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Isolation and Biochemical Characterization of Endophytic Bacterium *Gluconacetobacter diazotrophocus* from Native Sugarcane Cultivar of Middle Gangetic Plains of India

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***Abstract*:** The study focused on the isolation and characterization of *Gluconacetobacter diazotrophocus*, an endophytic bacterium obtained from various parts of a native sugarcane cultivar in India's middle Gangetic plains. Using conventional culturing methods, isolated and screened 115 isolates, ultimately selecting 15 highly efficient isolates based on their production of growth-promoting hormones. Among these, GdS08S, isolated from sugarcane cultivar CoP-9301, exhibited the highest nitrogen-fixing ability, with 153µg of N/mg of carbon used. Isolate GdS15S, from sugarcane cultivar Co-0238, produced the highest concentration of gibberellic acid (8.19µg/25ml). Additionally, GdS07R displayed notable phosphorus and zinc solubilization zones, measuring 4.22 cm and 4.52 cm, respectively. *G. diazotrophocus*, residing within sugarcane tissues, plays a pivotal role in promoting rooting, cell elongation, and overall sugarcane growth through the biosynthesis of indole-3-acetic acid (IAA) and gibberellic acid (GA). Furthermore, it actively fixes atmospheric nitrogen, offering a significant nitrogen supply to the growing crops. This research underscores the significance of *G. diazotrophocus* as a potential biofertilizer for native sugarcane cultivars in the middle Gangetic plains of India, contributing to sustainable agriculture practices.

Keywords: *Gluconacetobacter,* Sugarcane, Nitrogen fixation, Phytohormones, Organic acids

The endophytic bacteria *Gluconacetobacter diazotrophicus*, initially discovered in sugarcane fields (Euan et al 2001), has been a pivotal model for studying plant-bacterial interactions. It has also been identified in coffee and in wild and salt-tolerant rice varieties. This endophytic nitrogen fixation has gained significant attention, offering a solution for nitrogen deficient soils. *G. diazotrophicus* has demonstrated the remarkable ability to produce auxins and gibberellins, which promote efficient plant growth (Figueroa Viramontes et al 2011). Evaluating microorganisms for phosphorus solubilization is a critical trait for endophytic bacterial isolates. Globally, many soils lack essential micronutrients like Zn, Fe and Mn, with Zn being particularly crucial (Alloway 2001). Unfortunately, the soluble form of Zn applied to the soil undergoes transformations into inaccessible forms due to soil reactions. Out of the total phosphorus fertilizers applied to the soil, only 15-20% can be utilized by the standing crops, while the remaining portion becomes locked in the soil as phosphates of Ca, Al, Mn, Zn and Fe complexes in acidic and calcareous soils respectively (Ibrahim et al 2022), even before plant roots have the opportunity to absorb it (Vikram 2007). This isolate possesses the ability to convert insoluble forms into soluble ones and its use can significantly enhance sugarcane productivity. Several studies have revealed that in grassy plants, these microorganisms naturally solubilize phosphorus, zinc, iron, potassium and magnesium (Eshaghi et al 2019). Despite the widespread application of phosphorus fertilizers to soils, a substantial portion of this nutrient becomes immobilized in the soil, hindering its absorption by plants (Paredes Villanueva et al 2020). Specifically, *G. diazotrophicus* can solubilize phosphates in significant amounts (Restrepo et al 2017), which represents a crucial trait for economically significant crops. Investigations on native isolates of *G. diazotrophicus* in sugarcane crops have demonstrated positive impact on growth and thereby reducing the need for chemical fertilizers (Ferreira et al 2019). This endophyte produces gluconic acid in significant amounts, through the direct oxidation of glucose by a pyrroloquinoline-quinone-linked glucose dehydrogenase within this bacterium, which further promotes plant growth (Carlos et al 2014). The subtropical region of the Indo-Gangetic plain comprises 55% of the area and contributes to 45% of the total sugarcane production.

Among the twelve significant biodiversity hotspots globally, India is endowed with two: the North-eastern region and the Western Ghats (Rajaram and Suri 2000). Therefore, investigating the presence and impact of *G. diazotrophicus* in the subtropical region holds considerable importance. Presently, there is limited information available on this crucial endophyte *Gluconacetobacter* from the subtropical region of India, recognized as one of the global biodiversity hotspots. Given the substantial ecological and economic significance of *G. diazotrophicus*, this study was conducted to isolate, screen, characterize and estimate growth-promoting parameters. Factors such as nitrogen-fixing ability, growth hormone production and phosphorus and zinc solubilization were evaluated to identify the most efficient strains of endophytic *Gluconacetobacter* in sugarcane crops grown in the north-east alluvial plains of Bihar.

