

Exploring the potential of cow-based preparation Ghanajeevamrit in salinity stress

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ABSTRACT

Salt stress is a crucial factor limiting soil productivity and has become a significant global barrier to crop production, mainly due to the excessive use of chemical fertilizers. One promising alternative is Ghanajeevamrit, a native cow-based organic formulation that offers a wealth of macronutrients, micronutrients, plant growth-promoting substances, and beneficial microorganisms like plant growth-promoting bacteria (PGPB). This study focuses on the isolation and screening of plant growth-promoting bacteria from Ghanajeevamrit with the aim of their application in sustainable agricultural practices. A total of nine bacterial isolates were obtained using nutrient agar and were qualitatively tested for their plant growth-promoting activities. In comparison to the control, plants treated with Ghanajeevamrit and GJ02 showed 12% and 26.08% increases in root length and 18% and 31.08% increases in leaf length under 1.4% salinity stress. The GJ02 bacterial isolate could act as a potent plant biostimulant, promoting plant growth and resilience under salinity stress.

Keywords: Agriculture, Salinity stress, Ghanajeevamrit, Plant growth promoting bacteria (PGPB), Phosphate solubilization, *Brassica nigra*, Biostimulant

INTRODUCTION

The global population has steadily risen, with projections of 9.7 billion by 2050 (Grafton *et al.*, 2015). This increase in population significantly drives up the demand for food, creating a complex challenge for global food systems. Crop yields are frequently increased by farmers using chemical fertilizers such as potassium chloride, ammonium nitrate, and urea. Chemical fertilizers provide essential nutrients like

nitrogen, phosphorus, and potassium for plant growth, but their overuse can lead to salinity stress by increasing soil salt levels. Numerous chemical fertilizers, mainly those rich in potassium and nitrogen, can raise the salt level in the soil (Pahalviet *et al.*, 2021). Excessive application can disrupt the osmotic balance in plants, leading to salt buildup in the root zone, which hinders water and nutrient absorption and ultimately causes salinity stress (Mbarki *et al.*, 2018). An eco-friendly

and affordable approach is needed to achieve sustainable agriculture practices.

The increasing trend of organic farming reflects its growing importance in promoting sustainable agriculture, environmental protection, and public health. By avoiding synthetic pesticides and fertilizers, organic farming enhances soil health, conserves biodiversity, and reduces pollution, making it an eco-friendly alternative to conventional methods (Sener 2022). Nutrient-rich organic produce offers health benefits, while the absence of chemical residues lowers health risks. Organic farming benefits small-scale farmers economically and meets the rising consumer demand for ethically produced sustainable food. Organic farming is essential for building a resilient and sustainable food system that aligns with environmental and ethical values.

Cow-based preparations like Panchagavya, Kunapajala, Beejamrit, Jeevamrit, and Ghanajeevamrit are increasingly being used by farmers as Biofertilizers (Sornalatha *et al.*, 2018). These natural formulations, derived from cow products and other organic materials, are evaluated for their ability to enhance soil fertility, promote plant growth, and improve crop yields. Cow manure, urine, and composted cow products are rich sources of organic matter and nutrients essential for soil health. They improve soil structure, water retention, and plant nutrient availability, fostering a healthy soil microbiome (Sodani and Kumar, 2017). The application of organic manures, including cow dung, significantly increased soil organic carbon, microbial biomass carbon, and soil enzyme activities, contributing to improved soil fertility. Enriching the soil with beneficial microbes and nutrients, these bioformulations support sustainable farming

practices, reduce dependence on chemical inputs, and contribute to healthier, more resilient agricultural systems.

Ghanajeevamrit is a traditional organic formulation that originated in India and is known for its soil rejuvenation properties (Lakshman *et al.*, 2024). The primary ingredients include cow dung, urine, jaggery, gram flour, soil, and water. In Ghanajeevamrit, the mixture (cow dung, cow urine, jaggery and gram flour) is allowed to ferment for 15-20 days to prompt the beneficial microorganisms in cow dung and urine. During fermentation, bacteria in the cow dung and urine use the nutrients from the flour and multiply. A handful of soil is taken precisely from the field where it is to be applied because the beneficial microorganisms of the same soil can be multiplied (Veerana *et al.*, 2023). These Ghanajeevamrit increase the microorganism population in the soil, including bacteria, fungi, and actinomycetes, and help to convert the organic matter into readily available plant nutrients, thereby improving soil chemical and physical conditions. The composition of Ghanajeevamrit is mentioned in Table 1.

