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Phytoremediation of lead in sunflower (Helianthus annuus L.) by growth-promoting rhizobacterium and neem cake

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Abstract

Lead causes toxicity and affects plant growth by disturbing photosynthesis and the functions of enzymes. Its removal from the soil is a challenging issue. The present investigation aims to evaluate the effectiveness of organic amendment in soil with neem cake and a plant growth-promoting rhizobacterium (PGPR), *Pseudomonas aeruginosa*, to reduce the effects of lead toxicity in sunflower when cultivated with and without neem cake amended soil. The experiment was performed in earthen pots (10 cm diam) containing 1 kg sandy loam soil, five sunflower seeds per pot, induced with 0.2 mM and 0.5 mM lead at 100 mL. After 30 days, the combination of organic amendment with PGPR improved the growth and the physiological performance of sunflower plants subjected to lead stress. The reduction % of lead was reported as 99% by *P. aeruginosa* at a specific concentration of 200 ppm on an Atomic Absorption Spectrophotometer. The plants treated with PGPR in amended soil showed healthier roots and shoots and improved carbohydrates. The PGPR-treated plants demonstrated better scavenging of free radicals, with improved H_2O_2 scavenging activity and peroxidase (POD) activity. Neem cake and *P. aeruginosa* individually and in combination significantly enhanced sugar contents and POD activity while lowering stress-induced elevated levels of phenols and ascorbic acid. The combination of neem cake and *P. aeruginosa* could be a promising solution for growing sunflower plants in lead-contaminated soil.

Highlights

1. Investigated the efficacy of neem cake and *Pseudomonas aeruginosa* in reducing lead toxicity in sunflower
2. Neem cake along with rhizobacterium improves seedling growth in lead-contaminated soil.
3. They enhanced the activities of peroxidase and H_2O_2 scavenging activity and soluble sugars
4. They reduced ascorbic acid, total phenolic content, and proteins in neem cake and bacterial inoculation

Introduction

Lead, being one of the most noxious soil contaminants, is generally found in the industrial areas of Pakistan (Waseem et al. 2014). The wastewater irrigated vegetables have a higher lead concentration in their above-ground parts (Iqbal et al. 2020). Uncontaminated soil contains lead below 50 mg kg-1, while the contaminated soil contains 400–800 mg/kg. The residential area of Karachi City demonstrated 1000 mg/kg in their tarry deposits and soils (Farid et al. 2017, Farid et al. 2015, Shams and Beg 2000). Lead inhibits vital functions, viz., nitrogen metabolism to reduce plant growth (Naz et al. 2018). It damages chloroplast ultrastructure and impedes plastoquinone, electron transport system, and calvin cycle to decrease photosynthesis (Shen et al. 2016). Lead gives rise to oxidative stress by generating reactive oxygen species (ROS) such as hydroxyl, superoxide, and hydrogen peroxide (Sharma and Agrawal 2005). Some plants possess few physiological mechanisms which help them thrive in the contaminated environment.

Compared to synthetic growth regulators, organic amendment in the soil is proved a more cost-effective and environment-friendly method to enhance plant growth in a polluted environment. Neem cake provides protein, fiber, ash, and nutrients like N, P, K (Garuba and Oyinlola 2014), and neem cake amended soil increases organic acids, aeration, and water holding capacity. It is an effective and economical sorbent to remove metals like Cu, Cd, Pb, and Cr from water (Hlihor and Gavrilescu 2009). Neem cake combined with *P. aeuginosa* improves the growth of cotton plants and induced resistance against pathogens and environmental stress (Rahman et al. 2016). The application of metal tolerant beneficial bacteria is another strategy for enhancement of plant growth as they increase nutrient availability by augmenting solubility of minerals like phosphorus, potassium, iron, zinc, and nitrogen in soil and water (Dimkpa et al. 2009). Plant growth-promoting bacteria help biosynthesis for growth regulators such as auxin, cytokinin, and gibberellins. They support plants to reduce the deleterious effects of abiotic stress (Singh et al. 2015) by promoting the activity of POD and SOD antioxidants (Zainab et al. 2021). *Pseudomonas* has the potential to change lead into non-toxic forms and adsorb metals to their cell surface. The characteristic helps in the bioremediation of heavy metals like Hg, Pb, Cu, Cd, Cr, Cd, Ni, and Fe (Tiwari and Lata 2018, Imam et al. 2016, Oaikhena et al. 2016, Akhtar et al. 2013). PGPR augments phytoremediation by improving lead bioavailability in soil and its uptake by the plant (Pietrini et al. 2021, He et al. 2020). Kalita and Joshi (2017) isolated distinct species of exopolysaccharide producing lead tolerating *Pseudomonas.* Its strains isolated from the sites having high lead concentration induce stress tolerance in rice seedlings. Sunflower showed better photosynthetic performance, antioxidant activity, and lead uptake. (Nath et al. 2014, Saleem et al. 2018).

