ORGANIC ANIONS PRODUCTION BY *BACILLUS* SP. TO ENHANCE MAIZE AND MILLET GROWTH

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ABSTRACT

In this study, four bacterial isolates, YIId (KT764030), YIC (KT764029), S2 (KT764028) and O2 (KT764027) were selected on the basis of their broad range Zinc Oxide and Zinc Phosphate solubilising and antimicrobial activity against indicator bacterial strains. All four isolates were able to solubilise Zinc Phosphate and Zinc Oxide. The solubilisation activity is mostly related to the production of organic anions, so the production of Gluconic acid was confirmed by HPLC. Plant growth experiments were performed and the findings revealed very promising results from selected bacterial isolates YIC and YIId. The Maize seedlings showed 51% increase in seedling length, when treated with bacterial isolate YIC. The maximum increase approximately 39% and 36% in Millet seedling length was recorded as compared to the control when treated with bacterial isolate YIC, Zinc oxide and bacterial isolate YIId, zinc oxide and zinc phosphate, respectively. The result showed an increase in seedling biomass. Millet plants showed maximum soluble protein content (357.7µg ml^{-1}) when treated with YIC, ZnO and $Zn_3(PO_4)_2$ as compared to control (99.8µg ml^{-1}). The free proline content of Maize plants was significantly increased when inoculated with bacterial isolate YIId (562.68µg ml^{-1}) as compared to control (316.52µg ml^{-1}). It was concluded that selected PGPR can be used to enhance plant yield and biomass by producing organic anions to solubilize zinc and phosphate salts.

Key words: Zinc Solubilisation, HPLC, Gluconic acid, Plant Growth Promoting Bacteria, Proline.

INTRODUCTION

The increasing global requirement of agricultural products from continuously decreasing and degrading land resources has placed substantial stress on the delicate balance of ecosystem. Biological fertilisation is environmentally and economically recommendable, because it results in sustainability of applied nutrients (Miransari, 2013). The use of mineral fertilizers is considered to be the quickest and surest way of enhancing crop production, but their cost and other limitations discourage farmers from using them in suggested quantities as mentioned by Tilak *et al*., (2005). Micronutrients are important for the growth of plants, animals and also for the microbes (Tilak *et al*., 2005). Plant growth promoting rhizobacteria make micronutrients such as phosphorus and zinc available for plant uptake, fix nitrogen, sequester iron for plant uptake by siderophores, and also produce plant hormones such as auxins (Lucy *et al.,* 2004). The main mechanism for mineral phosphate and other mineral solubilisation useful for plant production is the production of organic acids (Rodríguez and Fraga, 1999). The drop in pH may result from production of organic acids such as gluconic acid or α-ketoglutarate and uptake of NH_4^+ ions which may release proton to the medium (Rodriguez, Gonzalez, Goire and Bashan, 2004).

In living systems, Zn is important in the activity of more than 300 enzymes (Sarathambal, Thangaraju, Paulraj and Gomathy, 2010).The organic based zinc nutrition is best since its Zn use efficiency is more. A bacterial based approach was developed to solve the micronutrient deficiency problem (mSaravanan, 1999; Raj, 2002). The basic principle behind this approach is decreasing the pH to5 or below and making zinc soluble and as a consequence the available zinc will get increased in the soil system (Sarathambal *et al*., 2010). A term called zinc solubilising bacteria (ZSB) was devised for those bacteria that are capable of solubilising the insoluble zinc compounds/ minerals in agar plate as well as in soil (Saravanan1999; Raj, 2002).Zinc is a limiting factor in crop production in alkaline and calcareous soil (Bapiri *et al.,* 2013). Zinc deficiency in fungi and bacteria is accompanied by deficiency of the formation of pigments such as melanin, chrisogenin, prodigiosin, subtilin and others. Exogenous use of soluble zinc sources, similar to fertilizer application, has been encouraged by various crops. This causes transformation of about 96-99 percent of applied available zinc to various unavailable forms (Bapiri *et al*., 2013).Plant enzymes activated by Zn are involved in carbohydrate metabolism, maintenance of the structure of cellular membranes, protein synthesis, regulation of auxin synthesis and pollen formation (Marschner and Marschner, 2012). The gene expression required for the tolerance of environmental stresses in plants is regulated

by Zn (Cakmak, 2000). Its deficiency results in abnormalities in plants which become visible as deficiency symptoms such as stunted growth, chlorosis and smaller leaves, spikelet sterility. Micronutrient Zn deficiency can also badly affect the quality of harvested products; plants susceptibility to injury by high light or temperature intensity and to infection by fungal diseases can also increase (Cakmak, 2000; Marschner and Marschner, 2012).

