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Phytoremediation of agricultural soils contaminated with Pb and Cd assisted by amendments of Zn Oxide nanoparticles stabilized with *Schinus Molle*

Daniel Neciosup Gonzalesa, Rita Cabello-Torresa\*, Ruben Munive Cerrónb, Eduardo Espinoza Farfana, Edison Romero-Cabelloc

aUniversidad César Vallejo, Grupo de Investigación ICAMB, San Juan de Lurigancho, Lima, Perú

bUniversidad Nacional del Centro del Peru

cUniversidad Nacional Agraria la Molina, Av. La Molina,La Molina Lima Peru

rcabello@ucv.edu.pe

The application of zinc nanoparticles is an efficient alternative to improve plant tolerance in the phytoremediation process of soils contaminated with Pb and Cd. A phytoremediation process of soils contaminated with Pb (11121 mg/kg) and Cd (5.9 mg /kg) using *Helianthus annuus* assisted by soil amendments and NPZn-SCM at the foliar level. For this, combinations of biochar, urea in soil and application of ZnO NPs produced by green synthesis were prepared. The process lasted 30 days. The results showed tolerance rates between 32.8 to 95.2%, with the best combination being Urea+Biochar, followed by Urea+NP and the foliar application of NP to the plant. In addition, 10.8 to 12.21% of Pb and 6.4 to 25% of Cd were removed. Regarding the treatments, only Cd showed a differentiated behaviour. Between the U+NP and U+NP+B treatments, the first achieved lower Cd removal but greater plant shoot length, contrary to the second case. The pH was a relevant factor that limited the availability of metals in the soil. This means that the results depend on the specific soil conditions. The comparative t-student analysis of the samples pointed out certain mobility difficulties for heavy metals, due to the alkaline pH of the soil studied. It is necessary to evaluate different doses of NP at the foliar level to improve the removals and response of *H. annuus* to high concentrations of Pb in the soil.

* 1. Introduction

Phytoremediation is an ecological technique applied to eliminate heavy metals from the soil, although contaminants can have a negative effect on plants (Gavrilescu, 2022). It is necessary to improve tolerance to heavy metals in the plant by improving the response in the process (Gavrilescu, 2022). *Helianthus annus* is a plant considered a hyper accumulator of metal ions and is used to clean contaminated environments, as it is a low-cost technique. Esta planta es elegida debido a su rápido crecimiento, densa biomasa y capacidad para eliminar metales pesados del suelo contaminado. When heavy metals are available in the soil and are found in high quantities, the roots of plants can adsorb these and stress it (Pérez-Hernández et al., 2024).

On the other hand, it has been reported that zinc oxide nanoparticles (ZnO NP) can stimulate seed germination and plant growth (Singh et al., 2019). So its application can improve the tolerance of *H. annuss* to heavy metal stress, and if the process is assisted by urea and biochar in the soil, a slower nutrient release process could be achieved, helping in plant survival. However, the immobilizing capacity of biochar could also affect the availability process of Pb and Cd, but the combination with soluble urea could balance the plant-soil system, improving the treatment. It is necessary to know the response of the plant in the removal of these metals assisted by the use of ZnNP at the foliar level, urea and amendments in the soil to improve its tolerance.

The objective of the research was to evaluate the application of ZnO nanoparticles stabilized with *Schinus Molle* extract (NPZnO-SM) used as an amendment to the foliar part of *Helianthus annuus* at the beginning of the phytoremediation process of soils contaminated with Pb and Cd. This research is part of Project P2022-53 of the ICAMB Group financed by the César Vallejo University.

* 1. Methods

**2.1 Study area and soil collection**

Approximately 600 kilos of fertile soil were collected from the arable layer between 0 to 20 cm deep, in the district of Muqui in the Province of Jauja in the department of Junín in Peru, located at 11º50´13.1” SL – 75º26´10.3” WL . These soils are contaminated with Pb and Cd, the soil sample was homogenized and sieved to a particle size of 2 mm. Four kilos of soil were placed in each pot, giving 84 experimental units. In addition, soil subsamples were analysed in the laboratories of the Lima East Campus of the César Vallejo University, to determine the physicochemical properties of the soil, as part of the activities of the ICAMB group.

