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Research Article

Synergistic impacts of synthesized zero-valent iron nanoparticles (nZVI) on phytoremediation of lead (Pb) contaminated soil using *Tagetus erecta* L.

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Abstract
Rapid industrialization, particularly in developing countries, has increased lead (Pb) levels in soil and the environment. This study explores the impacts of nanoscale zero-valent iron (nZVI) on the phytoremediation of Pb using *Tagetus erecta* L. as a plant. A three-month pot experiment was tested with nZVI amendments of 100, 300, and 500 mg/kg, with a Pb metal concentration of 400 mg/kg. The nZVI was characterized with the FE-SEM (Field Emission Scanning Electron Microscopy), UV-visible spectroscopy, and Zeta Potential Analysis. The study assessed various plant growths, and physiological and biochemical parameters. Characterizations of nZVI revealed that synthesized nZVI were porous in structure, having good stability with a zeta potential of -11.8 mV. Results showed that 500 mg/kg nZVI amendments in Pb treated pots significantly ($P<0.05$) increased root length by 41%, shoot length by 22 %, relative water content (RWC) by 20%, and total chlorophyll by 25 % as compared to Pb only treatments. The findings also suggested that nZVI amendments resulted in decreased proline content to overcome the Pb stress. The Pb accumulation in *T. erecta* at 500 mg/kg nZVI amendments was 610 mg/kg in roots and 150 mg/kg in shoots. These were significantly ($P<0.05$) higher as compared to Pb only treatments. The Pearson correlation analysis revealed that plant growth parameters were negatively correlated with proline content. Hence, the integration of nZVI improved Pb phytoremediation efficiency and mitigated Pb-induced stress in *T. erecta*.

Keywords: Pb stress, Phytoremediation, *Tagetus* erecta, and Zero valent iron nanoparticles

INTRODUCTION

Due to the rapid expansion of industrialization alongside agricultural activities, soil contamination by heavy metals has emerged as a significant global environmental issue (Chaoua *et al*., 2019). Heavy metals exhibit a half-life exceeding twenty years, demonstrating their persistent nature (Kapoor and Singh, 2021). Elements with densities greater than 5 g/cm³ are classified as heavy metals and are recognized as pervasive pollutants (Arora *et al*., 2024). Lead (Pb) is a significant anthropogenic contaminant introduced into the environment since the onset of the Industrial Revolution. Consequently, Pb has become a primary focus of remediation efforts due to its extensive distribution, persistence, and toxicity, particularly concerning human health (Chen *et al*., 2024). Furthermore, the Agency for Toxic Substances and Disease Registry (ATSDR, 2005) ranks Pb as the top heavy metal pollutant and the second most hazardous.

A number of conventional, physical, and chemical techniques (such as burning, oxidation, and soil washing) have been developed to extract heavy metals from polluted soil (Zaid *et al*., 2020). However, because of their high costs and negative environmental effects, such as creating secondary waste, these technologies encounter numerous difficulties (Mokarram-Kashtiban *et al*., 2019). Phytoremediation, a different and potentially effective remediation method, uses plants in direct contact with the soil (Arora *et al*., 2024). Plants encounter various difficulties in environments contaminated with Pb, such as deficits in nutrients and minerals and disturbances to physiological and biological processes. Remediation times can be minimized and efficiency increased by combining phytoremediation with other technologies (Deb *et al*., 2020). Furthermore, mitigating heavy metal contamination in soil has proven to be beneficial when non-biological amendments based on nanotechnology are applied (Song *et al*., 2019). According to research, heavy metals in soil can be effectively immobilized by nanoparticles (Baragaño *et al*., 2020).

Numerous researchers have proposed using nanoscale zero-valent iron (nZVI) as a viable and promising method for remediating heavy metal-contaminated soils (Arora *et al*., 2024; Zand *et al*., 2021). The nZVI has garnered significant attention compared to other nanoparticles due to its non-toxic nature, low cost, abundance, and ease of production. Its nanoscale size enhances its reactivity towards various contaminants, particularly heavy metals and organic pollutants (Lefevre *et al*., 2016). The primary mechanism through which nZVI achieves remediation objectives at metal-contaminated sites is the immobilization of heavy metals in the soil. However, the effectiveness of nZVI in immobilizing metals depends on several factors, including soil properties, the dose of nZVI applied, and the presence of non-target pollutants. As a potential option for phytoremediation of Pb-contaminated soil, the ornamental plant "*Tagetes erecta* L." also known as Aztec Marigold, has been chosen for this investigation. It is a member of the Asteraceae family and is rarely grazed by herbivores. The plant exhibits a high propagation rate, a short life cycle, and rapid growth. (Madanan *et al*., 2021).

