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Isolation and Identification of N_2- Fixing, Phosphate and Potassium Solubilizing Rhizobacteria and Their Effect on Root Colonization of Wheat Plant

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Abstract: Nitrogen, phosphorus and potassium are known to be essential elements for plant growth. Thorough farming practices high yield require chemical fertilizers, which are not only expensive but may also produce environmental problems. The present study presenting the isolation and identification of rhizosphere bacteria with nitrogen fixing bacteria (NFB), phosphate solubilizing bacteria (PSB) and Potassium Solubilizing Bacteria (KSB) capacities from six different region in Egypt. A total of fifty two bacterial strains were isolated, characterized and classified to twenty two NFB, eighteen PSB and twelve KSB. Free NFB were tested for nitrogenase activity, the active NFB isolate 1N exhibited higher nitrogenase activity (330.2 n moles C_2H_4 /ml/h) followed by isolates no. 7N recording 209.6 nmoles C_2H_4/ml /h. Qualitative and quantitative methods were used to evaluate the phosphate and potassium solubilization effectiveness of the isolates. Results indicated that isolate no. 2P gave a large clear zone followed by isolates 12P. All of the eighteen PSB exhibited difference in their qualitative and quantitative efficiency to solubilize phosphate ranged from 65.9 to 220.2 % qualitatively; isolate no. 2P recorded 26.89 µg / ml after 14 days from incubation period, a slow decline in pH of medium was also observed from initial value of 6.21 to 5.10. All KSB isolates were able to K-solubilization mica supplemented to media as a sole source of K and formed variation of clear zones on Aleksandrow's agar media *in vitro*. Isolate no. K5 gave large clear zone, solubilizing efficiency ranged from 50.44 to 230.11%. The amount of K is increased with increasing the incubation period 4-5 days at 35°C. Isolate no. 5K gave highest amount of K recorded 54.14 µg/ml and pH decreased through incubation periods. The top isolates strains from NFB, PSB and KSB (1N, 2P and 5K) were identified using 16S rDNA gene sequence analyses. The similarity sequence analysis indicated that no. 1N, no. 2P and no. 5K isolates have 99% similar sequence identify to *Paenibacillus polymyxa*, *Bacillus nakamurai* and *Bacillus pacificus respectively*. The colonization pattern of those strains were characterized using wheat plants on pots experiments. Overall the results indicates that the effect of NPK bacteria for enhancing the growth and increased the Vigor index recorded 70.83. Providing a great potential of using NPK bacteria for sustainable growth of wheat plants.

Key words: Sustainable agriculture · Root colonization · Rhizosphere microbes · Wheat and PGPR

INTRODUCTION

Nitrogen is the most essential and restrictive nutrient for plant growth and a key subject of agriculture. Most studies show that nitrogen [1], phosphorus [2] and potassium [3] fertilizers add to solving the challenge the world in facing, feeding the human population. High yield production of agriculture was accompanied by an enormous increase in the application of nitrogen fertilizer. Biological Nitrogen Fixation is a natural practice whereby the atmospheric nitrogen is changed to ammonia by enzyme known as nitrogenase [4]. Nitrogen enters living organism through nitrogen fixation [5]. Diazotrophic bacteria are the referee of this process; it is the prokaryotic organisms, which have the capacity to fix atmospheric nitrogen [6]. Ninety genera of specialized

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microorganisms are identified to have the enzyme nitrogenase and fix atmospheric N_2 into NH_3. Three species of *Azotobacter*, *A. chroococcum, A. beijerinckii,* showed high growth, nitrogen fixation and *in vitro* production of phytohormones [7]. N_2-fixing could be main for plant nutrition by increasing N uptake by the plants and playing a significant role as plant growth promoting rhizobacteria (PGPR) in the biofertilization of crops [8] and reported as plant growth promoters [9].

Phosphorus (P) in insoluble compounds is unavailable to plant. In calcareous soils application as inorganic phosphorus is quickly converted into less available procedures by making a complex with Al or Fe in acid soils or with Ca^{2+}, though becomes unavailable to plant [10]. After nitrogen, Phosphorus (P) is the second main essential nutrient element for plants growth. Phosphorus is one of the main macronutrients which plays an important role in plants in many biological activities like, photosynthesis, development of good root system, respiration, energy storage and transfer, cell division, cell enlargement and also numerous other processes in the living plant [11]. Plants absorb less amounts from phosphatadic fertilizer and the rest is fast converted into insoluble like tricalcium phosphate [Ca3 $(PO_4)_2$], $FePO_4$ and $AlPO_4$ [12]. In environment, a diverse variety of microorganisms keep variation of different phosphates solubilization mechanisms [13]. Rhizosphere has been found as the extreme zone with microbial community compare with non-rhizosphere [14]. Some bacteria have variety of different phosphates solubilization mechanisms [15]. It have been found to control the property of mineralization and solubilization of soil organic and inorganic P [16, 17]. P-solubilizing bacteria are generally mentioned as plant-growth-promoting rhizobacteria (PGPR), it can effect on plant directly or indirectly, direct effects of (PGPR) bacteria are the induction of plant growth by solubilizing inorganic phosphate and synthesizing some vitamins and phytohormones such as auxins [18] while the indirect effects of PGPR are the inhibition and reduction of the toxic effects of pathogenic microorganisms by producing siderophores [19].

