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# Plant Growth–Promoting Potential of Endophytic Bacteria Isolated from Asparagus racemosus Willd and their role in improving Chickpea (Cicer arietinum L.) Germination

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**Title page**

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**Author contributions**

**Shilpee Dhali, Chinmay Pradhan and Madhusmita Pradhan**: Formal analysis, Investigation, Review, Writing – original draft & review & editing, Project administration, Supervision.

**Chinmaya Kumar Swain**: Edited the Manuscript

**Arpita Das:** Formal analysis, Data curation

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**Abstract**

Endophytic bacteria inhabiting internal plant tissues are increasingly recognized for their role in enhancing plant growth and stress tolerance. The present study investigated the plant growth-promoting potential of root-associated endophytic bacteria isolated from the medicinal plant *Asparagus racemosus* Willd. and evaluated their effect on seed germination of desi chickpea (*Cicer arietinum* L.). Healthy tuberous roots were surface sterilized, and endophytic bacteria were isolated using standard microbiological techniques. Seven morphologically distinct bacterial isolates were obtained and characterized through morphological, biochemical, and molecular approaches. The isolates were screened for important plant growth-promoting traits including indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation, siderophore production, ammonia production, hydrogen cyanide production, ACC deaminase activity, and extracellular hydrolytic enzymes. Among the isolates, strain AR1 exhibited the most comprehensive PGP profile, showing positive responses for IAA production, phosphate solubilization, nitrogen fixation, siderophore production, ACC deaminase activity, and multiple hydrolytic enzymes. Molecular identification based on 16S rRNA gene sequencing revealed that AR1 belonged to *Enterobacter wuhouensis*, while amplification of the nifH gene confirmed its nitrogen-fixing potential. Seed inoculation experiments demonstrated a substantial enhancement

in chickpea germination, with inoculated seeds achieving 85.19% germination compared to 29.63% in uninoculated controls. Enhanced radicle growth and seedling vigor were also observed. These findings highlight *A. racemosus* as a valuable reservoir of beneficial endophytic bacteria and demonstrate the potential of strain AR1 as a bioinoculant for sustainable crop production. Further investigations involving whole-genome sequencing, transcriptomic analyses, and multilocation field evaluations are warranted to validate its agricultural applicability under diverse environmental conditions.

*Keywords:* Endophytic bacteria; *Asparagus racemosus*; Plant growth promotion; Chickpea germination; Phosphate solubilization.

## 1. Introduction

Endophytic bacteria can be defined as a varied class of microorganisms that can colonize the internal parts of plants such as leaves, roots, stems, inflorescence, fruits, buds, and even dead cells causes no harm, also called as endosymbionts forming mutualistic relationship with its respective hosts (Specian et al., 2012). Reports from various sources suggest approximately 30,000 plant species have provided habitat to different endophytic bacteria in its different parts. The endophytes occupy three different life cycle-based niches. One of them are obligate endophytes who transmits through seeds and are majorly dependent on the hosts for their survival. In the rhizosphere mostly facultative endophytes are found, who lives outside of plant surface but once favourable conditions occur, they move to plant internal tissues (Hardoim et al., 2008). Lastly opportunistic endophytes who are least common, weakest and inactive endophytes that enters plant tissue and only receives benefits (Rosenblueth et al., 2006). Bacteria endophytes generally spread and move inside the interior of plants parts through vascular tissues (Raizada et al., 2011). Endophytes diversity is determined by parameters of hosts such as species, growth stages, plant parts, and environmental conditions subjected to the hosts.

Plant Growth promoting bacteria commonly known as PGPB are multiple class of microorganisms having multi beneficial effect on host plants throughout their life cycles. They assist their hosts by reducing biotic stress caused by bacteria, fungi, virus and nematode pathogens, protects from drought, salinity conditions, and sinking etc. These combined stresses result in yearly crop loss, soil fertility loss; hence the synthetic biofertilizer and chemical pesticides are so much more commercialized in modern agricultural practices; promising more yield, instant pathogen removal, which then again gets mixed in soil, lynched into deep water and ultimately creates pollution. These pollutions rise in food web creating multiple diseases, causes ecosystem disruption, and harms environmental sanity. Hence the need of PGPB infused biofertilizer which contribute to sustainable agricultural practices and environment is these hours need (Sessitch et al., 2012). PGPE are a ubiquitous group of PGPB which enhances plant growth, stress tolerance and resistance against different types of pathogens (Glick et al., 2013). These activities are supported by the potentials to produce phytohormones, reactive oxygen species, volatile organic compounds, exopolysaccharides, extended rooting networks, increases sugar and amino acid production, enhanced osmotic adjustment. Studies till date suggest that roots associated endophytic strains can promote plant health and growth through various mechanisms such as over phytostimulation, biofertilization, and biocontrol activity (Ahemad et al., 2014).

The symbiotic association result in enhanced physiological effects on growth and development of plant species such as phytohormone production especially auxin (IAA i.e., indole-3- acetic acid, gibberellin, cytokinins and ethylene etc). IAA producing endophytic bacteria have developed more adaptive root colonization ability. The increased level of IAA can trigger ethylene production making fruit ripening and provides more adaptation to salt and metal pollutions (Iqbal et al., 2014). Tryptophan dependent and independent pathways, ex: Indole-3-pyruvate (IPyA) and Indole-acetamide (IAM) etc., are the illustrated pathways bacteria uses for IAA production (Spaepen et al., 2007).

Siderophore is a low molecular weight iron chelating compound (Ahemad et al., 2014), which solubilizes iron resulting in iron acquisition in low iron environments and detoxify the environment by binding to heavy metals resulting in bioremediation. Siderophore producing microbes can contribute to plant growth by increasing iron availability which is necessary for TCA and ETC mechanism, ATP and glucose synthesis (Fardeau et al., 2011). Microbe uptake iron by Fe-siderophore complex after which siderophore scavenges and reduces ferric form iron into ferrous form inside the cell (Dimkpa et al., 2016). Phosphate solubilizing endophytic bacteria can solubilize and mineralize soil inorganic phosphate pools such as rock phosphates, hydroxyapatites, tricalcium phosphates, and dicalcium phosphates into simple available phosphates such as monobasic and dibasic forms. They produce strong industrial acids like citric acid and malic acid which helps them to dissolve insoluble phosphates into soluble ones (Whitelaw et al., 1999). Plant readily absorbs these simplified phosphates through their roots by using organic ligands (Hinsinger et al., 2001), which later on increases plant biomass, more yield, activate stress responsive genes. The chemically available phosphate-based fertilizer often found to react with iron and aluminum in the soil after application and become insoluble so an alternative but constant source is a necessity.

