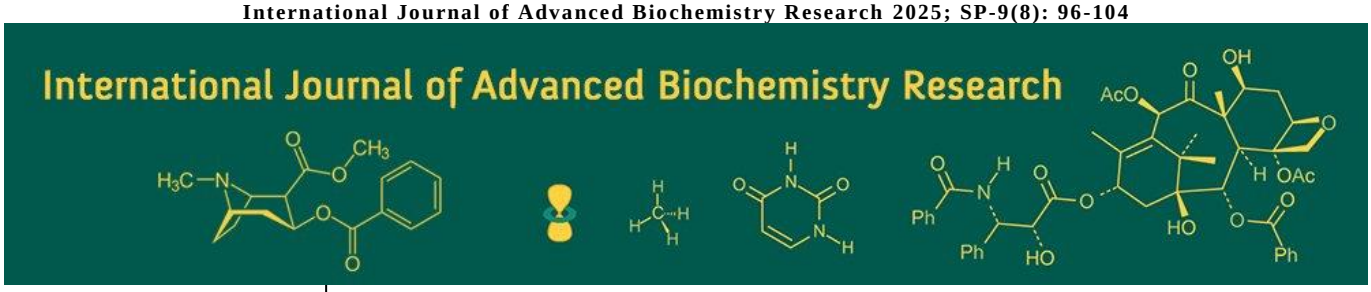

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Isolation, molecular identification, and screening of endophytic bacteria *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* from plant root tissues with the capacity for plant growth promoting traits

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Abstract
The microbes that colonize the plant inter or intracellular, referred to as endophytes, establish symbiotic relationships within plant cells, thereby augmenting their resilience to multiple stress factors. In the present study, endophytic bacteria, namely *Gluconacetobacter* sp. and *Herbaspirillum* sp., were isolated from the tissues of surface-sterilized roots and stems of two crops: sugarcane and rice. Six endophytic isolates were used for morphological, and biochemical observations. Furthermore, they were screened for plant growth-promoting traits, including phytohormone production, nitrogen-fixing ability, mineral solubilization, biocontrol activity, and stress tolerance capacity. The isolates GA 1 and HS 1 produced a maximum concentration of Indole acetic acid and Gibberellic acid, fixed maximum nitrogen i.e., 85.62 and 32.53 mg of N/g of carbon used, respectively. These two isolates also showed the maximum mineral solubilization zone, inhibition zone against the rice pathogens, ACC deaminase enzyme activity, and tolerance to various stresses viz., salt, thermal, and drought. The efficient isolates GA 1 and HS 1 which were selected based on screening results further examined for molecular characterization by the 16S rRNA gene sequencing method. The isolate GA 1 was identified as *Gluconacetobacter diazotrophicus* and HS 1 was identified as *Herbaspirillum seropedicae*. The results of this study demonstrated that these endophytic bacteria are good repositories of plant growth-promoting activity, which should be investigated further.

Keywords: Endophytic bacteria, isolation, screening, IAA and GA production, FIXING nitrogen, biocontrol activity, stress tolerance capacity, molecular identification

Introduction
Bacteria or fungi known as endophytes invade plant tissues either intracellularly or intercellularly without harming the host [1]. They are creating facultative or obligatory relationships with plants, which may be antagonistic or mutualistic [2]. Plants will completely regulate colonization [3]. They increase plant growth in several forms, mainly by producing and secreting plant growth regulators, fixing nitrogen, improving the availability and absorption of minerals like phosphate [4], and biocontrol of phytopathogens [5, 6, 7] by the production of antifungal or antibacterial agents, siderophores, nutrient competition, and the rise of immunity or resistance in systemically acquired hosts [8].
In an unfavorable environment, they support plant growth by the production of exopolysaccharides (EPS), enzymes like 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), improving the availability of minerals [7], and regulating the expression of stress-responsive genes through the release of metabolites [9]. These nitrogen-fixing endophytes are known as endophytic diazotrophs, and since 1990, they have been used as plant growth-promoting rhizobacteria (PGPR) predominantly belonging to the genera *Gluconacetobacter*, *Azoarcus*, *Azospirillum*, *Klebsiella*, *Serratia*, *Rhizobium*, and *Herbaspirillum*.
Endophytic bacteria such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. were isolated from rice and sugarcane and may be involved in BNF [10, 11]. *G. diazotrophicus* was first isolated in sugarcane plants (*Saccharum officinarum* L.) [12] in Brazil. It initially recovered from sugarcane crops [13], which belong to Alphaproteobacteria. After that, it was discovered in a number of crops, including sorghum [14], pineapple, maize, carrot [15], coffee [16], and wild rice.

