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Evaluation of Plant Growth Promoting Attributes and Characterization of *Micrococcus luteus* SNSr7 isolated from Spinach (*Spinacia oleracea* l.) Phyllosphere

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Abstract: We examined plant growth promoting properties of phyllospheric bacterial strain SNSr7 and characterized it. It was found to be gram positive *Micrococcus luteus* bacterial strain NH54PC02 and the sequences were submitted to NCBI (Accession No. KT340107). Maximum phosphate solubilisation was observed at 12 g/L of Ca_3PO_4 at 37 °C in 7 days and final pH was recorded as 3.26. Further, by maintaining the Ca_3PO_4 at constant concentration, maximum solubilization was observed around 160 µg mL^{-1} at 37 °C in 5 days with decrease in pH was 3.38. Quantitatively, 145.2 µg/mL of ammonia was produced by SNSr7. About 98 µg/mL IAA was produced by SNSr7 in 4 days at 37 °C. Beside this SNSr7 also showed promotive effect on *in vitro* seed germination of *Trigonella feonum-graecum* (Methi) and *Vigna radiata* (Mung bean) seeds. In this seed germination experiment we observed that root/shoot length, wet/dry weight and seedling height, % seed germination, vigour index was increased in comparison to untreated seeds. Along with this this bacterial strain's positive effect was also checked by using *Vigna radiata* and *Spinacia oleracea* seedlings in pot house experiment. Increase in growth parameters was also observed in these after treatment with SNSr7 like increase in chlorophyll content, no. pods (*V. radiata*), no. leaves (*S. oleracea*). The findings suggest that SNSr7 (*Micrococcus luteus*) appears to be a potential PGP bacterial strain for future use regarding sustainable agricultural practices.

KEYWORDS: Bio-inoculant, Biological Nitrogen Fixation, IAA, PGPB, Phyllosphere, Spinach

1. Introduction

Phyllosphere is the above surface of the plant including leaf surface, stem *etc.* except root surface. Along with rhizosphere, phyllosphere also includes various bacterial species which have important role in agriculture. The phyllospheric communities involve colonization, growth and survival of microorganisms, which is regulated by various environmental factors and physico-chemical properties of leaf. The phyllosphere has many features which help to make it as an excellent habitat. Bacterial communities endure on the phyllosphere of various plants include epiphytic on the plant surface and endophytic bacteria within plant tissue. Some of these are harmful while, others are beneficial for plants as they help in providing some additional nutrients and by competing with plant pathogens for space and nutrients. Various bacteria have been isolated from phyllosphere communities which degrade specific pollutants in culture, including various volatile organic detonators (Aken *et al.,* 2004; Saharan and Nehra, 2011) and some hydrocarbons (Ilori *et al.,* 2006). Plant growth promoting bacteria (PGPB) are the bacteria that inhabit on plant surface or around the plant surface (as rhizobacteria lie on root region or surface and some phyllospheric bacteria that lie on leaf surfaces) and help in plant growth and development by production and secretion of various types of regulatory chemicals. These bacteria assist this activity directly or indirectly for plant growth promotion. Direct plant growth promoting (PGP) mechanisms by bacteria may involve production of plant growth regulators such as auxins, cytokinins and gibberellins; suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase activity); biological nitrogen fixation and the assemblage of unavailable nutrients such as insoluble form of phosphorus and other mineral nutrients. Indirect mechanism is

performed by inhibiting the various pathogens by production of hydrolytic enzymes, antibiosis through inhibition of pathogen by producing hydrolytic enzymes or toxic compounds (Weyens *et al.,* 2009). Spinach is very important plant which is a dark green leafy, common dietary vegetable. It is mostly grown in the tropical regions of the world and is native to central and south-western Asia. It is considered as one of the very important functional foods due to its anti-cancer, antimutagenic, anti-inflammatory, antioxidant (flavonoids *i.e.,* glucuronides, acylated di-glycosides and acylated triglycosides of methylated molecules and derivatives of methylenedioxy as 6-oxygenated flavonols) and nutritional constituents (Aritomi and Kawasaki, 1984; Aritomi *et al*., 1985; Ferreres *et al.,* 1997; Gil *et al.,* 1999; Lomnitski *et al.,* 2000; Nyska *et al.,* 2001; Ren *et al.,* 2001). It helps in protection of central nervous system and also useful in treatment of various disorganization of cells and tissues *e.g.,* anaemia, insomnia, obesity, osteoporosis, tumours, neuritis and also effective in the treatment of kidney and liver disorders (Lomnitski *et al.,* 2003). Its tender, crispy, dark green leaves are used as ingredients in a variety of cuisines.