The study addresses gaps in phytoremediation research by investigating the combined use of nZVI and *T. erecta* for remediating Pb-contaminated soils. Unlike previous studies that examined either nZVI or plant-based remediation alone, this research explores their effects and the mechanisms of toxicity reduction. The objectives of the study were i) to evaluate the synergistic impacts of nZVI amendments on morphological, physiological, and biochemical parameters of *T. erecta* grown in Pb-contaminated soil and ii) to assess the impacts of nZVI on Pb phytoremediation potential of *T. erecta*.

MATERIALS AND METHODS

Characteristics of soil's physio-chemistry

The physicochemical properties of the soil, including electrical conductivity, pH, organic matter, soil texture, organic carbon, potassium, phosphorus, and total nitrogen were measured according to Dewis and Freitas (1970). Walkley and Black method was used to measure organic carbon (1934). Total nitrogen content was estimated with the help of kjeldahl method (1883), phosphorus with the Olsen P method (1982), and available potassium with the help of flame photometry. Stokes' law (Miller and Miller, 1987) method was used to determine soil texture.

Materials

All chemical compounds used in this experimental study were of analytical grade. Lead nitrate $Pb(NO_3)_2$ from Loba Chemie was utilized to prepare the stock solution of Pb. All experiments were performed in triplicate to ensure maximum precision.

Preparation of zero-valent iron nanoparticles

To synthesize nanoscale zero-valent iron (nZVI), 0.5406 g of ferric chloride hexahydrate ($FeCl_3.6H_2O$) was dissolved in a 4:1 ethanol-water mixture and stirred. A 0.1 M sodium borohydride ($NaBH_4$) solution was prepared by dissolving 0.37 g of NaBH4 in 100 ml of deionized water. The $NaBH_4$ solution was added dropwise to the solution of $FeCl_3$ with continuous stirring, resulting in the immediate formation of black solid particles. The remaining $NaBH_4$ powdered solution was added to accelerate the reduction reaction, and the mixture was stirred for 10-12 minutes. Black iron nanoparticles were collected using vacuum filtration with Whatman filter papers, washed 2-3 times with absolute ethanol to prevent oxidation, and dried in an oven at 323 K overnight. For storage, ethanol was added to prevent oxidation (Yuvakkumar *et al*., 2011).

Characterization of zero-valent iron nanoparticles

The FE-SEM was used to analyze the structure and size of the nZVI sample qualitatively. The zeta potential of nZVI was evaluated using a Zeta sizer (Malvern Nano ZS). This parameter indicates the degree of repulsion between charged particles in dispersion. UV–visible spectrophotometer was used to examine the elemental as well as structural properties of nZVI in the wavelength of 190 to 600 nm.

Pot experimental setup

A completely randomized pot experiment of one-factor factorial design in triplicate was conducted in a natural environment at MDU (Maharshi Dayanand University) in Rohtak City of Haryana, India. The sample soil was sieved through a 2 mm sieve to remove coarse materials and air-dried at 24 °C for one week, and 4 kg of the sieved soil was placed in polythene-lined pots. The soil in the pots was artificially contaminated with $Pb(NO_3)_2$ to achieve Pb concentration of 400 mg/kg by giving Pb solution of 1 liter. The soil was incubated at 70% field capacity for 2-3 weeks. The nZVI was added after incubation at 100, 300, and 500 mg/kg concentrations and thoroughly mixed. Various treatments given to pots were control treatment (without any contamination), T1 (400 mg/kg Pb only), T2 (400 mg/kg Pb + 100 mg/kg nZVI), T3 (400 mg/kg Pb + 300 mg/kg nZVI), and T4 (400 mg/kg Pb + 500 mg/kg nZVI). *Tagetus erecta* seeds were obtained from Haryana Agriculture University, Hisar, germinated in a 5 x 5 m field plot, and seedlings of identical size were transplanted into each pot. All pots were regularly watered using tap water to maintain field capacity and the total duration of experiments was three months.