**2.2 Production of Zn nanoparticles (NPZn-SM)**

The native plant *Schinus Molle* (SCM) was obtained in the markets of Junín; the washed and dried leaves were pulverized. Then 20 g of the dry powder (< 180 um) was weighed, 200 ml of deionized water was added, the mixture was stirred at 300 rpm at 60°C / 30 min. The extract was filtered (Whatman 1) according to Sing et al. (2019). The method of Abdelbaky et al. (2022) was applied, for the green synthesis, 5 ml of extract and 95 ml of a Zn acetate solution (10 mM) were mixed and brought to 90 °C, 300 rpm for 60 hours. Confirmation of the shape of ZnNP-SCM was performed by UV-visible spectrophotometry (Thermo SCIENTIFIC model GENESYS 10S UV-VIS) using an energy scan between 200 and 500 nm. This product was developed as part of the activities of the ICAMB-UCV Research Group.

**2.3 Obtaining biochar from the vine**

The biochar made from the vine waste was obtained from the ICAMB Research Group of the Cesar Vallejo University. In the field, 21 kg of dry waste was dried in the sun and pyrolyzed at 500 °C for 1 h. The cold biochar was then crushed and sieved to 2 mm (WS TYLER RX - sieve brand 29-16). The particle size was 2 mm. The performance achieved was 38%.

**2.4 Experimental design**

An Atomic Absorption Spectrophotometer (thermoscience AA SERIES) was used to quantify the Pb and Cd of each soil sample. The randomized block design was applied for 30 days of harvest time with an intermediate measurement at 15 days. Five experimental combinations were proposed in triplicate, considering the application of ZnNP-SCM at the foliar level (dose: 10 mg/L). Biochar at a rate of 100 mg/kg and urea (100 mg) were applied to the soil as fertilizer with a commercial content of 46% nitrogen. The following combinations were tested: urea and biochar (U+B), urea and NP (U+NP), NP alone, biochar and NP (B+NP), biochar, urea and NP (B+U +NP) making 84 experimental units in addition to the corresponding controls. The tolerance of the plant was also measured.

**2.5 Tolerance index of *H. annuus***

*H. annuus* was chosen due to its rapid growth, dense biomass, and ability to remove heavy metals from contaminated soil. The IT was calculated from the weights (g) of the fresh aerial biomass of stems and leaflets; in each case, it consisted of the division of the aerial biomass treated to remove Pb and Cd on the aerial biomass of the treatment control.

**2.6 Data analysis**

All data were tabulated using Microsoft Excel and IBM SPSS Statistics v 24 software, to calculate the mean values of each treatment and compare them with each other using Tukey's multiple comparison test (p≤0.05). Likewise, ANOVA was applied to evaluate the difference between the treatments in the plant at the foliar level and the use of urea/biochar in the soils through the five combinations applied at random.

* 1. Results and discussion
		1. Identification of ZnNP-SM

The formation of NPZnO-SM was observed by the color change from yellowish in the first hours, to orange after 24 hours and whitish at 72 (90 °C). The NPZnO-SMs were recognized by UV–vis absorption spectrometry at 378 nm by the formation of a peak. This is due to the plasmonic resonance band of the formed nanoparticles (Abomuti et al., 2021). Then, Schinus Molle extract stabilized the growth of the nanoparticles. Sing et al. (2019) showed spectra with intense peaks between 350 and 361 nm, Narath et al. (2021) demonstrated peaks at 376 nm of ZnONPs stabilized with S. officinalis extract and Abomuti et al. (2021) identified an absorption peak at 368 nm for ZnNP. In the same way Abdelbaky et al. (2022) reports that green synthesis to obtain ZnNPs subjected to a pH of 12 produces better results and allows the identification of biomolecules that protect and stabilize ZnO NPs.

* + 1. Physicochemical properties of contaminated soil (S) and grape vine biochar

Table 1 presents the physicochemical properties of the initial Muqui soil and the biochar made from grape vine.