Nitrogen fixating endophytic bacteria use specialized nitrogenase enzyme complex to convert atmospheric nitrogen into ammonia; the plant then uses this ammonia to produce amino acids and proteins which shapes and forms plant. Ethylene is a gaseous plant hormone also known to be ageing hormone or stress responsive hormone of plants (Abeles et al., 1992). Its direct effect processes such as seed germination, leaf abscission, leaf senescence, flowering, wilting, fruit ripening, rhizobia nodule formation aiding in nitrogen fixation, increased root length and branching leading to better nutrient and water uptake etc. ACC stands for 1-aminocyclopropane-1-carboxylic acid act as a parent for plant ethylene synthesis. Ethylene at low concentration activates defensive genes which protects hosts against biotic and abiotic stress conditions such as drought, salinity, heavy metals, radiations, injuries and infection by different pathogens

(Ciardi et al., 2000). Ethylene at high concentration leads to abnormal growth patterns and premature senescence (Glick, 2014). Certain microorganisms address this issue by producing the ethylene degrading ACC deaminase enzyme, which breaks down ACC into ammonia and then into α-keto butyrate, thereby lowering ethylene levels and easing its adverse effects (Belimov et al., 2009). Global food security and sustainable agricultural practices is constantly threatened by the activities of pests, insects, and phytopathogens. Hydrogen cyanide (HCN), is a major volatile secondary metabolite produced by certain endophytic bacteria, which functions as a natural biocontrol agent by suppressing the growth of pathogenic fungi, microbes, insects and nematodes (Ramette et al., 2003) by mechanisms such as acting as a signaling molecule which inhibits cytochrome c oxidase and ATP in disease causing agents, by inducing competition and other defense mechanisms such as systemic acquired resistance (SAR) and induced systemic resistance (ISR). Endophytic bacteria also possess the ability to produce hydrolytic or cell wall-degrading enzymes, directly breaking cell walls of pathogenic microorganisms. It was reported that cellulases and pectinases produced by them facilitate root penetration and colonization (Gupta et al., 2019). Proteases and chitinases have reported to suppress fungal pathogens (Zahir et al., 2003).

Medicinal plants tend to attract selective endophytes based on the criteria of the secondary metabolites they produce and compositions of their root exudates. Studies have revealed the dual metabolite producing capabilities of these endophytes both in plants and in themselves. Sometimes these endophytes aid in increasing plant's chemical diversity and biological potential by synthesizing unique metabolites not produced by the host medicinal plants. Therefore, medicinal plants provide a rich reservoir of endophytic microorganisms, with valuable applications in biotechnology and drug discovery. The medicinal plants falling into rare category are capable of growing and surviving diverse, unfavourable and harsh environments making their endophytic habitat capable of growth enhancement, drought tolerant, salinity resistant and antimicrobial in nature.

The selected plant for this study is *Asparagus racemosus* Willd., a rare member of the Asparagaceae family. It is naturally distributed across the Himalayan regions of India, as well as in Nepal and Sri Lanka. Traditionally known as Satavar, Shatavari or Shatamull. It grows in sandy or loamy soils reach upto 1-2 meters in length, with fine needle/slender/delicate like leaves (phyloclades) that are actually modified stems arranged in tufts along the branches. Small aromatic white flowers blooming in rainy seasons, which then turn into globular, purple to black berries. Fleshy, tuberous roots growing in clusters is the main part used traditionally to solve gynecological problems, enhances fertility and promote lactation. Additionally, it functions as a calming tonic for the nervous system, helping to alleviate conditions such as anxiety, anorexia, insomnia, irritability, disturbing dreams, hyperactivity in children, underweight issues, and cardiac weakness (Alok et al., 2013). It has high containt of sapogenin, sarsasapogenins, flavonoids and poly phenols making it a great source used as immunostimulatory, immunomodulatory, adaptogenic, antioxidant and antiinflamatory.

All of the above encouraging aspects fulfils the immediate needs of current sustainable plant cultivation programmes by methods that can reduce the use of heavy chemical fertilizers and pesticides and aid in long term conservation of soil biology (Granada et al., 2018). Therefore, selecting and using these microbes with biotechnological application can be a strategic alternative, which will positively influence the plant developments, as well as soil health and nutrient cycling. Farmers views regarding popularizing biotechnological products such as using live microorganisms as inoculant has been created by providing them adequate knowledge about the relationship plant and soil shares and pernicious effects that the excessive use of chemical and synthetic fertilizers have on environment (Choudhary et al., 2022). As a result, they are more popularized now due to their proven agronomic effects. In Odisha the farmers are mostly underfunded, often operate as family run units and face heavy responsibilities; so, they have very limited access to synthetic outputs as these fertilizers can be expensive, so to afford these in every growing season can be a hassle. Desi chickpea or kala chana (*Cicer arietinum* L.) a member of

fabaceae was selected as a model crop to evaluate bio efficacy of selected isolates. It's extremely nutritious contain, easy cropping, drought tolerance profile, pest/disease tolerant profile, higher market demand, shelf stable nature, makes it an ideal choice. Recently prime Chickpea serving as base variant for many values added products and can increase the source of income in rural areas makes it a benevolent choice. The biofertilizers infused with these multipurpose beneficial endophytic strains should be identified, produced, generalized, commercialized and disseminated thus, we can promote green and ecofriendly agriculture.

While its phytochemical and pharmacological properties have been studied extensively, the exploration of its roots associated endophytic microbial communities especially bacterial endophytes remain vastly underexplored. Very few studies have focused on isolating, characterizing and evaluating potentials of the endophytic bacteria from Shatavari plant' roots. This presents a significant research gap and an opportunity to discover novel bacterial strains with potential plant growth-promoting (PGP) activities. The present study is among the first few efforts made to explore endophytic bacteria from the roots of Shatavari, highlighting their biotechnological and agricultural potential. Various studies revealed that the roots serve as primary site of colonization and own rich bacterial diversity compared to other plant organs. Considering these facts, the present study focuses on obtaining endophytic bacteria from root tubers of *A. racemosus*.

## 2. Material method

### *2.1 Collection of plant material*

The *Asparagus racemosus* Willd. (Shatavari) plant was collected from its natural habitat at the one of the gardens in Bhadrak, Odisha, a site having shatavari diversity. The sample collection was done during winter season, January 2025, to ensure optimal maturity, vigour and microbial activity in its tuberous roots. It has been given special focus to the plants age i.e., at least 1 year. The plant collected was healthy and disease free and uprooted as whole including roots by using

a sterile spade and gloves to minimize soil contamination. The plant was then transported to the laboratory the same day after being placed in a sterile polythene bag, which is labelled with collection date, location coordinates, and sample ID. The taxonomic identity of the plant was verified using standard taxonomic keys and online databases (Figure 1 & 2).