Sunflower (*Helianthus annuus* L.), an important oilseed crop grown during the spring and autumn seasons, is moderately tolerant to environmental stresses like drought and salinity (Pal 2011). The enhanced activity of enzymatic and non-enzymatic antioxidants helps the plant cope with oxidative stress (Singh et al. 2004). High lead concentrations decrease the growth and physiological functions in the seedlings, while its translocation and accumulation in tissues increase (Alaboudi et al. 2018). Due to this ability of hyper-accumulating metals, sunflower is one of the potential candidates for phytoremediation (Al-Jobori et al. 2019). Earlier studies suggest the potential of organic amendments and beneficial microbes to minimize heavy metal stress in plants. The present study investigates the efficacy of neem cake and *P. aeruginosa*, individually and combined, to reduce lead toxicity and improve physiological functions in sunflower plants under lead stress.

Materials And Methods

Bacterial culture collection

The pure culture of bacterial strain *Pseudomonas aeruginosa* (ABPL-251), obtained from the Agricultural Biotechnology and Phytopathology Laboratory, Department of Botany, University of Karachi, was used later in the experiment. The bacteria were grown using King's B broth media. This broth was incubated for ten days and had attained a cell density of approx. $8x10^8$cells/mL.

Experimental layout:

A screen house, made at the Department of Botany, the University of Karachi, experimented in the clay pots of 10 cm diameter having a capacity of 1 kg soil with pH = 7.2 and EC= 4mS/m in September-November. Before sowing, the pH of amended soil was 7.6, and electrical conductivity was 5.2mS/m. Neemex powder (@1%) was mixed in the soil seven to ten days before sowing and was kept moist. A local market provided the sunflower seeds (*Helianthus annuus* L.) that were held on for 2-3 minutes in 1% sodium hypochlorite solution for surface sterilization and afterward washed thrice with distilled water and then air-dried. Ten seeds in each pot were sown, drenched with 25 mL broth of *P. aeruginosa* to respective treatment banks, and 25 ml of distilled water for control. After a week of germination, four seedlings per pot were treated with different concentrations (0.2mM and 0.5mM) of lead by using lead nitrate salt [$Pb(NO_3)_2$]. When the seedlings attained four leaves stage (two weeks of sowing), lead treatment started using lead nitrate solutions via irrigation in the morning. They were treated on alternate days during the first week but later treated daily till the 45^{th} day. Each treatment was replicated four times for the study, and the treatments included were:

C= control

Ps= *P. aeruginosa* inoculation in soil

N= Neem cake amendment in soil

Ps+N= Neem cake amendment + *P. aeruginosa* inoculation

0.2 mM= 0.2 mM Pb stress

0.5 mM= 0.5 mM Pb stress

Ps+0.2 mM= *P. aeruginosa* inoculation + 0.2 mM Pb stress

Ps+0.5 mM= *P. aeruginosa* inoculation + 0.5 mM Pb stress

N+0.2 mM= Neem cake amendment + 0.2 mM Pb stress

N+0.5 mM= Neem cake amendment + 0.5 mM Pb stress

Ps+N +0.2 mM= Neem cake amendment + *P. aeruginosa* inoculation + 0.2 mM Pb stress

Ps+N +0.5 mM= Neem cake amendment + *P. aeruginosa* inoculation + 0.5 mM Pb stress

Morphological data

The replicated data of the fresh weight and root and shoot lengths of all treatments were noted to demonstrate their growth, then oven-dried at 80°C for 48 hours to obtain dry weights of plants.

