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Characterization of Nutrient Solubilizing Bacteria and Their Efficacy Studies on Oryza Sativa

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ABSTRACT:

Nitrogen potassium and phosphorus are vital macronutrients for plant growth. Nitrogen in the soil can be lost due to denitrification, leaching, and volatilization. Rock phosphate is the only phosphate source in the world which is being depleted. When it is applied to soil, phosphate and potassium were quickly immobilized and cannot be absorbed by the plants. In this experiment total 8 free living N2 fixing bacterial isolates were isolated from different soil samples. Among the all isolates, isolate NP-2 and NP-7 produced more than 600 µg/ml ammonia. The isolated microorganisms 16s rRNA gene sequence was showed 99.9% similarity with Azotobacter vinelandii. The four soils showed clear difference in PSB bacterial diversity, virgin soil had completely 81 colonies and 16 different isolates in that isolate B7 and B6 were in highly prevailed. Plants were treated with different isolated organisms and growth parameters were evaluated in rice plants.

Keywords: Nitrogen, Phosphorus, Potassium, PSB

1. Introduction

Besides being important in biogeochemical cycling of nutrients, microbes play vital role in maintenance of soil fertility and in crop protection. Microbes are being exploited in two important ways as biofertilizers, and creating new nitrogen-fixing organisms. Most of the chemical reactions that take place in the soil, leading to increased availability of several major and micronutrients often have active contribution of microbes. The nitrogen-fixing bacteria, blue green algae, and phosphate solubilizing bacteria are well known to enhance availability of major nutritional elements like nitrogen and phosphorus to plants whereas the decomposer bacteria are instrumental for recycling, and thereby increasing, the availability of carbon and several micronutrients from plant residues to soil. (Adesmoye AO, Kloepper JW 2009)
Among all the elements nitrogen is a critical limiting element for plant growth and development. It is a major component of chlorophyll as well as amino acids, the key building blocks of proteins and also found in other important biomolecules, such as ATP, nucleic acids, growth regulatory substances etc(Gyaneshwar et.al.,2002).Nitrogen is an essential and major component of every cell, and autotrophic organisms such as plants usually utilize ammonium or nitrate as the nitrogen source. (Akira Suzuki , David B Knaff, 2005).

1.1 Nitrogen Fixation

The ability of a plant to capture nitrogen from the soil and atmosphere depends on soil type, environment and species. It has been estimated that 50– 70% of the nitrogen provided to the soil is lost .The use of nitrogen by plants involves several steps including uptake, assimilation, translocation, recycling and remobilization. In the ecosystem, biological nitrogen fixation can also take place in many forms, including blue green algae, lichens and free-living soil bacteria. These types of nitrogen fixing systems contribute significant quantities of available N to natural ecosystems (Lamo et.al.,,2012; Kloepper JW, Schroth MN ,1978)

1.2 Phosphate Solubilization

Phosphorus has been called “the key of life” because it is directly involved in most of the life processes. It serves as a primary energy source for microbial oxidation. It is a constituent substance in life processes. Soil cannot give high yields if it is deficient in phosphate. Plants take phosphate in the form of soluble orthophosphate ions but due to the presence of Ca, Mg, K, Na, Al and Fe ions in soil, the soluble orthophosphate is converted to insoluble form. Because of this process plants utilize very little amount of phosphate, even though phosphorus containing fertilizers are added (Balamurugan et.al.,2010). Many researchers have tried to increase the plant-available phosphate fraction by means of Phosphate solubilizing microorganisms (PSMs) such as Achromobacter sp, Agrobacterium sp, Alcaligens sp, Bacillus cereus, B. polymyxa, B. megaterium, B. subtilis, Pseudomonas striata and Xanthomonas sp and Fungi like Aspergillus niger, A. flavus, A. fumigatus, Penicillium sp and Rhizopus sp (Sonboir, H.L. and Sarawgi, S.K. 1998).

1.3 Potssium Solubilization

Potassium is the most important plant nutrient that has a key role in the growth, metabolism and development of plants. In addition to increasing plant resistance to diseases, pests, and abiotic stresses, K is required to activate over 80 different enzymes responsible for plant and animal processes such as energy metabolism, starch synthesis, nitrate reduction, photosynthesis, and sugar degradation (Hassan Etesami et.al.,2017; Verma, et.al;2016). This study deals with screening of nitrogen fixing, Phosphate Solubilizing and potassium solubilizing microbes from soil and its utilization of microbes for growth of rice plants.

