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Effects of mixed bacterial biofertilizer on beans and maize plants

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ABSTRACT: *The effect of mixed bacterial biofertilizers containing nitrogen fixers, phosphate solubilizers and potassium* solubilizers was investigated against individual applications. One strain each of *Rhizobium*, *Azotobacter* and two strains each of phosphate and potassium solubilizers were isolated, prepared and applied individually while a consortium of the inoculum was prepared by mixing each strain with the carrier. Each preparation was applied as seed treatment to study their effects on beans and corn plants. The shoot length, pod size, flowering, stalk formation, yield and other growth parameters were monitored. The mixture of bacterial preparations enhanced the plants' health, growth parameters and yield of beans and maize plants significantly at a significant value of $p<0.05$, when compared to the single bacterial applications and control. The results proved that the mixed biofertilizers increased the growth, and yield in addition to shorter crop cycle compared to the control and individual bacterial biofertilizers.

KEYWORDS: Bacteria, biofertilizer, nitrogen fixers, phosphate solubilizers, potassium solubilizers, beans, corn, Leguminous and non-leguminous plants.

INTRODUCTION

Biofertilizers are living organisms that improve soil fertility and make nutrients available to plants. They possess the ability to convert nutrients that are unavailable for plant uptake into readily available form when they are applied to the seed or soil (Rokhzadi *et al*.,2008). Soil erosion remains the major way by which soil loose it nutrients coupled with the use of chemical fertilizers that inhibit the growth of naturally occurring microorganisms in the soil. Soil erosion in India has negatively imparted the soil fertility which is gradually diminishing in nutrients, accumulates toxic elements, water logging and unbalanced nutrient compensation (Ritika and Utpal, 2014). In constrast, the potential biological fertilizers would play key role in productivity and sustainability of soil and also protect the environment as ecofriendly and cost-effective inputs for the farmers (Khosro Mohammadi and Yousef Sohrabi, 2012).

Major nutrients required by the plants are nitrogen (N), phosphorus (P) and potassium (K). Nitrogen is required in large amount due to its role as a component of the DNA. It is available in abundance in the atmosphere but not readily available for plant uptake. Nitrogen fixing bacteria play major role in the conversion of atmospheric nitrogen into organic nitrogen by nitrogen fixation (Fischer *et al*., 2007) which is carried out by two major groups of organisms; *Rhizobium* and *Azotobacter* using nitrogenase enzyme.

Rhizobium is a soil habitat gram negative bacteria that can replicate in the root nodules of leguminous plants and then fix atmospheric nitrogen by symbiotic association which is initiated when bacteria in the soil attach to the root hairs (Gomare,*et al.* 2013). *Rhizobium* fixes nitrogen much more efficiently and it is most studied and important nitrogen fixing bacteria (Odame, 1997).

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The other groups of nitrogen fixing bacteria are free living bacteria that do not form any symbiotic association with the plants. They exist as free-living bacteria in the soil; they are *Azotobacter* and *Azospirillum* (Garcia-Fraile *et al*. 2015). Okon (1985) reported that *Azospirillum* spp. increased yield of cereal and forage grasses by improving root development in properly colonized roots thereby increasing the rate of water and mineral uptake from the soil by biological fixation.

Phosphorus (P) is another plant nutrient that is essential for plant growth and productivity as well as cell division, photosynthesis, and development of good root system in addition to carbohydrate utilization (Sharma, *et al.* 2011). Phosphorus exists in the soil as insoluble phosphate and its assimilation from the soil by plants is brought about by the help of microbes through the enzyme phosphatase. Phosphatase is present in wide range of soil microorganism which converts the insoluble form of phosphorus into soluble form for plant uptake (Sharma, *et al*. 2011; Yosef, *et al*. 1999). Phosphorus deficiency leads to physiological defects such as small leaves, weak stem and slow development (Rajan*et al*., 2015)

Another important plant nutrient is potassium (K). It is the most abundantly absorbed element having roles in the growth, metabolism, and development of plants (Parmar and Sindhu, 2013). It is also available in the soil in an insoluble form and constitutes about 2.5% of the lithoscope with actual soil concentration ranging from 0.04 to 3.0% (Sparks and Huang, 1985). Application of imbalanced fertilizer and potassium deficiency is gradually becoming a major drawback in crop production (Parmar and Sindhu, 2013).

