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Zinc Metal Solubilization by *Gluconacetobacter diazotrophicus* and Induction of Pleomorphic Cells

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Abstract *Gluconacetobacter diazotrophicus* strain PAl5 exhibited a minimum inhibitory concentration value of 11 mM in an LGI medium amended with $ZnCl_2$. When an LGI medium was amended with Zn metal, solubilization halos were observed in a plate assay, and further solubilization was confirmed in a broth assay. The maximum solubilization was recorded after 120 h with a 0.1% Zn metal amendment. During solubilization, the culture growth and pH of the broth were indirectly correlated. Using a Fourier Transform Infrared Spectroscopy analysis, one of the agents solubilizing the Zn metal was identified as gluconic acid. When the Zn-amended broth was observed under a bright field microscope, long involution cells were observed, and further analysis with Atomic Force Microscopy revealed highly deformed, pleomorphic, aggregate-like cells.

Keywords: *Gluconacetobacter diazotrophicus*, Zn metal solubilization, FT-IR, aggregate-like cells, gluconic acid

Zinc is a metal that is commonly regarded as a double-edged sword in living systems. At trace concentrations, it acts as a cofactor in enzymes belonging to all six classes recognized by IUBMB nomenclature [26] and in DNA binding proteins [10]. However, at higher concentrations, Zn incites debilitating effects by intercepting other metal cations and inhibiting their respective physiological functions [20]. In nature, noticeable microbial interaction with Zn and phosphorus compounds frequently manifests itself through immobilization or mobilization [2, 7, 21]. Metal immobilization can be through cellular sequestration and bioaccumulation or through extracellular precipitation. Conversely, solubilization is mediated by the production of certain organic acids that act as ligands, or through proton activity or polysaccharide production. Solubilization has commercial applications in the form of bioleaching, which involves the use of autotrophic *Acidithiobacillus ferroxidans* and *Acidithiobacillus thiooxidans* to solubilize Zn metal from various ores [23]. However, pure Zn metal solubilization by heterotrophic bacteria has not been documented in previous studies. The biocontrol fungus *Trichoderma harzianum* Rifai 1295-22 has the ability to dissolve Zn metal based on its oxidative potential and the chelating power of certain complexing agents produced by this fungus [2], plus the bacterial solubilization of insoluble or sparingly soluble Zn compounds has already been documented [24, 27]. Furthermore, the effect of available Zn on soil microbiota has also been extensively investigated [16, 17, 22], yet its impact on endophytic bacteria has received less attention. Certain endophytic *Methylobacterium* species with a higher tolerance to Zn metal and a minimum inhibitory concentration (MIC) of more than 5 mM of Zn have been observed in isolates recovered from the shoots of the Zn-hyperaccumulating plant *Thlaspi caerulescens* subsp. *calaminaria* [13]. The *Gluconacetobacter diazotrophicus* used in the present study is an endophytic bacterium belonging to the acetic acid group of bacteria [5, 8], and the current authors have already reported on the efficient solubilization of different Zn compounds by *G. diazotrophicus* in a culture broth [24]. Accordingly, the present study was designed to determine the potential of *G. diazotrophicus* to solubilize metallic Zn and the impact of Zn ions on the cell morphology.

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MATERIALS AND METHODS

Organism and Maintenance

Gluconacetobacter diazotrophicus strain PAl5 (ATCC49037) isolated from sugarcane roots was obtained from Johanna Döbereiner (EMBRAPA, Itagui, RJ, Brazil) and maintained in LGI agar slants [5] [g/l composition: K_2HPO_4 0.2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.02, Na_2MoO_4 0.002, $FeCl_3 \cdot 6H_2O$ 0.01, glucose 100.00, bromothymol blue (BTB) 5 ml (0.5% solution in 0.2 mol/l KOH solution), pH 6.0] at 28±1°C.

Minimum Inhibitory Concentration (MIC) Determination

The MIC was determined as per the method described by Leigh-Emma [12]. The resistance of *G. diazotrophicus* to Zn metal was tested using test tubes containing LGI broth amended with $ZnCl_2$ at one concentration per tube. The zinc salt ($ZnCl_2$) was prepared as a 100 mM stock, then concentrations of 6, 7, 8, 9, 10, and 11 mM were obtained by dilution with LGI broth. The broth was inoculated with *G. diazotrophicus* exponential phase culture (6×10^8 cells/ml) and maintained in a gyratory rotating shaker at 120 rpm at 27±2°C. The growth was then recorded after 24 and 48 h at 620 nm using a Colorimeter (CL157 ELICO).

