Biological Forum – An International Journal **13(4): 988-996(2021)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Plant Growth Promotion and Nif H Gene Amplification of Bacteria Isolated From Different Agro-Climatic Zones of Odisha

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(Received 22 September 2021, Accepted 20 November, 2021)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Indiscriminate use of chemical fertilizers has led to environmental contamination including deterioration of soil health and nitrogen status. Hence it is imperative to inculcate biological agents in order to reduce the ill effects of chemical fertilizer. Use of potent bacteria as bio-fertilizer can improve the soil nitrogen status leading to improved soil health and increased productivity. From the rhizospheric soil of different agro-climatic zones of Odisha, 63 bacteria were isolated and their plant growth promoting traits were studied. Sixteen potent bacteria showing maximum PGP traits were subjected to RT-PCR for nif H gene quantification to ascertain biological nitrification. Two isolates BS 2 and BS45 showing highest copy number increased growth performance viz. shoot length, root length, fresh & dry biomass and no. of leaves of green gram, black gram and pigeon pea. After the 16s r-DNA sequencing BS2 and BS45, were identified as *Klebsiella quasipneumoniae* and *Enterobacter roggenkampii* respectively.

Keywords: Acid Soil, Agroclimatic Zone, Growth of Pulses, Nif H Amplification, Plant Growth Promoting Traits.

INTRODUCTION

In order to maximize agricultural production under the prevailing climatic condition and available resources, need-based and location specific technology is required to be developed. For sustainable production, one of the steps is delineation of agro climatic zones based on soil physiography, climate, geological formation, cropping patterns, mineral resources etc. Agro climatic environment primarily refers to the soil types, rainfall, temperature and water availability which influence cultivation. It is a land unit in terms of major climates, suitable for a certain range of crops and cultivars (Hoda *et al.,* 2017). The temperature, humidity and soil type of the agro climatic zones determine the microbial diversity and their activity. In Odisha, moist and sub-humid climate with an optimum temperature is the climate which influences microbial activity including growth of diazotrophs and biological nitrogen fixation, which is an integral part of the soil ecosystem (Bohlool *et al.,* 1992; Burris *et al.,* 1993).

Odisha is known to grow and cultivate pulses with an annual production of 10.6 lakh tons, at the rate of 508 kg/h (RKBO, 2019). The major pulses grown in the state are green gram, black gram, pigeon pea etc. in symbiotic association with nitrogen fixing bacteria. The Nitrogen fixing efficacy of bacteria can be ascertained by determining the presence of nitrogenase enzyme (Hoffman *et al.,* 2014), where the potential activity, structural and functional data can be linked to the molecular measurements of functional nif gene abundance (Pogoreutz *et al.,* 2017).

The nif genes are the marker genes which encode primarily for the nitrogenase enzyme present in both free-living & symbiotic bacteria and can specify the effectiveness of the organism in the nitrogen fixation process (Lin *et al.,* 2021).

In addition to nif H, plant growth promoting traits can determine the potential of isolates in enhancing growth and crop yield (Pradhan and Mishra, 2015; Backer *et al.,* 2018) through various direct and indirect mechanisms viz.: potassium and phosphate solubilisation, nitrogen fixation, phytohormone & siderophore production, hydrolytic & antimicrobial enzymes biosynthesis, induced systemic resistance, antioxidative defense mechanisms etc. (Bhatacharyya *et al.*, 2012; Ahemed *et al.*, 2014; Goswami *et al.,* 2016; Islam *et al.,* 2016; Pahari *et al.,* 2017; Backer *et al.,* 2018). In view of this, the present study aims to evaluate the nitrogen fixing efficiency through nif H gene amplification of plant growth promoting bacteria isolated from the rhizospheric region of pulse crops

from different agro-climatic zones of Odisha and their effect on growth of different pulse crops.

MATERIALS AND METHODS

A. Collection of samples

Soil samples from the rhizospheric region of green gram, black gram and pigeon pea were collected aseptically from various pulse hubs situated in different agro-climatic zones of Odisha and subjected to physico-chemical analysis. The experiment was conducted during the year 2018-19, in the laboratory of Department of Microbiology, CBSH, OUAT, Bhubaneswar, Odisha, India.