It possesses important traits for promoting plant growth, including biological nitrogen fixation, the production of phytohormones such as auxins (indole compounds) [17], and the natural solubilization of minerals including potassium, magnesium, zinc, phosphorus, and iron [18]. Additionally, according to Muthukumarasamy *et al.*, 2002 [19], it has negative properties against a variety of bacterial and fungal pathogens.

H. seropedicae was the first species of this genus, *Herbaspirillum*, which comes under the class of Betaproteobacteria [20]. It was first isolated from surface-sterilized roots of maize, sorghum, and rice [20]. They are found in forage grasses, pineapples, and bananas, but they do not fare well in soil [21, 22] as well as from sugarcane (*Saccharum* hybrid) leaves [23]. They can colonize on both epiphytic and endophytic plant tissues [24], although they mostly do so on the apoplast intercellular space [25]. They produced gibberellins and indole acetic acid (IAA) [26] and solubilized the phosphorus [24]. In *H. seropedicae* SmR1, the genome was found to have 18 genes encoding siderophore receptors in addition to the acdS gene, which codes for ACC deaminase [27].

Materials and Methods

1. Collection and Preparation of root samples

Rice and sugarcane root samples were collected from the Cuddalore districts in Tamilnadu, India. Tap water was used to remove the unwanted soil particles. After that, the roots were surface sterilized five times using sterile distilled water and for a period of five minutes using 5% sodium hypochlorite (NaOCl).

2. Isolation of endophytic bacteria

Endophytic bacteria *Gluconacetobacter* sp. was isolated from sugarcane roots by using LGI medium [12, 15]. *Herbaspirillum* was isolated from rice roots by using Jenson's nitrogen-free JNFB medium [28, 29].

Small pieces of the roots of the sample were washed separately with tap water. They were disinfected with 70% alcohol (v/v) for 30 seconds, followed by washing with sterile distilled water. They were again surface sterilized with 0.1% $HgCl_2$ (w/v) for one minute and washed through sterilized distilled water [30]. One gram of each root sample was homogenized (using a sterilized pestle and mortar) in a 10% sucrose solution/saline solution, and the extract was made and utilized for serial dilutions. The suspension of extract 1-2 ml was poured into sterilized petri plates on selective medium LGI and JNFB media for the isolation of *Gluconacetobacter* and *Herbaspirillum*, respectively. The inoculated LGI and JNFB plates were incubated at 30 and 33ºC for 3-5 days [28, 31].

After the incubation period, we observed the pure orange to yellow colonies on LGI media indicating the growth of *Gluconacetobacter* [12]. The small, moist, and slightly white to green-centered colonies on JNFb solid medium indicate the growth of *Herbaspirillum* sp. [32]. Further, they were used for successive subculturing and purification.

3. Phenotypic characterization of endophytic bacteria

Preliminary identification of endophytic bacteria was performed based on morphological and biochemical tests such as microscopic features, Gram's staining, H_2S production, Urease test [33], Starch hydrolysis test [34], Gelatinase activity, and Catalase test [35]. According to the biochemical and morphological characterization, the selected isolates have been identified up to the genus level.

4. Screening of isolates for plant growth-promoting activities

Production of Phytohormone

The isolates were cultured overnight in DF salt minimum media [36] and added with 1000 µg/ml of L-tryptophan in order to quantify indole acedic acid (IAA). The culture was centrifuged afterwards the incubation time. Four ml of Salkowski reagent were combined with one ml of culture supernatant. The OD value was measured at 535 nm using a UV-VIS spectrophotometer after 20 minutes of the pink color development [37].

