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ORIGINAL ARTICLE

Assessing the Zinc solubilization ability of *Gluconacetobacter diazotrophicus* in maize rhizosphere using labelled ^{65}Zn compounds

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Abstract Solubilization of insoluble zinc compounds like $ZnCO_3$ and ZnO by *G. diazotrophicus* was confirmed using radiotracers. The zinc compounds ($ZnCO_3$ and ZnO) were tagged with ^{65}Zn. $^{65}ZnCO_3$ and ^{65}ZnO was effectively solubilized and the uptake of zn by the plants also more in *G. diazotrophicus* inoculated treatments compared to the uninoculated treatments. Three types of soils (Zn deficient-sterile, Zn deficient-unsterile, and Zn sufficient-sterile) were used in experiment. Among the three soils, Zn deficient-unsterile soil registered maximum zinc solubilization compared to other two soils. This may be due to other soil microorganisms in unsterile soil. Application of ZnO with *G. diazotrophicus* showed better uptake of the nutrient.

Keywords *G. diazotrophicus* · $^{65}ZnCO_3$ · ^{65}ZnO · Radioassay

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Introduction

The global necessity to increase agriculture production from a steadily decreasing and degrading land resource base has placed considerable strain on the fragile ecosystems. While the use of mineral fertilizers is considered the quickest and surest way of boosting crop production, their cost and other constraints deter farmers from using them in recommended quantities [24]. Micronutrients are important for the growth of plants animals and also for the microbes [6]. In biological systems, Zn is involved in the activity of more than 300 enzymes.

The zinc deficiency scenario is some how analogues to the phosphorus deficiency, since both the nutrients are deficient in Indian soils with an average of about 40 percent [13, 21]. The water soluble zinc applied as zinc sulphate gets transformed into different forms like Zn (OH) and Zn $(OH)_2$ at a pH of 7.7 and 9.1 [22]; zinc carbonate in calcium rich alkali soils [10]; zinc phosphate in near neutral to alkali soils of high phosphorus application [4, 11]; and zinc sulphide under reduced conditions. Zyrin et al. [25] reported that zinc in soils associated mainly with hydrous Fe and Al oxides (14 to 38 per cent of total Zn) and with clay minerals (24 to 63 per cent). There was also a finding that movement of fertilizer Zn to lower layers was found negligible in cultivated soils [21]. All this findings strongly supports that Zn gets transformed into insoluble forms after application similar to phosphorus and this leads to appearance of Zn deficiency.

The organic based zinc nutrition is best since its Zn use efficiency is more. A bacterial based approach was devised to solve the micronutrient deficiency problem [17, 2]. The basic principle behind this approach is decreasing the pH to

5 or below and making zinc soluble and as a consequence the available zinc will get increased in the soil system [22]. A term called 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 [17, 2]

A preliminary idea to solve zinc nutrition using a bacterium was put forward and further the mechanism behind the solubilization was elucidated under in vitro conditions by *G. diazotrophicus* – a prolific gluconic acid producing bacterium [3, 18]. So far *G. diazotrophicus* was widely recognized because of its nitrogen fixing ability [20].

Materials and methods

Black cotton soil was used for the pot culture experiments. The available Zn content of the soil was found at the below critical level and hence the collected soil was treated as Zn deficient soil. In order to conduct the experiment in Zn sufficient soil, simultaneously, the soil collected was enriched with Zn by addition of $ZnSo_4$ and the Zn content was monitored for one month.

Seeds and sowing

Maize seeds (Co1) obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore was used.

Estimation of total zinc content in soil

Total zinc in the soil was estimated in HCl extract following the method of Lindsay and Norvel [12].

Estimation of available zinc content in soil

The soil sample was collected following the standard method, air dried, powdered and sieved to pass through a 2 mm mesh British standard sieve (BSS). The available zinc content of the soil was estimated using the diethylene triamine penta acetic acid extract following the standard procedure of Lindsay and Norvell [12].