B. Isolation and Screening of PGPR traits

Soil samples were serially diluted, cultured in Nutrient agar plates and incubated at 37°C for 48 hrs. A total of 63 bacteria were isolated and named as BS 1 to BS 63. The obtained rhizobacterial isolates were screened further for various plant growth promoting traits. Organisms with maximum plant growth promoting traits were taken for the further study.

C. Characterization and the identification of the isolates

Freshly cultured colonies of 16 isolates showing maximum PGP were subjected to gram reactivity and biochemical characterization by Bergey's manual of Determinative bacteriology (Holt *et al.,* 1994; Gupta *et al.,* 2000). The bacterial isolates were subjected to different pH ranging from 5-7 and temperature ranging from 15, 28, 37 and 50°C (Enebe and Babalola, 2018). They were also grown onto nutrient agar medium amended with 2% NaCl to study their salinity tolerance (Upadhyay and Singh, 2014).

D. Extraction and purification of DNA

The 16 bacterial isolates were subjected to DNA extraction. The cultures were inoculated in sterile luria bertani broth and incubated at 35 ± 2°C for 72 hrs and DNA was extracted using the bacterial DNA extraction kit (Sigma-Aldrich-GDI3 MSDS), quantified through Nano-3100 Analytical Spectrophotometer and kept at -80°C for further analysis.

E. Quantification of nifH gene copy number by Real-time PCR

NifH gene copy numbers were quantified and measured in Real-time polymerase chain reaction (RT-PCR) (Quant Studio® 5, Singapore) by the addition of an intercalating fluorescent dye SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan). Quantitative PCR (qPCR) protocol was carried out using universal primer sets (in 10 µM concentration) *nifH*-R (5 TTGTTSGCSGCRTACATSGCCATCAT3) and *nifH*-F (5 AAAGGYGGWATCGGYAARTCCACCAC3) (Kumar *et al.,* 2019; Rosch *et al.,* 2002).

Quality of plasmid DNA was quantified and evaluated through Nano-3100 (Analytical®) spectrophotometer and sequenced thereafter. To evaluate and assess the copy number of *nifH* gene, plasmid DNA was diluted and a standard was prepared ranging from 3×10^1 to 3×10^8 copies. The nif H copies were calculated from a standard constructed by plotting plasmid DNA concentrations versus quantification cycles which produced linear $R^2 > 0.97$ standard curve.

F. Effect of potential isolates on growth performances of pulses

Two organisms with maximum nif H gene copy numbers were taken to study their effect on growth performance of green gram, black gram and pigeon pea under pot culture. Three treatments viz., 1. Seed treatment with soil application of the bacterial culture (T1), 2. Seed treated with the bacterial culture (T2) and 3. Soil treated with the bacteria culture (T3). A control set was run with only garden soil only (C). Total 2kg of sterile soil was taken in each pot with soil: water 1:2 in triplicate. The level of water was marked and maintained. Seeds of black gram, green gram and pigeon pea were rinsed with 3% Sodium hypochlorite solution for 3minutes followed by repeated washing with sterile distilled water for about 5-7 times and soaked in 10mL of bacterial suspension (10^9 cfu/mL) amended with 2% Carboxy Methyl Cellulose CMC as an adhesive. Seeds treated with sterile nutrient broth containing CMC were taken as control. Three seeds were sown with 4-5cm depth of soil in each pot and watered. Growth parameters like root and shoot length, number of leaves, total biomass of the plants were observed.

G. Amplification and Evolutionary analysis of 16s r-DNA

The extracted DNA was sent to Eurofins Genomics India Pvt. Ltd. Bangalore, India, for 16s r-DNA sequencing. The quality of extracted DNA was evaluated on 1.2% gel using 1kb ladder. 16s r-DNA fragments were amplified and a single discrete PCR amplicon band resolved on the agarose gel. The amplified product was then purified in order to remove any contaminants. Forward and reverse DNA sequencing reaction was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730x1 Genetic Analyzer. Consensus of 16s r-DNA was analysed by Bio-Edit software v7.0.5.3. By using Clustal W v 1.6, the submitted 16s r-DNA sequence of the bacterial isolates were subjected to multiple sequence analysis. The phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987) of MEGA 6.06 (Molecular Evolutionary Genetics Analysis) software (Tamura *et al.,* 2013). Evaluation of the resultant phylogenetic tree topologies were done by bootstrap analysis of neighbor-joining data sets.