Table 1. Physicochemical characteristics of Muqui soil and grape vine biochar

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|   | Organic Matter (%) | Redox (mv/ORP) | pH  (24oC 1:2) | Electric conductivity (dS/m 1:2 24oC) | Bulk density (g/cm3) | Moisture (%) | P (mg/kg) | Pb(mg/kg) | Cd (mg/kg) |
| S | 1.67 ± 0.2 | 202.35 ± 0.25 | 7.9 ± 0.01 | 0.27 ± 0.0005 | 2.58 ± 0.02 | 3.99± 0.15 | 73.63 ± 0.215 | 1121.17 ± 39.26  | 5.9 ± 0.5  |
| Biochar | 90.71 ± 0.12 | 117.06 ± 0.35 | 10.07 ± 0.5 | 7.07 ± 0.23 | 1.6 ± 0.002 | 16.79 ± 0.15 | 695.85 ± 2.56 |  |  |

The soil texture of Muqui (S) is sandy loam in the first 20 cm of arable soil (Munive et al., 2020), and is composed of 73% sand and 19% silt. This characteristic influences the development of phytoremediation plants. The pH is slightly alkaline, it has a discrete organic matter content, a high available P content and a positive redox potential, indicating an aerobic or oxidizing environment. Furthermore, the electrical conductivity presented a discrete value.

Regarding vine biochar, a higher content of organic matter is observed, a high content of P that provides an important nutrient to the soil and the plant, the high electrical conductivity reflects a higher content of salts and a high pH value. Fresh biochar loses hydrogen and oxygen atoms in the pyrolytic process (Chen et al., 2021), resulting in a loss of moisture and volatile components. Therefore, changes in biochar increase pH, electrical conductivity, and carbon (C) content (Li et al., 2019). The functional groups that contain hydrogen (H), oxygen (O) are also increased.

About the content of heavy metals in the soil of Muqui, the results showed an average concentration of 1121.17 ± 39.26 mg Pb/Kg and 5.9 ± 0.5 mg Cd/Kg. These values demonstrate the serious contamination problem of the soils of Muqui located on the right bank of the Mantaro River, since during the rainy season (January to March) these are flooded by this river. At the end of the season, the river leaves its sediments on part of the agricultural soils, increasing the levels of contamination by Pb and Cd.

**3.3 Muqui soil treatment results**

Table 2 presents the analytical results of the K, Cd and Pb content at the beginning and end of each treatment.

Table 2. Analytical results of Muqui soils treated under 05 combinations

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment | K (mg/kg) | t | p-value\* | Cd (mg/kg) | t | p-value\* | Pb (mg/kg) | t | p-value\* |
| Before treatment |  |  |  |  |  |  |  |  |
| S | 1061.8 ± 0.32 | - | - | 6.0 ± 0.54 | - | - | 1108.6 ± 44.5 | - | - |
| U+B +S | 1015.9 ± 27.15 | - | - | 6.4 ± 0.21 | - | - | 1132.2 ± 17.1 | - | - |
| U+NP +S | 1033.975 ± 15.97 | - | - | 6.2 ± 0.28 | - | - | 1132.2 ± 17.1 | - | - |
| NP +S | 1002.6 ± 19.59 | - | - | 6 ± 0.84 | - | - | 1125.95 ± 46.6 | - | - |
| B+NP +S | 1021.9 ± 35.7 | - | - | 6.1 ± 0.28 | - | - | 1132.2 ± 14.1 | - | - |
| U+ B+ NP +S | 1041.3 ± 8.9 | - | - | 6.05 ± 0.21 | - | - | 1132.2 ± 17.1 | - | - |
| Post treatment |  |  |  |  |  |  |  |  |
| S | 971.9 ± 57.82 | 2.2 | 0.2728 | 5.4 ± 0.56 | 3 | 0.2048 | 973.2 ± 81.4 | 5.2 | 0.1212 |
| U+B +S | 892.7 ± 27.15 | 3.5 | 0.1795 | 4.8 ± 0.9 | 3.2 | 0.1928 | 999.6 ± 72 | 2.1 | 0.2824 |
| U+NP +S | 942.5 ± 7.62 | 15.5 | 0.0410 | 5.8 ± 0.28 | 1 | 0.0000 | 1009.9 ± 10.6 | 6.2 | 0.1012 |
| NP +S | 917.5 ± 26.30 | 18.0 | 0.0354 | 5.5 ± 0.14 | 1 | 0.5000 | 1000.1 ± 24.5 | 2.5 | 0.2420 |
| B+NP +S | 933.4 ±37.8 | 58.4 | 0.0109 | 5.4 ± 0 | 3.5 | 0.1772 | 1007. ± 24.4 | 4.3 | 0.1469 |
| U+ B+ NP +S | 925.7 ± 9.15 | 606.7 | 0.0011 | 5 ± 0.28 | 21 | 0.0303 | 1009.8 ± 10.4 | 25.8 | 0.0247 |

