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EFFICACY OF PB-TOLERANT *BACILLUS SUBTILIS* AND NEEM (*AZADIRACHTA INDICA*) SEED CAKE IN ATTENUATION OF LEAD STRESS IN *HELIANTHUS ANNUUS* L.

AFSHAN RAHMAN[1*], NAWALGUL SHAIKH[1], HABIBA[2], FOUZIA NASEER[1], SYEDA AIMAN FATIMA ZAIDI[1] AND SHUMAILA MOIN[1]

[1]Agricultural Biotechnology and Phytopathology Laboratory, Department of Botany, University of Karachi, Karachi 75270, Pakistan.
[2]Food Science Laboratory, School of Public Health, Dow University of Health Sciences, Karachi 75270, Pakistan
**Corresponding author's email: a.rahman@uok.edu.pk*

Abstract

One of the most consequential soil concerns is the accumulation of heavy metal pollutants. Present research aims to investigate the effectiveness of neem cake soil amendment, lead tolerant plant growth promoting rhizobacteria *B. Subtilis* inoculation and their co-application to improve physiological performance of *Helianthus annuus* L., against lead (Pb) stress. The experiment was conducted in pots containing 1 kg sandy loam soil with neem cake amendment (10 g/kg), *B. Subtilis* (inoculated via soil drench method) alone and in combination and were irrigated with two levels of Pb (0.2 mMand 0.5 mM). *In vitro* metal biodegradation assay confirmed that *B. subtilis* was able to immobilize 99.2 % of Pb when grown in broth containing 0.6 mM lead nitrate (incubated for 24 hrs at 150 rpm in a rotary shaker).In pot experiment, the co-application of *B. subtilis* and neem cake in stressed plants significantly improved carbohydrates (µg/mL), peroxidase (µgH_2O_2/min/gFW) activity and H_2O_2(%) scavenging activity while reduced phenolic contents (µg Gallic acid/mL), proteins (µg/gm) and ascorbic acid (µg/mL) indicating alleviation of stress. Considering results, present study advocates that neem cake with *B. subtilis* inoculation may be an excellent treatment to make use of contaminated soil for agricultural practices as they both significantly (p<0.05) improved plant growth in Pb contaminated environment.

Key words: Sunflower, *Azadirachta indica*, *Bacillus subtilis*, lead, Photosynthesis and quality.

Introduction

Pakistan is one of many countries that have reported that the concentration of Pb in soils has steadily increased and become a major factor in the decrease of crop yields (Aslam *et al*., 2022). Lead (Pb) pollution in the industrial parts of Pakistan has been growing at an alarming rate of as much as 1000 mg/kg in Karachi reported by several studies (Farid *et al*., 2017; Shams & Khwaja, 2019). Pb is one of the most found metal pollutants in soil and water, making its way to the food chain and get accumulated in fruits and grains. Lead enters the plant body through roots from soil where it is present as free ions as well as with complex formed with organic ligands (Collin *et al*., 2022). Inside the cell, the accumulation of Pb ions up to toxic concentration interferes with the important biochemical processes like DNA replication, cell division, electron transport chain, nitrogen metabolism (Malik *et al*., 2017; Naz *et al*., 2018; Usman *et al*., 2020). In leaf cells, it decreases carbon dioxide levels which reduces stomatal conductance ultimately transpiration rate drops (Shah *et al*., 2021). Heavy metal toxicity generally leads to accumulation of reactive oxygen species (ROS) such as OH^-, H_2O_2, O^{-2} which cause membrane disintegration via lipid peroxidation and distort membrane bound organelles (Kohli *et al*., 2020). Pb is responsible for the formation of stable complexes like phosphates, carbonates, sulfates, and hydroxides, all of which reduce crop yield and soil fertility (Aslam *et al*., 2022). Similarly, it interacts with various phosphate groups (ATP or ADP) of metabolic enzymes to substitute essential ions and generate phytotoxicity, which changes the permeability of cell membranes. Pb poisoning thus prevents the synthesis of ATP, which in turn causes lipid peroxidation and DNA distortion from an excess of reactive oxygen species (Tiwari & Lata, 2018).