### *2.2 Isolation, Purification and Preservation of Endophytic Bacteria*

Healthy roots were washed under tap water for 15 min to remove surface contaminants, then detached using sterile forceps and rinsed with sterile distilled water. Intact root segments (5–10 cm) were excised with sterile scissors and surface-sterilized by immersion in 70% ethanol for 1 min followed by 3–4% sodium hypochlorite for 3 min, with three final rinses in sterile distilled water (Kumar et al., 2016). Surface sterilization efficacy was confirmed by plating the final rinse on nutrient agar and incubating at 30°C for 2 days. Sterilized root segments were cut into 1-cm pieces with a sterile scalpel, macerated in 9 mL sterile PBS (pH 7.0) using a sterile homogenizer, and serially diluted ($10^{-1}$ to $10^{-5}$) in sterile PBS. Aliquots (100 µL) from each dilution were spread onto TSA agar plates. Plates were sealed with paraffin and incubated at 30°C for 3 and 7 days, with colony growth monitored daily (Pavlo et al., 2011). Morphologically distinct colonies were subcultured on fresh TSA plates to obtain pure cultures. Pure isolates were stored at standard freezing temperature for future use. All procedures were conducted under aseptic conditions in a laminar air flow cabinet.

### *2.3 Morphological Characterization*

The purified endophytic bacterial isolates were phenotypically examined for colony characteristics and Gram staining using standard methods described by Holt *et al.* (1994). Each bacterial isolates of the pure cultures were streaked on TSA plates to obtain single colonies. Incubated at 28 ± 2°C for 24 to 48 hours. Colony characteristics observed such as size, shape, margin, filamentous, elevation, texture, opacity, and colour. Colony morphology was studied under a compound light microscope and noted down. Gram's reaction tests were conducted on a clean glass slide containing thin smears of each bacterial isolates which were prepared by

suspending a loop of 24 hours old culture in a drop of sterile distilled water. Each smear was air-dried, heat-fixed, and stained using the Gram staining protocol (Benson, 2002). Each smear was 1 minute flooded with crystal violet, washed, followed with 1 minute Gram's iodine, and again washed. The smear was decolorized with 95% decolorizing ethanol for 10–20 seconds, promptly washed with water, followed by counterstaining with safranin for 45 seconds, rinsed again, and allowed to air dry. Each slide was observed under a 100x oil immersion lens to determine Gram's reaction: positive or negative, cell shape: cocci, bacilli, spiral, and arrangement: single, pairs, chains, clusters. All of the collected data were summarized in a table providing phenotypic profile to differentiate isolates.

### *2.4 Biochemical Enzymatic Activities*

Endophytic bacterial isolates were screened for extracellular enzymes (cellulase, pectinase, protease, chitinase) by spot-inoculation or streaking on substrate-specific agar media (pH 7.0), followed by incubation at 28–30°C for 2–7 days. Enzyme activity was indicated by clear halos around colonies after staining: Congo red (0.1%, 15 min) followed by 1 M NaCl destaining for cellulase on CMC agar (Teather & Wood, 1982); iodine (IKI) flooding for pectinase on pectin agar (Kasana et al., 2008; Hung, 1986); halo zones on skim milk agar for protease (Smibert & Krieg, 1994); and halos on colloidal chitin agar (from crab shells, HCl-extracted; Hsu & Lockwood, 1975; Priya et al., 2011). These assays confirmed substrate degradation, aiding plant colonization and nutrient cycling (Sindhu et al., 2001).

### *2.5 Plant Growth-Promoting Traits (PGPTs)*

Isolates were evaluated for PGPTs including IAA production, siderophore, phosphate solubilization, ammonia, nitrogen fixation, HCN, and ACC deaminase. IAA was detected by color change (reddish) in Salkowski reagent after growing in L-tryptophan-supplemented LB broth (Patten & Glick, 2002). Siderophores showed orange/yellow halos on CAS agar (Schwyn & Neilands, 1987; Rashid et al., 2012). Phosphate solubilization formed clear zones on

Pikovskaya's agar at 37°C (Jena et al., 2016). Ammonia production yellowed with Nessler's reagent in peptone water (Cappuccino & Sherman, 1992; Rashid et al., 2012). Nitrogen fixation yielded growth on Jensen's N-free agar (Jensen, 1951). HCN shifted filter paper to brown/red on glycine-TSA (Baker & Schippers, 1987). ACC deaminase enabled growth on DF-SEC agar with ACC as N-source (Dworkin & Foster, 1958).

### *2.6 Molecular Characterization of Bacterial Isolate*

Molecular identification of the selected endophytic isolate was carried out by 16S rRNA gene amplification and Sanger sequencing, a widely accepted approach for bacterial classification and taxonomic assignment. Detection of the nifH gene was outsourced to a SYBR Green–based qPCR service (personal communication, 2024), providing additional evidence for putative nitrogen-fixing capability. Genomic DNA was extracted from 2 mL of overnight culture by pelleting the cells, resuspending in TE buffer, treating with lysozyme, and incubating at 30°C, followed by proteinase K and RNase A lysis at 60°C for 20 minutes with intermittent mixing. DNA was then purified using a silica column–based protocol: the lysate was mixed with binding buffer and absolute ethanol, loaded onto a spin column, centrifuged to bind DNA, washed with the supplied wash buffer, and finally eluted in pre-warmed elution buffer after brief incubation at room temperature to obtain high-quality genomic DNA suitable for PCR. PCR products were resolved on 0.8–1.5% agarose gels prepared in 1× TAE buffer, run at 50 V for 30–45 minutes, stained, and visualized using a gel documentation system to confirm amplicon size and integrity. For 16S rRNA gene amplification, PCR was performed under standard conditions: an initial denaturation step was followed by 35 cycles of denaturation, annealing at 52°C, and extension at 72°C, with a final extension to complete synthesis of the amplicons. The resulting single, distinct bands of expected size on agarose gel indicated successful amplification and were subsequently submitted for sequencing and downstream phylogenetic analysis.