2. Materials and methods

Soil samples were collected from different river areas.

Sample preparation by serial dilution: - Serial dilution is a method to identify the number of viable micro-organisms in a fixed amount of a liquid. It can also be fairly easily modified to give results with solid substances. Six test tubes for each sample were taken. All the test tubes were filled with 9ml of deionised water. Then 1gm of sample to the first test tube with 9ml of deionised water added. Then the content of first test tube mixed thoroughly. Then 1ml of mixed content to the next test tube with 9ml of deionised water transferred. Then again the same procedure was done to the very next test tubes, one after one.

Nitrogen free Media used for isolation of bacteria
Jensen's Medium is recommended for detection and cultivation of nitrogen fixing bacteria. Add 36 gms of media and mix in 1000 ml of water and sterilise under autoclave at 121^0C temperature and 15 LB pressure. After isolation of pure culture by streak plate technique, the ammonia production by microbes can be checked by Nessler's reagent (Thiammaiah SR (2004; Suslow TV (1982))

Isolation of phosphate solubilizing Microorganism
These soils were mixed and subjected to serial dilution technique using the stock soil suspension which contains 10 g of soil sample in 100 ml of sterile distilled water. From the serial diluted suspension, 1 mL of suspension from 10^{-3} to 10^{-6} dilution was taken for pour plate technique in Pikovskaya agar medium. The Nutrient agar plates were observed after 2 days of incubation for enumeration of bacterial colonies. After 2-3 days of incubation, the plates were observed for phosphate solubilizing bacterial cultures from Pikovskaya agar medium (Walpola,2013). The bacterial cultures were then transferred to nutrient agar slants for further studies. And based on the morphological characteristics i.e colony colour, shape, texture and size, basic diversity of the phosphate solubilising bacteria in the four agriculture soils were enumerated (Balamurugan et.al.,2010; Rodriguez H, Fraga R 1999;)
Pure fungal cultures from Sabouraud agar slants were transferred to Sabouraud agar plates. After four days of incubation at 27 ºC, two 8 mm disc were cut from the plates by sterile borer from all three fungal plates and were inoculated into 100 ml Pikovskaya broth separately. The flasks were incubated at 27 ºC. Uninoculated Pikovskaya broth served as control in each case. Each experiment was done in triplicate set.

Isolation of K soluble bacterial strains
A modified Gopalakrishnan et al. (2011) protocol was used to isolate bacterial strains. Briefly, five grams of soil from each sample were suspended in 45 ml of physiological solution (0.85% of NaCl) in a flask and placed on a rotary shaker at 100 rpm and at room temperature for 2 h−1 . After shaking, the soil samples were serially diluted up to 10−5 dilution in physiological solution. Fifty µl aliquots of dilutions 104 –105 was placed on nutrient broth (NB) medium by the spread plate technique and incubated at 28 ± 2 °C for 48 h−1 . The most prominent colonies were isolated, based on macro morphological characteristics.

Pot experiment:
Effect of isolated PSB and nitrogen fixing bacteria on the growth of paddy crop plant:-
Experiments for studying the phosphorous mobilization and nitrogen fixation in the plants were made on paddy crop plant. Each pot was filled with 100grams of soil. The experiment

design was performed as follows: Treatment 1: inoculated soil (control) Treatment 2: soil inoculated with 0.2% NPK, Treatment 3: soil inoculated with bacterial + 0.2% NPK and Treatment 4: soil inoculated with only with bacteria. For the treatment, the NPK fertilizer was mixed thoroughly with the soil. 20 seeds were placed in each pot at 1cm depth. After that percentage germination and growth parameters were analysed (Poonguzhali S, Madhaiyan M,2007) .

3. Results and Discussion

3.1 Isolation of nitrogen fixing bacteria and its solubilzation:

The soil samples of different river areas were collected and used for the isolation of nitrogen fixing bacteria. Total 8 bacterial isolates isolated. These isolates were purified, identified and maintained for further use.