The PGP attributes are useful, because soil phosphate is present in insoluble form and is important for plant growth thus these PGPB helps in to make the easily availability of these insoluble phosphate molecules into soluble form for plants. IAA is very important PGP precursor molecules of auxin hormone. Bacteria form auxin during metabolism as secondary product. It helps in initiation of root, cell division, and cell enlargement (Salisbury, 1994). Other plant hormones like gibberellins and cytokinins *etc.,* are also helpful in plant growth by performing cell division, cell enlargement and tissue expansion. Ammonia helps in increasing the activity of plant for biological nitrogen fixation as this is used by plant to convert ammonia into nitrate or nitrite (available form for plant).

The available literature show that a lots of work has been done on rhizospheric bacteria but there are only few reports on phyllospheric bacteria as PGPB. Keeping these points in mind, in present investigation, we have selected the phyllosphere of spinach (*S. oleracea*) for the study of beneficial bacterial isolates exhibiting PGP attributes. The results obtained from the present investigation suggest that like Rhizobacteria, Phyllospheric bacteria may also exhibit PGP attributes. We isolated and characterized *Micrococcus luteus* SNSr7 from spinach phyllosphere and studied its role as PGPB for various properties including phosphate solubilization, IAA production, ammonia production, *in vitro* biological nitrogen fixation *etc*.

2. Materials and Methods

2.1. Sample Collection

Spinach leaves were collected from different regions of Haryana (Chika, Karnal, Kurukshetra and Shahabad fields) and Punjab (Kapiyal and Sangrur fields), in India. Fresh leaves were plucked, stored in sterile propylene bags and analysed for phyllospheric bacteria isolation within 24h (Ceballos *et al.,* 2012).

2.2. Isolation

The Spinach leaves were surface sterilized in sterile distilled water and then vortexed for 10-20 min. Leaf Samples were serially diluted and 100 µL of each dilution was plated on nutrient agar media. Total phyllospheric bacterial population was counted after 48h of incubation at 35-37 °C (Ceballos *et al.,* 2012).

2.3. Morphological and Biochemical Characterization

The bacterial isolates that showed best PGP properties were examined for their morphological features including colony size, elevation, shape, surface, colour and or pigmentation *etc.* Further, these were characterized using staining methods (Ashrafuzzaman *et al.,* 2009). Various biochemical tests like catalase activity, indole test, IMVIC test, Sugar fermentation, gelatin liquefaction, urease producing ability, Simmon citrate agar utilization and nitrate reduction were also performed using available standard methods (Dubey and Maheshwari, 2006).

2.4. Plant Growth Promotion Attributes

2.4.1. Phosphate solubilization

The phosphate solubilization capacity of the spinach phyllospheric bacterial strain was detected by plate assay using National Botanical Research Institute's phosphate (NBRIP) growth medium along with addition of bromo-thymol blue dye (Nautiyal, 1999). The plates containing Pikovskaya medium were streaked with bacterial culture and incubated at 35-37 °C for up to 7 days. The presence of clear zone around the microbial colonies was observed. The diameter of respective zone was measured and area of clear zone was calculated by the formula shown as

$$\text{Pi Solubilization efficiency} = \frac{\text{Solubilization diameter}}{\text{Colony diameter}} \times 100$$

$$\text{Phosphate solubilization index (SI*)} = \frac{\text{Colony diameter + Clearing zone}}{\text{Colony diameter}}$$

Quantitatively, phosphate solubilization was estimated in Pikovskaya's broth in 250 mL flasks for up to 4 days. Bacterial isolate that showed positive result for phosphate solubilisation (on basis of qualitative test) were inoculated into Pikovskaya's broth containing known amount of tri-calcium phosphate (Ca_3PO_4) or TCP and incubated at 35-37 °C. The concentration of the soluble phosphate was estimated after 24 h interval up to 10 days by stannous chloride ($SnCl_2.2H_2O$) method (Nautiyal, 1999). After every sample collection, culture broth was centrifuged. About 10 mL ammonium molybdate solution was added to 10 mL of clear supernatant by constantly shaking to remove CO_2. About 2 mL of distilled water was added. Then 0.25 mL of freshly prepared $SnCl_2$ solution was added and volume was made up to 50 mL with distilled water. The change in colour intensity was recorded at 600 nm and simultaneously change in pH of supernatant was recorded. Effect of various concentrations of TCP in the range of 2-20 g/L was also studied.

2.4.2. Ammonia Production

Freshly grown cultures were inoculated in 10 mL peptone water and incubated for 48-72 h at 35-37 ºC. Nessler's reagent (0.5 mL) was added and the test tubes were observed for the appearance of yellow to brown colour (Cappuccino, 2006).

