Production and Application of Phosphate Solubilizing Bacteria as Biofertilizer: Field Trial at Maize Field, Uchalan, Burdwan District, West Bengal.

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***Abstract*—** *Soil microorganism plays an important role in regulating the levels of carbon, nitrogen, phosphorus and sulphur at the rhizosphere. Solubilization of macronutrient is an important aspect in plant growth and development research. Phosphorus is one of the vital nutrient required for optimum growth of plant. Phosphate Solubilizing Bacteria (PSB) plays an important role in increasing the phosphate uptake by the plants. Pikovskaya medium containing TCP or tricalcium phosphate helps in isolation of PSB from soil sample. Isolated strains were tested and screened by Halo zone formation and pH test. The selected strains showing marked decrease in pH and clear zone formation was selected for field trial. The objective of this study was to ascertain that PSBs isolated from the soil sample can be used as biofertilizer. Application of PSBs with a carrier mixed with maize seeds in the field of study and compared against a control field and against commercially available fertilizer showed that PSBs can improve the quality of soil and in turn improved the growth and development of the plants.*

Keywords*— *Biofertilizer, Phosphate Solubilizing Bacteria, chemical fertilizer, plant growth, soil fertility, field trial.

I. INTRODUCTION

Development of a nation is directly proportional to the amount of food or nutrient available to the population. Growth of the human population creates demand for more food grains. To supply food grains according to the demand, fertilizers are used. A fertilizer is any substance that is used for increasing the productivity of the soil. It promotes soil fertility by adding nutrients in the soil, which helps in plant growth. Fertilizers which are composed of raw chemicals in solid or liquid form manufactured in factories targeting the nutritional requirement of the plants are by definition called a “chemical fertilizer”. Nitrogen, Phosphorous, Potassium together called NPK are normally present in these chemical fertilizers along with other nutrients (Youssef et al.,2014). The substances are normally present in a form that is easily absorbed by the plant. But the use of chemical fertilizers has some harmful side effect on the environment (Usman et al.,2015). Important issues include groundwater contamination especially nitrogen compounds, they break down into nitrate and accumulates in the groundwater. Biofertilizers can be used as supplements of chemical fertilizers; they are relatively inexpensive and renewable sources of plant nutrient. Biofertlizers are selected strains of microorganisms which are beneficial to the growth of the plants. These microorganisms are cultured in laboratory, mixed with suitable carrier materials and then applied to the fields. They maintain soil health, minimizes pollution of the environment by lowering the use of chemicals (Tripti et al., 2012). Biofertilizers are used to treating seeds, plantlets, grown plants. The popularity of the biofertilizers are due to its eco-friendly, non hazardous and non- toxic nature. The living microorganisms colonizes the rhizosphere or colonizes the interior of the plant, they promotes growth by increasing the availability of the nutrients and helps in the breakdown of inorganic substances into organic form, increasing the supply of growth stimulus to the seeds of crops, plant surfaces and even in the soil can help in greater productivity. Examples of bio fertilizers are numerous and varied like, Rhizobium, Azospirillium and Azotobacter – Nitrogen fixing biofertilizers. Pseudomonas, Bacillus, Aspergillus, are examples of PSB or Phosphate solubilising biofertilizer. Mycorrhiza is an example of Phosphate mobilizing biofertlizer. Pseudomonas species are also commercialized as Plant growth promoting biofertilizers. Soil microorganisms play an important role in maintaining the ecological balance, they participate actively in carbon, nitrogen, phosphorus, potassium, sulphur recycling in nature and thus facilitate uptake in the plants (Ansari et al., 2015).

