Synergistic Effect of Biofertilizer Containing Nitrogen Fixing and Zinc Solubilizing Bacteria on Plant Growth

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ABSTRACT

Biofertilizer is regarded as a potential alternative to chemical fertilizer for aiding in improving soil fertility, crop yield and in establishing sustainable farming. The objective of current study is to observe the synergistic effect of biofertilizer containing nitrogen fixing and zinc solubilizing bacteria on plant growth. Nitrogen fixing biofertilizer prepared and its nitrogen fixing efficiency is determined. Pre-prepared zinc solubiling biofertilizer is used in this study. Pot plant trials is conducted on okra (*Abelmoschus esculentus*) plants, fenugreek (*Trigonella foenum-graecum L.*) plants and seedlings of chili (*Capsicum annuum L.*). The application treatments included control, treatment 1 with nitrogen fixing biofertilizer and Treatment 2 with both biofertilizers. The plants co-inoculated with both fertilizers exhibited significant increase in length of shoot, length of root, number of leaves, width and length of leaves when compared with controls. This showed that the co- inoculation of plants with nitrogen fixing and zinc solubilizing biofertilizer provide an alternative to the use of chemical fertilizers the excessive use of which has led to greenhouse gas emissions and water leaching.

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INTRODUCTION

The rapid increase in population and reduction in fertile land should be countered with increase in soil productivity. All over the world, groundwater pollution has increased due to toxic chemicals of chemical fertilizers and pesticides. To provide safe alternatives to chemical biofertilizers and pesticides, scientists are forced to look into natural methods. Besides, the chemical fertilizers production could not meet the demand for growing agricultural development. This has led to the use of biofertilizers and nutrients obtained through recycling, manuring and wastes. Formulations of either alive or the dormant cells of different beneficial species of bacteria that fix nitrogen and microorganisms that solubilize phosphorus, are utilized for inoculation on soils and seeds to enhance nutrient availability to plants and are known as biofertilizers (Somani *et al.*, 2011). There are different types of biofertilizers based on metabolisms and properties. Most often utilized include, N fixing biofertilizer, K solubilizing biofertilizer, P solubilizing biofertilizer alongwith the microorganisms that promote growth of plants. Zinc solubilizing bacteria release various organic acids which can solubilize fixed zinc into its available form hence increasing yield, fertility level of soil and promoting plant growth. In several crops rotation and systems the native zinc solubilizing bacteria have been proved far more beneficial realitive to the foreign microbes (Kumar *et al.*, 2019).

Commercially microbial biofertilizers were launched by rhizobium based microbial inoculant known as Nitrogin. It was the first introduced biofertilizer in world (Patil and Solanki, 2016). Commonly used microorganisms for biofertilizers are microorganisms that fix N2, cynao bacteria that can fix N2, the bacteria which dissolve phosphate also the arbuscular mycorrhizal fungus.

Growth promoting hormones producing bacteria and cellulytic microbes can also be present in biofertilizers. On application to soil the biological processes of plants are increased aiding the maturity of plants. Hence, sustainability of the microbial inoculants during production and application is important to ensure plant growth promoting property of biofertilizer.

Materials and methods Sample Collection

The soil sample was taken from grounds of Food and Biotechnology Research Center of PCSIR, Labs. Complex, Lahore, Pakistan. (Hajra *et al*, 2023).

Purification of culture

Purification was then carried out by streaking on selective nitrogen free (N-free) *Azotobacter* medium until single isolated bacterial colonies were observed. The process of identifying the isolated strains of bacteria was done after comparing their morphologic and organic properties to the standards. The microscopic analysis was performed using staining technique of gram staining and microscopy and other tests were performed on isolated strains to identify them (Usman *et al.*, 2023).

Microscopic analysis of bacterial strain

The microscopic analysis was done by use of a microscope.

Biochemical analysis of bacterial strain

The biochemical analysis of the bacterial strain was carried out by performing some biochemical tests.

Catalase test:

This test was conducted to find out about the capability of selected strain to produce catalase enzyme. This enzyme has ability to breakdown the hydroxyl peroxide compound to H_2O and O resulting in release of bubbles. Bacterial culture is considered positive for catalase test if bubbles are observed when bacterial suspension is added to H_2O_2. .