Determination of *In Vitro* Zinc Solubilization

G. diazotrophicus strain PAl5 grown in LGI broth for 48 h (6×10^8 CFU/ml) was spotted at 10-µl volume onto plates containing LGI medium amended with Zn metal powder (0.1%). The plates were incubated at 28±1°C for 5 days and observed at 24-h intervals. The solubilization halo was visualized against an opaque background. For the broth assay, *G. diazotrophicus* PAl5 was grown in 100 ml of LGI broth amended with a 0.1% concentration of Zn metal at 28°C in a controlled environment incubator shaker (Model G27; New Brunswick, NJ, U.S.A.) at 120 rpm. *G. diazotrophicus* PAl5 originally cultured in LGI broth for 24 h was used as the mother inoculum. One-ml samples diluted to 50 ml with sterile distilled water were filtered through a 0.4-µm filter, and the soluble Zn present in the culture broth was determined using an atomic absorption spectrophotometer (AAS-Model Varian C) at different periods of growth. The pH of the broth was also monitored, and the growth of the culture was measured at 620 nm using a colorimeter (CL157, ELICO). Furthermore, the number of colony forming units (CFU/ml) in the broth was assessed using the pour plate method. Three replications were maintained for each treatment, and uninoculated media with insoluble Zn sources served as the control.

Fourier Transform Infrared (FT-IR) Spectroscopy and Identification of Culture Supernatant Component

For the FT-IR analysis, Zn-amended and control flask culture filtrates (5 ml) were both collected after 24 h and centrifuged (20 min, 6,000 ×*g*). Seven-hundred µl of the culture supernatant was added to 3-ml vials and frozen at −50°C using a lyophilizer (Christ LOC, 1M, Germany). A standard procedure was used for processing the dried samples into KBr disks, where 1 µg of the freeze-dried product was mixed with 300 mg of dry KBr powder using a pestle and mortar. The resulting mixture was then transferred to a stainless steel holder (13 mm inner diameter) that had been placed in a hydraulic press and evacuated with an attached vacuum pump. The press was adjusted to 10,000, 8,000, 6,000, 4,000, or 2,000 kg, generating a pressure of approximately 7,500, 6,000, 4,500, 3,000, or 1,500 kg cm^{-2} on the pellet within the holder, respectively. The pressure was maintained for approximately 3 min.

The infrared spectra of the culture supernatant was recorded using a Nicolet FT-IR (AVATAR-330) equipped with a narrow-band mercury/cadmium/telluride liquid-nitrogen-cooled IR detector. The optical bench was continuously purged with dry air, and the acquisition parameters were 4 cm^{-1} resolution, 32 co-added interferograms, within a range of 4,000–400 cm^{-1}.

Analysis of Cell Morphology Using Atomic Force Microscopy (AFM)

Cells from both the zinc-amended and control flasks were preliminarily analyzed using bright field microscopy, where the cells were unstained, and then observed at 40×, followed by 100× to identify any changes in the cell morphology. As changes were observed in the cells from the Zn-amended flask (Fig. 5B), the Zn-amended and control culture broths were both subjected to AFM analysis.

For the AFM, samples (10 ml) were taken from 120 h broth cultures of the Zn-amended and control treatments.

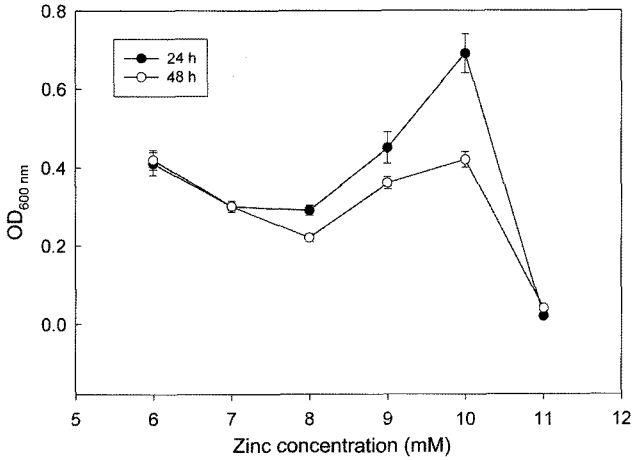

Fig. 1. Changes of population (OD) in LGI medium amended with different concentrations of $ZnCl_2$ during incubation with *G. diazotrophicus* PAl5 for 24 and 48 h.
Values are means of three replicates. Error bars (±SE) are shown when larger than the symbol.

