Global NEST Journal, Vol 26, No 4, 05820

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

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Received: 13/02/2024, Accepted: 19/03/2024, Available online: 21/03/2024
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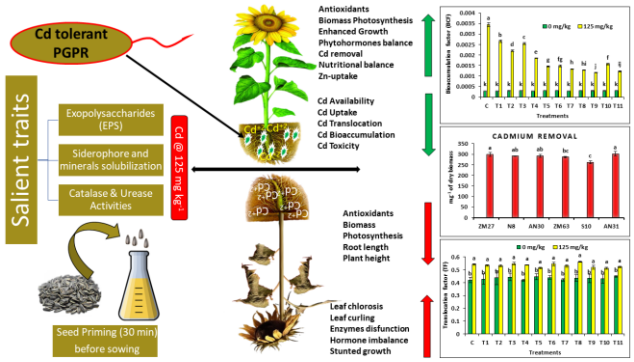
https://doi.org/10.30955/gnj.005820

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; Carry 60; Agilent, Santa Clara, CA, 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:

$$\%\ Siderophore = \frac{Ar - As}{Ar} \times 100 \quad (1)$$

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°C 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).

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

2.7. 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.

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.

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

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).

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

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.

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

Where: Cshoot and Croot are metals concentration (mg kg^{-1}) in shoot and root of sunflower plant, respectively. Moreover the TF > 1 represents effective translocation of metals from shoot from root (Yoon *et al.* 2006).

$$BCF = \frac{Cshoot}{Csoil} \quad (5)$$

Where: Cshoot and Croot are metals concentration (mg kg^{-1}) in shoot and root of sunflower plant, respectively. BCF was categorized further as hyperaccumulators as the accumulated metal concentration exceeds >1 mg kg^{-1} (Cui *et al.* 2007).

2.12. Statistical analysis

The data was statistically analyzed using completely randomized design (CRD) under factorial arrangement by analysis of variance (ANOVA) through Statistix 8.1® computer-based software (Steel 1961). The treatment means were compared by using LSD at 5% level of probability (Duncan 1955).

3. Results

Pre-isolated and pre-identified zinc solubilizing bacterial strains were screened for cadmium tolerance in laboratory under controlled conditions. They were refreshed using General purpose media (GPM) on agar plates and used for experimentation.

3.1. Determining minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration was used to screen the pre-isolated rhizobacteria for Cd resistance (Table 2). To identify their best resistance capacity, all of these (Cd) resistance rhizobacterial strains ZM27, ZM63, ZM31, AN24, AN30, AN31, ZE15, ZE11, ZR19, S10, N8, and ZR3 isolates were screened using the MIC approach and their MIC values were calculated. The results showed that out of 12 isolates, eight isolates AN31, ZM27, ZM31, N8, ZE11, AN33, ZE1,5, and S10 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	+++	+++	++	–

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

3.2. 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.

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).

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.

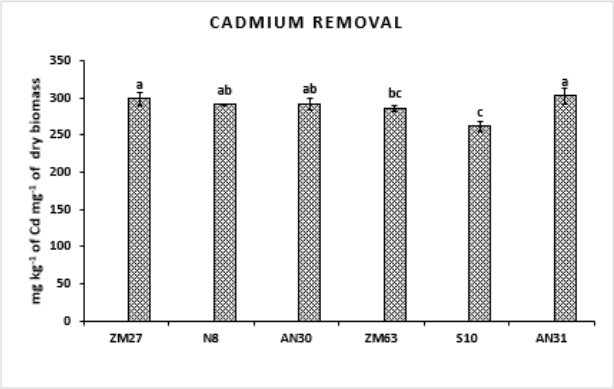


Figure 1. Cadmium removal by zinc solubilizing bacterial strains

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 Figure 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) (Figure 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.

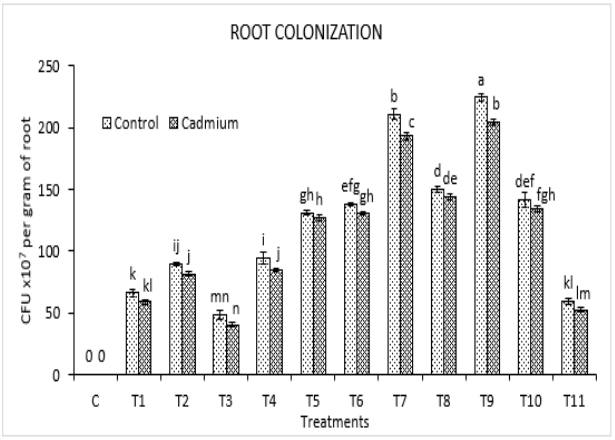


Figure 2. Effect on root colonization on the application of ZSB strains under cadmium stress. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

