JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

DIAZOTROPHIC ENDOPHYTE *Klebsiella* sp. N5 FROM SORGHUM SHOWS CROSS-COLONIZATION AND PLANT GROWTH PROMOTION IN RICE

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https://doi.org/10.55251/jmbfs.10361

ARTICLE INFO

Received 12. 7. 2023
Revised 27. 4. 2024
Accepted 3. 5. 2024
Published 1. 8. 2024

Regular article

OPEN ACCESS

ABSTRACT

Rice necessitates the highest levels of N fertilizers during its cultivation increasing demand for nitrogen fertilizers as well as negatively affecting the environment. Endophytic diazotrophic bacteria were isolated from sorghum, out of 35 isolates *Klebsiella sp.* N5 showed maximum potential for biological nitrogen fixation. Primary screening of strain N5 was done with acetylene reduction assay indicating dry root activity of 638.55 nmol C_2H_4 h^{-1} g^{-1}. That was further confirmed by *nifH* gene amplification. The strain was identified morphologically, biochemically and 16S rDNA sequencing analysis and identified as *Klebsiella sp*. strain N5. This strain exhibited other plant growth-promoting (PGP) traits, such as phosphate solubilization, production of indole acetic acid, siderophore activity, ACC deaminase activity, and anti-fungal activity. PGP endophyte N5 was inoculated into rice seedlings to investigate its interaction under axenic conditions and to characterize its ability to colonize non-native host plant rice. Extensive colonization of rice roots was corroborated by Scanning electron microscopy. In-planta experiments with rice seeds inoculated with *Klebsiella* sp. N5 showed significant improvements in various agronomic traits such as increase in shoot length, root length, fresh and dry weight of root and shoot, chlorophyll content, and nitrogen content of the plant and soil compared to the untreated plants. *Klebsiella sp.* N5, a cross colonizer from sorghum (C4 plant) to rice (C3 plant) plays a crucial role in facilitating the acquisition of essential resources such as nitrogen, phosphorus, and iron through direct mechanisms. This study expands the horizon for its potential as a valuable bioinoculant for sustainable agricultural practices, particularly in non-native host rice, paving the way for future field trials.

Keywords: Endophytes, nitrogen fixation, nitrogenase, plant growth promotion, cross colonization

INTRODUCTION

Rice (*Oryza sativa*) is the utmost essential cereal crop in the world. It is used to feed more than half of the world's population. It ingests around 16–17 kg N to produce 1ton dry weight of rough rice, counting straw **(De data,1981; Sahrawat, 2000)**. Majority of the agricultural soils around the globe are deficient in N, which becomes a constraint for agricultural productivity **(Mahmud *et al.*, 2020)** and thus, applications of N based fertilizer becomes crucial for augmentation of crop yield. Urea is regarded as the paramount nitrogen source amended to the soil, 25% of applied urea is lost by processes of denitrification, volatilization and leaching from the plant soil system **(Prasertsak *et al.*,2001; Halvorson *et al.*, 2002)**. These processes eventually pollute the atmosphere through the emission of greenhouse gases such as N_2O, NO and NH_3 and consequent leaching of NO_3–N which contributes to the groundwater toxicity **(Shrestha and Ladha, 1998)**. Extensive practice of using chemical fertilizers may decline the soil biodiversity and organic matter as well **(Wairiu and Lal, 2003)**. Biological nitrogen fixation is the promising alternative to overcome such difficulties in addition to the restoration of the soil fertility.

Sorghum [*Sorghum bicolor* (L.) Moench] is the world's fifth most important cultivated cereal after wheat, rice, maize and barley, with a global production of 60 million tons per year **(Dicko *et al.*, 2006)**. It is the dietary staple of more than 500 million people in 30 countries across the world and is the third most consumed cereal grain in India after rice and wheat **(Ali *et al.*, 2021)**. Sorghum is majorly included in routine diet as it is rich in fiber. Other uses of sorghum are as fodder and production of biofuels such as bio-ethanol and biodisel **(Rao *et al.*, 2010)**. Naturally, sorghum is a resilient and drought tolerant crop by virtue of its own genetic architecture and interactions mainly with its rhizospheric and endophytic beneficial microbes in its growing environment, which partly might be contributing to its tolerance mechanisms and adaptations.

Plant growth-promoting (PGP) endophytic diazotrophic bacteria stimulate plant growth and reinforce plant development and survival under nitrogen limiting conditions. A renewed interest in endophytic diazotrophs, such as *Acetobacter*, *Azoarcus*, and *Herbaspirillum*, in gramineous plants has arisen because of their occurrence mainly within plant tissues and evidence for significant nitrogen fixation **(James *et al.*, 1998)**. Despite the additional contributions to plant growth promotion, special considerations should be taken to ensure the compatibility of the endophytic strain with its host (native/non-native) plants, and its competency to colonize host tissue without causing any disease or stress symptoms. Diazotrophic *Klebsiella* (Family *Enterobacteriaceae*) has been found dominant in maize, sorghum, sugarcane, and other gramineous plants and has an efficiency to colonize other plants by increasing nitrogen availability **(Biaosheng *et al.*, 2019; James, 2000)**. Solicitation of diazotrophic bacteria to the foreign host, widen their application for variety of crops. In our laboratory *Klebsiella sp.* N5 isolated from roots of sorghum has demonstrated substantial nitrogen fixation potential. Rice having a prime prerequisite of nitrogen, the competent colonization of *Klebsiella sp.* N5 to rice may benefit the farmers by improving yield and thus reducing the need of chemical fertilizer applications. The present investigation therefore, focused on testing the cross colonization potential of *Klebsiella sp.* N5 between sorghum (C4 plant) to its non-native host rice (C3 plant), so that it can serve as a biological alternative for chemical N fertilizer in rice.