Helianthus annuus L., is grown as an important oil crop in Pakistan (Rauf, 2019). It is cultivated on an area of 151 thousand acres in spring and autumn seasons producing 33000 tonnes of oil (Anon., 2021). According to Altaf *et al*., (2020), there was a noticeable increase in sunflower crop yield over the first ten years of this century. However, after 2010, the average crop yield has steadily declined. There could be several reasons for this decline, including the limited availability of mineral fertilizers, overuse of marginal land, increased N/P fixation, and toxic heavy metal damage in the soil (Aslam *et al*., 2022). Plants can mitigate the detrimental effect of heavy metals by applying growth regulators, nutrient and organic amendments. Plant's defense mechanisms against metal stress include reduced metal absorption, increased antioxidant activity, and phytochelatin binding. Activation of enzymatic and non-enzymatic antioxidants is the first line of defense against heavy metals. To trigger their root exudates and cell wall is the second and phytoremediation is the third line of defense (Ali & Gill, 2022). The secret of sunflower remarkable endurance to environmental variance lies in its significantly effective antioxidant system including both enzymatic antioxidants; ascorbate peroxidase, catalase and superoxide dismutase and non-enzymatic antioxidants; ascorbic acid, phenols, proline and glutathione (Abd El-Hameid & Sadak, 2020; Alishah *et al*., 2022). Conversely, with increasing challenges in agriculture and cultivation due to global climate changes, efforts are being made to improve its tolerance via various approaches including breeding, exogenous nutrient applications and soil enrichment (Hladni *et al*., 2022).

Neem cake, the defatted residue of *Azadirachta indica* seeds is used for its insecticidal and medicinal

potential however, its nutrient rich composition including protein, fats and fiber makes it an excellent fertilizer (Rahman, 2016). It also enriches soil, inhibits the growth of pathogenic bacteria and other soil pests, supplies macronutrients needed for all plant growth, and increases plant yield over time. It is also an excellent soil conditioner that decomposes naturally (Lokanadhan *et al*., 2012). As a soil amendment, it improves aeration, water holding capacity, organic carbon and pH (Lalnunpuia *et al*., 2018). In addition to the soil nourishment, it is reported to be a potent and low-cost bio-sorbent for metallic contaminants including lead, cadmium, copper and chromium (Govarthanan *et al*., 2019; Sireesha and Sreedhar, 2022). Salma and Hossain (2021) reported improvement in soil pH, N, P, S content and yield of spinach crop when neem cake was applied as soil amendment. Rahman *et al*., (2016) documented that neem seed cake and PGPR in co-application induced resistance in cotton plants against pathogen attack and environmental stress. Ali & Gill (2022) documented that many organic amendments helped to improve plant growth and strengthened their resistance to metal stresses.

Beneficial bacteria employ several defensive mechanisms to counteract the toxic effects of heavy metals, which include biosorption, effluxing, production of metal chelators such as siderophores or metallothioneins that are synthesized exopolysaccharides, extracellular and intracellular bioaccumulation (Mitra *et al*., 2021). Many researchers (Tiwari & Lata, 2018; Rahman *et al*., 2021; Bhat *et al*., 2023) documented that plant growth promoting rhizobacteria (PGPR) produces siderophores and enhance mineral solubilization thus augmenting the availability of macro and micronutrients. Among PGPR, *Bacillus* species are one of the most acknowledged microbes for their bioremediation potential for metals like Pb, Cd, Ni, Hg and Cr (Qadir *et al*., 2022). The phenomenon involves complex formation, precipitation and adsorption of metallic ions to cell surface and thus checks metal availability to plants and prevents their accumulation in tissues (Khanna *et al*., 2019). Alotaibi *et al*., (2021) noticed intracellular and extracellular metallic precipitates where resistant strains of *Bacillus subtilis* reduced hexavalent chromium to trivalent form via enzymatic reduction. To prevent contamination in both soil and water, one of the most convenient methods is to convert metal contaminants to the forms with lower solubility and mobility. Pb-tolerant *Bacillus* strains produce siderophores and exopolysaccharides for metal chelation and solubilize nutrients which improve growth of plants grown in contaminated soil (Najm-ul-Seher *et al*., 2020). Bio-agents aid phyto-hormones synthesis and enhance SOD, POD, APX, CAT and GR levels which results in improvement of overall growth and productivity of crops (Backer *et al*., 2018; Saleem *et al*., 2018). Therefore, Mitra *et al*., (2021) illustrated that Pb remediation technology using bacteria may appear as a potential advantageous alternative to conventional physical or chemical processes owing to specificity, suitability to apply in situ conditions and feasibility of upgrading via genetic engineering.

Underpinning these precedents, this study was conducted to investigate the efficiency of metal adsorbing *Bacillus subtilis* and neem cake in alleviation stress and improving physiological performance and nutrient availability of sunflower growing in Pb contaminated environment.

Material and Methods

Cultivation of *Bacillus subtilis*: The pure bacterial culture of *Bacillus subtilis* (BS- 21) was grown on nutrient agar media that was later used in the experiment (Fig. 1).