Analysis of various plant parameters

In this study, morphological, physiological, and biochemical parameters were comprehensively analyzed to assess the effects of Pb and nZVI on plant samples. Parameters measured included shoot and root length (cm), relative water content (%), biomass (gm), total chlorophyll content content (mg/gFW), total protein content (mg/gFW), and proline content (mg/gFW). Specifically, the chlorophyll content of *T. erecta* was quantified using UV-visible spectrophotometry by measuring absorbance at wavelengths of 480, 645, and 663 nm, with 85% acetone as the blank reference (Arnon *et al*., 1949). The total protein content was determined using the Lowry method (1951), employing a calibration curve created with bovine serum albumin (BSA). Proline content was estimated by constructing a calibration curve with L-proline and measuring absorbance at 520 nm (Bates, 1973).

Analysis of Pb in plant

Fresh weights of plants were recorded after cleaning with running tap water. Plant samples were divided into roots and shoots and oven-dried for 48 hours at 60°C to achieve a constant weight. The dried samples were wet-digested with a mixture of nitric acid and perchloric acid in a 3:1 (v/v) ratio to measure Pb content using a microwave digester. Accumulation of Pb^{2+} (mg/kg) was examined using an AAS (Atomic Absorption Spectrophotometer) (Wu *et al*., 1997).

Statistical analysis

The mean of various treatments given to plants was assessed, and at 0.5% statistical level, Tukey's test was utilized to analyze variance (ANOVA) and the Dunnett test for the control. Different letters represent significant differences (P<0.05) between various treatments.

RESULTS AND DISCUSSION

Characteristics of soil's physio-chemistry

The findings showed that the soil used in this experiment had a sandy texture and was neutral in pH (6.97). Its low electrical conductivity of 1.21 ds/m shows the soil's non-saline nature. The sample soil had high levels of nitrogen (76.2 mg/kg) and potassium (53.4 mg/kg), but low levels of phosphorus (0.5 mg/kg). It had low levels of organic carbon (0.88%) and organic matter (1.96%).

Characterization of synthesized nZVI

Fig. 1a represents the SEM image of the nZVI. The nanoparticles have sizes ranging within 100 nm. The image highlights that the iron nanoparticles are spherical in shape and had a porous morphology, which may enhance their reactivity and effectiveness in environmental remediation. The primary mechanism for the aggregation of these iron nanoparticles is magnetic dipole-dipole interactions, which are impacted by the large surface area of each individual particle (Zhang *et al*., 2020). Nanoparticles' surface charge can affect their stability, aggregation, and interactions with other surfaces and particles. The zeta potential of the particles can show this charge. Zeta potential of nZVI is shown in Fig. 1b and was found to be -11.8 mV, showing good stability of the synthesized nZVI (Zeledón-Toruño *et al*., 2007). The excitation of surface plasmon vibrations inside the nZVI sample solution was manifested through a specific absorption peak found at wavelengths of 236 nm, as demonstrated by the UV-visible spectra of the nZVI particle (Fig. 1c). This absorption peak indicates that nZVI particles were successfully produced because it closely matched the UV–visible spectra linked to metallic iron (Ansari *et al*., 2021).

Impact on various plant parameters

Exposure of plants to Pb causes substantial metabolic damage, leading to plant death. This study evaluated the toxicity of Pb and the effects of nZVI in phytoremediation by measuring various plant parameters (morphological, physiological and biochemical parameters).

Root and shoot length

Fig. 2a reveals that root length in control plants was 25.76±1.8 cm. While Pb contamination of 400 mg/kg, significantly reduced root length to 12±33 cm. To test the efficacy of nZVI, soil was also amended with 100, 300, and 500 mg/kg nZVI in addition to Pb. Results showed that nZVI application significantly increased root length (P<0.05). The highest root length was observed in 500 mg/kg nZVI with an increment of 43% as compared to Pb only treatment. A similar trend was reported in the case of shoot length also (Fig. 2b). The nZVI amendment in Pb-treated soil significantly enhanced shoot length (P<0.05) by 19% as compared to Pb-only pots in the current study. It was due to the fact that the nZVI converted Pb2+ ions into less bioavaila-