The isolates were cultivated in nutrient broth added with 1000 µg/ml of L-tryptophan to quantify gibberellic acid (GA). After incubation, 5 ml of the culture was mixed with potassium ferrocyanide and zinc acetate before being centrifuged. 30% HCl was added to 3 ml of supernatant and allowed to incubate. A UV-VIS spectrophotometer was used to determine the absorbance of the sample and blank at 254 nm [38].

Nitrogen-fixing capacity

The micro-Kjeldahl method [39] was used to examine the ability of endophytic bacteria to fix atmospheric N_2 in a culture medium. The formula for estimating Nitrogen (N_2) is:

N_2 (mg/g) = (ml of H_2SO_4 in the sample x Normality of H_2SO_4 x 14.01)/Weight of the sample (Carbon used in grams).

Solubilization of Mineral

To assess the potential of isolates to solubilize the P, K and Zn by using plate assays in Pikovskaya media containing insoluble phosphorous hydroxyl apatite [40], Tris minimal medium (0.1%) containing ZnO [41], and Aleksandrov agar medium (0.5%) containing insoluble potassium aluminum silicate [42], respectively, the diameters of the clear zones surrounding the colonies were measured after the incubation period, expressed in centimeters.

Siderophore production

For the detection of siderophore production by isolates, the chrome azurol sulfonate (CAS) agar media was utilized. The diameter of the orange colored zone around the isolates was examined visually and graded as a narrow clear zone < 2 cm (+) and a wide clear zone > 2 cm (+ +). Diameter of the zone was expressed in cm [43].

Biocontrol Activity

The ability of the isolates to suppress major pathogens of paddy, such as *Rhizoctoniasolani*, *Pyriculariaoryzae*, and *Xanthomonas oryzae pv. Oryzae* was tested by the dual culture method [44]. After the incubation period, the size of inhibition zone (mm) was measured.

Stress tolerance capacity

On their particular growing medium enriched with 1%, 2%, and 3% concentrations of NaCl, the endophytic isolates were examined for salt tolerance [45]. One ml of a 15-day-old culture suspension was added to a particular isolate's medium, and the isolates were incubated for 24 to 48 hours

at 45° C, 50° C and 55° C to assess their capacity to withstand high temperatures [46]. The population level of the bacteria was expressed in log CFU/ml.

For testing moisture stress tolerance, the isolates were inoculated into a particular medium containing PEG 6000 at 5%, 10%, 15%, and 30% concentrations [47]. Using a spectrophotometer to measure the OD value at 600 nm, growth was estimated [45]. Further, the isolates were tested for their capacity to produce the ACC deaminase enzyme [48]. Based on the visible growth in the plate, they are classified into three groups, i.e., + + + (high growth), + + (moderate growth), and + (less growth).

5. Genotypic characterization of endophytic bacteria

Using 16S rRNA sequencing, the most efficient strains of endophytic bacteria were molecularly identified up to the species level. The strains were submitted to Bangalore's Medauxin Biotechnological Company for for molecular identification. The 16S rRNA gene was amplified using universal primers 27F and 1492R [49] after genomic DNA was extracted [50]. BLAST was performed using the NCBI Genbank database and gene sequence. To compute the evolutionary distances of organism Maximum Composite Likelihood approach was used [51]. MEGA XI software was used to create phylogenetic trees after the distance matrix was generated. Each bacterial isolate's sequencing was added to GenBank in order to obtain an accession number.

Results

Fresh plant material (root and stem tissue) of sugarcane and rice plants was used for the isolation of bacterial endophytes (Fig. 1). The population of bacteria in sugarcane plant parts collected from the Cuddalore district consists of 6.67 and 6.49 log CFU per gram root weight and stem weight, respectively, in LGI medium. The density of *Herbaspirillum* populations recovered in JNfb medium were 5.94 and 5.95 log CFU per gram of fresh root and stem weight of rice plant, respectively. Isolated *Gluconacetobacter* and *Herbaspirillum* colonies are shown in Fig. 2 and 3. A total of 6 isolates (Four from root tissue and two from stem tissue of both sugarcane and rice plants) were selected and designated as GA 1, GA 2, GA 3, and HS 1, HS 2, and HS 3, those considered as a genus of *Gluconacetobacter* and *Herbaspirillum*, respectively (Table 1). They are used for further characterization and screening procedures.