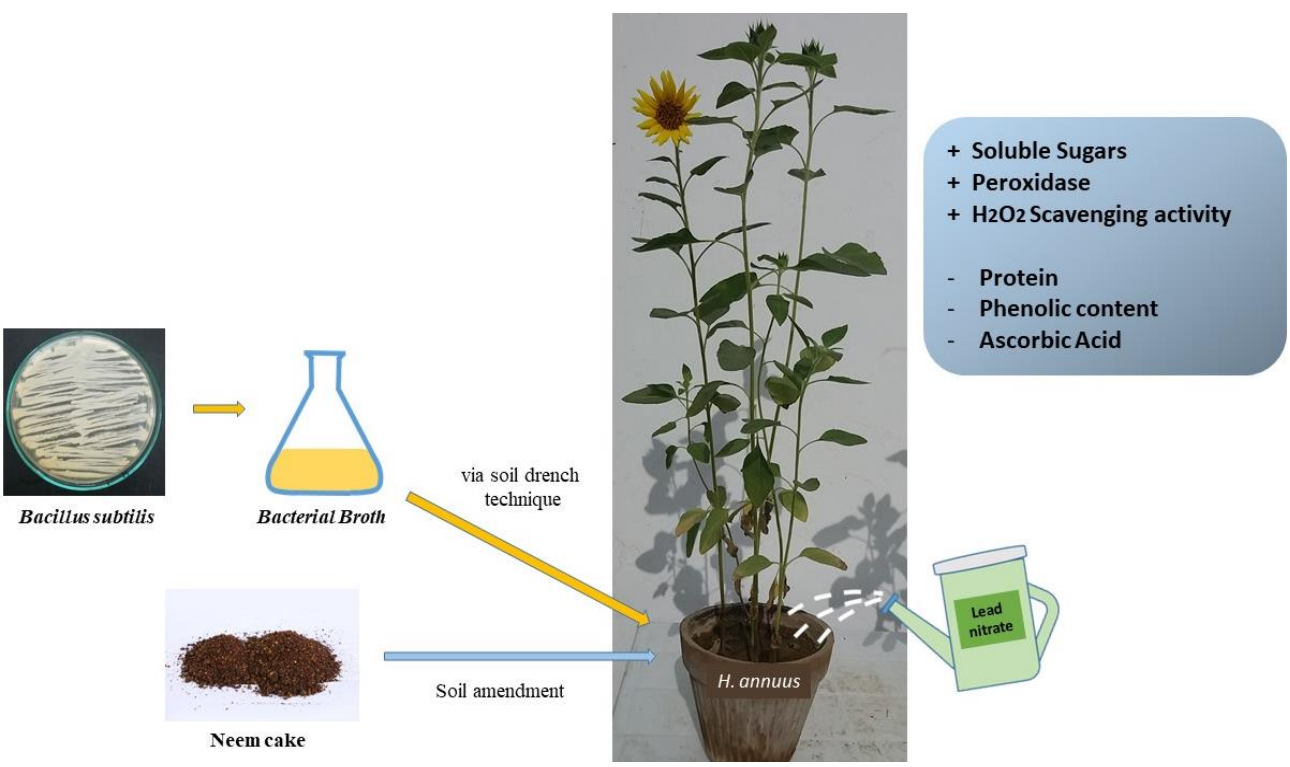

Fig. 1. Application of *B. subtilis*and neem cake amendment against lead stress in *Helianthus annuus.*

Lead (Pb) removal capacity assay: The removal and adsorption capacity of *Bacillus subtilis* was tested by the following method described by Huang *et al*., (2020) with slight modifications. The bacteria cultured in 50 mL LB broth medium (pH 7) at 37°C and when the absorbance value reached to 1.0 at 600 nm lead (10 mg) was added in broth (0.6 mM) as lead nitrate salt and was again incubated for 24 hours at 150 rpm in a rotary shaker. Following a 15-minute centrifugation at 2000 g of the culture, the pellet of bacterial colonies was discarded. Collected supernatants were analyzed using atomic absorption spectrophotometer for the Pb quantification after acid digestion as described by Marzan *et al*., (2017). The supernatants were mixed with double volume of concentrated $HNO_3$and heated on a hot plate until it was reduced to the initial volume. The removal of lead by *B. subtilis* was measured and computed with control sample by the formula:

$$\text{Pb removal efficiency (\%)} = C_0 - C_s / C_0 \times 100$$

where C_0 was the initial concentration of Pb^{2+} added to broth and C_s was the final concentration of Pb^{2+} detected in supernatant.

Experimental design and treatments: The potted experiments were conducted in the months of September-November in the Department of Botany, University of Karachi. Neem cake and sunflower (*Helianthus annuus* L.) seeds were purchased from the local market. Along with control, two levels of lead stress (0.2 mM and 0.5 mM), *B. subtilis* inoculation, neem cake amendment and their co-application, in total 12 treatments were laid out with three replications (Fig. 1; Table 1).