According to this result there was a clear difference between amount of ammonia produced with and without presence of urea in the media. The isolates which producing high amount of ammonia in the presence of urea might be having urease activity. All 8 isolates converting environmental nitrogen to plant utilizable ammonia in different levels. Among the all isolates isolate A7 produced more than 600 µg/ml ammonia (Table 1)

The microorganisms 16s rRNA gene sequence was showed 99.9% similarity with Azotobacter vinelandii (Hong JP,2010)). and in UPGMA dendrogram the two organisms were in same group with no distance(. Because of this reason we confirmed that the organism was Azotobacter vinelandii(Figure 1)

3.2 Isolation of Phosphate Solubilising Bacteria and its solubilization

The same serial diluted suspension were inoculated into Pikovskaya agar the plates were observed after 3 days of incubation for phosphate solubilizing microorganisms (PSB). The plates were inoculated with the suspensions such as 10^3 and 10^4 dilution, showed more colonies and were too numerous to count for both bacteria and fungi (Pikovskaya RI 1948). The four soils showed clear difference in PSB bacterial diversity, virgin soil had completely 81 colonies and 16 different isolates in that isolate B7 and B6 were in highly prevailed. All the isolates were screened for the phosphate solubilisation on PVK media. They had formed clearance zone (Hariprasad,2009) Among all the isolates, isolate-B6 and Isolate-B7 produced high zone of clearances in the PVK plates(Figure 2)

After 7 days of incubation all 16 isolates soluble some amount of phosphate in variable concentrations. And these values and scenario was almost similar to the primary screening zone of clearance values (Tabatabai MA, Bremner JM ,1969). In secondary screening isolate-A3, B2, B4, B6 and B7 were solubilised maximum amount of calcium tri phosphate into soluble inorganic phosphate have been observed. After 7 days of incubation all 16 isolates soluble some amount of phosphate in variable concentrations. And these values and scenario was almost similar to the primary screening zone of clearance values(Anbuselvi S,et.al.,2015). In secondary screening isolate-A3, B2, B4, B6 and B7 were solubilised maximum amount of calcium tri phosphate into soluble inorganic phosphate which have crossed more than 50 µg /ml soluble phosphate (Table 2).

3.3 Potassium Solubilisation estimation

After one week of the incubation few isolates were changed the medium color, those isolates were called potassium solubilising isolates(Table 3)

3.4 Application of NPK Solubilizing microbes for growth of rice

Plants were treated with different organisms and growth parameters were evaluated in rice plants (Hong et.al.,2010; Kim et.al.,2009; Tilak,2005) They revealed the effect of the effect of the bacteria on the plant growth(Anbuselvi, et.al.,2015. Apart from control treatment all treatments showed 100% germination. In case of root and shoot lengths treatment III showed positive growth effects. Root length and shoot length in treatment III were 5.9 cm and 10.1 cm, at the same time the control treatment Root length and shoot length were 4.3 cm and 8.1 cm. That means ~ 15-20% growth was increased in treatment III.

Nitrogen fixation is one of the major way by which atmospheric nitrogen is introduced into the ecosystem and the capability to carry out this process is restricted to a certain group of microorganisms (Tilman D et.al;2005) Nitrogen is a vital element for plant growth and development (Kaneko et al., 2002) which is usually absorbed as nitrate or ammonium, taking part in proteins, enzymes and chlorophyll structure (Cecílio Filho et.al., 2015). Biological nitrogen fixation is thought to explanation for more than 97% of "new" N inputs into unmanaged terrestrial ecosystems (Vitousek et al., 2002). Biological nitrogen fixation (BNF) is an efficient source of nitrogen and an important part of the microbial processes, is carried out only by prokaryotes, which may be symbiotic or free living in nature (Suslow TV 1982). It can be estimated at about 175 million metric tons per year or about 70% of all N fixed on the Earth per year, the remaining is by some micro-organisms, autotrophs or heterotrophs 'free' fixers (Janssen et al., 2002). this amount is much higher compared to the 85 t N / year consumed by nitrogenous fertilizers all over the world in 2002 .

4. Summary and conclusion

NPK bacteria isolated from six different zones and screened to 12 N -fixing bacteria, 4 PSB and 6 to KSB.$_2$ We studied the nitrogenase activity to select active free nitrogen fixing bacteria, phosphate solubilizing effectiveness for PSB and potassium solubilizing effectiveness for KSB. N -fixer isolate N 7 was the best active free nitrogen fixing,) is very efficient Phosphate solubilize and isolate no. B7showed very efficient potassium solubilizing. The phylogenetic analyses confirm the identity of the strains to *P. polymyxa, B. nakamurai*.