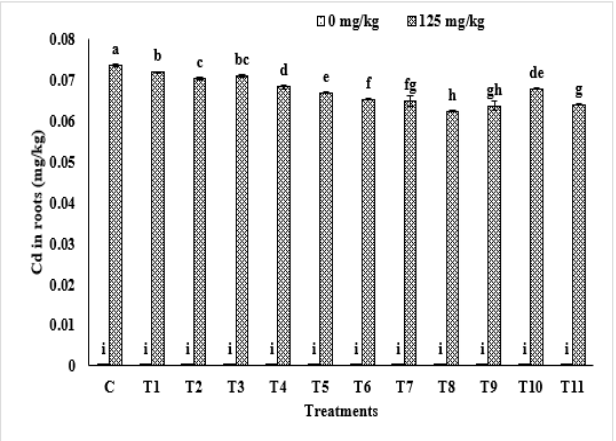


Figure 3. Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in roots of sunflower under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

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.

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

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 ($p \leq 0.05$).

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: $ZnSO_4$										

3.6. 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.

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 ZM27+AN31 (T_9) treatment exhibited the highest shoot length (31%) compared to the control. The bacterial strains AN31 and ZM27 exhibited superior performance within the consortium, as well as in individual treatments, in comparison to other bacteria that were solely applied in terms of shoot length (9% and 17%). However, the sole application of $ZnSO_4$ did not exhibit a significant increase in the shoot length in comparison to the control. The inoculation was also effective in enhancing the shoot length under Cd-stressed conditions. The highest (26%) shoot length found by the ZM27+AN31 (T_9) matched to the control.

The Cd-stress decrease the root length of the sunflower. All ZSB strain alone and co-inoculation treatments improve root length compared to control. Under normal conditions, the bacterial consortium ZM27+AN31 (T_9) showed the highest (46%) root length than the control. The strains AN31 and ZM27 outperformed the consortium and other solely applied bacteria in root length (15 and 25%) compared to the control. However, the sole inoculation of $ZnSO_4$ did not enhance root length compared to the control. The inoculation also increased the root length by 30% under Cd-stressed conditions with ZM27+AN31 (T_9) compared to the control.

The exposure to Cd-induced stress has been observed to result in the shoot fresh weight reduction. The results indicate that both the ZSB strain alone and co-inoculation treatments exhibit a significant enhancement in shoot fresh weight as related to the control. Under normal conditions, the consortium ZM27+AN31 (T_9) increase the shoot fresh weight that was higher (20%) than that of the control under cadmium stress. The results indicate that strains ZM27 and AN31 solely also exhibited superior performance in terms of shoot fresh weight, with a 9% and 13% respectively, increase in comparison to the control and other sole applied bacteria. The application of $ZnSO_4$ did not exhibit any significant improvement in the shoot fresh weight compared to the control. The experimental results indicate that the ZM27+AN31 (T_9) significantly increased the shoot fresh weight by 27% in the presence of Cd-induced stress, as compared to the control.

The Cd stress decreases the shoot dry weight of the sunflower. All ZSB strain through sole and co-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 $ZnSO_4$ 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.

Data showed that Cd-stress decrease the chlorophyll content of sunflower. The inoculation of ZSB through sole and co-inoculation significantly improved the results compared to the control. Under normal conditions, a maximum 30% increase was observed through ZM27+AN31 (T_9). The bacterial strains AN31 and ZM27 outperformed in the consortium as well as solely bacterial strains with respect to SPAD value (10 and 14%). However, the sole application of $ZnSO_4$ showed (3%) increase in SPAD value as compared to the control (Table 7). Under stressed conditions, a maximum 26% increase was recorded through AN31+ZM27 compared to the uninoculated control.

The Cd-tolerant ZSB strain exhibited a significant increase in the SVI of sunflowers under normal and Cd-stressed. Under normal conditions, the ZM27+AN31 (T_9) showed a 40% 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 15%, The inoculation of $ZnSO_4$ did not result in a significant increase in the SVI when compared to the control. The inoculation was found to be significant even under Cd stress, with a 36% increase in inoculation with ZM27+AN31 (T_9) compared to the control.