In the present study, the physical properties of Ghanajeevamrit were recorded, and plant growth-promoting bacteria were isolated from fermented Ghanajeevamrit. Further, they were analyzed for their plant growth-promoting abilities, such as indole acetic acid, gibberellic acid, ammonia, exopolysaccharides, and phosphate solubilization.

MATERIALS AND METHODS

Sample collection

The Ghanajeevamrit sample was obtained

from a natural farming practising farmer via Srishti NGO near the Gujarat University campus, Ahmedabad, Gujarat, India.

Isolation of bacteria from Ghanajeevamrit

1 gm of Ghanajeevamrit sample was suspended in 10 ml sterile distilled water and serially diluted up to 10^{-7} . 0.1 ml from each dilution was spread onto sterile nutrient agar plates incubated at 37°C for 24 hours. The plates were examined for isolated bacterial colonies, and pure cultures were transferred onto sterile nutrient agar plates. The pure bacterial cultures were grown on nutrient agar medium and preserved by periodic transfer onto nutrient agar slants and stored in a refrigerator at 4°C.

Cultural and morphological characterization

To study the cultural characteristics of bacterial isolates, they were streaked on nutrient agar plates by the four-flame method, incubated at 37°C for 24 hrs, and their colonial characteristics recorded. Gram staining was performed for morphological analysis of isolated bacterial colonies.

Inoculum preparation

The bacterial cultures from slants were inoculated in sterile nutrient broth and incubated at 30°C for 24 hrs. Cells were separated by centrifugation at 5000 rpm for 10 min, and the supernatant was removed. The cell pellet was resuspended in sterile normal saline to get the optical density 1.0. The prepared culture suspension was used as a 1.0% (v/v) inoculum to study the plant growth-promoting parameters like IAA, GA, NH_3 , EPS production and phosphate solubilization. The absorbance was measured in a spectrophotometer at 600 nm after 24 hours. The standard calibration curve

of BaCl_2 (1.0 O.D $\sim 8 \times 10^8$ cells/ml) was prepared to calculate the number of cells. Seven sets of flasks were prepared to study each plant growth promoting parameters. One flask (uninoculated broth) was used as a control. The other six flasks were inoculated with 1% (v/v) inoculum of each of the six isolates and incubated in the dark at 37°C, 120 rpm on an orbital shaker for further estimation of various plant growth-promoting parameters.

Screening of bacterial isolates for their plant growth-promoting ability

All the bacterial isolates were tested qualitatively and quantitatively for plant growth-promoting traits like indole acetic acid (IAA), gibberellic acid (GA), ammonia, exopolysaccharides, and phosphate solubilization.

Indole acetic acid production (IAA)

For IAA production, 30 ml of sterile Luria-Bertani broth (pH 7.5) supplemented with 0.1% (w/v) L-tryptophan was taken in a 100 ml Erlenmeyer flask. The spectrophotometric estimation of IAA was done using the Salkowski method (Bric *et al.*, 1991).

For IAA estimation, a 2 ml sample was taken aseptically in a sterile Eppendorf tube and centrifuged at 5000 rpm for 20 minutes. 1.0 ml supernatant was mixed with 2.0 ml Salkowski reagent and incubated in the dark for 30 minutes. The development of pink colour indicates the production of IAA, and its optical density was recorded at 540 nm. A standard calibration curve of IAA (0-20 $\mu\text{g/ml}$) was prepared to calculate the IAA production.

Gibberellic acid production (GA)

For Gibberellic acid production, 30 ml of sterile Nutrient broth (pH 7.4) was taken in a 100 ml Erlenmeyer flask. Spectrophotometric

estimation of gibberellic acid was performed using the DNPH method (Graham and Thomas, 1961).