After centrifugation (20 min, 6,000 ×*g*), the clear supernatant was discarded and the pellet was washed twice with 70% ethanol. Using a micropipette, the pellets were then suspended inside an Eppendorf tube, and a 50-µl suspension droplet was placed over a freshly cleaved mica sheet glued to a 15-mm metal stub. The cells were then allowed to dry on a mild heat source. Thereafter, the stub was manually positioned on the AFM head holder, approaching the tip perpendicularly to the mica surface. Scans were immediately performed on the dried samples in air, at room temperature and pressure, using an AFM (Shimadzu SPM 9500-2J). The cantilever used for scanning was an Olympus-type OMCL-TR 800 PSA: DC contact mode, with lever thickness 800 nm, lever length 200 µm, spring constant 0.15 N/m, resonance frequency 24 KHz, and tip height 2.0 µm. The images were obtained using different scan sizes (800–12,000 nm) at a 1–4 Hz scan speed and 0 V set point voltage, and the data collected with 400 points per line and 400 lines in a constant height mode. A number of different tips and scan series were used to obtain a sufficient number of replications.

RESULTS

Minimum Inhibitory Concentration (MIC) of Zn for *G. diazotrophicus* PAl5

The effect of various concentrations of $ZnCl_2$ on the *G. diazotrophicus* PAl5 population is presented in Fig. 1. A population reduction was obvious after both sampling intervals. The culture tolerated up to 10 mM of $ZnCl_2$, and then growth became inhibited at 11 mM. Furthermore, a plate count analysis revealed a complete absence of bacteria at that particular concentration. Thus, 11 mM of $ZnCl_2$ was taken as the MIC value for *G. diazotrophicus* PAl5 in LGI medium.

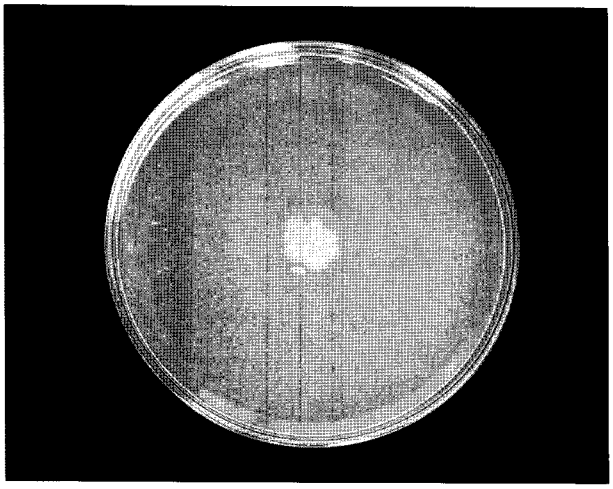

Fig. 2. Solubilization zone produced by *G. diazotrophicus* PAl5 spotted in LGI medium amended with Zn metal at 0.1% concentration.

Solubilization of Zn Metal by *G. diazotrophicus* PAl5

The solubilization of Zn metal was first assessed in a solid LGI medium amended with 0.1% Zn metal, and a solubilization halo observed after 24 h (Fig. 2).

The solubilization potential was also tested in a broth assay. Although no solubilization halo was present after 12 h in the plate assay (Fig. 3A), the broth assay showed clear evidence of the solubilization of Zn metal after 12 h, based on the presence of Zn ions (Fig. 3B). After 12 h, the

