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" Isolation and Screening of Zinc Solubilizing Rhizosphere Bacteria and Their Impact on Growth of *Vigna unguiculata* (Black Eyed Pea Plant) "

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Abstract:

Zinc is one of the eight essential trace elements that plants need for normal growth and reproduction. Plants require Zinc in small but critical concentrations. Zinc is present in soil in their complex organic form, but plants require their simple ionic form for fulfilling their requirements. Soil microorganisms play a crucial role in solubilization and mobilization of this element for plant growth and increase crop yield. In the present investigation, there were 5 Zinc Solubilizing Bacterial isolates (ZnSB) obtained from *Solanum lycopersicum* (Tomato), *Zea maize* (Maize), and *Sorghum bicolour* (Sorgum) rhizosphere soil samples, using growth medium supplemented with insoluble source of Zinc such as Zinc oxide (ZnO) 0.1%. All ZnSB isolates were further tested for their identification up to genus level in VITEK 2 automated microbiology system and results revealed that they all were *Bacillus thuringiensis*. They are further investigated for their biochemical activities and effect on *Vigna unguiculata* **(**Black eyed pea) plant's growth. Experiment revealed that all 5 ZnSB isolates were promising in exhibiting almost all Plant growth promoting characters. As Plant Growth Promoting Rhizosphere (PGPR) bactetia are environmental friendly and offer sustainable approach to increase production of crops and health. Therefore, these isolates can be utilized for biofertilizer formulation under local agroclimatic conditions which decreases the application of chemical fertilizers.

Keywords: Zinc as essential trace element, Zinc solubilizing rhizosphere bacteria, *Solanum lycopersicum* (Tomato), *Zea maize* (Maize), and *Sorghum bicolor* (Sorgum), Experimental *Vigna unguiculata* (L.) Walp. (Black eyed pea) Plant, Plant Growth Promoting Rhizosphere (PGPR) Bacteria, Biofertilizer

1. INTRODUCTION

The plants require several macro and micro nutrients for their growth and reproduction. These nutrients supplemented through inorganic or organic forms are taken up by the plant roots along with water. Zinc (Zn) is one of the essential micronutrient required for optimum plant growth and plays a vital role in metabolism (Hughes and Poole, 1989). Zinc is essential for the normal healthy growth and reproduction of plants, animals and humans and when the supply of plant-available Zinc is inadequate, crop yields are reduced and the quality of crop products is frequently impaired (Alloway, 2004).

In plants, Zinc plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and proteins in many important biochemical pathways and these are mainly concerned with: carbohydrate metabolism, both in photosynthesis and in the conversion of sugars to starch, protein metabolism, and acts as a significant anti-oxidant, Zn-finger transcription factors play an important role in the normal development of floral tissues, flowering, fertilization, fruiting (Epstein and Bloom, 2005), auxin (growth regulator) metabolism (Alloway, 2004), pollen formation, the maintenance of the integrity of biological membranes, and the resistance to infection by certain pathogens (Singh et al., 2005).

Plants can uptake Zinc as divalent cation (Kabata-Pendias and Pendias, 2001) but only a very minor portion of total Zinc is present in soil solution as soluble form. Rest of the Zinc is in the form of insoluble complexes and minerals like ZnO, $ZnCO_3$ and Zinc phosphate (Alloway, 2008). This could only be compensated by the application of costly chemical fertilizers, either as foliar or soil applications (Reyes and Brinkman, 1989). But indiscriminate use of fertilizers, has led to substantial pollution of soil, air and water. Excessive use of these chemicals exerts deleterious effects on soil microorganism, affects the fertility status of soil and also pollutes environment (Youssef and Eissa, 2014). The application of these fertilizers on a long term basis often leads to reduction in pH and exchangeable bases thus making them unavailable to crops and the productivity of crop declines. A better alternative to all these approaches is the use of Zinc solubilizing rhizosphere bacteria. These bacteria improve the plant growth and development by colonizing the rhizosphere and by solubilizing complex Zinc compounds into simpler ones, thus making Zinc available to the plants. Zinc solubilising bacteria are capable of solubilizing ZnO,

$ZnCO_3$ and Zinc phosphate through production and excretion of organic acids (Alexander, 1997).

