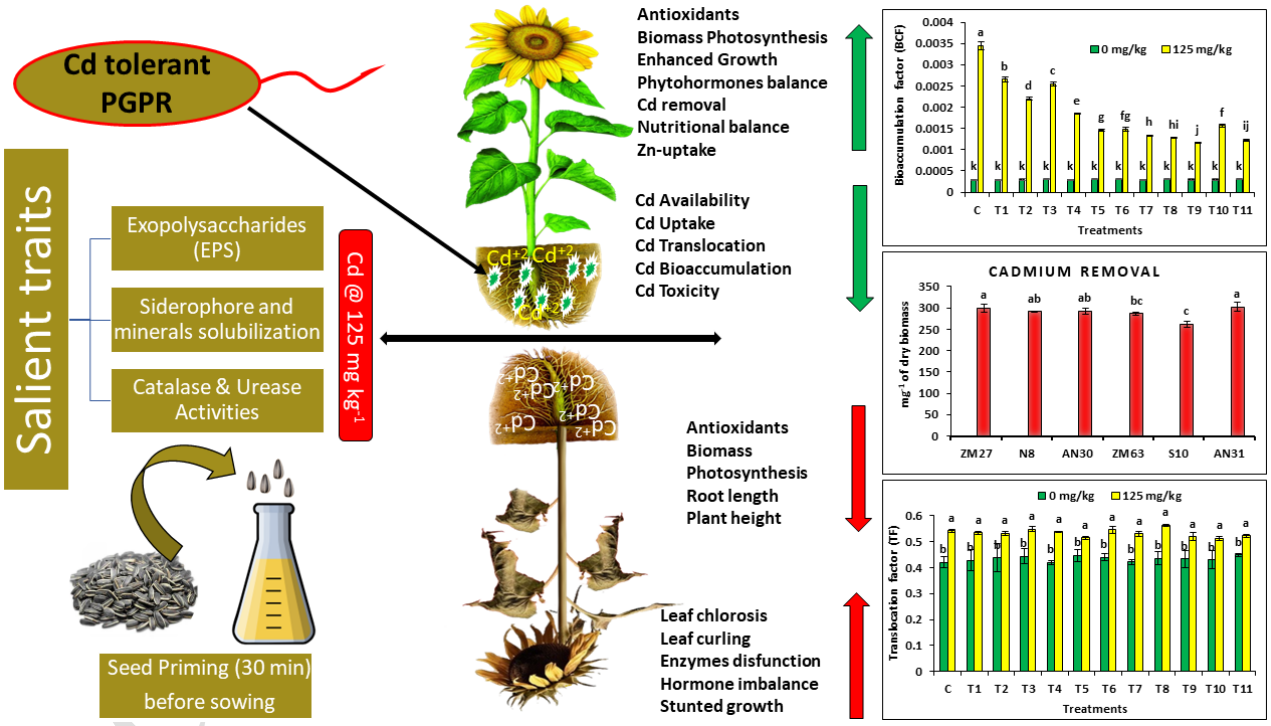
# Enhancing sunflower resilience: Zinc-solubilizing bacteria mitigate cadmium uptake and translocation

**Ayesha Aimen [1], Azhar Hussain [1]*, Maqshoof Ahmad[1], Abubakar Dar [1], Moazzam Jamil[1], Rashid Iqbal[2]*, Dunia A Al Farraj[3], Mohamed Ragab AbdelGawwad[4]**

[1]Department of Soil Science, The Islamia University of Bahawalpur, 6300, Pakistan.
[2] Department of Agronomy, The Islamia University of Bahawalpur, 6300, Pakistan.
[3] Department of Botany and Microbiology, College of Science, King Saud University, P.O. 2455, Riyadh 11451, Saudi Arabia.
[4] Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, International University of Sarajevo, 71210 Sarajevo, Bosnia and Herzegovina

*Corresponding author: Azhar Hussain and Rashid Iqbal
E-mail: azharhaseen@gmail.com and rashid.iqbal@iub.edu.pk

## GRAPHICAL ABSTRACT




## ABSTRACT

Cadmium (Cd) is a highly hazardous metal known for its easy absorption and accumulation in plants, particularly in edible vegetable oils. Sunflower (*Helianthus annuus* L.) holds global significance as an oil crop. This study aims to assess the potential of zinc-solubilizing bacterial strains in reducing cadmium uptake by enhancing zinc availability in sunflower plants. For this investigation, we selected twelve pre-isolated zinc-solubilizing bacterial (ZSB) strains for screening against cadmium tolerance

and were evaluated for their exopolysaccharides (EPS) and siderophore production capabilities. Four strains exhibiting excellent tolerance to cadmium, EPS production, and siderophore production were subsequently inoculated into sunflower plants to determine the cadmium concentration to determine EC50 at various cadmium levels (ranging from 0 to 200 mg $kg^{-1}$). Following the compatibility assessment of these strains as single and consortium inoculation, sunflower seeds were cultivated in jars containing soil with two cadmium levels (0 and 125 mg $kg^{-1}$), totaling 600 g of soil. Our findings demonstrated that the application of ZSB significantly enhanced sunflower growth while simultaneously reducing cadmium uptake. Particularly noteworthy was the performance of the bacterial consortium, composed of strains ZM27 and AN31, which resulted in a remarkable 96% reduction in cadmium translocation, an 86% decrease in cadmium bioaccumulation, and a 97% reduction in overall cadmium uptake compared to the control group. Moreover, the same treatment has caused increase in root length (21.6%), shoot length (18.8%), chlorophyll contents (23.5%), Zn in shoot (30.8%), root Zn contents (13.5%), seedling vigor (49.6%) and leaf area (23.1%) of sunflower under Cd stress as compared to control. The present study concludes that zinc-solubilizing bacterial strains, in consortium ZM27+AN31 were found effective in mitigating the adverse effects of cadmium uptake in sunflower plants. These results suggest that bacterial inoculation strategies hold significant promise for reducing cadmium content in sunflower crops, thereby enhancing the safety and suitability of sunflower-derived edible oils for human consumption and safeguarding human health.

**Keywords:** Cadmium, Translocation, Bioaccumulation, Zinc solubilizing bacteria, Microbial remediation

## 1. Introduction

Various agricultural and industrial activities, such as the usage of agrochemicals, manufacturing of steel, vehicle exhausts, waste disposal, and waste incineration, cause heavy metal accumulation in soil, posing significant risks to food safety and associated health hazards (Sarim et al. 2022). Previous

research has also shown that cultivating various crops in metal-contaminated soil results in significant ecological issues and substantial health risks (Wu and Ma, 2015; Rehman et al. 2021). The contamination of soil with hazardous heavy metals like cadmium (Cd) causes serious dangers to plant life and human health (Palansooriya et al., 2020). Cd is placed seventh among the top 20 most hazardous metals and is recognized as a class-I carcinogen (Jaishankar et al., 2014). It is one among the most dangerous metals due to its high toxicity and extensive bioaccumulation. It also harms plant growth and can induce oxidative stress (Hawrylak-Nowak et al., 2018). Cadmium toxicity symptoms are evident in plants, probably as leaf curling or chlorosis (Gallego et al., 2012) photosynthetic pigments in various crops (Rizwan et al., 2016a). Cadmium also interferes with stomatal conductance and suppresses several enzyme activities in different plants (Saifullah et al., 2014). In human's cadmium exposure led to renal damage, bone deformation issues, and certain types of cancers (Zulfiqar et al., 2023).

Plants have developed specific mechanisms to tolerate a certain amount of Cd exposure, including cell wall binding, compartmentalization of Cd in plant's inactive parts, metal chelation, enhancing specific enzyme activities (Rizwan et al., 2016b), and activation of metal transport genetics (Martos et al., 2016). However, when the levels of Cd exceed the plants' defense capabilities, it can lead to toxic effects at various levels (Gallego et al., 2012). Given the harmful consequences of Cd contamination, it is crucial to address the problems associated with its presence to safeguard plant health and productivity.

A number of factors influence Cd accumulation in plants, including plant species, genotype, soil properties, environmental conditions, and the presence of other minerals and nutrients (Quezada-Hinojosa et al., 2015). The importance of microorganisms in alleviating the Cd uptake in plants has received very little attention in the past.

Several studies suggest that bioremediation methods, such as phytoremediation and microbial remediation, are environmentally safe and practical approaches to mitigating plant cadmium toxicity (Rasool et al., 2023; Dhaliwal et al., 2019). Bioremediation can be more successful after inoculating

selected plant species with Plant growth promoting rhizobacteria (PGPR), which improves plant growth and survival in metal-contaminated soils (Grandlic et al., 2008), reducing the need for amendments and their related costs (Becerra-Castro et al., 2012). Some of the mechanisms proposed for the amelioration of HM toxicity in plants by PGPR include reduced metal uptake by the plant and increased plant growth (Vivas et al., 2006).

Plant-associated microorganisms are recognized to play an essential role in stimulating plant development through a variety of mechanisms (Zhang et al., 2017), particular emphasis has been paid to a category of bacteria known as plant growth-promoting rhizobacteria (PGPR) (Nadeem et al., 2014). Aside from promoting plant growth and food productivity, plant-associated microbes have a variety of additional applications, including soil remediation from organic and metal contaminants (Saha et al., 2021). Many studies have been conducted on the role of PGPR in heavy metal mobilization and phytoextraction from soil (Manoj et al., 2020), with a focus on techniques for Cd removal from soils via phytostabilization and hyperaccumulation (Sharma et al., 2016). Plant growth-promoting (PGP) bacteria that promote phytoextraction of metals are often metal tolerant, increasing Cd bioavailability and enhancing metal accumulation in plants, allowing the metal to be successfully extracted from soils (Li et al., 2022). These bacteria effectively boost Cd mobilization by solubilizing metal phosphates, increasing root surface area for Cd absorption and promoting Cd translocation from root to shoot. Such organisms, however, may increase the metal concentration of edible plant parts (Wu et al., 2022). In recent years, there has been an increase in research on plant-associated microbes that stimulate plant development in metal-polluted soils while preventing or reducing metal accumulation in edible plants (Alves et al., 2022). *Bacillus* genus, as one of the most common PGPR, has shown great potential to restore heavy metal-polluted soil due to its quick growth and robust resilience (Huang et al., 2020). Saran et al., (2020), discovered that *Bacillus proteolyticus* ST89 enhanced sunflower biomass by 40% while considerably reducing the quantity of bioavailable Cd in contaminated soil, enhancing Cd phytoremediation efficacy.

Zinc-solubilizing bacteria (ZSB) also play an essential role in the nutrient cycling for plant uptake. Zn solubilization by soil microorganisms offers more excellent prospects than chemical fertilizers (Gontia-Mishra et al., 2017). The application of beneficial microorganisms in sustainable agriculture and soil restoration is gaining popularity. Plant growth-promoting rhizobacteria (PGPR) have been shown to solubilize insoluble Zn compounds [ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$] in recent years (Krithika and Balachandar, 2016). Zn-solubilizing bacteria can solubilize Zn by various mechanisms, including acidification, siderophores generation, and oxidoreductive systems on cell membranes (Saravanan et al., 2004). In soil, these bacteria generate organic acids that sequester zinc cations while simultaneously chelating zinc and increasing zinc solubility (Jones and Darrah, 1994).