MATERIAL AND METHODS

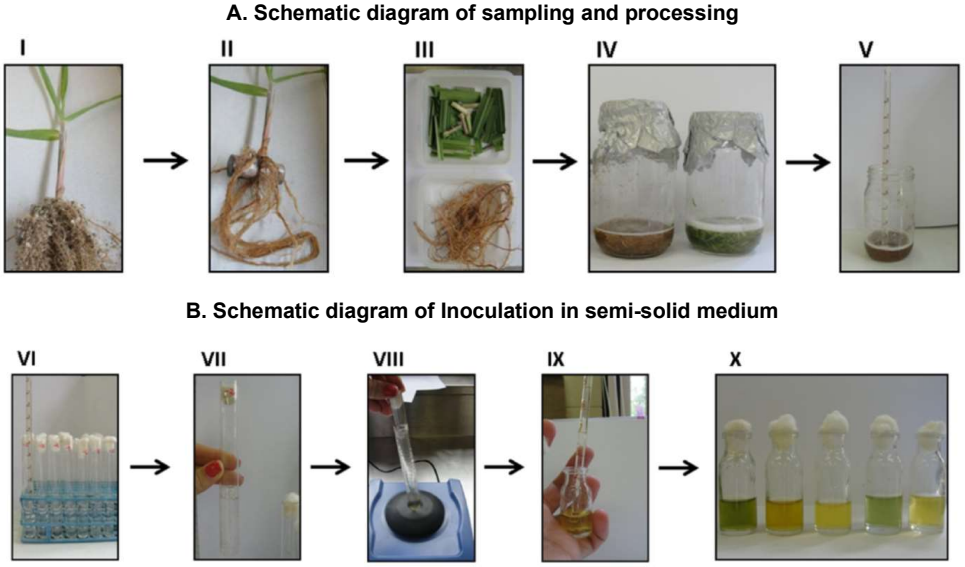
Location of experimental/sampling sites: The study was conducted at experimental site near the Burhi Gandak River in Bihar, situated at coordinates 26.0039° N, 85.6753° E. The research farm located at an altitude of 52.0 m above sea level. This site experiences an annual rainfall of 1909 mm, a relative humidity of 80.45%, and an average temperature of 22.45 °C. The experiment was carried out in both in vitro and in vivo conditions on a medium upland terrain with consistent topography. This site falls within the Ustic moisture regime of the subtropical climatic. The experimental soil is classified as Entisols order, Fluvents suborder and Typic Ustifluvent great group. It has a sandy loam texture and a bulk density of 1.52 Mg m^{-3}.

Collection of samples: The root, shoot and leaf samples of different elite varieties of sugarcane viz CoP-16437 (RG-1), CoP-09437 (RG-2), CoP-18437 (RG-3), Co-P 9301 and Co-0238, collected from the gene park of Sugarcane Research Institute, RPCAU, Pusa, located in office premises.

Preparation of media and cultures: An N-free semisolid LGI medium enriched with 0.5% sugarcane juice at a pH of 4.5 was utilized. For isolation and culture development, acetic acid LGI agar plates supplemented with yeast extract (50 mg/l) and potato agar plates with 10% cane sugar were used, as per Cavalcante and Dobereiner's methods (1988).

Preparing root, stem and leaf samples: Since *G. diazotrophicus* is an endophyte, so root, stem and leaf samples were used in the isolation process. The plants were uprooted, and the various plant parts were separated and rinsed with sterile distilled water. The dissected plant parts were surface sterilized for 5 minutes using a 5% sodium hypochlorite solution and then rinsed 5-7 times with sterile distilled water (Fig. 1).

Isolation of *G. diazotrophicus:* The surface sterilized different plant parts, viz., root, stem and leaf samples were weighed, massrate and homogenized separately in a sterile sucrose solution (1%) using a sterile pestle and mortar. Aliquots of 500 ml each were inoculated in semisolid LGI and were then incubated at a temperature of 28±2°C for a period of 5-7 days. Five samples from each plant part were



I: Sampling of plants; II: Root free from soil and substrate; III: Root and shoot tissue separated, IV: First dilution of sample (10^{-1}) on saline solution; V & VI: Serial dilution; VII & VIII: Vortexing sample before inoculation; IX & X: Inoculation of 0.1 ml into semisolid media

Fig. 1. Schematic diagram of sample preparation and inoculation in media for isolation of *G. diazotrophicus* (A and B)

inoculated into semisolid LGI tubes. The yellowish bacterial growth from the tubes was streaked onto LGI plates (Cavalcante and Dobereiner, 1988) and then incubated at a temperature of 28±2°C for duration of 5-7 days.