Setting of pot culture experiment

The Zn deficient soil was a divided in to two portions. One portion was sterilized in the autoclave at 20 lb for 2 hrs for two consecutive days. The other portion was used as such (unsterile soil). Similarly Zn sufficient soil was sterilized and taken as sterile soil. All the three types of soils (Zn deficient – sterile, Zn deficient – unsterile and Zn sufficient – sterile) were filled in the 18 tubular pots each at 750 g/pot.

Inoculation of *G. diazotrophicus*

Tenth day after sowing 24 hrs old fresh culture of *G. diazotrophicus* (8×10^8/ml) inoculated at 10 ml per pot as per the treatment.

^{65}Zn tagging with the fertilizer and application

Two zinc sources viz., $ZnCO_3$ and ZnO were taken for the isotopic experiment. The ^{65}Zn compound required for tagging with $ZnCO_3$ and ZnO was obtained from Bhabha Atomic Research Centre, Trombay. The tagging was done at 1mCi/g of Zn, using the principle of Isotope Dilution Technique (IDT). Carbon flux was fixed as 100 and the duration of the irradiation was about 6 days. The labelled $ZnCO_3$ and ZnO were applied on 17^{th} day after sowing at the rate of 0.117 g and 0.18 g respectively as per the treatment schedule.

Intercultural operations

The pots were watered with double distilled water as judged by the soil moisture. Plant protection measures were adopted as and when required.

Radioassay of plant materials

At 15 days interval after the application of Zn sources, one plant from each treatment was uprooted, shade dried and then oven dried. One gram of the plant material from each treatment was ashed in silica crucible and transferred to scintillation vial. The vial was then placed in a T1- activated Nal crystal, well type gamma ray spectrometer (Type GRS, 23B, Electronics Corporation of India Ltd., Hydrabad) and the radioactivity of the samples was determined by integral counting, keeping the single channel analyzer at optimal window and lower level settings. The radioactivity of the fertilizer standards was also determined.

Analysis of soil and plant samples for zinc

The total Zn content, available Zn content in soil and Total Zn content of maize seedlings was analyzed at 15 days interval in the pot culture experiment up to 45 days. Total zinc content of maize seedlings [8].

Results

The initial N, P, K, total zinc and available zinc content of soil used for experiment were estimated. The available nitrogen content of soil was in the range between 295 kg/ha and 304 kg/ha in their soils. Similarly the available phosphorus content was from 10.2 kg/ha to 13.5 kg/ha and potassium was between 215 kg/ha and 265 kg/ha. Though the total Zn content showed not much variation, the available Zn content was more (1.8 ppm) in Zn sufficient soil compared to Zn deficient (0.4 ppm) soil (Table 1).

A gradual decrease in the total zinc content of soil was noticed with the growth of maize. At all the three stages and in the three samples used, the reduction was more in the *G. diazotrophicus* inoculated treatments. The reduction in total soil zinc was found maximum in the zinc deficient unsterile soil than sterile zinc sufficient and deficient soils. Among the zinc sources ^{65}ZnO application with *G. diazotrophicus*

Table 1 Initial nutrient status of soil used for the pot culture experiment

Soil	Available soil nitrogen (Kg/ha)	Available soil phosphorus (Kg/ha)	Available soil potassium (Kg/ha)	Soil total zinc (ppm)	Soil available zinc (ppm)
Zn sufficient soil (sterile)	304	13.5	265	63	1.8
Zn deficient soil (sterile)	298	10.2	215	64	0.4
Zn deficient soil (unsterile)	295	11.2	224	67	0.4

Table 2 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on the total Zn content of soil grown with maize