H. Statistical Analysis

The treatments were analysed by ANOVA at a significant level of 5% Tukey's HSD Honestly significant difference.

RESULTS AND DISCUSSION

A. Physico-chemical analysis of the soil samples

The physico-chemical parameters of the soil samples are depicted in Table 1. The pH of the soil was acidic and ranged between 5.77 to 6.61, redox potential (Eh) between 22.5-29.8 mV and Electrical Conductivity

0.039-0.088 dSm^{-1}. The TDS levels was reported between 18.92 – 47.5 ppm. The physico-chemical studies of the soil from different agroclimatic zones depict the soil pH ranging from 6.02 to 6.6 which is considered as acidic. Nayak and Mishra (2014) reported the acidic nature of Odisha soil, and isolated acidophilic bacteria with potential antimycotic activity against phytopathogens (Nayak and Mishra, 2012). With the Eh, EC and TDS in normal range, Dora Neina, (2019) opined that such acidic soil is suitable for crop production.

B. Isolation, identification and screening of plant growth promoting traits of the bacterial isolates

A total number of 63 bacteria were isolated from the rhizospheric region of black gram, green gram and pigeon pea form different agro-climatic zones of Odisha. Of the isolates, 16 showed production of IAA, 12 were phosphate solubilizers, 19 were positive for siderophore production, 59 isolates were positive for ammonia production and no isolate was positive for HCN production. Sixteen isolates exhibiting more PGP traits were further characterized for biochemical assays and also for the detection and quantification of nif H gene (Table 2).

Table 1: Physico-chemical analysis of the soil samples.

Soil sample	pH	Eh (mV)	EC (dSm^{-1})	TDS (ppm)
Boudh green gram	6.023	25.1	0.068	32.9
Ganjam black gram	5.775	29.8	0.039	18.92
Angul arhar	6.457	26.5	0.081	41.6
Boudh arhar	6.174	22.6	0.041	21.6
Angul black gram	6.617	22.5	0.088	47.5
Ganjam arhar	5.979	27.5	0.047	25.2
Ganjam green gram	6.065	22.5	0.087	29.7

Table 2: Plant growth promoting traits of the 16 potent rhizobacterial isolates.

Organism	Ammonia	IAA	PSB	HCN	Siderophore
BS 2	+	+	+	-	+
BS 7	+	-	+	-	+
BS 16	+	+	+	-	+
BS 17	+	+	-	-	+
BS 19	+	+	-	-	+
BS 24	+	+	+	-	+
BS 25	+	+	+	-	+
BS 26	+	-	+	-	+
BS 29	+	+	+	-	+
BS 37	+	+	-	-	+
BS 45	+	-	+	-	+
BS 46	+	+	-	-	+
BS 50	+	+	+	-	+
BS 51	+	-	+	-	+
BS 52	+	+	-	-	+
BS 53	+	+	+	-	+

-: Negative +: Positive

The isolates were screened for various plant growth promotion traits. Out of the 63, 16 isolates were IAA positive with BS 2, BS 16, BS 26 and BS 28 showing higher IAA production. Pradhan and Mishra, 2015, reported and quantified IAA production by bacteria isolated from acidic soil of Odisha. They further opined that the isolates increase germination percentage and growth variable of green gram, groundnut and rice.BS 7 showed maximum solubilization of Phosphate in the medium. Ngomle *et al.,* (2014) reported crop rhizospheric bacteria have the potential to solubilise phosphate. Pahari *et al.,* 2017 reported solubilization of phosphate by bacteria isolated from acidic soil; a plant growth promoting trait.

Siderophore are iron (metal) chelating proteins produced by certain bacteria at the rhizospheric region and supplement iron to the crop plant by preventing availability of iron to the soil born fungal pathogens (Loper and Henkels, 1997; Pradhan *et al.,* 2017). The 19 isolates, found positive for siderophore production, can be used to promote growth of crop plants as reported by Pahari *et al.*, (2017). Similarly, the isolates showing ammonia production can supplement nitrogen to the crop plant (Pahari et al, 2020).