*Helianthus annuus* (sunflower), belongs to the Asteraceae family, it is one of the world's most important crops, is a plant having not only food and bioenergy product, but also phytoremediation potential (Bashir et al., 2021). It is used in phytoremediation research because of its large biomass, fast growth, and high tolerance to heavy metals (Benavides et al., 2020). Moreover, the ZSB can play a significant role in alleviating Cd stress by competing with Cd for uptake as the bacteria solubilize Zn more in the rhizosphere which promotes Zn uptake by roots rather than the Cd (Singh et al., 2024). However, no Cd accumulation-related studies have been conducted assessing the efficacy of cadmium-tolerant zinc-solubilizing bacterial strains to minimize cadmium absorption in sunflowers. In this study it was hypothesized that zinc- solubilizing bacterial strains have the capacity to endure elevated cadmium levels and can enhance zinc bioavailability by reducing cadmium accumulation in sunflower plants subjected to cadmium-induced stress. We predict that the application of these bacterial strains will lead to a reduction in cadmium uptake and translocation, consequently promoting improved sunflower growth and reduced cadmium-related stress effects. The results of this study will give valuable information and new perspectives for further studies on properly repairing Cd-polluted soil without compromising sunflower crop safety.

## 2. Materials and Methods

### 2.1. Collection of rhizobacterial strains

The pre-identified and characterized rhizobacterial strains (ZM63 (*Bacillus subtilis*), S10 (*Bacillus aryabhattai*), ZM27 (*Paenibacillus polymyxa*), ZM31 (*Bacillus aryabhattai*) (Mumtaz et al., 2017), AN31, AN30 (*Bacillus megaterium*), AN24 (*Bacillus megaterium*) (Naseer et al., 2020), ZE15 (*Bacillus subtilis*), ZE11 (*Paenibacillus* sp.), ZR19 (*Bacillus megaterium*), ZR3 (*Bacillus subtilis*) (Hussain et al., 2020), under accession numbers KX788861, KX788862, KX788859, KX788860, MN005929, MN005926, MN003400, MN003399, MN003401, MN007185, respectively) were collected from culture bank of Soil Microbiology and Biotechnology Laboratory and were subjected to screening for Cadmium tolerance in laboratory under controlled conditions.

**2.2. Determination-of-minimum-inhibitory-concentration (MIC)**

The tolerance of Zinc Solubilizing Bacterial (ZSB) strains to cadmium was evaluated using the agar dilution method in the study. Then, 48-hour-old bacterial cultures were introduced onto agar plates containing Luria-Bertani (LB) medium. The LB medium was supplemented with cadmium chloride at 500 mg $L^{-1}$ concentration. The cadmium concentration was increased gradually until the point with no visible growth of bacteria was observed and considered as MIC for these strains (Vela-Cano et al., 2014).

**2.3. Cadmium Tolerance Assay**

To assess the cadmium tolerance of specific rhizobacterial strains, cadmium (1000 mg $kg^{-1}$) was added to LB broth according to the minimum inhibitory concentration (MIC) results. The prepared broth was poured into autoclaved conical flasks (25 ml) and inoculated separately with freshly grown bacterial colonies of each selected strain. The flasks remained sealed with parafilm and incubated at 30°C and 100 rpm, while spectrophotometer measurements of optical density (OD600) were periodically recorded. Bacterial strains that exhibited growth under cadmium stress were selected for further investigations.

**2.4. Exopolysaccharides (EPS) production (quantitative) by ZSB strains under Cd stress**

To evaluate the EPS production by the selected zinc solubilizing bacterial strains the fresh cultures of each strain was inoculated in sterilized Luria Bertani (LB) broth at varying concentrations of

cadmium (0, 600, 1200, 1800 mg $L^{-1}$) in triplicate. The cultures were incubated for 24 and 48 hours, and the OD of the cultures was calculated using a UV spectrophotometer (Model Cary 60, Agilent, USA), following the method described by Raja et al., (2006). Furthermore, after 72 hours, the production of Exopolysaccharides (EPS) by the PGPR strains was quantified using a spectrophotometer, following the procedure outlined by Dubois et al., (1956), for producing quantifiable exopolysaccharide (EPS) in which After cultivating the bacterium, the EPS was extracted from the supernatant, purified, and precipitated with phenol-sulfuric acid. A spectrophotometer (Model AAS 240 FS, Agilent, USA) was used to determine carbohydrate concentration, and EPS output was computed per unit volume or mass of the original culture.

**2.5. Siderophore production by ZSB under cadmium stress**

Siderophore production at different levels of Cd was determined on Chromo Azurol Sulphonate (CAS) as described by Dworkin and Foster (DF) medium spiked with Cd (Schwyn and Neilands, 1987). After 48 hours of incubation, the contents were centrifuged at 9000*g* for 10 minutes at room temperature, followed by filtration with 0.22-µm sterilized membrane filters. The supernatant after centrifugation was mixed with CAS dye and incubated for 20 min for color development. The samples readings were taken at 630 nm by using reference solution (CAS dye + Media) and necessary blank (media). The siderophore were quantified by using the formula:

$$\% \, \boldsymbol{Siderophore} = \frac{\boldsymbol{Ar-As}}{\boldsymbol{Ar}} \boldsymbol{100} \ldots\ldots\ldots\ldots\ldots\ldots \textbf{(1)}$$

Where,
Ar is a reference solution reading
As is the sample reading

**2.6. Compatibility test**

To evaluate compatibility, the bacterial strains with the highest EPS and siderophore production were chosen. LB media were prepared, autoclaved, and poured into sterilized petri plates. The selected strains were cross streaked on the plates in all possible combinations. Three replicate plates were then incubated at a temperature of 30±1 ºC for 48 hours. Compatibility was determined by observing the growth at the intersection point of the streaks. If the strains exhibited growth, they were considered

compatible, whereas the absence of growth indicated incompatibility as described by Fukui et al., (1994).

### 2.7. Collection of Soil Samples for Soil Physiochemical Properties

A composite soil sample from 0-15 cm depth was taken from the research area; the Department of Soil Science, the Islamia University of Bahawalpur and taken to the laboratory, where the soil was dried under shade, ground, and thoroughly mixed. Subsequently, it was passed through a stainless mesh sieve with a mesh size of 2 mm to ensure uniformity. The soil was then subjected to analysis using the procedure outlined by Hoagland and Arnon (1950), and Ryan et al., (2001). The results of the soil analysis are presented in the accompanying table1.

**Table 1. Physio-Chemical Characteristics of the experimental soil**

| Analysis | Unit | Soil Sample |
|---|---|---|
| Textural class | | Sandy Loam |
| Ece | dS $m^{-1}$ | 6.6 |
| pH | | 8.0 |
| Saturation Percentage | % | 31.3 |
| Organic matter | % | 0.58 |
| Available phosphorus | mg $kg^{-1}$ | 0.35 |
| Extractable potassium | mg $kg^{-1}$ | 77.0 |
| Total nitrogen | % | 0.03 |

### 2.8. Screening of crop tolerance for cadmium and determining EC50 for sunflower

The collected soil, as described earlier, underwent autoclaving before being used in the experiment. Various levels of cadmium (0, 25, 50, 75, 100, 125, 150, 175, and 200 mg $kg^{-1}$) were spiked to the soil using $CdCl_2$ as the source of cadmium. To ensure the stabilization of the metal, the soil was kept for 15 days in jars as per the method described by Ryan et al., (2001). Plastic jars were filled with 600 grams of the treated soil and irrigated with tap water of good quality to achieve field capacity. The jars were placed in a growth room with controlled conditions, including a 12-hour day period with 1000 lx light intensity at a temperature of 30 ± 1 °C, followed by a 12-hour dark period with a temperature of 25 ± 1 °C. Humidity was kept at 70%. The jars were replicate for 3 time and organized in a completely randomized design (CRD).

Seeds of the latest variety of hyperaccumulator sunflower were obtained from the local market in Bahawalpur. Before sowing, the seeds were surface sterilized using sodium hypochlorite (5%) and 70% ethanol. Ten surface sterilized seeds were sown in separate jars with three replications for each crop. For irrigation and nutrient supply, Hoagland solution of half strength was used periodically. The germination percentage was calculated using the formula provided by Jing et al., (2012), after 48 to 72 hours.

$$\text{Germination percentage (\%)} = \frac{\text{No of seeds germinated}}{\text{Total no of seeds}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots (2)$$

A plant population was maintained to 3 plants per $jar^{-1}$ for 25 days after complete germination. On the completion of the trial, seedlings were harvested, growth and physiological parameters were measured. The cadmium level that reduced 50% of the growth of seedlings was selected for further investigation.

**2.9. Biosorption/Removal experiment**

During the incubation process, the bacterial isolates were subjected to shaking in a liquid medium for one hour. Subsequently, sterilized Cd solution ($CdCl_2$) was introduced to the medium at a concentration of 100 milligrams per liter. The mixture underwent further incubation for 24 hours. To assess the amount of Cd retained by the bacteria, the bacterial culture was subjected to centrifugation. This process resulted in the separation of supernatants, which were subsequently treated with concentrated $HNO_3$, filtered, and subjected to analysis using an Atomic Absorption Spectrophotometer (Model AAS 240 FS, Agilent, USA) (Gontia-Mishra et al., 2017). To ensure the reliability of the findings the treatments were replicated thrice. By comparing the initial concentration of the metal (added during inoculation) with the amount of metal utilized, the extent of metal biosorption by the bacteria was estimated using the following formula given by Zafar et al., (2007).

$$\frac{\text{Biosorption}}{\text{Removal}}(\text{mg g}^{-1}) = \frac{(\text{Co} - \text{Ce}) \times \text{V}}{\text{M}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where:

Co= Initial Conc. Of metal in in the solution(mg/L)

Ce= Final Conc. Of metal in in the solution (mg/L)

V= Volume of metal solution used

M= Dry biomass of bacteria used for biosorption (g).

**2.10. Preparation of inoculum and seed inoculation**

The selected zinc solubilizing strains, identified based on their minimum inhibitory concentration (MIC), underwent further evaluation. To prepare a bacterial inoculum, a loopful of a specific bacterial strain was added to 250 mL of LB broth in a 250 mL flask. The flask was then placed in a rotary shaker at 28 ± 2 °C and 100 rpm for 48 hours to achieve a uniform bacterial population. The optical density (OD) of the culture was measured at a wavelength of 600 nm using a UV-visible spectrophotometer to ensure a population of $10^7$ colony-forming units (CFU) $mL^{-1}$ in the broth. For consortium application equal volumes of the homogenous cultures were taken and vortexed for 1 min prior to seed application (Dar et al., 2020). Before sowing, surface sterilization of sunflower seeds was performed by treating them with 70% ethanol for 60 seconds, followed by a sodium hypochlorite (5%) solution for 5 minutes. The seeds were then rinsed three times with distilled water. Subsequently, the sterilized seeds were immersed in a bacterial inoculum and a sugar solution (20%) in a ratio of 1:1 (w/v) for 30 min. In the control treatment, seeds underwent the treatment with co application of sterilized broth and sugar. For the jar trial, ten seeds were sown in each pot (Zahir et al., 2018).

**2.11. Jar Experiment to Evaluate the Effectiveness of ZSB Strains on Growth of Sunflower under Axenic Conditions**

In order to enhance sunflower seedling growth under controlled conditions contaminated with cadmium (Cd), a series of in vitro experiments and laboratory bioassays were conducted to identify four strains that solubilize zinc, tolerate Cd, and produce exopolysaccharides and siderophores. These strains were then tested in jar trials under axenic conditions. Each jar was filled with 600 grams of soil, and ten seeds were inoculated following the aforementioned procedure. The experiment consisted of multiple treatments, each replicated three times. The temperature was maintained at 25 ± 1 °C during the day and 22 ± 1 °C at night, while the relative humidity was 75 ± 1% during the day and 65 ± 1% at night. Two levels of cadmium were tested: 0 mg $kg^{-1}$ (control set) and 125 mg $kg^{-1}$ (selected based on the EC50 of the crops). The treatments included the control (C1), individual strains (T1: ZM31, T2: ZM27, T3: N8, T4: AN31), and various strain combinations (T5: ZM31+ZM27, T6: ZM31+N8, T7: ZM31+AN31, T8: ZM27+N8, T9: ZM27+AN31, T10: N8+AN31). Additionally, a treatment with a recommended dose at 6 kg/acre of $ZnSO_4$ (33%) was included as T11, while the other sets received only the recommended level of zinc through the half strength Hoagland solution. After a specific growth period (25 days for sunflower) different physiological and growth parameters were assessed, including root length, shoot length, root fresh biomass, root dry biomass, shoot fresh biomass, shoot dry biomass, root diameter, root colonization, chlorophyll content, cadmium concentration in shoot and root, zinc concentration in root and shoot, translocation factor, bioaccumulation factor, and cadmium uptake in root and shoot. These evaluations aimed to determine the impact of different zinc-solubilizing bacterial strains, both individually and in consortia, on plant growth and cadmium reduction.