Oxidase test:

This test was performed for determining the isolated bacterial strains capacity to synthesize CcO enzyme. If the color changes to blue the bacterial strain is considered oxidase positive (Talaiekhozani, 2013).

Inoculum preparation

The Stock culture was maintained in nutrient broth and the glycerol cultures of the nutrient broth were stored at -80°C. *Azotobacter* inoculum was prepared in the Erlenmeyer flasks using

selective media which is nitrogen free *Azotobacter* medium and then was incubated for 24 hours at 30°C in rotary shaker at 360 rpm (Din *et al.*, 2019).

Production of Azotobacter on small scale

For the small scale fermentation of inoculum, total 1 Liter Nitrogen free *Azotobacter* media was made in the 1 Liter bioreactor. Then 50 ml of the *Azotobacter* inoculum which was maintained in the Nitrogen free media was transferred to the bioreactor. For 3 days, Every 24 hours samples were taken from the bioreactor to be analyzed for the nitrogen.

Screening of nitrogen fixation activity

The efficiency of Azotobacter in fixing nitrogen equals milligrams of N which are made per the milligrams of C which are utilized. The nitrogen fixing efficiency was evaluated through the kjeldahl method.

Physical analysis of liquid inoculum

The liquid inoculum (Biofertilizer) was analyzed for its pH. Its color was noted and the odor of liquid inoculum was also observed (Ngampimol and Kunathigan, 2008).

Microbial analysis of liquid inoculum

For the microbial analysis of liquid biofertilizer the plate counting technique was used. It is a method which is used to analyze the amount of viable bacterial cells or colony forming unit cfu which can enhance and form colonies on solid agar media.

Evaluation of biofertilizers

The Outcome of the biofertilizers on length of plant, width and the length of plant leaf was tested and evaluated on plants. Different treatments were applied. In the first treatment (T1), soil was inoculated with 15ml nitrogen fixing biofertilizer (NFB), in the second treatment (T2), soil was coinoculated using 15ml nitrogen fixing biofertilizer and 15ml zinc solubilizing biofertilizer (ZSB) which was obtained from FBRC of PCSIR, Lab. Lahore, Pakistan. And untreated soil with same number of seeds was used as control (C) (Hajra *et al.*, 2023).

Seed germination study

The study of seed germination was performed in plant pots (Ansari *et al.*, 2015). The time taken by different plants for germination varies.

Statistical analysis

All studies were conducted in triplicates. The data presented was mean of all replicates with ± indicating standard deviation. One-way ANOVA test was also done, the P value was than calculated for indicating significant variation among the applied trials and standard the control.

Results Isolation of nitrogen fixing bacteria

The isolated white sticky colonies were noted after primary isolation. For this purpose of isolation and the identification of nitrogen fixing bacteria *Azotobacter* the sample was isolated on selective nitrogen free *Azotobacter* medium. A series of dilutions were made of soil from 10-1 - 10-7 on the *Azotobacter* media. Spread plate method was used and plates with the soil sample were incubated at 30℃ for 24h.

Screening of selected colonies

The bacterial colony was selected and further cultured in the selective Nitrogen free *Azotobacter* media. Isolation was done by utilizing the streak plate method and pure culture was obtained in form of isolated single white sticky colonies.

Microscopic analysis of bacterial strain

Screening of the nitrogen fixing bacterial culture was done by gram staining and observing the smear under microscope at 100x and oil emulsion. The bacterial cells were observed as oval and pink in color which shows them as gram negative bacteria.

Biochemical analysis of bacterial strain

For the purpose of identifying the bacterial culture biochemical tests were performed. The catalase test was positive indicated by immediate formation of bubbles and oxidase test was also positive shown by appearance of purple color which is true for *Azotobacter* bacteria.

Preparation of stock culture

Sub-culturing was also done and the isolated bacterial culture was preserved in nutrient broth at

4℃ and also than stored at -80℃ in nutrient broth containing about 20% glycerol.

Inoculum preparation

The liquid inoculum was than prepared in the Erlenmeyer flasks by using *Azotobacter* medium. It was incubated for 24 hours at 30℃.

Production of *Azotobacter* liquid inoculum on small scale

Biofertilizer was produced on small scale after fermentation on Bioreactor. For its production 1 liter of *Azotobacter* medium was prepared in bioreactor and 50ml of *Azotobacter* inoculum which was developed in *Azotobacter* medium was transferred to the bioreactor. Samples were collected every day from bioreactor for determination of nitrogen fixing efficiency of *Azotobacter*.