The soil used in the experiment was sandy loam with pH 7.2, EC 4 dS/m and organic matter 1.30%. For the inoculation of *B. subtilis* in soil (B), nutrient agar broth culture with cell density of approximately $8x10^8$cells/mL was used via soil drenching technique (25 mL of broth per pot) at the time of seed sowing in respective treatment pots. For neem cake amendment (N), 10 g of neem cake was mingled in the soil and incubated for a period of 10 days before seed spreading as well as being kept wet. This amended soil had pH 7.6 and EC 5.2 dS/m. Sunflower seeds were surface sterilized using 1% $NaClO_3$ for 2-3 minutes and washed with sterilized water and air dried. Ten seeds were sown per pot and after 7 days of sowing each pot was thinned to 4 seedlings. For lead stress, two levels of lead treatment 0.2mM (T1) and 0.5mM (T2) were prepared using $Pb(NO_3)_2$ salt. Two-week old seedlings were subjected to lead stress via irrigation, on alternate days during the first week after that they were treated daily till the 45^{th} day.

Morphological data collection: The plants were harvested 45 days after sowing. To demonstrate the growth parameters of fresh and dry shoot, root lengths (cm) were recorded by measuring tape and weights (g) were obtained in triplet by electric balance of all treatments (Somaddar *et al*., (2023). The dry weight of plants was recorded after drying in the oven for 48 hours at 80°C.

Biochemical analysis

Carbohydrates assessment: Carbohydrates were extracted by crushing 1 g leaves in 10 ml distilled water, centrifuged at 1300 g for 10 minutes. One mL leaf aliquot was mixed with (3 mL) Anthrone reagent (freshly prepared via mixing of 0.2 g antrone in 100 mL of H_2SO_4 Conc.) and was heated for 30 minutes using a water bath. According to Hamid *et al*., (2011), a spectrophotometer was used to read the cooled contents at 680 nm. The control was prepared with distilled water and freshly anthrone reagent. Glucose was used as the standard.

Protein determination: Proteins were extracted by crushing leaf material (1 g) in 10 mL of 0.1 M (pH 7) phosphate buffer and centrifuging (1300 g) for 10 minutes following Bradford method (1976). The optical density was measured at 595 nm after mixing 1 mL of cooled extract with 5 mL of Bradford test reagent. Standard BSA (bovine serum albumin) was employed for calibration curve and phosphate buffer with Bradford reagent was used as a blank.

Phenolic content determination: For the determination of total phenolic content, the method described by Rahman *et al*., (2017) was followed keeping Gallic acid as standard. Leaf extract (0.1 mL) was combined with 2% Na_2CO_3 (2 mL) and incubated for 2 minutes at room temperature. Afterwards (0.1 mL) Folin-Ciocalteau Phenol reagent diluted 50% was mixed with samples and placed the test tubes in dark (to avoid oxidation) for 30 minutes. For quantification of phenol the optical density was measured at 720 nm on a spectrophotometer.

Ascorbic acid determination: A technique described by Sofy *et al*., (2020) was used to extract and analyze ascorbic acid. 1 mL leaf water extract (10% w/v) and 2 mL of (2, 4 dinitrophenyl hydrazine 2% in concentrated H_2SO_4) was mixed up, afterwards 1 drop of thiourea (10%) was put in the sample tubes and heated in a water bath for 15 minutes. After chilling, 5 mL (80% H_2SO_4) was added, and a spectrophotometer read the mixture at 530 nm.

Table 1. Treatments applied in experiment and their abbreviations.

Treatments	Tap water irrigation (no stress)	0.2 mM Lead stress irrigation	0.5 mM lead stress irrigation
Control	T0	T1	T2
B. subtilis inoculation	B	BT1	BT2
Neem cake amendment	N	NT1	NT2
Neem cake and *B. subtilis* co-application	BN	BNT1	BNT2

Peroxidase activity determination: Leaf material (0.5 g) was crushed in a 10 mL potassium phosphate buffer (0.1 M, pH 6.5) centrifuged at 12000 g for 20 minutes. Leaf extract 0.1 mL was added in a test tube containing 3 mL of pyrogallol solution (0.05 M) and mixed well. Afterwards, 0.5 mL of H_2O_2 solution (1% prepared in 0.1 M potassium phosphate buffer) was mixed with the test solution and was recorded enzymatic activity by observing the change in absorbance at 430 nm after 30 seconds up to 3 minutes (Shaikh *et al*., 2022).

Percentage of H_2O_2 scavenging activity: H_2O_2 scavenging activity was determined by a method described by Ruch *et al*., (1989). Working hydrogen peroxide (H_2O_2) solution (40 mM was made in 0.1 M, 7.4 pH phosphate buffer) and 0.6 mL of leaf extract (10 mg/mL) were mixed, and the final volume was made up to 3 mL using phosphate buffer. The absorbance was recorded at 230 nm against blank (test tube without H_2O_2 solution). The percentage of H_2O_2's scavenging activity was estimated using the formula below:

$$H_2O_2 \text{ scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100$$

A_0 = Abs. of control
A_1 = Abs. of test sample

Statistical investigation: One-way ANOVA was used for statistical assessment. Duncan's multiple range test was applied as a follow-up to the ANOVA to compare the treatment means (CoStat version 6.4). At $p<0.05$, the mean values showed a significant difference by LSD. The figures were plotted using software SigmaPlot (version 12.5).