Biochemical Analysis

Determination of Phytoremediation of Lead

The capacity of *Pseudomonas aeruginosa* to immobilize lead and reduce its bioavailability in a liquid medium was determined by culturing LB broth medium (50 mL), incubated for 60 minutes in a rotary shaker at 150 rpm (7.0 pH and 37°C) (Marzan et al. 2017). After achieving the optical density of 0.6 at 600 nm, 200 ppm Pb solution was added using [Pb $(NO_3)_2$] salt in a flask and then incubated for 24 hours. The culture was then centrifuged at 2000 g for 15 minutes to separate bacterial colonies and media. The pellet discarded and supernatants collected were mixed with double volume of concentrated HNO_3 to carry out acid digestion of the lead present in the supernatant. The mixtures were heated on a hot plate to reduce them to the initial volume. The lead concentration was analyzed using atomic absorption spectrophotometer. Phytoremediation of lead by *P. aeruginosa,* was quantified and compared with control by the formula

Phytoremediation (%) = (Lead in broth - Lead in culture) / (Lead added to broth) × 100

Estimation of carbohydrates

Anthrone reagent determined carbohydrates content (Hamid et al. 2011). Dried leaf samples were homogenized in ethanol ratio (1:10) and centrifuged at 1300 g for 10 minutes. The 1 mL supernatant with 3 mL of Anthrone reagent (prepared by mixing 0.2 g in 100 mL conc. H_2SO_4) was kept in a water bath for 30 minutes. The cooled contents were then read at 680 nm using a spectrophotometer. The distilled water and anthrone reagent made the control. A calibration curve of carbohydrate content (µg/mL) was prepared by glucose as a standard.

Determination of protein

The Bradford method (1976) determined the protein content. The leaf material was extracted using phosphate buffer (0.1 M with pH 7) and centrifuged at 1300 g for 10 minutes. For estimation, 1 mL chilled extract and 5 mL Bradford assay reagent were mixed, and the optical density was read at 595 nm, taking Bradford and phosphate buffer as blank. BSA (Bovine Serum Albumin) was used as a standard.

Total phenolic content

Gallic acid was used as a standard to estimate total phenolic content (Rahman et al. 2017). Leaf aliquots (0.1 mL) mixed with 2 mL 2% Na_2CO_3 were left at room temperature. After 2 minutes, 50% Folin-Ciocalteau Phenol reagent (0.1 mL) was added and then kept the samples in darkness for 30 minutes at 720 nm optical density.

Ascorbic acid

The ascorbic acid was extracted and analyzed as described protocol (Sofy et al. 2020). To estimate ascorbic acid, 1 ml leaf extract and 2 mL 2, 4 dinitrophenyl hydrazine (2% in concentrated H_2SO_4) were mixed. One drop of 10% thiourea (1 g in 10 mL distilled water) was prepared and kept in a water bath for 15 minutes. After chilling, 5 mL 80% H_2SO_4 was added and read at 530 nm by a spectrophotometer.

Peroxidase activity

Peroxidase activity was estimated as described by Reddy et al. (1995). 20% homogenate was prepared in 0.1 M phosphate buffer with 6.5 pH to extract the enzyme. 0.1 mL enzyme extract was made with 3 mL of 0.05 M pyrogallol solution to determine peroxidase activity. 0.5 mL of 1% H_2O_2 solution (made in 0.1 M potassium phosphate buffer) was added to the test solution, and the absorbance was read every 30 seconds up to 3 minutes at 430 nm.

Percentage of H_2O_2 scavenging activity

Leaf aliquot (10 mg/mL) and 0.6 mL of 40 mM H_2O_2 solution (prepared in 0.1 M phosphate buffer of 7.4 pH) were mixed, and phosphate buffer was applied to obtain the final volume of 3 mL to determine H_2O_2 scavenging activity (Ruch et al. 1989). A blank test tube was prepared without H_2O_2, and O.D was measured at 230 nm. The following formula calculated the percent scavenging activity of H_2O_2.

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

A_0 = Absorbance of control

A_1 = Absorbance of plant extract

Statistical analysis

For statistical analysis, one-way ANOVA means triplicated data, and standard errors were performed using the program COSTAT. The mean values are of significant difference by LSD at $p<0.05$.

Results And Discussion

The soil organic amendment (neem cake) and endophytic plant growth-promoting rhizobacterium, *P. aeruginosa*, individually and together, increased various growth parameters of the sunflower plants under control as well as lead-stress conditions. The application of neem cake and *P. aeruginosa* decreased the detrimental effects of lead on the growth parameters of the plants (Table 1). Both the dry and the fresh weights of the plants were significantly reduced ($p < 0.05$) when exposed to 0.5 mM lead stress. The plants demonstrated the highest shoot length in the neem-cake amended soil, with both leaded and lead-free conditions. However, the root length did not differ significantly. Neem cake and P. aeruginosa equally improved the fresh and dry weight of the plant ($p < 0.05$) when treated individually. Furthermore, they demonstrated a synergistic effect when treated in combination (Table 1). Warwate et al. (2017) reported