Potassium (K) is one the essential element for the plant growth, metabolism and progress of crop [20]. Potassium (K) is one of the key plant macronutrients inducing plant growth, increase and grain quality, it plays a key role in the synthesis of cells, enzymes, proteins, starch, cellulose and vitamins, also, K does not only play a part in nutrient moving and uptake, but also confers resistance to a biotic and biotic stresses, leading to improved production of quality crops and make available resistance to plant diseases [21, 22]. K is absorbed by plants in enormous quantity than any other mineral, synthetic K fertilizers are the largest available sources of K rhizosphere, thus, larger amounts of K fertilizers can be used to encourage the availability of K for plant uptake [23]. Soil microorganisms have played the main role in keeping Potassium balance in soil, this microorganism helps to change complex potassium current in soil into simple form and make them accessible to plants [24]. Some bacteria such as Bacillus, *Thiobacillus*, *Pseudomonas*, *Acidothiobacillus,* has been found to reduce and secret potassium from potassium-bearing complex minerals in soils [25]. Potassium solubilization efficiency depends upon the environment of bacteria and condition of the mineral where it is alive, so, the yield of the crops can be increased by adding biofertilizer keeping Potassium containing minerals along with K-solubilizing bacteria, therefore it is essential to identify microbial strains which shows to be helpful over damaged soil and reparation its quality along with good fertility of soil lessen the environmental pollution affected by application of chemical fertilizers. Colonization of wheat rhizosphere in addition to treated seedling of wheat with Plant growth promoting bacteria demonstrated its ability to live, adjust and recolonize the rhizosphere area [26]. The aims of this study were to isolate, identify and characterize the rhizosphere bacteria with the potential capacitate to fix nitrogen and to both solubilize phosphate and potassium. Moreover, to evaluate efficiency of bacterial to colonization seedling of wheat plants.

MATERIALS AND METHODS

Collocation of Rhizosphere Soil Samples: Six soil samples were collocated from different zones of Egypt such as Giza, Sahl El-Tina, Nubaria, Sahl El-Hussina-El-Sharkia Governorate, Menoufia and Ismailia Governorate of the rhizosphere of wheat, Alfalfa and pepper plants. The root system were collected in aseptic bags and carried to the laboratory for further studies. Rhizosphere soil was separated genially from the roots by vigorous shaking. The physicochemical properties of the soils were determined by using the methods previously described [27, 28] and particle size according to Piper [29].

Total Count of Bacteria, N_2-Fixer Bacteria, Phosphate and Potassium Solubilizing Bacteria: Total count of bacteria in rhizosphere was done by dilutions method plating on nutrient agar media [30], nitrogen fixing bacteria

were done on Glucose Mineral Media (NFGMM) as described by Zaw *et al.* [31]. Phosphate Solubilizing bacteria were done using Pikovskaya medium [32], also the total count of Potassium Solubilizing bacteria was estimated by using modified Aleksandrov medium [33] then the plates were incubated at 30°C for 48 hours.

Isolation and Purification of N_2- Fixing Bacteria: 5g soil samples were transferred to a conical flask containing 45ml of sterile water and then dilution was carried out from 10^{-1} to 10^{-7} prepared under aseptic condition, 0.1 ml of the suspension of each dilution was cultured on Nitrogen Free Glucose Mineral Media (NFGMM) as described by Zaw *et al.* [34] and the plates were incubated at 28 ± 2°C for 7days. After incubation period, representative bacterial colonies from nitrogen fixer's bacteria were biked up and re- inoculated in flask with equivalent enrichment medium [30], successfully growing isolates were transferred and stored in 35% glycerol (w/v) at -80°C until used it.

Isolation and Purification of Phosphate Solubilizing Bacteria (PSB): Serial dilation was made under aseptic condition to isolate PSP and Pikovskaya's Agar media [32] was using; plates were incubated at 30±5°C for 48-96h. Single colonies having clear solubilizing zones were isolated separately to a new Pikovskaya's agar plates.

Isolation and Purification of Potassium Solubilizing Bacteria KSB: 5g of the same soil samples were transferred to 45 mL of sterile distilled water, then shacked and heated at 75°C for 5 min. Suitable dilutions 10^{-1} to 10^{-6} was prepared under aseptic condition, modified solid Aleksandrov medium [33] with 0.5% mica powder obtained from (sigma Aldrich) and used to screen KSB in the soil. The Petri dishes were incubated at 27±2°C for 7 days. After incubation period, potassium solubilizing colonies were selected and pure colonies were transferred to sterile slants on nutrient agar medium.

Nitrogen Fixation: Nitrogenase activity was tested by acetylene reduction assay. Pure colonies from culture were inoculated individually into 100 ml of (Glucose Mineral Media (NFGMM)) broth. All of nitrogen fixing isolates was incubated at 30±2°C in shaking incubator for 3 days. Acetylene (10% v/v) was injected to the vials and incubated at 28±2°C for 16 h and 100 µL of gas samples from the vials were analyzed on a gas chromatograph according to protocol described by Hardy *et al.* [35] and Somasegaran and Hoben [36]. Three replicates of the experiment were done and mean was calculated.

Phosphate Solubilizing Effectiveness

Qualitative Assessment of Phosphate Solubilization: Phosphate solubilizing qualitative were done to all isolates by added spot inoculation of single bacterial colonies on the center of plates with Pikovskaya medium (PVK) counting insoluble tricalcium phosphate (TCP) is considered as a classical compound for measuring the potential or absolute rates of microbial solubilization of insoluble inorganic phosphate compounds, after incubation at 30±5°C for 48-96 h, a clear zone about colony was investigated as indicator for positive phosphate solubilization [37] then measuring the zone around the colony of growth and calculated the solubilization efficiency by the relation according to formula [38]:

Solubilization efficiency (% S.E) = Colony diameter + Halo zone diameter /Growth diameter × 100.

Quantitative Assessment of Phosphate Solubilization: Solubilization of P was quantified using PVK broth medium with (TCP). Flasks 100 mL containing 50 ml from PVK broth supplemented with (0.5ml of 10^8 CFU ml^{-1}) were incubated at orbital shaker 30°C for 3, 7 and14 days, Un inoculated flasks containing the same medium were conventional as the controls and then the culture was centrifuged at 1300rpm for 20 min. Then supernatant was used to measure the soluble P content calorimetrically as described by Jackson [39], pH was measured using a pH meter

Potassium Solubilizing Effectiveness

Potassium Solubilizing Ability of KSR: Modified Alexsandro's medium plus 0.5% mica powder as a sole source of K, was used to screen KSB bacteria at plates. After incubation periods 4-5 days at 35°C, clear zones were detected for isolates [40] we have measured the diameter of hale zone and growth to isolates at plates according to Khandeparkar's selection ratio.