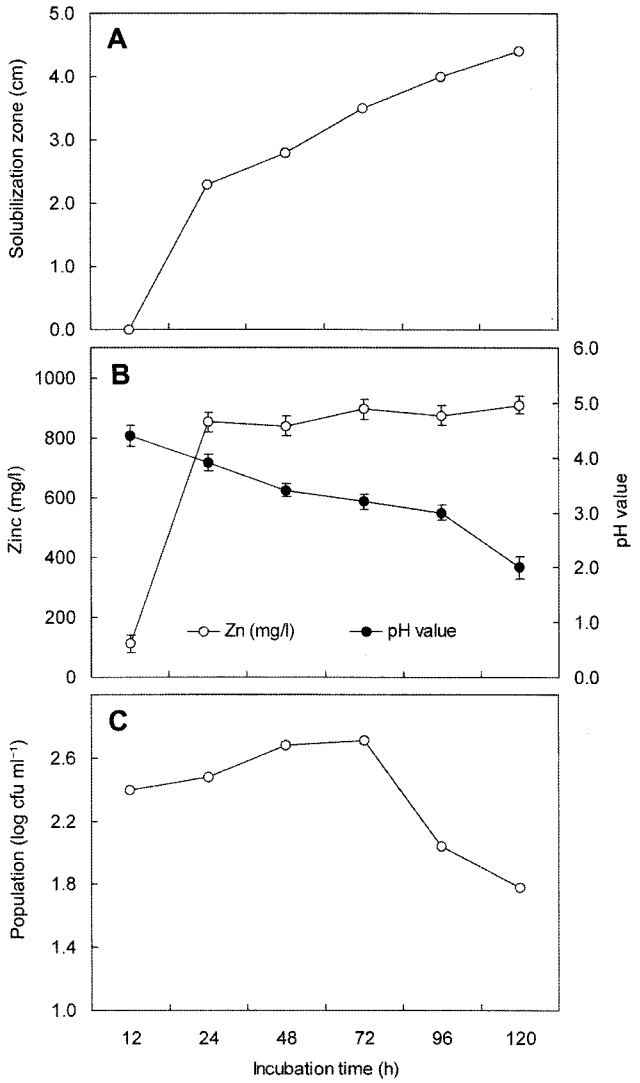

Fig. 3. A. Solubilization halo in LGI medium amended with Zn metal during growth of *G. diazotrophicus* PAl5. **B.** Changes in pH and available Zn in LGI medium amended with Zn metal during growth of *G. diazotrophicus* PAl5. Values are means of three replicates. Error bars (±SE) are shown when larger than the symbol. **C.** Changes in population of *G. diazotrophicus* PAl5 culture grown in Zn metal (0.1%)-amended LGI medium.

pH of the broth was reduced to 4.4 from the initial pH value of 5.8. Concurrently, an increased concentration of available Zn (111.8 mg Zn/g Zn metal) was observed after 12 h when compared with the uninoculated control flask's available Zn concentration of 0.002 (data not shown).

After 24 h, a seven-fold increase in the available Zn was evident, and this increase continued up to 120 h of incubation, at which point the available Zn concentration was 910 mg. The pH was also drastically reduced to 2.0 during the last sampling.

The Zn-amended broth alone was also subjected to population analysis (Fig. 3C), where the number of viable cells during the course of the experiment was estimated as the number of CFU/ml. An early increase in the population was noted from 12 to 72 h; then at 96 h, the number of CFU was found to decrease to 1.1×10^2 CFU/ml, with a further reduction to 0.6×10^2 CFU/ml during the final interval of the experiment.