Ammonia production was quantified by growing freshly grown cultures in peptone water for 2-5 days at 35-37 °C. The amount of ammonia in the supernatant was estimated by means of Nesslerization reaction. About 1 mL Nessler's reagent was added to 1 mL of supernatant and total volume was made 10 mL using distilled water. Development of brown to yellow colour was positive indication and O.D. was measured at 600 nm. The conc. of ammonia was calculated using standard curve of ammonium sulphate (Demutskaya and Kalinichenko, 2010).

2.4.3. Estimation of IAA Production

LB broth containing standard conc. of L-tryptophan was used to inoculate the bacterial culture at 35-37 ºC for 48 h-72 h. Further, it was centrifuged at 10,000 rpm for 15 min. About 2 mL of supernatant was mixed with 2 drops of O-phosphoric acid and 4 mL of the salkowski reagent (50 mL of 35% perchloric acid and 1 mL of 0.5M $FeCl_3$ solution) and allowed to stand for 0.5-2 h at room temperature and O.D. was taken at 530nm. Pink colour appearance after addition of the reagents indicated the IAA production (Brick *et al.,* 1991).

Various conc. of L-tryptophan (20-500 µg/mL) were used to find the effect of tryptophan conc. on IAA production. About 1 mL of supernatant was collected after centrifugation and mixed with 4 mL conc. of Salkowski reagent. The appearance of pink colour in test tubes indicated IAA production described by Gordon and Weber (1951). O.D. of the resultant pink colour was recorded after 30 min. at 530 nm and amount produced was calculated by using standard curve of IAA (Brick *et al.,* 1991).

2.4.4. *In-vitro* Biological nitrogen fixation (BNF)

Nitrogen free Jensen's media was prepared and bromothymol blue was added to it. The bacterial culture was then inoculated and incubated at 35-37 ºC for 24-48 h. Yellow zone was formed by the positive nitrogen fixer. Quantitatively, *in vitro* BNF was estimated by keeping the inoculum in nitrogen free broth medium with constant sucrose conc. and incubation at 35-37 ºC for 1-7 days. The sucrose percentage (%) was also varied from 2-10% in order to check the effect of sugar conc. on BNF and O.D. was taken at 630nm (Jensen, 1942). The BNF was calculated by following the formula:

$$\text{BNF (\%)} = \frac{\text{O.D. of Reference} - \text{O.D. of Inoculated culture}}{\text{O.D. of reference}} \times 100$$

2.4.5. Seed Germination Assay

Seeds of economically useful plants *i.e., Vigna radiata* (mung bean) and methi were collected and soaked in sodium hypochlorite solution for 5 min. and washed with sterile distilled water 3-5 times (Abdul and Anderson, 1973). About 10-12 seeds were treated with bacterial strain for 1 h and kept in 1% soft agar plate for 3-5 days in the dark at 30 ºC. Seeds without bacterial treatment were designated as control. Various parameters like seed germination (%), root length, shoot length, vigour index, wet and dry weight of seedlings after germination were reported.

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated} \times 100}{\text{Total No. of seeds}}$$

$$\text{Vigour index} = \text{Germination (\%)} \times \text{Seedling length (cm)}$$

2.5. Pot experiment

Pot house experiment was performed by treating the seeds with selected bacterial isolates having maximum PGP attributes. The seeds of *V. radiata* were sown into pots filled with sandy loamy soil. About 30 surface sterilized seeds were bacterized with 24 h old bacterial culture (10^8 CFU mL^{-1}). The seeds were treated with individual pure culture as well as consortium of different isolates. The seeds were treated with these combinations of bacterial isolates for 1 h and used for sowing in pots. The various parameters were recorded/calculated upto 14 days in case of *S. oleracea* and 30-40 days in *Vigna radiata.* During the experiment, the pots containing treated and un-treated seeds were irrigated with sterilized water every day.

2.6. Determination of Chlorophyll content

The chlorophyll content in the leaves of *Spinacia oleracea* as well as *Vigna radiata* was estimated. In each case, about 0.08 g of fresh leaves was taken and 10 mL acetone (90%) was added and kept at -4 °C overnight. About 1 mL of DMSO was added in it by continuous mixing for 2 min. and centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and the pellet was used. The process was repeated two times using 1 mL DMSO. O.D. was taken at 645 and 663 nm within 20 min. and DMSO was used as blank (Arnon *et al.,* 1949). The amount of chlorophyll was calculated as follows:

Chl a (mg L^{-1}) = $0.0127A_{663}$-$0.00269A_{645}$

Chl b (mg L^{-1}) = $0.0029A_{663}$-$0.00802A_{645}$

Total Chl (mg L^{-1}) = $0.0202A_{663}$+$0.00802A_{645}$

2.7. Statistical analysis

Results were expressed as mean (±) standard deviation (SD) was calculated by using SPSS software (version 16.00).