### **2.7 Seed Germination Assay with Endophytic Bacterial Inoculation**

Isolates found positive for PGP activity have the potential to enhance seed germination in crops like desi chickpea (*Cicer arietinum* L.). This study assessed the impact of isolated endophytic bacterial strains on desi chickpea seed germination, using, sterile distilled water to compare the effects of bacterial inoculation against standard treatments. The assays measured germination percentage. Each treatment had been carried out in triplicates with 9 seeds per replicate i.e., 27 seeds per treatment with 9 seeds per control were taken. Healthy, uniform sized desi chickpea of a local cultivar JG 11 variety were collected from a certified supplier. Desi chickpea seeds were cleaned and surface sterilized following standard procedure described above with continuous shaking. Seeds were washed five to six times with sterile distilled water in aseptic chamber to remove residual chemicals (ISTA, 2015). Seeds were Blot dried using whatman 42 filter paper under aseptic conditions for 30 minutes. To check effective sterilization 100 µL of the final seed rinsed water was plated onto nutrient agar and incubated at 28°C for 48 hours; absence of microbial growth confirmed effective sterilization.

**2.8 Preparation of Bacterial Inoculum**

Promising strain was left to grow onto nutrient agar broth and left for overnight with shaking incubator (150 rpm). Optical density was adjusted at 600 nm, $OD_{600} \approx 0.8–1.0$, approximately $10^8–10^9$ CFU/mL by using a spectrophotometer. A single mL of culture was centrifuged for pallet formation. Pellets were washed with PBS and suspended with sterile PBS (pH 7.4) to prepare inoculum. Control treatments included sterile distilled water. Sterilized and blot dried chickpea seeds were inoculated with bacterial suspensions following a modified method (Glick *et al.,* 1999). 9 seeds per replicate were soaked in 10 mL of bacterial suspension ($10^8$ CFU/mL in PBS) for hours at normal temperature in sterile tubes with gentle shaking to ensure uniform coating. Control group seeds were soaked in sterilized distilled water prior to further experimentation.

**2.9 Seed Germination Assay**

The assay was conducted using filter paper (ISTA, 2015). Petri dishes were kept in rows with two layers of Whatman filter paper moistened with 2-3 mL sterile distilled water. 9 inoculated

and control seeds were placed evenly on the filter paper using a sterile forcep, ensuring no contact between seeds. To preserve moisture, the Petri dishes were packed and left to grow at temperatures ranging from 25 to 28°C, with equal dark and light photoperiod. Filter papers were kept moist by adding 1–2 mL sterile water daily as needed. Germination was monitored daily for 7 days and data recording was started from day 2 post- incubation.

Germination Percentage (%) = Number of seeds germinated /Total seeds ×100

## 3. **Result & Discussion**

### *3.1 Isolation, Purification, and Preservation of Endophytic Bacteria*

A total of seven isolated strains exhibiting distinct morphologies were obtained from surface-sterilized roots of *Asparagus racemosus*. No microbes grew on the control plates with final rinse water, showing that the surface sterilization worked well. Colonies appeared within 3–5 days after enough growth on tryptic soy agar (TSA). Each isolate exhibited unique colony characteristics such as differences in colour, shape, margin, elevation, and surface texture, indicating a diverse community of culturable endophytic bacterial presence (Table 1). Pure cultures were obtained through repeated sub-culturing and were preserved at 4°C for further characterization. The successful isolation of endophytes validates the presence of endophytic bacterial populations in the root tissues of A. racemosus, in reference with the previous findings in other medicinal plant (Kumar et al., 2016). Surface sterilization with ethanol and sodium hypochlorite followed by sterile PBS maceration effectively eliminated epiphytes that contaminates; while preserving internal microbial communities intact.

### **3.2 Morphological Characterization of Bacterial Isolates**

All seven purified endophytic bacterial isolates exhibited distinct colony morphologies on TSA plates after two days of growth. Differences were seen in colony size, colour, shape, margin, elevation, texture, and opacity. Gram stain analysis revealed distinct differences in Gram response, cellular shape, and structural arrangement across the bacterial isolates. Colony colours ranged from white to cream and yellow, with shapes commonly varying from circular to irregular.

Margins of the colonies were observed to vary from entire to lobate, and elevations ranged from flat to convex. Both Gram +ve and Gram -ve reactions were recorded, with bacilli being the most common cell shape.

### 3.3 Hydrolytic enzyme activity

The seven endophytic bacterial isolates showed a diverse portfolio of extracellular enzymes and plant growth–promoting traits, indicating their strong functional potential as bioinoculants. Most isolates produced cellulase and pectinase, with five and six positives respectively, suggesting an enhanced ability to degrade plant cell wall polymers and facilitate endophytic colonization, nutrient release, and tissue penetration. Protease and chitinase activities were more restricted (two positives each), pointing to a selective subset capable of protein and chitin turnover, which can contribute to nutrient cycling and, in the case of chitinase, potential antagonism against fungal pathogens. Such combinations of hydrolytic enzymes are widely reported among endophytes and are considered key determinants of successful internal colonization and host compatibility. All isolates synthesized IAA and ammonia, while one isolate each showed strong siderophore production, phosphate solubilization, and nitrogen fixation, and two expressed ACC deaminase, highlighting complementary mechanisms for nutrient acquisition and modulation of plant hormone and stress responses. Universal IAA production indicates a shared capacity to stimulate root growth and architecture, whereas siderophore production, P solubilization, and nitrogen fixation collectively support improved iron and phosphorus availability and supplemental nitrogen input to the host. The detection of HCN in all isolates, albeit at low to moderate levels, alongside occasional chitinase and protease activity, suggests an additional biocontrol dimension through suppression of soilborne pathogens, a feature well documented for plant growth–promoting bacteria. ACC deaminase–positive isolates are particularly valuable under stress conditions because they can lower plant ethylene levels and thereby sustain growth under suboptimal environments. Taken together, the multi-trait profile observed in these Asparagus-derived endophytes aligns with current literature on PGPR/endophytic consortia and

supports their candidacy as eco-friendly inoculants for legumes like chickpea, especially where both growth promotion and disease suppression are desired (Table 2).

### 3.4 Molecular Characterization of Bacterial Isolate

The multifunctional endophytic isolate i.e. strain AR1 was molecularly identified by performed 16S rRNA gene sequencing. Genomic DNA was extracted and PCR-amplified (~1.5 kb) using universal primers 27F and 1492R (Figure 4 & 5). Sanger sequencing followed by BLASTn analysis revealed a close relationship with both *Enterobacter wuhouensis* (99.44% identity in the NCBI rRNA database). The isolate most likely belongs to the genus Enterobacter, with the closest match being *Enterobacter wuhouensis*. The results strongly support its taxonomic placement within Enterobacteriaceae. The sequence was submitted to NCBI GenBank (accession no. [PV639654]). Phylogenetic analysis (Figure 6 & 7) using Neighbor-Joining with 500 bootstraps and MAFFT alignment clustered the isolate within the E. cloacae complex with strong support (>90%). The closer match to *Enterobacter wuhouensis*, a less common strain, highlights the isolate's unique position within this complex. This molecular evidence confirms its taxonomic placement and supports its potential as a plant growth-promoting agent, reinforcing its application in sustainable agriculture for *Cicer arietinum*.