Solubilization efficiency = Diameter of zone of clearance/ Diameter of growth × 100

Quantitative Assessment of Potassium Solubilization: Available K content of all isolates was measured by flame photometric methods according to Sugumaran and Janarthanam [41]. Three flasks for each isolate containing Modified Alexsandro's medium with o.5% from mica as a source of K, were incubated (0.5mL of 10^8 CFU ml^{-1}) at 150 rpm at 30°C for 3, 7 and 14 days, the control without inoculated bacteria. 50 mL from broth solutions were vortexed for 10 min to remove difficult materials, after that we centrifuged it at 10, 000 rpm for 10 min for separate the supernatant from growth cells and insoluble mica. Then K content was determined by flame photometry. pH was measured in broth media using a pH meter.

Molecular Identification: Three isolates of the bacteria that exhibited active nitrogen fixing bacteria (1N), best Phosphate (2P) and Potassium Solubilizing bacterial (5K) isolates respectively were selected to be identified by 16S rRNA gene sequencing, using PCR master mix (Promega, Madison, WI, USA) with bacterial universal primer sets 27F and 1492R (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R: 5'-TACGGYTACCTT GTTACGACT T-3'). Resolved 16S rRNA gene sequences were BLAST searched against the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database [42]. Multiple alignments of the nucleotide sequences were performed with the program MUSCLE [43]. The phylogenetic tree was constructed by the Neighbor-Joining method [44], based on the Kimura 2-parameter model (Kimura 1980), with bootstrap analysis (1, 000 replications) using the software MEGA (version 7) [45].

Germination Experiment: Three selected bacterial strains were verified of their efficacy on growth and colonization of wheat plants. Strains (1N, 2P and 5K) were grown individually on nutrient broth medium [30] with shaking on rotary shaker at 150 rpm for 48 hours (28±2°C). Cells were harvested by centrifugation at 6000 rpm for 15 min and bacterial cells were re-suspended in phosphate buffer (10 mM, pH=7.0). The cell density was adjusted to 10^8 cfu/ml. Seeds of wheat (*Triticum aestivum*) cultivar Giza 168, which obtained from field Crops. Research Inst., (ARC), Giza, Egypt, were placed in sandy pots. Pots capacity was 1 Kg sand with a diameter of 7 cm. 5 seeds per pot were in the sand aseptically and three replicates for each treatments. Seeds were coated individually for bacterial strain with isolates cells1×10^8 cfu per seed. Where the control was treated with broth medium only for each strain. After thinning the pots three seeding for each pot was left. Initially the pots were irrigated with water at 60% WHC. The treatments were following: 1- Inoculation with strain 1N (, 2- Inoculation with *2P*, 3- Inoculation with *5K*, 4-Inoculation with mixed of all strain (1N, 2P and 5K), 5- control, (non inoculated). After 30 days from sowing, growth parameters were determined as described by Gouzou *et al.* [46], like Shoot length, Root length, Shoot fresh weight, Root fresh weight, Shoot dry weight, Root dry weight were determined. Germination percentage was estimated. Vigor index was calculated by using the formula as described by Kharb *et al.* [47]:

Vigor index = Percent germination× Seedling length (shoot length + root length).

Statistical Analysis: Data were statistically analyzed using software; CoStat (CoHortSoftware, U.S.A) version 21. Comparison between mean values of treatments was made by least significant difference to test significant differences at $p \leq 0.05$.

RESULTS AND DISCUSSION

The NPK potential of Rhizobacteria isolated from rhizosphere of wheat, alfalfa, Pepper, Lupines and maize grown in the Giza, Sahl El-Tina, Nubaria, Sahl El-Hussina–El-Sharkia Governorate, Menoufia and Ismailia Governorate regions were examined and characterized . The beneficial effect of NPK in conserving sufficient levels of mineral nutrients NPK in crop production had been previously reported [48].

Characteristics of Rhizosphere Soil Samples: In the present study we collected six rhizospheric soil samples and determined the physicochemical properties of the soil, we noticed that the pH and organic matter have an effect on soil texture Soil samples were studied and classified in types as order Clay loam, Loamy sand, clay, Sandy clay loam, Clay loam and Sandy texture, (Table 1). The lowest value pH recorded with sandy soil (7.61) while highest pH recorded with Loamy sand (8.45). pH ranged from 7.61 to 8.45 with EC 1.7 to 20.38 ds/m. The variation in organic matter (OM%) had 0.43% in *Nubaria* regions while in Giza was 1.34 % and in Sahl El-Tina recorded 0.48%.Highst (OM%) recorded with soil obtained from Giza and Menoufia regions were 1.34 and 1.12% respectively, while lowest (O.M%) was recorded with Nubaria region 0.43%, these result are in harmony with Najm adeen *et al.* [49] who cleared that type soil clay loam had pH 6.6 and found that soil texture had a marked effect on the structure and

Table 1: Morphological characteristics of rhizosphere soil under investigated

	Host	Texture	pH (1:2.5)	EC dS/m	OM (%)	Geographic Coordinates
Giza	Wheat	Clay loam	7.9	1.95	1.34	30°01'13.6"N- 31°12'30.4"E
Sahl El-Tina	Alfaalfa	Loamy sand	8.45	10.86	0.48	31° 02' 16" N- 32° 35' 21" E
Sahl EL-Hussinia	Pepper Wheat	Clay	8.34	20.38	0.62	28°2.033' N- 1°39.578' E
Nubaria	Maize	Sandy clay loam	8.2	1.48	0.43	30° 43' 54" N- 30° 33' 01" E
Menoufia	Lupines	Clay loam	7.85	1.70	1.12	30.5972° N- 30.9876° E
Ismailia	Wheat	Sandy	7.61	2.15	0.52	30.5965° N- 32.2715° E

activity of microbial population, Soil pH is directly affected in growth of plant by limitation of nutrients supply and toxicity. Soil pH less than 5.5 led to increase Al^{3+} and Mn^{2+} concentration and induced reduction in plant growth due to the toxicity [50]. Organic matter binds soil particles into aggregates and increases the water holding capacity of soil; most soils contain 2 to 10% organic matter [51].