Screening of nitrogen fixation activity

The nitrogen fixing activity of liquid inoculum (biofertilizer) was determined. Samples were collected after every 24 h for 3 days during fermentation. The results indicated an increase in carbon utilization and consequently an increase in nitrogen fixing efficiency of *Azotobacter* (Table

1).

Table 1. Nitrogen fixing efficiency of Azotobacter liquid inoculum at different temperatures during fermentation in bioreactor.

Fermentation time	Measured nitrogen (mg/100ml)	Mean of Measured nitrogen (mg/100ml)	Carbon utilized (g/100ml)	Mean of Carbon utilized (g/100ml)	Efficiency (mg of N/ g of C)
24h	17.33	18.21±0.77	0.26	0.39±0.13	46.69
	18.51		0.38		
	18.8		0.53		
48h	20.19	21.08±0.91	0.31	0.42±0.09	50.19
	21.04		0.46		
	22.01		0.49		
72h	22.63		0.39		

	22.91	22.64±0.26	0.42	0.44±0.06	51.45
	22.39		0.51		

The mean values are the average of triplicate values and ± shows standard deviation in the replicate values.

Physical analysis of liquid inoculum

The pH level of the biofertilizer was 7.4. The odor of biofertilizer was strong and pungent and it was turbid and of yellow color in appearance. For the physical analysis of the liquid inoculum (Biofertilizer) all these different parameters were noted.

Microbial analysis of liquid inoculum

Plate counting method was utilized to conduct the microbial analysis of the liquid biofertilizer. The plates with the colonies from 30-300 were used to calculate colony forming unit CFU of the biofertilizer. The CFU of liquid Biofertilizer was $5.14x10^{9}$ which is ideal for Biofertilizer.

Evaluation of biofertilizers

Pot trials of Biofertilizers were conducted on the selected plants. The growth of these plants is represented in Figures 1 –3. The factors that were studied in present study include length of shoot, leaf length, width of leaves, root length and no. of leaves (Table 2-4). A significant increase in length of shoot ($2.46x10^{-9}$, $7.539x10^{-6}$ and $2.68x10^{-10}$), number of leaves ($4.22x10^{-4}$, $1.562x10^{-2}$ and $1.682x10^{-6}$), length of root ($4.42x10^{-8}$, $4.638x10^{-2}$ and $1.951x10^{-7}$), leaf length ($2.77x10^{-5}$, $1.843x10^{-2}$ and $2.7x10^{-2}$), and leaf width ($4.78x10^{-8}$, $3.212x10^{-3}$ and $8.567x10^{-3}$) was observed in Okra, fenugreek and Chili seedlings respectively, as compared to that of controls (Figure 4-13). Additionally, the plant pots co-inoculated with both NFB and ZSB gave significantly better results than of plants only inoculated with nitrogen fixing *Azotobacter* in both types of plants.

Figure 1. The Effect of Biofertilizer application on growth of *Abelmoschus esculentus* (Okra) plants after 10 weeks of sowing seeds. From left to right Control, Treatment 1 with NFB and Treatment 2 plants having both NFB and the ZSB.

Figure 2. Effect of Biofertilizers on growth of *Trigonella foenum-graecum* L. (Fenugreek) plants after 2 weeks of sowing (a) Control plant (b) Treatment 1 plant (c) Treatment 2 plant.

Figure 3. Effect of Biofertilizers on growth of *Capsicum annuum* L. (Chili) seedlings from left to right Control, Treatment 1 and Treatment 2 chili seedlings after application of Biofertilizers.

Germination study

Seed sprouting in pot plants was carried out. The sprouting of seeds was observed about 10 -15 days after sowing in okra plants and after about 3-5 days in fenugreek plant.

Table 2. Impact of Biofertilizers on *Abelmoschus esculentus* (Okra) plants growth parameters after 10 weeks of sowing.