The parameters, TF (translocation factor) and BCF (biological concentration factor of Cd) as defined in Equations (1) and (2), which were computed from the biomass of plant parts and accumulation of Cd in treatments, were used to discuss the results.

$$\mathrm{TF} = \frac{\mathrm{Cshoot}}{\mathrm{Croot}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

277 Where: Cshoot and Croot are metals concentration (mg $kg^{-1}$) in shoot and root of sunflower plant,
278 respectively. Moreover the TF > 1 represents effective translocation of metals from shoot from root
279 (Yoon et al., 2006).
280
281
282 $$BCF = \frac{Cshoot}{Csoil} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$
283
284 Where: Cshoot and Croot are metals concentration (mg $kg^{-1}$) in shoot and root of sunflower plant,
285 respectively. BCF was categorized further as hyperaccumulators as the accumulated metal
286 concentration exceeds >1 mg $kg^{-1}$ (Cui et al., 2007).
287 **2.12 Statistical Analysis**
288 The data was statistically analyzed using completely randomized design (CRD) under factorial
289 arrangement by analysis of variance (ANOVA) through Statistix 8.1® computer-based software
290 (Steel, 1961). The treatment means were compared by using LSD at 5% level of probability (Duncan,
291 1955).
292 **3. Results**
293 Pre-isolated and pre-identified zinc solubilizing bacterial strains were screened for cadmium tolerance
294 in laboratory under controlled conditions. They were refreshed using General purpose media (GPM)
295 on agar plates and used for experimentation.
296 **3.1.Determining minimum inhibitory concentration (MIC)**
297 The Minimum inhibitory concentration was used to screen the pre-isolated rhizobacteria for Cd
298 resistance (Table 2). To identify their best resistance capacity, all of these (Cd) resistance
299 rhizobacterial strains ZM27, ZM63, ZM31, AN24, AN30, AN31, ZE15, ZE11, ZR19, S10, N8, and
300 ZR3 isolates were screened using the MIC approach and their MIC values were calculated. The results
301 showed that out of 12 isolates, eight isolates AN31, ZM27, ZM31, N8, ZE11, AN33, ZE1,5, and S10
302 showed more resistance against Cd-stress. According to the table, AN31 and ZM27 strains

demonstrated the highest MIC (1500 mg $L^{-1}$) for Cd and ceased up to 2000 mg $L^{-1}$. While other strains such as those showed the lowest values of MIC (500 mg $L^{-1}$).

**Table 2. Minimum inhibitory concentrations (mg $L^{-1}$) of heavy metals for selected rhizobacterial strains.**

| Bacterial Strains | 500 | 1000 | 1500 | 2000 |
|---|---|---|---|---|
| ZM63 | +++ | +++ | +++ | _ |
| ZM27 | +++ | +++ | +++ | _ |
| ZM31 | ++ | + | - | - |
| AN24 | ++ | ++ | ++ | - |
| AN30 | +++ | +++ | ++ | - |
| AN31 | +++ | ++ | + | - |
| ZE15 | ++ | + | - | - |
| ZE11 | +++ | + | + | - |
| ZR19 | ++ | + | - | - |
| ZR3 | + | + | - | - |
| N8 | +++ | +++ | ++ | - |
| S10 | +++ | +++ | ++ | - |

### 3.2.Cadmium Tolerance Assay

Cadmium tolerance for zinc solubilizing bacterial strains were confirmed in liquid culture amended with 1000 mg $kg^{-1}$ of cadmium using $CdCl_2$ as cadmium source. Measurements of bacterial growth were performed using spectrophotometers. The results have indicated that 6 out of 12 bacteria has shown optimum growth in the liquid culture (Table 3) and the best performing bacterial strains are ZM63, AM27, AN30, N8, S10 are selected for further evaluation**.**

**Table 3. Growth of ZSB strains ($OD_{600}$) in DM media at (1000 mg $L^{-1}$) of cadmium.**

| ZSB stains | Values for $OD_{(600)}$ |
|---|---|
| ZM63 | 1.03±0.04 |
| ZM27 | 1.15±0.03 |
| ZM31 | 0.56±0.03 |
| AN24 | 0.675±0.02 |
| AN30 | 1.32±0.05 |
| AN31 | 0.94±0.03 |
| ZE15 | 0.843±0.02 |
| ZE11 | 0.92±0.04 |
| ZR19 | 0.739±0.03 |
| ZR3 | 0.896±0.04 |
| N8 | 1.06±0.04 |
| S10 | 0.907±0.03 |

### 3.3.Zinc-solubilizing bacteria with growth-promoting properties

In the Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur, zinc-solubilizing bacteria were characterized for Zn Solubilization, CAT

activity, urease activity and siderophore production. The results depicted that AN30 and ZM27 strains possess highest potential for CAT and urease activities, siderophore production and Zn solubilization followed by N8 and ZM63 strains (Table 4).

**Table 4. Zn- solubilizing bacterial strain with plant growth-promoting properties.**

| Growth promoting characteristics | Zinc Solubilizing Bacterial Strains | | | | | |
|---|---|---|---|---|---|---|
| | ZM63 | AN30 | ZM27 | N8 | S10 | AN31 |
| Zn Solubilization | ++ | +++ | +++ | +++ | ++ | + |
| CAT activity | ++ | +++ | +++ | ++ | ++ | ++ |
| Urease activity | ++ | +++ | +++ | +++ | ++ | ++ |
| Siderophore producing ability | +++ | +++ | +++ | ++ | ++ | ++ |

(+++) denotes the best growth, (++) with lesser value and (+) with lowest possible visible growth of bacteria.

### 3.4.Cadmium effects on growth, siderophores, and EPS production of zinc-solubilizing bacteria

Different levels of Cd stress were applied to selected ZSB strains with multiple PGP traits form 24 hours up to 72 hours, to monitor their EPS production ability. Rhizobacterial growth was significantly reduced as Cd stress increased (Table 5). Additionally, the log phase was shortened as Cd stress increased, resulting in an earlier stationary phase. The growth rate was not significantly different in most cases. AN31, however, showed a significantly higher growth rate than ZM27, even when Cd stress was applied. Siderophore production ability varies greatly between strains under normal and Cd stress. Within 24-hour, 48-hour, and 72-hour time intervals, the EPS and siderophore production was monitored for different bacterial strains under various levels of Cd stress. A higher level of Cd inhibits the production of EPS and siderophores. In the strain AN31, the maximum siderophore production was after 48 hours of growth, while the minimum production was after 72 hours of growth. Tested strains produced more siderophore and EPS up to 48 hours of growth, but after 72 hours, their production decreased. In terms of EPS production ability, strains differ significantly. Strain AN31 gives (108 mg/kg), ZM27 (95mg/kg) followed by AN33, ZM63, S10, and N8 with (96mg/kg) EPS per liter after 48 hours of growth at lower level of Cd stress i.e., 600 mg $L^{-1}$ of Cd. Following 24 hours, 48 hours, and 72 hours of growth, all strains demonstrated a significant decrease in EPS production ability.

### 3.5 Cadmium removal by bacterial strains

All selected bacterial isolates behaved differently regard to their ability to accumulate Cd as indicated by their Cd removal efficiency data. In Fig 4, the maximum Cd removal efficiency (299 Cd mg $g^{-1}$ of dry biomass) for AN31 followed by ZM27 (292 Cd mg $g^{-1}$ of dry biomass), (291 mg of Cd per gram of dry biomass for N8), and SS10 (286 Cd mg $g^{-1}$ of dry biomass for ZM31) (Fig. 1).

On the basis of bioassays in the laboratory and in vitro study results four ZSB strains (AN30, ZM27, N8 and S10) with the ability to produce exopolysaccharides, and siderophore were selected for further evaluation. Moreover, a hyper-accumulator (sunflower) and a non-hyper accumulator (chilies) crops were selected to determine $EC_{50}$ of seedlings under cadmium stress at different levels.

348 **Table 5. Zinc Solubilizing bacteria potential for Growth OD (540), EPS and Siderophore production.**

| | | Time | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hours | | | | | | 48 hours | | | | | | 72 hours | | | | | |
| | | Zinc Solubilizing Bacterial Strains | | | | | | Zinc Solubilizing Bacterial Strains | | | | | | Zinc Solubilizing Bacterial Strains | | | | | |
| | | ZM27 | ZM63 | N8 | AN30 | S10 | AN31 | ZM27 | ZM63 | N8 | AN30 | S10 | AN31 | ZM27 | ZM63 | N8 | AN30 | S10 | AN31 |
| | Levels | Growth ($OD_{540}$) Of ZSB bacterial strains | | | | | | | | | | | | | | | | | |
| Cd level (mg/L) | 0 | 0.81a | 0.71bc | 0.69cd | 0.69cd | 0.61e | 0.73b | 1.04a | 0.94bc | 0.92c | 0.92c | 0.84d | 0.96b | 1.14a | 1.04bc | 1.02c | 1.02c | 0.94d | 1.06b |
| | 600 | 0.71bc | 0.68cd | 0.62e | 0.68cd | 0.59hi | 0.63g | 0.83d | 0.8e | 0.74g | 0.8e | 0.71h | 0.75i | 0.88e | 0.85f | 0.79g | 0.85f | 0.76i | 0.80g |
| | 1200 | 0.66d | 0.6e | 0.49e | 0.41h | 0.39hi | 0.49g | 0.77f | 0.71h | 0.60i | 0.52j | 0.5k | 0.6i | 0.8g | 0.74h | 0.63j | 0.55k | 0.53l | 0.63j |
| | 1800 | 0.54f | 0.48g | 0.37i | 0.29j | 0.27j | 0.37i | 0.59i | 0.53j | 0.42l | 0.34m | 0.32n | 0.42l | 0.68i | 0.62j | 0.51l | 0.43m | 0.41n | 0.51l |
| | | Siderophore Production | | | | | | | | | | | | | | | | | |
| Cd level ( mg/L ) | 0 | 2.58a | 1.16e-g | 2.23ab | 0.95f-h | 0.91f-h | 1.63c-e | 3.60ab | 2.7cd | 3.76a | 2.49c-e | 2.45c-e | 3.16a-c | 3.64ab | 2.73cd | 3.8a | 2.52cde | 2.48c-e | 3.2a-c |
| | 600 | 1.9bc | 0.8gh | 1.55c-e | 0.75gh | 0.9bc | 0.95f-h | 2.9b-d | 1.8e-g | 2.55c-e | 1.75e-g | 1.9h | 1.95fg | 2.93b-d | 1.83e-h | 2.58c-e | 1.78e-h | 1.93gh | 1.98f |
| | 1200 | 1.83b-d | 0.76gh | 1.48c-e | 0.72gh | 0.66h | 0.88f-h | 2.5c-e | 1.43fg | 2.15d-g | 1.39g | 1.32g | 1.55fg | 2.54c-e | 1.47f-h | 2.19d-g | 1.43f-h | 1.36h | 1.59f-h |
| | 1800 | 1.73b-d | 0.66h | 1.38d-f | 0.62h | 0.56h | 0.78gh | 2.56c-e | 1.49fg | 2.21d-f | 1.45fg | 1.39fg | 1.61fg | 2.59c-e | 1.52f-h | 2.24d-f | 1.48f-h | 1.42gh | 1.64f-h |
| | | Exopolysaccharides (mg $kg^{-1}$) production | | | | | | | | | | | | | | | | | |
| Cd level ( mg/L ) | 0 | 109a | 102.7b | 95.0c | 80i | 105ab | 106.3ab | 111a | 104.7b | 97cd | 82j | 107ab | 108.3ab | 111a | 104.7b | 97cd | 82j | 107ab | 108.3ab |
| | 600 | 106.7ab | 94.2c | 87.3d-g | 77.8ij | 94.7cd | 97.0c | 108.7ab | 96.2c-e | 89.3g-i | 79.8jk | 96.7cd | 99cd | 108.7ab | 96.2c-e | 89.3g-i | 79.8jk | 96.7cd | 99.0cd |
| | 1200 | 94.3c | 89.7de | 84.3gh | 73.7j | 89.3def | 91.3cd | 96.6cd | 91.9e-h | 86.6i | 75.9k | 91.6f-h | 93.6g | 96.6cd | 91.9e-h | 86.6i | 75.9k | 91.6f-h | 93.6d-g |
| | 1800 | 86.7e-g | 85.3fg | 80.3hi | 69.3k | 84.3gh | 86.5e-g | 98.2c | 93.5d-g | 88.2hi | 77.5k | 93.2d-g | 95.2c-f | 98.2c | 93.5d-g | 88.2hi | 77.5k | 93.2d-g | 95.2c-f |