Enumeration of Total Biomass: Enumeration of total biomass of bacteria, nitrogen fixers, phosphate solubilizing and Potassium solubilization bacteria are shown in (Table 2). Data showed that in Menoufia region the total count of bacteria recorded highest number followed by Giza region recorded 3.49 and 3.18× 10^7 cfu respectively, while Sahl EL-Hussinia region recorded lowest total count compared with other regions being 22.7 × 10^7 cfu. Total count of nitrogen fixers bacteria recorded highest number with Giza region it was 17.2 × 10^6 cfu followed by Menoufia region recorded 15.7× 10^6 cfu and the total nitrogen fixers bacteria was in order 17.2 = 15.7 =12.8 = 12.2 =10.2 = 10.0 with Giza = Menoufia = Sahl El-Tina = Sahl EL-Hussinia = Nubaria = 10.0 cfu × 10^6 respectively. Highest total count from nitrogen fixer's bacteria recorded with Giza region in spite of the lowest total bacteria recorded with Ismailia region. The bacteria count of PSB and KSB isolates are presented in Table 2. The phosphate solubilizing bacteria (PSB) in rhizosphere soil of Menoufia region recorded highest count 11.8 cfu × 10^5 while Ismailia region recorded lowest count PSB 8.2 cfu × 10^5, PSB count were in order Menoufia = Sahl El-Tina = Sahl EL-Hussinia = Nubaria = Giza = Ismailia recorded 11.8, 11.3, 11.1, 9.2, 8.9, 8.2 cfu × 10^5 respectively. Potassium solubilization bacteria (KSB) recorded highest number also in Menoufia region 12.8 cfu× 10^5 while Sahl El-Tina region recorded lowest total count recorded 3.1 cfu× $10^{5.}$ Here too, soil had highest organic matter have higher number of bacteria, PSB and KSB compared to soil have lower organic matter. The highest organic matter in soil led to increase in total count of bacteria this result is similar with that obtained by Zhao *et al.* [52] and Patrick and Adeniyi [53] cleared that this comment may be as a result of the added nutrients (N, P, K and micronutrients) provided by the extracts analyzed by bacteria for plant to usage, this breakdown produce from plant in turn start to release much of exudates for use by the rhizosphere bacteria. Therefore, increase in rhizosphere bacterial count [54] also, the distribution of microorganisms are related to soil moisture and nutrient contents, Menoufia and Giza regions have higher number of bacteria than other regions, this due to textured clay loam soils type than coarse textured soils, sandy soils cannot hold water very well and drain fast. In contrast, clay loam protected water and catch nutrients for longer period of time, the diazotrophic population growth is highly correlated with sugars and amino acids from the root exudates [55].

Nitrogen Fixation: Acetylene Reduction Assay for twenty two isolates was subjected to ensure their ability to exhibit nitrogenase activity for isolates are shown in (Figure 1). Most of isolates are capable to nitrogenase activity except of (4N, N8, 10N, 13N and 14N). Isolate 1N exhibited higher nitrogenase activity 330.2 nmoles C_2H_4/ml /h followed by isolates 7N recorded 209.6 n moles C_2H_4/ml /h and isolate 12N recorded 202.1 nmoles C_2H_4/ml/h respectively. Lowest nitrogenase activity recorded with isolate No. N12 was 33.9 nmoles C_2H_4/ml /h. Isolate 1N recorded highest nitrogenase activity isolate so, it was selected to identify by 16SRNA and further study. Nitrogenase is a key display of the capacity of diazotroph which able to fix dinitrogen and discharge the nitrogen to soil and become accessible for the plant [56]. Nitrogenase enzyme is sensitive to the presence of O_2, it catalyzes the reduction of 2N to NH3 [57]. Nitrogen fixing bacteria are capable to fix nitrogen under high O_2 condition because of the respiratory, the protection nitrogenase enzymes is located in the cells, therefore the nitrogenase activity remains high [58], the nitrogenase activity of isolates was higher than the result obtained by Abo-Koura [59] where the highest nitrogenase activity recorded was 55.59 (µmoles C_2H_4/ml/h), in spite of the results were lower than obtained by Myongsu *et al.* [60] the highest nitrogenase activity was recorded as 3677.81 nmol/h/mg protein.

Table 2: Enumeration of total number of bacteria, N_2-fixing bacteria, phosphate solubilizing and Potassium solubilization bacteria in rhizosphere of soil samples investigated

Location	Total number of bacteria cfu × 10^7	N-fixing bacteria CFU × 10^6	Phosphate solubilizing bacteria cfu × 10^5	Potassium solubilization bacteria cfu × 10^5
Sahl El-Tina	29.8	12.8	11.3	3.1
Sahl EL-Hussinia	22.7	12.2	11.1	10.9
Nubaria	23.9	10.2	9.2	8.9
Menoufia	34.9	15.7	11.8	12.8
Giza	31.8	17.2	8.9	7.3
Ismailia	28.2	10.0	8.2	4.2

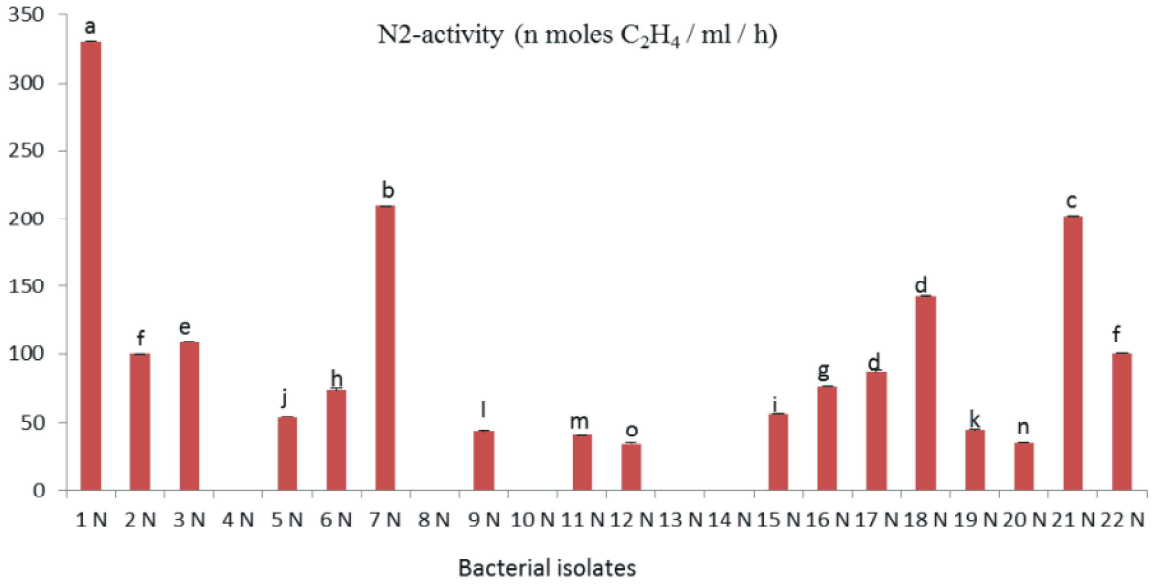

Fig. 1: Nitrogen fixation by isolates. Error bars represent the standard error of the means for three replicate