The role of microorganisms in converting insoluble form of nutrient into soluble form is well known. After nitrogen, phosphorus is second in terms of importance for growth in plants. Phosphorous is 0.2% of the dry weight in plants. Phosphorus is obtained by the plant as phosphate anions. Phosphate solubilizing bacteria possess capability to convert phosphorus from insoluble to soluble form (Keneni et al., 2010). Phosphatic fertilizer when applied to the soil it has been seen that only a small amount is actually utilized by the plants. In India, it has been estimated that about 98% of the soil have some amount of deficit in phosphorus. Chemical fertilizer having phosphorus have a disadvantage, inorganic phosphates when applied to the soil are immobilized and thus not totally available to the plant (Karpagam and Nagalakshmi, 2014). PSBs or Phosphate solubilizing bacteria helps in converting phosphorus into soluble forms by acidification by organic acids, chelating oxo acids from sugars. They also produce enzymes like phosphatase enzymes that help in further degradation. Inoculation of PSBs in soil or near the rhizosphere of the plants has shown to promote growth of plants as a stimulatory effect. Plant roots can take up different forms of phosphorus like H_2PO_4, HPO_4^{2-}, this take up normally depends upon the soil pH, temperature, moisture content and other nutrients or minerals present in the soil (Rajsekaran et al,2012).

II. MATERIAL AND METHOD

2.1 Collection of samples

The soil sample was collected from agricultural field areas of Burdwan district, West Bengal. Around 7- 10 areas were selected and the soil sample was collected from depth of 15-20cm. the soil was packaged in sterile container and brought to the laboratory. After drying and crushing the soil by means of mortar pestle, the soil was ready for isolating phosphate solubilizing bacteria.

2.2 Serial dilution of the soil sample

1gm of soil sample was weighed in a weighing balance and dissolved in 10 ml of distilled water in a test tube, making the stock solution. To the remaining nine test tubes, 9 ml of water was added. 1ml of stock solution was transferred to test tube with 9ml of distilled water with the help of a pipette. This yielded 10-1 dilution. Further series of dilutions yielded upto 10^{-9} dilutions. The process was performed under Laminar Air Flow to maintain sterile conditions.

2.3 Isolation of Phosphate Solubilizing Bacteria

The selective media prepared for isolation and growth of Phosphate Solubilizing Bacteria had the following constituents: Yeast extract, Dextrose, Tricalcium Phosphate, Ammonium Sulphate, Potassium Chloride, Magnesium Sulphate, Manganese Sulphate and Ferrous Sulphate, Agar and Distilled water. The media was sterilized by autoclaving at 121^0celsius for 15 minutes. The media was poured into Petri plates and allowed to solidify. The soil solution of about 0.1 ml was spread on to the plate by Spread-plate technique. The plates were incubated for 24 hours at 37^0 Celsius (Walpola and Yoon 2013).

After incubation for 24 hours, the plates were taken out and growth of microorganisms was seen on the plates. Plates of dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-7} were chosen for further screening and formation of halo zone around the selected colonies.

2.4 Halo zone test

Isolated colonies from the isolation plates were chosen and utilized for screening and halo zone test. The isolates were screened for their capability of TCP solubilizing activity. These were performed in PKV plates. Isolated colonies were streaked on PKV plates. They were kept in a incubator for 5-7 days at 37^0 Celsius. Clear zone around the growth of the microorganism was regarded as a positive test. All the observations and experiments were carried out in triplicates for better results. The strains that developed clear zones around the colonies of microorganism growing on the plate were easily identified as PSBs (Panhwar et al., 2012).

2.5 Characterization of the isolated microroganism

2.5.1 Morphological characterization:

The isolated strains of microorganisms was grown on EMB plates, Eosin Methylene Blue helps to identify whether the strain is a gram positive or gram negative (Collee et al.,1996). Only gram negative strains grows on EMB plates. It is thus selective in nature. EMB agar plates were prepared and autoclaved for 15 minutes at 121^0celsius. The media was poured in the Petri plates and allowed to solidify. Isolated strains from plates of dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-7} were streaked in the EMB plates and kept overnight for incubation at 37^0 Celsius. Identification and characterization of the isolated strains were done following standard bacteriological techniques (Cheesbrough, 2000; Holt et al., 1994).