Treatment	Length of Shoot (cm)	Mean of Length of Shoot (cm)	Length of Root (cm)	Mean of root length (cm)	Number of leaves	Mean of number of leaves	Length of leaves (cm)	Mean of length of leave (cm)	Width of Leaves (cm)	Mean of width of leaves (cm)
T1 NFB	25.8	26±0.26	10.8	11±0.26	9	10±1	6.4	7±0.55	8.2	8.4±0.26
	25.9		10.9		10		7.1		8.3	
	26.3		11.3		11		7.5		8.7	
T2 NFB+ZSB	33.6	34±0.36	17.7	18±0.26	13	14±1	10.8	11±0.26	13.8	14±0.26
	34.1		18.1		14		10.9		13.9	
	34.3		18.2		15		11.3		14.3	
Control	15.6	16±0.36	6.5	7±0.43	6	7±1	6.2	6.5±0.43	6.9	7±0.1

	16.1		7.2		7		6.3		7	
	16.3		7.3		8		7		7.1	

Table 3. Effects of Biofertilizers on *Trigonella foenum-graecum* L. (Fenugreek) plants after 2 weeks of sowing seeds.

Treatment	Length of Shoot (cm)	Mean of shoot length	Length of Root (cm)	Mean of root length (cm)	Number of leaves	Mean of number of leaves	Length of leaves (cm)	Mean of leaf length	Width of Leaves (cm)	Mean of leaf width (cm)
T1 NFB	4.9	5±0.1	1	1.1±0.1	1	2±1	0.9	1±0.1	0.7	0.8±0.1
	5		1.1		2		1		0.8	
	5.1		1.2		3		1.1		0.9	
T2 NFB+ZSB	6.8	7±0.2	1.8	1.9±0.1	3	4±1	0.9	1.2±0.26	0.7	1±0.26
	7		1.9		4		1.3		1.1	
	7.2		2		5		1.4		1.2	
Control	3.9	4±0.1	0.5	0.5±0	2	2±0	0.4	0.5±0.1	0.2	0.2±0
	4		0.5		2		0.5		0.2	
	4.1		0.5		2		0.6		0.2	

Depicted values are the average values of three different replicates of same treatment and the ± symbol shows standard deviation in values of replicates.

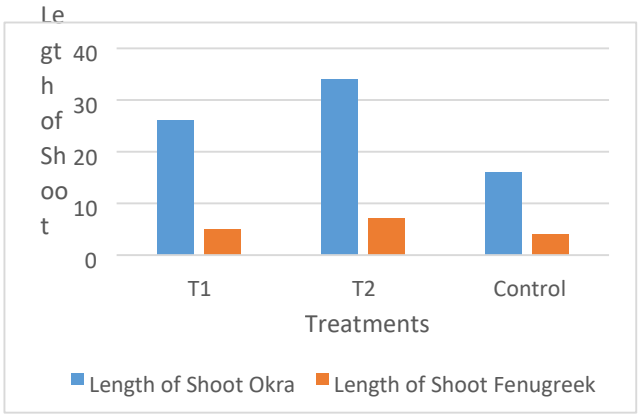

Figure 4. Effect of different biofertilizer treatments on length of plant Shoot.

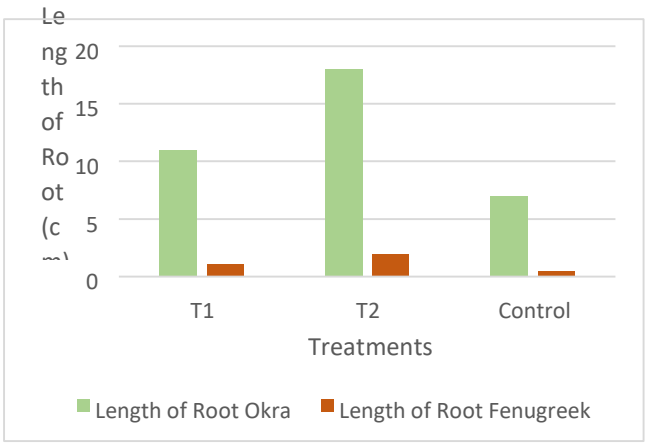

Figure 5. Effect of different biofertilizer treatments on length of plant Root.