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Nitrogen Fixation:
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Potassium Solubilization:
Potassium is an essential plant nutrient that has a vital role in the growth, metabolism and development of plants. Apart from these, it has the potential to increases the resistive nature of plants towards diseases, pests, and abiotic stresses. It is required to activate over 80 different enzymes responsible for plant and animal processes which includes energy metabolism, starch synthesis, nitrate reduction, photosynthesis and sugar degradation. This study focuses on screening of nitrogen fixing, Phosphate Solubilizing and potassium solubilizing microbes from soil and its utilization of microbes for growth of rice plants.

Materials and Methods:
Soil samples were collected from different river areas.

Sample preparation by Serial dilution:-
Serial dilution is a method to identify the number of viable micro-organisms in a fixed amount of a liquid. It can be quite easily altered to provide results with solid substances. Six test tubes for each sample were taken. All the test tubes were filled with 9ml of deionized water. Then 1gm of sample to the first test tube with 9ml of deionized water added. Then the content of first test tube mixed thoroughly. Then 1ml of mixed content to the next test tube with 9ml of deionized water transferred. Then again the same procedure was done to the very next test tubes, one after one.

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Isolation of Phosphate Solubilizing Microorganism:
These soils were mixed and subjected to serial dilution technique using the stock soil suspension which contains 10 g of soil sample in 100 ml of sterile distilled water. From the serial diluted suspension, 1 mL of suspension from 10-3 to 10-6 dilution was taken for pour plate technique in Pikovskaya agar medium.
The Nutrient agar plates were observed after 2 days of incubation for enumeration of bacterial colonies. After 2-3 days of incubation, the plates were observed for phosphate solubilizing bacterial cultures from Pikovskaya agar medium. The bacterial cultures were then transferred to nutrient agar slants for further studies. And based on the morphological characteristics i.e colony colour, shape, texture and size, basic diversity of the phosphate solubilising bacteria in the four agriculture soils were enumerated.
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Isolation of Potassium Soluble Bacterial Strains:
Five grams of soil was taken from each sample and were suspended in a flask containing 45 ml of physiological solution (0.85% of NaCl). The flask was placed on a rotary shaker at 100 rpm and at room temperature for 2 h−1 . After shaking, the soil samples were serially diluted up to 10−5 dilution in physiological solution. Fifty µl aliquots of dilutions 104 –105 was placed on nutrient broth (NB) medium by the spread plate technique and incubated at 28 ± 2 °C for 48 h−1 . The predominant colonies were isolated, based on macro morphological characteristics.

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Effect of isolated PSB and nitrogen fixing bacteria on the growth of paddy crop plant:- Experiments for studying the phosphorous mobilization and nitrogen fixation in the plants were made on paddy crop plant. Each pot was filled with 100grams of soil. The experiment design was performed as follows:

Treatment 1: Inoculated soil (control)
Treatment 2: Soil inoculated with 0.2% NPK
Treatment 3: Soil inoculated with bacterial + 0.2% NPK
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20 seeds were placed in each pot at 1cm depth. After that percentage germination and growth parameters were analyzed.

Results and Discussion:

Isolation of Nitrogen Fixing Bacteria and its Solubilization:
The soil samples of different river areas were collected and used for the isolation of nitrogen fixing bacteria. Total 8 bacterial isolates isolated. These isolates were identified, purified and stored for further analysis. According to this result there was a clear difference between amount of ammonia produced with and without presence of urea in the media. The isolates that produced higher amount of ammonia in the presence of urea, has the potential of urease

activity. All 8 isolates had converted environmental nitrogen to plant utilizable ammonia at different levels. Among the all isolates isolate A7 produced more than 600 µg/ml ammonia (Table 1).

Table 1: Ammonia fixed by Nitrogen fixing bacterial isolates from soil

S. No.	Bacterial strain	Ammonia (µg/ml)					
		(3rd day)		(5th day)		(7th day)	
		With urea	Without urea	With urea	Without urea	With urea	Without urea
1	A1	356.21	102.33	528.96	200.33	600.96	120.33
2	A2	469.83	8.33	497.46	6.41	398.46	10.42
3	A3	260.71	235.46	480.4	258.96	468.21	361.96
4	A4	315.46	30.08	236.96	39.8	499.46	46.71
5	A5	354.21	16.96	472.08	21.08	534.46	25.96
6	A6	305.46	22.08	473.21	8.71	499.46	21.96
7	A7	496.46	130.33	526.96	174.96	601.96	173.8
8	A8	315.46	90.58	404.46	152.96	501.96	281.46

The microorganisms 16s rRNA gene sequence was showed 99.9% similarity with Azotobacter vinelandii and in UPGMA dendrogram the two organisms were in same group with no distance. Because of this reason we confirmed that the organism was Azotobacter vinelandii (Figure 1).