Keeping in view the above facts, this study was designed to identify and characterize pre-isolated bacteria from the rhizosphere soil of Tomato, Sorgum and Maize for plant growth promoting (PGP) abilities, Zinc solubilizing ability using plate assays and to evaluate the contribution of Zinc solubilizing strains on growth and of *Vigna unguiculata* (Black eyed pea plant) through pot experiments. It is member of the legume family. It is an excellent source of vitamins, minerals, fiber, folate and a good source of iron and has various health benefits like it can improve digestion, prevent anemia, lower blood pressure, increase folate intake, boosts skinand eye health in human (“Show foods” Ndb.nal.usda.gov. Retrieved 2014, 06 April).

2. MATERIALS AND METHODS

2.1. Collection of Soil Samples :

The rhizosphere soils of different crop plants like Tomato, Maize and Sorgum were collected from a depth of 0-10 cm. Soil samples were collected from the agriculture field of Rajkot District, Gujarat, India.

2.2 .Isolation of Zinc Solubilizing Bacteria :

Zinc Solubilizing Agar Medium containing glucose-10.0g, ammonium sulphate-1.0g, potassium chloride-0.2g, dipotassium hydrogen phosphate-0.1g, magnesium sulphate-0.2g, distilled water-1000ml, pH-7.0 and 0.1% Zinc oxide (ZnO) as insoluble Zinc source was prepared (Jorgensen et al., 2015) and autoclaved at 121°C for 20min and then plated out in sterilized glass plates. The soil samples were serially diluted and streaked on the plates. The plates were put in inverted position and incubated for 48 hrs at 35 ºC temperature in Incubator for proper growth of Zinc solubilizing bacteria (Di Simine et al., 1998).

2.3.Screening of Zinc Solubilizing Bacteria :

After 48 hrs incubation, the isolated colonies which were observed on Zinc Solubilization Agar Medium were separated on the basis of colony characteristics and transferred to another plate, which were containing the Zinc Solubilization Agar Medium. For this, the isolated colonies were picked up with the help of wire loop and spotted singly on the plates. The plates were put in inverted position and incubated for 48 hrs at 35 ºC temperature in Incubator for proper growth of Zinc solubilizing bacteria (Di Simine et al., 1998). After 48 hrs, the Zinc solubilizing colonies were screened by observing transparent halo zones on Zinc Solubilization Agar Medium. The diameter of zone of solubilization was measured and expressed in millimeter. The diameter of colony and clear zone around the colony was measured for calculating the solubilization efficiency in percentage and area in mm^2 (Fasim *et al.,* 2002).

Zinc Solubilization Efficiency (ZSE) Equation :

ZSE (Zinc Solubilization Efficiency) = Z / C x 100

Where, Z- Clearance zone of Zinc solubilization including bacterial growth

C- Colony diameter

Area in mm^2 for Zinc Solubilization :

Area mm^2 for Zinc solubilization = $\pi r^2 h$

Where, Π =22/7

r =Radius of zone of solubilization

h = Height of the Zinc agar medium from the bottom of plate

2.4.Colony Characterization of Bacterial Isolates :

The isolated colonies were characterized on the basis of their colony characteristics like : size, shape, margin (border), elevation, texture, opacity, consistency, and pigmentation (Anonymous 1957 and Barthalomew, Mittewer 1950).

2.4.Morphological Characterization of Bacterial Isolates :

Morphological characterization of bacterial isolates was done on the bases of gram staining and capsule staining.

2.5.Biochemical Characterization of Zinc Solubilizing Bacteria :

The isolated colonies for Zinc solubilization were further characterized on the basis of their biochemical characteristics like : Methyl red test, Voges proskauer’s test, Indole production test, Nitrate reduction test, Urea hydrolysis test, Citrate utilization test, Catalase test, Oxidase test, Gelatin hydrolysis test, Hydrogen cyanide test, Ammonia (NH_3) production test, Starch hydrolysis test, pH tolerance test etc. (Cappuccino and Sherman, 1992).

2.6.Plant Pot Trial Experiment :

2.6.1.Sowing of *Vigna unguiculata* (Black Eyed Pea) Seeds and Treatments Given to Seeds :

Based on Zinc solubilizing ability, five strains; ZnSB-1, ZnSB-2, ZnSB-3, ZnSB-7, and ZnSB-10 were selected for pot trial experiment (Cheng et al., 1997).The physical and chemical properties of soil used for pot experiment according to soil health card tested by Department of Agriculture, Gujarat State are given in Table 1. The temperature of climate was 28 ± 2 ºC with 8 to 10 hrs sun light. To provide moisture, plants were watered daily using tap water (Hoagland and Arnon, 1950).The *Vigna unguiculata* (Black Eyed Pea) plant pot trial composition and experimental details are given in Table 2.