From the biochemical characterization, the 16 potent isolates were identified to be the species of *Klebsiella* sp. and *Enterobacter* sp.

C. Morpho-physiological and biochemical characterization of the isolates

All the 16 bacteria were found to be gram negative rods in microscopic morphological study. They were subjected to pH tolerance ranging from 5-7, temperature at 15, 28, 37 and 50°C (Fig. 1 and 2) and salt tolerance at 2% NaCl. Out of the 16, twelve bacterial isolates were found to be resistant to 2% NaCl (Table 6) with optimum growth in pH 6 at 28°C. Biochemical assays such as catalase, oxidase, nitrate reduction, citrate and carbohydrate utilization etc were studied to ascertain the generic level identification of the isolates (Table 3). The isolates were studied for enzymatic activities and sugar utilization which is depicted in Table 4 and 5.

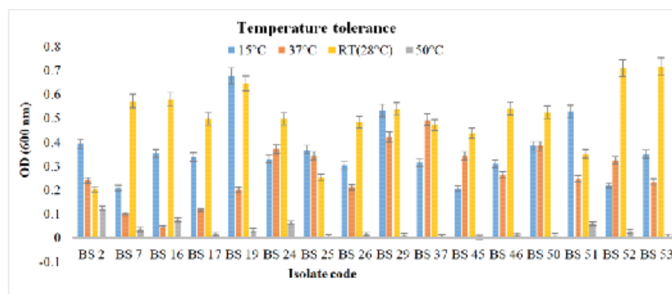

Fig. 1. Study of temperature tolerance of the bacterial isolates (Vertical bars represent Standard Error of the Mean values).

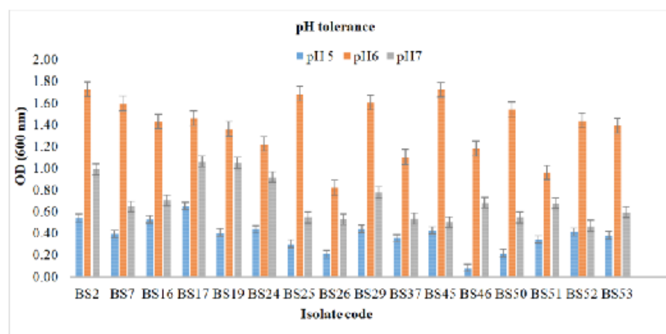

Fig. 2. Study of pH tolerance of the bacterial isolates (Vertical bars represent Standard Error of the Mean values).

Table 3: Morphological and biochemical analysis of the 16 potential plant growth promoting rhizobacterial isolates.

Organism	Gram staining	Methyl red	Vogues-Proskauer	Indole	Citrate utilisation	Nitrate reduction	ONPG	TSI Gas/ slant/ butt	TTC
BS 2	-ve rods	+	-	-	+	+	+	+/RY	+
BS 7	-ve rods	+	-	-	+	-	+	+/RY	+
BS 16	-ve rods	-	-	-	+	-	+	+/RY	+
BS 17	-ve rods	-	-	-	+	-	+	-/RY	+
BS 19	-ve rods	-	-	-	-	+	+	-/RY	-
BS 24	-ve rods	+	-	-	-	+	+	-/RY	+
BS 25	-ve rods	-	-	-	-	+	+	+/RY	+
BS 26	-ve rods	-	-	-	+	+	+	+/RY	+
BS 29	-ve rods	-	-	-	+	+	+	-/RY	+
BS 37	-ve rods	+	-	-	+	+	+	+/RY	+
BS 45	-ve rods	+	-	-	+	+	+	+/RY	+
BS 46	-ve rods	-	-	-	+	+	+	-/RY	+
BS 50	-ve rods	-	-	-	-	-	+	+/RY	-
BS 51	-ve rods	-	-	-	-	+	+	+/RY	+
BS 52	-ve rods	-	-	-	+	+	-	+/RY	+
BS 53	-ve rods	-	-	-	+	+	-	+/RY	+

-: negative; +: positive; R: red; Y: yellow

Table 4: Enzymatic analysis of the 16 potent plant growth promoting rhizobacterial isolates.