similar results in *P. aeruginosa* treated plants. They suggest that the increment in weight and shoot length of the plants may be due to the potential of *P. aeruginosa* to produce growth regulators within plants such as gibberellic acid, indole acetic acid, and salicylic acid, which improved the nutrients available in the soil. Many studies revealed neem cake amendment with and without *P. fluorescens* improved the overall growth of plants by enhancing soil fertility and chlorophyll content (Rizvi et al. 2012, Eifediyi et al. 2017). Neem cake adds organic matter, protein, and minerals like N, Ca, K, Mg, P, d, S in the soil (Lokanadhan et al. 2012), which may compensate for lead that causes a nutrient deficiency in plants leading to decreased chlorophyll content, photosynthetic activity and biomass. (Figlioli et al. 2019; De Abreu et al. 2016). The endophytic *P. aeruginosa* caused 99% phytoremediation of lead (Table 2). 200 ppm lead was added to the broth, and with centrifugal removal of the bacterial culture, lead concentration in the supernatant was 1.875 ppm, determined by Atomic absorption spectrophotometry. The endophytic *P. aeruginosa* reduced the lead concentration and its detrimental effects by synthesizing biosurfactants such as rhamnolipids (Kukla et al. 2014) that removed lead from groundwater up to 81% (Elhosary et al. 2019). Furthermore, neem cake adsorbs Cd, Pb, and Cu ions and decreases their availability in soil and water (Rao and Khan 2007, Abdelkrim et al. 2019).

Table 1
Evaluation of growth parameters of *Helianthus annuus* induced by PGPR inoculation and neem cake amendment under heavy metal stress of lead. (Control, Ps = *P. aeruginosa*, N = neem cake, Ps + N = *P. aeruginosa* with neem cake, 0.2 mM = 0.2 mM lead concentration, 0.5 mM = 0.5 mM lead concentration).

Treatments	Shoot length (cm)	Root length (cm)	Shoot Fresh weight (g)	Root Fresh weight (g)	Shoot Dry weight (g)	Root Dry weight (g)
Control	48.16 ± 0.09	10.23 ± 0.14	11.84 ± 0.02	1.25 ± 0.005	3.17 ± 0.11	0.31 ± 0.01
Ps	57.56 ± 0.23	14.03 ± 0.14	14.69 ± 0.17	1.28 ± 0.005	3.41 ± 0.11	0.35 ± 0.005
N	49.00 ± 0.35	13.03 ± 0.20	13.22 ± 0.04	1.35 ± 0.01	3.36 ± 0.23	0.32 ± 0.01
Ps + N	56.50 ± 0.25	14.36 ± 0.31	14.66 ± 0.08	1.41 ± 0.02	3.20 ± 0.10	0.35 ± 0.01
0.2 mM	46.50 ± 0.28	16.56 ± 0.11	9.70 ± 0.08	1.00 ± 0.05	1.53 ± 0.28	0.17 ± 0.005
Ps + 0.2 mM	56.53 ± 0.28	13.36 ± 0.14	12.07 ± 0.03	1.27 ± 0.01	2.53 ± 0.28	0.27 ± 0.01
N + 0.2 mM	52.36 ± 0.28	12.63 ± 0.25	11.63 ± 0.11	1.30 ± 0.01	2.30 ± 0.17	0.32 ± 0.01
Ps + N + 0.2 mM	54.50 ± 0.28	13.96 ± 0.18	13.41 ± 0.11	1.38 ± 0.01	2.86 ± 0.08	0.32 ± 0.011
0.5 mM	45.60 ± 0.32	11.56 ± 0.29	8.31 ± 0.08	0.88 ± 0.05	1.24 ± 0.14	0.12 ± 0.005
Ps + 0.5 mM	50.20 ± 0.36	12.76 ± 0.21	11.57 ± 0.21	1.16 ± 0.02	2.15 ± 0.09	0.20 ± 0.005
N + 0.5 mM	50.80 ± 0.41	12.60 ± 0.23	11.63 ± 0.13	1.15 ± 0.02	2.36 ± 0.08	0.18 ± 0.01
Ps + N + 0.5 mM	52.00 ± 0.57	13.77 ± 0.20	13.21 ± 0.10	1.23 ± 0.02	2.46 ± 0.23	0.27 ± 0.005
$LSD_{0.05}$	2.14	0.66	3.08	0.082	0.548	0.028