Characterization of isolates through biochemical analysis: Isolates that were presumably identified as *G. diazotrophicus* based on morphology using LGI media underwent further characterization through a range of biochemical tests. These included gram stain, motility, catalase, gelatin hydrolysis, over oxidation of ethanol, brown pigment production on GYC agar, growth on various carbon sources, growth at different sugar concentrations, and growth at various temperatures (Dong et al 1995).

Assessment of Plant Growth-Promoting Activities

Evaluating nitrogen fixation capability: The nitrogen-fixing ability was determined by using the micro-Kjeldahl method. Cultures aged 48 hours were inoculated into 5 ml of N-free semi solid broth of LGI medium and incubated for another 48 hours. Then, 1 ml of this broth was inoculated into 50 ml of semisolid medium and left to incubate for 15 days. The nitrogen was estimated from 10 ml of this culture following the standard procedure of the Micro-Kjeldahl technique (Reis et al 1994).

$$N_2 \frac{mg}{g} = \frac{\text{ml of } H_2SO_4 \text{ in the sample} \times \text{Normality of } H_2SO_4 \times 14.01}{\text{The weight of the sample (the amount of carbon used, in grams)}}$$

Evaluating phytohormones (IAA and GA): Conical flasks containing 50 ml of Czapeck's solution (composed of 1000 ml distilled water, 30 g cane sugar as an energy source and the only carbon source, 1 g dipotassium phosphate as a buffering agent, 0.5 g magnesium sulfate as a cation source, 0.5 g potassium chloride as an essential ion source, and 0.01 g iron sulfate as a cation source) were prepared and autoclaved. These flasks were inoculated with 500 µl of a 72-hour old culture from each isolate and incubated at 28±2°C for 7 days. The cultures were then centrifuged at 3300 rpm for 20 minutes. The supernatant was used for the estimation of Indole-3-acetic acid (IAA) and Gibberellic acid (GA). The quantity of IAA was determined using a spectrophotometer (Ivanova et al 2001), while GA was estimated using the method described by Bastian et al (1998).

Solubilization of phosphorus and zinc: To evaluate the potential for phosphorus and zinc solubilization, assays were conducted both on plates and in broth using LGI medium. For the plate assay, glucose was selected as the carbon source at a 1% concentration, and the medium was supplemented with insoluble zinc compounds, namely, zinc oxide (ZnO), zinc carbonate ($ZnCO_3$), and zinc phosphate ($ZnPO_4$), each at a 0.1% concentration as separate treatments. The media supplemented with insoluble nutrient compounds were added to sterile petri dishes. After solidification, 48-hour-old cultures of *G. diazotrophicus* strains ($6x10^8$ CFU mL^{-1}) at a 10 µl concentration were placed over the media and incubated at 28±2°C for 3 days. Post incubation, the diameter of the solubilization zone was measured in cm. The amount of solubilization was assessed following the procedure described by Fasim et al (2002). The measurement of halozone diameter was done for screening and assessing the phosphate and zinc solubilizing activity of isolates.

Measurement of low molecular weight organic acids: The production of low molecular weight organic acids was quantified in broth using the paper chromatography technique. These organic acids play a crucial role in the solubilization of insoluble zinc and phosphorus. For quantification, the yellow-colored spots were cut from the paper using scissors and placed in a petri dish. Approximately 3 mL of n-butanol was spread over the cut part of the chromatograph to dissolve the compounds from the yellow spots. Once the paper strip became colorless, the filtrate was poured into a 5 mL volumetric flask and topped up to 5 mL with n-butanol. The intensity of the light yellow-colored spots from each sample was measured at a wavelength of 426 nm, where it yields maximum absorbance (%). Since most of the isolates produced tartaric acid, a standard was prepared with this acid. Standard curves were plotted with the reaction product of tartaric acid (L.R grade) and bromophenol blue at different concentrations of tartaric acid, namely 2.5, 5.0, 10, 15, 20, and 25 mg L^{-1} dissolved in butanol. The percentage absorbance of the sample was measured, and the actual amount produced was calculated from the standard curve.