Deficiency of potassium in plants is characterized by poorly developed roots, slow growth, and production of small seeds as well as low yield. (Sparks and Huang, 1985).

A wide range of bacteria that have ability to release potassium from inaccessible form to accessible form are; *Pseudomonas, Barkholderia, Acidothiobacillusferooxidans, Bacillus mucilaginosus, Bacillus edaphicus, Bacillus circulans* and *Paenibacillus spp*. (Li,*et al*. 2006; Lian,*et al*. 2002; Liu, 2012; Sheng, 2005;). However, the information on the mechanism of solubilization and effect of potassium solubilizing bacteria (KSB) inoculation is not yet well understood.

Chemical fertilizers are the main cause of losing soil fertility and are actually causing huge amount of soil and land degradation (Liu,*et al*., 2012.).The fact that chemical fertilizer continues to cause more havoc to the soil than good, an alternative, biofertilizer, with the ability to supply the three major required nutrients is invaluable to reduce the cost of fertilizer and most importantly to restore the soil nutrient to its natural state and to make it more suitable for better yield in agricultural practices without affecting the environment negatively. Hence, this investigation was carried out to identify the nitrogen fixing, phosphorus and potassium solubilizing bacteria and to evaluate their effects individually and in consortium on the growth and productivity of bean and maize plants.

MATERIALS AND METHODS

ISOLATION OF BACTERIAL CULTURES

Rhizobium isolates were obtained from the root nodules of beans plant (Phaseolus vulgaris) collected from Prof. G.M. Research foundation agricultural garden (Fig. 1), Hyderabad, India. The nodules were washed and surfaced sterilized with ethanol (95%), then crushed in sterile water using pestle and mortar. A loop full of nodule paste was inoculated on freshly prepared yeast extract mannitol agar medium (YEMA) containing (g/l): Mannitol, 10; Yeast Extract, 1; K_2PO_4, 0.5; $MgSO_4$, 0.2; NaCl, 0.1; $CaCO_3$, 1 and Agar, 15 with methylene blue at a pH of 6.8 and incubated at 25°c for 7days. Pure cultures were isolated on YEMA broth, the *Rhizobium* was then inoculated after 7days on to perlite carrier.

Fig. 1.

Azotobacter was isolated by collecting rhizosphere soil samples of Capsicum (Capsicum annuum) and tomato plants (*Solanum lycopersicum*) at a concentration of 0.1g/ml in distilled water. Serial dilution was prepared up to 10^{-3} and then inoculation was carried out by spread plate method on Ashybys Mannitol Agar medium containing (g/l):Mannitol, 20; K_2HPO_4, 0.2; K_2SO_4 , 0.1; $MgSO_4$, 0.2; NaCl, 0.2; $CaCO_3$, 5; and Agar, 15at a pH of 7.4 The plates were incubated at 25°c for 7days after which the morphological appearances were observed under the microscope. After isolation of the pure culture, the isolates were inoculated onto Ashybys Mannitol broth medium and after 7days of incubation these cultures were applied into perlite carrier.

Three different strains of phosphate solubilizing bacteria (PS)B were isolated by serial dilution of rhizosphere soil samples from Jasmines (*Jasminum sambac*) and tomato (*Solanum lycopersicum*) plants at a concentration of 0.1g/ml in distilled water up to 10^{-3}.