Treatment	Total zinc content of soil (ppm)											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	63.0	62.5	61.0	62.6	64.0	63.2	62.0	63.06	67.0	66.2	65.2	66.13
$^{65}ZnCO_3$ alone	75.0	74.0	72.2	72.2	71.0	69.5	68.2	69.56	74.0	72.0	70.2	72.06
$^{65}ZnCO_3$ + *G. diazotrophicus*	60.0	56.0	51.0	55.6	58.0	52.0	48.0	52.66	56.0	69.0	42.0	55.66
^{65}ZnO alone	72.5	72.0	72.6	72.36	69.5	68.7	66.2	68.13	69.0	68.3	62.3	65.33
^{65}ZnO + *G. diazotrophicus*	61.2	57.1	51.4	56.56	55.0	51.5	47.4	51.30	67.0	52.5	43.4	54.30
G. diazotrophicus alone	65.0	63.0	60.0	62.66	67.0	64.2	62.2	64.46	66.0	62.4	60.2	62.86

Table 3 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on the available Zn content in soil grown with maize

Treatment	Available zinc content of soil (ppm)											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	2.20	1.80	1.75	1.91	0.40	0.30	0.25	0.31	0.4	0.35	0.28	0.34
$^{65}ZnCO_3$ alone	2.30	1.90	1.82	1.70	0.45	0.40	0.30	0.38	0.45	0.42	0.40	0.42
$^{65}ZnCO_3$ + *G. diazotrophicus*	2.10	2.00	1.70	2.00	0.80	0.60	0.46	0.60	0.90	0.70	0.56	0.72
^{65}ZnO alone	2.25	1.80	1.74	1.74	0.40	0.30	0.28	0.32	0.60	0.50	0.42	0.50
^{65}ZnO + *G. diazotrophicus*	2.15	2.10	1.96	1.96	0.90	0.80	0.71	0.80	0.95	0.82	0.59	0.78
G. diazotrophicus alone	2.20	1.70	1.90	1.90	0.70	0.60	0.50	0.60	0.60	0.50	0.42	0.50

(51.30 ppm) showed more reduction in total zinc content (Table 2).

A gradual decrease in the available zinc content of soil was noticed with the growth of maize. At all the three stages and in the three samples used, available zinc content of soil was more in the *G. diazotrophicus* inoculated treatments. Among the treatments $^{65}ZnCO_3$ with *G. diazotrophicus* treatment showed more available Zn (2.00 ppm) (Table 3).

Percent zinc derived from fertilizer showed a progressive increase up to 45th day with zinc and *G. diazotrophicus* inoculated treatments. Among the treatments $^{65}ZnCO_3$ with *G. diazotrophicus* and ^{65}ZnO with *G. diazotrophicus* registered the maximum %Zndff. Unsterile Zn deficient soil (0.172 ppm) showed higher %Zndff than other soils) (Table 4).

Zinc uptake from fertilizer showed a conspicuous increase with the sampling period. While comparing the treatments the zinc uptake from fertilizer was higher in $^{65}ZnCO_3$ with *G. diazotrophicus* (0.058 mg/pot) and ^{65}ZnO with *G. diazotrophicus* (0.062 mg/pot). The uptake of fertilizer was higher in the unsterile Zn deficient soil (0.062 mg/pot) when compared to other two types of soils (Table 5).

The percent zinc derived from soil showed a dramatic decline at growing stages of plant. With increasing Zndff, the %Zndfs showed a decline in treatments. Treatments without inoculation of *G. diazotrophicus* (99.98%, 100%) showed higher %Zndfs. Sterile Zn sufficient soil registered the highest %Zndfs (Table 6).