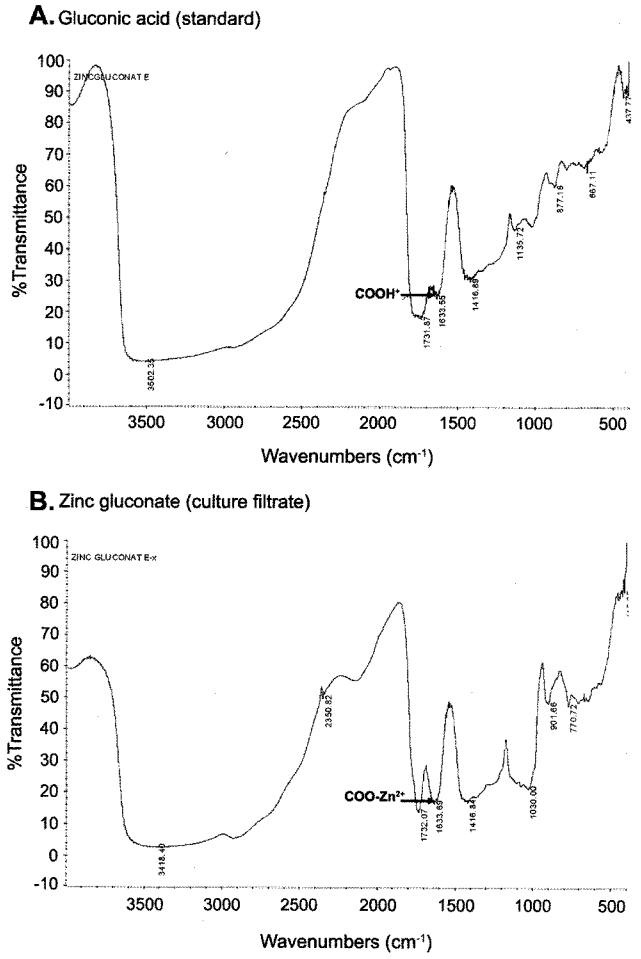

Fig. 4. FT-IR absorption spectra obtained from (**A**) gluconic acid (standard) and (**B**) Zn-amended *G. diazotrophicus* PAl5 culture supernatant. Shift in spectrum at 1633 wave number (cm^{-1}). Arrow indicates possibility of Zn^{2+} ions binding to carboxyl group.

FT-IR Analysis

The components of the culture broth were analyzed using FT-IR, and Fig. 4 shows the IR spectra for standard gluconic acid and the culture supernatants. For the standard gluconic acid spectrum (Fig. 4A), the characteristic absorption was obtained at 1633.55 cm^{-1}, which typically denotes the presence of COOH group in gluconic acid. For the 24-h culture broth amended with zinc, there was a slight shift in the spectrum (Fig. 4B), which was recorded at 1633.69 cm^{-1}, probably due to the chelation of some Zn ions in the carboxyl terminal of the gluconic acid.

Bright Field and AFM Observation

To observe any morphological variation, cells from both the Zn-amended and control flasks were subjected to bright field microscopy. The cells from the control flask were small and rod-shaped; however, the cells from the Zn-amended broth showed variations, such as long, continuous, and chain-like involution forms (Fig. 5B). Thus, the cells were further analyzed using AFM to ascertain whether any deformation had occurred. As a result, it was found that the chain-like appearance under the bright field microscopy was actually highly deformed, pleomorphic, disorganized, large-sized (approximately 10 times larger than normal size), and aggregate-like cells

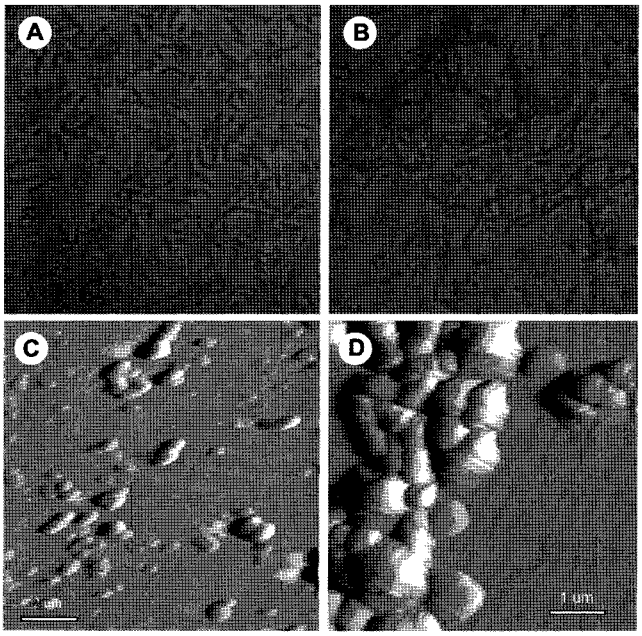

Fig. 5. Bright field microscopy and atomic force microscopy images of *G. diazotrophicus* cells. Bright field microscopic images recorded at 100× magnification.
A. Normal rod-shaped cells obtained from control flask. **B.** Cells grown in 0.1% Zn metal for a period of 120 h; note the presence of deformed long thread-like involuted cells. **C.** AFM images showing normal rod-shaped cells in control flask. **D.** Presence of large aggregate-like, deformed, pleomorphic cells, approximately 10 times larger than normal cells, in Zn metal-added broth. The horizontal bars indicate the scale.

(Fig. 5D) when compared with the normal rod-shaped cells in the control flask (Fig. 5C).