2.6.2.Plants Harvesting and Comparison of Different Physical and Chemical Parameters of Plants :

Plants were harvested after 1 month (30 days). Roots were washed using tap water. The plants were measured for the total shoot height, root length, number of leaves, length of leaves, leaf area index, total dry weight of plants and chlorophyll a, chlorophyll b and total chlorophyll content of the leaves. All the given parameters were checked and compared among all the experimental black eyed pea plants.

Formula for Chlorophyll Estimation:

- Chlorophyll a : $11.75 \times A_{663} - 2.35 \times A_{645}$
- Chlorophyll b : $18.61 \times A_{645} - 3.96 \times A_{663}$

3. RESULT AND DISCUSSION

3.1.Screening of Different Isolates for Zinc Solubilization (0.1% ZnO) by Plate Assay After 2 Days Incubation (2 DAI)

Total 5 ZnSB were isolated. The zone of solubilization observed is shown in Fig-1. The qualitative analysis of the isolates for Zn solubilization is presented in Table 3. The comparative estimation of zone of solubilization diameter (mm), solubilization efficiency (%), and area (mm^2) for Zinc solubilization is presented in Histogram 1. The qualitative analysis of ZnSB showed that among all bacterial isolates, all were able to solubilize Zinc from zinc agar media which contains ZnO (0.1%) in the range of 5 mm to 16 mm. ZnSB-1 showed highest zone of solubilization 16 mm and ZnSB-2 & ZnSB-7 showed same zone of solubilization 5 mm which is lowest among all isolates. ZnSB-3 showed 11 mm and ZnSb-7 showed 7 mm zone.

3.2.Biochemical, Morphological, and Colony Characterization of bacterial isolates

Colony characteristics of bacterial isolates is given in Table 4. Morphological characteristics of ZnSB isolates by gram staining and capsule staining procedure is given in Table 5. Biochemical characteristics of ZnSB isolates is given in Table 6. The optical density at 600 nm of ZnSB bacterial isolates at different pH (pH 5, pH 7, pH 9) is given in Table 7.

3.3.Effect of Inoculation of Zinc Solubilising Bacterial Isolates on Growth of *Vigna unguiculata* (Black Eyed Pea) Plants :

Among all ZnSB isolates ZnSB-1 showed highest plant height 7.26 cm, root length 1.96 cm, leaf number 17, leaf length 1.45 cm, leaf area index 0.0138 ds /m^2, dry weight of shoot 0.25 gm, dry weight of root 0.09 gm, chlorophyll a 5.619 mg/l, chlorophyll b 2.275 mg/l and total chlorophyll 7.894 mg/l. ZnSB-10 showed lowest plant height 5.53 cm, root length 1.46 cm, dry weight of shoot 0.13 gm, dry weight of root 0.05 gm, chlorophyll a 2.378 mg/l, chlorophyll b 1.301mg/l and total chlorophyll 3.679 mg/l. Where positive control plant, which is having consortium of all 5 isolates showed highest plant height 7.66 cm, root length 2.13 cm, leaf number 18, leaf length 1.46 cm, leaf area index 0.0140ds /m^2, dry weight of shoot 0.28 gm, dry weight of root 0.1 gm, chlorophyll a 9.661 mg/l, chlorophyll b 4.477 mg/l and total chlorophyll 14.138 mg/l. Un-inoculated control plant showed lowest plant height 3.66 cm, root length 1.13 cm, leaf number 7.33, leaf length 0.65cm, leaf area index 0.0026 ds

/m^2, dry weight of shoot 0.08 gm, dry weight of root 0.03 gm, chlorophyll a 2.178 mg/l , chlorophyll b 1.101mg/l and total chlorophyll 3.279 mg/l.

Effect on growth of *Vigna unguiculata* (black eyed pea) plants by inoculating Zinc solubilizing bacterial isolates is given in Table 8. Comparison of effect on growth of *Vigna unguiculata* (Black Eyed Pea) plants by inoculating ZnSB Isolates is given in Histogram 2 (a), 2(b) and 2(c).