2.8. Molecular Characterization

DNA was isolated from each sample by using Biopure™ kit. 16S rRNA gene was amplified by PCR from DNA of SNSr7 bacterial isolate. The primers used for the amplification of 16S rRNA gene: F = AGAGTTTGATCHYGGYTYAG (where Y and H represent mixed base pair (Y=C/T, H= A/C/T); R = ACGGCTACCTTGTTACGACTT; PCR Conditions: 94 °C for 5 min (Initial denaturation); 35 cycles includes: 94 °C for 60 sec. (denaturation); 53 °C for 45 sec. (annealing); 68 °C for 90 sec. (extension); 68 °C for 10 min. (final extension)

1. Amplified PCR product was subjected to electrophoresis using Agarose gel (1%) in TAE buffer and visualized by staining with ethidium bromide.

2. PCR product was purified by washing with Sodium acetate and ethanol (70%) and eluted from the gel.

3. Forward and reverse sequencing reactions of PCR amplicon were carried out on ABI 3730XL sequencer to obtain the sequence.

4. The assembled DNA sequence was used to carry out BLAST with the nuclear database of NCBI.

3. Results

3.1. Isolation

About 200 bacterial isolates were obtained from spinach phyllosphere on nutrient agar media from 19 samples of Haryana and Punjab in India. Out of these, 15 isolates exhibited maximum PGP activities. Finally, the bacterial isolate SNSr7 having diverse PGP activities are discussed here in detail. Verma *et al.* (2014) had reported a total of 89 bacteria from wheat phyllosphere and studied the bacterial diversity and their PGP attribute and therefore favour our findings.

3.2. Morphological and Biochemical Information

The SNSr7 bacterial isolate showed creamish, spherical with raised surface and entire margin colonies (Fig. 1a). SNSr7 was observed to be gram positive micrococci (Fig.1b). Biochemical tests like catalase, indole, methyl red, sugar fermentation, citrate utilization were found positive.

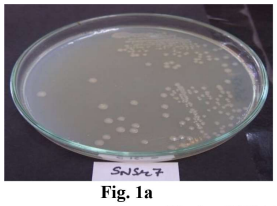


Fig. 1a

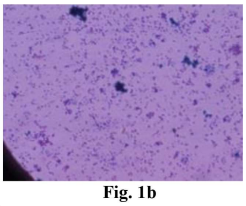


Fig. 1b

Fig 1a. SNSr7 on nutrient agar
Fig 1b. Gram Staining of SNSr7

3.3. Plant Growth Promoting Attributes:

3.3.1. Phosphate Solubilization

SNSr7 showed positive reaction for phosphate solubilization (Fig. 2a). The colony diameter was

recorded as 11.3 mm and zone size was observed to be 18 mm. The solubilization efficiency was reported as 159.29 and index was found to be 2.59.

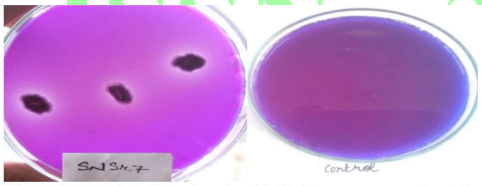


Fig. 2a Phosphate solubilization by SNSr7 as compared with control

It was observed that SNSr7 solubilized maximum of 150 µg mL^{-1} of phosphate at 12 g/L of Ca_3PO_4 and pH value was decreased up to 3.26 after 3 days at 37 °C (Table 1). We also estimated that the maximum of 160 µg mL^{-1} Ca_3PO_4 was solubilized after 5 days of incubation by *Micrococcus luteus* SNSr7 and decrease in pH was less *i.e.,* 3.38 (Fig. 2b). This decrease in pH may be due to the acidic environment (formation of certain organic acid) in pikovskaya broth during phosphate solubilization. Our results are sustained by findings of Yadav *et al.* (2011) and Goswami *et al.* (2015). Yadav *et al.* (2011) reported that maximum of 495 µg/L of phosphate was solubilized by *A. niger* and decrease in pH was recorded as 3.08. While Goswami *et al.* (2015) stated that *Pseudomonas aeruginosa* BS8 solubilized 13 mg/mL Ca_3PO_4 therefore favours our result outcomes. Mehta *et al.* (2013) also reported that *Bacillus circulans* CB7 solubilized maximum of 832 µg/L of phosphate that is also in accordance to our findings. The reports of Tahir *et al.* (2013) also supported our findings by conveying the description that *Azospirillum* strainWS-1 solubilized maximum of 218.1 µg/mL phosphate. The findings related to effect in pH during phosphate solubilization are supported by the earlier literature study of Farhat *et al.* (2015). They reported that phosphate solubilization leads to the production of some acidic compounds like gluconic, oxalic and citric acid *etc.* and thus pH of pikovskaya broth decreases.