In morphological characterization, the endophytic bacterial isolates exhibited diverse shapes and colony colors, i.e., rod to vibrioid shape and orange/yellow color to green-centered, moist, white color. Regarding Gram staining, all isolates were found to be gram-negative in the Gram reaction. In biochemical characterization, all the isolates were found positive towards catalase, the H_2S production test, and the urease test, and negative towards gelatin liquefaction and the starch hydrolysis test (Table 2).

All endophytic isolates were able to produce indole acetic acid and gibberellins at different quantities. In *Gluconacetobacter* sp. The highest IAA and GA3 were produced by an isolate GA1, i.e., 18.44 and 0.78 µg/ml, respectively. In *Herbaspirillum*, isolate HS 1 produced maximum phytohormones (i.e., 11.75 µg/ml-IAA and 0.82 µg/ml-GA3) when compared to isolates HS 2 and HS 3. The minimum GA3 production was observed in GA 2, HS 2 (Fig. 3a). The ability of isolates to fix the nitrogen was tested by the Microkjeldhal method. The isolate GA 1 fixed a higher amount of nitrogen than other strains (85.62 mg N/g of carbon), and the isolate HS 2 fixed the lowest amount of nitrogen, i.e., 29.83 mg N/g of carbon. Strain HS 2 fixed about 29.83 mg N/g of carbon (Fig. 3b).

All six isolates solubilized P and Zn. The larger P solubilization zone was observed in isolate GA 1, i.e., 1.6 cm, and HS 1 i.e., 1.3 cm. The diameter of the largest zone (i.e., 1.2 cm) for zinc solubilization was recorded with GA 1 followed by isolate GA 2. While the lowest solubilization (i.e., 0.3 cm) was recorded with strain HS 2. Potassium solubilization was not observed in any plates (Fig. 3c). All isolates were able to produce siderophores on CAS agar by the formation of orange-colored halo zones around bacterial colonies ranging from 2.82 to 1.64 cm (Fig. 3d).

All endophytic isolates showed inhibition activity against *Rhizoctonia solani* except HS 2, ranging from 3.78 to 7.22 mm of the zone of inhibition. All *Gluconacetobacter* isolates showed biocontrol activity against bacteria *Xanthomonas oryzae* since HS 1, HS 2, and HS 3 isolates don't show any activity. The rice pathogen *Pyricularia oryzae* wasn't inhibited by any of the isolates (Fig. 4a).

The endophytic isolates were tested for salt tolerance in respective media supplemented with 1-3% NaCl. All the isolates showed growth on a medium containing 1% NaCl ranging from 0.81 to 3.73 log CFU/ml of inoculum. In 2% NaCl concentration, only *Gluconacetobacter* isolates showed growth. No isolate showed tolerance against 3% NaCl. Higher levels of NaCl suppressed the growth of endophytic isolates (Fig. 4b). Similarly, all isolates showed growth from 45°C to 50°C, which ranges from 2.93 to 4.75 log CFU/ml of inoculum. In high temperatures (55°C) during incubation, only the isolates HS 1 and HS 2 showed growth when no others did (Fig. 4c).

In the drought-tolerant test, all isolates showed growth at 5-10% of PEG (6000) concentration, while only five isolates (GA 1, GA 2, GA 3, HS 1, and HS 3) showed growth at 15% of PEG. The isolates with the highest O.D. values at 15% PEG concentrations were considered potential plant growth promoters under water deficit conditions. The growth rate wasn't observed in 30% of PEG concentration except in isolate HS 1 (Fig. 4d). All isolates grew on plates having ACC as the sole source of nitrogen, exhibit their capacity to produce the ACC deaminase enzyme. The isolates that showed high visible growth were GA 1 and HS 1. Less visible growth was observed by strain HS 2, while others showed moderate growth on plates (Table 3).

Out of 6 bacterial isolates, the most efficient strains, GA 1, and HS 1 were molecularly identified by using 16S rRNA sequencing, and the sequences were submitted to GenBank to get accession numbers. The isolate GA 1 was identified as *Gluconacetobacter diazotrophicus* (PQ137891), and HS 1 was identified as *Herbaspirillum seropedicae* (PQ148469). The phylogenetic trees are shown in Figures 5 and 6.