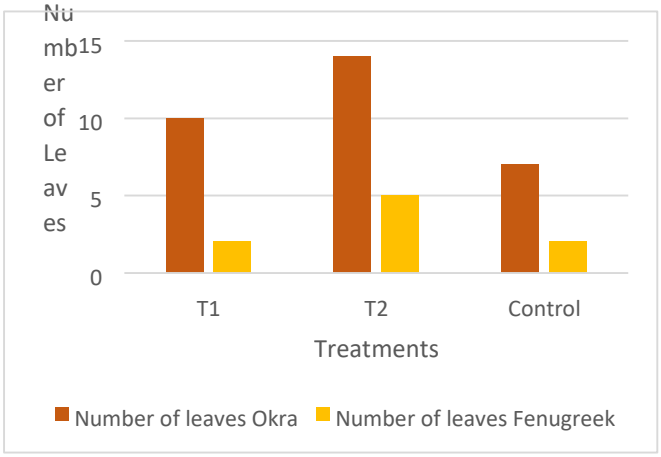

Figure 6. Effect of different biofertilizer treatments on Number of plant leaves.

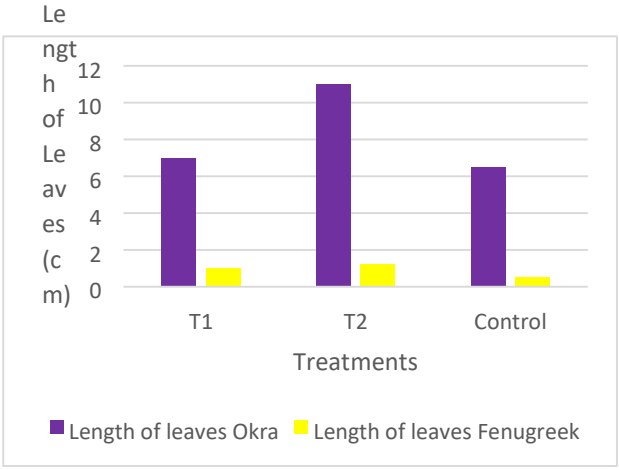

Figure 7. Effect of different biofertilizer treatments on length of plant Leaves.

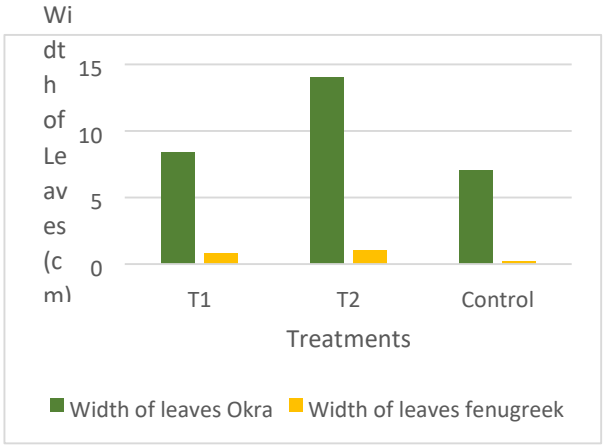

Figure 8. Effect of different biofertilizer treatments on width of plant Leaves.

Table 4. Effects of Biofertilizers on growth of *Capsicum annuum* L. (Chili) seedlings.

Treatment	Length of Shoot (cm)	Mean of shoot length (cm)	Length of Root (cm)	Mean of root length (cm)	Number of leaves	Mean of number of leaves	Length of leaves (cm)	Mean of leaf length	Width of Leaves (cm)	Mean of leaf width (cm)
T1	19.9	20±0.1	5.5	6±0.45	51	52±1	4	3±1	1.3	1.4±0.1
NFB	20		6.1		52		3		1.4	

	20.1		6.4		53		2		1.5	
T2 NFB+ZSB	26.8	27±0.2	13.6	14±0.45	83	88±5	4.8	5±0.17	2.9	3±0.1
	27		13.9		88		5.1		3.1	
	27.2		14.5		93		5.1		3	
Control	15.9	16±0.1	4	4±0	23	26±3	1	2±1	1.1	1.5±0.4
	16		4		26		2		1.5	
	16.1		4		29		3		1.9	

Calculated numbers are average calculations of the three replicates of a treatment and the ± symbol is to indicate the standard deviation in values of replicates.