Table 1: Estimation of phosphate solubilization efficiency along with Ca_3PO_4 conc. (g/L)

Ca_3PO_4 (g/L)	Amount of Phosphate Solubilized (µg mL^{-1}) (Mean ± S.D)	pH of PVK broth along with phosphate Solubilization (Mean ± S.D)
2	30±0.01	5.94±0.11
4	80±0.02	4.98±0.01
6	80±0.03	4.39±0.30
8	100±1.01	3.96±0.02
10	120±1.00	3.86±0.21
12	150±0.10	3.26±0.01
14	80±0.03	4.08±0.05
16	62±0.002	4.87±0.03
18	60±0.21	4.98±0.11
20	40±0.001	5.11±0.02

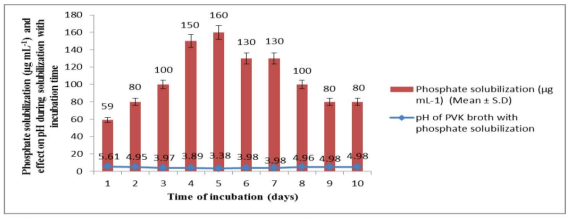


Fig. 2b. Phosphate solubilization by SNSr7 and change in pH of PVK after solubilization with time incubation

3.3.2. Ammonia Production

SNSr7 produced maximum of 145.2 µg/mL of ammonia after 5 days at 37 °C (Fig. 3a & 3b) and these are favoured by various earlier literature reports of ammonia production (Majeed *et al.*, 2015; Shahid *et al.*, 2012; Demutskaya and Kalinichenko, 2010). As ammonia is produced during nitrogen cycle and the useful PGPB breaks down nitrogenous materials (peptones) into ammonia which were then released into soil and taken up by plants as a nutrient. This leads to the improvement of plant growth and development. Our findings are supported by result outcomes of Goswami *et al.* (2015) who reported that maximum of 27 mg/mL of ammonia was produced by *Pseudomonas aeruginosa* strain BS8. Similarly, Dutta *et al.* (2015) reported that *Rhizobacteria* named *Pseudomonas aeruginosa* strain KH45 isolated from tea plants produced 4.9 µmol mL^{-1} of ammonia after 72h and significantly increased the biomass plants. Therefore, favours our findings as our result outcomes are also comparable to these findings.

Fig. 3a

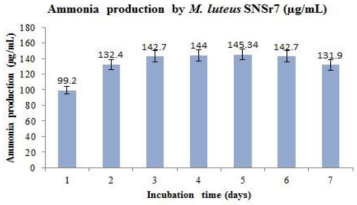


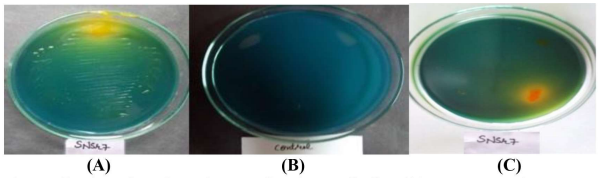
Fig. 3b

Fig 3a. Ammonia production by SNSr7

Fig. 3b. Quantitative estimation of ammonia production by SNSr7

3.3.3. *In vitro* biological nitrogen fixation (BNF)

In vitro biological nitrogen fixation by SNSr7 was confirmed by formation of yellow zone in nitrogen free Jensen's media. The amount of nitrogen fixation was estimated by recording the difference between the total colony size along with yellow zone and colony size. The size of zone was recorded as 19 mm (Fig. 4a).



(A) (B) (C)

Fig. 4a. ***In vitro*** **biological nitrogen fixation by SNSr7 (A) compared with control plate (B); C) showing positive BNF through yellow zone formation around colony**

Quantitatively, it was found that SNSr7 fixed maximum of 88.05% BNF after 3 days of incubation at 37 °C (Fig. 4b). Along with this we also observed that about 84.95% of *in vitro* biological nitrogen fixation by SNSr7 after utilizing 8% of sucrose (Fig. 4c) and these are in accordance to the earlier literature studies (Khan and Doty, 2009; Attar *et al.*, 2015; Majeed *et al.*, 2015). Khan and Doty (2009) reported that Spa bacterium is a good nitrogen fixer in comparison to *Azotobacter vinelandii* and *Rahnella aquatilis* as plant associated nitrogen fixer and therefore supported our result outcomes. Verma *et al.* (2016) also reported in favour of our findings that several bacterial strains like *Achromobacter, Alcaligenes, Bacillus, Delftia, Providencia, Pseudomonas, Rhodobacter, Salmonella, A. faecalis, B. subtilis, D. acidovorans* and *M. mesophilicum* showed *in vitro* BNF and bacterial strain IARI-NIAW2-29 fixed maximum of 87.5% *in vitro* BNF in wheat plant and therefore promote the growth of plant.