The nifH gene was successfully amplified, producing a clear ~360 bp band on agarose gel, confirming the strain AR1' s nitrogen-fixing potential (Figure 7). This aligns with previous studies linking nifH presence to plant growth promotion via nitrogen fixation (Poly et al., 2001). The effective DNA extraction and PCR protocols ensured reliable detection. These results support the isolate's role as a plant growth-promoting bacterium for *Cicer arietinum*, guaranteeing further quantitative studies on nifH expression.

To validate the functional attributes observed during phenotypic screening, molecular characterization of strain AR1 was performed through sequence-based bioinformatic analysis. Phylogenetic reconstruction based on the 16S rRNA gene sequence confirmed its affiliation with *Enterobacter wuhouensis*. Furthermore, nifH gene analysis revealed conserved nitrogenase-

associated regions, supporting the nitrogen-fixing capability of the isolate. Genome-based functional annotation (where available) may provide further evidence for the presence of genes associated with auxin production, phosphate solubilization, siderophore biosynthesis, ACC deaminase activity, and stress tolerance, thereby explaining its multifaceted plant growth-promoting properties.

The comparative analysis of plant growth-promoting (PGP) associated genes revealed the presence of multiple functional determinants in strain AR1, supporting its multifunctional role as a potential bioinoculant (Figure 8). The strain showed the presence of key genes associated with phytohormone production (*ipdC/IAA pathway*), phosphate solubilization (*gcd* and *pqq* genes), nitrogen fixation (*nifH/nifD*), siderophore biosynthesis (*ent* genes), and stress tolerance mechanisms (*acdS*, *sod*, and *kat* genes). The detection of these genetic traits correlates with the observed phenotypic activities, including IAA production, phosphate solubilization, nitrogen fixation, ACC deaminase activity, and enhanced chickpea seed germination. The presence of extracellular enzyme-associated genes further indicates the ability of AR1 to improve nutrient cycling and rhizosphere interactions.

### 3.5 Mechanism of AR1-mediated chickpea growth promotion

The proposed model illustrates the multifaceted mechanisms through which *Enterobacter wuhouensis* AR1 enhances chickpea growth (Figure 9). Following seed inoculation, AR1 colonizes the root region and internal tissues, where it promotes plant development through several synergistic pathways. IAA production stimulates root elongation and improves nutrient acquisition, while phosphate solubilization and siderophore production increase the availability of essential nutrients such as phosphorus and iron. The presence of nitrogen fixation-related genes enables biological nitrogen enrichment, reducing dependence on external nitrogen sources. Furthermore, ACC deaminase activity may alleviate abiotic stress by regulating ethylene levels in plants. Collectively, these mechanisms contribute to improved germination percentage, enhanced seedling vigor, and better plant establishment under sustainable agricultural conditions.

### 3.5 Seed Germination Assay with Endophytic Bacterial Inoculation

The germination test highlighted a significant disparity between the treated samples and the controls. Seeds inoculated with the selected endophytic bacterial strain AR1 exhibited a more germination rate of i.e. 23 out of 27, while the control group treated with sterile distilled water showed only 8 out of 27 germinations. Each treatment was performed in triplicates with 9 seeds per plate (n = 30) (Table 3). In the control (treated with sterile distilled water), only around 29.63% of seeds successfully germinated. In contrast, seeds inoculated with selected bacterial strains achieved a germination rate of approximately 85.19%. Furthermore, inoculated seeds exhibited enhanced radicle elongation and greater early seedling vigour. This enhanced germination effect could be linked to the isolate's PGP traits, particularly IAA synthesis, phosphate solubilization, and efficient nutrient uptake. These attributes support seed vigour and early establishment. This result supports earlier findings where beneficial endophytes improved germination in legumes and highlights the potential of this isolate as a sustainable bioinoculants for chickpea cultivation.

## Conclusion

This study investigated the root endophytic bacterial community of *Asparagus racemosus* Willd., with emphasis on isolating strains exhibiting plant growth–promoting (PGP) traits. Several isolates demonstrated key functional attributes, including phytohormone production, phosphate solubilization, nitrogen fixation, and siderophore synthesis. Seed inoculation assays in desi chickpea (*Cicer arietinum* L.) revealed enhanced germination rates compared with uninoculated controls, highlighting their potential as biofertilizer candidates for sustainable agriculture. The selection of *A. racemosus*, a medicinally important yet microbiologically underexplored species, enabled the identification of beneficial root endophytes with promising agronomic applications. These findings support the utilization of native microbial resources as environmentally friendly alternatives to synthetic fertilizers, particularly in low-input farming systems. Future research should focus on multilocation field trials to evaluate strain stability, colonization efficiency, and

bioefficacy under diverse agro-climatic conditions. Advanced molecular approaches, including genomics and metagenomics, are recommended to elucidate functional genes, regulatory mechanisms, and plant–microbe interaction dynamics. Overall, this study establishes A. racemosus as a valuable reservoir of beneficial endophytes with potential applications in developing sustainable and ecologically resilient agricultural systems.

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**Conflict of interest**

The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Data availability**: All data generated or analyzed during this study are included in this published article. Data sharing is applicable to this article wherever any request is done to the authors.

**Author contributions**

**Shilpee Dhali, Chinmay Pradhan and Madhusmita Pradhan**: Formal analysis, Investigation, Review, Writing – original draft & review & editing, Project administration, Supervision.