Zinc seems to affect the capacity for water uptake and transport in plants and also reduce the adverse effects of short periods of heat and salt stress (Hafeez*et al.,* 2013). As Zn is required for the synthesis of tryptophan which is a precursor of IAA, it also has an active role in the production of an essential growth hormone auxin (Alloway, 2004). The importance of solubilisation of insoluble metals cannot be underestimated. Its role in energy transfer is critical to plant metabolism. Many soil microorganisms make metal more available to plants by solubilising. The zinc solubilising activity of *Bacillus* sp. and *Pseudomonas* sp. was assessed using zinc oxide, zinc sulphide and zinc carbonate, the solubilisation might be due to acid production (*Saravana et al., 2004*). Proline mounts up in plants when subjected to wide range of stress like water deficiency, salt stress and temperature fluctuations. Proline is a very useful amino acid. It protects mature protein molecules form degradation, help phospholipids to stabilize the membrane structure, it also serves as hydroxyl group forager and often functions as nitrogen and energy source (Paleg *et al.,* 1984). Higher concentration of Zinc significantly reduce growth but increase the production of free radicals and accumulation of proline which is related to non-enzymatic detoxification of free radicals that are generated excessively produced under stress (Prasad *et el.,* 1995).

The purpose of this study was to isolate bacteria having the ability to produce antibiotics along with mineral solubilisation property.

MATERIALS AND METHODS

Bacterial strains and Growth condition: Bacteria were isolated from soil, rotten fruits (apple guava, orange. banana), dairy products (homemade yogurt) and were screened for the production of antimicrobial activity and Zinc solubilisation. The isolates were grown in Tris Minimal salt media in presence of 2.5mM $Zn_3 (PO_4)_2$ and 7Mm of ZnO at 37°C for 48 to 72 hours at shaking. Antimicrobial activity of isolated bacterial strains were checked against two indicator strains, representing gram positive and gram negative bacteria (Parekh and Chanda, 2006). The time of production of active compounds was determined with the help of time induction assay. Cultures of test strain were grown in Nutrient-broth for 24 hours at 37°C with 150 rpm. After 24 hours of incubation the optical density of the cultures was estimated. Two flasks were taken, each containing Tris minimal salt media2.5mM$Zn_3(PO_4)_2$ and 7Mm of ZnO and optical density of cultures were maintained at 0.5 at 600nm according to Fasim *et al.,*(2002),

Plant Growth Experiment

Surface Sterilization of Seeds: Randomly selected healthy seed of Maize and Millet were soaked in 0.1% $HgCl_2$ solution for 30 minutes. After 10 minutes seed were washed with autoclaved distilled water 4-5 times to remove any traces of $HgCl_2$(Batool *et al.,* 2015)

Preparation of Inoculums: Bacterial strains were grown, each in 100 ml N-broth at 37°C with 150 rpm. After 24 hours pallet was thoroughly washed with autoclaved distilled water. Optical density of each culture was optimized at 600nm.Plant growth experiment was conducted in petri dishes. The experiments were performed in replica and following different treatments were used to check their effect on plant growth promotion. i) Control ii) Bacterial strains iii) Bacterial strain, Zn3(PO4)2 (0.5mM) iv) Bacterial strain, ZnO (14mM) v) Bacterial strain, $Zn_3(PO_4)_2$ and ZnO vi) $Zn_3(PO_4)_2$ vii) Seed, ZnO. For inoculation 20-25 seed were aseptically transferred to bacterial suspensions and soaked for 20-25 minutes. For control seeds were soaked in autoclaved distilled water for the same period of time. Petri plates with filter papers were autoclaved and then oven dried. Seeds were transferred aseptically to plates, for each treatment two petri dishes were taken. Plates were kept in dark for 2-3 days and then transferred to plant growth chambers at 25°C, 59% humidity, 16 h photoperiod with light intensity of 180-200 µ E m^2 S^{-1}. After germination, 10 ml of Hoagland's solution was added to fulfil the nutritional requirements of seedling, twice (Mooji *et al.,* 2015).

Harvesting of seedlings: Harvesting was done after 14 days and plant Growth parameters (seed germination, seedling length, root and shoot number) were estimated for biochemical characterisation soluble protein and free proline content were estimated.

Soluble Protein Estimation: One g of plant material was taken and 4ml of 0.1M phosphate buffer was added in it (pH: 7). Crushed the above mixture in a cold pastel and mortar and centrifuged for 10 minutes at 4°C. The palette was discarded and 400µl of supernatant was taken for further experimentation. Two ml of lowery's solution was added in the above supernatant and incubate at room temperature for 15 minute. Folin'sCiocalteus phenol reagent (200µl) was added. The above solution was thoroughly mixed and incubated for 45 minutes. Optical density was taken at 750nm (Lowry *et al.,* 1951).

Proline Content: The estimation of proline content was done by taking 0.5g of plant material was taken and

homogenized with 10ml of 3% Sulfsalicylic acid. The mixture was filtered using a Whattman filter paper no.4. The filtrate (2ml) was taken and 2ml of acid ninhydrin and 2ml of glacial acetic acid was added in it. The mixture was incubated at 100°C for 1 hour. The reaction was stopped by transferring the mixture to ice. Four ml of toluene was added, vortexed for 20 seconds, shaken vigorously and aspirated the toluene from aqueous layer. The mixture was brought at room temperature and optical density was taken at 520nm. (Saradhi, 1991)

High Pressure liquid Chromatography (HPLC): HPLC (propyl paraben method) was performed for Gluconic acid production by the bacterial isolates. To perform this experiment, modified versions of two experiments were used. The mobile phase used for gluconic acid HPLC was 0.1 M sodium hydroxide (Larcher *et al.,* 2009). The standard used was 1% gluconic acid. The run time was set at 10 minutes and the injection volume used was 10µl. The solute elution was monitored at 210 nm (Doyon *et al.,* 1991) and flow rate was kept at 1ml per minute.