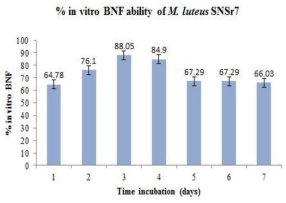


Fig. 4b

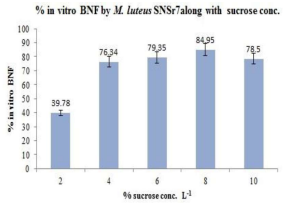


Fig 4c

Fig 4b. ***In vitro*** **BNF by** ***Micrococcus luteus*** **SNSr7**
Fig 4c. ***In vitro*** **BNF at different conc. of sucrose by SNSr7**

3.3.4. IAA production

IAA production ability of SNSr7 was confirmed by the formation of deep pink colour in LB broth due to utilization of L-tryptophan amino acid (Fig. 5a).

Fig. 5a IAA production (Deep pink)

About, 89 µg/mL of IAA was produced by SNSr7 after utilization of 200 µg/mL of L-tryptophan at 37 °C (Fig. 5b). Along with this, 98 µg/mL of IAA was produced after 4 days at 37 °C by keeping the L-tryptophan conc. constant (Fig. 5c). This IAA production is in accordance with the findings of Patil *et al.* (2011) who reported that *Acetobacter diazotrophicus* L1 produced maximum IAA at the 0.2-1.0 g/L of tryptophan conc. and then declined from 1.2-1.6 g/L of L-tryptophan and therefore favours our result outcomes. Majeed *et al.* (2015) also reported in accordance to our findings by stating that *Stenotrophomonas* sp. strain AJK-9 produced maximum of 77.98 µg/mL of IAA.

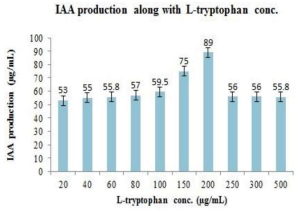


Fig. 5b

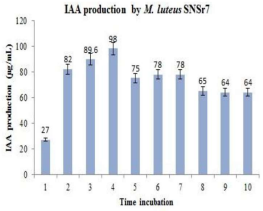


Fig. 5c

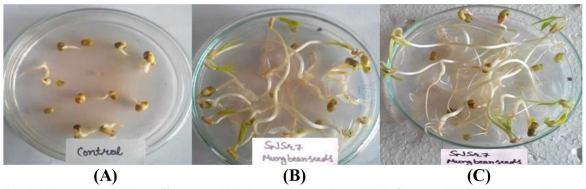
Fig 5b. IAA production with L-tryptophan conc.

Fig 5c. IAA production by SNSr7 with time incubation

3.3.5. *In vitro* Seed Germination assay

In present study, SNSr7 bacterial strain's plant growth promoting effect was checked on the seeds of *T. feonum-graecum* (methi) and *V. radiata* (mung bean) seeds. It was observed that bacterial treatments to the seeds had shown a stimulatory effect on all germination parameters like in vigor index, % germination, seedling height, wet/dry weight and root/shoot length in comparison to the untreated plants (Table 2). We found maximum % germination in mung bean (Fig. 6) and methi seeds (Fig. 7; Fig. 8) after treatment with *M. luteus* SNSr7, we observed that like PGPR this phyllospheric bacterial strain had also the positive effect in seed germination of plants as compared to control. Our findings are supported by the report of this seed germination effect of *M. luteus* SNSr7 was supported by Ashrafuzzaman *et al.* (2009). They stated that PGPR treated rice plant enhance the seed germination by 2.3-14.7% in comparison to control and other growth parameters also. The findings of Kefela *et al.* (2015) also favoured our result outcomes by reporting that *Bradyrhizobium japonicum* IRAT FA3,

Paenibacillus polymyxa and *Bacillus licheniformis* promote the seed germination rate up to 145% higher than the untreated control. The root/shoot length, seedling height, wet/dry weight and vigor index were also increased in SNSr7 treated mungi and methi seeds as compared to control. We also observed that during seed germination, mung bean and methi seeds that were not treated with SNSr7 (control seeds) suffered with fungal infection and growth was less as compared to the seeds treated with *M. luteus* SNSr7 strain.