The dilutions were spread on plates containing Pikovskaya (PVK) medium containing (g/l): Glucose, 10; Yeast Extract, 0.5; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; $Ca_3(PO_4)_2$, 5; NaCl, 0.2; KCl, 0.2; $MnSO_4.2H_2O$, 0.002; $FeSO_4.7H_2O$, 0.002; and Agar, 15at a pH of 7.0. After 7days of incubation at 30^{o}C, the bacterial colonies were observed for holozone formation. Five colonies were identified and subcultured on King's B medium containing (g/l): Proteose peptone, 20; K_2HPO_4, 1.5; $MgSO_4.7H_2$, 1.5; and Agar, 20 at a pH of 7.2 and incubated at 25^{o}C. Two of the colonies were also sub-cultured to obtain pure culture base on their halozone formation. The pure isolates were sub-cultured on broth of Pikovskaya's medium and inoculated on to the carrier after 7 days of incubation. The cultures were examined for morphological, biochemical and microscopic identification.

KSB were isolated from the rhizosphere of tomato plant after serial dilution at a concentration of 0.1g/ml of the soil sample in distilled water. The dilutions were inoculated on to Aleksandrov's media containing (g/l): Glucose, 10; $MgSO_4.7H_2O$, 0.5; $FeCl_3$, 0.005; $CaCO_3$, 0.1; $CaPO_4$, 2; Potassium Alluminium Silica (mica), 5; and Agar, 30at a pH of 6.5 by spread plate method and incubated for 7days at 25°c. The colonies having holozones were subcultured to obtain pure isolates after which the pure culture was then inoculated on broth medium of Aleksandrov medium. The broth was incubated for 7days at 25°c and then mixed with the perlite carrier.

Perlite carrier was purchased from a commercial vendor and then 700g was weighed and autoclaved. Later, it was transferred into laminar hood and inoculated with the isolated pure broth cultures of the *Rhizobium*, *Azotobacter*, PSB and KSB at ratio 1:2 to make a consortium. The 10^8 viable cells/gm was used that is (50%) 50ml of each broth was mixed with 100g of perlite. The same procedure was followed for all bacterial isolates individually and packed in polythene bags. The carrier was incubated for 21 days at room temperature. The carrier was applied on the field of beans and corn plants after testing for the viability of bacterial isolates inoculated.

At first the carrier with viable bacteria were mixed with sterile distilled water in the ratio 1:2 to make it into slurry. This was applied to the seeds. The seeds of beans and maize (*Phaseolus vulgaris and Zea mays*) were prepared by mixing each inoculated perlite carriers with the seeds (30%). Each of *Rhizobium*, *Azotobacter*, consortium (mixed preparation containing *Rhizobium*, *Azotobacter*, KSB isolate-1 from tomato(KSB $2T^1$), KSB isolate-2 from tomato (KSB $3T^1$), PSB isolate-1 from tomato (PSB T^1), and PSB isolate-2 (PSB T^2), and Control (perlite only) treatments were used for bean plants. Consortium, KSB $2T^1$, KSB $3T^1$, PSB T^1, PSB T^2 and control treatments were followed for maize plants. The treated bean and maize seeds were planted and observed for growth, shoot length, flowering, pod formation, yield as well as growth rate and results were recorded. The average of the 10 replicates is calculated along with statistical analysis using students't-test

RESULTS AND DISCUSSION

The *Rhizobium* inoculum on YEMA medium with bromophenol blue showed only one type of colony with smooth milky white, opaque, elevated and raised colonies with irregular edges (Fig 2). The microscopic observation after grams staining showed a pink color, rod shaped, gram negative bacteria which was in line with the results obtained by Shahzad*et al.* (2012) confirming the strain to be *Rhizobium meliloti.*

Fig. 2.

Morphological observation of the non-symbiotic bacterial inoculums on Ashybys Agar medium from both tomato and capsicum showed the same properties of whitish, smooth irregular, shining and elevated colonies. When observed under the microscope, it was also observed to have a pink color rod shape gram negative character which coincides with the results obtained by Jimenez et. al. (2011) suggesting the morphological and microscopic characteristics of *Azotobacter vineland i*(Fig 3).