RESULTS

Isolation and selection of bacterial strains: A total of 17 strains were isolated from soil, yogurt and rotten fruits. The morphological characteristics of these strains were characterized using colony morphology determination and Gram staining procedures. Out of 17 isolates, 8 were Gram positive while 9 isolates were Gram negative. 41% isolates were Gram positive rods, 23% isolates were Gram negative rods, and 29% bacterial isolates were Gram negative cocci while only one isolate was Gram positive cocci. Four strains, YIId, YIC, S2 and O2, on the basis of their antimicrobial and zinc solubilisation ability, were identified using 16s ribosomal RNA. All of these strains come out to be Bacillus such as *Bacillus safensis*(KT764027)**,** *Bacillus subtilus*(KT764030, KT764029, KT764028). Ability of microorganism to multiply up to 48 hours was checked. For most of the strains, lag phase persisted for 1 to 2 hours. All of the four selected strains showed gradual increase in growth and there was no decline phase even after 48 hours. To check whether these traits are plasmid encoded or not, plasmid isolation was done for four selected strains. All of the four isolates showed presence of plasmid which indicates that the traits for production of organic acids might be present on plasmids. In the present study, a total of 17 strains was subjected to solubilisation of Zinc Oxide and Zinc Phosphate. Plate assay was performed for all the strains, appearance of clear zones around wells indicated solubilisation of salts. To qualitatively measure the solubilisations, zones of clearance were measured in mm. 23% isolates solubilised both zinc Oxide and Zinc Phosphate while 76% did not solubilise these salts. The largest zone of clearance against Zinc Phosphate was 19mm shown by YIId. The maximum zone of clearance for Zinc Phosphate was of 21mm shown by O2.

High pressure liquid chromatography: The organic compounds which were responsible for antimicrobial activity and solubilisation of Zinc, HPLC of the supernatant of bacterial cultures was done. Gluconic acid was used as standard. The retention time of 1% Gluconic acid was 2.690 minutes while of samples YIId, YIC, S2 and O2 was 2.627, 2.713, 2.590 and 2.608 minutes respectively as shown in figure 6. These results showed that the compounds which were responsible for antimicrobial activity and salt solubilisation were nearly related to Gluconic acid.

Plant microbe interaction: In the present study, plant microbe interaction experiments were done in the presence of salts as well as in the absence of salts. Two different seeds Millet and Maize were used for this study and their relative growth parameters were studied such as seedling length. In the first treatment seeds were inoculated with bacterial strains solely. Four isolates, YIId, YIC, S2, and O2 were selected for plant microbe interaction due to their broad range antimicrobial activity and salt solubilising activity.

Seed germination and seedling length parameters: The percentage germination of seeds inoculated with bacterial strain YIC and S2 as shown in figure 1.The largest increase in seedling length in Millet was observed in case of strain S2 which was 7% (8.19cm) more than that of control (7.61cm) as described in figure 2. While in case of Maize plant the seedling length increased up to 20.4375cm (51%), the seed was inoculated with YIC, while that of control's length only 10.0625cm (figure 3). It seems from the above data that YIC showed greater synergism with Maize seedling, While S2 showed marked increase in length in case of both plants, Maize and Millet.

Bacterial Isolates and Zinc Oxide:The seedling length in case of seeds treated with bacterial isolates and Zinc Oxide was also observed. The plants showed a significant increase in length when treated with strain YIId and Zinc Oxide. The seedling length in case of YIId and Zinc Oxide was 15.85cm (37%) while that of control was only 10.06cm. Strain O2 and YIC also showed marked increase in seedling length as compared with control.

In case of Millet plant YIC and S2 showed a marked increase in length, 12.35cm (39%) and 12.15cm (38%) respectively while length of control was only 7.61cm. It is clear from the above data that strain S2 and YIC are showing very promising results when used alone and when used in combination with Zinc salts.

Bacterial isolates and Zinc Phosphate: When the seeds of Maize and Millet were treated with bacterial isolates and Zinc Phosphate a marked increase in seedling length was observed as compared with control plants. In case of Maize plant the largest plant was seen when treated with strain YIId and YIC. The seedling length in case of YIID and YIC along with zinc phosphate was 16.5625cm (40%) and 15.5cm (36) respectively. Strain S2 and O2 did not show any marked increase in length as compared with control plant. In case of Millet plant strain YIC and S2 showed noticeable increase in seedling length. The seedling length in case of YIC was 10.9cm (30%) while in case of S2 it was 10.67cm (29%). Strain O2 also showed obvious increase in length. So far, it is clear that strain YIC and S2 are very much capable of enhancing plant growth when applied alone and when used in the presence of salt stress.

Bacterial Isolates, Zinc Oxide and Zinc Phosphate: When both salts were used in combination with bacterial strains, least increase in growth was seen which indicates that due to increased salt stress plant showed less increase in length, even when they proved to be helpful when used alone. The largest increase in length in case of Maize plant was shown by strain O2 which was 15.35cm (34%) while this strain when used alone did not encouraged the growth of plants very much, while other strains did not showed any obvious increase in seedling length when used in combination with Zinc Oxide and Zinc Phosphate. In case of Millet plant strain YIId showed marked increase in seedling length, which was 11.88cm (36%) as compared with control, 7.61cm. While other strains also showed increase in seedling length which was very much evident. In this study different bacterial strains were used with the ability to solubilise Zinc and Phosphate and they clearly promoted growth of Maize and Millet plants.