Results and Discussion

Lead (Pb) degradation capacity: Synthetic fertilizers and metal containing wastes dumped contaminants like heavy metals usually get accumulated in edible plant parts due to the application of metal containing wastes and synthetic fertilizers (Meenambigai *et al*., 2016). Several bacteria are reported to show resistance to hazardous metals like lead (Pb) and they have capability to degrade these metals (Marzan *et al*., 2017). In this study, PGPR *B. subtilis* was tested for metal degradation capability and was found to have 99.2% lead degradation capacity when grown in 0.6 mM lead-containing broth (Table 2). Syed and Chinthala (2015) reported the Pb remediation capability of *B. subtilis* subjected to 1000 ppm to be 86% which may help in the reduction of the bioavailability of metallic ions in soil water. The microbes immobilize metals by intracellular as well as extracellular precipitation or by transforming them into inconsumable forms as Govarthanan *et al*., (2013) reported that *Bacillus* sp. KK1 isolated from mine soil showed the efficacy of converting Pb $(NO_3)_2$ into water insoluble forms; PbS and $PbSiO_3$ caused calcite precipitation. Similarly, neem cake possesses excellent metal adsorptive capability and is therefore known for effective removal of metallic ions like Cr, Cu, Pb and Ni from soil as well as aqueous medium (Govarthanan *et al*., 2019; Sireesha & Sreedhar, 2022). The sorption of Pb by bioadsorbents is dose and time dependent which is needed for the binding of metals to functional groups on adsorbent surface (Gautam *et al*., 2020; Ighalo *et al*., 2020).

Table 2. Leaddegradation capability of *B. subtilis*.

Bacterial strain	Pb added to broth(C_0)	Pbin culture (C_s)	Removal efficiency %
B. subtilis	10 mg	0.078 mg	99.2%

Growth assessments: Neem cake amended soil is reported to have improved fertility, mineral content, and plant growth (Eifediyi *et al*., 2017). Based on these reports, a study was conducted to evaluate the individual and combined effect of *B. subtilis* and neem cake in alleviation of lead stress in sunflower and it was evident that these treatments helped *Helianthus annuus* in combating strain and performing physiologically well under stress. The amendment of neem cake and *B. subtilis* decreased adverse effects of lead on sunflower growth (Fig. 2). Plants growing under 0.5mM lead stress (T2) were found to have significantly reduced ($p<0.05$) dry weight and fresh weight while both *B. subtilis* (B) and neem cake amendment (N) improved weight and lengths of shoot and root in combined treatment (BN, BNT1 and BNT2) than individual application (Fig. 2).

Carbohydrate contents: Neem cake as organic amendment improves pH and organic content in the soil and adds nutrients which help plant to grow under lead stress and recover up nutritional deficiencies and improve chlorophyll content, photosynthesis and biomass (Figlioli *et al*., 2019). *B. subtilis*is designated as plant growth promoters for their ability of nutrient solubilization such as Zn and P (Najm-ul-Seher*et al*., 2020), produce proteases, amylase and phytohormones under control as well as stressed conditions (Bashir *et al*., 2022). Shah *et al*., (2021) reported that application of *B. subtilis* improved IAA content in *S. melongena* grown in Pb contaminated soil. Both T1 and T2 lead treatments decreased plant growth. The carbohydrate contents were decreased in T1 (43.67 µg/ml) and T2 (35.67 µg/ml) compared to T0 (51.33 µg/ml) and was significantly ($p<0.05$) improved by *B. subtilis*in BT1 (47.33 µg/ml) and BT2 (47.33 µg/ml). Similar trend was seen in neem cake application in NT1 (47.66 µg/ml) and NT2 (55.32 µg/ml) while co-application showed better results than alone treatments, BNT1 (54.34 µg/ml) and BNT2 (59.33 µg/ml) (Fig. 3). Similar results were reported in *B. subtilis* treated groundnut by Mathivanan *et al*., (2018) and in sugar cane by Fonseca *et al*., (2022).