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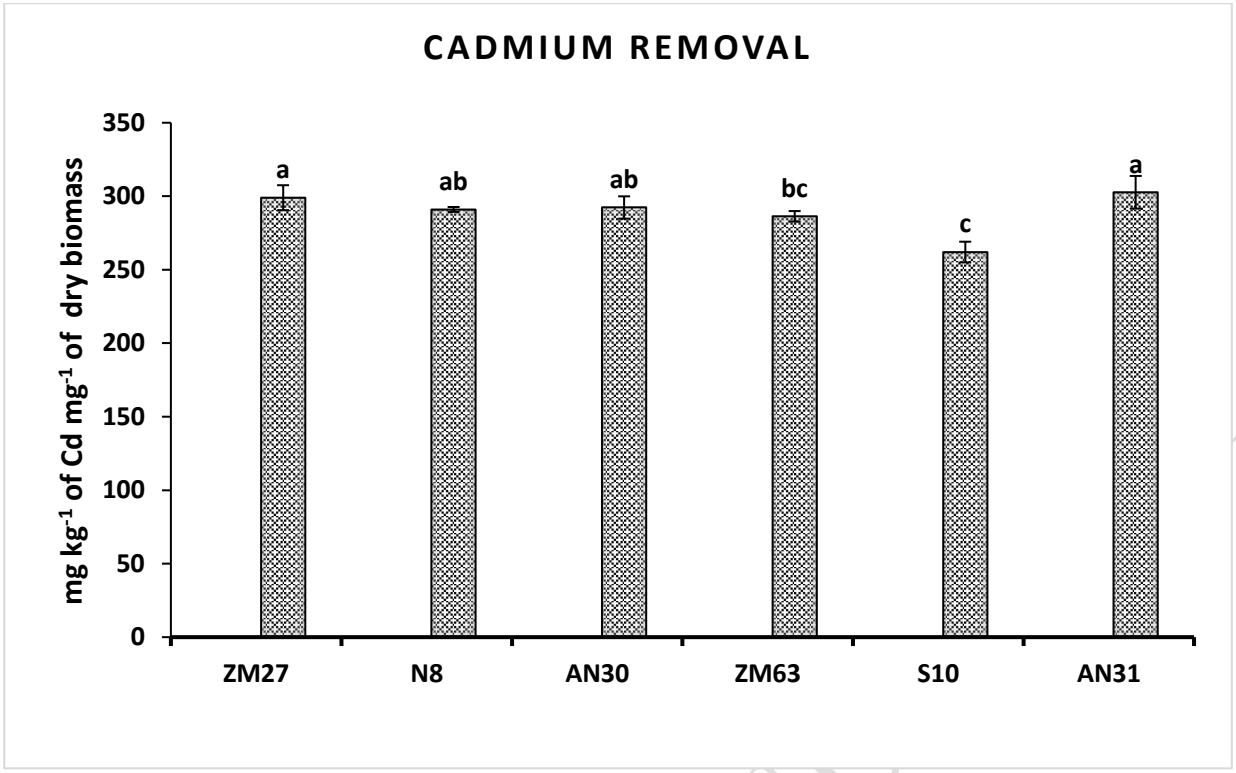


**Figure 1.** Cadmium removal by zinc solubilizing bacterial strains.

### 3.5.Assessment of the $EC_{50}$ of sunflower seedlings

From the local market, seeds of the newest variety of sunflower collected, and their $EC_{50}$ was determined at different levels of cadmium concentration (0, 25,50, 100, 125, 150, 175, 200 mg $kg^{-1}$). As shown, in Table 6. germination %, root/shoot length, shoot/root fresh weight, and dry weight was recorded 100% without cadmium stress ($T_o$), however by increasing the Cd level the results were significantly decreasing. In $T_5$ (125 mg $kg^{-1}$) the germination percentage was reduced by 57.52%, which was further reduced to 25.57% and 3.23% at 175 mg $kg^{-1}$ ($T_7$) and 200 mg $kg^{-1}$ ($T_8$). The shoot and root length were reduced to (51.3%), shoot/root fresh weight was reduced to (50.6%) and dry biomass (51.8%) at (125 mg $kg^{-1}$), respectively. Therefore, the $EC_{50}$ recorded for sunflower seedlings was 125 mg $kg^{-1}$ The growth of sunflower seedlings was evaluated further with respect to Cd stress.

**Table 6. Growth Parameters of sunflower under cadmium stress to find EC50 @(0, 25, 50, 75, 100,125, 150, 175, 200 mg $kg^{-1}$ ).**

| Treatments | Root fresh weight (g) | Root dry weight (g) | Shoot fresh weight (g) | Shoot dry weight (g) | Root length (cm) | Shoot Length (cm) | Germination % |
|---|---|---|---|---|---|---|---|
| **T0 (0 mg $kg^{-1}$)** | 1.13a | 0.57a | 3.36a | 1.90a | 5.00a | 13.70a | 83.0a |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **T1 (25 mg kg$^{-1}$)** | 0.96b | 0.48b | 3.01b | 1.53b | 4.00b | 11.97b | 77.0ab |
| **T2 (50 mg kg$^{-1}$)** | 0.76c | 0.38c | 2.69c | 1.30c | 3.53c | 10.02c | 63.0bc |
| **T3 (75 mg kg$^{-1}$)** | 0.68d | 0.33d | 2.34d | 1.07d | 3.10d | 8.43d | 50.0cd |
| **T4 (100 mg kg$^{-1}$)** | 0.6e | 0.25e | 1.96e | 0.78e | 2.70e | 7.24e | 43.0de |
| **T5 (125 mg kg$^{-1}$)** | 0.56e | 0.27e | 1.66e | 0.91f | 2.43e | 6.67e | 43.0de |
| **T6 (150 mg kg$^{-1}$)** | 0.26f | 0.127f | 1.32f | 0.55g | 0.93f | 4.7f | 33.ef |
| **T7 (175 mg kg$^{-1}$)** | 0.15g | 0.07fg | 1.11fg | 0.35h | 0.53g | 3.45g | 23.0ef |
| **T8 (200 mg kg$^{-1}$)** | 0.05h | 0.03g | 0.967g | 0.11i | 0.36g | 2.94g | 17.0g |

Values sharing the same letters do not differ statistically from one another ($p \leq 0.05$).

### 3.7 In vitro experiments for plant growth promotion of sunflower by zinc solubilising bacterial strains

Based on the results, four ZSB strains (AN30, ZM27, N8 and S10) with the ability to produce exopolysaccharides, and siderophore were selected for further evaluation. Moreover, a hyper-accumulator sunflower was selected to determine $EC_{50}$ of seedlings under cadmium stress at different levels. Which was considered as the level that reduces the growth up to 50% under cadmium stress and that was found to be level $T_5$ (125 mg kg$^{-1}$ Cd) as shown in Table. 6.

The data regarding the impact of Cd-tolerant, ZSB strains on the physiology of sunflowers showed that Cd-stress negatively affects the germination and growth of crop (Table 7). All the applied treatments whether sole or co-inoculation of ZSB strains increased the germination percentage compared to the control. The highest increase in germination percentage (33%) was recorded by consortium of ZM27+AN31 ($T_9$) under cadmium stress. The strains AN31 and ZM27 outperformed in the consortium and also performed better in sole treatments than other solely applied bacteria in terms of germination percentage (25, 14% respectively) as compared to the control. However, the sole application of $ZnSO_4$ didn't show any significant increase in germination percentage (5%) as compared to the control. Inoculation was also effective in enhancing the germination percentage under unstressed conditions where the maximum increase (14%) in germination percentage was observed by ZM27+AN31 ($T_9$).

The data regarding the influence of Cd-tolerant, ZSB strains on the physiology of sunflowers indicates that the presence of Cd-stress has an adverse impact on shoot length. The application of treatments involving sole and co-inoculation of ZSB strains has been observed to result in a higher shoot length as compared to the control. Under normal conditions, the experimental results indicate that the

390 ZM27+AN31 ($T_9$) treatment exhibited the highest shoot length (31%) compared to the control. The
391 bacterial strains AN31 and ZM27 exhibited superior performance within the consortium, as well as
392 in individual treatments, in comparison to other bacteria that were solely applied in terms of shoot
393 length (9% and 17%). However, the sole application of ZnSO4 did not exhibit a significant increase
394 in the shoot length in comparison to the control. The inoculation was also effective in enhancing the
395 shoot length under Cd-stressed conditions. The highest (26%) shoot length found by the ZM27+AN31
396 ($T_9$) matched to the control.
397 The Cd-stress decrease the root length of the sunflower. All ZSB strain alone and co-inoculation
398 treatments improve root length compared to control. Under normal conditions, the bacterial
399 consortium ZM27+AN31 ($T_9$) showed the highest (46%) root length than the control. The strains
400 AN31 and ZM27 outperformed the consortium and other solely applied bacteria in root length (15
401 and 25%) compared to the control. However, the sole inoculation of $ZnSO_4$ did not enhance root
402 length compared to the control. The inoculation also increased the root length by 30% under Cd-
403 stressed conditions with ZM27+AN31 ($T_9$) compared to the control.
404 The exposure to Cd-induced stress has been observed to result in the shoot fresh weight reduction.
405 The results indicate that both the ZSB strain alone and co-inoculation treatments exhibit a significant
406 enhancement in shoot fresh weight as related to the control. Under normal conditions, the consortium
407 ZM27+AN31 ($T_9$) increase the shoot fresh weight that was higher (20%) than that of the control under
408 cadmium stress. The results indicate that strains ZM27 and AN31 solely also exhibited superior
409 performance in terms of shoot fresh weight, with a 9% and 13% respectively, increase in comparison
410 to the control and other sole applied bacteria. The application of $ZnSO_4$ did not exhibit any significant
411 improvement in the shoot fresh weight compared to the control. The experimental results indicate
412 that the ZM27+AN31 ($T_9$) significantly increased the shoot fresh weight by 27% in the presence of
413 Cd-induced stress, as compared to the control.
414 The Cd stress decreases the shoot dry weight of the sunflower. All ZSB strain through sole and co-
415 inoculation treatments improves their results compared to the control. Under normal conditions, the

bacterial consortium ZM27+AN31 ($T_9$) showed the highest (32%) shoot dry weight than the control. The strains ZM27 and AN31 outperformed the consortium and other solely applied bacteria in dry biomass of shoot (19 and 12%) related to the control. However, the sole inoculation of $ZnSO_4$ did not enhance shoot dry weight compared to the control. The inoculation also increased the shoot dry weight by 30% under Cd-stressed conditions with ZM27+AN31 ($T_9$) compared to the control.