Table 1: Isolation of Endophytic bacteria from different crops

Isolation source	Habitat	Population of bacteria (log CFU/ g of sample weight)	Isolate code
Sugarcane	Root tissue	6.67	GA 1, GA 2
Sugarcane	Stem tissue	6.49	GA 3
Rice	Root tissue	5.94	HS 1, HS 2
Rice	Stem tissue	5.95	HS 3

Table 2: Morphological and Biochemical characteristics of Endophytic isolates

Particulars	*Gluconacetobacter* sp.			*Herbaspirillum* sp.		
	GA 1	GA 2	GA 3	HS 1	HS 2	HS 3
Cell shape	Rod	Rod	Rod	Vibrioid	Vibrioid	Vibrioid
Colour	Light yellow	Yellowish orange	Yellowish orange	Slimy white	Slimy white	Moist white
Gram reaction	Gram-	Gram-	Gram-	Gram-	Gram-	Gram-
Gelatin liquefaction	-	-	-	-	-	-
H2S production	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-
Urease test	+	+	+	+	+	+

+ Positive;-Negative

Table 3: Screening of bacterial isolates for ACC deaminase activity

Isolates	ACC deaminase activity
GA 1	+ + +
GA 2	+ +
GA 3	+ +
HS 1	+ + +
HS 2	+
HS 3	+ +

Visible growth on plates: + (less growth), + + (moderate growth), + + + (high growth)

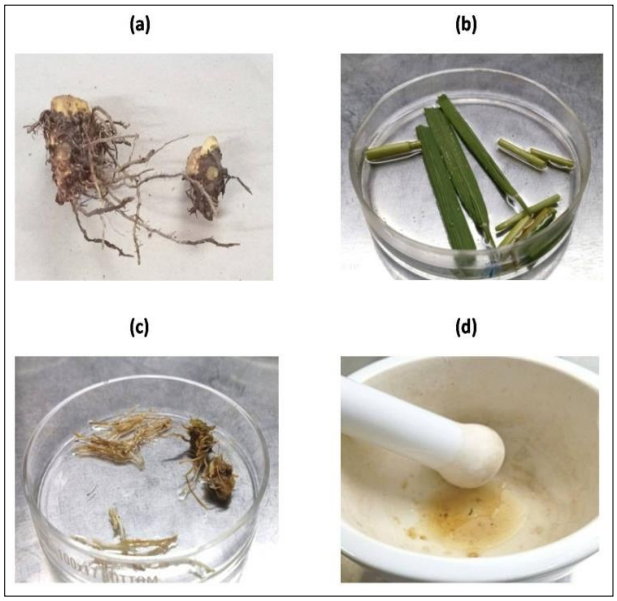


Fig 1: Collection and Preparation of plant samples (a) Collection of Sugarcane roots sample (b) Surface sterilization of Rice stem sample using 5% sodium hypochlorite (NaOCl) (c) Surface sterilization of Sugarcane root sample (d) One gram of each sample was homogenized using a sterile pestle and mortar.