Determination of titratable acidity: It is a measurement of the concentration of total dissociated plus undissociated H^+. The titratable acidity (mmol H^+ $Liter^{-1}$) was determined by titrating 10 mL of the culture filtrate with 0.05 M NaOH solutions using phenolphthalein indicator.

RESULTS AND DISCUSSION

Isolation, identification, screening, and biochemical characterization of endophytic bacterial isolates: For initial screening, the cultures were isolated using LGI medium. Out of the 115 strains that were isolated from five different sugarcane varieties (CoP-9301, RG-1, RG-2, RG-3, Co-0238), 15 efficient isolates were identified: GdS25R, GdS08S, GdS19L, GdS17S, GdS07R, GdS26L, GdS5R, GdS13S, GdS16L, GdS04R, GdS11S, GdS18L, GdS02R, GdS15S, GdS21L, based on their nitrogen-fixing capacity, phytohormone production, phosphorous and zinc solubilization, and production of low molecular weight organic acids in broth. These were selected based on their

morphological and biochemical characteristics, as well as their plant growth-promoting traits. The formation of an orange-colored colony on LGI media was the primary criterion to identify the *G. diazotrophicus* isolates (Fig. 1, Fig. 3). *G. diazotrophicus* isolates were collected from sugarcane plants, roots, stems and leaves. Notably, the CoP-9301 variety provided the highest number of isolates, totaling 29, isolated from the root, stem and leaf portions. The RG-1 variety also produced a substantial number, with 28 isolates, while the RG-3 and Co-0238 varieties provided 19 and 22 isolates, respectively. In contrast, the RG-2 variety provided the lowest number of isolates (17). This comprehensive isolation and distribution of *G. diazotrophicus* isolates from different sugarcane varieties (Fig. 2) serve as a valuable resource for future research on the ecological and agricultural significance of this endophyte. These isolates underwent further biochemical characterization to confirm specific characteristics of *G. diazotrophicus*, in accordance with Bergey's Manual of Systematic Bacteriology (Table 1, Fig. 3). The colonies of *G. diazotrophicus* typically have a circular shape with a smooth texture. The color can vary, but is often pale yellow or cream-colored. The edges of the colonies (margins) are typically entire, slightly raised and opaque. All the isolates tested positive for most of the traits. Additionally, all isolates tested negative for the biochemical characteristic of gelatin liquefaction hydrolysis. The endophytic colonization of sugarcane by *G. diazotrophicus* is a ground breaking discovery that provides substantial fixed nitrogen for plant growth. This finding is the uniqueness of this relationship in the plant kingdom, where typically, numerous bacterial endophytes have been isolated from a single plant species (Zinniel et al 2002). The type of plant tissue and the location of the endophytes can influence the frequency of colonization. Kaur and Vyas (2017) isolated thirty five bacterial endophytes, focused on the isolation and characterization of *G. diazotrophicus* from various parts of sugarcane cultivars.

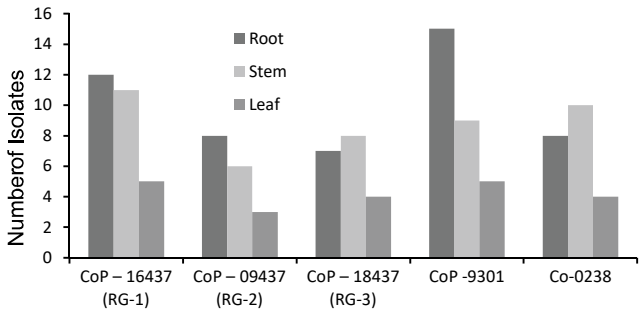


Fig. 2. Isolates of *Gluconacetobacter diazotrophicus* from different varieties of sugarcane

Plant growth-promoting traits: The cultures were evaluated for nitrogen fixation ability, production of phytohormones as well as phosphorus and zinc solubilization capabilities.