(A) (B) (C)

Fig. 6 Untreated *V. radiata* seed (A) as control and *M. luteus* SNSr7 treated seeds (B) after 5 days of incubation while (C) showing growth of treated *V. radiata* seeds after 7days of incubation

Fig. 7 Methi (*Trigonella feonum-graecum*) seeds without *M. luteus* SNSr7 strain as control (A) and methi seeds (B) after 3 days of *M. luteus* SNSr7 treatment

(A) (B)

Fig 8. Methi seeds treated with *M. luteus* SNSr7 (A) and untreated seeds (B)

Table 2: Effect of bacterization on germination of seeds

Seed(s)	Germination %	Seedling height (cm)	Root length (cm)	Shoot length (cm)	Wet weight (g)	Dry weight (g)	Vigor index
M. luteus SNSr7 treated mung bean	97.22	18.5 ±2.40	8.97 ±1.20	11.62 ±1.00	0.318 ±0.03	0.016 ±0.00	1798
M. luteus SNSr7 untreated mung bean	70.22	2.1 ±1.60	0.3 ±0.61	0.8 ±0.68	0.122 ±0.05	0.046 ±0.06	147.46
M. luteus SNSr7 treated Methi	98.99	13.88 ±1.99	5.78 ±1.30	10.24 ±1.00	0.284 ±0.07	0.0141 ±0.00	1373
M. luteus SNSr7 untreated methi	69.11	2.1 ±1.00	0.5 ±0.34	1.1 ±0.64	0.07 ±0.04	0.011 ±0.009	145.13

3.3.5.1. Pot experiment

In vivo seed germination of *V. radiata* and *S. oleracea* seedling was done for the study of beneficial PGP effect of SNSr7 bacterial strain using pots experiment. We observed the significant increase in all these plant growth parameter like seedling height, root/shoot length (cm), wet/dry weight (g) and vigor index of these plants seedlings that were treated with SNSr7 as compared to untreated plants (Table 3; Table 4; Fig. 9a, b; Fig. 10a, b). It was implemented that 100% seed germination was recorded in mung bean and spinach plants after treatment with SNSr7 in comparison to untreated mung bean (60%) and untreated spinach plants (75%). Similarly, the seedling vigor index of treated mungi plants and spinach plants were also increased significantly in comparison to the vigor index of untreated plants. These result outcomes are supported by the findings of Babu *et al.* (2015) as they reported the significant increase in seed germination and seedling vigor of PGPR treated tomato seeds as compared to untreated seeds. Dasgupta *et al.* (2015) also reported in favour of our findings by describing the effect of three PGPR bacterial strains namely *Escherichia coli* DACG2+*Pseudomonas fluorescens* DACG3+*Burkholderia sp* DACG1 on the growth of Chickpea plant. Total chlorophyll content of SNSr7 treated mung bean and spinach leaves in the form of chl A and chl B was also observed to be high as compared to untreated leaves. That is favoured by findings of Kumar *et al.* (2015) who reported that the combined effect of *Pseudomonas*+*Rhizobium* could help to improve the chlorophyll contents (58.7±2.7) of plants that is used in the form of nutrient and also on growth of plants in comparison to control. The findings of Jain *et al.* (2015) also favoured our work by reporting the increase in plant height, total biomass, nodules, number of leaves and secondary roots, total chlorophyll and yield of plants treated with microbial consortia (*Pseudomonas aeruginosa* PJHU15, *Trichoderma harzianum*TNHU27 and *Bacillus subtilis* BHHU100) under green house in comparison to control and individual bacterial effect on growth of pea plants.

Fig. 9a

Fig. 9b

Fig. 9a. Improvement in growth of *Vigna radiata* plant after treatment with SNSr7 in pot

Fig. 9b. Improvement in growth of *Vigna radiata* plant after treatment with SNSr7 in comparison to control

Fig. 10a

Fig. 10b

Fig. 10a. Effect of SNSr7 treatment on the growth of *S. oleracea* plant in pot
Fig. 10b. Effect of SNSr7 treatment on the growth of *S. oleracea* plant

Table 3: Effect of SNSr7 treatment on *S. oleracea* seeds in pot experiment

SNSr7 treated Plant's seed	Seed germination (%)	Seedling height (cm)	Root length (cm)	Leaf length	No. of leaves	Wet weight (g)	Dry weight (g)	Vigour index
Treated *S. oleracea* seedlings	100^{A}	25.5 ± 1.06^{AA}	$12.5\pm0.03^{A-A}$	13.9 ± 0.28^{AC}	26 ± 1.15^{CA}	$8.7\pm0.05^{A-B}$	0.98 ± 0.01^{DA}	2550^{C-A}
Untreated Control plant	75	16.4±0.11	5.2±0.01	8.2±1.01	9.2±0.01	3.1±0.11	0.35±1.00	1230

Mean ± Standard deviation; *All values are (P ≤0.05) significant as compared to control; Same letters are not significantly different at 0.05 levels