Qualitative Assessment of Phosphate Solubilization: Phosphate solubilization is measured to be most important point of plant growth promoting rhizobacteria. In this study a total of eighteen isolates from phosphate solubilizing bacteria obtained from rhizosphere of soil samples were screened for their ability to solubilize phosphate using PVK medium are shown in (Table 3). Most of isolates were able to solubilize tri-calcium phosphate (TCP) as source of phosphate. Isolates 2P (Fig. 2.) giving a large clear zone followed by isolates 5P, 8P, 10P, 11P, 12P, 13P , 16P and 17 P and highest percentage of P solubilization while isolate no. 6P, 7P, 14P and 15P giving a small clear zone but isolates 1P, 3P, 4P and 9P did not give any visible solubilization zone in PVK medium.. Isolate no. 2P gave a large clear zone with solubilization efficiency 220.2 % followed by isolate 12P giving 200.6 %. The solubilization efficiency of isolates is in order No. 2P, 12P, 8P, 13P, 11P and 10 Precorded 220.2 = 200.6 = 200.0 =190.9 =144.2 = 142.9 % respectively. Isolate 2No. P, 8P and 12P are categorized as high solubilizes while isolate No. 5P, 10P, 11P, 13P, 16P and17P are classified as medium solubilizers. Nutrients in soil are not available for plant root absorption due to the complex structures. Several bacterial species have been found to overcome the properties of phosphate solubilization between the rhizosphere microbes are higher in number with more might than non rhizosphere [61]. We used PVK medium to create clear halo zone of phosphate solubilization and as indicator of the decreases in pH of the medium due to the release of organic acids during the phosphate solubilization activity of the isolates [62]. The variances in clear zone between isolates are probable because the qualitative nature of method , it depends on the phosphate solubilization and the colony of bacteria over the bottom of the Petri dish. Similar results have been reported by Nautiyal *et al.* [63] reported that many isolates show clear zone in qualitative methods while other results obtained by Baig *et al.* [64] cleared that many microorganisms did not show any clear zone in qualitative method on agar plate assay.

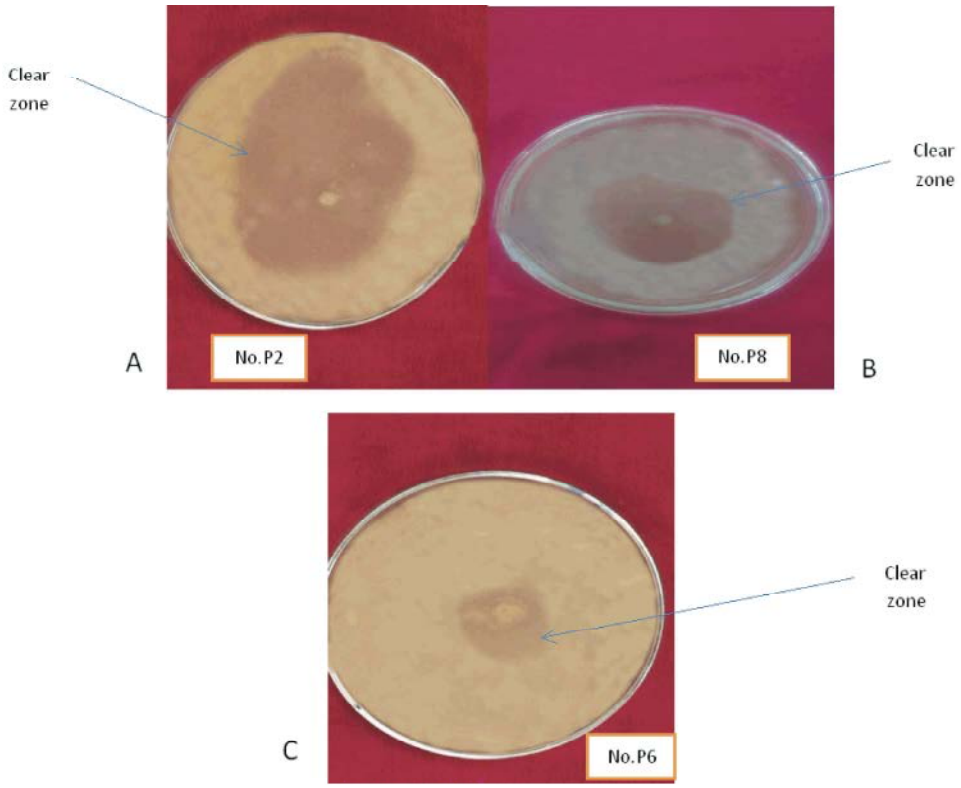

Fig. 2: Clear Zone around the growth of strains No.P2 , P 8 and P 6 on PKV medium as indictor phosphate solubilization

Table 3: Screening isolates for phosphate solubilizing

Bacterial isolates	Phosphate production	Solubilization efficiency (E)
1P	-	-
2P	+++	220.2
3P	-	77.9
4P	-	-
5P	++	100.9
6P	+	80.0
7P	+	76.9
8P	+++	200.0
9P	-	-
10P	++	142.9
11P	++	144.2
12P	+++	200.6
13P	++	190.9
14P	+	65.9
15P	+	66.9
16P	++	101.1
17P	++	100.1
18P	+	77.3

Clearance index: Good growth +++, medium growth, ++, low growth+, negative growth -