Soluble protein content: Soluble protein content of plants was also calculated with the help of Lowery's method. Most of the plants showed higher soluble protein content as compared to the control plants. The soluble protein content of control came out to be 563.408µg/ml and highest soluble protein content was of Maize seedlings inoculated with bacterial isolate O2 and Zinc phosphate which was 1087.04µg/ml, while when Millet seedlings were inoculated with YIC, ZnO and zinc phosphate showed maximum protein content (357.7 µg/ml) as compared to the control (99.8 µg/ml) as shown in figure 4.

Free proline content: The free proline content of control plant was 316.52µg/ml while the highest proline content was of plant treated with YIId which was 562µg/ml. Plants treated with bacterial isolate YIC, zinc phosphate and zinc oxide also showed elevated level of proline content which was 555µg/ml (figure 5).

Table 1. Antimicrobial activity and Zinc solubilisation by bacterial isolates.

Strain	Zone of Antimicrobial activity (mm)		Zone of Zinc solubilisation (mm)	
	Gram +Ve	Gram –Ve	$Zn_3(PO_4)_2$	ZnO
P1	-	-	-	-
Pc1	-	-	-	-
YIId	10	08	19	10
YIc	21	18	13	19
YIIc	-	-	-	-
O2	15	12	09	21
Apb	-	-	-	-
YIIa	12	-	-	-
Apc	10	06	-	-
S1	-	-	-	-
S2	19	13	11	17
S3	05	-	-	-
S4	09	-	-	-
S5	-	-	-	-
S6	-	-	-	-
S7	-	-	-	-
O1	08	05	-	-