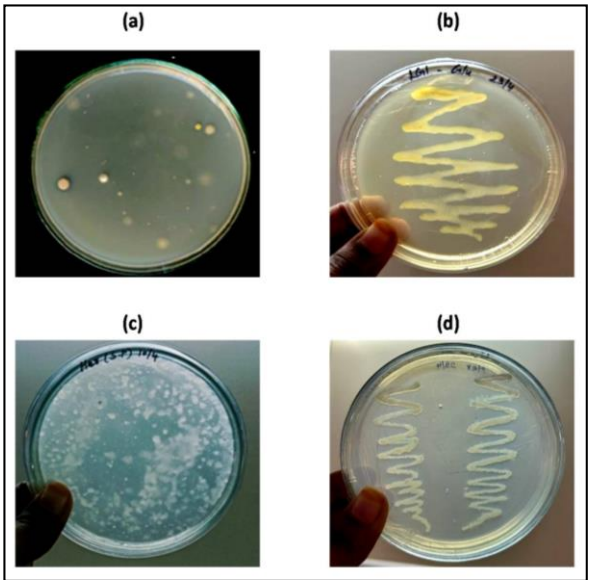
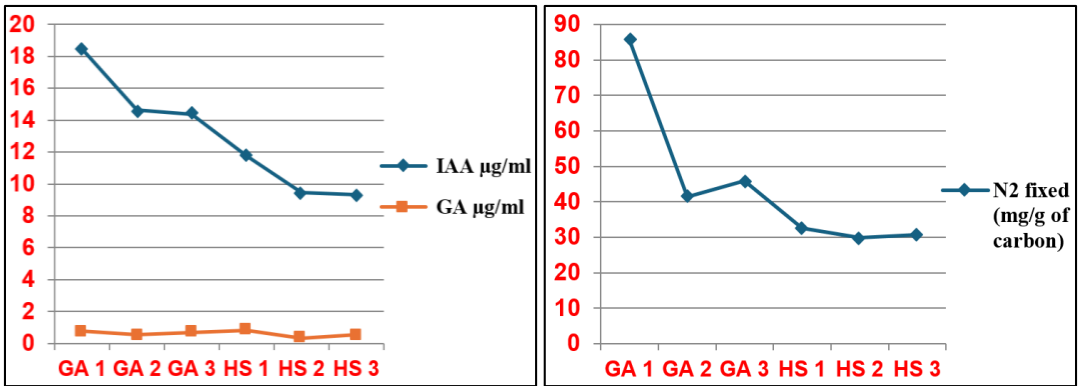
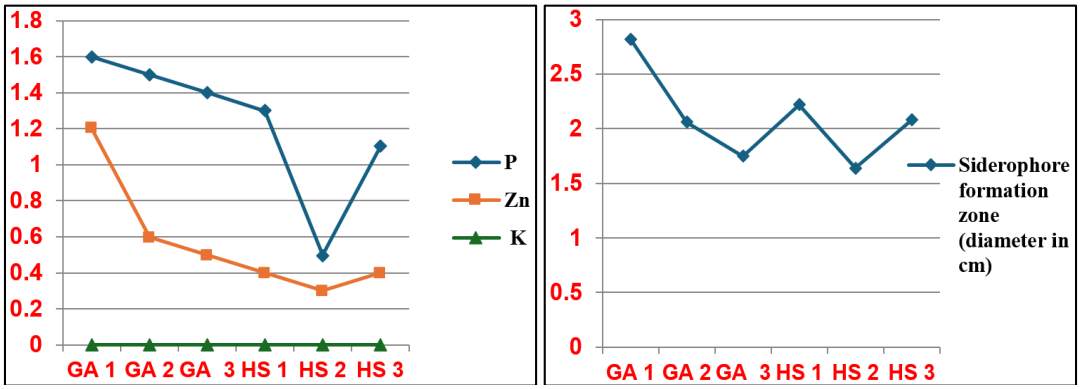


Fig 2: Isolation of Endophytic bacteria (a) Colony morphology of *Gluconacetobacter*-Yellow color colony on LGI agar plate (b) Subculture of isolate GA1 (c) Colony morphology of *Herbaspirillum*-Moisty colony on JNFB agar plate (d) Subculture of isolate HS1.

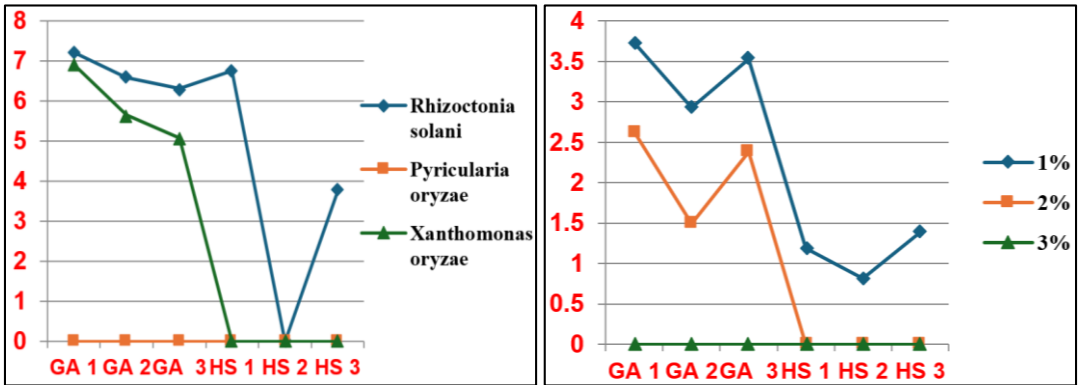


(a) (b)