MATERIALS AND METHODS

Isolation of endophytic diazotrophic bacteria

Sorghum plant samples were collected from 5 different regions of South Gujarat, India *i.e.* Surat, Dandi, Navsari, Valsad and Vapi. Roots of selected plant samples were surface-sterilized with 70% ethanol for 3 min followed by 1% chloramines treatment for 30 min. Treated samples were washed thoroughly with sterile water. Surface-sterilized plant materials were thoroughly crushed in 4% sucrose solution using mortar and pestle and suspension was streaked on Liquid Glucose Ivo (LGI) plates for purification **(Cavalcante and Dobereiner, 1988]**. For sterility check, 1 ml from the final washing was transferred to 9 ml of LGI broth and 100 µl of this solution was spread on LGI plates.

Screening and characterization of selected isolate/s

Screening for nitrogen fixation ability: Screening of bacterial isolates obtained from 5 different regions, were done based on presence of *nifH* gene to select an efficient nitrogen fixing endophyte. Amplification of *nifH* region having size of 360 bp was done using degenerate primer sequences: Pol F primer- TGC GAY CCS AAR GCB GAC TC and Pol R primer-ATS GCC ATC ATY TCR CCG GA **(Saikia and Jain,2007*).*** PCR reaction in 50 µl reaction volume was set with: 5 µl of genomic DNA, 5 µl of PCR buffer (10x), 3 µl of $MgCl_2$, 1.25 µl of dNTP mixture, 0.5 µ mol of forward and reverse primers each and 0.2 µl of Taq DNA polymerase. PCR reaction was performed in a thermocycler (eppendorf) and programmed as follows: Initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min, extension at 72ºC for 2 min. PCR amplicon (s) were run on 1 % agarose gel and visualized under UV gel documentation system.

***Biochemical characterization*:** Selected bacterial isolate was grown on LGI agar plate at 37ºC and pure culture was used for morphological and biochemical characterization as per Bergey's Manual of Systematic Bacteriology **(Krieg, 1984)**. Biochemical tests such as Voges-Proskauer test**,** Citrate utilization test, Urea hydrolysis test, Catalase test, Oxidase test, Starch hydrolysis, Casein hydrolysis test and sugar utilization tests were performed to classify and differentiate the bacterial species (**Singh *et al.*, 2013).**

Molecular characterization of endophytic isolate*:** The selected endophytic isolate N5 was identified by 16S rRNA gene sequencing. Genomic DNA was extracted by CTAB method **(Ivanova *et al.*, 2001)** and the concentration of total genomic DNA was adjusted to a final concentration of 20 ng μl^{-1} for PCR amplification. The 16S rRNA gene of the isolate was amplified using two universal primers namely: 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGYTACCTTGTTACGACTT3') **(Weisburg *et al.*, 1991*). Standard PCR conditions were maintained as: Initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 1 min, annealing at 52°C for 45 sec, and elongation at 72°C for 1 min. At the end of 30 cycles, the final extension step was at 72°C for 8 min **(Sun *et al.*, 2010)**. Cycle sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out using AB prism Sequencer, 3130 Genetic analyzer (Applied Bio systems) with 4 capillaries. Further, BLAST was carried out for similarity search of the selected isolate against the GenBank database (website: http://www.ncbi.nih.gov/BLAST).

Study of PGPR traits (in vitro tests)

Indole Acetic Acid (IAA) production: *Klebsiella sp*. N5 was inoculated in LB medium (Luria bertani) supplemented with 1 $mgml^{-1}$ of tryptophan and incubated at 28ºC for 24 h under shaking condition. Further, 2 ml culture of isolate was centrifuged at 1000 rpm for 10 min. Then 2 - 3 drops of orthophosphoric acid and 4 ml of Salkowski's reagent were added to the collected Cell Free Supernatants (CFS) and were kept at room temperature for 20 min. IAA production was indicated by the development of pink colour. Quantification of Indole-3-acetic acid (IAA) was done by measuring absorbance at 530 nm using spectrophotometer (Dynamica Halo DB-20, Australia) **(Singh *et al.*, 2017)**.

Siderophore production: *Klebsiella* sp. N5 culture was inoculated in the center of CAS agar plate for assessing siderophore production. Orange purple or dark purplish-red halos around the colonies on blue agar were indicative of siderophore production **(Schwyn and Neilands**, **1987)**.

Phosphorous solubilisation: Actively growing bacterial culture was spot inoculated on Pikovaskya agar plates with composition (gL^{-1}): (Yeast extract 0.5, Dextrose 10, $(NH_4)_2SO_4$ 0.5, $Ca_3(PO_4)_2$ 5, KCl 0.2, $MgSO_4$ 0.1, $MnSO_4$ 0.0001, $FeSO_4$ 0.001 and Agar 15, at pH 7.0), and incubated at 30ºC for 3 days. Development of transparent zone against white opaque background was suggestive of the positive results.