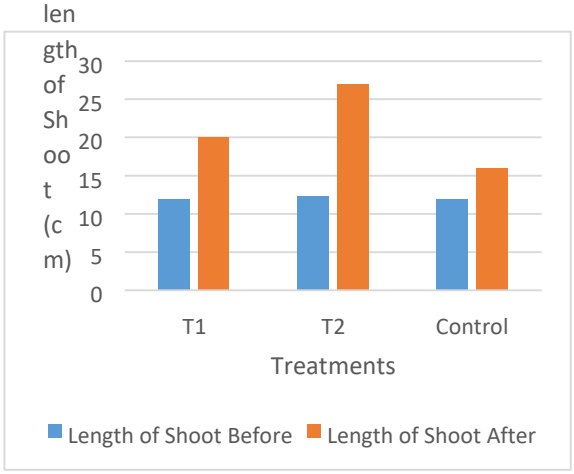

Figure 9. Effect of different biofertilizer treatments on length of chili seedlings Shoot.

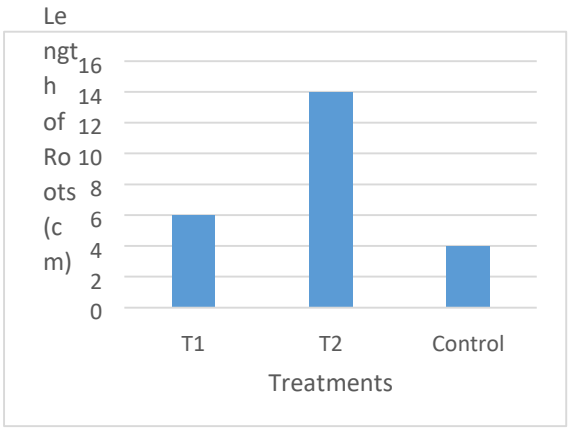

Figure 10. Effect of different biofertilizer treatments on length of Chili seedlings Root.

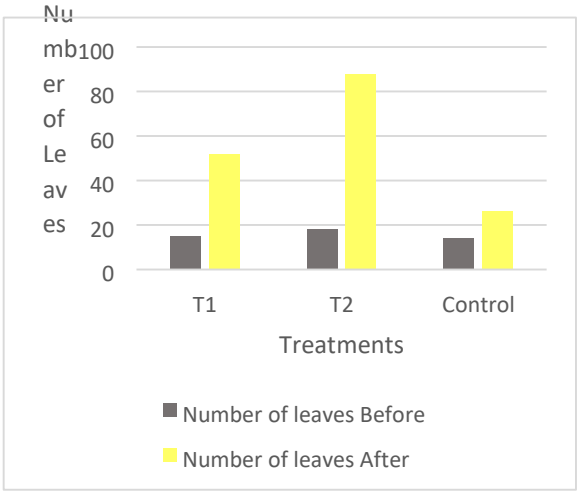

Figure 11. Effect of different biofertilizer treatments on Number of leaves of Chili seedlings.

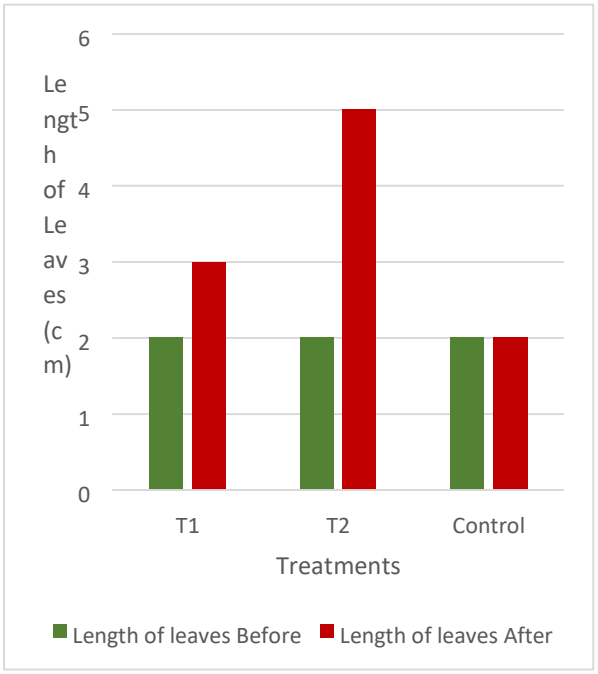

Figure 12. Effect of different biofertilizer treatments on length of Leaves of Chili seedlings.