Table 2
Phytoremediation of Lead (Pb) by *P. aeruginosa.*

Bacterial strain	Pb added to broth (ppm)	Pb in culture (ppm)	Percentage %
P. aeruginosa	200	1.875	99

In the present study, a significant ($p < 0.05$) rise in soluble sugar was noticed in individual and combined treatments of *P. aeruginosa* and neem cake when compared to 0.2 mM and 0.5 mM lead stressed plants

with healthier plants (Fig. 1). Mathivanan et al. (2018) found enhanced production of sugars content and antioxidant status in groundnut by inoculation of PGPR. The neem cake amended soil, and *P. aeruginosa* improved protein content compared to healthy control. Both the lead concentrations (0.2 mM and 0.5 mM) significantly ($p < 0.05$) increased proteins and were the highest among all treatments and control (Fig. 1). The increase in protein content in lead-stress sunflower plants may be due to the activation of metallothionein genes and elevated level of glutathione or accumulation of ubiquitin conjugated proteins (Marzena et al. 2015). Neem cake and *P. aeruginosa* treatments under lead-stress declined proteins (Fig. 1), which indicates the alleviation of stress or suppression of stress proteins.

Phenols are secondary metabolites produced by plants and have defensive and antioxidant properties. In the present experiment, lead (0.2 mM and 0.5 mM) stressed plants demonstrated the highest levels of phenolic content compared to a healthy plant. According to Michalak (2006), heavy metal stress increases phenolic content in plants as they can chelate metals, scavenge ROS, and stabilize membranes. Thus, the higher levels of total phenolic content under lead stress indicate activation of systemic resistance in plants (Azad et al. 2011). The neem cake and *P. aeruginosa* decreased significantly the phenolic content in the plants grown under both lead concentrations ($p < 0.05$) (Fig. 1). Polyphenolic compounds and flavonoids from neem extracts divulged strong free radical scavenging and metal chelating properties and inhibition of lipid peroxidation (Ghimeray et al. 2009). This decrease in the phenol levels in bacterial and neem cake treatments indicates mitigation of the stress (Azad et al. 2011).

Ascorbic acid scavenges reactive oxygen species (ROS), protects against oxidative stress, and acts as an electron donor to reduce H_2O_2. The present study showed that both stress levels enhanced ascorbic acid in plants while the neem cake and *P. aeruginosa* treatment reduced it to 0.2 mM Pb stress. However, the ascorbic acid levels did not differ significantly at 0.5 mM concentration. Lead disturbs the electron transport chain and increases free radical production, which results in increased antioxidants like ascorbic acid and phenols. In this research, Pb stressed plants showed the lowest peroxidase (POD) and H_2O_2 scavenging activity while neem cake combined with *P. aeruginosa* significantly ($p < 0.05$) enhanced these antioxidants under lead (0.2 mM and 0.5 mM) stress (Fig. 1). Zainab et al. (2021) reported similar results in PGPR-inoculated *S. sesban*, which showed improved POD activity in response to heavy metal stress. Prolonged exposure to a higher concentration of metals causes a reduction in the biosynthesis and activity of antioxidants (Bielen et al. 2013) meanwhile frequent generation of ROS and scavenged via the production of enzymatic and non-enzymatic antioxidants is necessary to improve stress tolerance (Janmohammadi et al. 2013).

Conclusion

The present study concludes that neem cake amended soil together with plant growth-promoting rhizobacterium, *P. aeruginosa* improves seedling growth of sunflower in the lead-contaminated soil. The enhanced antioxidant activity, viz., peroxidase and H_2O_2 scavenging activity, and soluble sugars confirm the phytoremediation in sunflower seedlings, which implies the activation of resistance mechanisms in

the plant. The reduction in ascorbic acid, total phenolic content, and proteins in neem cake or bacterial inoculation compared to control plants suggests the phytoremediation of lead in the contaminated soil.

Declarations

Authors' contribution

Nawal Gul Shaikh designed and performed the experiment and wrote the original draft. **Afshan Rahman** supervised the overall study and helped in writing the manuscript. **Mehwish Sadiq** analysed the data and helped in performing the experiment. **Syed Ehteshamul-Haque** provided resources, technical assistance and laboratory facilities. **Zafar Iqbal Shams** reviewed, edited and validated the manuscript.

Data availability

The datasets generated during and/or analysed during the present study are available from the corresponding author on reasonable request.