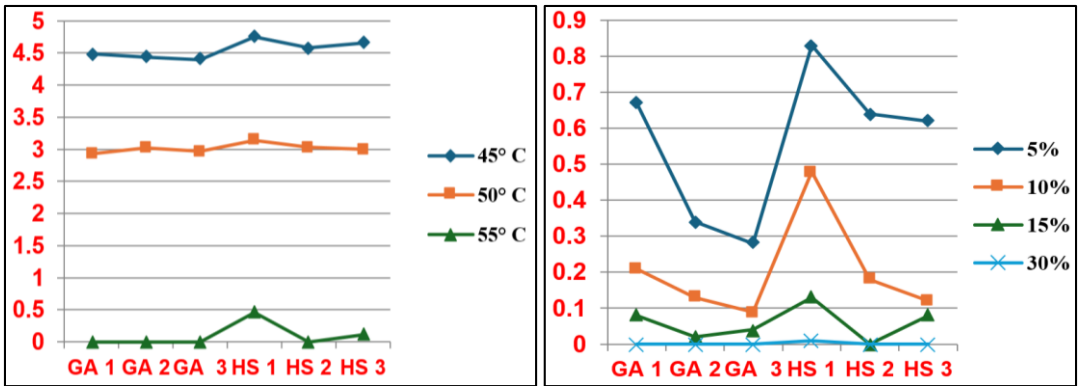


(c) (d)

Fig 3: Screening of Endophytic bacteria for plant growth promoting activity (a) Production of Indole acetic acid and Gibberellic acid in µg/ml of the medium (b) Ability to fix atmospheric N_2 in mg/ gram of carbon used in medium (c) Solubilization of minerals (P, K, Zn)-Halo zone formation expressed in cm (d) Siderophore production-Orange colour zone around colony in cm



(a) (b)



(c) (d)

Fig 4: Screening of Endophytic bacteria for plant growth promoting activity (a) Antagonistic activity measured by size of the inhibition zone (mm) (b) Population level of the bacteria (log CFU/ml) in 1-3% of salt stress (c) Population level of the bacteria (log CFU/ml) in 45-55 °C thermal stress (d) Growth level of bacteria (OD value at 600 nm) in 5-30% of PEG 6000

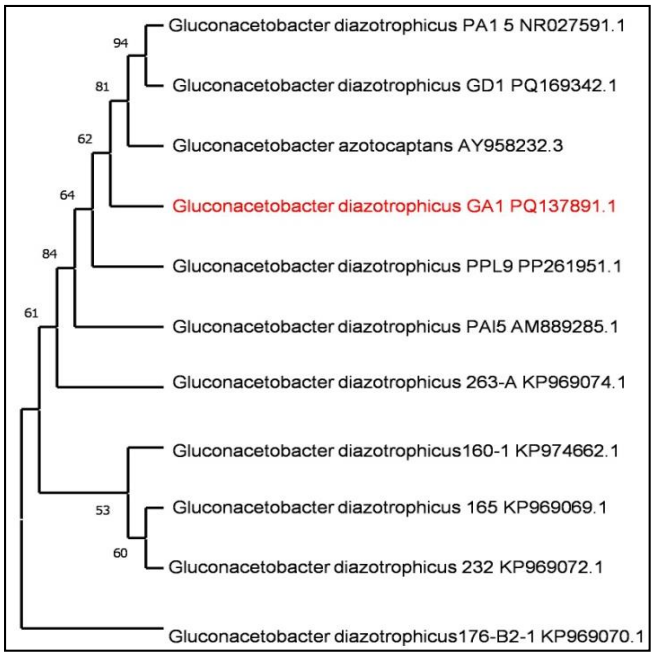


Fig 5: Phylogenetic tree of isolate GA1 (*Gluconacetobacter diazotrophicus*)