The Cd-tolerant, ZSB increased sunflower root fresh weight under normal and Cd-stressed conditions. Uninoculated root fresh weight was lowest during Cd stress. Under normal conditions, ZM27+AN31 showed a 39% increase in root fresh weight compared to the control The strain ZM27 and AN31 outperformed the consortium and other solely applied bacteria with respect to root fresh weight (14 and 21%). However, the sole inoculation of ZnSO4 did not enhance root fresh weight compared to the control. The effect of inoculation on root fresh weight was also effective under Cd-stress with (25%), inoculated with ZM27+AN31 ($T_9$) compared to control.

The Cd-tolerant ZSB strain exhibited a significant increase in the root dry weight of sunflower under normal and Cd-stressed. Under normal conditions, the ZM27+AN31 ($T_9$) showed a 37% increase in comparison to the control. The strains ZM27 and AN31 exhibited superior performance in the consortium and other solely applied bacteria, with increases of 12% and 19%, The inoculation of $ZnSO_4$ did not result in a significant increase in the root dry weight when compared to the control. The inoculation was found to be significant even under Cd stress, with a 25% increase in inoculation with ZM27+AN31 ($T_9$) compared to the control.

The Cd-tolerant ZSB strain significantly increased sunflower root diameter under normal and Cd-stressed conditions. Under normal conditions, ZM27+AN31 ($T_9$) outperformed the control by 62% comparative to control. The strains ZM27 and AN31 outperformed both in the consortium and other solely applied bacteria with 38% and 43% increases. However, the inoculation of $ZnSO_4$ did not increase root diameter compared to the control. Under Cd-stressed conditions, the consortium ZM27+AN31 ($T_9$) gives 58% more increase than the control.

441 Data showed that Cd-stress decrease the chlorophyll content of sunflower. The inoculation of ZSB
442 through sole and co-inoculation significantly improved the results compared to the control. Under
443 normal conditions, a maximum 30% increase was observed through ZM27+AN31 ($T_9$). The bacterial
444 strains AN31 and ZM27 outperformed in the consortium as well as solely bacterial strains with
445 respect to SPAD value (10 and 14%). However, the sole application of $ZnSO_4$ showed (3%) increase
446 in SPAD value as compared to the control (Table 7). Under stressed conditions, a maximum 26%
447 increase was recorded through AN31+ZM27 compared to the uninoculated control.
448 The Cd-tolerant ZSB strain exhibited a significant increase in the SVI of sunflowers under normal
449 and Cd-stressed. Under normal conditions, the ZM27+AN31 ($T_9$) showed a 40% increase in
450 comparison to the control. The strains ZM27 and AN31 exhibited superior performance in the
451 consortium and other solely applied bacteria, with increases of 12% and 15%, The inoculation of
452 $ZnSO_4$ did not result in a significant increase in the SVI when compared to the control. The
453 inoculation was found to be significant even under Cd stress, with a 36% increase in inoculation with
454 ZM27+AN31 ($T_9$) compared to the control.
455

**Table 7**. Influence of ZSB strains on growth and physiology of sunflower seedling under axenic conditions. Values sharing the same letters do not differ statistically from one another ($p \leq 0.05$).

| Treatments | Root fresh wt. (g Pot$^{-1}$) | Root dry wt. (g Pot$^{-1}$) | Shoot dry wt. (g Pot$^{-1}$) | Shoot fresh wt. (g Pot$^{-1}$) | Root length (Cm) | Shoot length (Cm) | Root diameter (Cm$^3$) | SPAD value | Seedling vigor index | Germination (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **C1 (-Cd)** | 1.20n | 0.61no | 1.45k | 3.25g | 2.93i | 7.3j | 0.30j | 53.7g | 55.93fg | 76.6ce |
| **T1** | 1.33m | 0.65lm | 1.54ij | 3.43f | 3.06h | 7.66op | 0.42g | 61.8e | 56.16fg | 73.3df |
| **T2** | 1.40kl | 0.70g-j | 1.60gh | 3.57ef | 3.1g-i | 8.16gh | 0.49ef | 60.23ef | 57.4ef | 70.0ge |
| **T3** | 1.28m | 0.69-jk | 1.57hi | 3.5f | 3.26e-g | 7.86hi | 0.46f | 58.53e | 49.7gh | 63.3gh |
| **T4** | 1.47ef | 0.76d-f | 1.62fg | 3.73de | 3.36d-f | 8.3fg | 0.53d | 62.86f | 66.4cd | 80.0bd |
| **T5** | 1.58bc | 0.79bc | 1.7cd | 3.89c | 3.46cd | 8.63ef | 0.73b | 65.33de | 66.2cd | 76.6ce |
| **T6** | 1.52de | 0.73fg | 1.67de | 3.86cd | 3.43de | 8.8de | 0.75b | 66.7cd | 73.4bc | 83.3ac |
| **T7** | 1.54cd | 0.80bc | 1.76b | 4.06ab | 3.63bc | 9.1cd | 0.63c | 68.33c | 78.8b | 86.6ab |
| **T8** | 1.57b-d | 0.77c-e | 1.73bc | 3.85cd | 3.73b | 9.4bc | 0.80a | 69.9bc | 72.06bc | 76.6ce |
| **T9** | 1.63a | 0.80a | 1.76a | 4.1a | 3.96a | 9.83a | 0.81a | 71.4ab | 88.5a | 90.0a |
| **T10** | 1.6ab | 0.79ab | 1.74b | 3.93bc | 3.8ab | 9.56ab | 0.61c | 61.9a | 73.36bc | 76.6ce |
| **T11** | 1.46fg | 0.7g-j | 1.66ef | 3.66e | 3.23f-h | 8.03g-i | 0.43g | 57.96e | 64.33de | 80.0bd |
| **C2 (+Cd)** | 1.2n | 0.59n | 1.2q | 2.18n | 1.31n | 4.03p | 0.15m | 31.1f | 24.16n | 60.0h |
| **T1** | 1.28m | 0.64mn | 1.27p | 2.35m | 1.35mn | 4.3ij | 0.21l | 32.96o | 27.16mn | 63.3gh |
| **T2** | 1.34kl | 0.67j-l | 1.30op | 2.41k-m | 1.38l-n | 4.53m-o | 0.31j | 34.2m-o | 31.73m | 70.0eg |
| **T3** | 1.06o | 0.66k-m | 1.29op | 2.39lm | 1.42k-n | 4.43no | 0.25k | 32.83l-n | 28.1ln | 63.3gh |
| **T4** | 1.34kl | 0.7g-j | 1.33no | 2.52j-l | 1.47k-n | 4.8l-n | 0.31j | 34.86no | 35.23il | 73.3df |
| **T5** | 1.42g-i | 0.70g-i | 1.4lm | 2.63h-j | 1.52jk-m | 4.9k-m | 0.40g | 36.46i-l | 39.2ij | 80.0bd |
| **T6** | 1.26m | 0.67j-l | 1.36mn | 2.55i-k | 1.49jk-m | 5.13kl | 0.36i | 35.86k-m | 39.33i | 76.6ce |
| **T7** | 1.45fgh | 0.75ef | 1.46k | 2.67hij | 1.55jkl | 4.83lm | 0.36hi | 37.9hij | 40.33i | 83.3ac |
| **T8** | 1.36jk | 0.71gh | 1.41l | 2.69hi | 1.58jk | 4.96kl | 0.39gh | 39.1hi | 37.96ik | 76.6ce |
| **T9** | 1.62ab | 0.79bcd | 1.44j | 2.76h | 1.67j | 5.33k | 0.50de | 40.63h | 48h | 90.0a |
| **T10** | 1.37ijk | 0.76def | 1.38lm | 2.62hij | 1.54jkl | 5.06kl | 0.39m | 37.6ijk | 33.9i-k | 66.6fg |
| **T11** | 1.29lm | 0.66klm | 1.37mn | 2.52kl | 1.43klmn | 4.3op | 0.21l | 32.06no | 31.5kl | 73.3df |

Values sharing the same letters do not differ statistically from one another ($p \leq 0.05$).

Where; T1: ZM31, T2: ZM27, T3: N8, T4: AN31 T5: ZM31+ZM27, T6: ZM31+N8, T7: ZM31+AN31, T8: ZM27+N8, T9: ZM27+AN31, T10: N8+AN31, T11: $ZnSO_4$

459 The results revealed that Cd stress decreases root colonization Fig.2 All sole and co-inoculated ZSB
460 treatments improved the root colonization compared to the stressed treatment. The maximum (8.9%)
461 root colonization was observed through a consortium AN31+ZM27 ($T_9$). Both strains AN31 and
462 ZM27 outperformed in the consortium and solely applied bacteria with regard to root colonization.
463 However, the sole application of $ZnSO_4$ also showed substantial rise in root colonization (11%) as
464 compared to the control (Fig.2). Effect of sole and consortium of Cd-tolerant bacterial strains on root
465 colonization of sunflower seedlings under control and cadmium stress in jar trial.
466 It was observed that Cd-stress increased the Cd concentration in the roots of the sunflower (Figure
467 3.). The Cd-tolerant, ZSB decreased stress-induced Cd in roots of sunflower seedling. Maximum Cd
468 concentration was shown in uninoculated control plant roots. Under non-stressed situations, all
469 treatments were statistically non-significant compared to the uninoculated control. The maximum
470 reduction (15%) was observed by AN31+ZM27 ($T_9$). However, the sole application of $ZnSO_4$ also
471 gave same value in Cd reduction as compared to the control. The bacterial strains AN31 and ZM27
472 outperformed in the consortium as well as solely bacterial strains for Cd reduction with (7 and 4%
473 reduction) compared to control.
474 The results showed that exposure to Cd stress resulted in a significant elevation of Cd concentration
475 in the shoots of sunflowers as shown in Figure 4. The Cd-tolerant ZSB exhibited a reduction in
476 cadmium ($Cd^{2+}$) stress-induced cadmium accumulation in the shoots of sunflowers. Under non-
477 stressed situations, all treatments were statistically non-significant compared to the uninoculated
478 control.