Organism	Oxidase	Catalase	Urease 24h/72h	Amylase	Lysine decarboxylase	Arginine decarboxylase	Gelatinase	Caseinase
BS 2	+	+	+/++	+	+	+	+	-
BS 7	-	+	-/++	+	+	+	-	-
BS 16	-	+	++/++	+	+	+	-	-
BS 17	-	+	-/++	+	-	+	+	-
BS 19	-	+	-/++	-	-	+	-	-
BS 24	-	+	-/+	-	-	+	-	-
BS 25	+	+	++/++	+	-	+	+	-
BS 26	+	+	-/++	-	+	+	+	-
BS 29	-	+	++/++	-	+	+	-	-
BS 37	-	+	-/+	+	-	+	-	-
BS 45	-	+	-/+	+	-	+	-	-
BS 46	-	+	-/++	-	-	+	-	-
BS 50	+	+	++/++	+	+	+	-	-
BS 51	+	+	-/++	+	+	+	+	-
BS 52	+	+	-/++	-	+	+	-	-
BS 53	+	+	++/++	-	+	+	-	-

-: negative +: positive ++: highly positive

Table 5: Sugar utilization of the 16 potent plant growth promoting rhizobacterial strains.

Organism	Glucose	Lactose	Sucrose	Maltose	Inositol	Dulcitol	Arabinose	Fructose	Dextrose	Mannitol
BS 2	+	+	+	+	+	+	+	+	+	+
BS 7	+	+	+	+	+	+	+	+	+	+
BS 16	+	+	+	+	+	+	+	+	+	+
BS 17	-	+	+	+	-	+	+	+	+	+
BS 19	+	-	+	+	+	+	+/gas	+	+	+
BS 24	-	+	+	+	+	+	+	+	+	+
BS 25	+	+	+	+	+	+	+	+	+	+
BS 26	+	+	+	+	+	+	+/gas	+	+	+
BS 29	-	+	+	+	-	+	+	+	+	+
BS 37	+	-	+	+	+	+	+	+	+	+
BS 45	+	+	+	+	+	+	+	+	+	+
BS 46	+	+	+	+	+	+	+	-	-	-
BS 50	+	+	+	+	+	+	+/gas	+	+	+
BS 51	+	+	+	+	+	+	+	+	+	+
BS 52	+	+	+	+	+	+	+/gas	+	+	+
BS 53	+	+	+	+	+	+	+/gas	+	+	+

-: negative +: positive gas: gas production observed

D. Absolute quantification of nif H gene

The nitrogen fixing ability of the isolated bacteria was assessed through a quantitative estimation of the nif H gene. The efficiency of fixing the atmospheric nitrogen can be determined by absolute quantification of nif H gene through qPCR wherein, maximum copy numbers indicated their efficiency for effective BNF (Kumar *et al.,* 2020). The two strains with highest copy numbers of nif H, BS2 and BS45 were found potent for efficient BNF and were selected for pot culture application.

Out of the 16, 5 isolates BS2, BS25, BS26, BS29 and BS45showed maximum nif H copy numbers. By plotting with best three points of plasmid DNA concentrations *versus* critical threshold (*Ct* mean), the gene copy number (nifH) per mL culture was calculated from a linear standard curve ($R^2 = 0.987$), where slope m= -2.382 (Fig. 3-5).

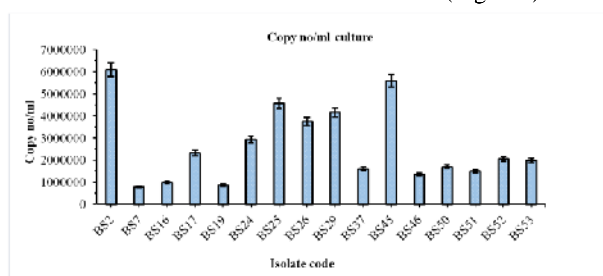

Fig. 3. nif H gene quantification in the 16 potent rhizobacterial isolates (Vertical bars represent Standard Error of the Mean values).

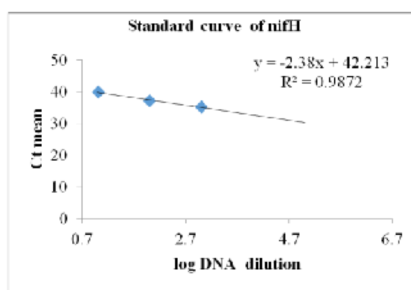

Fig. 4. Standard curve for nif H gene.