2.5.2 Gram Staining

Gram staining is one of the fundamental test done to characterize a isolated microorganism. Isolated strains from plates of dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-7} were smeared on clean and sterile slides. They were then heat fixed. Each glass slide was marked. The smears were stained according to protocol. The slides were examined under the 100 x oil immersions microscope.

2.6 pH test

The measurement of the phosphate solubilization for the strains that were isolated as PSBs were further tested. This was a quantitative test where the isolated strains were analyzed on their capability of phosphate solubilization in liquid media. The isolated strains were grown in PKV broth. The broth was autoclaved at 121^0celsius for 15 minutes, then allowed to cool down to a suitable temperature. The isolated strains were inoculated by the help of loops. Then the broths were incubated for 5-7 days at 37^0 Celsius. The pH of the media was noted as a measure of phosphate solubilization in the media.

2.7 Biochemical characterization of the organism

Biochemical analysis for the isolates was done to examine activities of MR-VP test, Hydrogen sulphide test, Starch hydrolysis test, Catalase test. The above tests were performed as per Bergey's Manual of Systematics Bacteriology (Claus and Berkery, 1986).

2.8 Preparation & Production of PSB biofertilizer

Selected strains were picked from respective Petri plates (10^{-3}, 10^{-4}, 10^{-6}) and then prepared for growth in production media. 100ml of PKV broth was prepared; each strain was inoculated separately in respective conical flask. This served as the starter culture. This starter culture was incubated for a week in a B.O.D shaker for mass multiplication of PSB bacteria.

The growth rate of the starter culture was measured at regular interval, when the viable count of the culture reached 6.8 x 10^{9} CFU/ml, a loopful of starter culture for each strain was inoculated into a 100 ml conical flask containing PKV broth, when growth reached a maximum point the culture was transferred to 500ml broth. The process was repeated in a 1000ml conical flask, which was kept for 2 weeks for mass multiplication in a B.O.D shaker. After the stipulated time the viable count was seen to be 7.8x 10^{11} Cfu/ml. The culture was mixed with carrier material.

The carrier material keeps the bacterial strain viable and active for a long duration. In this experiment cow dung is used as a carrier material. The ratio of biofertilizer and carrier material was kept at 1:1. Cow dung was used as a carrier material because of its abundant supply and it is inexpensive, thus can be made popular among farmers. The mixture was packaged and kept for storage at 4 degree Celsius overnight.

2.9 Field trial of the biofertilizer on maize seeds

The mixture containing the bacterial strain mixed with the carrier was further mixed with maize seeds. The seeds were then inoculated in a field, at Uchalan situated in Burdwan district. The field of study was divided into segments. Each segments was demarcated and divided into grids. The grids denoted the Control field (C), Fertilizer field (N) and the PSB biofertiliser field (P). Each grid was denoted as C1, C2, P1, P2, N1, and N2. The maize seeds were planted at a distance of 20-30 cm apart from each other. The seeds were regularly watered and growth was measured in terms of plant height, number of tassels, flowers and fruit that grew on the maize plant.

III. RESULT

Morphological characterization showed the isolated PSB strain from plates 10^{-3}, 10^{-4}, 10^{-6} showed that the strains from 10^{-3} are gram positive, whereas the strains isolated from 10^{-4}, 10^{-6} is gram negative.

pH test revealed that after incubation for 5-7 days, the pH of the broth steadily declined from the initial 7 to 3.48 and 4.6 at an average for all the isolates. The decrease in the pH indicates production of organic acids in the medium that may help in the phosphate solubilization by the isolated microorganism.

Biochemical tests showed that strains isolated from 10^{-3} was Methyl Red positive, VP negative, Catalase positive, Starch hydrolysis yielded a positive result, Hydrogen Sulphide test yielded a negative test.

Strains isolated from plate 10^{-4} was Methyl Red positive, VP negative, Catalase positive, Starch hydrolysis yielded a positive test, Hydrogen Sulphide test was positive.