The results revealed that Cd stress decreases root colonization Figure.2 All sole and co-inoculated ZSB treatments improved the root colonization compared to the stressed treatment. The maximum (8.9%) root colonization was observed through a consortium AN31+ZM27 (T_9). Both strains AN31 and ZM27 outperformed in the consortium and solely applied bacteria with regard to root colonization. However, the sole application of $ZnSO_4$ also showed substantial rise in root colonization (11%) as compared to the control (Figure 2). Effect of sole and consortium of Cd-tolerant bacterial strains on root colonization of sunflower seedlings under control and cadmium stress in jar trial.

It was observed that Cd-stress increased the Cd concentration in the roots of the sunflower (Figure 3). The Cd-tolerant, ZSB decreased stress-induced Cd in roots of sunflower seedling. Maximum Cd concentration was shown in uninoculated control plant roots. Under non-stressed situations, all treatments were statistically non-significant compared to the uninoculated control. The maximum reduction (15%) was observed by AN31+ZM27 (T_9). However, the sole application of $ZnSO_4$ also gave same value in Cd reduction as compared to the control. The bacterial strains AN31 and ZM27 outperformed in the consortium as well as solely bacterial strains for Cd reduction with (7 and 4% reduction) compared to control.

The results showed that exposure to Cd stress resulted in a significant elevation of Cd concentration in the shoots of sunflowers as shown in Figure 4. The Cd-tolerant ZSB exhibited a reduction in cadmium (Cd^{2+}) stress-induced cadmium accumulation in the shoots of sunflowers. Under non-stressed situations, all treatments were statistically non-significant compared to the uninoculated control.

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 stress. However, the $ZnSO_4$ showed (19%) Cd reduction in comparison to the control. The bacterial strains AN31 and ZM27 exhibited superior performance in both the consortium and as individual bacterial strains for reducing Cd levels by 8% and 6% as compared to the control.

It was observed that Cd stress increased the Cd concentration in the soil (Figure 5). The ZSB through sole and co-inoculation increased soil cadmium contents. Under non-stressed situations, all treatments were statistically non-significant compared to the uninoculated control. The consortium (AN31+ZM27) increases the Cd in soil by 59% compared to the control. The strain AN31 and ZM27 both outperformed in the consortium and solely inoculated bacteria with respect to Cd concentration in soil (32 and 41%). However, the strain $ZnS0_4$ showed (57%) decrease in Cd concentration as compared to the control.

Cd-tolerant ZSB in sole and consortium improved zinc content in shoots (Figure 6). All the applied treatments including sole and consortium of ZSB strains increase Zn concentration as compared to the control treatment under stress and non-stress conditions. However, the application with recommended dose $ZnSO_4$ showed the highest Zn concentration (40%), and ZM27 and AN31 also increased results by 20 and 16% respectively. However,

AN31+ZM27 (T_9) showed 30% increase compared to the control. Under Cd-stressed conditions, 52% increase in Zn concentration was recorded by AN31+ZM27 compared to the control.

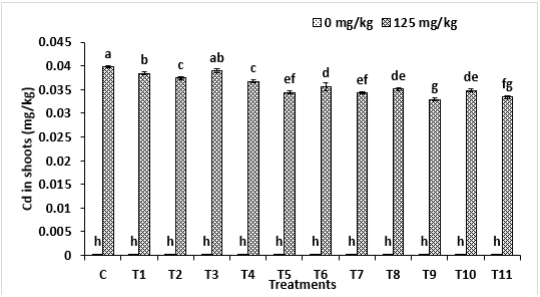


Figure 4. Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in shoots of sunflower under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

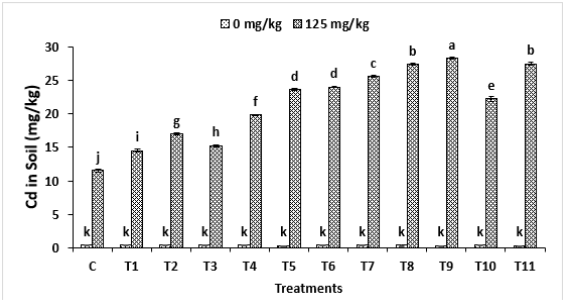


Figure 5. Effect of sole and consortium of Cd-tolerant bacterial strains on cadmium concentration in soil under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

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.

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.