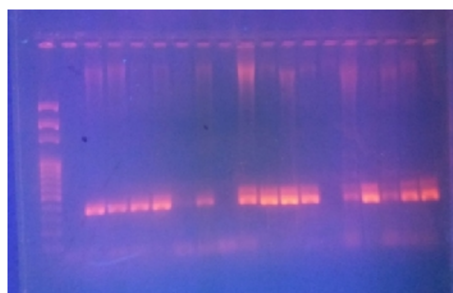

Fig. 5. nif H amplification and quantification.

E. Pot culture experiment

A significant improvement in the growth parameters and plant biomass was obtained after the bacterial inoculation. Three types of treatments were demonstrated viz., dual inoculation with seed and soil treated with the bacterial culture (T3), seed treated with the bacterial culture (T2) and soil treated with the bacteria culture (T3).With respect to control, treatment-1 (T1: soil + seed inoculation) in case of green gram, black gram and pigeon pea, depicted better results than the other treatments. With a significant increase in growth parameters, the dual inoculation technique where both, the seed and soil, are treated with the bacterial culture can apparently be practiced effectively. The combined PGPR attributes of the bacterial isolates which enhanced the plant growth by effective production of siderophores, phosphate solubilisation as well as IAA and other enzymes (Pereira *et al.,* 2016).The increased biomass, root and shoot length, nodulation efficacy and other growth parameters indicated the efficiency of the strains to help with the increment in yield and productivity.

Table 6: 2% NaCl salinity tolerance of the 16 potent plant growth promoting rhizobacteria.

Organism	Observation
BS 2	Resistant
BS 7	No growth/ sensitive
BS 16	Resistant
BS 17	Resistant
BS 19	Resistant
BS 24	Resistant
BS 25	Resistant
BS 26	Resistant
BS 29	Resistant
BS 37	No growth/sensitive
BS 45	Resistant
BS 46	Resistant
BS 50	Resistant
BS 51	Resistant
BS 52	Resistant
BS 53	Resistant

The organisms BS2, BS45 portraying maximum nif H gene expression were taken for pot experiment with green gram, black gram and pigeon pea. Effect of the isolates on their root length, shoot length, biomass of root and shoot and no. of leaves are presented in Table 7, 8 and 9. With respect to control, treatment-1 (T1: soil + seed inoculation) in case of green gram, black gram and pigeon pea, depicted better results than the other treatments. In green gram under T1, BS2 showed highest shoot length (+91.30%), root length (+115.23%), fresh weight (+186.86%) and dry weight (+146.42%) whereas BS45 showed maximum no. of leaves (+58.70%). In case of black gram under T1 shoot length (+76.23%), root length (+72.53%), fresh weight (+328.03%), dry weight (+422.9%) and no. of leaves (+98.36%) was maximum in case of BS2. The result of pot experiment in case of pigeon pea was also similar to others as under T1, BS2 depicted maximum shoot length (+33.25%), root length (+49.31%), fresh weight (+102.95%), dry weight (+42.30%)& no. of leaves (+76.14%)

F. Molecular identification and phylogenetic analysis of the isolates

Molecular identification of the two potent isolates: BS2 & BS45 was done by 16s r-RNA DNA sequencing.

Consensus sequence of 16s r-RNA was obtained from the forward and reverse sequence data with help of Aligner software, and further analysis was carried out through BLAST search tool of NCBI GenBank for identification of bacterial isolates.

From the molecular sequencing and multiple sample similarity tree the isolates BS2 & BS45 are identified as species of *Enterobacter roggenkampii* Ed-982 and *Klebsiella quasipneumoniae* KP18-31 respectively and the phylogenetic tree is given in Fig. 6. They are present either in the soil or as endophytic organisms and help in boosting the plant growth promotion through various mechanisms. Neilson *et al.,* (1976) showed the nitrogen fixing ability of the *Enterobacteriaceae* by estimation of acetylene reduction assay. An *et al*., (2017) isolated *Enterobacter* sp. from maize roots, which resulted in being an opportunistic endophytic diazotroph. Kreutzer *et al*., 1991 showed the genetic characterization of nitrogen fixation in *Enterobacter* strains isolated from the rhizospheric soil of cereals.

