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# ZINC SOLUBILIZING NATURE OF *GLUCONACETOBACTER DIAZOTROPHICUS*

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**Abstract:-**Zinc is an essential trace element required in minor amount by prokaryotes and Eukaryotes whereas in the case of plants proper concentration is needed to have regular plant physiological pathway to function normally. The entrance of zinc in pathways plays a vital role in photosynthesis, sugar formation protein synthesis and defense against diseases. Microbes solubilize different element in soil and it leads to addition of zinc, the present research aimed to investigate efficiency of G.diazotrophicus on zinc solubilization. In the present investigation about four G.diazotrophicus isolates were isolated from different parts of sugarcane, characterized and used for zinc solubilizing invitro studies. The outcome of research interestingly showed efficiency of G.diazotrophicus isolates (GdSR, GdSS, GdSB and GdSL) and reference strain (PAL-5) on zinc solubilization and recorded maximum clear zone on the zinc added LGI medium.

**Keywords:**Gluconacetobacter diazotrophicus, zinc, zince solubilization.

## INTRODUCTION:

Zinc is an important minor key element which limits the productivity of different agricultural crops and zinc deficiency is one of the most widespread limiting factors to crop production affecting 30% of the agricultural soils. According to a survey made on the available status of micronutrients in India, zn recorded as the micronutrient deficient in all the 20 states assessed and also deficient in all the agro-ecological regions of India. The deficiency ranges from 86 per cent in soils of Maharashtra to 8 per cent in Pondicherry (Singh, 2001). Tamil Nadu soils recorded 56 per cent deficiency.

The correction of zn deficiency through addition zn fertilizers is a common practice. The application of 62.5 kg ZnSo4 to the cereal based cropping system is sufficient to meet the zn requirement for three years (Bhupinder Singh, 2005). Most common soil application ranged from 2.5kg zn/ha to 22kg zn/ ha for inorganic forms such as ZnSo4 and 0.3-6kg zn/ha for chelated forms. However this approach is neither economical nor ecofriendly in the long run as only 20% of the applied zn is available for plant uptake, while the remind gets absorbed on soil, which is an environmental concern. With regard to human zn nutrition, fortification of zn in food is practiced but expensive and difficult to implement in developing countries like India (Allowaay, 2001). G.diazotrophicus a known diazotroph fixes appreciable amount of nitrogen, solubilize phosphorus in maximum amount as well as this organisms induces growth in sugar rich crop by producing growth promoting substances and being a multibenificial diazotroph some more researches needs to exploit the potentiality of this organisms for other beneficial nature.

Even though it may be a endophytic organism there is enough literature to say it as a rhizosphere organism. Fuentes-Ramirez et al., (2001) were able to isolate some new species viz., Gluconacetobacter johannae and Gluconacetobacter azotocaptans from coffee rhizosphere in Mexico. A slightly higher population in rhizosphere compared to endophytic population was observed by Munoz-Rojas and Cabellero-Mellado (2003). The presence of G.diazotrophicus in the rhizosphere and its ability to solubilise zn are important characters of this organism to utilize it as a candidate for solubilizing zn.

## ZINC SOLUBILIZING MICROBES:

The common soil microbes pseudomonas can enhance the dissolution of insoluble minerals in neutral pH (Vandevivere et al., 1994). Bacterial organisms produce low molecular weight organic acids and high molecular weight

polysaccharides they will affect dissolution. Solubilizations of phosphorus by microorganisms and its role on plant growth is an age old study dates back from 1940's (Gerretsen, 1948). But microbial zinc solubilization was mainly focused as leaching of metal ores, employing some strong acidophilic bacteria like Thiobacillus ferroxidans that has been widely studied for dissolution of zinc compounds (White et al., 1997). Another approach is solubilization of native zinc in the rhizosphere region of a plant system and hyper accumulation of zinc solubilization (whiting et al., 2001 and Coles et al., 2001).

Meyer and Linderman (1986) reported that growth promoting rhizobacteria like pseudomonas putida inoculation with VAM had increased concentration of Fe, Cu, Al, Zn, Co and Ni in the shot tissue; they further hypothesized that P. putida may produce 2-ketogluconic acid that made native micronutrients soluble, which in turn taken inside the plant with the help of mycorrhizal association. Altomare et al., (1999) reported that Trichoderma harzianum has the ability to solubilize insoluble or sparingly soluble minerals such as MnO2, Fe2O3, CuO, granular metallic zinc via acidification of medium, producing chelating metabolites and redox activity. Graham and Webb(1991) reported that solubilization of Fe and Mn by soil micro flora are important since these nutrients have some effect on plant disease control and zinc was also found to improve the bio control and nematicidal activity of fluorescent pseudomonads population(Duffy and Defago,1997 and Siddiqui and Shaukat,2002).