Quantitative Assessment of Phosphate Solubilization: All of isolates after qualitatively examined were further screened for their quantitative possible to solubilize the phosphate after 3, 7 and 14 days (Table 4) All isolates showed variation in their possible to phosphate solubilization ranging between 1.78 to 11.07 µg/ml after 3 days, 4.31 to 16.07 µg/ml after 7 days and 7.80 to 26.89 µg/ml after 14days. The highest amount of phosphate recorded with isolate 2P was 11.07 µg/ml at 3th from incubation while the lowest amount recorded with

Table 4: Quantitative valuation of Phosphate Solubilization (µg/ ml) after 3, 7 and 14 days with pH changes exhibited by the Phosphate Solubilizing bacteria

	Incubation periods					
	3 days		7 days		14 days	
Bacterial isolates	P Solubilized µg / ml	pH	P Solubilized µg/ml	pH	P Solubilized µg /ml	pH
1P	1.78^{j}	6.41	4.31^{i}	5.90	7.80^{j}	5.41
2P	11.07^{a}	6.21	15.07^{b}	5.81	26.89^{a}	5.10
3P	8.89^{c}	6.31	12.67^{cde}	5.90	19.04^{fg}	5.41
4P	7.69^{d}	6.29	11.19^{fg}	5.70	18.33	5.40
5P	11.03^{a}	6.11	16.07^{a}	5.31	26.04^{a}	4.90
6P	7.53^{d}	6.81	14.07^{c}	5.70	19.88^{ef}	5.33
7P	5.53^{g}	6.61	12.16^{def}	5.45	18.27^{g}	5.22
8P	8.04^{d}	6.45	12.80^{cde}	5.76	21.37^{cd}	5.10
9P	2.3^{i}	6.66	10.17^{g}	5.80	18.94^{fg}	5.20
10P	9.04^{c}	6.81	13.67^{c}	5.60	24.97^{b}	5.42
11P	10.16^{b}	6.40	13.50^{cd}	5.55	22.26^{c}	5.33
12P	9.90^{b}	6.89	12.70^{cde}	5.60	20.53^{de}	5.30
13P	5.97^{f}	6.28	11.16^{fg}	5.40	19.03^{fg}	5.20
14P	5.36^{g}	6.60	12.00^{ef}	5.33	18.00^{g}	5.20
15P	8.08^{d}	6.40	13.47^{cd}	5.20	21.44^{cd}	5.10
16P	4.83^{h}	6.30	10.10^{g}	5.44	19.49^{efg}	5.33
17P	6.13^{f}	6.37	8.74^{h}	5.39	16.41^{h}	5.28
18P	7.01^{e}	6.78	10.13^{g}	5.51	14.02^{i}	5.31
Control	-	7.01	-	7.01	-	7.01

isolate No. 1P was 1.78 µg ml at 3th. Generally, quantitative phosphate solubilization increased by increasing time of the incubation periods while pH decreased gradually with increasing the incubation period especially with isolate No. 5P compared to control the pH was 7. A decrease in pH value of the isolate No. 2P from 6.21 to 5.10 up to the 14th from incubation period. There was significant difference in the solubilization of phosphate of all selected isolates during the whole period of incubation. These results are harmony with Mini et *al.* [65], they found quantitative phosphate solubilization were 54.01 to 139.12 µg /l and 52.03 to 118.79 µg /l. There was significant difference in the solubilization of phosphate of all selected isolates during the whole period of incubation. There is converse relationship between pH value and soluble-Phosphate in media, may be because indirect production of organic acid by bacterial strains an important part in the acidification of the medium causing the solubilization of Phosphate. The results on the decreased of pH level from early value of 6.21 to 5.81 to 5.10 due to the acidification of supernatant's culture [66] and the organization of phosphate solubilization with acidification of broth media can be established [67-69].

From the result study it shows that bacterial isolate no 2P showed larger halo zone producing and higher quantitative of Phosphate was selected for identification and further study.

Potassium Production and Efficiency: Data in (Table 5) showed the potassium production and solubilizing efficiency (E) for twelve bacterial isolates screened for K solubilizing ability. Aleksandrov medium added mica powder as a sole source of K was used to screen K-solubilizing rhizobacteria. Most of bacteria isolates are able to solubilizing K and formed variation of clear zones on Alekandrove's agar media. Isolate No.K5 (Fig. 3) showed largest zone followed by isolate No. 3K, 7K, 8K and No. 10K.These halo clear halo zones around the bacterial growth were considered as potassium solubilizing. Isolate No. 1K, 2K, 4K, 6K, 9K, 11K and No. 12K showed lower clear zone around the growth of bacteria. Solubilizing efficiency (E) ranged from 50.44 to 230.11. Isolate No.K5 showed most obvious ability to solubilize K recorded 230.11 followed by isolate No.K3 recorded 190.12 respectively. However, isolate No.K9 solubilized least amount of K as detected by weak zone of 50.44 compared with other isolates. Several studies reported that rhizosphere soil contains a variety of solubilizing K as also found out in our study [70]. These results are in agreement with the outcomes of Parmar and Sindhu [33] who reported that microorganism's helps solubilization of unsoluble metal compounds in soil by protons, chelate ligands, enzymes, organic acids and by oxide reduction nutrients such as potassium systems. Twelve K-solubilization rhizobacteria were isolated from

Table 5: Potassium solubilization value of bacterial isolates

Bacterial isolates	Potassium production	Solubilization efficiency (E)
1K	+	100.70
2K	+	92.30
3K	++	190.12
4K	+	60.80
5K	+++	230.11
6K	+	72.89
7K	++	143.81
8K	++	133.12
9K	+	50.44
10K	++	120.71
11K	+	68.41
12K	+	78.90

Clearance index: Good growth +++, medium growth, ++, low growth+

Fig. 3: Potassium solubilizing is indicated by clear zone around the colony grown in Alekandrove's agar media

rhizosphere of common Kharif crops (maize, banana, sugarcane, potato, pigeon pea and tobacco) based and tested on their ability to solubilize waste mica (muscovite and biotite) in plate assay. All these KSR were capable of K- solubilization from mica in both solid and liquid medium *In vitro* [71].