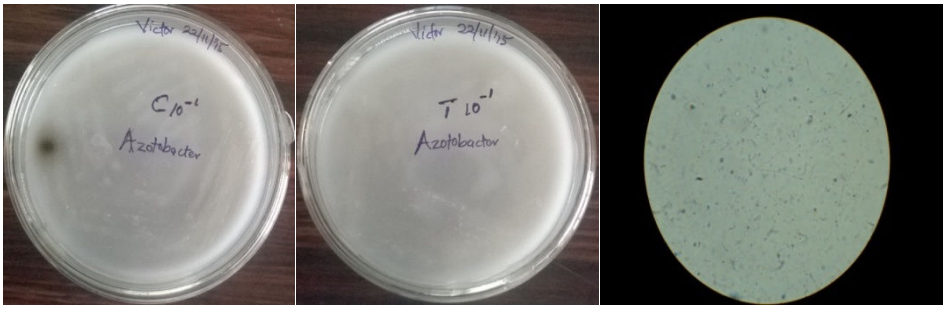

Fig. 3.

The counting of the colonies formed on the plate of Pikovskaya medium for the PSB isolated from tomato revealed 80 colonies out of which only five colonies showed holozones formation and were designated PSB(T^1,T^2, T^3, T^4, T^5). Among these, T^2, T^3, T^5 colonies were very similar to each other such that the number of the cultures for PSB were now grouped into three (PSB T^1 and PSB T^2). These isolates were selected because they showed better clear zones. Characterization of the PSB on Kings' B medium confirmed T^1 to be *Pseudomonas fluorescence* as it illuminates when the medium containing the isolate was briefly exposed to ultraviolet light under UV trans-illuminator (Fig 4) while others gave a negative result to the test.

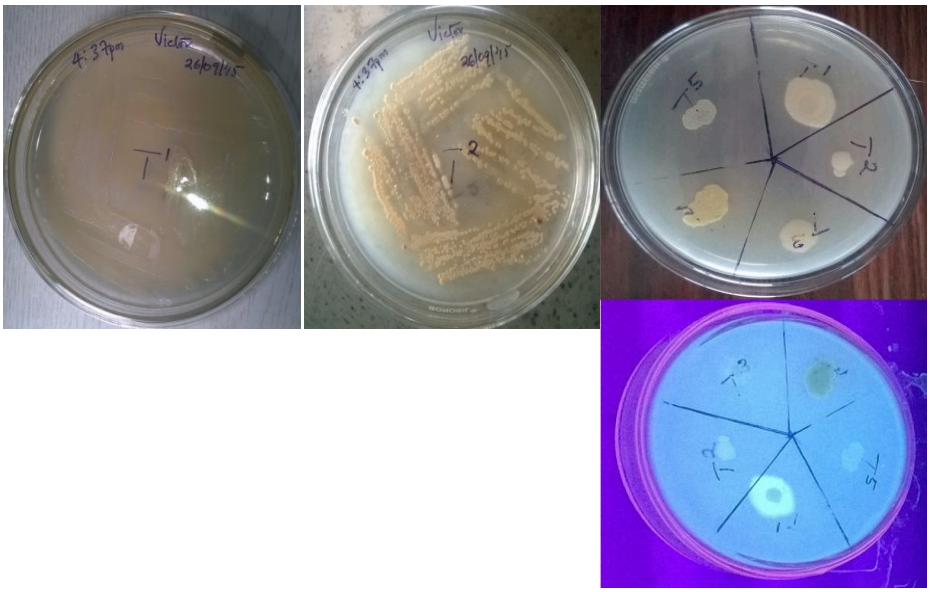

Fig. 4.

All the initial five PSB isolates tested positive on starch hydrolysis and negative on catalase test. These results are in line with the results obtained by Rajan,*et al.* (2015).

All the bacteria isolated in this study showed capabilities to solubilize phosphate as some produced white, yellow and some brown colonies. While T^1 was confirmed to be *Pseudomonas fluorescence*, T^4 proved to be a *Bacillus*spp, while T^2, T^3, and T^5 belong to *Streptococcus* spp.