Protein contents: The significantly ($p<0.05$) improved protein content was noted in T1 (87.33 µg/g) and T2 (120. 66 µg/g) compared to T0 which was 40.33 µg/g. The elevated levels of proteins in stressed plants were decreased in BT1 (66.65 µg/g), BT2 (103.33 µg/g), NT1 (62.67 µg/g) and NT2 (92 µg/g). Combined treatments showed even lower protein levels, 63.66 µg/g in BNT1 and 68.33 µg/g in BNT2 (Fig. 3). According to Wong *et al*., (2017), the increase in protein content in plants under lead toxicity is ascribed to the synthesis of metal binding proteins particularly two cysteine-rich peptides i.e., metallothioneins and phytochelatins, glutathione and ubiquitin conjugated proteins which help in heavy metals detoxification. While in BNT1 and BNT2 the decreased levels of protein are a clear indication of stress alleviation where the plant no longer produces and needs stress proteins.

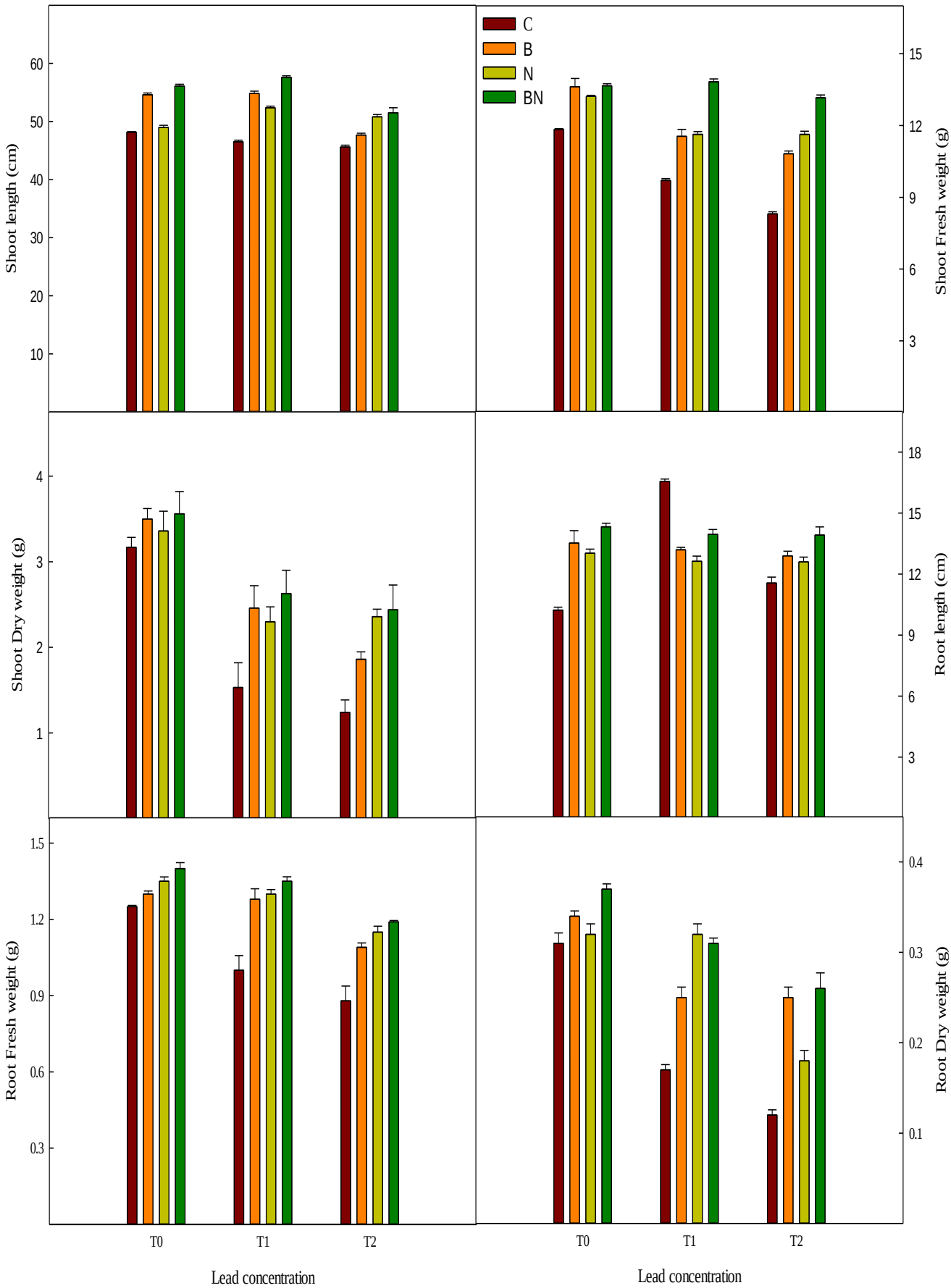

Fig. 2. Growth parameters of *Helianthus annuus*inoculated with*B. subtilis* and neem cake amendment under Pb stress. (T0= water control, T1= 0.2 mM lead, T2= 0.5 mM lead, B= *B. subtilis*, N= neem cake, BN= *B. subtilis* with neem cake). The data is presented as mean (n=3) and error bars show standard error.