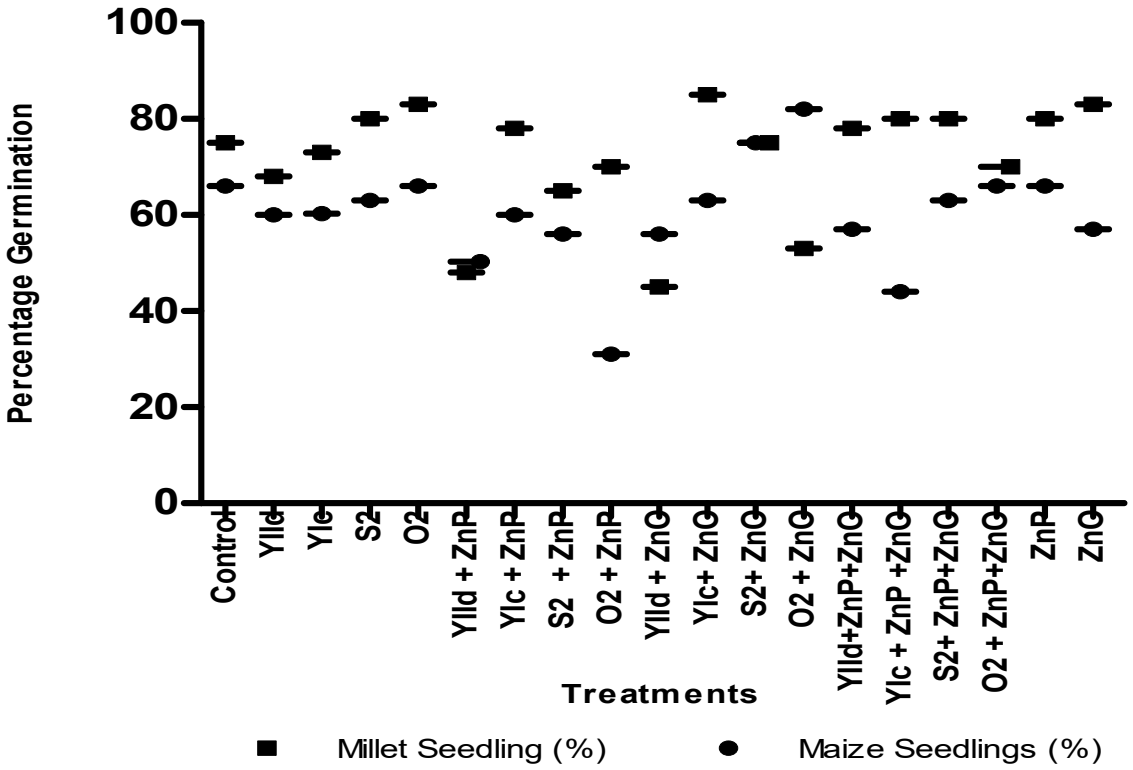

Figure 1 . Percentage Germination of Maize and Millet Seedlings

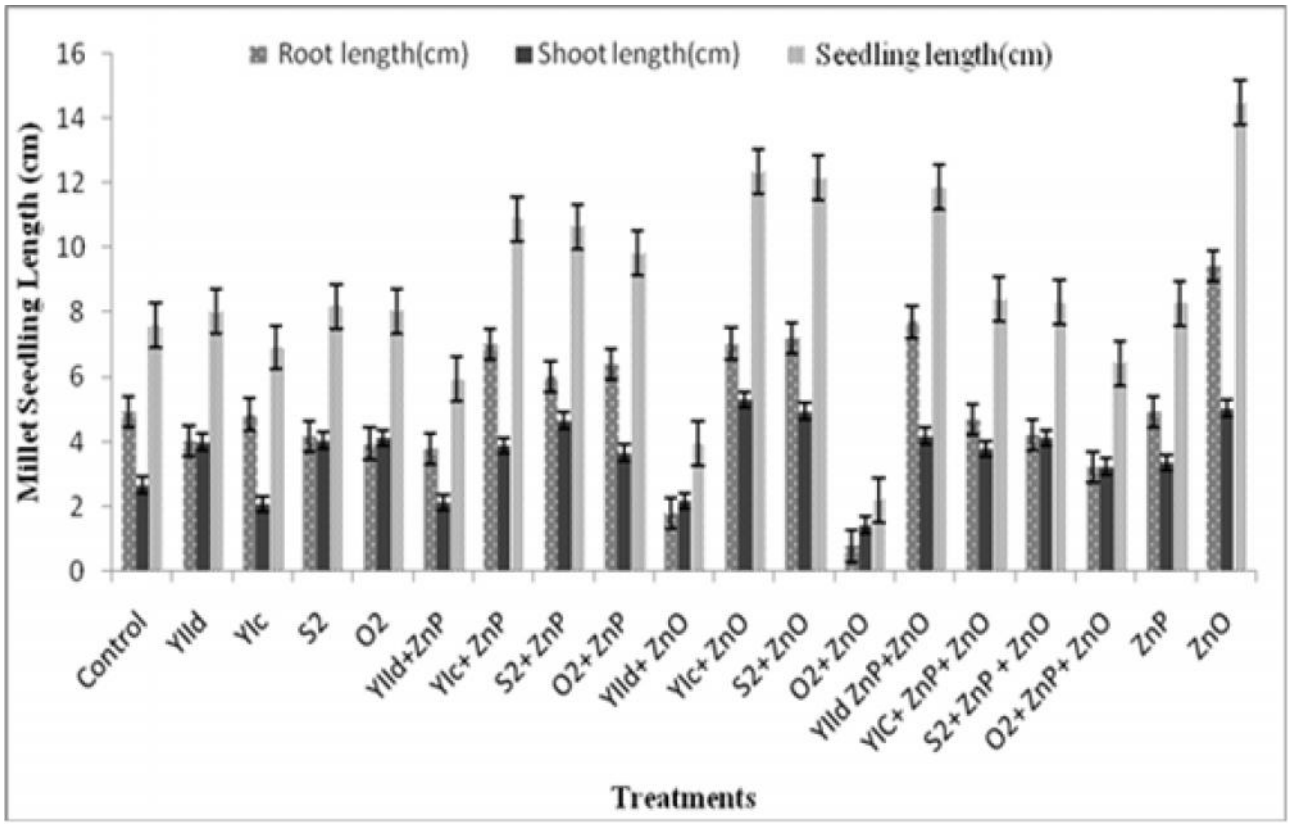

Figure 2. Growth parameters of Millet seedlings

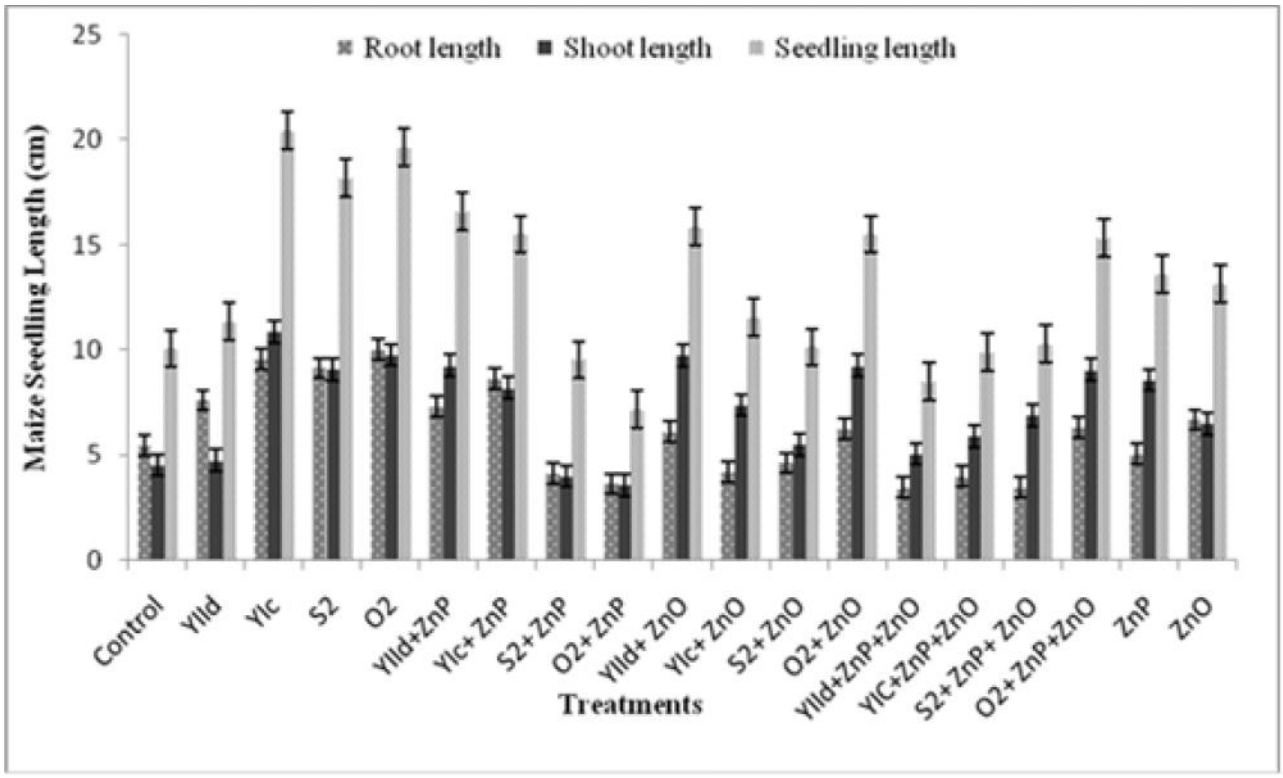