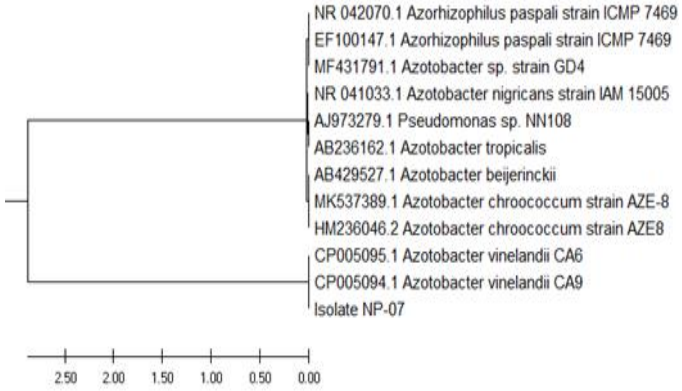

Figure: 1 16s rRNA gene sequence of Azotobacter vinelandii

Isolation of Phosphate Solubilizing Bacteria and its Solubilization:

The same serial diluted suspension were inoculated into Pikovskaya agar. The plates were observed after 3 days of incubation for phosphate solubilizing bacteria (PSB). The plates were inoculated in the suspensions with 103 and 104 dilution, expressed more colonies and were innumerable for both bacteria and fungi (Pikovskaya RI 1948). The four soils showed clear difference in PSB bacterial diversity, virgin soil had completely 81 colonies and 16 different isolates in that isolate B7 and B6 were in highly prevailed. All the isolates were screened for the phosphate solubilization on PVK media. They had formed clearance zone. Among all the isolates, isolate-B6 and Isolate-B7 produced high zone of clearances in the PVK plates (Figure 2).

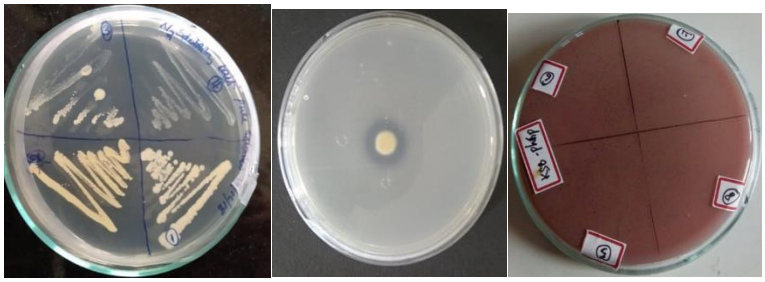

Figure 2: Isolation and screening of NPK Solubilizers from soil

After 7 days of incubation all 16 isolates, had some amount of Phosphate being soluble in variable concentrations. The values were found to be almost similar to the primary screening zone of clearance values. In secondary screening isolate-A3, B2, B4, B6 and B7 were solubilized and maximum amount of calcium tri phosphate into soluble inorganic phosphate conversion was observed. After 7 days of incubation all 16 isolates, had some amount of Phosphate being soluble in variable concentrations. The values were found to be almost similar to the primary screening zone of clearance values. In secondary screening isolates-A3, B2, B4, B6 and B7 had solubilized maximum amount of calcium tri phosphate into soluble inorganic phosphate which had crossed more than 50 µg /ml soluble phosphate (Table 2).

Table 2: Formation of inorganic phosphate in different isolates

Isolate	**Soluble phosphate (mg/ml)**		
	3rd day	**5th day**	**7th day**
A1	21.81	23.6	24.1
A2	24.8	26.3	28.7
A3	52.76	53.1	56.9
A4	41.8	57.1	53.7
A5	23.8	24.8	26.1
A6	40.8	42.2	45.9
A7	44.6	45.1	45.9
A8	30.8	32.1	36.7
B1	21.5	26.0	29.5
B2	50.8	54.1	56.0
B3	21.8	23.5	28.9
B4	52.71	52.9	55.5
B5	22.18	25.9	29.4
B6	54.15	57.9	59.2
B7	58.91	62.3	64.8

Potassium Solubilization Estimation:

After one week of the incubation few isolates were changed the medium color, those isolates were called potassium solubilizing isolates (Table 3).