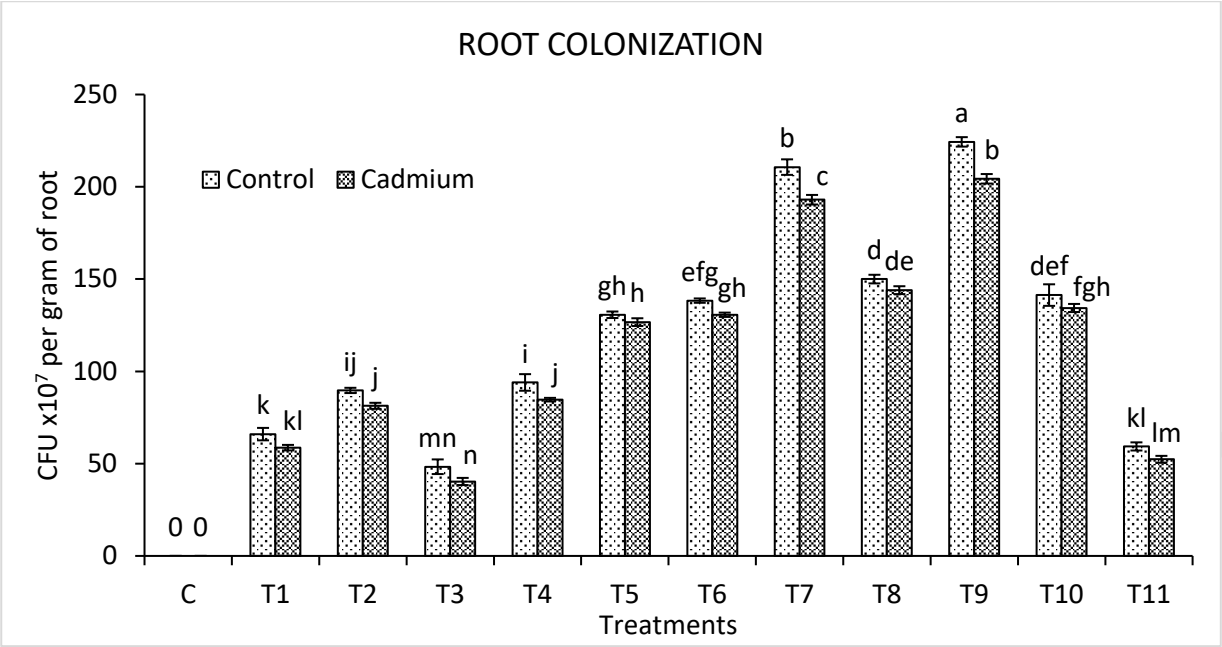


**Figure 2.** Effect on root colonization on the application of ZSB strains under cadmium stress. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11}$: $ZnSO_4$

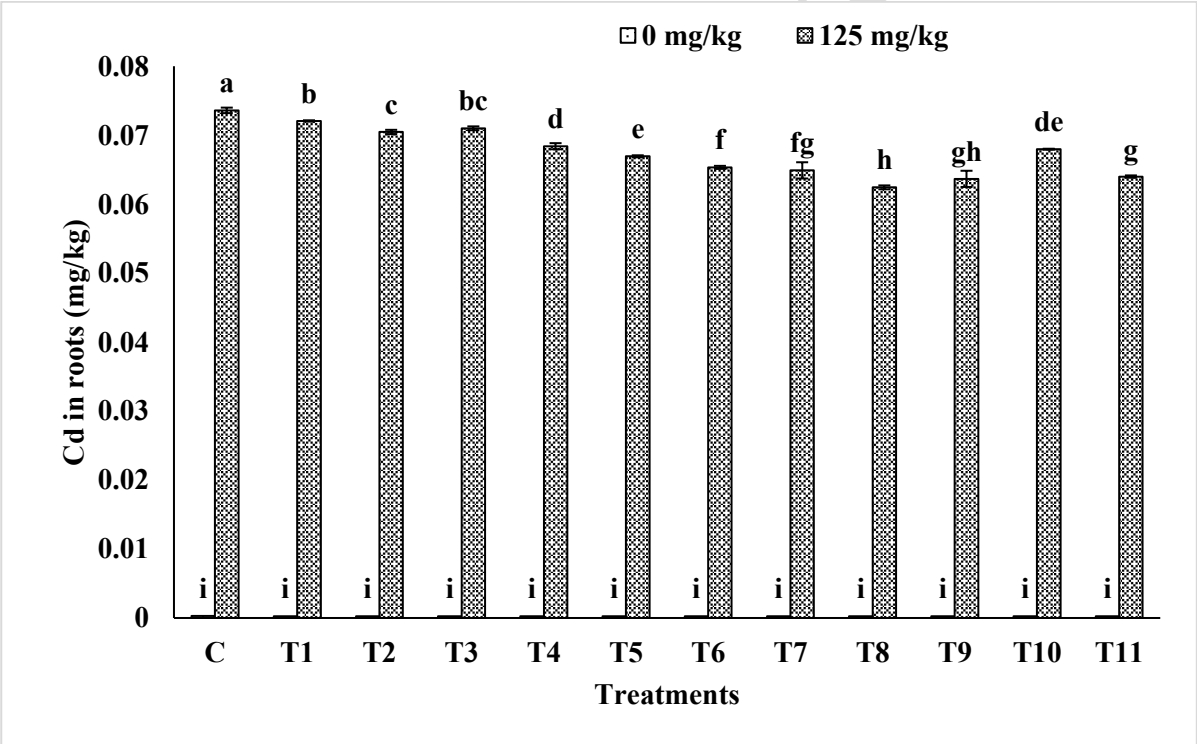


**Figure 3.** Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in roots of sunflower under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11}$: $ZnSO_4$

The highest concentration of Cd was observed in the shoot of plants that were not inoculated. AN31+ZM27 ($T_9$) resulted to the highest level of reduction (20%) compared to the control under

492 stress. However, the $ZnSO_4$ showed (19%) Cd reduction in comparison to the control. The bacterial
493 strains AN31 and ZM27 exhibited superior performance in both the consortium and as individual
494 bacterial strains for reducing Cd levels by 8% and 6% as compared to the control.
495 It was observed that Cd stress increased the Cd concentration in the soil (Figure 5.). The ZSB through
496 sole and co-inoculation increased soil cadmium contents. Under non-stressed situations, all
497 treatments were statistically non-significant compared to the uninoculated control. The consortium
498 (AN31+ZM27) increases the Cd in soil by 59% compared to the control. The strain AN31 and ZM27
499 both outperformed in the consortium and solely inoculated bacteria with respect to Cd concentration
500 in soil (32 and 41%). However, the strain $ZnS0_4$ showed (57%) decrease in Cd concentration as
501 compared to the control.
502 Cd-tolerant ZSB in sole and consortium improved zinc content in shoots (Figure 6.). All the applied
503 treatments including sole and consortium of ZSB strains increase Zn concentration as compared to
504 the control treatment under stress and non-stress conditions. However, the application with
505 recommended dose $ZnSO_4$ showed the highest Zn concentration (40%), and ZM27 and AN31 also
506 increased results by 20 and 16% respectively. However, AN31+ZM27 ($T_9$) showed 30% increase
507 compared to the control. Under Cd-stressed conditions, 52% increase in Zn concentration was
508 recorded by AN31+ZM27 compared to the control.
509

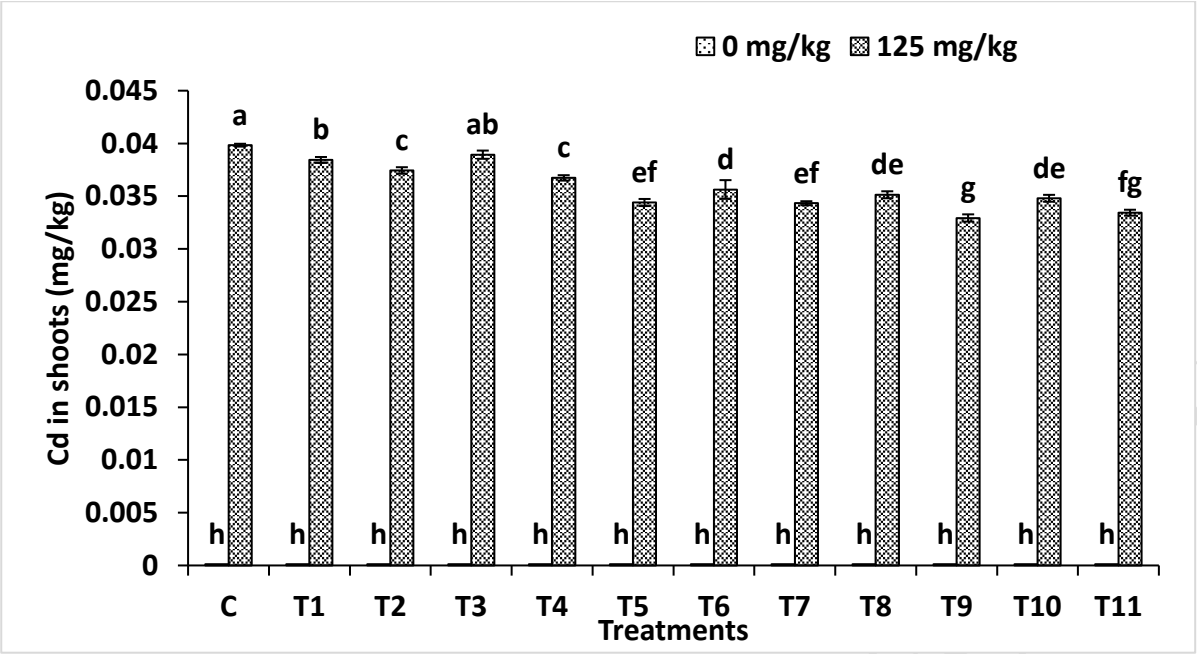


**Figure 4.** Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in shoots of sunflower under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11:}$ $ZnSO_4$

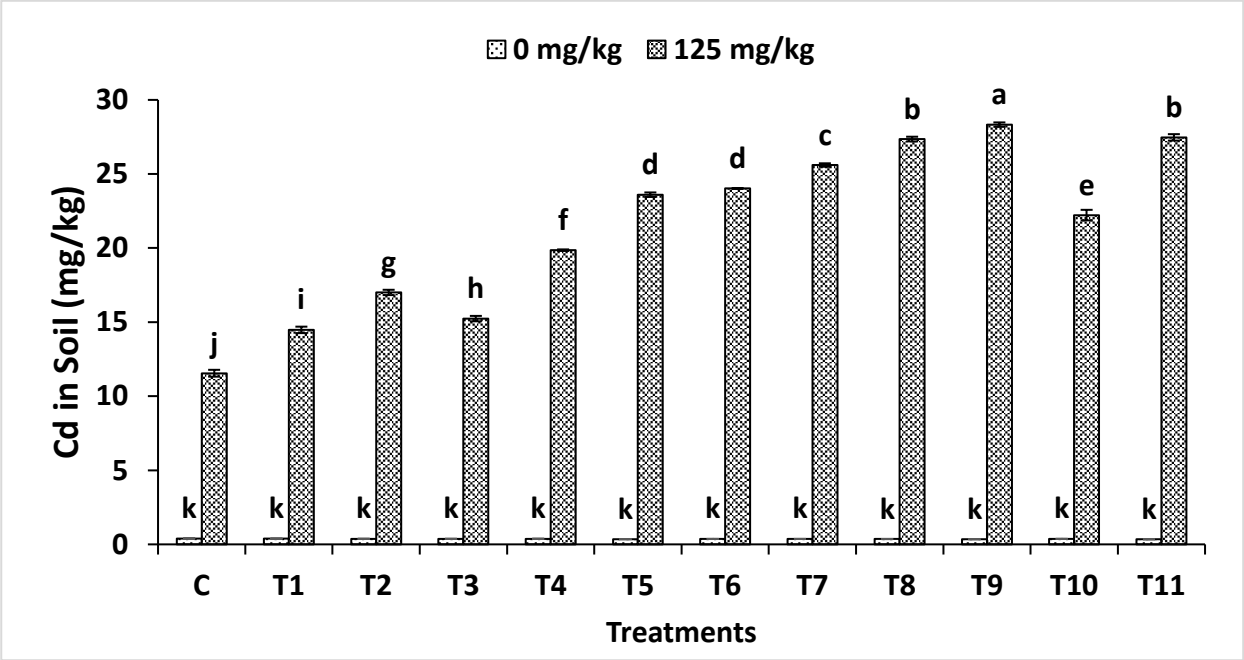


**Figure 5.** Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in soil under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11:}$ $ZnSO_4$

Cd-tolerant ZSB in sole and consortium improved zinc content in roots (Figure 7). All the applied treatments including sole and consortium of ZSB strains increase Zn concentration as compared to the control treatment. The l inoculation with $ZnSO_4$ showed the highest Zn concentration (14%), and ZM27 and AN31 also increased results by 4 and 3% respectively. However, AN31+ZM27 ($T_9$)

showed t 13% increase compared to the control. Under Cd-stressed conditions, a maximum 14% increase in Zn concentration was recorded by AN31+ZM27 compared to the control.