Figure 3. Growth parameters of Maize Seedlings

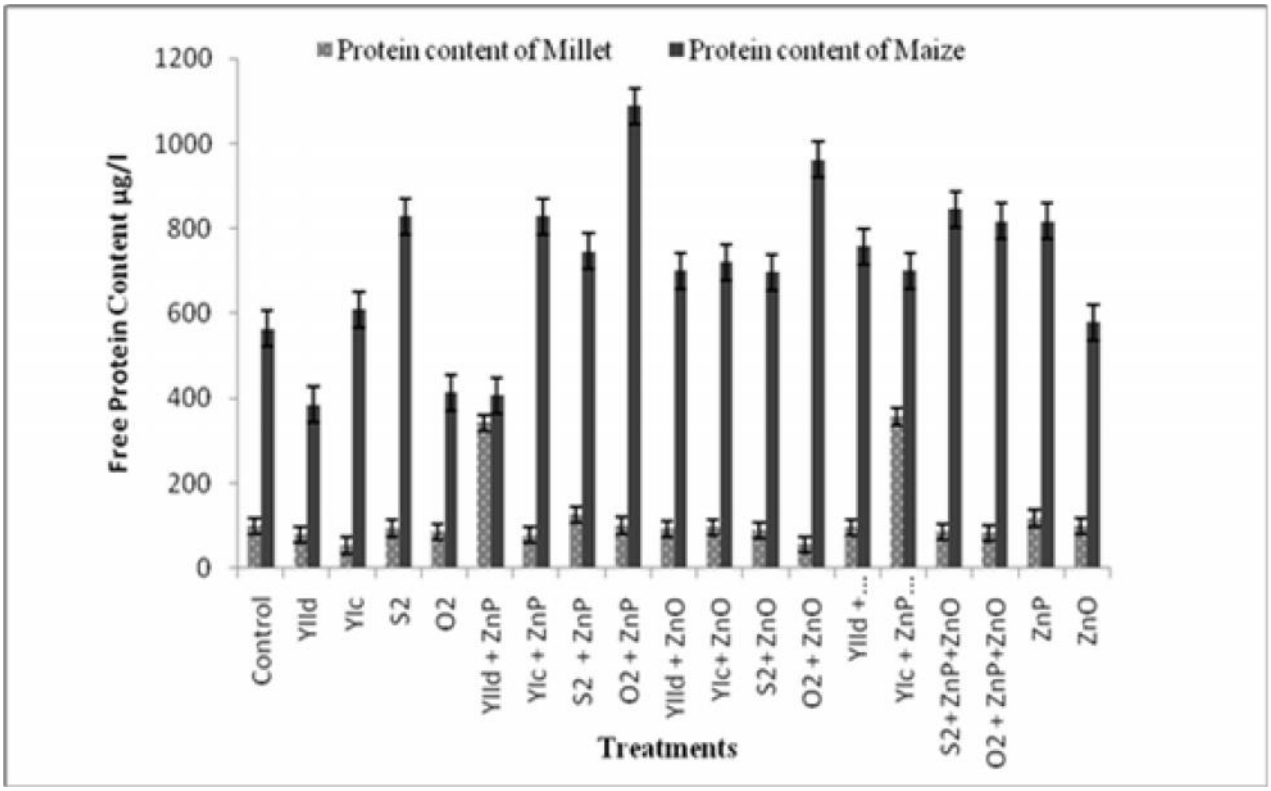

Figure 4. Soluble protein content of Millet and Maize Seedlings

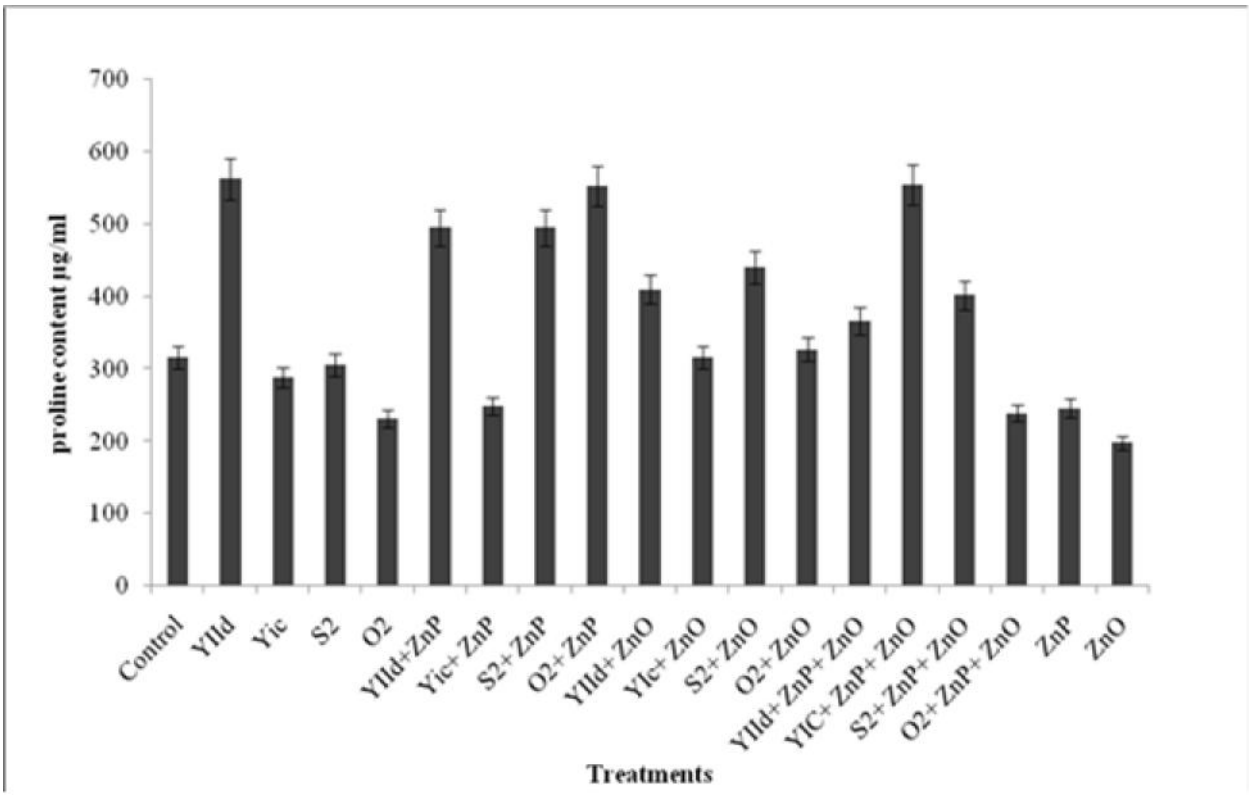

Figure 5. Production of Proline by Maize seedlings