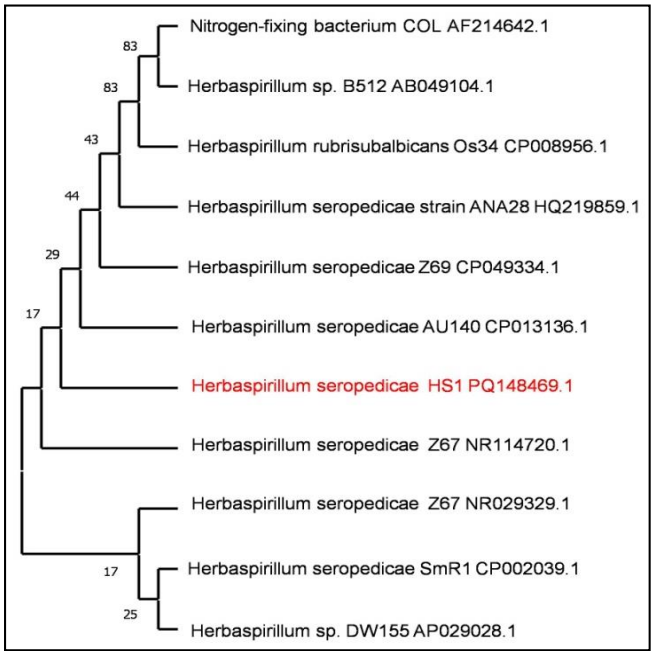


Fig 6: Phylogenetic tree of isolate HS1 (*Herbaspirillum seropedicae*)

Discussion

Microbial endophytes colonize virtually all tissues of the host plant, in the intercellular spaces of the cell walls and xylem vessels, root, stem, flowers, fruits, and seeds [52]. The plant provides primary nutritive components and a protective niche for the endophytic organisms whereas, the endophytes produce useful metabolites and systemic signals [53]. Growth-promoting endophytes get restored in crops which could result in a reduction of agrochemical inputs used for controlling various pests and diseases. As a result, it could lead to the generation of crops that can tolerate biotic and abiotic stresses more efficiently [54].

In this present investigation, endophytic bacteria *G. diazotrophicus* and *H. seropedicae* were isolated from different plant parts of Sugarcane and Rice, respectively. This was based on a similar study by and Cavalcante and Dobereiner, 1988 [12] and Baldani *et al.*, 1986 [20] who first isolated *G. diazotrophicus* and *H. seropedicae*, respectively. A total of six morphologically different strains were selected from the larger collection of isolates based on the colony morphology, i.e., GA 1, GA 2, GA 3, HS 1, HS 2, and HS 3. In biochemical characterization of isolates is similar to the findings of Rao and Savalgi, 2017 [15] and Baldani *et al.*, 1996 [55].

All *Gluconacetobacter* isolates had the ability to produce phytohormone, solubilization of phosphorous and zinc, nitrogen-fixing capacity, and biocontrol activity against pathogens. These results were in harmony with the findings of Rao and Savalgi, 2017 [15] and Kumar *et al.*, 2024 [56]. The *Herbaspirillum* isolates also produced the plant growth regulators [57], fixed the nitrogen [58, 59], siderophore [27], solubilize the minerals [24].

Endophytes were present inside different tissues help to the survival of plants in extreme environments [60]. Their competitive nature to thrive within the host tissue away from microbial competition and environmental stress [61]. All isolates had the ability to withstand salinity, high temperature, and drought conditions and also produced the ACC-deaminase enzyme which was similar to the findings of Ganie *et al.*, 2022 [9] and Eid *et al.*, 2021 [7].

Conclusion

This study creates a foundation for future research into Endophytic bacteria that have potential plant growth augmenting property like, phytohormone, nitrogen fixation, mineral solubilization, production of siderophores, protection against phytopathogens, stress tolerance capacity, ACC deaminase enzyme activity. There is a need to increase the focus on utilizing endophytic microbes as a sustainable agricultural practice to induce crop yield. Because it is critical to carry out systematic study related to biology of endophytic bacteria within the host plant.

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