Biochemical test of strains from plate 10^{-6} yielded a positive Methyl Red test, Negative VP test, Catalase positive, Starch Hydrolysis test yielded a positive result and Hydrogen Sulphide test was positive.

TABLE 1
RESULTS AFTER STAINING

Plates	Observation
1. 10^{-3} Strain 1&2	Purple colored, Rods in chain formation. Gram Positive.
2. 10^{-4} Strain 1&2	Pink colored, Rod shaped. Gram Negative
3. 10^{-6} Strain 1&2	Pink in color, Rod shaped. Gram Negative

TABLE 2
RESULTS OF BIOCHEMICAL TESTS

Plates	Strain No.	Methyl Red Test	VP Test	Catalase Test	Starch Hydrolysis Test	Hydrogen Sulphide Test
10^{-3}	1 &2	Positive	Negative	Positive	Positive	Negative
10^{-4}	1&2	Positive	Negative	Positive	Positive	Positive
10^{-6}	1&2	Positive	Negative	Positive	Positive	Positive

IV. FIELD TRIAL RESULTS

4.1 Results for plant height for 20 maize plants measured in each field segment (cms).

C1	C2	P1	P2	N1	N2
52	46	78	62	62	68
47	49	72	60.5	66.5	37
68	64	62	52	63	71.5
66	31	68	58	54.2	44
68	28	76	23.5	43	73
72	19	72.5	66	74	53
35	48	65	75	73	32
51	31	72	70	41.6	43.5
63	38	76	78	45.5	71.8
62	21	66	52	82	63.2
26	32	63	74	35.5	53.7
65	47	72	68	43.2	35.2
53	49	69.5	40.5	51.8	34
70	35	49	74	61.7	82
57	39	69	71	70.2	72
29	39	68	55	78.3	67.8
51	49	68	71	36	64
32	64	79	43.5	76	73.4
59	67	78.5	69	67	36.7
73	68	35.5	63	72	76

4.2 Results for number of tassels seen for 20 maize plant measured in each field segment.

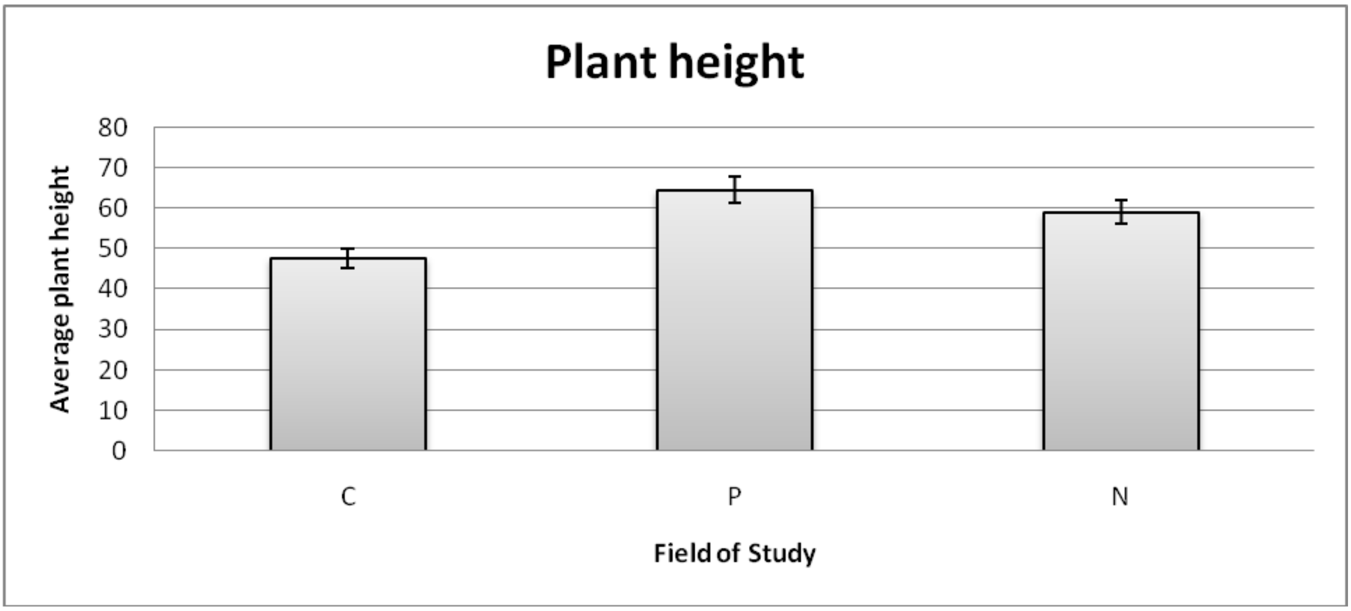
C1	C2	P1	P2	N1	N2
12	9	15	15	17	12
11	13	16	11	12	10
11	11	11	14	13	17
12	7	11	11	11	13
13	8	13	8	9	15
16	5	17	13	13	9
13	9	10	12	14	7
10	6	13	10	9	10
16	10	17	13	9	13
17	5	11	8	10	12
8	7	9	17	18	8
14	11	11	11	6	7
10	18	18	9	9	6
18	9	12	15	11	18
12	11	14	13	12	15
10	10	13	10	14	16
16	11	17	19	16	10
11	3	13	16	9	11
16	7	19	15	12	5
18	13	10	13	17	14