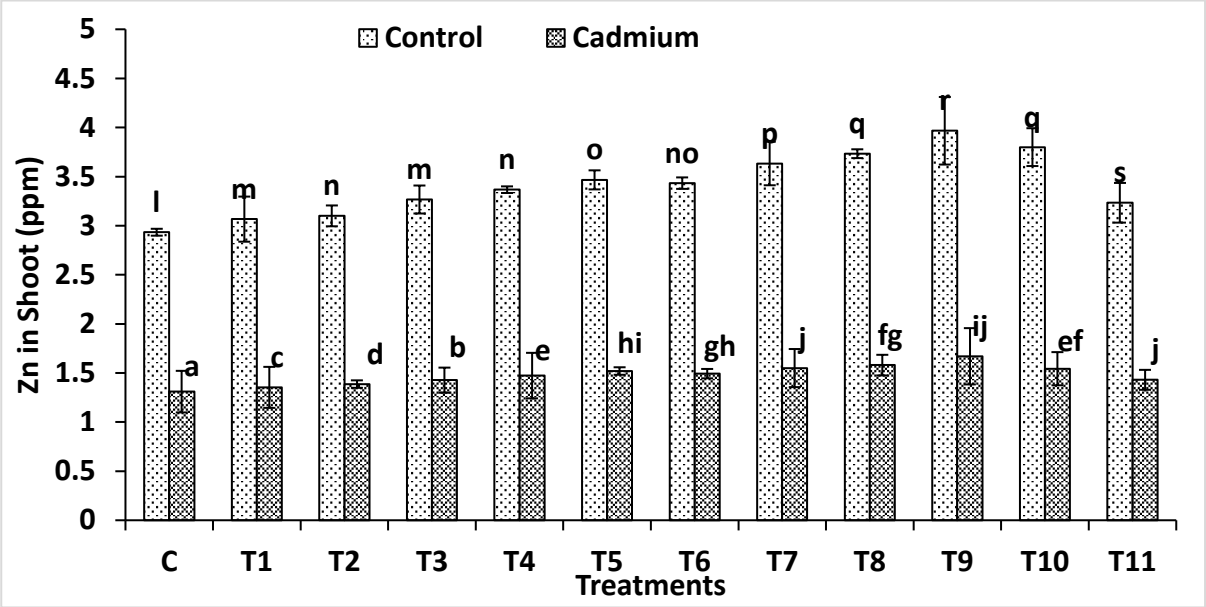


**Figure 6.** Effect of sole and consortium of Cd-tolerant bacterial strains on zinc concentration in shoots of sunflower under control and cadmium stress in jar trial.

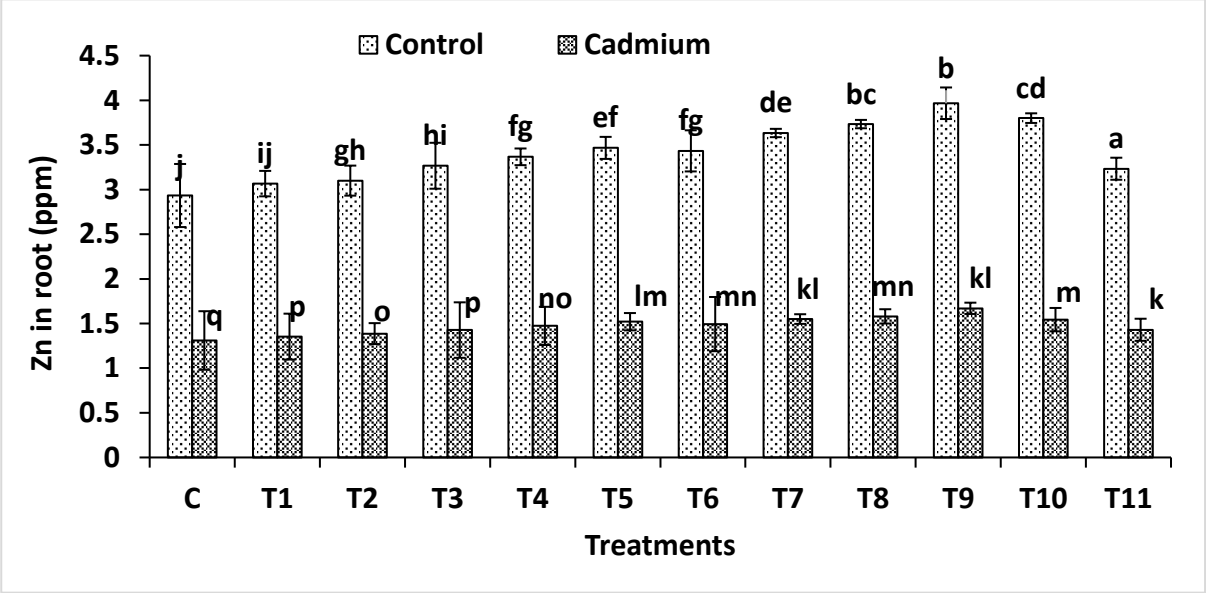


**Figure 7.** Effect of sole and consortium of Cd-tolerant bacterial strains on zinc concentration in roots of sunflower under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11:}$ $ZnSO_4$

The ZSB lessen the cadmium uptake in shoots through solo and co-inoculation (Figure 8). All treatments were statistically non-significant under normal conditions. Under stressed conditions, the consortium AN31+ZM27 ($T_9$) reduced the Cd uptake in shoots by 97%. The strains AN31 and ZM27 both outperformed in the consortium and solely inoculated bacteria with respect to Cd uptake in

shoots (94 and 96%). The strain $ZnS0_4$ gave (96%) a discernible decrease in Cd content compared to the control.

The cadmium uptake in roots was enhanced by the ZSB through solo and co-inoculation (Figure 9). Under normal conditions, all treatments showed statistically non-significant results. The consortium AN31+ZM27 ($T_9$) has been observed to decrease (93%) the uptake of Cd in roots by 14% under Cd-stressed. Both strains AN31 and ZM27 exhibited superior performance in the consortium as well as in the inoculated bacteria alone, in terms of their ability to reduce (92%) Cd in roots. The experimental results indicate that $ZnSO_4$ gave (96%) decrease in uptake of Cd as compared to control treatment.

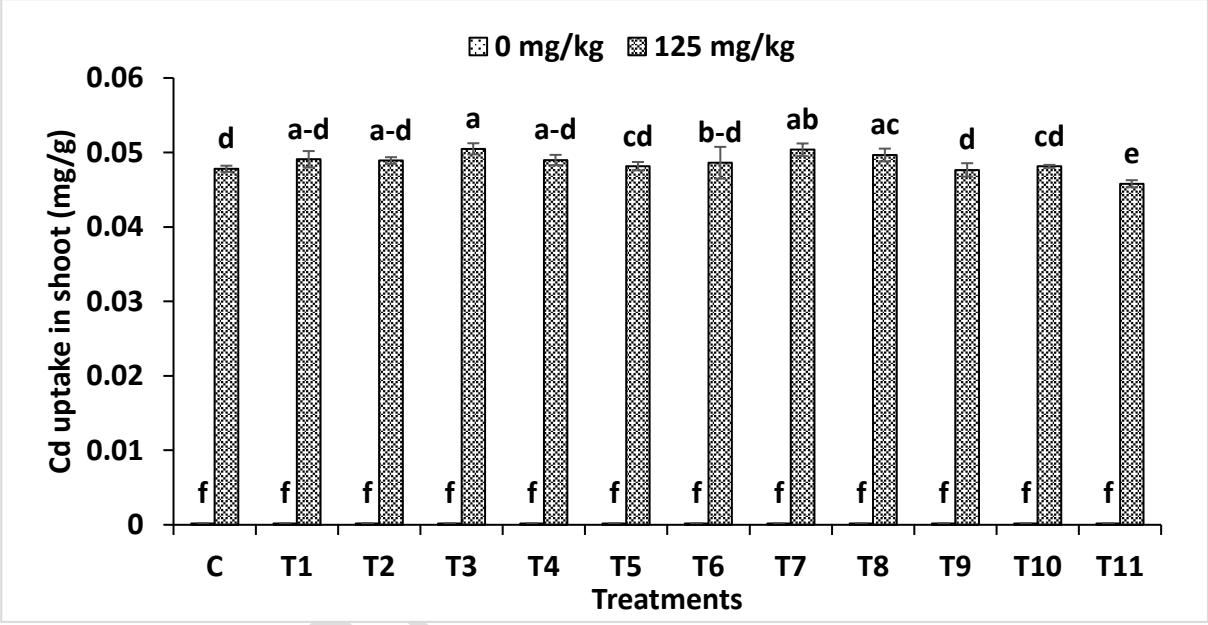


**Figure 8.** Effect of sole and consortium of Cadmium tolerant bacteria on Cd uptake in shoots of sunflower under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11}$: $ZnSO_4$

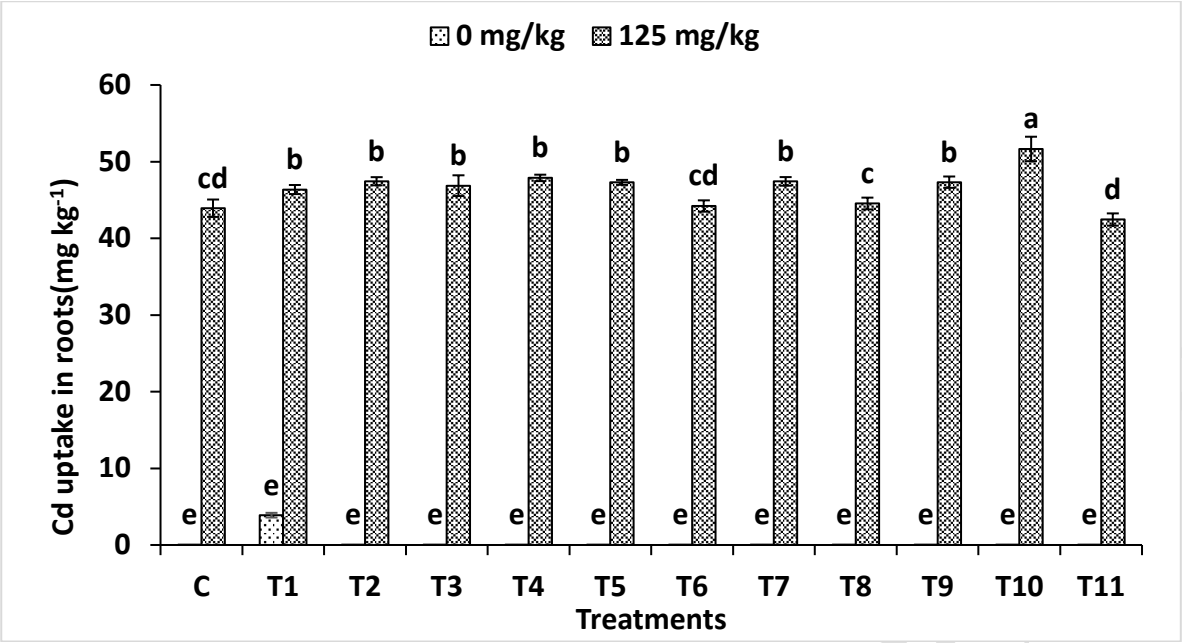


**Figure 9.** Effect of sole and consortium of Cd-tolerant bacterial strains on Cd uptake in roots of sunflower under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11}$: $ZnSO_4$

The results indicate a negative correlation between the translocation factor and Cd concentrations (Figure 10). The translocation factor of Cd was reduced when Cd-tolerant, ZSB was used to inoculate sunflower seedlings under 125mg $kg^{-1}$ Cd stress. The AN31+ZM27 ($T_9$) exhibited the highest level of reduction (96%). However, the $ZnSO_4$ exhibit a reduction of (94%) in comparison to the control. The bacterial strains AN31 and ZM27 exhibited superior performance both as a consortium and as individual bacterial strains in terms of translocation factor resulting in a reduction of compared to the control.