1-Aminocyclopropane-1-Carboxylate (ACC) deaminase production: The isolate was point inoculated on salt minimal medium containing ACC as sole nitrogen source. Development of dark red color was considered as positive test for ACC deaminase production **(Dworkin and Foster**, **1958).**

Ammonia production: Bacterial isolate was grown in peptone water for 24 h. 1 % inoculum was added to 5 ml of peptone water in each tube and incubated for 72 h at 30ºC. Nessler's reagent (0.5ml) was added in each tube. Development of brown to yellow color indicates positive test for ammonia production **(Cappuccino and Sherman, 1992)**.

***In planta* experiments**

***Evaluation of Agronomic traits and Nutrient Uptake of N5-Coated Rice Seeds*:** Healthy seeds of variety Jaya were obtained from main rice research centre, Navsari Agricultural University, and washed thoroughly with distilled water followed by surface sterilization using 0.1% $HgCl_2$ solution for 4 min and 70% ethanol for 10 min. Then seeds were washed thoroughly with sterile distilled water and coated with bacterial culture by incubation for 3 h followed by drying. Culture coated seeds were sown in pot and maintained under green house conditions. All recommended practices and plant protection measures were adopted to obtain healthy plants **(Ji et al., 2014**). The agronomic traits like root length, shoot length, fresh weight of root and shoot, dry weight of root and shoot, chlorophyll content, leaf area and nitrogen content were recorded in plant and soil samples after 60 days of inoculation.

Measuring nitrogenase activity*:** Nitrogen fixation capacity of nitrogen fixers were quantified indirectly by Acetylene reduction assay (ARA) measuring the reduction of acetylene to ethylene by *Klebsiella* sp. N5 inoculated plants roots **(Ladha *et al.*, 1986*). Ten seedlings from each treatment were taken at panicle initiation and grain-filling stages and roots were separated and washed twice with sterile water to remove loosely associated bacteria. The roots were then transferred to fresh, N-free liquid Jensen's medium **(Somasegaran and Hoben ,1994)**. Test-tubes containing the plant's root were sealed with a rubber seal and 10% of the headspace volume was replaced with acetylene. Non inoculated plant roots and test-tubes not injected with acetylene served as controls. They were returned to growth chamber and incubated in the dark for 12 h at 30oC. Acetylene reduction activity was determined using a Gas Chromatography (Shimadzu GC-14A with Porapak-N 80/100 – INOX column).

Scanning Electron Microscopy (SEM): Plant tissues collected 7 days post inoculation were washed twice with sterile water, fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2-7.4, for 2 h at 28°C. Fixed tissues were washed three times with phosphate buffered saline (PBS, pH 7.2) and cut to separate roots and aerial parts. Plant segments were post-fixed in 1% (w/v) osmium tetroxide in PBS, for 2 h at 4°C, dehydrated in a 50-100% (v/v) gradient ethanol series, and dried by the CO_2 critical point method in a Balzers apparatus, model CDP-20. Samples were subsequently mounted on aluminum stubs with double coated carbon conductive tape (Pelco Int.) and sputtered with gold in a Balzers apparatus, model FL-964. Observations and micrographies were made in a Jeol JSM-5310 scanning electron microscope.

Statistical Analysis

Each experiment was conducted in three replications and later mean and standard deviation were calculated for all experiments. Data were analyzed by analysis of variance (ANOVA) using Duncan`s multiple range test (DMRT) by Statistical Analysis System (SAS 9.1).

RESULTS

Isolation and screening of selected isolates: Thirty-five endophytic nitrogen fixing bacteria were isolated from the sorghum root samples, collected from various regions of the South Gujarat using N-free LGI medium supplemented with 0.5% sugarcane juice at pH 4.5. Out of 35 isolates cultured from sorghum root, N5 was selected based on *nifH* gene amplification using a pair of *nifH* specific universal primers. PCR amplification of *nifH* gene showed that isolate N5, produced the expected 360-bp amplification product (Fig. 1).

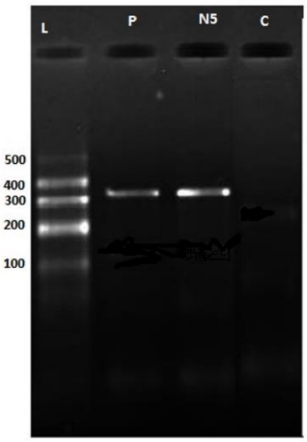


Figure 1 Amplification of *nifH* gene; L-ladder, P-Positive control (*Azotobacter* sp.), N5- *Klebsiella sp*. N5, C-negative control

Identification of the selected isolate: Further, morphological and biochemical characterization of this isolate was carried out. The colony appears small, round with a smooth surface and orange colored. The organism is Gram negative, rod shaped, non-motile and positive for starch hydrolysis, casein hydrolysis and catalase activity. Though, it was found to be negative for H_2S production.
Molecular identification: Molecular identification of diazotrophic bacterial isolate N5 was done using the 16S rRNA gene amplification and sequencing using

universal 16S primers. NCBI-BLAST online homology search program confirmed the isolate as *Klebsiella sp*. N5. The sequence has been submitted to GenBank and the accession number has been assigned as MF590169 to the isolate, *Klebsiella sp*. N5.