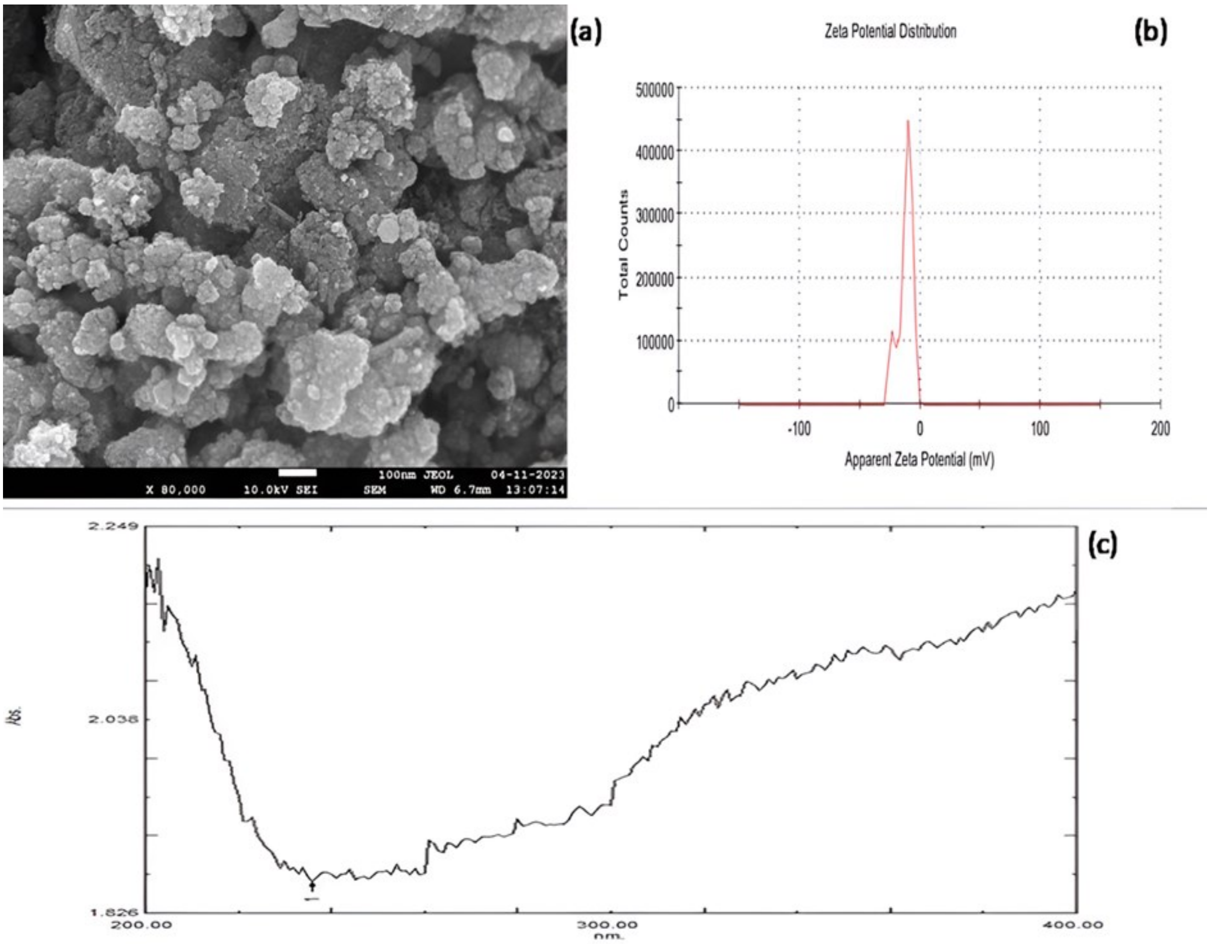

Fig. 1. *(a) FE-SEM image, (b) Zeta potential, and (c) UV-visible spectra of nZVI*