Zinc solubilizing bacteria (ZSB) was coined for those bacteria that are capable of solubilizing the insoluble zinc compounds/minerals in agar plate as well as in soil (Saravanan, 1999; Anthoni Raj, 2002) this is the only study so for in literature focusing on bacterial based approach to supply Zn as crop nutrient and these bacteria even found to increase zinc content inside soybean plant when inoculated in the rhizosphere as observed in Mn reducers (Marschner et al., 1991).

**ZINCE SOLUBILIZATION EFFICIENCY:**

G.diazotrophicus was compared with p.fluorescens, Bacillus sp. (ZSB) and B.megaterium (PSB). The G.diazotrophicus was found to solubilize all the insoluble compounds tested and p.fluorescenes solubilized all except manganese dioxide, whereas ZSB was effective against zinc oxide and zinc carbonate and PSB solubilized zinc phosphate and completely inefficient towards other insoluble compounds.

The present study was undertaken with the aim to exploit the potentiality of G.diazotrophicus for the solubilization of zince under laboratory condition.

**MATERIALS AND METHODS:**

The laboratory experiments were carried out at department of Microbiology, Faculty of agriculture, Annamalai University.

**Isolation of G.diazotrophicus:**

The Gluconacetobacter diazotrophicus were isolated from different parts of sugarcane by using LGI and acetic LGI medium with acidic pH all the cultures were maintained in LGI slants.

**Characterization of G.diazotrophicus:**

The isolated cultures were grown in acetic LGI medium and single colony was streaked on acetic LGI agar slants and the young cultures at exponential phase i.e. on 7th day were taken for further characterization.

**Motility:**

The presence of motility in the isolated cultures was observed by hanging drop technique using a cavity slide as described by Aneja (1993).

**Brown pigment production property (Cavalcante and Donereiner, 1988)**

The potato agar medium designed by (Cavalcante and Donereiner, 1988) was used. After solidification of the media, G.diazotrophicus cultures maintained in LGI slants were streaked on the agar plates and incubated at 290C±10C for 15 days. The plates were observed for the presence of brown colour water soluble pigments.

**Pigmentation on the acetic LGIP agar Medium (Cavalcante and Donereiner, 1988)**

Acetic LGIP agar medium was prepared by increasing the agar concentration of semi-solid acetic LGIP medium from2.2to 20g per litre and the final pH was maintained at 5.5 by acetic acid. The isolates were streaked on the medium and observed for the growth.

**Growth on the glucose yeast calcium carbonate agar (Micales et al., 1985)**

The isolates were streaked on the glucose yeast extract agar medium. The Gluconacetobacer colonies were observed for the characteristics growth on the medium with water soluble brown colonies.

**Over oxidation property (Frateur, 1950)**

The medium described by Frateur (1950) was prepared in 50ml lots in 250ml flasks. To this medium, ethanol was added at 1 per cent concentration before pouring the medium into the Petriplates. While pouring the medium, care was taken to transfer the entire content of the medium completely to the Petriplates. After solidification of the medium, G.diazotrophicus cultures maintained in the LGI slants were streaked in the plates and incubated at 290C±10C. After 5 days, the acetic acid bacterial colonies were identified based on the formation of clear zone.

**Oxidase tests (Collins and Lyne, 1970)**

Small pieces of filter paper were soaked in 1 per cent aqueous tetra methyl-p-phenylene diamine and placed in a petridish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after 10seconds indicated positive oxidase test where as development of a light violet colour indicated negative oxidase test.

**Nitrate Reductase test (Beishir, 1987)**

Cultures were inoculated in test tubes containing nutrient glucose broth with 1 per cent KNO3 and incubated at 370C for 48h. Test for the presence of nitrate reductase was carried out by adding 1 drop of sulfanilic acid and 1 drop of alpha naphthylamine reagent to each of the nutrient broth cultures. Development of distinct red colour indicated positive test and no colour development indicated negative test.

**Reagent**

5N Acetic acid: 294.0ml of glacial acetic acid+ 706.0ml of distilled water
Alpha Naphthylamine reagent: 5.0ml α-naphthylamine+1000ml 5N acetic acid
Sulfanilic reagent: 8g Sulfanilic acid+1000ml of 5N acetic acid

**Test for hydrogen sulphide formation (Beishir, 1987)**

Peptone iron broths in tubes were inoculated with cultures and incubated at 370C for 48h. Black precipitation in the medium indicated hydrogen sulphide formation.