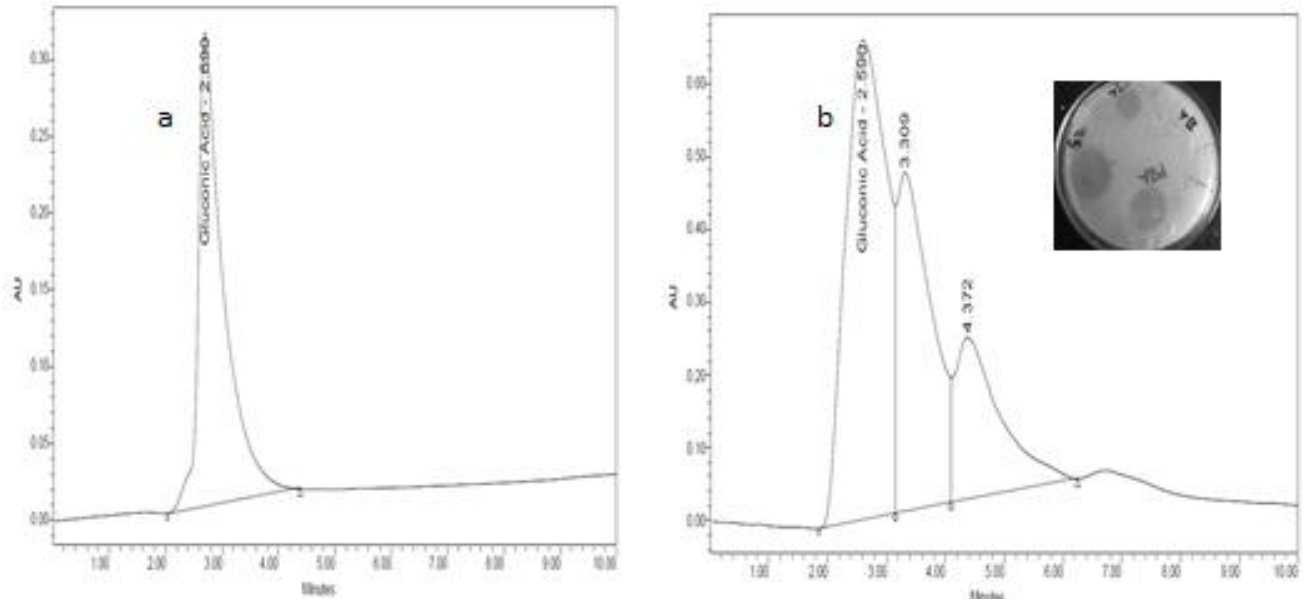

Figure 6. HPLC results, The comparison of (a) standard Gluconic acid retention time (2.690) and (b) Gluconic acid produced by Bacterial isolate YIId, (retention time 2.690)