Table 1. The Physical and Chemical Properties of Soil Used for Pot Trial Experiment According to Soil Health Card Tested by Department of Agriculture, Gujarat State

Sr no.	Soil Property	Values
1	Type of soil	Black
2	Nitrogen	Medium
3	Phosphurus	18.00 kg/hec (Low)
4	Potassium	531.00 kg/hec (High)
5	Ph	8.02 (Medium)
6	Total solute: Desisiman/ miter	0.18 Desisiman/ miter (Low)
7	Organic carbon %	1.21 (sufficient)

Table 2. The *Vigna unguiculata* (Black Eyed Pea) Plant Pot Trial Composition and Experimental Details

Experimental Details	Pot Composition	No. of Plants
Un-inoculated Control	Seed + Natural Manure	3
Experimental Plants	Seed + Natural Manure + ZnSB-1	3
	Seed + Natural Manure + ZnSB-2	3
	Seed + Natural Manure + ZnSB-3	3
	Seed + Natural Manure + ZnSB-7	3
	Seed + Natural Manure + ZnSB-10	3
Positive Control	Seed + Natural Manure + ZnSB-1+ ZnSB-2+ ZnSB-3+ ZnSB-7+ ZnSB-10	3
Total no. of Plants		21

Table 3. Screening of Different Isolates for Zinc Solubilization (0.1% ZnO) by Plate Assay After 2 Days Incubation (2 DAI)

Sr No.	Isolates	Zone of Solubilization Diameter (mm)	Colony Diameter (mm)	Total Zone (mm)	Solubilization Efficiency (%) (Z / C x 100)	Area (mm^2) ($\pi r^2 h$)
1	ZnSB-1	23	7	16	328.57	2078.21
2	ZnSB-2	13	8	5	162.5	663.92
3	ZnSB-3	16	5	11	320	1005.71
4	ZnSB-7	10	3	7	333	392.85
5	ZnSB-10	12	7	5	171.42	565.71

Histogram 1 : Comparison of Zone of Solubilization (mm), Solubilization Efficiency (%) and Area (mm^2)Among Isolates