**Catalase test (Rangaswami and Bagyaraj, 1993)**

A loopful of bacterial culture to be tested was taken from the solid medium and mixed with a drop of 3 per cent hydrogen peroxide on a glass slide. Catalase positive organisms showed bubbles of oxygen.

**Solubilization studies:**

**Solubilization of insoluble Zn compounds by G.diazotrophicus plate assay (Fasim et al., 2002)**

The medium used in the study was LGI with 0.132 g1-1 of ammonium sulphate as N source. The medium was prepared by incorporating insoluble zinc sources viz., zinc oxide, zinc carbonate and zinc phosphate at 0.1 per cent and 0.2 per cent with the carbon source sucrose at 10 per cent. The pH was adjusted to 6.0 after sterilization, with sterilized 1N NaOH or 1N HCL and the medium was added to pertriplates. Care was taken for uniform distribution of the insoluble zinc source into the petriplates and the plates were prepared without air bubbles. The agar plates were allowed to cool and 10µ1 of G.diazotrophicus (6x106 cfuml-1) suspension was placed on the agar surface. After placing the culture on the agar surface, the plates were kept undisturbed for 10 min to get absorbed in the agar medium without spreading. Then the plates were incubated at 290C±10C and observed for solubilization zones up to 5 days. The diameter of the solubilization was measured and expressed in cm. Three replications were maintained for each treatment.

**Results and Discussion:**

Zinc is an essential trace element required in minor amount but proper concentration is needed by plants to have

regular plant physiological pathways to function normally. These corrections of zince in pathways plays vital role in photosynthesis, sugar formation, protein synthesis, growth regulation and defense against disease. Unlike other micronutrients deficiency, Zn deficiency is ubiquitous (Guerinot and Eide, 1999). Where zinc is deficient, morphological function will be impaired and the health and productivity of the plants will be adversely affected, resulting in lower yields (or even crop failure) and frequently in poor quality crop products.

In the present environment condition Zn deficiency is most widespread throughout the world. The application of Zn fertilizers is not totally successful strategy in alleviating Zn deficiency because of agronomic (i.e. subsoil constraints, disease interactions, economic (unavailability of Zn fertilizers for poor farmers) and environmental pollution associated with excessive use zinc deficiency in humans is recognized as a public health problem of global proportions. Correction of zinc deficiency in young children has been effective in reducing major causes of morbidity and mortality and in improving growth and development. In many populations, zinc deficiency has been attributed to an impaired bioavailability of dietary zinc (Adams et al., 2002).

**Isolation of G.diazotrophicus isolates:**

In the present study about four isolates of G.diazotrophicus were isolated from different parts of sugarcane and designated as GDSR, GDSS, GDSB and GDSL respectively from sugarcane root, stem, bud and leaves. (Table-1)

**Isolation of G.diazotrophicus**

| Source | Designation | Medium used |
|---|---|---|
| Sugarcane root | GDSR | LGI |
| Sugarcane stem | GDSS | LGI |
| Sugarcane bud | GDSB | LGI |
| Sugarcane leaves | GDSL | LGI |

**Characterization of G.diazotrophicus:**

The isolated G.diazotrophicus strains were confirmed by performing characterization tests viz., gram reaction, motility, catalase activity, oxidase activity, nitrate reductase activity, over oxidation property, hydrogen sulphide formation and growth under different conditions. The results are presented in table 2.

**Characterization of G.diazotrophicus isolates**

| Isolates | Gram reaction | Motility | Catalase Activity | Oxidase Activity | Over oxidation property | Nitrate reductase activity | $H_2S$ formation |
|---|---|---|---|---|---|---|---|
| GDSR | - | + | + | + | + | - | + |
| GDSS | - | + | + | + | + | - | + |
| GDSB | - | + | + | + | + | - | + |
| GDSL | - | + | + | + | + | - | + |
| PAL5 | - | + | + | + | + | - | + |

+ Positive
-Negative

All the strains (GDSR, GDSS, GDSB and GDSL) produced yellow to dark yellow orange SUB surface pellicles on semisolid LGI and acetic LGI medium. They developed brown pigment colonies on PGA medium. This was accordance with the finding of Cavalcante and Dobereiner(1988). The nitrate reductase activity was not observed in all the strains of G.diazotrophicus tested in the present investigation. The results were in conformity with the finding of Boddey et al., (1991) who reported that the G.diazotrophicus possessed no nitrate reductase activity. The tested strains were motile, catalase and oxidase positive exhibiting the reaction. The strains also produced hydrogen sulphide. The strains were gram negative and rod shaped as described by Cavalcante and Dobereiner(1988). This strains showed the over oxidation property when grown on the media developed by Frateur (1950). Similarly the strain also produced brown water soluble pigments on Glucose yeast extract calcium carbonate agar. The results are also in agreement with the finding of Cavalcante and Dobereiner(1988).