4.3 Results for number of fruits observed for 20 maize plant measured in each field segment.

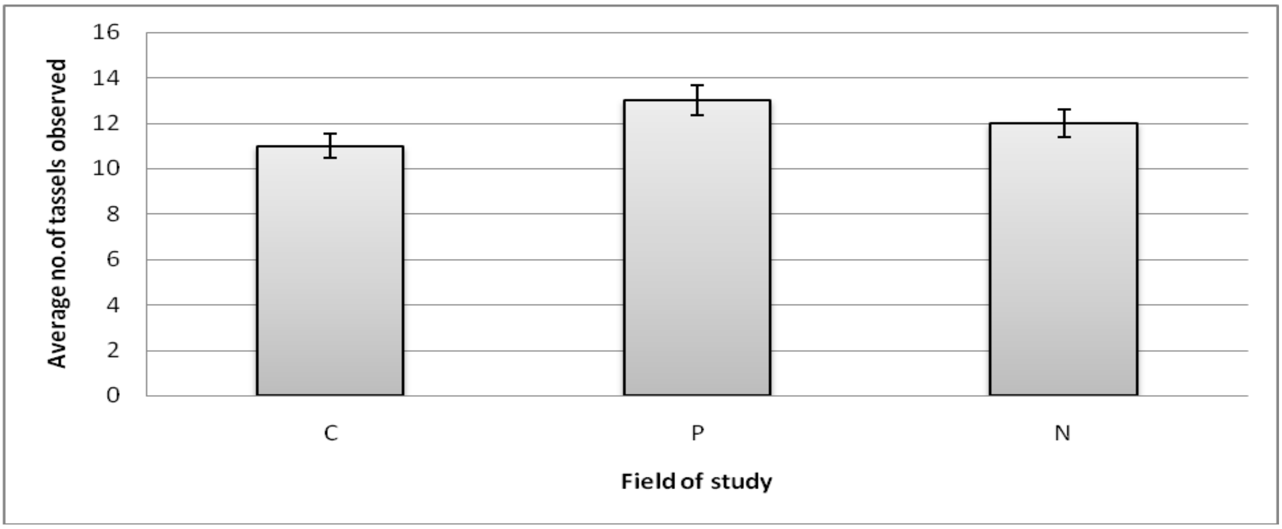
C1	C2	P1	P2	N1	N2
0	1	1	3	1	1
0	0	0	1	1	0
1	0	2	1	0	1
1	0	3	3	2	0
0	2	1	2	1	1
2	1	0	1	0	0
1	1	1	0	1	0
0	0	2	1	1	0
2	1	1	0	0	1
2	0	3	0	0	0
1	1	1	1	1	0
0	1	3	2	0	0
0	0	2	1	0	1
1	2	1	1	2	2
0	1	1	1	2	1
1	2	0	1	1	0
0	1	2	0	2	1
2	1	0	2	0	1
1	2	3	3	1	1
1	0	2	1	1	2