**Chinmaya Kumar Swain**: Edited the Manuscript

**Arpita Das:** Formal analysis, Data curation

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Figure 1: Collected plant sample of *Asparagus racemosus*



Figure 2: Tuberous roots of *Asparagus racemosus*

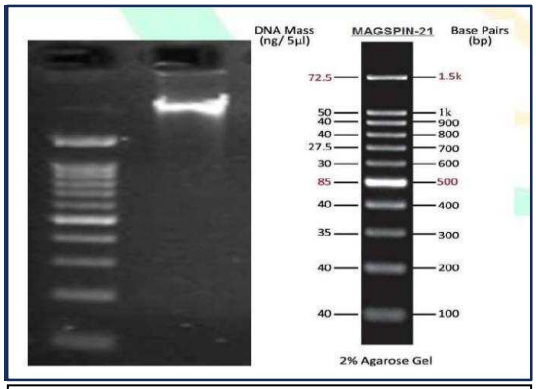



Figure 4: 8% agarose gel showing single 1500 bp of 16s amplicon

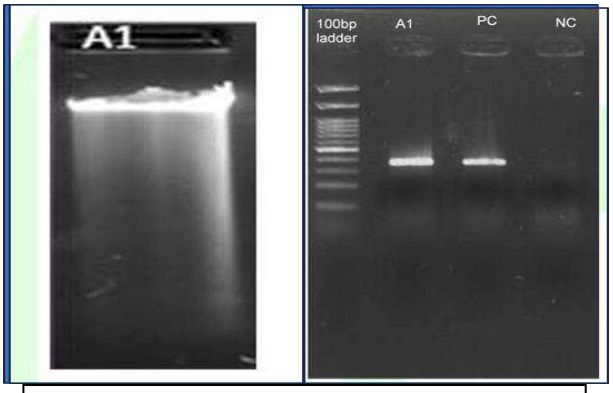



Figure 5: 8% agarose gel showing single 360 bp of *nif*H gene amplicon

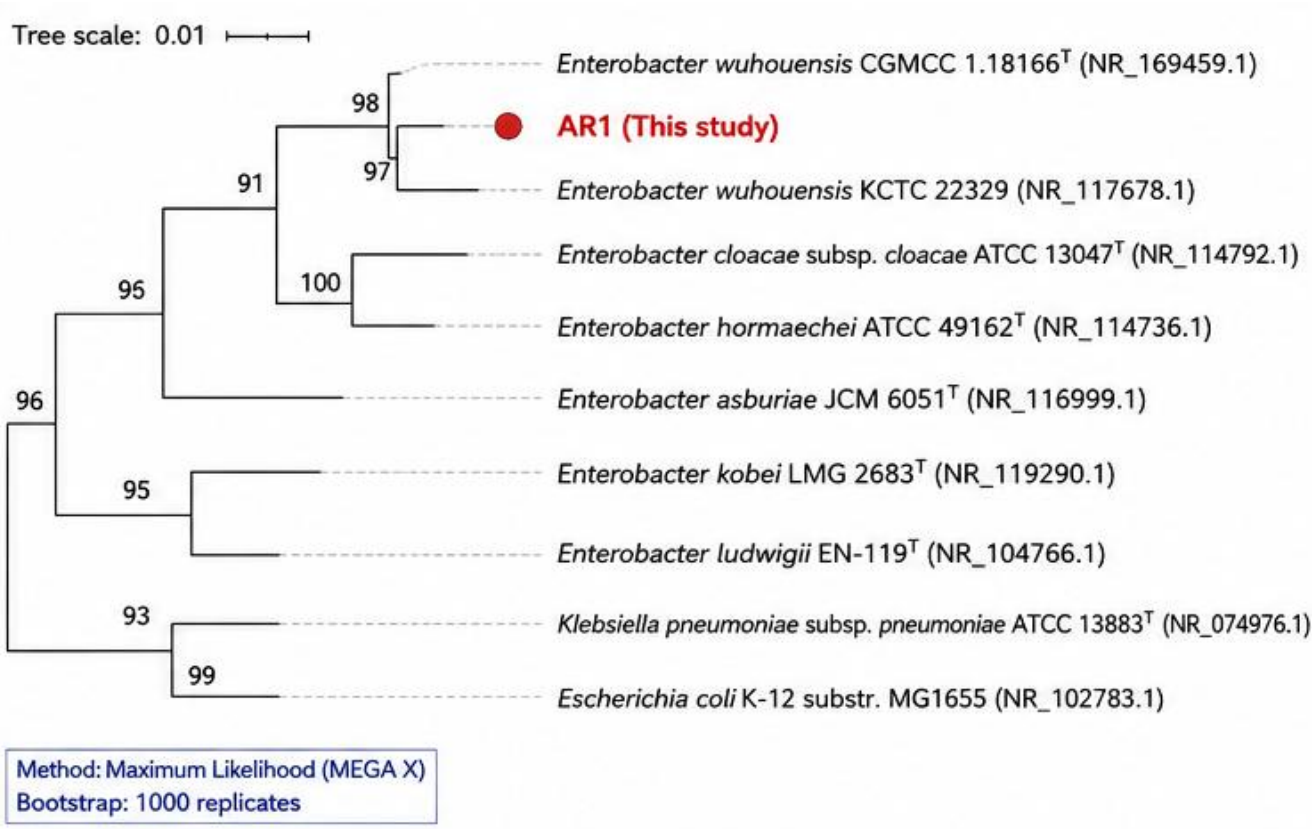