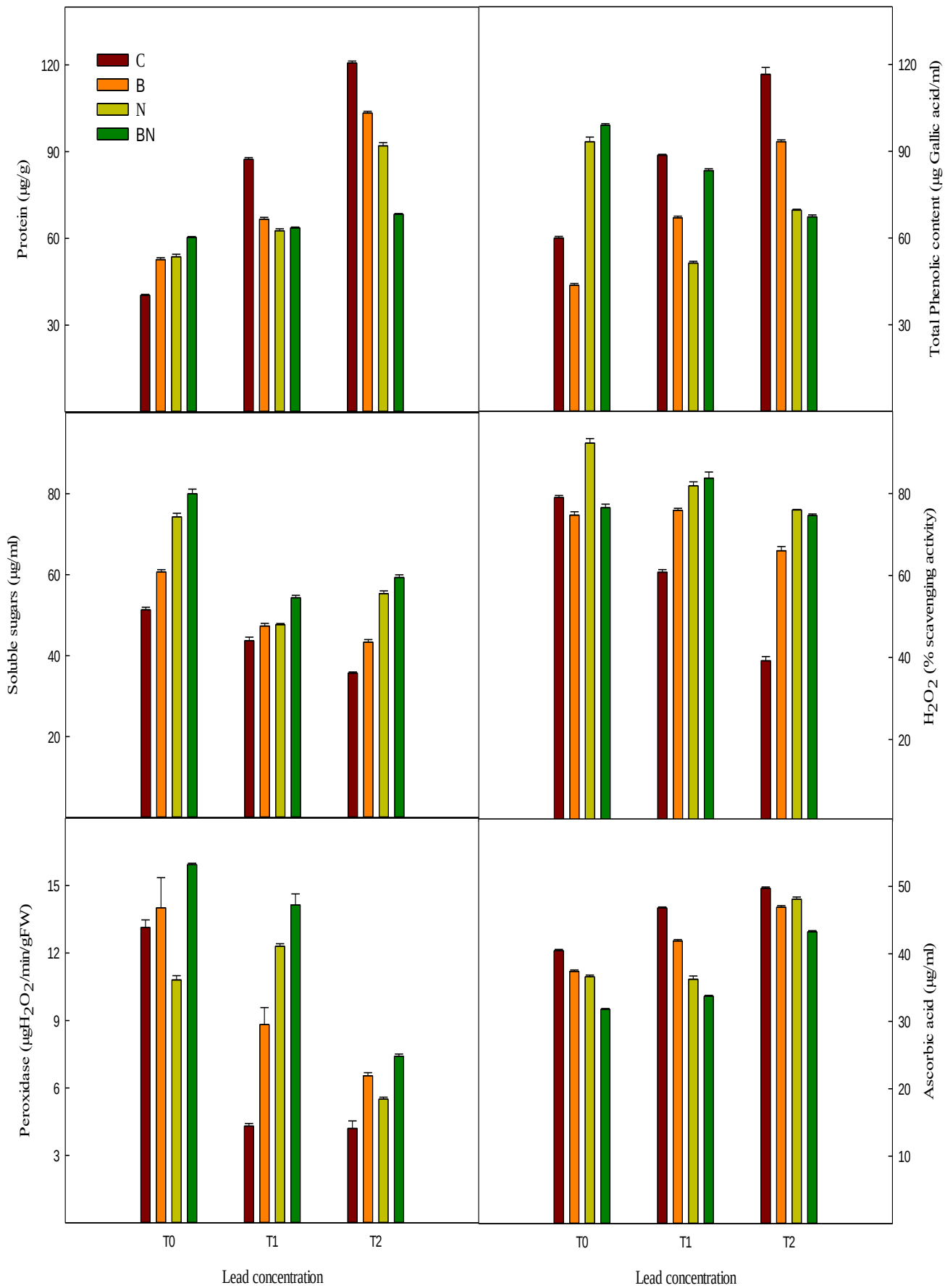

Fig. 3. Biochemical parameters of *Helianthus annuus*inoculated with *B. subtilis* and neem cake amendment under lead stress. (T0= water control, T1= 0.2 mM lead, T2= 0.5 mM lead, B= *B. subtilis*, N= neem cake, BN= *B. subtilis* with neem cake). The data is presented as mean (n=3) and error bars show standard error.