The isolates of the KSB on the Aleksandrov agar medium after the incubation period, showed three coloniess exhibiting zone clearance of potassium solubilisation (Fig 5).

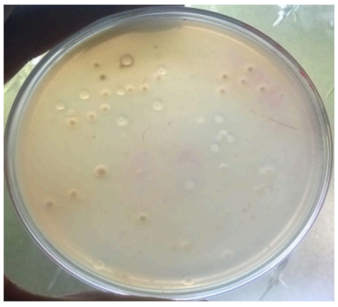

Fig. 5.

Sub-culturing of the three colonies that exhibited halo-zone formation revealed same morphological and microscopic features. The morphological characteristics of the colonies include smooth, small, creamy, transparent and elevated colonies which appeared to be small rod shaped, non-sporulating, motile gram negative bacteria. This result is consistent with the findings of Prajapati and Modi (2012). This isolate is presumed to be *Bacillus* spp.as its exact identification could not be ascertained.

Application of the biofertilizers on beans and maize plants showed tremendous positive result at a signicant level of $p<0.05$. It was observed that the bean seed treated with the biofertilizers (*Rhizobium*, *Azotobacter* and mixed biofertilizers) started germination on the third day after planting while for the control (only beans), germination was noticed on the sixth day. The shoot length biofertilizer treated bean plants showed a significant increase when compared with control at $p<0.05$ (Table 1) and (Fig. 6 and 7).

Table 1.

	T-test for BEANS SHOOT LENGTH (cm)			
Week	Azotobact	Rhizobiun	Mixture	Control
1	8.78	4.44	12.09	3.56
2	16.4	15.04	16.99	13.1
3	35.61	37.92	37.28	35.17
4	40.25	42.2	45.05	39.05
5	47	48.4	49.5	41.7
p value =	0.036667	0.035025	0.009087	

The seeds treated with mixed biofertilizers (*Rhizobium*, *Azotobacter*, PSB and KSB) showed a more significant increase when compared with control ($p<<0.05$) (Table 2).

Table 2.

	T-Test Comparison of Pod numbers			
	Rhizobium	Azotobacter	Mixed	Control
	4	4	4	2
	3	4	5	4
	3	3	4	1
	4	3	5	1
	4	2	7	2
	4	3	6	1
	3	4	5	2
	4	3	4	1
	3	3	5	3
	2	4	5	2
Average	3.4	3.3	5	1.9
p values =	0.009106154	0.001322951	3.2622E-05	

The pods produced in bacterial treatments were far better and significant in comparison to the control at $p<0.05$. The mixed biofertilizers resulted in bigger and more number of pods compared to the other treatments and control.

The plants in mixed biofertlizer treatment flowered on 26th day after planting while in both *Rhizobium* and *Azotobacter* treatments, flowering started on the 30th day, and in the control the flowering was on the 37th day after planting. Singly treated biofertilizers were PSBT^{1}, PSBT^{2}, KSB2T^{1} and KSB3T^{1}. The treatment with single bacterial culture on maize plants showed no significant difference when compared to the control (Table 3).

Table 3.

		T-test for CORN SHOOT LENGTH (cm)				
Week	Mixed	KSB2T1	KSB3T1	PSBT1	PSBT2	Contol
1	1.22	1.13	0.92	0.86	0.87	0.79
2	5.67	3.61	2.88	3.15	2.98	3.54
3	12.52	9.62	8.9	9.3	10.55	11.28
4	22.45	13.2	12.65	12.4	13.15	15.5
5	44.8	21.15	16.43	17.5	18	22.2
6	47.5	22.75	28.7	29.15	33.8	30.55
7	75	38.35	39.8	40.3	44	54.8
p value =	0.031932	0.123067	0.083722	0.098211	0.23928	

All bacterial treatments also showed high yield and healthy green leafy plants in comparison to the control which had lower yield and folded green leaves (Fig. 6).