Figure 6: 16S rRNA phylogenetic tree of Strain AR1

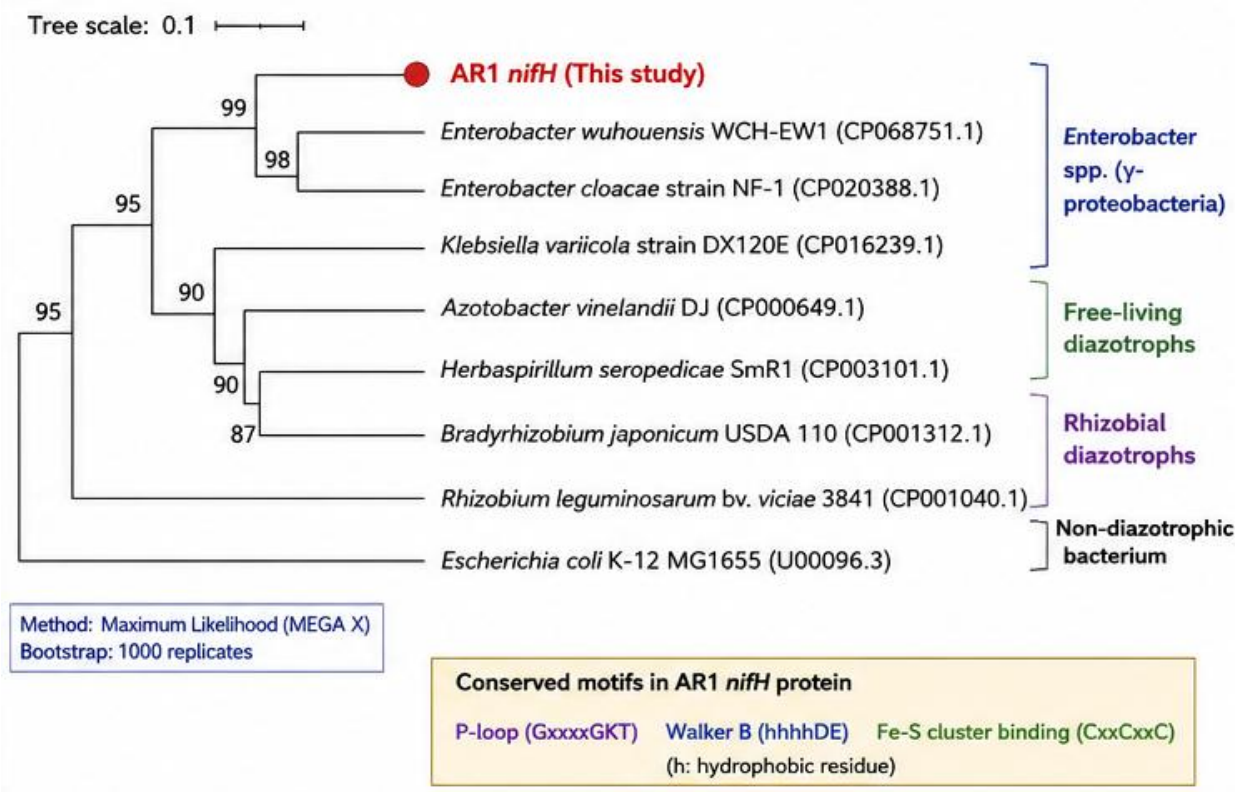



Figure 7: nifH gene phylogenetic tree

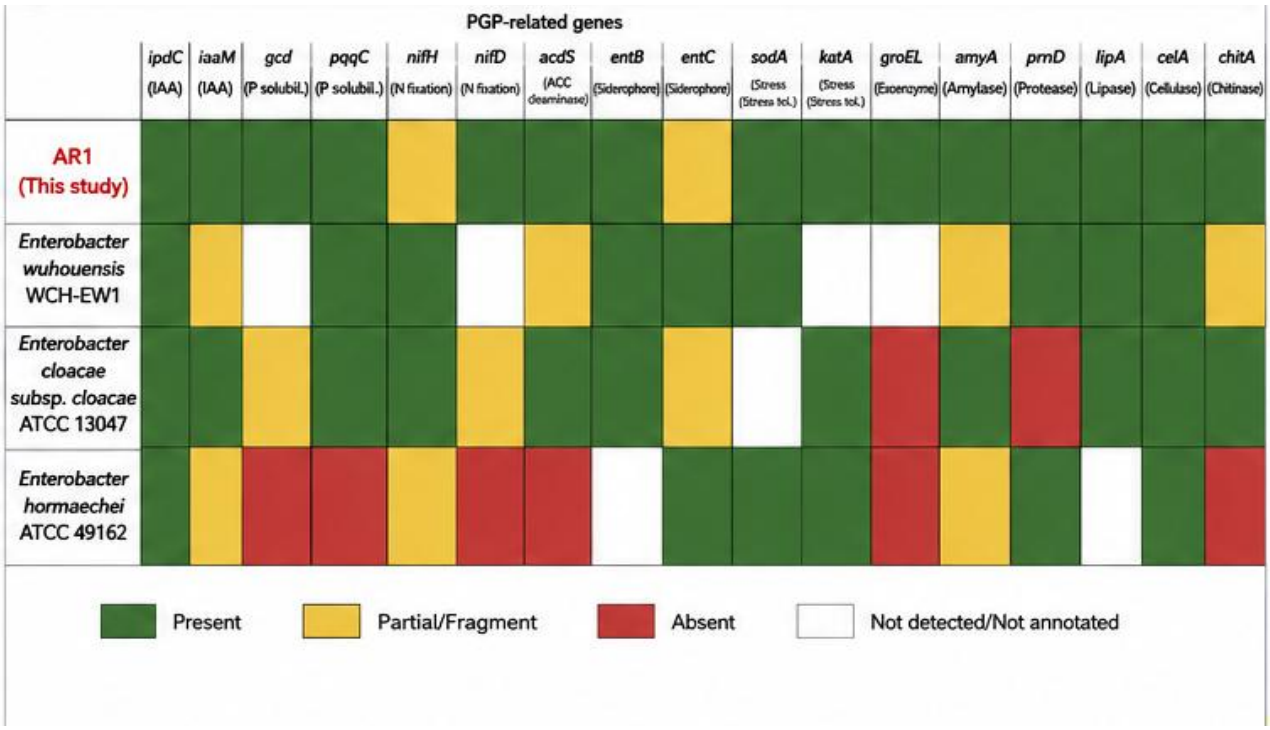



Figure 8: Heatmap showing the distribution of plant growth-promoting gene determinants associated with *Enterobacter wuhouensis* AR1 and related *Enterobacter* strains.

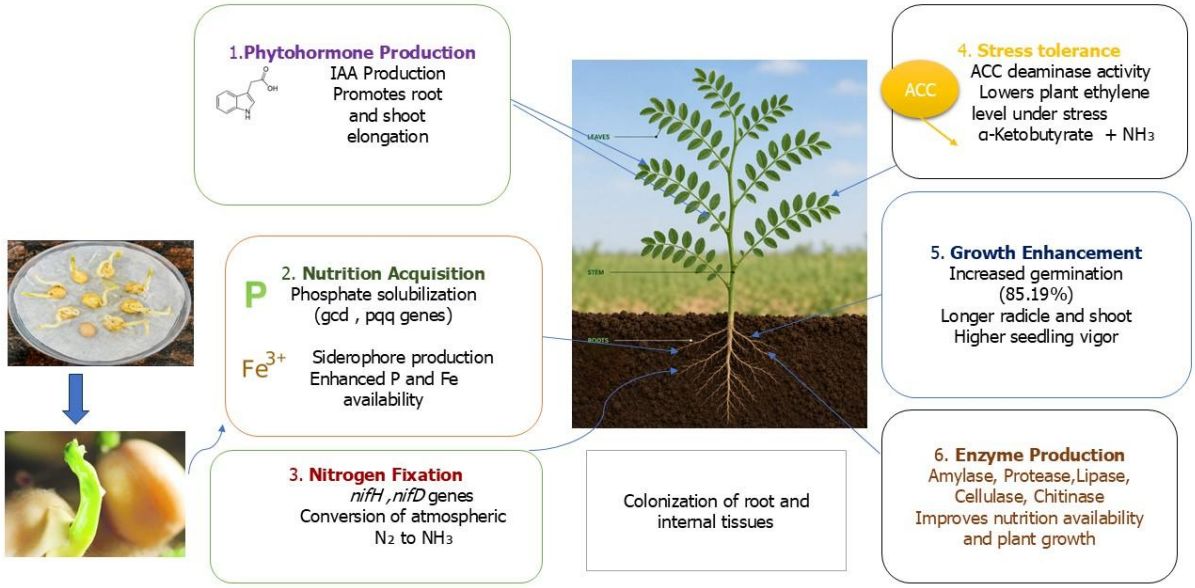



Figure 9: Enhanced chickpea emergence, improved root architecture, seedling vigor and stress resilience leading to better plant growth and yield