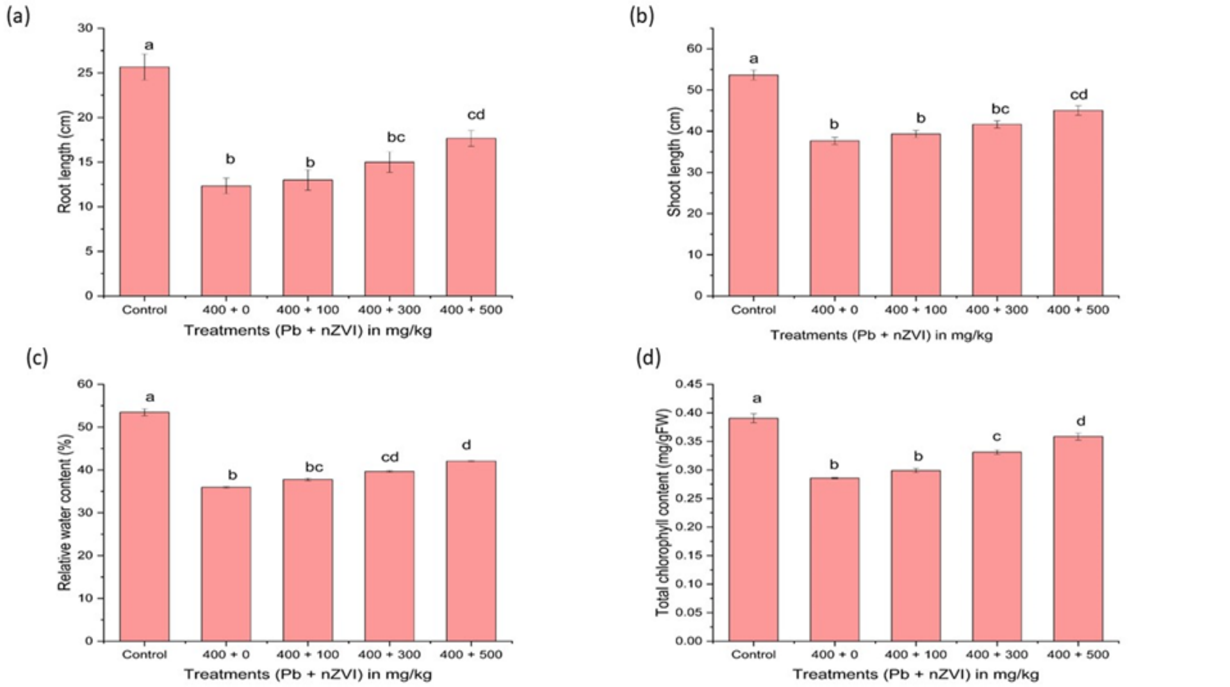

Fig. 2. *Impact of nZVI amendments on (a) root length, (b) shoot length, (c) relative water content, and (d) total chlorophyll content (different letters represent significant differences (P<0.05) between various treatments)*

ble forms through adsorption, co-precipitation, and redox reactions due to its large surface area and high reactivity (Xu *et al*., 2024). This interaction reduces Pb's mobility and bioavailability in soil, mitigating its toxic effects on plants and enhancing growth conditions. The nZVI also increases essential nutrient availability by reducing soil acidity and promoting beneficial microbial activity. The iron released during nZVI oxidation is a micronutrient vital for various plant physiological processes. The hydroxyl radicals produced can also improve soil structure loosen cell walls, and promote plant growth (Irshad *et al*., 2020).

Relative water content (RWC)

RWC is a key parameter for assessing the stress effects of heavy metals on plants. Lead significantly impairs plant hydration. In this study, Pb stress reduced RWC by 32% as compared to the control (Fig. 2c). However, adding nZVI to Pb-treated pots significantly ($P<0.05$) increased RWC, with higher nZVI doses correlating with greater water content. The highest RWC was 42.05±0.13% in 500 mg/kg nZVI amendments. Iron-based nanoparticles alleviate oxidative stress, enhancing plant water content. The oxidation of nZVI produces iron hydroxides, which improve soil structure by promoting aggregation. This improved structure aids in water retention and reduces soil compaction, facilitating efficient water uptake by roots. Enhanced soil aeration and porosity allow easier water access for roots, increasing plant RWC (Huang *et al*., 2021). The nZVI also supports root growth and development by providing essential micronutrients and improving soil conditions. Additionally, nZVI affects plant hormone levels, particularly those involved in stress responses and water regulation, such as abscisic acid (Alazaiza *et al*., 2022).

Total chlorophyll content

The content of total chlorophyll serves as an indicator of photosynthetic function and overall plant health in phytoremediation studies. In the control group, total chlorophyll content was 0.39±0.008 mg/gFW. However, exposure to Pb-only stress significantly ($P<0.05$) reduced chlorophyll content to 0.28±0.006 mg/gFW. The addition of 500 mg/kg nZVI to Pb-treated pots significantly increased chlorophyll content, with the highest values observed 0.35 ±0.005 (Fig. 2d). The nZVI enhances photosynthesis through two main mechanisms. Firstly, it increases the availability of essential nutrients like nitrogen and phosphorus, crucial for chlorophyll production (Liu *et al*., 2019). This nutrient availability boosts chlorophyll content and photosynthesis. Secondly, nZVI improves light-harvesting efficiency and electron transport in plants. Additionally, nZVI mitigates oxidative stress caused by Pb by reducing the accumulation of ROS (reactive oxygen species), which can damage chloroplasts and decrease photosynthetic efficiency (Srivastav *et al*., 2021).