Table 4 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on percent zinc derived from fertilizer

Treatment	% Zndff											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	0	0	0	0	0	0	0	0	0	0	0	0
$^{65}ZnCO_3$ alone	0.017	0.023	0.021	0.020	0.017	0.023	0.024	0.021	0.025	0.026	0.024	0.025
$^{65}ZnCO_3$ + *G. diazotrophicus*	0.035	0.039	0.040	0.038	0.039	0.143	0.043	0.075	0.048	0.049	0.043	0.046
^{65}ZnO alone	0.086	0.088	0.086	0.087	0.079	0.112	0.103	0.098	0.096	0.130	0.105	0.110
^{65}ZnO + *G. diazotrophicus*	0.157	0.151	0.140	0.140	0.159	0.167	0.154	0.16	0.179	0.180	0.158	0.172
G. diazotrophicus alone	0	0	0	0	0	0	0	0	0	0	0	0

Table 5 Influence of Zn^{65} application and *G. diazotrophicus* inoculation on uptake of Zn derived from applied fertilizer

Treatment	Zinc uptake (mg/pot)											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	0	0	0	0	0	0	0	0	0	0	0	0
$^{65}ZnCO_3$ alone	0.005	0.005	0.0061	0.005	0.0037	0.004	0.006	0.004	0.0037	0.005	0.015	0.007
$^{65}ZnCO_3$ + *G. diazotrophicus*	0.006	0.013	0.018	0.012	0.014	0.140	0.020	0.058	0.012	0.012	0.020	0.014
^{65}ZnO alone	0.020	0.022	0.027	0.027	0.016	0.027	0.032	0.025	0.021	0.029	0.040	0.030
^{65}ZnO + *G. diazotrophicus*	0.035	0.048	0.065	0.065	0.043	0.040	0.077	0.041	0.046	0.055	0.085	0.062
G. diazotrophicus alone	0	0	0	0	0	0	0	0	0	0	0	0

Table 6 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on percent zinc derived from soil

Treatment	% Zndfs											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	100	100	100	100	100	100	100	100	100	100	100	100
$^{65}ZnCO_3$ lone	99.98	99.97	99.96	99.97	99.98	99.97	99.96	99.97	99.98	99.97	99.96	99.97
$^{65}ZnCO_3$ + *G. diazotrophicus*	99.97	99.96	99.95	99.96	99.96	99.95	99.94	99.95	99.96	99.95	99.94	99.95
^{65}ZnO lone	99.94	99.91	99.90	99.93	99.92	99.88	99.87	99.89	99.90	99.87	99.80	99.89
^{65}ZnO + *G. diazotrophicus*	99.86	99.84	99.82	99.86	99.84	99.83	99.82	99.84	99.82	99.82	99.80	99.82
G. diazotrophicus alone	100	100	100	100	100	100	100	100	100	100	100	100

Table 7 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on uptake of Zn derived from soil

Treatment	Zinc uptake (mg/pot)											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	21.79	23.40	29.40	23.06	14.25	17.4	22.14	17.93	16.38	21.28	23.71	20.75
$^{65}ZnCO_3$ lone	23.99	26.69	29.39	26.43	21.95	23.56	27.09	22.86	17.85	22.43	36.10	25.46
$^{65}ZnCO_3$ + *G. diazotrophicus*	18.68	34.14	45.68	32.83	36.23	37.31	47.54	39.02	26.86	28.30	47.52	33.56
^{65}ZnO lone	24.28	25.40	31.47	27.05	20.84	24.17	32.36	25.79	22.77	22.85	39.96	28.52
^{65}ZnO + *G. diazotrophicus*	19.54	31.80	46.85	32.73	27.55	30.12	49.92	35.86	26.20	30.54	53.91	36.88
G. diazotrophicus alone	21.92	22.45	25.80	23.05	19.83	22.60	28.82	23.76	21.78	27.25	33.60	27.54

The zinc uptake from soil showed prominent increased with the progressive stages of sampling. The total Zn uptake from the soil was lower in control (17.93 mg/pot) and *G. diazotrophicus* alone applied treatments (23.05 mg/pot) in all the stages. $^{65}ZnCO_3$ with *G. diazotrophicus* (39.02 mg/pot) and ^{65}ZnO with *G. diazotrophicus* (36.88 mg/pot) registered the highest zinc uptake. In general ^{65}ZnO with *G. diazotrophicus* (39.02 mg/pot) recorded that maximum uptake of zinc up to 45 days. Among the three soils, sterile Zn sufficient soil registered maximum Zn uptake compare to other two soils (Table 7). Inoculation of *G. diazotrophicus* with ^{65}ZnO and $^{65}ZnCO_3$ enhanced the zinc utilization from the applied fertilizer, irrespective of the soils. However, the percent utilization was recorded maximum in the zinc deficient soil then the other two soils used (Table 8).