Table 1: Morphological and Microscopic Characteristics of Endophytic Bacterial Isolates from *Asparagus racemosus*

| Strain | Shape | Size | Color | Texture | Opacity | Arrangement |
|---|---|---|---|---|---|---|
| Strain 1 | Irregular | Small | Cream | Smooth | Translucent | Cocci (spherical) and some rods (bacilli), clusters and chains |
| Strain 2 | Irregular | Small to medium | Cream | Smooth | Translucent | Rod-shaped (bacilli), clusters and single |
| Strain 3 | Rod- shaped (bacilli) | Small | Cream | Smooth | Translucent | Mostly single and some in pairs |
| Strain 4 | Rod- shaped (bacilli) | Small | Cream | Smooth | Translucent | Single, short chains, and some in pairs |
| Strain 5 | Rod- shaped (bacilli) | Small | Cream | Smooth | Translucent | Single and in pairs |
| Strain 6 | Irregular | Small | Cream | Smooth | Translucent | Rod-shaped (bacilli), clusters |
| Strain 7 | Irregular | Small | Cream | Smooth | Translucent | Single and in pairs |

Note: Strain 1: *Enterobacter wuhouensis*

Table 2- Biochemical Characteristics of Strains 1–7

| Test | Strain 1 | Strain 2 | Strain 3 | Strain 4 | Strain 5 | Strain 6 | Strain 7 |
|---|---|---|---|---|---|---|---|
| IAA | + | + | + | + | + | + | + |
| Siderophore | + | - | - | - | - | - | - |
| Ammonia | + | + | + | + | + | + | + |
| Nitrogen Fixation | + | - | - | - | - | - | - |
| Phosphate Solubilization | + | - | - | - | - | - | - |
| HCN | + | + | + | + | + | + | + |
| ACC Deaminase | + | - | - | - | - | - | + |
| Cellulase | + | + | - | + | - | + | + |
| Pectinase | + | + | + | + | + | + | + |
| Protease | + | + | - | - | - | - | - |
| Chitinase | + | - | - | - | - | - | + |

**'+' = Positive, '-' = Negative**

Table 3: Germination Efficiency of Inoculated vs. Control Seeds

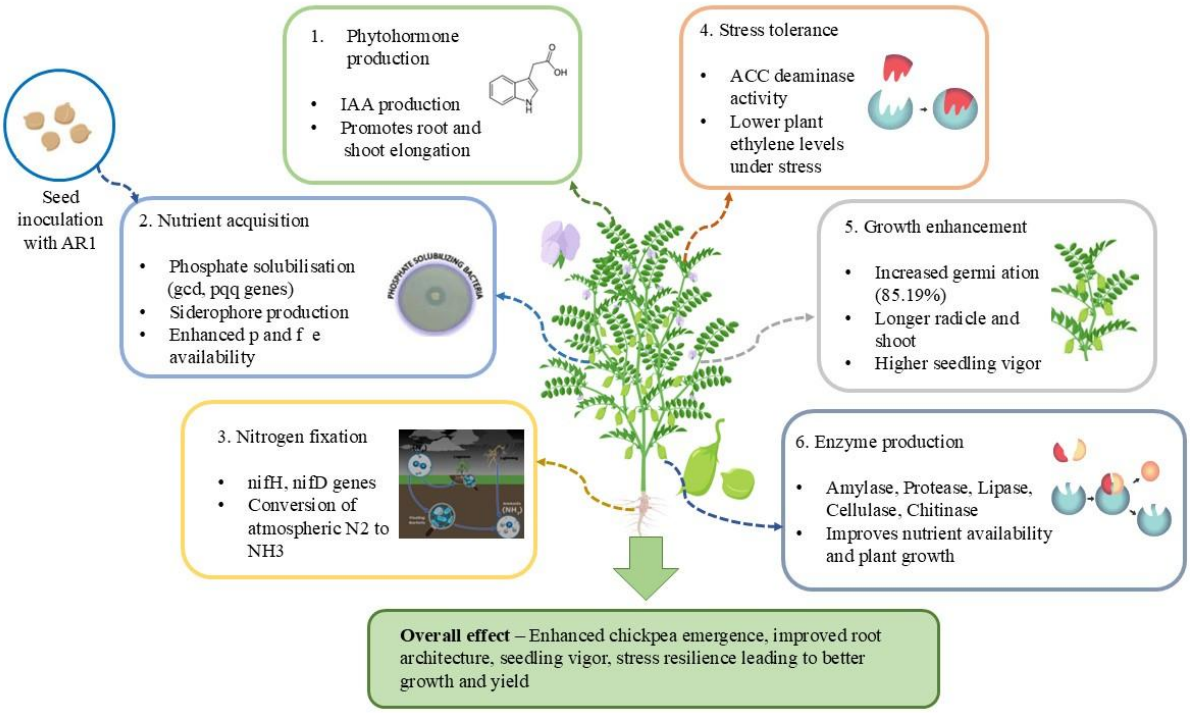
| Treatment | Seeds per plate | Total seeds (3 plates) | Germinated seeds | Germination % |
|---|---|---|---|---|
| Control | 9 | 27 | 8 | 29.63% |
| Inoculated | 9 | 27 | 23 | 85.19% |

## Highlights

- Endophytic bacteria were isolated from medicinal plant *Asparagus racemosus* roots and evaluated for plant growth-promoting traits.
- Seven bacterial isolates exhibited diverse PGP activities, including IAA production, phosphate solubilization, nitrogen fixation, and siderophore production.
- Strain AR1 showed the strongest multifunctional PGP potential with ACC deaminase and hydrolytic enzyme activities.
- 16S rRNA and *nifH* gene analysis identified AR1 as *Enterobacter wuhouensis* with nitrogen-fixing capability.
- AR1 inoculation improved chickpea germination (85.19%) and enhanced seedling growth.
- AR1 demonstrates promise as a sustainable microbial bioinoculant.

194 **Graphical Abstract**

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