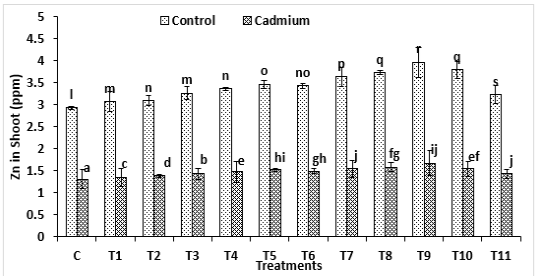


Figure 6. Effect of sole and consortium of Cd-tolerant bacterial strains on zinc concentration in shoots of sunflower under control and cadmium stress in jar trial

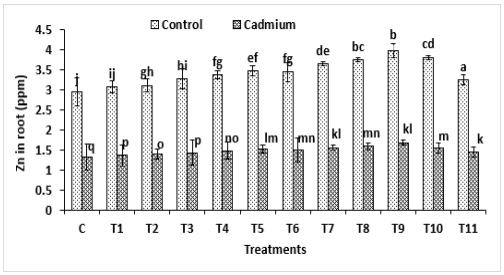


Figure 7. Effect of sole and consortium of Cd-tolerant bacterial strains on zinc concentration in roots of sunflower under control and cadmium stress in jar trial. (C_1: Control, T_1: ZM31, T_2: ZM27, T_3: N8 T_4: AN31 T_5 ZM31+ZM27 T_6 ZM31+N8 T_7 ZM31+AN31 T_8: ZM27+N8 T_9: ZM27+AN31 T_{10} N8+AN31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

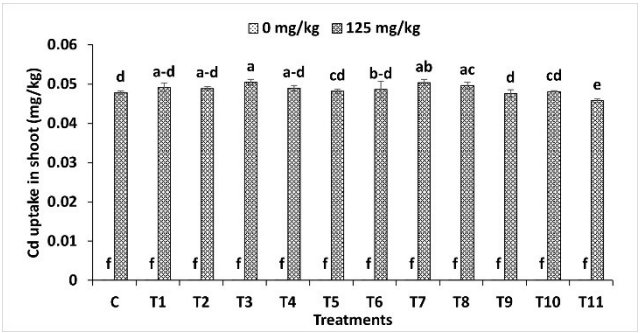


Figure 8. Effect of sole and consortium of Cadmium tolerant bacteria on Cd uptake in shoots of sunflower under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

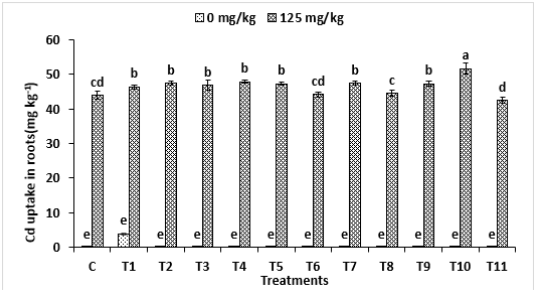


Figure 9. Effect of sole and consortium of Cd-tolerant bacterial strains on Cd uptake in roots of sunflower under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

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.

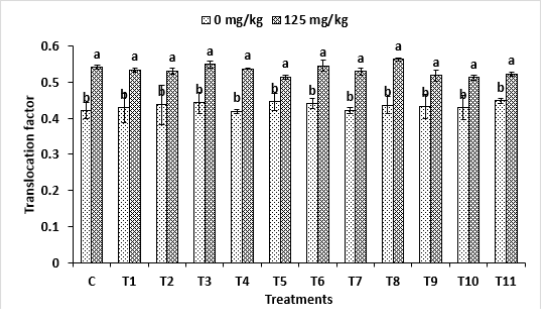


Figure 10. Effect of sole and consortium of Cd-tolerant bacterial strains translocation factor of sunflower under control and cadmium stress in jar trial

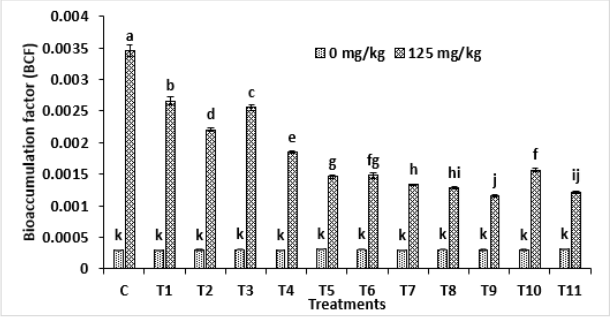


Figure 11. Effect of sole and consortium of Cd-tolerant bacterial strains on bioaccumulation factor of sunflower seedlings under control and cadmium stress in jar trial. (C1: Control, T1: ZM31, T2: ZM27, T3: N8 T4: AN31 T5 ZM31+ZM27 T6 ZM31+N8 T7 ZM31+AN31 T8: ZM27+N8 T9: ZM27+AN31 T10 N8+AN31 T11: $ZnSO_4$

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 from the root to the shoot. The two bacterial strains displayed contrasting abilities in curtailing the translocation of cadmium and the bioconcentration factor of cadmium in sunflower plants. The presence of strains resulted in increased cell density (indicated by OD600) and reduced water-soluble cadmium concentration in the cadmium-supplemented medium, suggesting the capacity of these strains to grow and immobilize cadmium in the solution (Table 4). The anionic functional groups of the two strains likely played a pivotal role in binding cadmium and facilitating its immobilization in the solution, as previously reported by (Halim *et al.* 2020). Moreover, as the two strains belong to different bacterial species, variations in growth ability and anionic functional groups on their cell walls could account for the divergent cell growth and cadmium immobilization observed in the cadmium-supplemented medium (Figure 1).