Total protein content

Fig. 3a illustrates the impact of Pb only treatment and (Pb +nZVI) amendments on the total protein content. The control treatment exhibited a total protein content of 24.4±0.8 mg/gFW. Treatments with 400 mg/kg Pb significantly reduced protein content ($P<0.05$). However, applying nZVI at 500 mg/kg caused a significant increment ($P<0.05$) in protein content by 64 % compared to non-amended treatment. Pb contamination in soils detrimentally affects plant growth and physiological processes, including protein synthesis, by inhibiting soil microbial activity, altering pH levels, and reducing nutrient availability. Pb disrupts nitrogen assimilation by

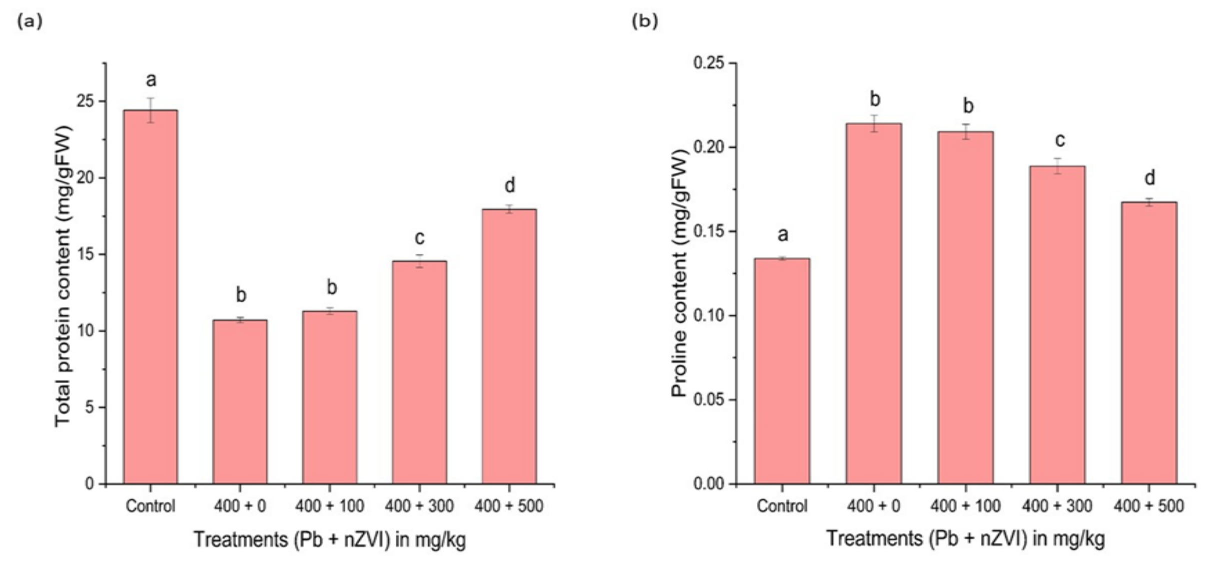

Fig. 3. *Impacts of nZVI amendments on (a) total protein content, and (b) proline content (different letters represent significant differences (P<0.05) between various treatments)*

inhibiting nitrate reductase activity and harming nitrogen-fixing bacteria, which are essential for amino acid and protein production. Additionally, Pb degrades chlorophyll and generates reactive oxygen species, which impair photosynthesis and nutrient uptake, thereby reducing protein synthesis (Sperdouli, 2022). Conversely, nZVI mitigates these adverse effects by adsorbing and stabilizing Pb, reducing its bioavailability and toxicity, and enhancing soil microbial activity and nitrogen availability (Guha *et al*., 2020). Its antioxidant properties help alleviate oxidative stress, preserving photosynthetic efficiency and overall plant health, thereby promoting increased protein content.

Proline content

Proline accumulation in plant cells is a defense mechanism against oxidative stress induced by heavy metals (Ashraf and Foolad, 2007). This parameter is crucial for assessing plant stress tolerance following Pb exposure. Fig. 3b shows that proline content increased significantly (0.13±0.002 mg/gFW) after Pb exposure compared to the control (0.21±0.005 mg/gFW). However, nZVI amendments significantly ($P<0.05$) alleviated stress, as evidenced by a decrease in proline content with increasing nZVI doses. The application of nZVI in contaminated soils decreases Pb availability to plants, thereby reducing stress and lowering proline accumulation.