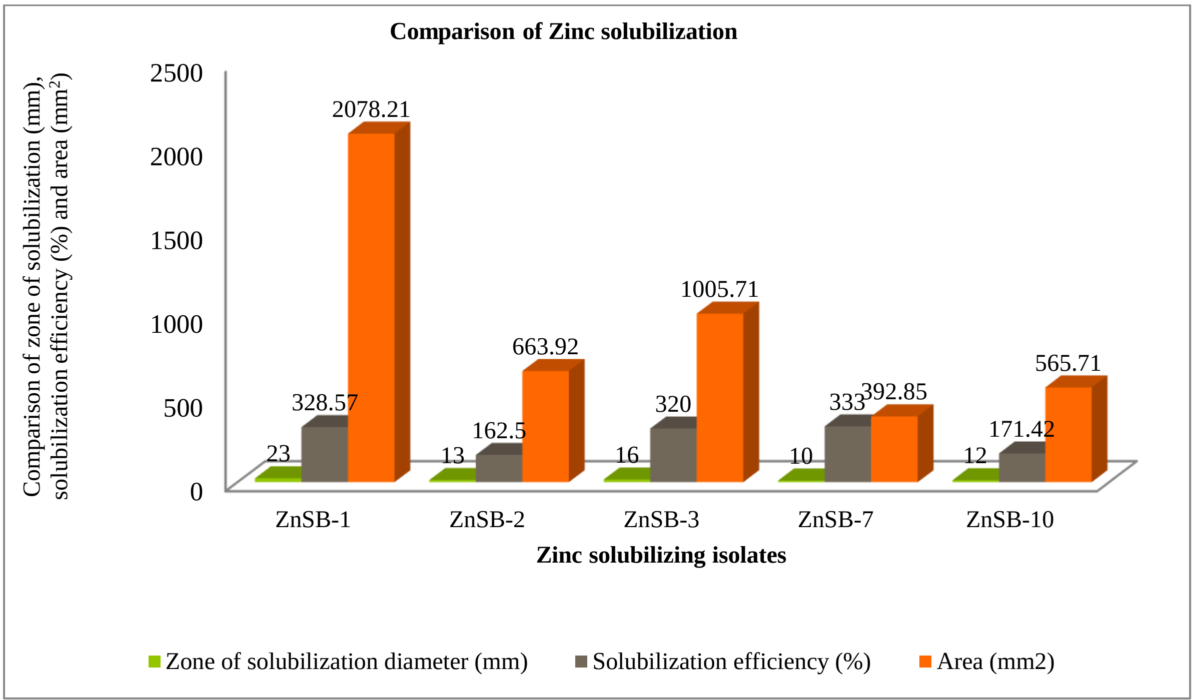

Table 4. Colony Characterization of Bacterial Isolates

Sr. no.	Colony characteristics	ZnSB isolates				
		ZnSB-1	ZnSB-2	ZnSB-3	ZnSB-7	ZnSB-10
1	Size	Big	Big	Big	Small	Big
2	Shape	Round	Round	Round	Round	Irregular
3	Margin	Entire	Filliform	Entire	Entire	Undulate
4	Elevation	Umbonate	Convex	Flat	Flat	Flat

5	**Texture**	Smooth	Rough	Smooth	Smooth	Smooth
6	**Opacity**	Opaque	Opaque	Opaque	Translucent	Opaque
7	**Consistency**	Sticky	Rough	Sticky	Sticky	Sticky
8	**Pigmentation**	No	White	Slight yellow	No	No

Table 5. Morphological Characterization of Bacterial Isolates by Gram Staining Procedure

Morphological Characteristics	**ZnSB Isolates**				
	ZnSB-1	**ZnSB-2**	**ZnSB-3**	**ZnSB-7**	**ZnSB-10**
Gram Staining	Gm – ve	Gm – ve	Gm + ve	Gm + ve	Gm – ve
Cell Shape	Long Rod	Short Rod	Chain Rod	Short rod	Short Rod
Capsule Staining	Negative	Negative	Negative	Negative	Negative

Table 6. Biochemical Characterization of ZincSolubilizing Bacteria

Sr. No	**Test**	**ZnSB-1**	**ZnSB-2**	**ZnSB-3**	**ZnSB-7**	**ZnSB-10**
1	**Methyl Red Test**	+	+	+	+	+
2	**Voges Proskauer's Test**	+	+	+	+	+
3	**Indole Production Test**	+	+	+	+	-
4	**Nitrate Reductiontest**	+	+	+	+	+
5	**Urea HydrolysisTest**	-	-	-	-	-
6	**Catalase Test**	+	+	+	-	+
7	**Oxidase Test**	+	+	+	+	+
8	**Gelatin HydrolysisTest**	+	+	-	-	-
9	**HCN Production Test**	-	-	-	-	-
10	**NH_3 ProductionTest**	+	+	+	+	+
11	**Starch Hydrolysis Test**	+	+	+	+	-

Table 7. The Optical Density at 600 nm of ZnSB Bacterial Isolates at Different pH (pH 5, pH 7, pH 9)

Sr. no.	Isolate	Optical Density at 600 nm		
		pH 5	pH 7	pH 9
1	**ZnSB-1**	0.231	1.131	0.863
2	**ZnSB-2**	0.051	0.395	0.807
3	**ZnSB-3**	0.106	0.695	0.782
4	**ZnSB-7**	0.304	0.742	0.636
5	**ZnSB-10**	0.453	1.145	0.514

Table 8. Effect on Growth of Black Eyed Pea Plants by Inoculating Zinc Solubilizing Bacterial Isolates

Experimental Plants	ZnSB-1	ZnSB-2	ZnSB-3	ZnSB-7	ZnSB-10	Un-inoculated Contrrol	Positive Control
Plant Height(cm)	7.26	6.36	7.1	6.76	5.53	3.66	7.66
Root Height (cm)	1.96	1.5	1.63	1.53	1.46	1.13	2.13
No. of Leaves	17	8.66	14.66	13.66	11.66	7.33	18
Leaf Length (cm)	1.45	1.14	1.19	1.14	1.04	0.65	1.46
Leaf Area Index (ds /m^2)	0.0138	0.0055	0.0098	0.0087	0.0068	0.0026	0.0140
Dry Weight of Shoot (gm)	0.25	0.15	0.22	0.17	0.13	0.08	0.28
Dry Weight of Root (gm)	0.09	0.06	0.08	0.07	0.05	0.03	0.1
Chl. a (mg/l)	5.619	3.560	5.027	3.751	2.378	2.178	9.661
Chl. b (mg/l)	2.275	1.518	2.065	1.926	1.301	1.101	4.477
Total chl. (mg/l)	7.894	5.078	7.092	5.677	3.679	3.279	14.138