**Zinc solubilizing efficiency of G.diazotrophicus :**

The zinc colubilization potential of G.diazotrophicus cultures in different concentration of insoluble zinc with sucrose as carbon source was assessed. The results are presented in table 3.

**Solubilization of insoluble Zn compounds by G.diazotrophicus cultures (Plate assay)**

| ***G.diazotrophicus* culture** | **Solubilization zone(cm)** | | | | | |
|---|---|---|---|---|---|---|
| | **Zinc oxide** | | **Zinc carbonate** | | **Zinc phospate** | |
| | **0.1%** | **0.2%** | **0.1%** | **0.2%** | **0.1%** | **0.2%** |
| **GDSR** | 2.8 | 2.6 | 3.7 | 2.1 | 2.0 | 1.4 |
| **GDSS** | 3.0 | 2.7 | 3.7 | 2.2 | 2.1 | 1.2 |
| **GDSB** | 4.2 | 2.8 | 3.8 | 2.6 | 2.4 | 1.3 |
| **GDSL** | 2.7 | 2.5 | 3.6 | 2.0 | 1.9 | 1.0 |

All the cultures of G.diazotrophicus established growth at both 0.1 per cent and 0.2 per cent concentration of ZnO, ZnCO3 and Zn3 (PO4)2. Among the zinc concentrations, 0.1 per cent supported more solubilization compared to 0.2 per cent for all the cultures. Among the cultures tested, GDSB showed high solubilization. Maximum solubilization was observed at 0.1 per cent ZnO(4.2cm) by. The solubilization of Zn3(PO4)2 was comparatively less. In general, the solubilization was more at 0.1 per cent than 0.2 per cent zinc compounds. At 0.1 zinc concentration GDSB exhibited the solubilization zone of 4.2, 3.8 and 2.4cm with zinc oxide, zinc carbonate and zinc phosphate respectively. This was followed by GDSS with 3.0,2.7 and2.0cm respectively with zinc oxide, zinc carbonate and zinc phosphate at 0.1 per cent zinc concentration.

**Zn solubilization under in vitro conditions:**

Microorganisms require various nutrients for their growth and development. Among the nutrients Zn is an element present in the enzyme system as co-factor and metal activator of many enzymes (Parisi and Vallee, 1969). The role of Zn in the nutrition and physiology of both eukaryotic and prokaryotic organisms is widely studied, especially its importance for activity of many enzymes (Hughes amd Poole, 1989). Zinc deficiency in fungi and bacteria is accompanied by impairment of the formation pigments such as melanin, chrisogenin, Prodigiosin, subtilin and others (Chernavina, 1970).

Zn solubilizing potential of few bacterial genera has been studied. Hutchins et al., (1986) reported that Thiobacillus thiooxidans, T. ferroxidans and facultative thermophyllic iron oxidizer soluilized Zn from sulphide ore. A Bacillus spp and a pseudomonas sp capable of solubilizing Zn were also isolated from a garden land soil. Both these organisms were found to solubilise insoluble Zn compounds invitro. The organisms were isolated based on the clearing zone produced around the colony similar to phosphate solubilization (Anthoni Raj, 2002). The solubilization of Zn by G.diazotrophicus cultures PAL5, L3 and S7 was assessed in both LGI and LGIM media (Saravanan, 2004). Insoluble Zn sources viz., ZnO, ZnCO3 and Zn3 (PO4)2 at 0.1% concentration was better solubilized by G.diazotrophicus than higher concentration. In present study also the Zn solubilization by G.diazotrophicus (GDSB, GDSS and PAL-5) was efficient at 0.1% concentration. Among the three sources of Zn (ZnO, ZnCO3 and Zn3(PO4)2 studied, more solubilization was observed in ZnO.

**SUMMARY:**

The isolated G.diazotrophicus strains were confirmed by performing characterization tests viz., gram reaction, motility, catalase activity, oxidase activity, nitrate reductase activity, hydrogen sulphide formation and growth under different conditions. The zinc solubilizing property of G.diazotrophicus cultures was assessed under in vitro condition. The strain GDSB was found to be effective in solubilizing the insoluble Zn compounds viz., zinc oxide, zinc carbonate and zinc phosphate. The present study showed the efficiency of G.diazotrophicus on zince solubilization. Hence there is a possibility to correct zn difficiency by employing G.diazotrophicus as biological means.

**FUTURE STUDY:**

Further researches are needs to exploit the potentiality of G.diazotrophicus on zinc solubilization in soil or in natural environment.

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