Study of PGPR traits (*in vitro* tests)

Klebsiella sp. N5 was found to produce IAA (245.9 μgml^{-1}) and siderophore (Figs. 2a and 2b) alongwith the other plant growth promoting traits such as phosphate solubilisation (Fig. 2c), ACC deaminase activity and ammonia (Fig. 2d). Interestingly, *Klebsiella* sp. N5 also inhibited growth of a fungal phytopathogen, *Fusarium oxysporum* and showed appreciable antifungal activity (Data not shown).

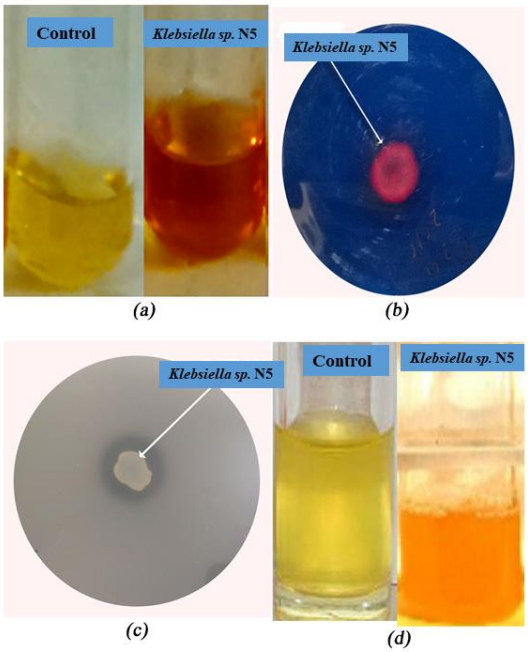


Figure 2 Plant growth promoting traits of endophytic *Klebsiella sp.* N5 a) IAA production b) Siderophore production c) Phosphate solubilisation d) Ammonia production**.**

***In planta* experiments (Pot studies)**

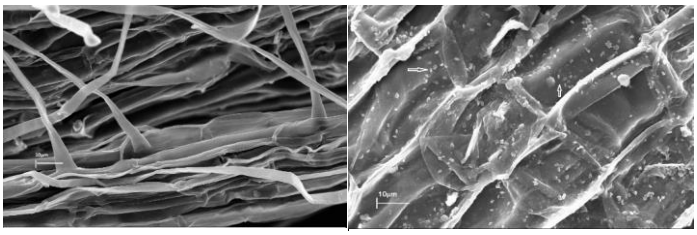
Effect of Endophytic inoculation on growth of rice plants: Rice seeds were inoculated with *Klebsiella sp.* N5 to analyze its effect on growth parameters after 60 days of inoculation. Plant growth promoting activity of *Klebsiella sp*. N5 was evaluated by measuring. Results showed that isolate has the potential to be used as plant growth promoting bacteria as it aided significantly to plant growth correlated with the significant improvement in various agronomic traits (Fig. 3 and Table 1) like shoot length, root length, shoot and root fresh and dry weight, chlorophyll content, N content of plant and soil, after 60 days of inoculation. Rice plants showed a substantial increase in all parameters as compared to control e.g. root length (15.46 cm), shoot length (57.06 cm), root fresh weight (0.76 gm), root dry weight (0.15 gm), shoot fresh weight (1.68 gm), shoot dry weight (0.69 gm), chlorophyll content (39 mgg^{-1}) and leaf area (0.63 cm^2). Surprisingly**,** the nitrogen content of the *Klebsiella sp.* N5 treated rice plant and soil was nearly 2.46 % and 21.41 $mgkg^{-1}$, which is again higher than the control plants (Table **1**).

Figure 3 Effect of *Klebsiella sp.* N5 inoculation on rice (GNR-4) plant physiology after 60 days of treatment.

Table 1 Effect of endophytic treatment of *Klebsiella sp.* N5 on rice plants. (Values are mean ± S.E. of three samples).

Treatments	Shoot length (cm)	Root length (cm)	Shoot		Root		Chlorophyll content SPAD	Leaf area $(cm)^2$	N content from plant (%)	N content from soil $mgkg^{-1}$
			Fresh weight (gm)	Dry weight (gm)	Fresh weight (gm)	Dry weight (gm)				
T1	51.26±0.01 [a]	13±0.01 [a]	1.18±0.01 [a]	0.44±0.01 [a]	0.54±0.01 [a]	0.12±0.01 [a]	35±0.01 [a]	0.45±0.01 [a]	1.22 [a]	17.51 [a]
T2	57.06±0.01 [b]	15.46±0.01 [b]	1.68±0.01 [b]	0.69±0.01 [b]	0.76±0.01 [b]	0.15±0.01 [b]	39±0.01 [b]	0.63±0.01 [b]	2.46 [b]	21.31 [b]

Estimation of nitrogen fixing efficiency of *Klebsiella* sp. N5: N-fixation efficacy of *Klebsiella sp*. N5 in rice plant was checked via pot studies. Acetylene Reduction Assay was performed and analyzed using GC. The *Klebsiella sp.* N5 showed appreciable amount of nitrogenase activity (638.55 n moles $C_2H_4h^{-1}$ g^{-1} dry root).
Study of endophytic Root colonization: Scanning Electron Microscopy (SEM) was performed to confirm the endophytic association of *Klebsiella sp.* N5 with roots of rice plants. Significant number and profuse growth of *Klebsiella sp.* N5 visible in the endophyte treated plants roots (Fig. 4b) compared with no growth in uninoculated control (Fig. 4a), confirmed the endophytic association of N5 in rice plants. Superior physiological growth of rice witnessed in plants treated with *Klebsiella sp.* N5 compared to control happened due to the positive endophytic association of selected strain with rice plants (Table 1).