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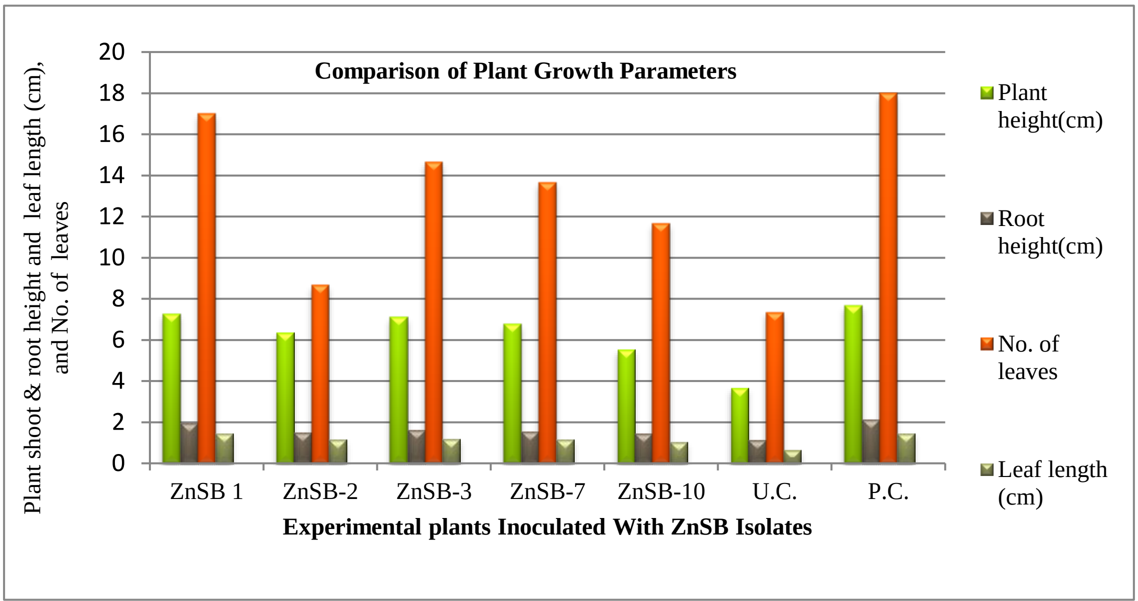
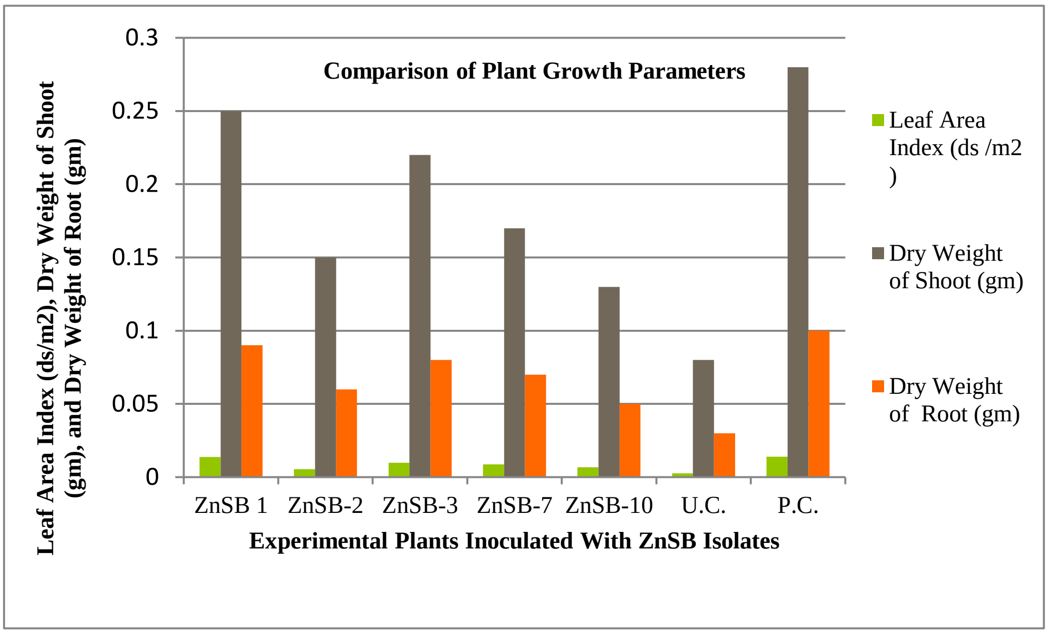
Comparison of Growth of *Vigna unguiculata* (Black Eyed Pea) plants by Inoculating ZnSB Isolates