For GA estimation, a 2 ml sample was taken aseptically in a sterile Eppendorf tube at an interval of 24 hours for three days and centrifuged at 5000 rpm for 20 minutes. An equal volume of cell-free extract and ethyl acetate was taken in a test tube and shaken vigorously. The ethyl acetate layer was separated in a glass beaker, and the extraction was repeated thrice. The separated ethyl acetate extract was allowed to evaporate at room temperature. The remains were dissolved in methanol and transferred into screw-cap tubes. To this, 1.0 ml of 2,4-dinitrophenylhydrazine (DNPH) was added, and the mixture was incubated at 100°C for 5 minutes. After incubation, it was cooled in ice water, and then 5.0 ml of 10% (w/v) alcoholic potassium hydroxide (KOH) was allowed to stand until red wine colour developed. Add 15 ml of distilled water. The colour intensity was measured at 430 nm. A standard calibration curve of gibberellic acid (0.8 mg/ml) was prepared to calculate GA production.

Ammonia production

Ammonia production was carried out using sterile peptone water (pH 7.2) in a 100 ml Erlenmeyer flask. Spectrophotometric estimation of ammonia was performed using Nessler's method with few modifications (Damodaran *et al.*, 2013).

For ammonia estimation, 2ml of the sample was taken aseptically in a sterile Eppendorf tube at an interval of 24 hours for three days, the tubes were centrifuged at 5000 rpm for 20 minutes, and 1 ml of culture supernatant was mixed with 0.1 ml. Nessler's reagent and the final volume of the mixture were made up to 5

ml by adding ammonia-free distilled water. The yellow-to-brown colour development indicates ammonia production, and its optical density was measured at 425 nm. The standard curve of ammonium hydroxide solution was prepared to calculate ammonia production (2µmol/ml).

Exopolysaccharide production (EPS)

For quantitative estimation of EPS, 100 ml sterile Brain Heart Infusion broth (pH 7.1) was supplemented with 4% w/v sucrose in a 100 ml Erlenmeyer flask. The solvent extraction method described by Azeredo and Oliveira (1996) was used for the quantitative estimation of EPS (Azeredo and Oliveira, 1996).

For EPS extraction, 50 ml of culture was withdrawn aseptically and centrifuged at 5000 rpm for 20 minutes. 25 ml of supernatant was taken in a tube, and 50 ml of chilled acetone was added. It was allowed to precipitate overnight at 4°C. Precipitates were collected on a pre-weighed aluminium foil, and the wet weight was recorded. The precipitates were allowed to dry, and the dry weight of the precipitates was recorded until three consecutive constant readings were obtained. Un-inoculated EPS broth was used as a control.

Phosphate solubilization

The concentration of the soluble phosphate was estimated using the stannous chloride method, with some modifications (King 1932). The amount of phosphate solubilized was measured after 3, 5 and 7 days. 30 ml of sterile Pikovskaya's broth (pH 7.0±0.2) was supplemented with 0.5% tricalcium phosphate in a 100ml Erlenmeyer flask.

For estimation, a 2ml sample was taken aseptically in a sterile Eppendorf tube; the tubes were centrifuged at 5000 rpm for 20

minutes. 1.0 ml sample was mixed with 1.0 ml chloromolybdic acid and 2.975 ml double distilled water. 25 µl of chlorostannous acid reagent was added, and the test tubes were mixed well until a blue colour was developed. Optical density (OD) was taken at 600 nm. A standard calibration curve of $K_2 HPO_4$ (10-100 µg/ml) was prepared to calculate the amount of phosphate solubilized.

Salt tolerance of selected six bacterial isolates

The salt tolerance of six selected bacterial isolates (GJ01, GJ02, GJ03, GJ04, GJ06, and GJ08) was checked on nutrient agar plates supplemented with different concentrations of NaCl (0.2, 0.4, 0.6, 0.8, and 1.0 g/L). Each isolate was spot-inoculated on nutrient agar plates.