(a) **(b)**

Figure 4 Scanning electron micrographs of root colonization by endophytic *Klebsiella sp.* N5 in **a)** Un inoculated control (Mag 3.4 K X) **b)** Treated with *Klebsiella sp.* N5 (Mag 4.03 KX).

DISCUSSION

Sorghum is a robust crop that can grow well in adverse climatic conditions even in less fertile soil. Different endophytic bacteria have been reported to live inside sorghum plants and support its growth by various growth promoting activities. A potential sorghum endophyte, *Enterobacter cloacae* has been characterized to impart stress tolerance and good root architecture in sorghum **(Govindasamy *et al*., 2020)**. Notably, *Paenibacillus* sp. strain B2 and *Pseudomonas* spp. have also been reported to inhibit the growth of phytopathogens and lessen the abiotic stress

(Budi *et al.*, 1999; Funnell-Harris *et al.*, 2013*;* Ali *et al.*, 2010) respectively. Hence, in the present study, isolation and characterization of the diazotrophic bacteria was carried out from root-endophytic zone of sorghum. Most potential isolate was selected and its plant growth promoting activity in non-native host rice was evaluated.

The most potential isolate obtained was identified as ARA-positive endophyte *Klebsiella sp.* N5. It was reported to promote plant growth by various activities such as biological nitrogen fixation (BNF), IAA production, ACC deaminase activity and improvement in the availability of nutrients to the host plant. Moreover, it was also reported to possess good biocontrol activity against *Fusarium oxysporum*. Thus, *in vitro* studies suggested that endophytic *Klebsilla sp*. N5, harboured great efficiency for plant growth promotion alongwith the plant protection functions.

The BNF actvitity of the *Klebsiella sp.* N5 was confirmed by the presence of *nifH* gene, an integral part of the nitrogenase enzyme complex. The *nifH* gene encodes for the iron (Fe)-protein subunit of nitrogenase enzyme and reported to remain conserved among all nitrogen fixing microorganisms **(Liu *et al.*, 2012*)***. Significant nitrogenase activity was exhibited by N5 (638.55 n moles of C_2H_4 h^{-1} g^{-1} dry root in rice) in Acetylene reduction assay (ARA). It reduced the inhibitory nitrogen concentrations in the soil and led to over production of root exudates that are beneficial to diazotroph growth and activity **(Boddey and Dobereiner, 1995)**. Eventually, P solubilization and other growth promoting activities helped *Klebsiella sp.* N5 to grow and colonize non-native host rice plants. P solubilization activity converted the unavailable form of P to available form for the host plant. It indicated that the origin of *Klebsiella sp.* N5 might be the rhizosphere region, from where it further migrated to the internal root tissues.

Endophytic bacteria express their nitrogen-fixing potential better inside plant tissues because there they get the required low partial pressure of oxygen. Further, N_2-fixation is actively expensive, requiring carbon and phosphate with at least 16 ATP per N_2 fixed [34], which is a restriction for endophytic N_2-fixation. P is a second most important nutrient after N **(Udvardi and Poole, 2013**). Its level in the soil regulates the biodiversity of rhizospheric microorganisms and help phosphate-solubilizing bacteria (PSB) in inital stages of colonization. Enhancement in P supply to N_2-fixing microbes could be an alternative approach to ameliorate N_2 fixation and P availability to favour plant system. Therefore, nature selects diazotrophic endophyte with P solubilisation capabilities that are competitively adequate to dominate in the plant root zone **(Anamika et al., 2007)**. The isolate, *Klebsiella sp.* N5 possessed N_2 fixation, P solubilisation and other PGP traits, which substantiated its role in growth promotion and induction of abiotic stress tolerance in the native host, Sorghum.

The endophytic *Klebsiella sp.* are reported to produce plant growth hormones and fix atmospheric nitrogen under agricultural conditions with broad range of host crops. *Klebsiella variicola* GN02 was reported as a potent endophytic organism isolated from *Pennisetum sinense* Roxb. It was reported to have complete set of nitrogen fixing and nitrogen metabolism genes such as *nif* and *amt*B genes **(Lin et al., 2019)**. *Klebsiella sp.* 342, isolated from maize showed nitrogenase production in presence of suitable carbon source and it was reclassified by 16srRNA sequencing using locus *rpoB, fusA, gapA, gyrA* and *leuS* genes. It was further confirmed with phylogenetic analysis, KpI as *Klebsiella sp.* and KpIII as *Klebsiella variicola* **(Iniguez *et al.*, 2004)**. Notably, every PGP endophyte may not harbour the ability to colonize non-native host plants, but different species of *Klebsiella* have been reported to colonize with the agronomically important crops like rice **(An *et al.*, 2001)**, sugarcane **(Ando *et al.*, 2005)**, maize **(Mowafy *et al.*, 2021**), sweet potato **(Reiter *et al.*, 2003)** and banana **(Martínez et al., 2003)** indicating its compatibility and survival with wide host range. BNF is limited to prokaryotes and rice demanding high nitrogen, needs association with prokaryotes to fulfil its nitrogen demand. Therefore, diazotrophic endophyte *Klebsiella sp.* N5 was investigated to analyse its potential in non-native host, rice.

