment.[40] Future research should focus on elucidating the molecular mechanisms underlying RAE13's stress tolerance and plant growth promotion capabilities, as well as investigating potential synergistic interactions with other beneficial soil microorganisms.

Conclusion

An endophytic bacterial strain, *Paenibacillus dendritiformis* strain RAE13, was isolated and studied in this work. The plants studied were *Tecomella undulata* (Roheda), which thrive in the harsh Thar Desert environment in the Bikaner region of Rajasthan, India. In summary, the study offers important insights into the role of desert endophytes as vital elements for sustainable agriculture, enhancing crop resilience against climate change and contributing to wider ecological and pharmaceutical progress. This study found endophytes that can be employed as biofertilizers to boost wheat, maize, and legume growth and productivity under abiotic and biotic stress. Further research in this field holds the key to unlocking innovative solutions for global challenges in food production, environmental sustainability and human health.

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Conflict of Interest

The authors do not have any conflict of interest

Data Availability Statement

The study includes the original contributions, which may be found in the article and accessible through the NCBI gene bank using the accession number of the deposited sequence. For any additional questions, please contact the corresponding author.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent statement

This study did not involve human participants, and therefore, informed consent was not required.

Author Contributions

- **Nisha Choudhary, Mahima Choudhary and Rakesh Kumar Verma:** contributed to the conceptualization of the work that was being done.
- **Nisha Choudhary:** was the one who carried out the research and penned the initial draft of the manuscript.
- **Nisha Choudhary and Rakesh Kumar Verma:** went through the manuscript and made their final revisions. All authors have read and agreed to the published version of the manuscript.

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