Streicher *et al.,* (1974) reported on the regulation of nitrogen fixation in *Klebsiella pneumoniae* depicting it as efficient for nitrogenase synthesis. *Enterobacter* has proven to be an efficient microbial consortium for enhancing the growth and production in wheat (Kumar *et al.,* 2014).

Table 7: Effect of bacterial strains of on the growth performance of green gram in Pot culture method (Values are mean of three samples & ± represent SEM. Values are highly significant at p<0.05 by ANOVA analysis).

Treatment	Strain	Shoot Length (in cm)	Root Length (in cm)	No. of Leaf	Fresh Weight (in gm)	Dry Weight (in gm)
No. of Treatment	Control	12.3±0.51	9.06±0.58	15.33±0.88	0.99±0.21	0.28±0.07
T1	BS2	23.53±3.32 (+91.30%)	19.5±1.65 (+115.23%)	20±1.73 (+30.46%)	2.84±0.19 (+186.86%)	0.69±0.06 (+146.42%)
	BS45	21.3±0.60 (+73.17%)	17.13±0.78 (+89.07%)	24.33±0.88 (+58.70%)	2.42±0.73 (+144.44%)	0.57±0.17 (+103.57%)
T2	BS2	20±0.87 (+62.60%)	16.5±0.98 (+82.11%)	22.66±0.88 (+47.8%)	2.32±0.72 (+134.34%)	0.60±0.14 (+114.28%)
	BS45	18.66±0.87 (+51.70%)	15.46±1.13 (+70.64%)	17.66±1.85 (+15.19%)	0.63±0.15 (-36.36%)	0.40±0.28 (+42.85%)
T3	BS2	17.96±0.39 (+46.01%)	15.06±0.08 (+66.22%)	10.33±0.88 (-32.61%)	0.91±0.17 (-8.08%)	0.27±0.06 (-3.57%)
	BS45	18.13±0.57 (+47.39%)	16.16±0.84 (+78.36%)	11±0.57 (-28.24%)	1.069±0.22 (+7.07%)	0.27±0.04 (-3.57%)

Table 8: Effect of bacterial strains on the growth performance of black gram in different treatment (Values are mean of three samples & ± represent SEM. Values are highly significant at $p<0.05$ by ANOVA analysis).

Treatment	Strain	Shoot Length (cm)	Root Length (cm)	No. of Leaf	Fresh Weight (gm)	Dry Weight (gm)
No. of Treatment	Control	17±0.79	14.2±0.83	18.3±1.76	2.64±0.53	0.96±0.24
T1	BS2	29.96±3.49 (+76.23%)	24.5±1.82 (+72.53%)	36.3±0.33 (+98.36%)	11.3±0.51 (+328.03%)	5.02±0.19 (+422.9%)
	BS45	29.8±0.80 (+75.29%)	22.5±0.72 (+58.45%)	31.3±4.05 (+71.03%)	8.09±2.14 (+206.4%)	4.00±1.41 (+316.6%)
T2	BS2	18.93±1.22 (+11.35%)	19.4±1.45 (+36.61%)	26.6±2.90 (+45.35%)	2.55±0.46 (-3.4%)	0.71±0.15 (-73.9%)
	BS45	25.83±0.98 (+51.94%)	19.76±1.02 (+39.15%)	26±3.60 (+42.07%)	5.50±0.63 (+108.3%)	2.16±0.32 (+125%)
T3	BS2	25.13±3.03 (+47.82%)	19.03±0.99 (+ 34.01%)	26.6±1.76 (+45.35%)	6.49±2.21 (+145.8%)	2.24±0.80 (+133.3%)
	BS45	27.93±0.37 (+64.2%)	20.13±1.14 (+41.76%)	25.3±0.66 (+38.25%)	6.28±0.79 (+137.8%)	1.79±0.42 (+86.4%)

Table 9: Effect of different bacterial strains on growth performance of Pigeon pea. (Values are mean of three samples & ± represent SEM. Values are highly significant at $p<0.05$ by ANOVA analysis).