Pérez-Monta˜no, et al. (2014) stated that plant growth-promoting rhizobacteria (PGPR) are free-living bacteria which actively colonize plant roots, exerting beneficial effects on plant development in their review. Earlier findings prove that the plants benefit from host–bacterial (PGPR) interactions which include plant health and growth, suppress disease-causing microbes and accelerate nutrient availability and assimilation (Yang,*et al*. 2009). This may be due to the fact that biofertilizers tend to protect plant from infections while supplying nutrients to the plants.

The maize plant treated in this study with a mixture of the biofertilizers showed a positive significance ($p<0.05$) in the shoot length when compared to the control and single treated plants.

The maize plant treated with mixed biofertilizer also showed stalk formation in the 6th week after planting. The leaves were lush green and the plant looks very healthy while plants that were treated with single strains showed yellow and curled leaves indicating nutrient deficiency and probably infection (Fig. 6). The control plant showed a mixture of both green and yellow leaves. The bacterial genera used in this investigation were reported as phosphate solubilizing bacteria (Mehnaz and Lazarovits, 2006;Sudhakar,*et al*.2000). In addition root colonizing bacteria like, *Azotobacter*, and *Pseudomonas* species are known to produce growth hormones which often leads to increase root and shoot growth. These reports explain the increased growth and productivity of the bean and maize plants in this study. The *Azotobacter*, *Bacillus*, and *Psuedomonas* known to produce certain plant hormones that directly affects the plant growth and certain antibiotics which inhibits the infection of pathogens (Paula Garcia-Fraile,*et al* 2015). In the present study when consortium of bacterial cultures was inoculated, a multiple factors at the site of action contributed to the better health, growth and productivity of bean and corn plants. This combined effect is lacking when single bacterial inoculation was used in this study.

CONCLUSION

The results of this work confirmed that any of the nitrogen biofertlizers (*Rhizobium and Azotobacter*) can be used for beans plants however a mixture of biofertilizer (*Rhizobium, Azotobacter*, PSB and KSB) has a potential of giving better results than a single biofertilizer application. The same is also true for the maize plants as the plant will have access to nitrogen from the non-symbiotic nitrogen fixing bacteria as well as phosphorus and potassium form PSB and KSB respectively. The plants also have a higher range of protection from diseases as a mixture of biofertilizer application tends to confer protection for the plants. Hence there is need to understand the relationship between all the microbes used for biofertilizer in order to get a better yield and nutrient supply to the soil at the same time the plant.

Fig. 6.

Comparison in the plant growth and health between the inoculated and non-inoculated plants with biofertilizers is showed in Fig.6.