Phenolic content: The results regarding antioxidant activity in both Pb levels T1 and T2 showed elevated phenols levels than control (T0). Several secondary metabolites are known to induce defensive activities in plants by scavenging free radicals and chelation of metallic ions which is why stressed plants may accumulate higher phenolic content (Do Prado *et al*., 2022). The phenolic content in T0 was 60 µg gallic acid/ml and was increased in stressed plants: T1 (88.65 µg gallic acid/ml) and T2 (116.65 µg gallic acid/ml). Both *B. subtilis* and neem cake lowered phenolic contents under stress. The NT1 showed lowest levels of total phenolic content (51.32 µg gallic acid/ml) under 0.2 mM lead stress as compared to BT1 (67 µg gallic acid/ml) and BNT1 (83.33 µg gallic acid/ml). While under 0.5 mM lead stress co-application of neem cake and *B. subtilis* (BNT2) was found more effective than their single applications as it significantly (p<0.05) decreased the total phenolic content (67.34 µg gallic acid/ml) (Fig. 3). Similar results were also reported by Bashir *et al*., (2022) in *B. subtilis* inoculated plants under Cd stress. Azad *et al*., (2011) stated that the lowered phenolic content in PGPR inoculated plants as well as the ones growing in neem cake amended soil was a clear indication that plants had turned on their systemic resistance pathways to cope with the stress. Also, polyphenols due to their ROS scavenging capability inhibit lipid peroxidation and are thus responsible for membranous stability (Chiappero *et al*., 2019).

Ascorbic acid: Ascorbic acid is a non-enzymatic antioxidant which reduces H_2O_2 and scavenges free radicals formed because of oxidative stress (Noreen *et al*., 2020). The accumulation of ascorbic acid in tissues may indicate the innate response of plants against stress (Akram *et al*., 2017). In this study, ascorbic acid levels were increased in T1 (46.8 µg/ml) and T2 (49.7 µg/ml) compared to control T0 (40.46 µg/ml). Similarly, Cai *et al*., (2022) also noted elevated ascorbic acid levels in Pb $(NO_3)_2$ treated turf grass. Both *B. subtilis* and neem cake decreased ascorbic acid contents in individual as well as co-application in stressed plants. The lowest ascorbic acid levels were recorded in BN (31.7 µg/ml) followed by BNT1 (33.7 µg/ml), NT1 (36.2 µg/ml), N (36.6 µg/ml), B (37.4 µg/ml), BT1 (41.9 µg/ml), BNT2 (43.2 µg/ml), BT1 (46.9 µg/ml) and NT2 (48.1 µg/ml).

Peroxidase scavenging activity: Peroxidase (POD) activity was greatly reduced in T1 (4.3 µgH_2O_2/min/gFW) and T2 (4.2 µgH_2O_2/min/gFW) as compared to T0 (13.13 µgH_2O_2/min/gFW). Decreased antioxidant enzyme activity was previously reported by Ashraf *et al*., (2017) as well where POD, SOD, APX and CAT activity was decreased with increasing concentration of lead. Highest POD levels were found in BN (15.9 µgH_2O_2/min/gFW) followed by BNT1 (14.13 µgH_2O_2/min/gFW) and B (14.01 µgH_2O_2/min/gFW) while lowest POD levels in stressed plants were recorded in NT2 (5.5 µgH_2O_2/min/gFW). Similar results were reported by Shah *et al.* (2021) where *B. subtilis* enhanced peroxidase (POD) levels along with other antioxidant enzymes including CAT and SOD in *S. melongena* subjected to lead stress. As a stress tolerance strategy organisms limit the ROS generation by enhancing their antioxidant activities (Bashir *et al*., 2022). In this study, no evidence was found which explains the exact mechanism by which neem cake as a soil amendment regulates plant's antioxidant activity but according to the phytochemical analysis of neem seed cake done by Vasudha Udupa *et al*., (2021), it contains alkaloids, flavonoids, phenols and tannins which may add to soil fertility and help plants thrive in stressed environment.

H_2O_2 scavenging activity: H_2O_2 % scavenging activity was reduced in T1 (60.8%) and T2 (39.1%) compared to control T0 (79.02%). The reduced antioxidant activity in stressed plants causes accumulation of H_2O_2 and MDA content in tissues indicating oxidative damage (Rabiya *et al.*, 2022). The highest H_2O_2 % scavenging activity was recorded in N (92.2%) followed by BNT1 (83.7%), NT1 (81.1%), BN (76.5%), NT2 (76%), BT1 (75.9%), B (74.7%), BNT2 (74.6%) and B (66.03%). Thus, both *B. subtilis* and neem cake improved H_2O_2 % scavenging activity in individuals as well as co-application in stressed plants.

Conclusion

The current study advocates that neem cake with *B. subtilis* inoculation may be an excellent treatment to make use of contaminated soil for agricultural practices as they both improve plant growth in Pb contaminated environments. The improved antioxidant activity exhibits the beneficial interaction of both treatments i.e. neem cake and *B. subtilis* in lead subjected sunflower. Future developments are anticipated to reduce the danger associated with the safe use of polluted soils through the application of beneficial microorganisms and soil treatment techniques.

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