The compatibility and colonization of endophyte *Klebsiella sp.* N5 in non-native host, rice was confirmed by scanning electron microscopy (Fig. 4b). The isolate inhabited the root tissues of rice plants without causing any damage. N5 significantly increased the root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, chlorophyll content and leaf area of rice as compared to the uninoculated control which was evident in the pot experiment (*in planta* studies). Hence, it was indicative of the positive effect of endophytic *Klebsiella sp.* N5 on its non-native host rice. Nitrogen content of the N5 treated rice plant and soil was approximately 2.46 % and 21.41 mg kg^{-1} which was again higher than the control plants (Table 1). A strong correlation between N absorption and root architecture in maize plants, was reported under N limiting condition and during optimum nitrogen assimilation **(Coque *et al.*, 2008)**. IAA and ACC deaminase production by bacteria helped to amend the physiological response and stress mitigation in plants. IAA is the main auxin in plants with no apparent function in bacterial cells, and it could be speculated that IAA production may improve the fitness of the plant–microbial interaction by governing many imperative physiological routes including cell enlargement and division, tissue differentiation, and responses to light **(Khalid *et al.*, 2004*;* Frey-Klett *et al.*, 2005**). Rhizospheric bacterial isolates having capability to produce ACC deaminase reduce the ethylene concentration and impart protection against various abiotic and biotic stresses **(Glick *et al.*, 2007*;* Chandwani and Amaresan, 2022)**. Siderophore production by *Klebsiella sp.* N5 could confer competitive advantages to colonize rice tissues and to exclude other microorganisms from the same ecological niche. Thus, inoculated *Klebsiella sp.* N5 into rice has improved the plant growth of its non-native host possible *via* either direct or indirect growth promoting mechanisms. Direct mechanism of *Klebsiella sp.* N5 like IAA production, ACC deaminase activity, exhibited a remarkable capability to facilitate the acquisition of resources such as N, P and Fe to the host plant. However, whether this improved plant growth was a result of better mobilisation of these nutrients or a secondary effect of improved root growth still needs to be investigated.

CONCLUSION

The diazotrophic endophyte *Klebsiella sp*. N5 isolated from sorghum displayed several plant growth promoting traits such as P solubilisation, siderophore production, phytohormone production and biocontrol activity in addition to a vital function of N2 fixation. The isolate is a promising cross colonizer of non-native host rice which was revealed during pot studies. Eventually, these properties assisted in improving the overall growth and development of the host plant by direct and indirect mechanisms. Thus, it is concluded from the present study that *Klebsiella sp.* N5 may serve as a promising biofertilizer agent and can be a suitable alternative of the hazardous chemicals for sustainable agriculture after conducting field trials.

Conflict of interest: The authors have no conflict of interest.

Acknowledgements: The authors would like to thank ASPEE College of Horticulture and Forestry and ASPEE SHAKILAM Biotechnology Institute, Navsari Agricultural University for necessary help and financial support.

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REFERENCES

Ali, B., Sabri, A.N., Hasnain, S. (2010) Rhizobacterial potential to alter auxin content and growth of *Vigna radiata* (L.) *World J Microbiol Biotechnol*. 26(8),1379-1384. https://doi.org/10.1007/s11274-010-0310-1

Ali, K., Valeria, M., Komala, V. V., Asgar, F., Christopher, L.B., Thomas, H.R. (2021) Sorghum in foods: Functionality and potential in innovative products. *Crit. Rev. Food Sci. Nutr*. https://doi.org/10.1080/10408398.2021.1960793

An, Q.L., Yang, X.J., Dong, Y.M., Feng, L.J., Kuang, B.J. (2001) Using confocal laser scanning microscope to visualize the infection of rice roots by GFP-labelled *Klebsiella oxytoca* SA2, an endophytic diazotroph. *Acta Bot Sin*. 43, 558–564. https://europepmc.org/article/cba/349869

Anamika Saxena, J., Sharma, V. (2007). Isolation of tricalcium phosphate solubilizing strains from semiarid agricultural fields of Rajasthan. *India. J. Pure Appl. Microbiol,* 1, 269-280.

Ando, S., Goto, M., Meunchang, S., Thongra-ar, P., Fujiwara, T., Hayashi, H., Yoneyama, T. (2005) Detection of *nifH* Sequences in Sugarcane (*Saccharum officinarum* L.) and Pineapple (*Ananas comosus* [L.] Merr.) *J Soil Sci Plant Nutr*. 51(2), 303-308. https://doi.org/10.1111/j.1747-0765.2005.tb00034.x

Biaosheng, L., Zhaozhao, S., Yulei, J., Yulong, Z., Lifang, W., Jinling, F., Zhanxi, L. (2019) Biological characteristics and genome-wide sequence analysis of endophytic nitrogen-fixing bacteria *Klebsiella variicola* GN02. *Biotechnol. Biotechnol. Equip*. 33(1), 108-117. https://doi.org/10.1080/13102818.2018.1555010

Boddey, R.M., Dobereiner, J. (1995) Nitrogen fixation associated with grasses and cereals: recent progress and perspectives for the future. *Fertilizer Res*. 42(1-3), 241-50. https://doi.org/10.1007/978-94-009-1706-4_24

Budi, S.W., Tuinen, D.V., Martinotti, G., Gianinazzi, S. (1999) Isolation from the *Sorghum bicolour* mycorrhizosphere of a bacterium compatible with Arbuscular Mycorrhiza development and antagonistic towards soilborne fungal pathogens. *Appl Environ Microbiol*. 65 (11), 5148-5150. https://doi.org/10.1128/AEM.65.11.5148-5150.1999

Cappuccino, J.C., Sherman, N. (1992) In: Microbiology: A Laboratory Manual, 3rd edn. Benjamin/cummings, New York, pp 125-179.