Treatment	Strain	Shoot Length (in cm)	Root Length (in cm)	No. of Leaf	Fresh Weight (in gm)	Dry Weight (in gm)
	Control	16.96±0.02	5.13±0.34	7±0.57	0.169±0.0008	0.052±0.002
T1	**BS2**	22.6±1.25 (+33.25%)	7.66±0.28 (+49.31%)	12.33±0.88 (+76.14%)	0.343±0.035 (+102.95%)	0.074±0.003 (+42.30%)
	BS45	20.83±0.28 (+22.81%)	6.58±0.40 (+28.26%)	11.66±0.88 (+66.57%)	0.245±0.0008 (+44.97%)	0.069±0.005 (+32.69%)
T2	**BS2**	19.90±0.20 (+17.33%)	5.6±0.30 (+9.16%)	10.33±0.88 (+47.57%)	0.234±0.061 (+38.46%)	0.060±0.005 (+15.38%)
	BS45	18.93±1.03 (+11.61%)	4.23±0.14 (-17.54%)	6.66±1.46 (-4.85%)	0.194±0.004 (+14.7%)	0.055±0.018 (+5.76%)
T3	**BS2**	18.96±0.03 (+11.79%)	5.56±0.34 (+8.38%)	11±1.16 (+57.14%)	0.240±0.009 (+42.01%)	0.062±0.003 (+19.23%)
	BS45	15.0±0.11 (-11.55%)	6.30±0.37 (+22.80%)	5.66±1.20 (-19.14%)	0.193±0.005 (+14.20%)	0.056±0.004 (+7.69%)

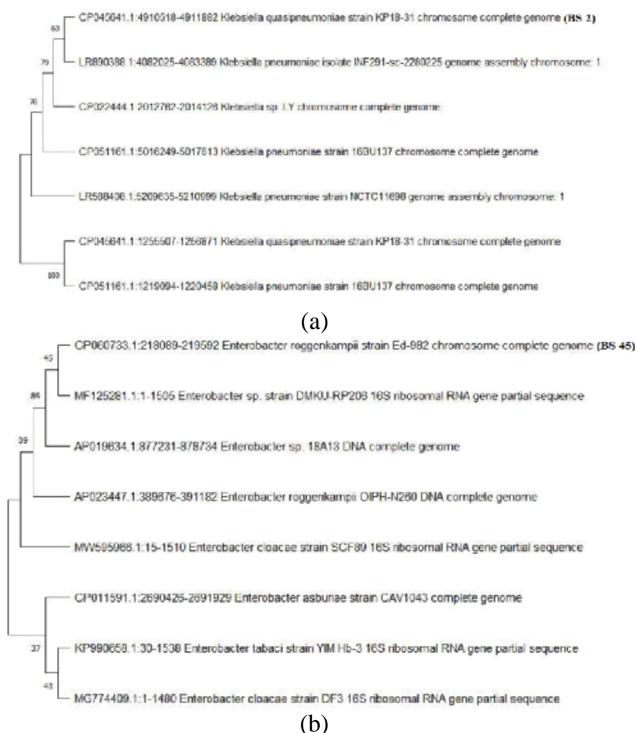

Fig. 6. Phylogenetic analysis of the bacterial isolate BS2(a) and BS45(b).

CONCLUSION

In this study, the role of *Enterobacter roggenkampii* and *Klebsiella quasipneumoniae* were found to be ameliorative under *in-vitro* pot culture applications where, a considerable enhancement of plant growth was observed in all the three legume crops. Therefore, the use of these plant growth promoting rhizobacterial strains as biofertilizer can be considered as a novel approach in replacing chemical fertilizers for attaining sustainable agriculture.

Acknowledgements. We sincerely acknowledge financial assistance by Department of Science and Technology, Government Of India through DST-Inspire Fellowship.

Conflict of Interest. There was no conflict of interest among the authors while contributing to the manuscript.

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How to cite this article: Priyadarshini, S.; Panda, A. and Mishra, B. B. (2021). Plant Growth Promotion and Nif H Gene Amplification of Bacteria Isolated From Different Agro-Climatic Zones of Odisha. *Biological Forum – An International Journal*, *13*(4): 988-996.