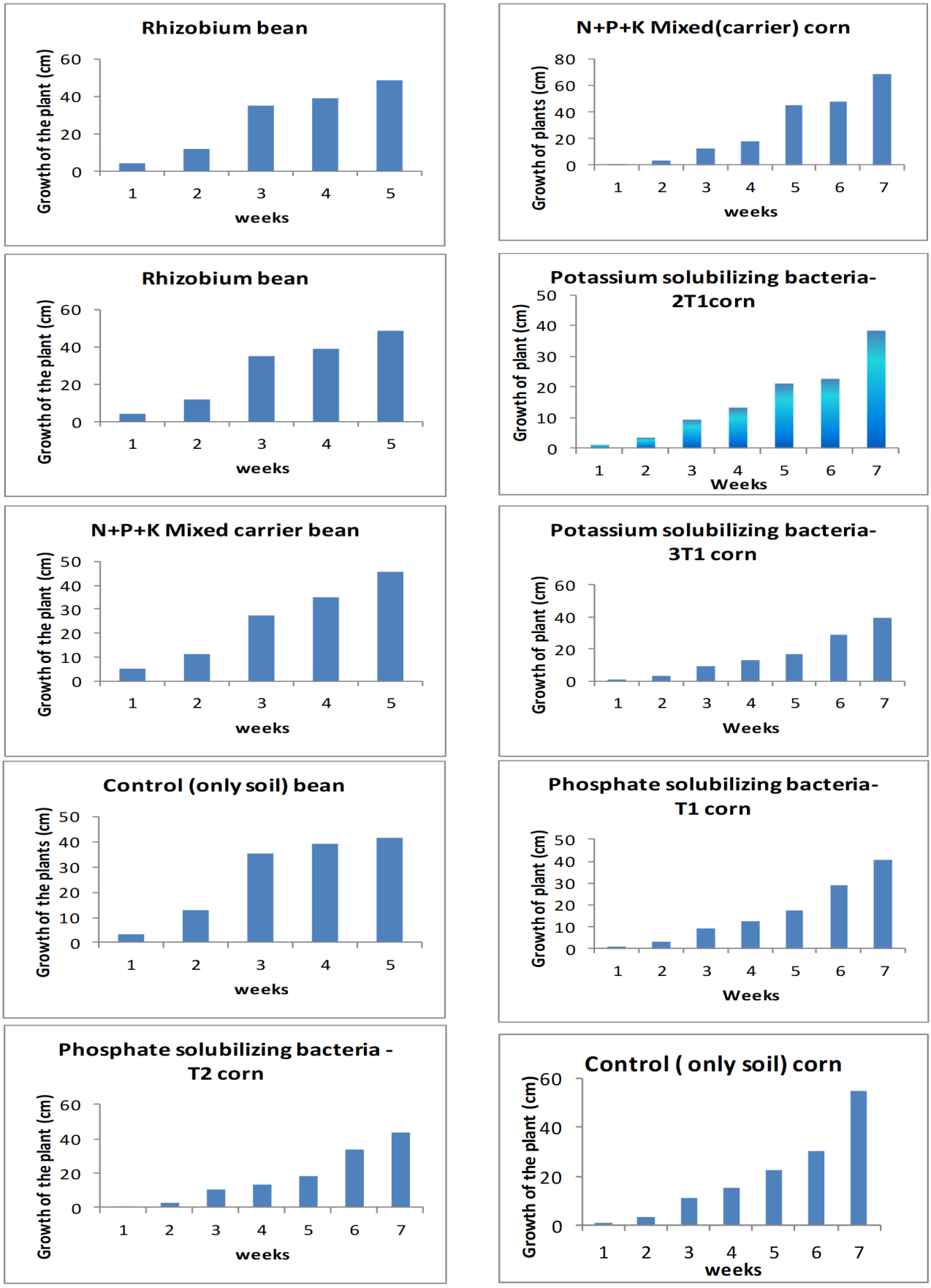

Fig. 7. Average shoot length of the plants per week

BIOGRAPHY:

Dr. Sujana is the Head of the Research and Development department of the Prof. G. M.Reddy Research institute where she coordinates research activities pioneered by the institute and she has been a guide to over 30 students at both undergraduate and postgraduate levels. She's happily married and blessed with a child. She and her family reside at Uppal.

REFERENCES

[1] Fischer, S. E., Fischer, S. I., Magris, S. and Mori, G. B. (2007). Isolation and characterization of bacteria from the rhizosphere of wheat. *World J MicrobiolBiotechnol* 23:895–903.

[2] Gomare, K. S., Mese, M. and Shetkar, Y. (2013). Isolation of *Rhizobium* and cost effective production of biofertilizers. *Indian J.L.Sci.*2(2):49-53.

[3] Jiménez, D. J., Montaña, J. S. and Martínez, M. M. (2011). Characterization of free Nitrogen fixing bacteriaof the genus Azotobacter in Organic Vegetable grown Columbian soil. *Brazilian Journal of Microbiology*. 42:846 – 858. (Title missing)

[4] Li, F. C., Li, S., Yang, Y. Z. and Cheng, L. J. (2006). Advances in the study of weathering products of primary silicate minerals, exemplified by mica and feldspar. *Acta Petrol Mineral*25: 440–448.