DISCUSSION

G. diazotrophicus culture PAl5 was found to have an MIC value of 11 mM in an $ZnCl_2$-amended LGI medium. However, the same culture was able to grow in an LGI medium amended with a 0.1% Zn metal concentration, *i.e.*, 15.3 mM Zn. The ability of the culture to grow in a slightly increased concentration of Zn may have been due to the amended nature of the Zn metal in the broth. Although the Zn metal was completely insoluble in the LGI medium during the initial phase of the experiment, the progress of solubilization made it available, possibly inflicting toxicity on the culture. Moreover, a difference in the MIC values in $ZnCl_2$-amended media was previously observed for *Gluconobacter*, a related genus of *Gluconacetobacter* [12], where it was postulated that the MIC value of a culture can differ based on the type of Zn source used, constituents of the medium, and methodology employed to test the MIC. In the present study, an increase in the concentration of available Zn was noted throughout the experimental period. Similar results were also observed in previous studies employing *Pseudomonas putida* and *G. diazotrophicus* with insoluble Zn compounds, like ZnO and $Zn_3(PO_4)_2$ [6, 24].

Because of bacterial growth, a reduction in pH was observed to the end of the experiment. A decrease in the pH of the broth amended with insoluble Zn compounds has been argued by previous authors to result from organic acid production and subsequent acidification of the medium [6, 24]. In the present study, the pH decrease was also likely due to organic acid or H^+ ions released in the broth *via* the culture metabolism. In the FT-IR spectral analysis, the shift in the wave number for the COOH group in gluconic acid was attributed to the chelation of Zn ions formed from the Zn metal due to the decrease in the pH caused by bacterial growth. Since the H^+ ion in the carboxylic group can be replaced with Zn^{2+} ions, the carboxylic acid (gluconic) formed may have chelated the metallic ions. Very recently, it was unequivocally proved that during the metabolism of glucose by *G. diazotrophicus*, gluconic acid and its derivative acids were the dominant and central products of the metabolic pathway [4, 24]. This acid is also commonly encountered during the process of microbial phosphorus solubilization [25]. In the present study, the effect of the solubilized Zn metal on the cell morphology was assessed using bright field microscopy and AFM [3, 19]. With the bright field microscopy, the Zn metal-treated cells were deformed and long and thread-like in contrast to the normal short, rod-shaped cells. AFM revealed the occurrence of pleomorphic, highly deformed, and aggregated cells after 120 h of the experiment. The deformed cells were approximately ten times larger than the normal cells (Fig. 5D), which may have been due to the presence of high levels of soluble Zn (910 mg/g of Zn metal) in the broth, resulting from solubilization. The formation of pleomorphic cells has already been observed with *G. diazotrophicus* when the cells were subjected to elevated concentrations of NH_4 or pesticides [15, 18]. A detailed description of the formation of pleomorphism by bacteria was previously presented by Krasil'nikov [11] in his book "Soil Microorganisms and Higher Plants", where *Azotobacter* spp. subjected to lithium chloride treatment produced cells that were greatly enlarged and deformed, described as involution forms. It is also interesting to note that in a previous study of *G. diazotrophicus*, its older cell forms in a culture broth were also referred to as long involution forms [8]. Krasil'nikov [11] further stated that this kind of cell could also be formed when increasing the concentration of salts, dyes, UV rays, antibiotics, bacteriophages, and the suboptimal temperature. In addition, these cells do not lose their viability, as under certain favorable conditions they can regenerate and produce normal progeny. This phenomenon is now refered to as a viable but nonculturable (VBNC) cell formation. Published evidence is already available regarding the induction of VBNC in *Agrobacterium tumefaciens*, *Rhizobium leguminosarum*, and *Escherichia coli* using Cu [1, 9]. Thus, a similar VBNC state was seemingly formed in the present study, although pleomorphic cells were only detected in the culture broth. It has also been suggested that the formation of VBNC by *G. diazotrophicus* may be a soil survival mechanism [4]. Nonetheless, the present study elucidated the solubilization of Zn metal and pleomorphism resulting from Zn toxicity in *G. diazotrophicus*. However, an indepth analysis is needed to understand the basic mechanisms behind the *G. diazotrophicus*-Zn metal interaction and the possible development of VBNC by this bacterium.

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