Cavalcante, V.A., Dobereiner, J. (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant and Soil*. 108(1), 23-31. https://doi.org/10.1007/BF02370096

Chandwani, S., Amaresan, N. (2022) Role of ACC deaminase producing bacteria for abiotic stress management and sustainable agriculture production. *Environ Sci Pollut Res.* 29, 22843–22859 https://doi.org/10.1007/s11356-022-18745-7

Coque, M., Martin, A., Veyrieras, J.B., Hirel, B., Gallais, A. (2008) Genetic variation for N-remobilization and postsilking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. *Theor Appl Genet.* 117,729–747. https://doi.org/10.1007/s00122-008-0815-2

De data, S.K. (1981) *Principles and Practices of Rice Production*. J Wiley & Sons, New York

Dicko, M.H., Gruppen, H., Travore, A.S., Voragen, A.G.J., Van Berkel, W.J.H. (2006) Sorghum grain as human food in Africa: relevance of content of starch and

amylase activities. *Afr J Biotechnol*. 5(5), 384-395. https://www.ajol.info/index.php/ajb/article/view/137858
Dworkin, M., Foster, J.W. (1958) Experiments with some microorganisms which utilize ethane and hydrogen. *J Bacteriol* 75(5), 592. https://journals.asm.org/doi/pdf/10.1128/jb.75.5.592-603.1958
Frey-Klett, P., Chavatte, M. (2005) Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent *pseudomonads*. *New Phytol*. 165, 317–328. https://doi.org/10.1111/j.1469-8137.2004.01212.x
Funnell-Harris, D.L., Sattler, S.E., Pedersen, J.F. (2013) Characterization of fluorescent *Pseudomonas* spp. associated with roots and soil of two sorghum genotypes. *Eur J Plant Pathol*. 136, 469–481. https://doi.org/10.1007/s10658-013-0179-6
Glick, B.R., Cheng, Z., Czarny, J., Duan, J. (2007) Promotion of plant growth by ACC deaminase-producing soil bacteria. In: Bakker P.A.H.M., Raaijmakers J.M., Bloemberg G., Höfte M., Lemanceau P., Cooke B.M. (eds) New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-6776-1_8
Govindasamy, V., George, P., Kumar, M. (2020) Multi-trait PGP rhizobacterial endophytes alleviate drought stress in a senescent genotype of sorghum (*Sorghum bicolor* (L.) Moench). *3 Biotech.* 10 (13). https://doi.org/10.1007/s13205-019-2001-4
Halvorson, A. D., Wienhold, B.J., Black, A.L. (2002) Tillage, nitrogen and cropping system effects on soil carbon sequestration. *Soil Sci Soc Am J*. 66, 906–912. https://doi.org/10.2136/sssaj2002.9060
Iniguez, A.L., Dong, Y., Triplett, E.W. (2004) Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. *Mol Plant Microbe Interact*. 17,1078–1085. https://doi.org/10.1094/MPMI.2004.17.10.1078
Ivanova, E.G., Doronina, N.V., Shepeliaskovskaia, A.O., Laman, A.G., Brovko FA, Trotsenko YA. (2001) Aerobic methylobacteria are capable of synthesizing auxins. *Microbiol*. 70, 392–397. https://doi.org/10.1023/A:1010469708107
Jagnow, G.C. (1983) Nitrogenase (C_2 H_2) activity in non-cultivated and cereal plants; influence of nitrogen fertilizer on population and activity of nitrogen fixing bacteria. Z Pflanzenernaehr Bodenkd. 146: 217-227. https://doi.org/10.1002/jpln.19831460211
James, E.K. (2000) Nitrogen fixation in endophytic and associative symbiosis. *Field Crop Res*. 65 (2-3), 197-200. https://doi.org/10.1016/S0378-4290(99)00087-8
James, E.K., Olivares, F.L., Sprent, J.I. (1998) Infection and colonization of sugarcane and other graminaceous plants by endophytic diazotrophs. *Crit Rev Plant Sci*. 17:77–119. https://doi.org/10.1080/07352689891304195
Ji, S.H., Gururani, M.A., Chun, S.C. (2014) Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. *Microbiol Res*. 169(1), 83-98. https://doi.org/10.1016/j.micres.2013.06.003
Khalid, A., Arshad, M., Zahir, Z.A. (2004) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *J Appl Microbiol*. 96(3), 473-480. https://doi.org/10.1046/j.1365-2672.2003.02161.x
Krieg, N.R. (1984) Bergey's manual of systemic bacteriology, Vol. I. Williams and Wilkins. Baltimore, U.S.A.
Ladha, J.K., Tirol, A.C., Daroy, M.L., Caldo, G., Ventura, W., Watanabe, I. (1986) Plant-associated N_2 fixation (C_2H_2-reduction) by five rice varieties, and relationship with plant growth characters as affected by straw incorporation. *J Soil Sci Plant Nutr*. 32(1), 91-106. https://doi.org/10.1080/00380768.1986.10557484