Ability to fix nitrogen: All 115 isolates were evaluated for their nitrogen-fixing capacity using the Micro-Kjeldahl method. Among them, 35 isolates showed efficient nitrogen fixation and were able to fix a considerable amount of nitrogen from these 15 top-performing isolates were selected for further characterization. Five isolates were chosen from each plant part, namely, the root, stem and leaf. Among the isolates, GdS08S, derived from the CoP-9301 sugarcane variety, fixed the highest amount of N_2, around 153.10 µg of Nitrogen/mg of Carbon use. This was closely followed by the GdS18L isolate from the RG-3 sugarcane cultivar, The isolate GDS02R, from the Co-0238 sugarcane variety, fixed the least amount of nitrogen at 68.23 µg/mg of carbon (Table 2). The *G. diazotrophicus* elucidate various growth promoting properties. As per Fisher and Newton (2005), the molybdenum dependent mechanism (Mo-nitrogenase) utilized by *G. diazotrophicus* for nitrogen fixation provide a significant amount of fixed nitrogen to its host plants. Various studies have shown that certain sugarcane cultivars may obtain up to 200 kg of nitrogen per hectare from *G. diazotrophicus*. This contribution can meet more than half of the crop's nitrogen requirement (Boddey et al 2001). Studies also highlight the unique feature of *G. diazotrophicus* - it does not contain a nitrate reductase protein. Therefore, this bacterium can effectively fix nitrogen that are supplemented

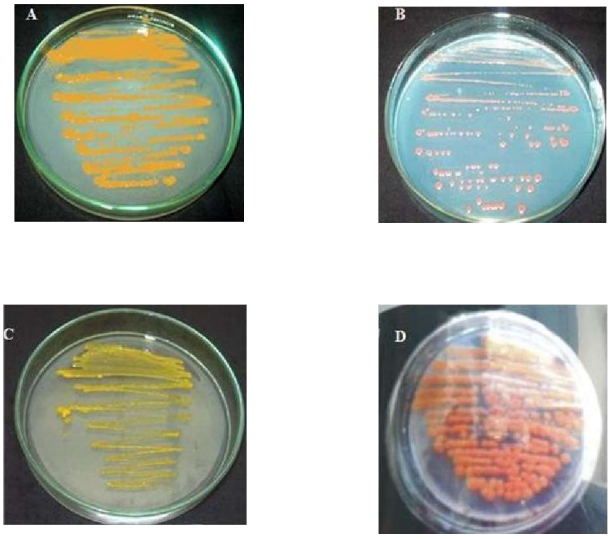


Fig. 3. Actual colony morphologies of the *Gluconacetobacter diazotrophicus* isolates A (GdS25R), B (GdS08S), C (GdS15S) and D (GdS07R) grown at 28°C for 72 h in LGI medium obtained from sugarcane varieties from CoP-9301 (both A & B), Co-0238 and RG-1, respectively

with either nitrate-based fertilizers or with low amounts of ammonium based fertilizers (Eskin et al., 2014). This finding underscores the potential of *G. diazotrophicus* in reducing the dependence on fertilizers. The strain of *G. diazotrophicus* exhibited increased nitrogen fixation activity, which is consistent with the findings of Nong et al (2022), who characterized the strain under various physiological and nutritional conditions. The nitrogenase activity of the strain was significant at temperatures between 28°C to 37°C. Additionally, different carbon sources were tested and the strain showed enhanced nitrogenase activity with 5% sucrose.

Phyto-hormone production: The highest IAA production was observed in the isolate GdS08S, (7.58 µg/ml). This isolate was derived from the sugarcane variety CoP-9301., the isolate GdS07R, obtained from the sugarcane variety RG-1, produced the lowest amount of IAA, i.e., 3.49 µg/ml. the pattern for GA production did not match that of IAA production. The isolate GdS15S, obtained from the sugarcane variety Co-0238, produced the highest amount of GA, around 8.19 µg/25ml and was closely followed by the isolate GdS17S from the RG-1 variety, (8.10 µg/25ml.) The lowest amount of GA (4.98 µg/25ml) was produced by the isolate GdS16L, derived from the RG-2 sugarcane variety (Table 3). A standout isolate from sugarcane, GdS08S, exhibited the highest amount of nitrogen fixation, approximately 153µg of N/mg of carbon used, and also produced the highest concentration of Indole Acetic Acid (7.58µg/ml). Meanwhile, the GdS15S isolate produced the maximum concentration of GA (8.19µg/25ml). The GdS07R isolate displayed the highest phosphorus solubilization zone with a diameter of 4.22 cm and a zinc solubilization zone of 4.52 cm. *G. diazotrophicus*, which resides in the intercellular space of plant tissues, contributes to N fixation and the biosynthesis of the IAA, promoting root development and enhancing sugarcane growth. The synthesis of GA aids in cell elongation, which ultimately increases cane length. The top 15 screened isolates were further examined for their production of various organic acids and titratable acidity, which play a crucial role in the solubilization of insoluble phosphorus and zinc. These findings highlight the significant potential of these bacterial isolates in promoting plant growth