Quantitative Assessment of Potassium Solubilization: Amount of K released from twelve isolates were studied at 3, 7 and 14 days after incubation period are presented in (Table 6). The amount of K is increased with increased the incubation period. Isolate No. K5 gave highest amount of K recorded 18.22, 30.22 and 54.14 µg mL^{-1} respectively after 3, 7 and 14 days from incubation period over all other isolates. The amount of K ranged from 10.04 µg mL^{-1} to 54.14 µg mL^{-1} after 14 days. Whereas, isolate 1K giving lowest amount of K recorded 4.18, 7.67 and 10.04 µgmL^{-1} respectively after 3, 7 and 14 days from incubation period. Available of twelve isolates tested two isolates No. K1 and No. 2K showed lower amount 4.18 and 5.17 µgml^{-1} respectively of potassium solubilizing ability. Some studies have shown that rhizosphere has a diversity of K Solubilizing rhizobacteria for example also found out in our results [72]. Also [73] found that *Bacillus megatherium* and *B. mucilaginosus* were able to solubilizing rock potassium, also *Bacillus* spp were possible in solubilizing potassium rocks, it was active in solubilizing the potassium compared to *Pseudomonas* spp. (9.09 µgml^{-1}) [74] also reported that maximum amount of K from *Bacillus* strain was (42.95 µgml^{-1}). Results cleared that there are reduced in pH during incubation period compared to control, this may be because of production of H^+ during the hydrolysis of added mica [75] found very little change in pH of mineral added broth with increase in incubation periods. pH value for all isolates decreased with increasing incubation period. Lower pH was recorded with isolate No. 5 recorded 5.70 and isolate No.4, 10 and 12 recorded 5.80 for isolates. Reduction in pH may be because of production of different types of organic and inorganic acids by K-solubilizes [76]. This declaration is in harmony to the results of some workers who have reported that

Table 6: Potassium solubilization activity (µg mL^{-1}) of KSB isolates in liquid medium after 3, 7 and 14 days with pH dynamics

	Incubation periods					
Bacterial isolates	3 days	pH	7 days	pH	14 days	pH
1K	4.18^{k}	6.90	7.67^{g}	6.77	10.04^{i}	6.07
2K	5.17^{j}	7.01	11.78^{f}	6.88	19.88^{h}	6.60
3K	12.34^{d}	6.88	15.84^{e}	6.55	20.01^{h}	6.00
4K	12.78^{c}	6.33	20.29^{d}	6.20	33.20^{f}	5.80
5K	18.22^{a}	6.60	30.22^{b}	6.33	54.14^{a}	5.70
6K	9.10^{g}	6.90	16.97^{e}	6.33	40.86^{d}	6.12
7K	15.37^{b}	6.80	31.70^{a}	6.31	39.85^{de}	5.80
8K	10.17^{f}	6.44	20.70^{d}	6.21	43.50^{c}	5.82
9K	11.67^{e}	6.77	21.58^{d}	6.55	48.51^{b}	6.33
10K	8.50^{h}	6.55	11.20^{f}	6.30	29.94^{g}	5.80
11K	7.14^{i}	6.80	16.14^{e}	6.22	38.74^{e}	5.88
12K	7.11^{i}	6.44	23.14^{c}	6.10	39.84^{de}	5.80

Table 7: Effect of inoculation with *Paenibacillus polymyxa, B. nakamurai* and *B. pacificus* on growth parameters (cm), vigor index and colonization of wheat

Treatment	SL	RL	SFW	RFW	SDW	RDW	Germination (%)	Vigor index	Colonization
P. polymyxa MSR H5	50.06^{b}	22.43^{b}	7.10^{b}	1.92^{a}	2.20^{b}	0.90^{a}	89	64.51	2.0×10^{7}
B. nakamurai MSR H1	43.70^{c}	22.70^{b}	5.26^{d}	1.61^{b}	1.36^{d}	0.56^{b}	87	57.86	1.4×10^{7}
B. pacificus MSR H3	48.90^{b}	21.73^{b}	6.43^{c}	1.50^{b}	1.83^{c}	0.66^{b}	89	62.86	1.5×10^{7}
Mixed of 3 isolates	53.47^{a}	25.23^{a}	7.60^{a}	1.99^{a}	2.79^{a}	0.95^{a}	90	70.83	2.2×10^{7}
Control	30.34 ^{d}	12.1 ^{c}	3.3 ^{e}	1.00 ^{c}	0.97 ^{e}	0.34 ^{c}	60	25.47	n.d

SL=Shoot length, RL=Root length, SFW= Shoot fresh weight, RFW= Root fresh weight, SDW= Shoot dry weight, RDW= Root dry weight, *n.d.* not detected

K-solubilizers produce mono-, di- and tri-organic acids i.e., gluconic, acetic, oxalic, fumaric, tartaric and citric, which caused in reducing the pH of the consumed medium [77, 78]. K- solubilizes isolates also might have produced several kinds of organic acids which probably destroyed mica construction to content their Si^{+} 4 and K^{+} supplies bringing them into solution, thus lowering the pH of the inoculated broth media [79].

From the result study it shows that bacterial isolate No. 5K showed larger halo zone producing and higher quantitative of potassium was selected for identification and further study.

Identification of the Rhizobacteria: According to the preceding results we select the best active three isolates from above results that have highest nitrogenase activity 1N, largest qualitative and quantitative assay from PSB and KSB bacteria (2P, 5K) respectively by 16S rRNA sequences technique and blast search in data base (Fig.4). 1N strain had the best hit with *Paenibacillus polymyxa* (99%) and we renamed as MSR H5, while the 2P show a high sequence identity with *Bacillus nakamurai* (99%) and the strain named as MSR H1 and finally the 5K showed a high similarity with identity with *Bacillus pacificus* (99%) and re names as MSR H3.