Conflict of interest: On behalf of all authors, the corresponding author states no conflict of interest.

Ethical approval and consent to participate: Not applicable since humans and or animals are not involved in this study.

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Figures

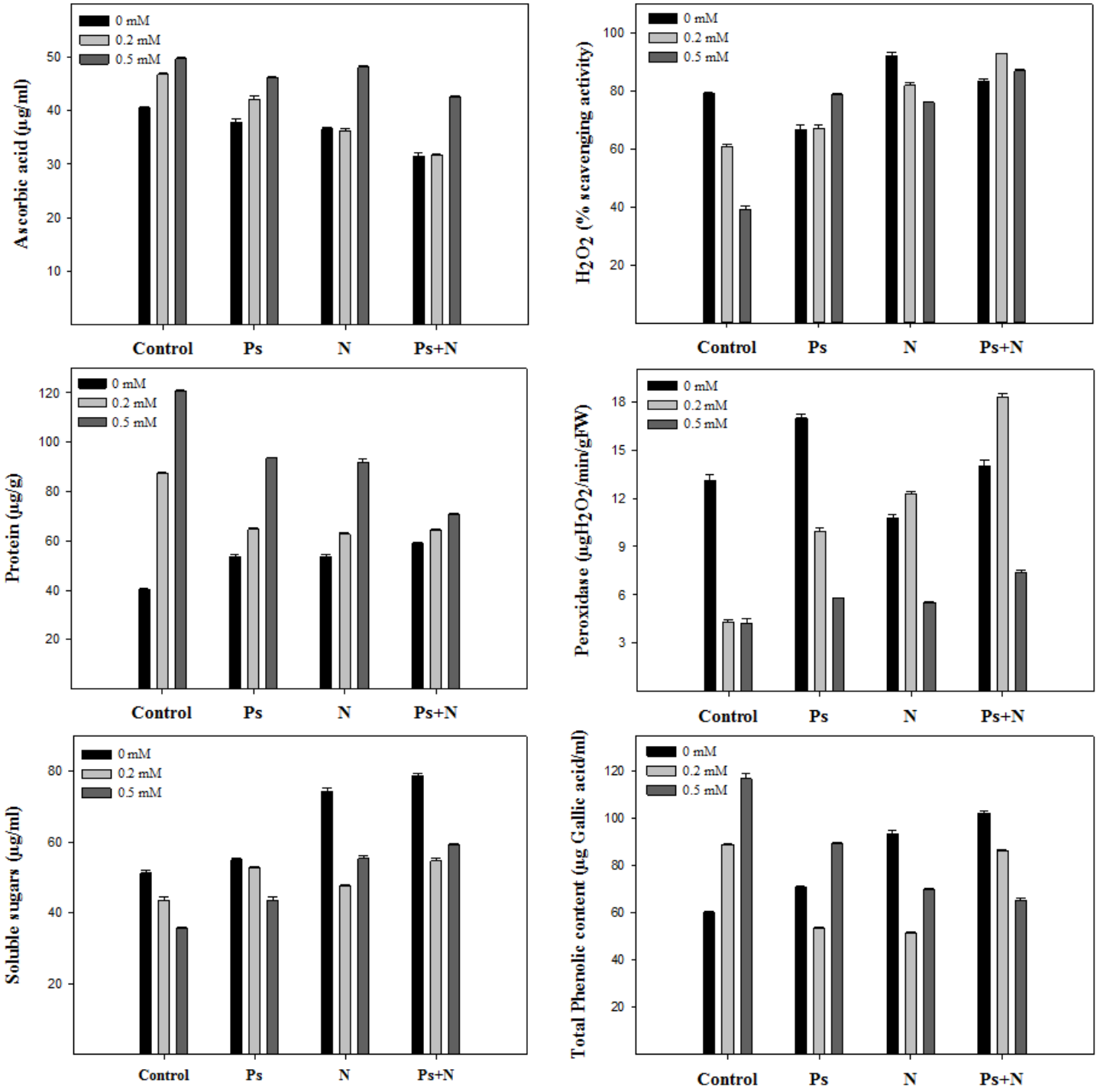

Figure 1

Evaluation of biochemical parameters of *Helianthus annuus* induced by PGPR inoculation and neem cake amendment under heavy metal stress of lead. (Control, Ps= *P.aeruginosa,* N= neem cake, N+Ps= neem cake with *P. aeruginosa* 0.2 mM= 0.2 mM lead concentration, 0.5 mM= 0.5 mM lead concentration).