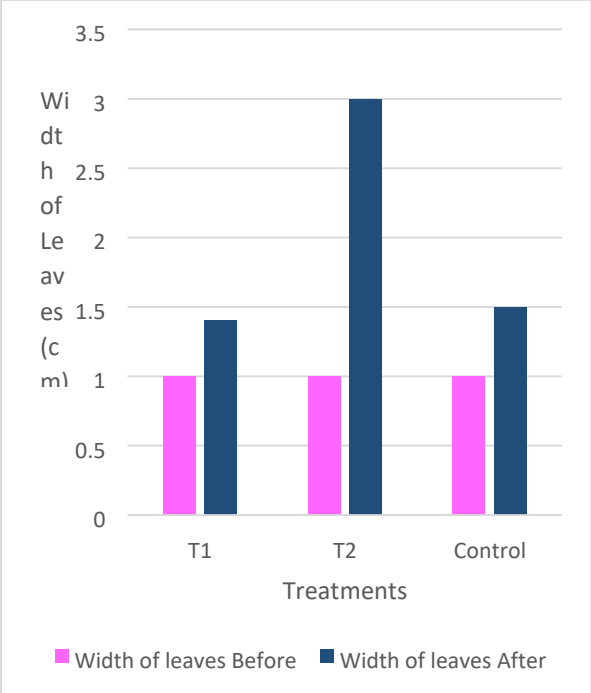

Figure 13. Effect of different biofertilizer treatments on width of Leaves of Chili seedlings.

Discussion

The rapid increase in population throughout the world results in a higher demand of food. Production of the Chemical fertilizer utilizes copious amount of energy, almost 1% of total energy consumed worldwide consequently playing part in the climate change. Hence establishment of maintainable agricultural products and practices is essential for keeping up with the increasing food demands of the constantly expanding world and to minimize environmental damage. Various studies have showed that bacteria can aid in the improvement of crop fields using several mechanisms. It is also possible to utilize a consortia of different microorganisms aiding in several plant benefits to be integrated to combine several microbial factors in one product (García-Fraile *et al.*, 2015). Current experiment shows, a consortia of biofertilizers containing NFB and ZSB was applied in different treatments on plants of Okra, Fenugreek and Chili for observing their influence on plant growth.

The biofertilizers are not a normal culture in broth rather they are special formulations containing nutrients that aid in microbial growth and special protectants which aid in their better shelf life. The liquid inoculants can be synthesized with less energy consumption, less labor and small space. In this study the selected plants were co-inoculated with bacteria that dissolve zinc and the NFB and increase in growing parameters in the plants relatively to control showed that the combination of these microbial inoculants is beneficial to plant growth.

For the purpose of quality control of biofertilizers the viable cell count is a key factor. A standard was developed in the developed countries to have regulations for the quality of inoculants. The

quantity of plant rhizobacteria in inoculant is between 10^7- 10^9 cfu/g of the inoculant. To count the viable cells common methods used include the plate counting method, ELISA, most probable no and immunoblot. The indian ministry of the agriculture and cooperation have given a specific standard for *Azospirillum, Rhizobium and Azotobacter.* For Azotobacter, The bacterial inoculants must have at least cfu of 10^8 per an ml of inoculant. The carrier must be in the form of powder or granules. It can also be in form of liquid inoculant. The pH level range is 6.5 – 7.5 and the percentage efficiency of strain must be able to fix minimum of 10 miligram mg of the N per a g of Sugar consumed (Brahmaprakash *et al.*, 2012).

In this study, the azotobacter biofertilizer produced had cfu of 5.14 x 10^9 which is good cfu level for biofertilizer. It was in form of liquid microbial inoculant. The pH level was 7.4 and it was able to fix an efficient amount of nitrogen.

The integration of seeds with zinc solubilizing rhizobacteria resulted in the enhancement in plant height, area of the leaf and dry weight of plant. Similar results were noted in plant growth parameters when inoculated with *Azospirillum, Azotobacter* and *Pseudomonas* strains. These results support the results of current study which depicted that there is a very good influence on plant development when co –inoculated with NFB and ZSB.

CONCLUSION

In this study nitrogen fixing biofertilizer (*Azotobacter*) has been prepared by a method which is both cost effective and suitable for sustainable agriculture. The better plant growth than control showed that the consortia of nitrogen fixing bacteria and zinc solubilizing bacteria had no harmful effect on selected plants rather they are beneficial to plants and are able to be used as an alternative to chemical fertilizers to augment plant growth.

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