Pot study on *Brassica nigra*

A pot study was conducted in pots of 20×20 cm size in March 2024, when the average temperature was 25-30°C. Farm soil was sun-dried for five days, and approximately 4 kilograms of soil was filled in the pots. The sorghum seeds were purchased from local market routinely used by farmers. The seeds were surface sterilized by shaking in 70% methanol for 2 minutes, dipped in 0.1% $HgCl_2$ for 1 minute, and rinsed twice with sterile distilled water. The seeds were sown in the pot up to a depth of 1 cm. To prepare the bacterial suspension for pot treatment, the GJ02 isolate was grown in sterile nutrient broth and incubated at 30°C on an orbital shaker at 120 rpm for 24 hours. 5 gm of Ghanajeevamrit was added directly into each pot. The soil was treated with Ghanajeevamrit and the GJ02 bacterial isolate at 5-day intervals. After ten days,

plants were uprooted. Vegetative parameters like root length, shoot length, leaf length, and wet and dry weight were measured.

RESULTS AND DISCUSSION

Physical characteristics of Ghanajeevamrit

The Ghanajeevamrit sample was brown in colour having mild foul odour. The pH of the sample was 7.8, and electrical conductivity (E.C.) was 0.84 dS m⁻¹ (Table 1&2).

Table 1: Composition of Ghanajeevamrit

Ingredients	Quantity
Cow dung	100 kg
Cow urine	10 L
Jaggery	100 gm
Gram flour	100 gm
Soil	A handful

Table 2 : Physical characteristics of Ghanajeevamrit

Ingredients	Quantity
Colour	Dark brown
Odour	Mild foul
pH	7.8
Electric conductivity (dS m ⁻¹)	0.84

Isolation of bacteria from fermented Ghanajeevamrit

Nine bacteria were isolated, and pure cultures were prepared by streaking onto sterile nutrient agar plates. Various plant growth-promoting activities (IAA, GA, ammonia, phosphate solubilization and EPS) of nine isolates were checked (Table 3).

Table 3 : Qualitative determination of plant growth-promoting traits

Isolates	IAA	GA	Ammonia	Phosphate solubilization	EPS
GJ01	++	+	+++	+++	+++
GJ02	++	+++	+++	+++	+++
GJ03	+	++	+++	+++	+++
GJ04	++	++	+++	+++	++
GJ05	+	+	++	++	-
GJ06	++	++	++	++	++
GJ07	+	+	++	++	-
GJ08	++	+++	+++	+++	+
GJ09	-	++	+	+	-

+++ = higher production, ++ = moderate production, + = lower production, - = no production

Cultural characteristics of selected bacterial isolates

Six potent isolates were selected for further study. Morphological and cultural

characteristics are shown in Table 4. GJ01, GJ02, GJ04, and GJ08 bacterial isolates observed Gram-negative, while GJ03 and GJ06 were Gram-positive.

Table 4 : Morphological and cultural characteristics

Colonial characteristics								
Isolates	Size	Shape	Elevation	Texture	Margin	Opacity	Pigment	Gram's reaction
GJ01	Big	Round	Slightly raised	Gummy	Entire	Translucent	Peach	Negative
GJ02	Big	Rhizoid	Slightly raised	Rough	Undulate	Opaque	Off white	Negative
GJ03	Small	Round	Flat	Smooth	Entire	Opaque	White	Positive
GJ04	Medium	Round	Flat	Smooth	Curled	Translucent	Cream	Negative
GJ06	Small	Round	Slightly raised	Gummy	Entire	Translucent	Yellow	Positive
GJ08	Small	Round	Slightly raised	Gummy	Entire	Translucent	White	Negative

Secondary screening for quantitative plant growth promoting ability of six potent bacterial isolates

Plant growth-promoting activities like IAA,

GA, ammonia, phosphate solubilization, and EPS were measured at different time intervals (Fig.1). Plant growth-promoting activities are crucial for enhancing plant

growth and development, and these activities are significantly influenced by plant growth-promoting bacteria (PGPB) isolated from Ghanajeevamrit. These bacteria play a vital role in various biological processes that support plant health, such as phytohormone production, ammonia production, phosphate solubilization and exopolysaccharide production.