Additionally, nZVI enhances the activity of antioxidant enzymes such as peroxidase and catalase, which mitigate Pb-induced oxidative stress and reduce the need for proline synthesis. It was due to the fact that iron-based nanoparticles enhance plant tolerance to stress by restoring chlorophyll levels and alleviating oxidative damage. Exposure to heavy metals often induces chlorosis and oxidative stress in plants, but iron nanoparticles improve nutrient uptake and activate the plant's antioxidant mechanisms, thereby reducing the accumulation of reactive oxygen species. Consequently, this reduces proline synthesis, leading to improved plant growth and physiological function (Abbas *et al*. 2019).

Pb accumulation in plant organs

The Pb accumulation in plant roots and shoots directly correlates with the plant's phytoremediation efficiency. Fig. 4a shows Pb accumulation in roots with and without nZVI amendments, where phytoextraction efficiency significantly increased ($p \leq 0.05$) with higher nZVI doses. Pb accumulation in roots was enhanced by 50 % after 500 mg/kg nZVI amendment. Similarly, Fig. 4b indicates increased Pb translocation in shoots (150.85 mg/kg) with nZVI amendments compared to Pb only treatment (120.45 mg/kg). The nZVI enhances Pb accumulation in *Tagetus erecta* through several mechanisms. Firstly, nZVI increases Pb bioavailability by converting Pb(II) to Pb(0), dissolving Pb complexes, and modifying soil pH and redox conditions, thus raising Pb concentration in the soil solution for root uptake (Rahmatizadeh *et al*., 2019). Secondly, nZVI affects root morphology and physiology, improving root surface area, permeability, and Pb uptake efficiency. These changes enhance Pb translocation from soil to plants through root uptake pathways influenced by nZVI-induced soil chemistry alterations. Additionally, nZVI interacts with plant physiological processes related to Pb uptake and accumulation, potentially boosting phytochelatin production involved in Pb detoxification, thereby promoting Pb accumulation in roots and shoots (Soni *et al*., 2023).

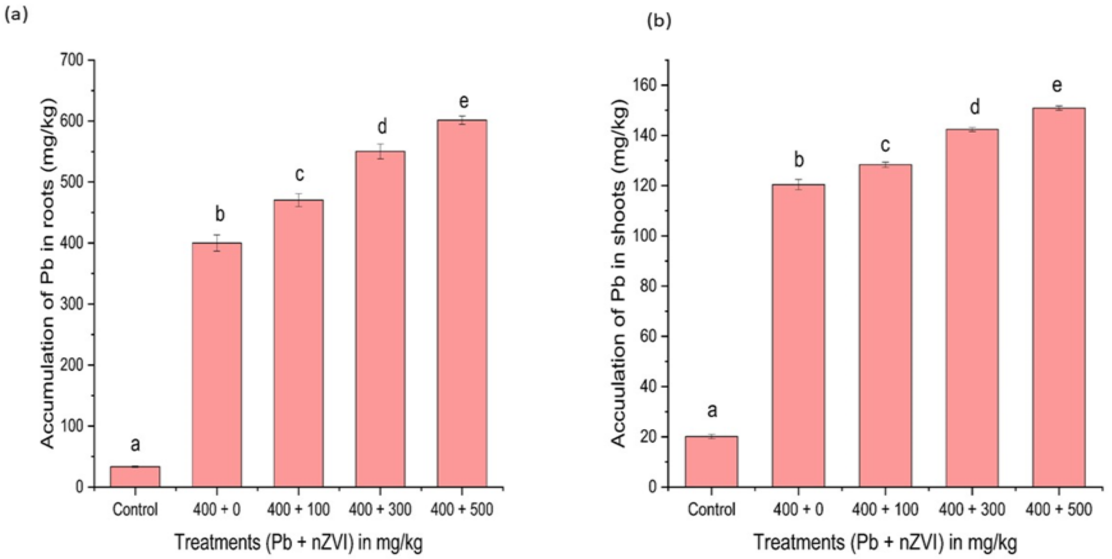

Fig. 4. *Accumulation of Pb in (a) roots, and (b) shoots (different letters represent significant differences (P<0.05) between various treatments)*