Table 4: Effect of SNSr7 treatment on *V. radiata* seeds in pot experiment

Name of SNSr7 treated Plant's seed	Seed germination (%)	Seedling height (cm)	Root length (cm)	Shoot length (cm)	No. of pods	Total Wet weight (g)	Total Dry weight (g)	Vigour index
M. luteus SNSr7 treated *V. radiata*	100^{A}	38.95 ± 1.96^{C}	15.76 ± 0.63^{AA}	23.9 ± 0.71^{BA}	6 ± 0.05^{P}	$5.9\pm1.01^{C-A}$	1.57 ± 2.01^{EA}	3895^{A-A}
Control plant	60	24.2±0.91	5.1 ±0.61	17.9±0.01	2±0.1 1	3.6±0.21	0.87±1.01	1452

Mean ± Standard deviation; *All values are (P ≤0.05) significant as compared to control; Same letters are not significantly different at 0.05 levels

3.3.5.2. Estimation of chlorophyll content

The chlorophyll content in plant's leaves helps to increase in plant growth through photosynthesis. Chlorophyll content of the treated and untreated *S. oleracea* and *V. radiata* leaves were calculated by taking the O.D. at 645 nm and 663 nm for chl A/chl B and total chlorophyll content. It was observed that chlorophyll content of SNSr7 treated mungi and spinach plants was high in comparison to untreated control plants (Fig. 11). The findings of Poupin *et al.* (2013) supported our result outcomes by reporting the role of *Burkholderia phytofirmans* strain PsJN in the improvement of various growth parameters, chlorophyll content and therefore increase in yield of *Arabidopsis thaliana* plants in comparison to control.

The report of Jain *et al.* (2015) also favoured our findings by discussing the increase in chlorophyll content and other plant growth parameters of pea seedlings after treatment with *Trichoderma harzianum*TNHU27, *Pseudomonas aeruginosa* PJHU15 and *Bacillus subtilis* BHHU100 bacterial strains in comparison to the chlorophyll content in control plants.

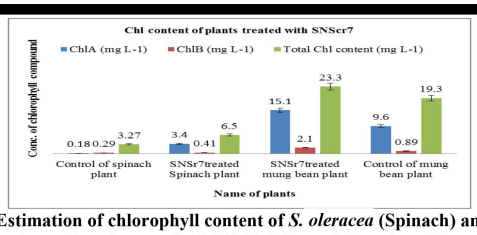


Fig. 11 Estimation of chlorophyll content of *S. oleracea* (Spinach) and *Vigna radiata* (mung bean) treated with SNSr7 bacterial strain and untreated

3.5. Molecular Characterization

Molecular sequencing of SNSr7 was performed after PCR- by using the DNA marker of 100 bp. PCR products were provided 500 bp 16S rRNA sequence. By performing molecular sequencing and matching with NCBI sequence it was found that SNSr7 showed maximum resemblance with *Micrococcus luteus* strain NH54PC02 having accession no. KT340107 when the sequences are submitted to NCBI. These 16S rRNA gene sequence were compared with other bacterial genome sequence using NCBI mega Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 5) and showed maximum resemblance with *M. luteus* SNSr7. The phylogenetic tree was also constructed with the help of MEGA 5.05 software (Fig. 12).

16S rRNA sequencing showed that SNSr7 is non-pathogenic bacterial strain having PGP activities by showing 100% similarity with *Micrococcus luteus* and promote seed germination.

Table 4: Identity of *Micrococcus luteus* SNSr7 with the sequence (submitted in NCBI)

Score	Expect	Identities	Gaps	Strand
1092 bits(591)	0.0	591/591(100%)	0/591(0%)	Plus/Plus

Inference

The sequence obtained shows 100% identity to the sequence of *Micrococcus luteus* strain NH54PC02.

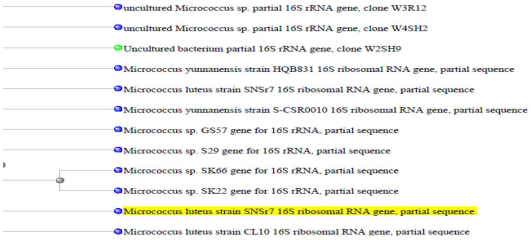


Fig. 12. Phylogenetic tree showing resemblance of SNSr7 with *Micrococcus* sp.

4. Conclusion

Our research findings suggest that SNSr7 (*Micrococcus luteus* NH54PC02) bacterial strain exhibit many plant growth promoting attributes therefore it can be used as potent bio-inoculant in future for sustainable agriculture. SNSr7 *Micrococcus luteus* bacterial strain NH54PC02 exhibit various properties like phosphate solubilization, IAA production, ammonia production, *in vitro* biological nitrogen fixation along with their positive effect in comparison to untreated plant as

discussed above using pot experiment. Therefore, this bacterial strain can be used as PGPB and applied in the form of bio-fertilizers in the fields and thus it can also replace the use of harmful chemical fertilizers.

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