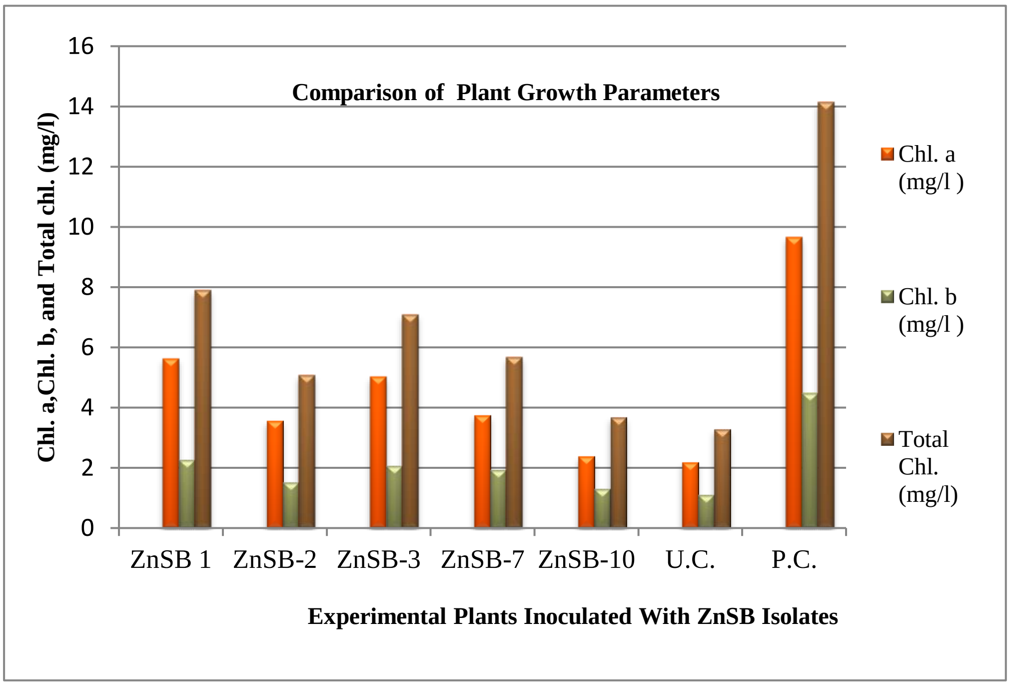
Histogram 2 (a) : Comparison of Plant Shoot Height, Root Height and Leaf Length (cm), and No. of Leaves



Histogram 2 (b): Comparison of Leaf Area Index (ds/m2), Dry Weight of Shoot (gm), and Dry Weight of Root (gm)



Histogram 2 (c): Comparison of Chl. a, Chl. b, and Total chl. (mg/l)



4. CONCLUSION

In agricultural practices, to circumvent the Zinc deficiency, chemical Zinc fertilizers are applied. However, the excessive and injudicious applications of these fertilizers leads to a severe threat to microbial diversity, soil microbial community structure, soil fertility and consequently the productivity of crops in different agro-ecosystems.

Microbiologists and soil scientists are thus searching for an alternative to these problems. Since the majority of soils the world over are deficient in plant available Zinc and since Zinc fertilizers are expensive, focus is placed on the use of soil microorganisms endowed with Zinc Solubilizing ability, which could be used as inoculants to mobilize Zinc from poorly available sources in soil. The use of such ZnSB(s) opens up a new horizon for better plant productivity besides reducing the reliance on chemical Zinc and protecting the agro-ecosystems from the hazards of agrochemicals. The protection of the soil environment by applying ZnSB(s) could become a major breakthrough for plants grown in derelict soils.

Moreover, the molecular engineering of these microbes has also provided a new insight into the promotion of crops in Zinc deficient soils. In this context, novel, genetically engineered and soil- and region- specific ZnSB(s) and technologies have to be developed, pilot tested and transferred to farmers in a relatively short time in order to improve plant Zinc nutrition and agro-ecosystem sustainability.

Thus, the use of bacterial consortium such as Bacillus sp. can effectively increase the growth attributes of black eyed pea plant. Black eyed pea plants inoculated with suitable combination of Zn solubilizing bacterial strains were found more efficient in acquiring Zn from Zn deficient soil as compared to un-inoculated plants.

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