Lin, B., Song, Z., Jia, Y., Zhang, Y., Wang, L., Fan, J., Lin, Z. (2019) Biological characteristics and genome-wide sequence analysis of endophytic nitrogen-fixing bacteria *Klebsiella variicola* GN02. *Biotechnol Biotechnol Equip.* 33(1),108-117. https://doi.org/10.1080/13102818.2018.1555010
Liu, J., Peng, M., Li, Y. (2012) Phylogenetic diversity of nitrogen-fixing bacteria and the *nifH* gene from mangrove rhizosphere soil. *Can J Microbiol*. 58(4),531-9. https://doi.org/10.1139/w2012-016
Mahmud, K., Makaju, S., Ibrahim, R., Missaoui, A. (2020) Current Progress in Nitrogen Fixing Plants and Microbiome Research. *Plants*. 9, 97. https://doi.org/10.3390/plants9010097
Martínez, L., Caballero-Mellado, J., Orozco, J. (2003) Diazotrophic bacteria associated with banana (*Musa* spp.). *Plant Soil* 257, 35–47. https://doi.org/10.1023/A:1026283311770
Mowafy, M. A., Fawzy, M ..M, Gebreil, A., Elsayed, A. (2021) Endophytic *Bacillus, Enterobacter*, and *Klebsiella* enhance the growth and yield of maize, *Acta Agric. Scand. - B Soil Plant Sci.* 71(4), 237-246. https://doi.org/10.1080/09064710.2021.1880621
Prasertsak, P., Freney, J., Saffigna, P. *et al.* Fate of urea nitrogen applied to a banana crop in the wet tropics of Queensland. *Nutrient Cycling in Agroecosystems* **59**, 65–73 (2001). https://doi.org/10.1023/A:1009806826141
Rao, P.P., Basavaraj, G., Ahmad, W., Bhagavatula, S. (2010) An analysis of availability and utilization of sorghum grain in India. *J of SAT Agricul Res*. 8: 1-8. http://oar.icrisat.org/97/
Reiter, B., Burgmann, H., Burg, K., Sessitsch, A. (2003) Endophytic *nifH* gene diversity in african sweet potato. *Can J Microbiol*. 49,549–555. https://doi.org/10.1139/w03-070
Sahrawat, K.L. (2000) Macro- and micronutrients removed by upland and lowland rice cultivars in West Africa. *Commun Soil Sci Plan.* 31 (5-6), 717-723. https://doi.org/10.1080/00103620009370472
Saikia, S.P., Jain, V. (2007) Biological nitrogen fixation with non-legumes: an achievable target or a dogma. *Curr Sci*. 92(3), 317-22. https://www.jstor.org/stable/24096726
Schwyn, B., Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem*. 160(1), 47-56. https://doi.org/10.1016/0003-2697(87)90612-9
Shrestha, R.K., Ladha, J.K. (1998) Nitrate in Groundwater and Integration of Nitrogen-Catch Crop in Rice-Sweet Pepper Cropping System. *Soil Sci Soc Am J*. 62(6),1610-1619. https://doi.org/10.2136/sssaj1998.03615995006200060019x
Singh, D., Geat, N., Rajawat, M.V., Prasanna, R., Saxena, A.K., Kaushik, R. (2017) Isolation and Characterization of Plant Growth Promoting Endophytic Diazotrophic Bacteria from Wheat Genotypes and their Influence on Plant Growth Promotion. *Int J Curr Microbiol Appl Sci*. 6(4),1533-40. https://doi.org/10.20546/ijcmas.2017.604.188
Singh, D., Sharma, A., Saini, G.K. (2013) Biochemical and molecular characterisation of the bacterial endophytes from native sugarcane varieties of Himalayan region. *3 Biotech*. 3(3), 205-12. https://doi.org/10.1007/s13205-012-0084-2
Somasegaran, P., Hoben, H.J. (1994) Counting rhizobia by a plant infection method. In Handbook for Rhizobia. Springer New York, pp 58-64. https://doi.org/10.1007/978-1-4613-8375-8_6
Sun, L., Qiu, F., Zhang, X., Dai, X., Dong, X., Song, W. (2010) Endophytic Bacterial Diversity in Rice (*Oryza sativa* L.) Roots Estimated by 16S rDNA Sequence Analysis. *Microb Ecol* 55, 415-424. https://doi.org/10.1007/s00248-007-9287-1
Udvardi, M., Poole, P.S. (2013) Transport and metabolism in legume-rhizobia symbioses. *Annu Rev Plant Biol*. 64,781-805. https://doi.org/10.1146/annurev-arplant-050312-120235
Wairiu, M., Lal, R. (2003) Soil organic carbon in relation to cultivation and topsoil removal on sloping lands of Kolombangara, Solomon Islands. *Soil Tillage Res*. 70 (1), 19-27. https://doi.org/10.1016/S0167-1987(02)00116-2
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 173(2), 697-703. https://doi.org/10.1128/jb.173.2.697-703.1991