[5] Lian, B., Fu, P. Q., Mo, D. M. and C. Q. Liu. (2002). A comprehensive review of the mechanism of potassium release by silicate bacteria. *Acta Mineral Sinica* 22: 179–183.

[6] Liu, D., Lian, B. and Dong, H. (2012). Isolation of *Paenibacillus*sp. and assessment of its potential for enhancing mineral weathering. *Geomicrobiology Journal* 29: 413–421.

[7] Mehnaz, S. and Lazarovits, G. (2006). Inoculation effects of *Pseudomonasputida*, *Gluconacetobacterazotocaptans*, and *Azospirillum*lipoferum on corn plant growth under greenhouseconditions. *MicrobEcol* 51:326–35.

[8] Odame, H. (1997). Biofertilizer in Kenya: Research, production and extension dilemmas. *Biotechnol. Dev. Monit*. 30:2023.

[9] Okon, Y. (1985). *Azospirillum* as a potential inoculants for agriculture. *Trends Biotechnol*. 3(9):223-228.

[10] Parmar, P., and Sindhu, S. S. (2013). Potassium solubilisation by rhizosphere bacteria: influence of nutritional and environmental conditions. *Journal of Microbiology Research*. 3(1):25-31.

[11] Paula García-Fraile, Esther Menéndez and Raúl Rivas. (2015). Role of bacterial biofertilizers in agriculture and forestry. *Bioengineering* 2 (3):183-205.

[12] Prajapati, K. B. and Modi, H. A. (2012). Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. *CIBTech J Microbiol.* 1: 8-14.

[13] Ranjan, A., Mahalakshmi, M. R., and Sridevi, M., (2013). Isolation and Characterization of phosphate solublizing bacterial species from different crop fields of Salem, Tamil Nadu, India. *Int'l J. of Nutrition, Pharmacology, Neurological Diseases*. 3:29-33

[14] Ritika, B. and Utpal, D. (2014). Biofertilizer, a way towards organic agriculture: A review. *African Journal of Microbiology Research*. 8 (24):2332–2342.

[15] Rokhzadi, A., Asgharzadeh, A., Darvish, F., Nourmohammadi, G. and Majidi, E. (2008). Influence of plant growth-promoting rhizobacteria on dry matter accumulation and yield of chickpea (*Cicer arietinum* L.) under field condition. *Am-Euras. J. Agric. Environ. Sci.* 3(2): 253-257.

[16] Shahzad, F., Shafee, M., Abbas, F., Babar, S., Tariq, M. M., and Ahmad, Z. (2012). Isolation and biochemical characterization of *Rhizobium meliloti* from root nodules of Alfalfa (*Medicagosativa*). *J. Animal Plant Sci.* 22(2): 522-524.

[17] Sharma, S., Kumar, V. and R. B. Tripathi. (2011). Isolation of Phosphate Solubilizing microorganisms (PSMs) from soil. *Journal of Microbiology and Biotechnology Research*1(2):90-95.

[18] Sheng, X.F. (2005). Growth promotion and increased potassium uptake of cotton and rape by a potassium releasing strain of *Bacillus edaphicus*. *Soil Biology and Biochemistry* 37: 1918-1922.

[19] Sparks, D. L. and P. M. Huang. (1985). Physical chemistry of soil potassium. In: *Potassium in agriculture.* R.D. Munson, (ed). American Society of Agronomy Journal, USA. pp. 201-276.

[20] Sudhakar, P., Chattopadhyay, G. N., Gangwar, S. K. and J. K.Ghosh. (2000). Effect of foliar application *of Azotobacter*, *Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). *J Agric Sci.* 134:227–34.

[21] Yang, J., Kloepper J. W. and C. M.Ryu. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14:1–4.

[22] Yosef, B. B., Rogers, R. D., Wolfram, J. H. and Richman, E. (1999). *Pseudomonas cepacia*–mediated Rock Phosphate Solubilization in Kaolinite and Montmorillonite Suspensions. *J. Soil sci. of America* 63: 1703-1708.