Discussion

Radio tracer technique is an important tool to investigate the nutrient uptake in crop plants. Studying plant -microbe interaction using isotope technology was reported (Jim et al. 2006). In the present study experiment was conducted to assess the zinc solubilization potential of *G. diazotrophicus* using labelled ^{65}Zn compounds (^{65}ZnO and $^{65}ZnCO_3$). The results showed that the inoculation of *G. diazotrophicus* with ^{65}ZnO and $^{65}ZnCO_3$ found to increase the available zinc content in the soil. However the available zinc content was more in zinc deficient unsterile soil than the sterile zinc sufficient and zinc deficient soils. This clearly indicated that microorganism other than *G. diazotrophicus* may also involved the solubilization of insoluble zinc sources. Zn

Table 8 Influence of ^{65}Zn application and *G. diazotrophicus* inoculation on percent utilization of applied fertilizer

Treatment	Percent utilization of applied fertilizer											
	Sterile soil								Unsterile soil			
	Zinc sufficient soil				Zinc deficient soil				Zinc deficient soil			
	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean	15th day	30th day	45th day	Mean
Control (without Zn and without *G. diazotrophicus*)	0	0	0	0	0	0	0	0	0	0	0	0
$^{65}ZnCO_3$ lone	0.028	0.020	0.020	0.022	0.016	0.020	0.022	0.019	0.020	0.022	0.041	0.027
$^{65}ZnCO_3$ + *G. diazotrophicus*	0.035	0.038	0.039	0.037	0.038	0.042	0.042	0.040	0.048	0.048	0.080	0.058
^{65}ZnO lone	0.082	0.086	0.085	0.084	0.058	0.111	0.098	0.098	0.095	0.126	0.100	0.107
^{65}ZnO + *G. diazotrophicus*	0.176	0.150	0.135	0.153	0.076	0.132	0.154	0.154	0.175	0.180	0.190	0.168
G. diazotrophicus alone	0	0	0	0	0	0	0	0	0	0	0	0

solubilizing potential of few bacterial genera namely Bacillus spp, Pseudomonas fluorescens, Thiobacillus ferroxidans, T.thiooxidans have been reported [7, 5]. Comparatively inoculated organism better in solubilization in zinc deficient soil than zinc sufficient soil and hence the growth of maize seedlings was better in the zinc deficient soil. Irrespective of soil types, the inoculation of *G. diazotrophicus* showed higher plant zinc content. The total zinc content was more in unsterile zinc deficient soil. This may due to more zinc available due to solubilization of insoluble zinc compounds by soil microorganisms or *G. diazotrophicus* in unsterile soil.

The per cent zinc derived from fertilizer (%Zndff) was found more in maize seedlings grown on zinc deficient soil. However %Zndff was evidently more in maize grown on the three soil types were *G. diazotrophicus* was inoculated compared to uninoculated counterparts. It is obvious from the results that *G. diazotrophicus* is the one which made available the zinc from insoluble sources for maize crop. Application of ZnO with *G. diazotrophicus* showed better uptake of the nutrient. ZnO is a sparingly soluble compound ($3–5 \times 10^4 g^{-1} 100^{-1}$ water at 25°C) [16] compared to $ZnCO_3$ might be the added advantage of ZnO.

In general from the results of present investigation, it is obvious that *G. diazotrophicus* is capable of solubilizing insoluble zinc compounds through the production of gluconic acid, and inoculation of this organism in zinc deficient soils will certainly help for the alleviation of zinc deficiency under sustainable crop production.

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