4.4 Results for number of flowers observed for 20 maize plant in each field segment.

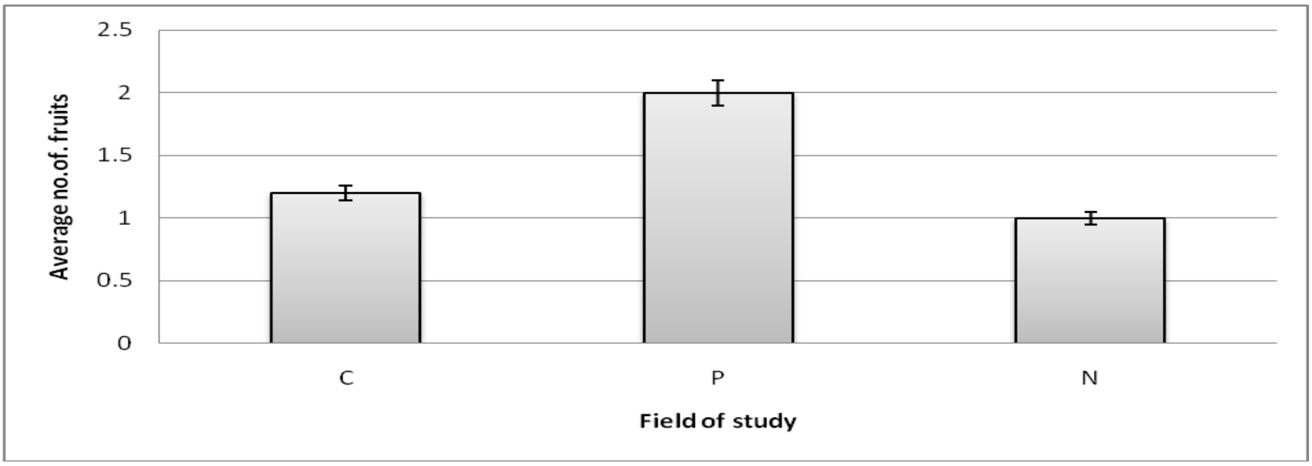
C1	C2	P1	P2	N1	N2
0	0	3	2	2	1
2	1	1	1	0	1
1	0	2	1	0	3
0	1	1	2	1	2
4	1	1	5	3	2
1	1	2	4	4	4
1	0	3	8	0	1
1	0	5	2	1	3
3	2	4	3	2	2
0	1	0	5	5	1
1	0	3	1	1	1
1	0	7	1	0	1
1	0	1	1	0	2
2	1	2	0	1	1
1	1	6	0	2	3
0	2	3	2	1	2
0	1	4	3	4	1
1	2	1	1	3	0
1	2	2	1	2	0
0	1	0	1	1	1