Table 3: Formation of Potassium solubilization in different isolates

Isolate	Soluble phosphate (µg/ml)		
	3rd day	**5th day**	**7th day**
A1	3.2	3.8	4.3
A2	4.6	5.0	6.1
A3	4.7	4.8	5.0
A4	1.0	1.2	1.6
A5	2.0	2.6	3.5
A6	4.0	5.3	6.2
A7	4.6	6.9	8.0
A8	1.4	1.9	3.2
B1	3.1	4.9	5.2
B2	14.9	19.5	24.3
B3	1.9	2.3	5.3
B4	2.1	2.5	3.3
B5	3.8	7.0	7.5
B6	15.1	18.3	22.4
B7	15.8	25.7	31.0
B8	5.3	6.1	7.0

Application of NPK Solubilizing Microbes for Growth of Rice:

Plants were treated with different organisms and growth parameters were evaluated in rice plants. They revealed the effect of the effect of the bacteria on the plant growth. Apart from control treatment all treatments showed 100% germination. In case of root and shoot lengths treatment III showed positive growth effects. Root length and shoot length in treatment III were 5.9 cm and 10.1 cm, at the same time the control treatment Root length and shoot length were 4.3 cm and 8.1 cm. That means ~ 15-20% growth was increased in treatment III. Nitrogen fixation is an integral method by which atmospheric nitrogen is introduced into the ecosystem and the capability to carry out this process is restricted to a certain group of microorganisms. Nitrogen is a vital element for plant growth and development which is usually absorbed as nitrate or ammonium, taking part in proteins, enzymes and chlorophyll structure. Biological nitrogen fixation is thought to explanation for more than 97% of "new" N inputs into unmanaged terrestrial ecosystems. Biological nitrogen fixation (BNF) is an efficient source of nitrogen and an important part of the microbial processes, is carried out only by prokaryotes, which may be symbiotic or free living in nature. It can be estimated at about 175 million metric tons per year or about 70% of all N fixed on the Earth per year, the remaining is by some micro-organisms, autotrophs or heterotrophs 'free' fixers. This amount is much higher compared to the 85 t N / year consumed by nitrogenous fertilizers all over the world.

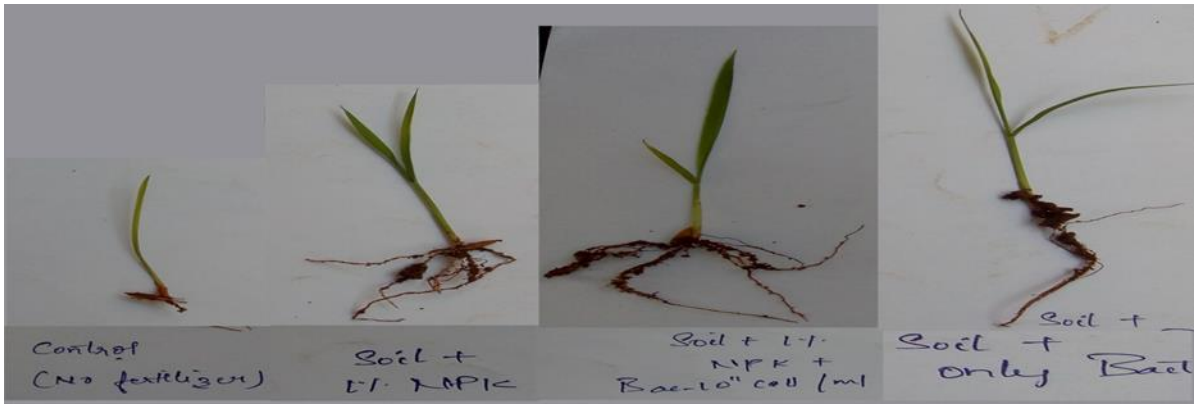

Figure 3: Plants were treated with different organisms and growth parameters were evaluated in rice plants.

Conclusion:

NPK bacteria isolated from six different zones and screened to 12 N -fixing bacteria, 4 PSB and 6 to KSB.2 We studied the nitrogenase activity to select active free nitrogen fixing bacteria, phosphate solubilizing effectiveness for PSB and potassium solubilizing effectiveness for KSB. N -fixer isolate N 7 was the best active free nitrogen fixing,) is very efficient Phosphate solubilize and isolate no. B7showed very efficient potassium solubilizing. The phylogenetic analyses confirm the identity of the strains to P. polymyxa, B. nakamurai.

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