IAA, a plant growth hormone, plays a crucial role in the signalling pathways that regulate the development of roots and shoots. It facilitates communication with plant growth-promoting bacteria (PGPB), enabling proper plant growth and development. The GJ06 produced the highest IAA in just 24 hours, which was 0.95 $\mu\text{g/ml}$, while the GJ04 produced 0.7 $\mu\text{g/ml}$ IAA in 24 hours. The GJ02 produced 0.48 $\mu\text{g/ml}$ IAA in 48

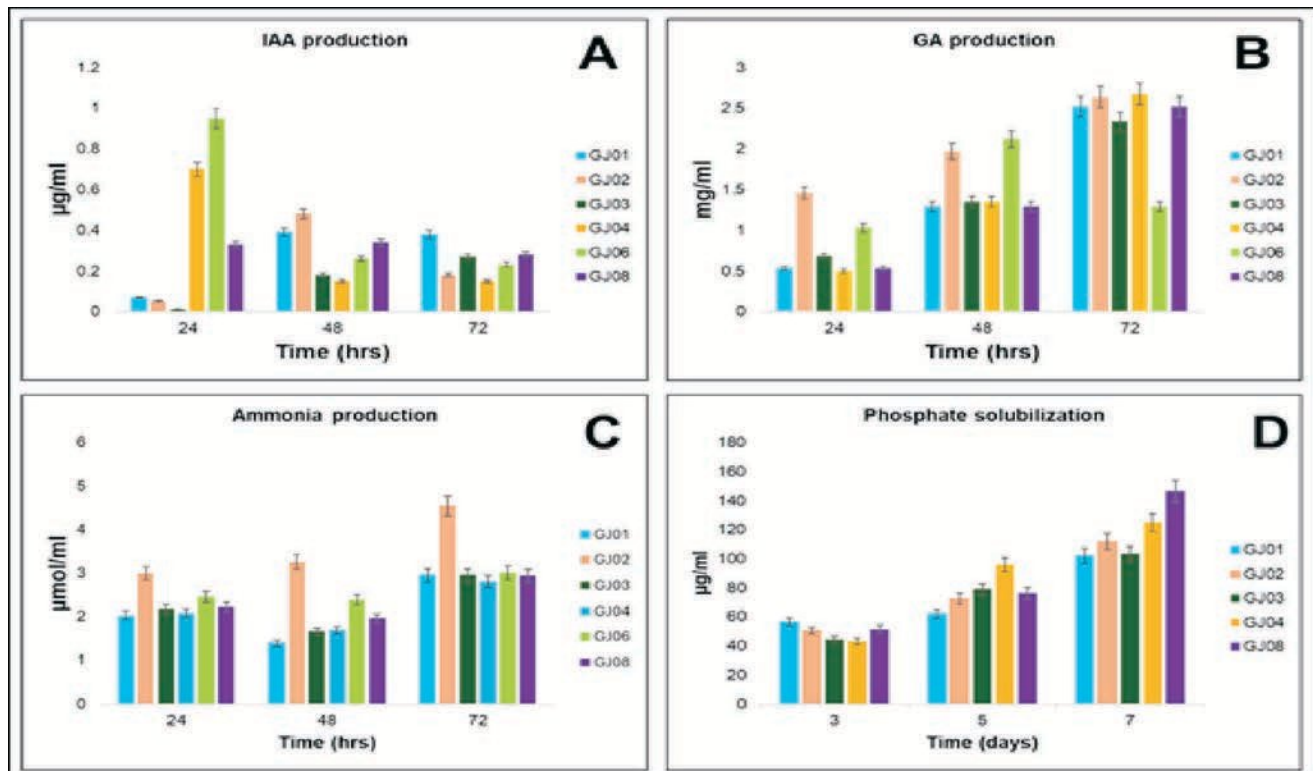


Figure 1 : Plant growth promoting activities at different time intervals A) IAA production B) Gibberellic acid production C) Ammonia production D) Phosphate solubilization

hours. *Bacillus subtilis* and *Bacillus simplex* showed 0.80 and 1.50 $\mu\text{g/ml}$ IAA production isolated from the Aegean region of Turkey (Bahadir et al., 2018). Gibberellic acids affect numerous aspects of plant growth and development, such as fruit set, seed germination, stem growth, meristem size of roots and leaves, cell division, and cell elongation. All the isolates showed significantly higher GA production in the 2.34–2.67 mg/ml range after 72 hours. The