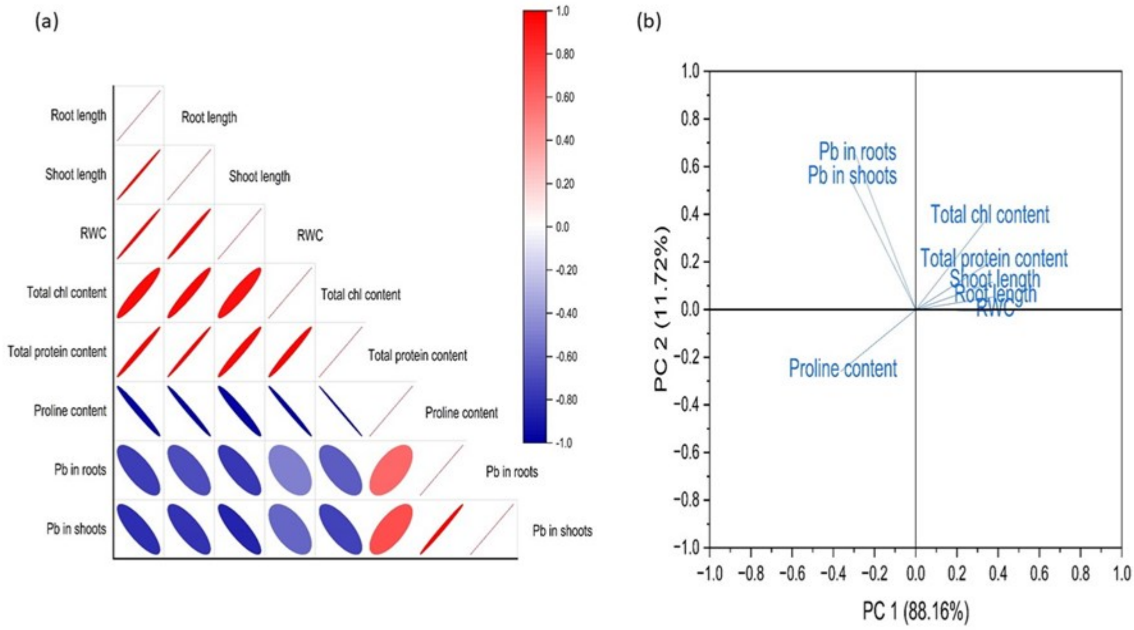

Fig. 5. *(a) Pearson correlation analysis, and (b) Principal component analysis*

Pearson correlation and principal component analysis

Pearson's correlation coefficients were calculated for various morphological, physiological, and biochemical parameters. Pb accumulation in plant tissues subjected to different Pb and nZVI treatments (Fig. 5a). The correlogram revealed a strong positive correlation between growth parameters, total chlorophyll content, and total protein content, indicating nZVI positively impacts overall plant growth by mitigating stress. In contrast, these parameters were negatively correlated with proline content. Meanwhile, Pb accumulation in shoots had a weaker correlation with proline content. The PCA (principal component analysis) was employed to reduce the dimensionality of complex datasets, identify patterns, and isolate key variables influencing the phytoremediation process (Fig. 5b). The results indicated that all the parameters were divided into two principal components (PC). First PC was associated with total protein, proline, and Pb accumulation in plant tissue. Second PC was associated with root length, shoot length, and chlorophyll content. The first PC explained the 88.16% of the variance, and 11.75% by the second PC.

Conclusion

This study investigated the applicability of nZVI in the phytoremediation process of Pb contaminated soil using *T. erecta* in a pot experiment. The synthesized nZVI particles were primarily spherical with a zeta potential of -11.8 mV, indicating good stability. The nZVI treatment increased plant chlorophyll and protein content in plants and reduced proline levels, suggesting that nZVI helps mitigate plant stress. Increasing nZVI dosage from 100 to 500 mg/kg significantly enhanced Pb accumulation in *T. erecta*'s plant organs. A correlogram showed positive correlations among growth parameters. The results suggest that nZVI significantly boosts Pb accumulation and reduces toxicity by positively affecting plant growth. No significant toxic effects of nZVI on the plants were observed, demonstrating its effective stress mitigation strategy. Thus, nZVI-assisted phytoremediation with *T. erecta* is a promising and eco-friendly method for Pb soil remediation. However, careful field application is needed to avoid environmental toxicity and ensure sustainability.

Conflict of interest

The authors declare that they have no conflict of interest.

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