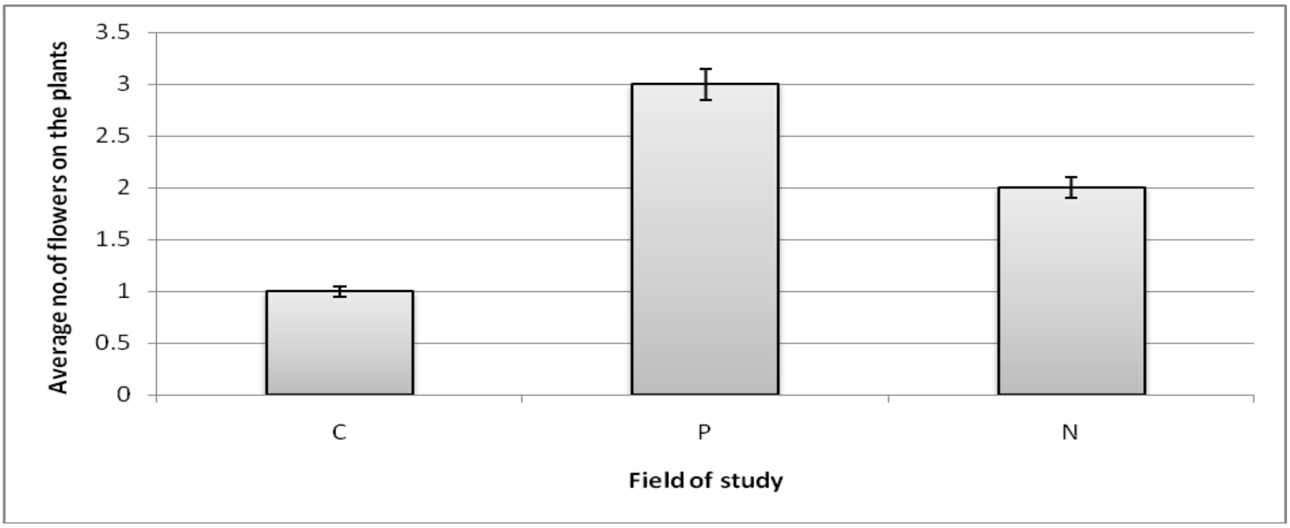
GRAPHICAL REPRESENTATION OF AVERAGE PLANT HEIGHT NOTED ON THE MAIZE PLANT WHEN PSB BIOFERTILIZER IS APPLIED TO THE FIELD WHEN COMPARED WITH OTHER COMMERCIALLY AVAILABLE FERTILIZERS AND AGAINST THE CONTROL.



GRAPHICAL REPRESENTATION OF AVERAGE NUMBER OF TASSELS NOTED TO GROW ON THE MAIZE PLANT WHEN PSB BIOFERTILIZER IS APPLIED TO THE FIELD WHEN COMPARED TO OTHER COMMERCIALLY AVAILABLE FERTILIZERS AND AGAINST THE CONTROL.



GRAPHICAL REPRESENTATION OF AVERAGE NUMBER OF FRUITS NOTED TO GROW ON THE MAIZE PLANT WHEN PSB BIOFERTILIZER IS APPLIED TO THE FIELD WHEN COMPARED WITH OTHER COMMERCIALLY AVAILABLE FERTILIZERS AND AGAINST THE CONTROL.



GRAPHICAL REPRESENTATION OF AVERAGE NUMBER OF FLOWERS OBSERVED TO GROW ON THE MAIZE PLANT WHEN PSB BIOFERTILIZER IS APPLIED TO THE FIELD WHEN COMPARED WITH OTHER COMMERCIALLY AVAILABLE FERTILIZERS AND AGAINST THE CONTROL.

V. CONCLUSION

The isolated strains of microorganisms 10^{-3} Strain 1 and Strain 2, 10^{-4} Strain 1 and Strain 2, 10^{-6} Strain 1 and Strain 2 showed significant phosphate solubilizing activity. The use of biofertilizers can help towards reduction in use of harmful chemical fertilizers, that are not environment friendly. The decrease in pH levels of the media shows the production of organic acids, enzymes by the microorganisms to help with the solubilization of the phosphate provided in the medium.

According to the results obtained from field trial, use of PSB biofertilizer shows marked difference in soil fertility, increased plant height, number of tassels observed in the maize plant. It was also observed that plants growing with the help of PSB biofertilizer had better quantity and quality of fruits and flowers, than those that grew on the control field and on the field which was supplemented with commercially available fertilizer.

ACKNOWLEDGEMENTS

Authors are very thankful to Techno India University for providing this infrastructure and also thankful to Mr. Biswarup Dawn, GM Agro pvt. ltd for providing field of our experiment.

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