Assessment Effect of Selected Three Strains on Wheat Plant Growth: *Paenibacillus polymyxa* MSR H5*, B. nakamurai* MSR H1 and *B. pacificus* MSR H3 were tested as a bio fertilizer for wheat plant in Table 7. All isolates stimulated significant increases the seed germination especially mixed with NPK bacteria. Highest growth parameters recorded with inoculation with mixed strains, germination percentage were 90% and Vigor index recorded 70.83, colonization with mixed strains recorded 2.2×10^{7}, followed by inoculation with *P. polymyxa* recorded 89% in germination percentage, Vigor index was 64.51and colonization was 2×10^{7}. The lowest growth parameters recorded with inoculation *B. nakamurai* was 43.70 cm in SFW, vigor index recorded 57.86 and colonization was 1.4 $\times10^{7}$. All of strains give positive effect on growth parameters and improvement the colonization in the rhizosphere of soil wheat compared with un-inoculated wheat. Wheat seed inoculation with all of strains has a positive effect on root growth. Highest root length of 25.23 cm obtained with inoculation mixed of all strains followed by 22.70 cm obtained with inoculation with *B. nakamurai* followed by 22.43 cm obtained with inoculation with *P. polymyxa* and 21.73cm obtained with inoculation with *B. pacificus*. N.P.K bacteria succeed to establish and colonize the roots of plant and led to

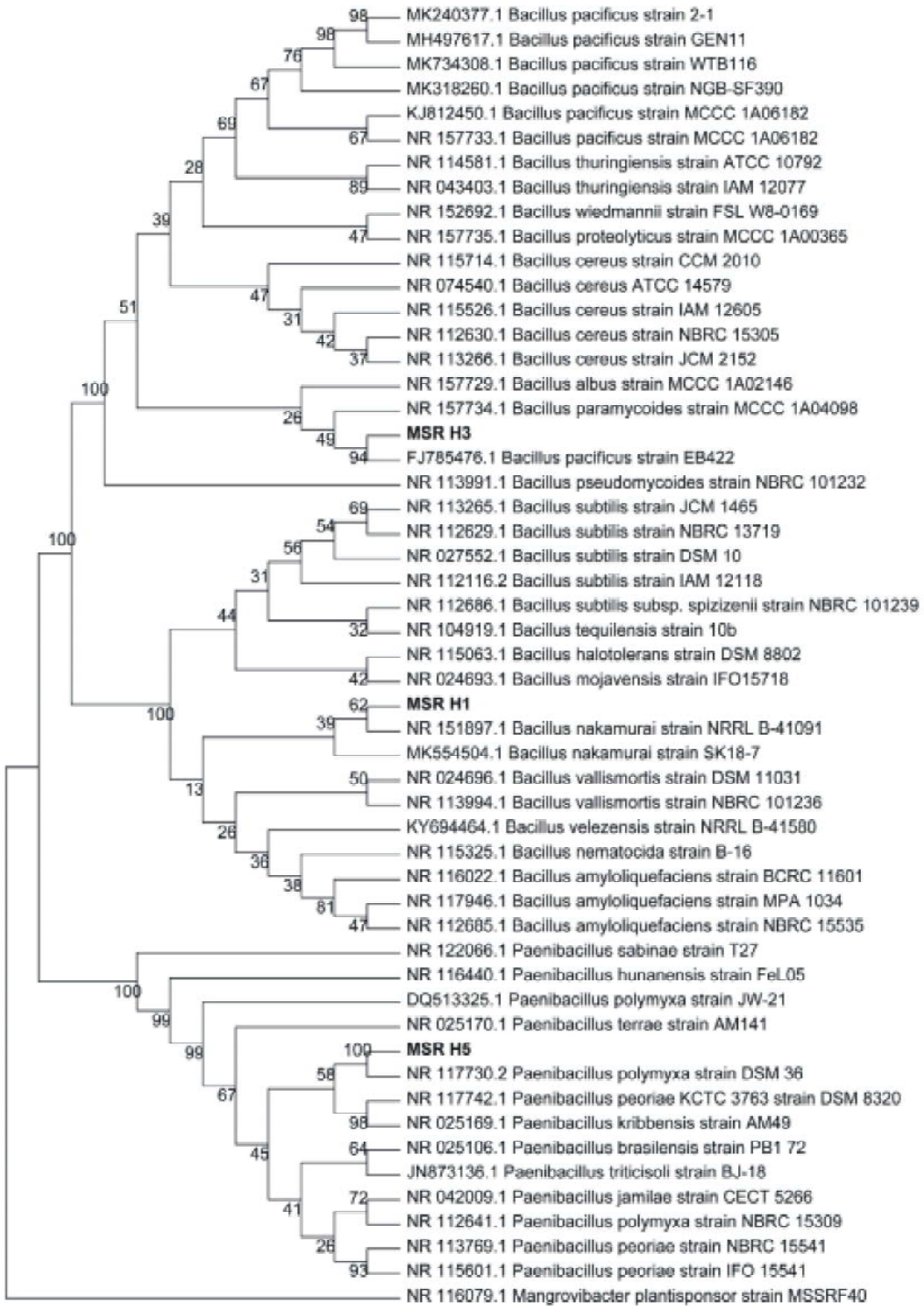

Fig. 4: Phylogenetic tree based of the nearly complete 16S rRNA sequence of the top three bacteria characterized with the potassium solubilizing bacteria, nitrogen fixing bacteria, phosphate solubilizing bacteria on 16S rRNA gene sequence comparison bacteria. Phylogenetic relation inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method

increase the growth parameters, this results are harmony with [80 and 81]. Root colonization is effective if the bacteria are capable of extent and spread along the root in the presence of good original rhizosphere microorganisms [26]. *P. polymyxa* can act either as a deleterious rhizobacterium (DRB) or, it induces the formation of biofilms around the root tip, as a root entering bacterium [82], so it colonizes in the rhizosphere soil and led to increase in growth parameters of wheat plant.

CONCLUSIONS

52 NPK bacteria isolated from six different zones and screened to 22 N_2-fixing bacteria, 18 PSB and 12 to KSB. We studied the nitrogenase activity to select active free nitrogen fixing bacteria, phosphate solubilizing effectiveness for PSB and potassium solubilizing effectiveness for KSB. N_2-fixer isolate No. 1N (MSR H5) was the best active free nitrogen fixing, isolate no. 2P (MSR H1) is very efficient Phosphate solubilize and isolate no. 5K (MSR H3) showed very efficient potassium solubilizing. The phylogenetic analyses confirm the identity of the strains to *P. polymyxa, B. nakamurai* and *B. pacificus*. Root colonization of wheat was done to evaluate these NPK strains in colonization, we found the three strains in case of alone or mixed are able to successful colonization the wheat plant and stimulated significant increases in growth parameters and Vigor index. Based on our data we advise in feature using these NPK bacteria as a bio fertilizer in field.

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