DISCUSSION

The results presented in this study indicate that microorganisms play a major role in plant growth promotion, either directly or indirectly. Beneficial effects of microorganism have been reported on plant growth (Gravel *et al.,* 2007).The aim of this study was to isolate bacterial strains from soil, yogurt and rotten fruits, and to screen these isolates for the production of antimicrobial compounds, salt solubilisation, and effect of these bacterial isolates and salt solubilisation activity on the

growth of Millet and Maize plants. Ability of microorganism to multiply depends on their ability to acquire essential nutrients. Under optimum conditions bacteria are able to multiply very rapidly and their number may double after every 20 minutes (Beaumont *et al.,* 1998).

The bacterial isolates showed antimicrobial as well as metal solubilisation activity. The isolates solubilised metal in solid as well as liquid media. According to time induction assay the bacterial isolates started producing antimicrobial compounds after two or four hours of incubation, similarly they started solubilising Zinc in early stage of their growth. So we can say that the compound which was responsible for Zinc solubilisation was also responsible of antimicrobial activity. Antimicrobial effect can be due to organic acids that cause a reduction in pH (Gould, 1991), so we can say that organic acids that are acting on one side as antimicrobial agents also cause solubilisation of heavy metals such as Zinc. As well during the observation of bacterial growth in broth media a gradual decrease in pH was also observed, which confirms the hypothesis that these isolates do produce organic acids. Microorganism need to solubilise insoluble metal ions from their environment prior to uptake and utilisation of associated nutrients. Such microbes which can solubilise metal or extract them from ores are very important for humans as they may be used in industry for the recovery many important metals from their reservoirs (Ehrlich, 2002). Microorganisms produce organic acids such as α-ketoglutarate and Gluconic acid as salt solubilizing agents (Fasim *et al*., 2002; Rodriguez *et al*., 2004). As discussed by Simne *et al*, that zinc solubilisation in Zinc Oxide and Zinc Phosphate by strain by Pseudomonas fluorescence was due to gluconic acid production from glucose (Di Simine *et al.,* 1998). HPLC results clearly showed that all of the selected strains were producing Gluconic acid. It might be responsible for decrease in pH along with many other compounds. Soil is a complex habitat where a large number of microorganisms including bacteria, fungi, protozoa and algae are found. Bacteria are the most abundant microorganism found in soil. Pathogenic microorganisms are major and chronic threat to plant health. To eliminate these pathogenic microbes chemicals are applied to the soil which cause a serious damage to environment and may also effect the beneficial flora of that particular soil to which these chemicals are applied and may poison the plant material. There are also some fastidious diseases for which chemical solutions are few, ineffective, or non-existent (Gerhardson, 2002). Biological control is thus considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture as suggested by Compant *et al.,*(2005). The numerous interactions between bacteria, fungi and roots may have beneficial, harmful, or natural effects on plant, the outcome being dependent on the type of symbiont interaction and the soil conditions (Lynch and Leij, 1990; Smith and Read, 1996).

Plant growth promoting Rhizospheric bacteria are being exploited commercially to protect the plants from various diseases. Bacteria exhibit many activities which cause an increase in plant growth and crop yield. Bacteria with multiple plant growth promoting activities can be used as bio fertilizers. According to Ramamoorthy *et al.,* (2001) bacteria used for the protection of the plants against different diseases are *Bacillus subtilus*. Production of Gluconic Acid and derivatives of Gluconic acid enhance plant growth and is considered one of the plant growth promoting ability of microorganisms due to their Zinc and phosphate solubilizing abilities (Saravana *et al.,* 2007). Free proline content of plants were also calculated as plants grown under stress condition show elevated levels of free proline as it act as a nitrogen source and protects plants from salt stress (Lin and Kao, 1996). Maize and Millet are very important food and fodder crops and are not very often studied. The present study emphasized on the growth promoting abilities of indigenous bacterial species in the presence of Zinc and Phosphate salts. In this study some of the strains showed depression in growth when co-inoculated with Zinc and Phosphate salts. It is not necessary that every isolated strain work in concordance with added salts. In this studies some strains worked best alone but when they were inoculated in the presence of salts, a depression in the growth was seen, which means that these strains might not be able to work in synergism with the particular crop or salt. On the other hand some strains alone were not very promising in increasing the seedling length but when inoculated in the presence of salts gave promising results. There is a great variation in the results obtained, but most of the results showed that the bacterial isolates can overcome the capital loss due to application of insoluble salts by making them soluble and available to the plants. The PGPR can be used in field to enhance the growth and availability of micronutrients to plants. This approach can easily overcome the threat posed by chemical fertilisers to the crops and environment (Roy Chowdhury *et al.,* 2016).

Conclusion: Maize and Millet are very important food and fodder crops and are not very often studied. The present study emphasized on the growth promoting abilities of indigenous bacterial species in the presence of Zinc and Phosphate salts. In the present study bacterial isolates significantly enhanced the growth and biomass of seedlings. The results obtained showed that the bacterial isolates can overcome the capital loss due to application of insoluble salts by making them soluble and available to the plants.

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