GJ04 and GJ02 showed higher production, which was 2.67 and 2.64 mg/ml after 72 hours. *Bacillus cereus* showed 0.39 mg/ml GA production isolated from the rhizospheric soil of sugarcane (Pandya and Desai, 2013). An essential characteristic of PGPBs like *Pseudomonas*, *Bacillus*, and *Rhizobium* is their ability to fix atmospheric nitrogen (N_2), making it accessible to plants. Additionally, they can mobilize nutrients bound in organic or mineral forms from the soil, enhancing

plant nutrient availability. GJ02 showed the highest ammonia production among all the isolates, which was 3.0, 3.26, and 4.55 $\mu\text{mol/ml}$ after 24, 48, and 72 hours, respectively. *Bacillus megaterium*, isolated from the rhizosphere of uncultivated weeds, produced 2.04 $\mu\text{mol/ml}$ of ammonia after 96 hours (Krunal and Sanjay, 2020). *Klebsiella pneumoniae* showed 1.00 $\mu\text{mol/ml}$ ammonia after 96 hours, which was isolated from the chickpea rhizosphere (Mazumdar et al., 2018). Phosphate-solubilizing bacteria can convert insoluble phosphates in the soil into forms that plants can absorb. These include the release of organic acids, enzyme production, and the excretion of siderophores, which chelate metal ions and form complexes that free phosphates for plant uptake. The isolates GJ-08 and GJ-04 showed the highest solubilization on the 7th day, which was 146.5 and 125 $\mu\text{g/ml}$, respectively. At the same time, GJ02 showed 112 $\mu\text{g/ml}$ phosphate solubilization after seven days. *Bacillus cereus* was able to solubilize 140 $\mu\text{g/ml}$ phosphate after three days isolated from *Tamarix ramosissi* marhizosphere (Abdelmoteleb and Gonzalez-Mendoza, 2020).

Bacterial extracellular polysaccharides (EPSs) play various roles in producing soil aggregates, enhancing the amount of water and nutrients in the soil. The EPS-producing PGPBs can boost water and fertilizer availability to the plant by increasing the volume of soil macro-pores and the rhizosphere soil aggregation. EPS production was estimated after three days (Fig.2). GJ-08 produced the highest 5.8 g/L of EPS, followed by GJ-06 and GJ-04 with 5.6 and 5.2 g/L, respectively. GJ01 showed a lower amount of EPS (1.4 g/L). *Bacillus tequilensis* showed 2.9 g/L exopoly

saccharide production isolated from fermented goat milk (Abid et al., 2019). Pawar et al. (2013) reported that 5.2 g/L of exopoly saccharide was produced by bacteria that were isolated from saline soil of the Baramati region of Maharashtra.

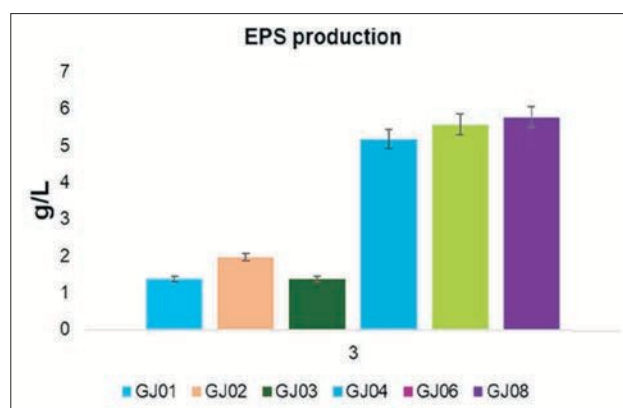


Figure 2 : Exopolysaccharide production

Salt tolerance of Ghanajeevamrit isolates

Many plant growth-promoting bacteria exhibit a remarkable ability to tolerate different salt concentration levels, which proves crucial for plant survival in saline environments. These bacteria enhance the resilience of plants by helping them cope with the adverse effects of salinity stress, which can otherwise inhibit growth, nutrient uptake, and overall plant health. GJ02 bacterial isolate can tolerate up to 10% NaCl concentration in the nutrient agar plate. All the other isolates showed a salt tolerance of 2% or less.