Table 1. Selective biochemical assays performed on *Gluconacetobacter diazotrophicus* isolates from sugarcane crop varieties

Biochemical characters		Varieties of sugarcane crop				
		RG-1	RG-2	RG-3	CoP-9301	Co-0238
Cell shape		Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Gram reaction		Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Motility		+	+	+	+	+
Brown pigment on GYC medium		+	+	+	+	+
Gelatin liquefaction		-	-	-	-	-
Catalase activity		+	+	+	+	+
Oxidation of ethanol		+	+	+	+	+
Growth on C - sources						
i.	Glucose	+	+	+	+	+
ii.	Sucrose	+	+	+	+	+
iii.	Ethanol	+	+	+	+	+
iv.	Mannitol	+	+	+	+	+
Growth at various concentration of sugar						
i.	5%	+	+	+	+	+
ii.	10%	+	+	+	+	+
iii.	20%	+	+	+	+	+
iv.	30%	+	+	+	+	+
Growth at various temperatures						
i.	4°C	-	-	-	-	-
ii.	28°C	+	+	+	+	+
iii.	32°C	+	+	+	+	+
iv.	37°C	+	+	+	+	+

GYC: Glucose, yeast and calcium carbonate

and nutrient utilization. Though *G. diazotrophicus* was initially isolated from sugarcane, numerous studies have documented its widespread presence in a variety of crops (Hernandez et al 2000). This bacterium has been isolated from sugarcane, maize, pineapple, and carrot. These plants, having significant amounts of sucrose in their cell sap,

Table 2. Nitrogen fixation ability of *Gluconacetobacter diazotrophicus* isolates from different sugarcane cultivar

Isolate code	Cultivar	isolated from			µg of Nitrogen/ mg of carbon
		Root	Stem	Leaf	
GdS25R	CoP-9301	-	-	√	135.82
GdS08S	CoP-9301	√	-	-	153.10
GdS19L	CoP-9301	-	√	-	139.42
GdS17S	RG-1	-	√	-	123.15
GdS07R	RG-1	√	-	-	109.06
GdS26L	RG-1	-	-	√	127.53
GdS05R	RG-2	√	-	-	91.06
GdS13S	RG-2	-	√	-	98.46
GdS16L	RG-2	-	-	√	108.32
GdS04R	RG-3	√	-	-	84.06
GdS11S	RG-3	-	√	-	98.19
GdS18L	RG-3	-	-	√	141.25
GdS02R	Co-0238	√	-	-	68.23
GdS15S	Co-0238	-	√	-	78.95
GdS21L	Co-0238	-	-	√	112.34
CD (p=0.5)		-	-	-	35.41

Gd represents *Gluconacetobacter diazotrophicus;* S – Sugarcane crop; Numerical number represents isolates serial number and last alphabet represents different plant parts viz R - root, S- stem and L- leaf

Table 3. The production of IAA, GA, phosphorus and zinc solubilization zone by the efficient strains of *G. diazotrophicus*

Isolate code	Cultivar	IAA (µg/ml)	GA (µg/25ml)	Diameter of solubilization zone	
				P-solubilization Zone (cm)	Zn-solubilization Zone (cm)
GdS25R	CoP-9301	5.53	6.75	2.80	3.81
GdS08S	CoP-9301	7.58	7.73	2.60	3.19
GdS19L	CoP-9301	6.43	5.63	2.91	3.05
GdS17S	RG-1	5.74	8.10	3.96	4.47
GdS07R	RG-1	3.49	6.53	4.22	4.52
GdS26L	RG-1	4.43	5.72	3.41	4.15
GdS05R	RG-2	5.68	7.95	3.08	4.11
GdS13S	RG-2	5.12	5.93	2.99	3.97
GdS16L	RG-2	4.08	4.98	2.76	3.59
GdS04R	RG-3	7.44	6.36	2.74	3.71
GdS11S	RG-3	4.54	7.87	2.87	3.54
GdS18L	RG-3	5.76	6.84	3.28	3.61
GdS02R	Co-0238	4.86	6.18	2.54	3.22
GdS15S	Co-0238	5.53	8.19	2.45	3.18
GdS21L	Co-0238	4.49	6.51	3.21	3.79
CD (p=0.05)		2.38	1.04	1.07	1.15

provide suitable environments for *G. diazotrophicus* to survive and proliferate. These endophytic strains showed considerable abilities in nitrogen fixation, phytohormone production and solubilization of zinc and phosphorus. The mechanisms for growth promotion are the production of growth hormones detected in the cultures of *G. diazotrophicus* (Bastian et al 1998). Different plant hormones interact synergistically or antagonistically through complex regulatory pathways to regulate plant development. It is noteworthy that IAA, produced by *G. diazotrophicus*, can exert its effects independently on nitrogen fixation (Lee et al 2004).