The application of Cd-tolerant PGPB with Zn-providing capabilities has been demonstrated to significantly increase root and shoot length in Cd-stressed plants as shown in Table 7. This might be due to the bacteria that facilitate Cd detoxification, minimizing its inhibitory effects on root elongation and enhancing nutrient uptake efficiency. Additionally, the provision of Zn by PGPB promotes cell division, elongation, and differentiation in root and shoot tissues, leading to improved plant growth even under Cd stress as proved by (Huihui *et al.* 2020).

Abiotic stress (metals stress) reduces the chlorophyll contents of plants, compromising photosynthesis and reducing crop growth (Saifullah *et al.* 2014). Cadmium ions replacing Mg ions in the chlorophyll structure can affect the chlorophyll a/b ratios, with chlorophyll a being more susceptible to damage than chlorophyll b, which reduces the efficiencies of light capturing photosystem-I and photosystem-II (Janeeshma *et al.* 2021). This might be due to ZSB, which reduces Cd uptake, enhances Zn availability through multiple mechanisms (EPS and Siderophore production), and reduces the alteration of Mg ions. As depicted, zinc application has been shown to positively effect gas interchange characteristics and chlorophyll content in wheat foliar application (Saifullah *et al.* 2014). Cadmium stress has been reported to decrease chlorophyll contents in various plants like peas (*Pisum Sativum* L.), soya beans, tomatoes, lettuce, and potatoes (Baruah *et al.* 2019).

Moreover, the reduction in chlorophyll content is attributed to the disruption of the photosynthetic electron transport chain, leading to a decline in biomass production (Bai *et al.* 2014), as shown in Table 7. However, the consortium of ZSB strains was found to increase fresh and dry biomass, which might be due to a reduction in metal uptake and repairing of the photosynthetic system of the plant under stress conditions (Bai *et al.* 2014; Chen *et al.* 2016).

Studies have established the beneficial impact of bacteria capable of producing extracellular polymeric substances (EPS) and siderophores on plant growth and their ability to reduce metal uptake in plants (Saifullah *et al.* 2014). These bacteria can decrease metal bioavailability by binding metals through anionic functional groups and chelating metal ions using metabolites such as extracellular polymers, siderophores, and organic acids, as Zhang *et al.* (2019), demonstrated the reduction of nickel and cadmium availability in soil and their subsequent accumulation in tomato roots and shoots through the

actions of *Magnaporthe oryzae* CBMB20 and *Burkholderia* sp. CBMB40. immobilizing heavy metals in the soil through bacterial inoculation is a critical attribute that enhances plant growth and reduces heavy metal uptake by plants (Madhaiyan *et al.* 2007). According to them metal-resistant bacteria effectively reduced cadmium accumulation in rice grains grown in soil with very low cadmium contamination (0.57 mg kg^{-1}) by decreasing the available cadmium content in the soil. Ahmad *et al.* (2014) compared the adsorption capabilities of *Pseudomonas aeruginosa, Bacillus subtilis,* and *Beauveria bassiana*, finding that *Pseudomonas aeruginosa* adsorbed more cadmium and demonstrated a higher capacity to reduce grain cadmium content in cadmium-contaminated soil. While the sole application of the strains in this study yielded superior results compared to the control treatment, their consortium exhibited a significantly lower cadmium bioconcentration factor than when applied individually (Zhang *et al.* 2019).

These findings indicate that bacteria resistant to heavy metals and possessing immobilization capabilities could be employed to mitigate cadmium accumulation in sunflower plants grown in 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.

Funding

This research received no external funding

Data availability statement

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

Acknowledgments

We would like to express our gratitude to the "Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur" for providing research facilities. The authors extend their appreciation to the Researchers supporting project number (RSP2024R190), King Saud University, Riyadh, Saudi Arabia and Higher Education Commission (HEC) for their support through the indigenous fellowship program for provision of funding.

Conflicts of Interest

"The authors declare no conflict of interest."

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