The results showed that the bioaccumulation factor increased as Cd levels increase as shown in Figure 11. Uninoculated plants have a negligible decline. Inoculating sunflower seedlings with Cd-tolerant, ZSB under 125mg $kg^{-1}$ Cd stress reduced Cd bioaccumulation by all the treatments. However, the maximum reduction (97%) was observed by AN31+ZM27 ($T_9$). However, the sole application of $ZnSO_4$ gave (83%) reduction in accumulation.

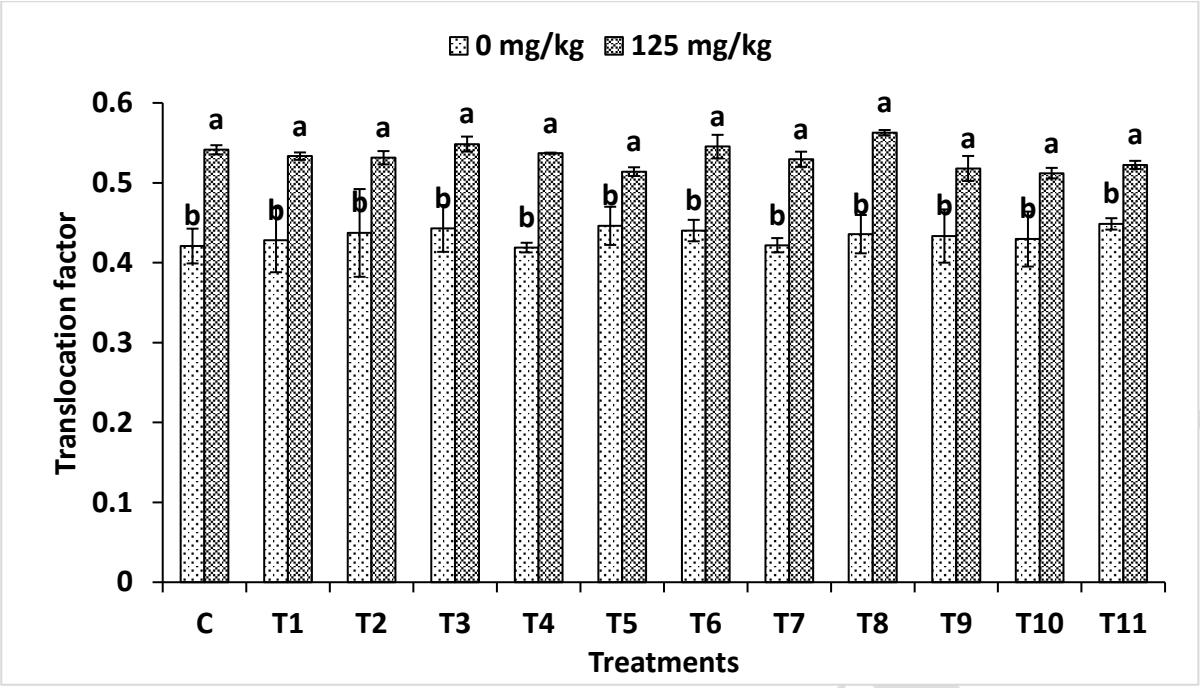


**Figure 10.** Effect of sole and consortium of Cd-tolerant bacterial strains translocation factor of sunflower under control and cadmium stress in jar trial.

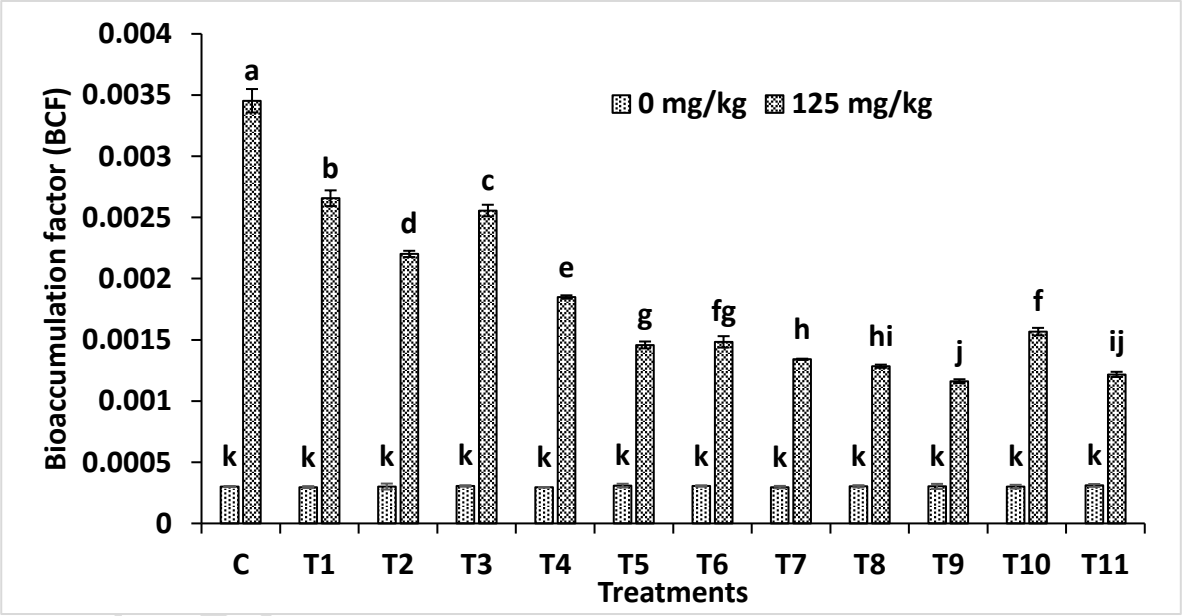


**Figure 11.** Effect of sole and consortium of Cd-tolerant bacterial strains on bioaccumulation factor of sunflower seedlings under control and cadmium stress in jar trial. ($C_1$: Control, $T_1$: ZM31, $T_2$: ZM27, $T_3$: N8 $T_4$: AN31 $T_5$ ZM31+ZM27 $T_6$ ZM31+N8 $T_7$ ZM31+AN31 $T_8$: ZM27+N8 $T_9$: ZM27+AN31 $T_{10}$ N8+AN31 $T_{11}$: $ZnSO_4$

## 4. Discussion

In this study, a consortium of zinc solubilizing bacteria (ZM27+AN31), which exhibited resistance to cadmium, demonstrated a notable enhancement in the fresh and dry biomass of sunflower seedlings. Additionally, this consortium effectively reduced the uptake and translocation of cadmium

585 from the root to the shoot. The two bacterial strains displayed contrasting abilities in curtailing the
586 translocation of cadmium and the bioconcentration factor of cadmium in sunflower plants. The
587 presence of strains resulted in increased cell density (indicated by OD600) and reduced water-soluble
588 cadmium concentration in the cadmium-supplemented medium, suggesting the capacity of these
589 strains to grow and immobilize cadmium in the solution (Table 4). The anionic functional groups of
590 the two strains likely played a pivotal role in binding cadmium and facilitating its immobilization in
591 the solution, as previously reported by (Halim et al., 2020). Moreover, as the two strains belong to
592 different bacterial species, variations in growth ability and anionic functional groups on their cell
593 walls could account for the divergent cell growth and cadmium immobilization observed in the
594 cadmium-supplemented medium (Figure 1).
595 The application of Cd-tolerant PGPB with Zn-providing capabilities has been demonstrated to
596 significantly increase root and shoot length in Cd-stressed plants as shown in Table 7. This might be
597 due to the bacteria that facilitate Cd detoxification, minimizing its inhibitory effects on root
598 elongation and enhancing nutrient uptake efficiency. Additionally, the provision of Zn by PGPB
599 promotes cell division, elongation, and differentiation in root and shoot tissues, leading to improved
600 plant growth even under Cd stress as proved by (Huihui et al., 2020).
601 Abiotic stress (metals stress) reduces the chlorophyll contents of plants, compromising
602 photosynthesis and reducing crop growth (Saifullah et al., 2014). Cadmium ions replacing Mg ions
603 in the chlorophyll structure can affect the chlorophyll a/b ratios, with chlorophyll a being more
604 susceptible to damage than chlorophyll b, which reduces the efficiencies of light capturing
605 photosystem-I and photosystem-II (Janeeshma et al., 2021). This might be due to ZSB, which reduces
606 Cd uptake, enhances Zn availability through multiple mechanisms (EPS and Siderophore production),
607 and reduces the alteration of Mg ions. As depicted, zinc application has been shown to positively
608 effect gas interchange characteristics and chlorophyll content in wheat foliar application (Saifullah et
609 al., 2014). Cadmium stress has been reported to decrease chlorophyll contents in various plants like
610 peas (*Pisum Sativum* L.), soya beans, tomatoes, lettuce, and potatoes (Baruah et al., 2019).

611 Moreover, the reduction in chlorophyll content is attributed to the disruption of the photosynthetic
612 electron transport chain, leading to a decline in biomass production (Bai et al., 2014), as shown in
613 Table 7. However, the consortium of ZSB strains was found to increase fresh and dry biomass, which
614 might be due to a reduction in metal uptake and repairing of the photosynthetic system of the plant
615 under stress conditions (Bai et al., 2014; Chen et al., 2016).
616 Studies have established the beneficial impact of bacteria capable of producing extracellular
617 polymeric substances (EPS) and siderophores on plant growth and their ability to reduce metal uptake
618 in plants (Saifullah et al., 2014). These bacteria can decrease metal bioavailability by binding metals
619 through anionic functional groups and chelating metal ions using metabolites such as extracellular
620 polymers, siderophores, and organic acids, as Zhang et al., (2019), demonstrated the reduction of
621 nickel and cadmium availability in soil and their subsequent accumulation in tomato roots and shoots
622 through the actions of *Magnaporthe oryzae* CBMB20 and *Burkholderia* sp. CBMB40. immobilizing
623 heavy metals in the soil through bacterial inoculation is a critical attribute that enhances plant growth
624 and reduces heavy metal uptake by plants (Madhaiyan et al., 2007). According to them metal-resistant
625 bacteria effectively reduced cadmium accumulation in rice grains grown in soil with very low
626 cadmium contamination (0.57 mg $kg^{-1}$) by decreasing the available cadmium content in the soil.
627 Ahmad et al., (2014) compared the adsorption capabilities of *Pseudomonas aeruginosa, Bacillus*
628 *subtilis,* and *Beauveria bassiana*, finding that *Pseudomonas aeruginosa* adsorbed more cadmium and
629 demonstrated a higher capacity to reduce grain cadmium content in cadmium-contaminated soil.
630 While the sole application of the strains in this study yielded superior results compared to the control
631 treatment, their consortium exhibited a significantly lower cadmium bioconcentration factor than
632 when applied individually (Zhang et al., 2019).
633 These findings indicate that bacteria resistant to heavy metals and possessing immobilization
634 capabilities could be employed to mitigate cadmium accumulation in sunflower plants grown in
635 cadmium-contaminated soil. Consequently, the utilization of metal-resistant and immobilizing

bacteria offers a viable means of ensuring the safe production of sunflower even in soils contaminated with cadmium, with the effectiveness of the approach contingent upon the specific strains employed.

## Conclusion

In conclusion, when compared to the control treatment, the bacterial consortium used in this study reduced cadmium levels and uptake in sunflower seedlings significantly. Furthermore, the presence of the consortium boosted zinc availability in the plant. On the other hand, large amounts of zinc alone had a synergistic impact with cadmium, resulting in decreased cadmium reduction efficacy. These findings show the potential of cadmium-tolerant zinc-solubilizing bacteria as a promising technique for reducing cadmium toxicity while also increasing zinc absorption in sunflower seedlings. However, caution should be exercised when administering zinc at high levels on its own, as it may interfere with cadmium reduction in plants. Furthermore, these strains (consortium) should be tested in field conditions for better recommendations to the farming community and development of a biofertilizer containing live bacteria to alleviate the Cd toxicity in food crops.

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**Data Availability Statement:**

The data presented in this study are available on request from the first corresponding author.

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