Ability to solubilize phosphorus and zinc: The largest phosphorus solubilization zone, measuring 4.22 cm, was for the GdS07R isolate from the root of the RG-1 sugarcane variety, the smallest solubilization zone, measuring 2.45 cm, was for the GdS15S isolate from the stem of the Co-0238 sugarcane variety. The pattern of zinc solubilization varied from that of phosphorus. The largest zinc solubilization zone, (4.52 cm) was for GdS07R isolate from RG-1, followed closely by the GdS26L isolate. The smallest zone, (3.05 cm), was for the GdS19L isolate from the CoP-9301 sugarcane variety (Table 3). Isolates, a strain from sugarcane (GdS08S) demonstrated the highest capacity for nitrogen fixation (153.10 µg of Nitrogen/mg of carbon) and the highest IAA production (7.58 µg/25 ml). This indicates its potential for use as a bio-inoculant. Based on morphological, biochemical, and functional characteristics, it can be concluded that the GdS08S, GdS15S, GdS07R, and Gds17S isolates, obtained from the sugarcane cultivars CoP-9301, Co-0238, and RG-1, proved to be the most potent. These isolates could be harnessed in the future as biofertilizers to enhance sugarcane productivity. Alloway (2001) also highlighted the beneficial role of solubilizing bacteria for phosphorus and zinc as microbial inoculants, where over 70% of soils suffer from zinc deficiency. Nutrients like phosphorus and zinc often transform into insoluble forms due to complex soil reactions. To enhance the nutrient status, *G. diazotrophicus* could be used in conjunction with less expensive materials, such as rock phosphate and zinc ores. Considering its ability to fix nitrogen, produce growth hormones and now solubilize essential nutrients like phosphorus and zinc.

Production of low molecular weight organic acids and titratable acidity: Organic acids were produced in LGI broth by *G. diazotrophicus* isolated from different sugarcane cultivar plant parts. The isolates produced a variety of organic acids including gluconic, tartaric, malonic, fumaric, citric, and lactic acids. The isolate GdS16L produced the highest total amount of organic acids (9.36 mg L^{-1}), followed by GdS15S, GdS08S and GdS19L, while GdS25R produced the lowest amount (2.27 mg L^{-1}). The most commonly produced organic acids were tartaric acid (13 isolates) and gluconic acid (5 isolates), while malonic and lactic acids were produced exclusively by GdS08S and GdS18L, respectively. Isolates GdS18L and GdS02R produced citric acid with moderate (++) and strong (+++) intensity, respectively. Strong yellow color intensity of gluconic acid spots in paper chromatographs was with GdS19L and GdS17S, while a lower intensity was with GdS08S, GdS16L, and GdS26L. All *G. diazotrophicus* isolates synthesized tartaric acid, except for GdS18L and GdS02R. The highest titratable acidity (34.5 mmol H+/liter) was for isolate GdS16L, followed by GdS15S. The lowest value (14.5 mmol H+/liter) was recorded for isolate GdS21L. The production of low molecular organic