Pot study on *Brassica nigra*

Ghanajeevamrut and GJ02 bacterial isolate were chosen for the pot study experiment in salinity stress. Plants were uprooted after ten days, and physico-vegetative parameters were analyzed (Fig.3 and Table 5). The plant treated with Ghanajeevamrit with 1.4% NaCl showed 6.45 and 12.30 cm root and shoot lengths, respectively. The leaf length (8.99

Table 5 : Physico-vegetative parameters after ten days

Parameters	A	B	C	D	E
Rootlength(cm)	5.70	6.80	7.10	6.45	7.19
Shoot length (cm)	15.0	13.0	18.60	12.30	18.76
Leaf length (cm)	7.40	9.0	9.30	8.99	9.70
Wet weight (gm)	0.77	1.38	1.17	1.29	1.19
Dry weight (gm)	0.07	0.15	0.12	0.14	0.16
A-Control, B-Ghanajeevamrut treated, C-GJ02 treated, D-Ghanajeevamrut + 1.4% NaCl, E-GJ02 + 1.4% NaCl					

cm), wet (1.29 gm), and dry weight (0.14 gm) were also measured. The plant treated with GJ02 isolate with 1.4% NaCl showed 7.19 and 18.76 cm root and shoot length,

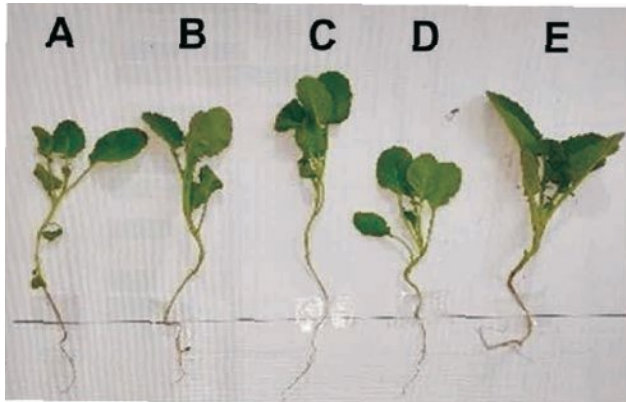


Figure 3: Uprooted plant after 10 days A) Control B) Ghanajeevamrut treated C) GJ02 treated D) Ghanajeevamrut + 1.4% NaCl E) GJ02 + 1.4% NaCl

Ghanajeevamrit significantly boosts soil microbial activity, increasing crop nutrient availability. Additionally, these beneficial microbes improve nutrient availability and uptake by the plant roots, leading to more robust root systems and enhanced overall plant vigour (Choudhary *et al.*, 2022). By fostering a more conducive environment for plant growth, the bacteria isolated from Ghanajeevamrit contribute to improved crop yields and resilience, making them an integral part of sustainable agricultural

practices. The application of Ghanajeevamrit on rice and wheat crops enhances soil health, boosts nitrogen uptake, and significantly improves crop yield (Darjee *et al.*, 2024). Widawati and Sulasih (2018) reported that inoculating sorghum seeds with *Azospirillum lipoferum* showed favourable root length and shoot length results. Certain halophilic bacteria have proven effective in reducing plant salt stress, thereby enhancing the growth and productivity of crops in saline conditions. Numerous studies have demonstrated that bacteria such as *Achromobacter*, *Arthrobacter*, *Bacillus*, *Chryseobacterium*, *Enterobacter*, *Ochrobactrum*, and *Pseudomonas*, which have been isolated from saline soils, can significantly promote plant growth in these challenging environments (Sharma *et al.*, 2016).

CONCLUSION

Ghanajeevamrit offers a sustainable, cost-effective alternative to chemical fertilizers that improves soil health, boosts plant immunity, promotes beneficial microbes, and enhances resilience to salinity. The bacterial isolate GJ02 exhibits potent plant growth-promoting traits, including phytohormone production, phosphate solubilization,

exopolysaccharide formation, and 10% salt tolerance. It significantly enhanced the growth of *Brassica nigra*, indicating its potential as an effective bioinoculant for improving crop performance in saline soils.

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DECLARATION

The authors have declared that no competing interest exists.

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