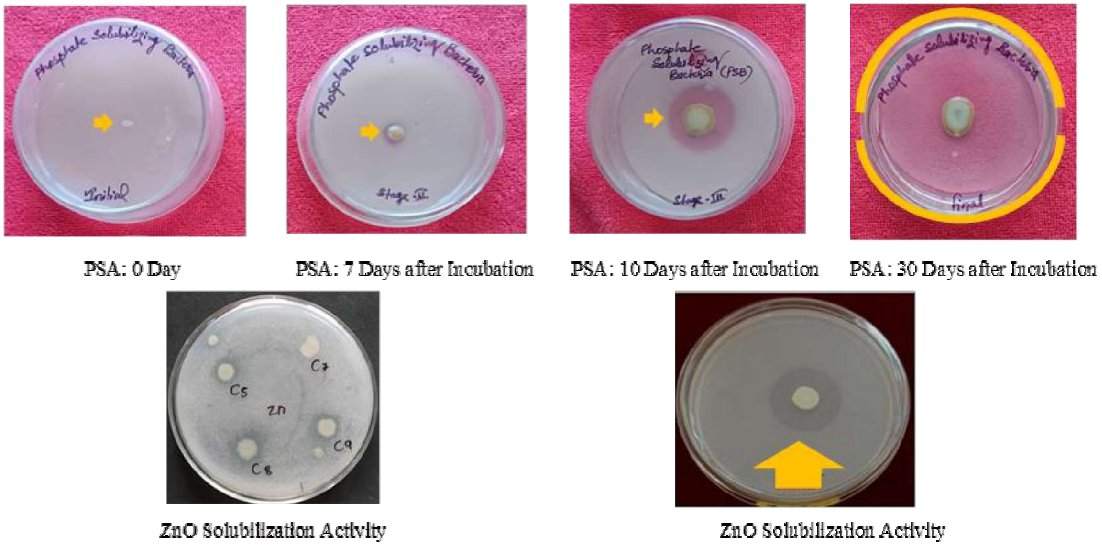


Fig. 4. Halozone formation of phosphorus and zinc solubilization activity by endophytic bacteria

Table 4. Low molecular weight organic acids produced by *Gluconacetobacter diazotrophicus* in broth and their impact on titratable acidity and lowering of pH

Isolate code	Organic acid produced	Colour (yellow) *intensity of organic acid spots in chromatograph	Total organic acid (mg/L)	Titratable acidity (m mol H^+ liter $^{-1}$)
GdS25R	Tartaric	++	2.27	16.0
	Fumaric	+		
GdS08S	Malonic	+	8.61	17.5
	Tartaric	++		
	Gluconic	+		
GdS19L	Gluconic	+++	8.07	23.0
	Tartaric	++		
GdS17S	Gluconic	+++	4.54	24.0
	Tartaric	++		
GdS07R	Tartaric	++	4.41	23.0
GdS26L	Gluconic	+	6.27	21.0
	Tartaric	++		
GdS05R	Tartaric	+++	4.24	22.0
	Fumaric	+		
GdS13S	Tartaric	+++	7.93	21.0
	Fumaric	+		
GdS16L	Tartaric	++	9.36	34.5
	Gluconic	+		
GdS04R	Tartaric	+++	7.26	17.5
GdS11S	Tartaric	+++	7.36	21.0
GdS18L	Citric	++	2.81	22.0
	Lactic	+		
GdS02R	Citric	+++	5.77	18.5
	Fumaric	+		
GdS15S	Tartaric	++	8.92	28.0
	Citric	+		
GdS21L	Tartaric	+++	3.36	14.5
SEM ±	---	---	0.21	1.21
CD (p= 0.05)	---	---	0.89	3.38

* Key: + = Low intensity
++ = moderate intensity and
+++ = strong intensity

acids reveals that *G. diazotrophicus* mainly produces tartaric, gluconic and fumaric acids. Illmer and Schinner (1995) also observed that *Aspergillus niger* produces modest quantities of gluconic acid but Whitelaw et al. (1999) indicated that the phosphate solubilizing microbial strains did not produce gluconic acid. The type and amount of organic acids produced by phosphate solubilizing strains can vary depending on the composition of the medium, and growth conditions. Glucose has been identified as a common carbon source used by microorganisms to produce organic acids. Nautiyal et al (1999) also observed increased organic acid production when glucose was used as a carbon source. Among the phosphate-solubilizing bacterial isolates, GdS15S exhibited the highest total amount of organic acids, followed by GdS02R and GdS18L. The effectiveness of these isolates in chelating calcium was attributed to the presence of citric acid, a tribasic acid known for its strong chelating properties towards calcium. In contrast, aliphatic monobasic acids showed less potent calcium chelating abilities. Wada et al (2021) supports these findings, showing that citric acid produced displayed the powerful Ca chelation compared to other organic acids.

CONCLUSION

The isolates from sugarcane roots performed best in terms of organic acid production, phosphorus, and zinc solubilization, while the isolates from sugarcane stems excelled in phytohormone production and nitrogen fixing ability. These findings indicate that *G. diazotrophicus* has the potential to be utilized as a biofertilizer to enhance nutrient (nitrogen, phosphorus and zinc) use efficiency in sugarcane, which is known as a high-input crop. By

harnessing the growth-promoting properties of *G. diazotrophicus*, may be able to reduce the reliance on costly nitrogenous fertilizers and mitigate the environmental risks associated with their overuse. The results indicate new possibilities for sustainable sugarcane production and offer insights into the diverse roles of *G. diazotrophicus* as an endophytic bacterium in promoting plant growth and nutrient acquisition.

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