Table 2 presents the analytical results of heavy metals and the K content has also been included because, as a specific nutrient of the plant, it has served to analyse absorption or bioavailability abilities. According to Table 2, the post-treatments that have shown a significant difference (p < 0.05) with the initial concentration of each trial have been given for K (with the exception of the B+NP treatment).

On the other hand, for Pb and Cd it has been lower. In the case of Cd, the treatments that stood out were U+NP and U+B+NP because these showed a significant difference with respect to the others (p<0.05). In the case of Pb, its elimination from the soil actually depended on the chemical speciation present in the medium. In our case, the original application to the soil has been similar to that followed by Yao et al. (2023), only a small amount of Pb was exchangeable type and bioavailable to be absorbed by *H annuss*. This is explained by an approximate 10% decrease in its content (100 mg/kg) in the treated soils. Figure 1b shows similar periodic values between each treatment followed.

Biochar B had a high content of organic matter and the presence of clays favoured a lower initial mobility of Pb, forming complex compounds and decreasing its bioavailability. It is known that B has a large surface area with functional groups, a high content of nutrients that are released slowly, its high porosity increases the water retention capacity, increasing the stability of soil aggregates.

Likewise, soil pH was a determining factor that affected Pb solubility. In our case, the pH was 7.9, indicating a limitation for its solubility since when the soil pH increases from 6 to 9, insoluble precipitates can form in the soils (Yao et al., 2023). Abbas et al. (2020) used rice straw biochar and immobilized Cd from the soil, this was associated with increasing soil pH, similar to what occurred in our research. Unlike the method of Abbas et al. (2020), the B was assisted with urea (B+U); a better response was achieved in the final levels of Cd in soil (Figure 1 c). The plant developed its shoots and roots but the bioavailability of Cd in the soil decreased. The case of K as a nutrient is different, since it is necessary for plant growth and as a mono-cation, its mobility was more defined and it was consumed by the plant. This element acts as a regulator, controlling the functions of essential elements and enzymes that activate plant development (Hemeid et al., 2020).



Figure 1. Decrease in the concentration of K and the contaminants Pb and Cd in soils cultivated with Helianthus *annuus*

* + 1. Plant growth and morphological analysis: Treatments and effects of ZnNP-SCM

A morphological analysis was carried out to evaluate the effects of Pb and Cd on the length and weight of the root and shoot of *H. annuss*, and to determine the tolerance of the seedlings (see figure 2).

*Figure 2. Development of Helianthus annuus reinforced with ZnNP-SCM, under different soil treatments*

The results showed that the heights of the plant varied depending on each application made to the soil and the plant, also considering the real levels of Pb and Cd contamination of the Muqui soil. After 30 days of treatment, the maximum shoot length (Figure 2 a) was 15 cm for the treatment with U+ZnNP-SCM, followed by NP (14 cm) and U+B (13 cm); while the minimum length corresponded to the U+B+NP treatment (4 cm) followed by the untreated soil (8.5 cm). In most cases, ZnO NPs increased plant height, root size, and fresh biomass. This situation suggests that the nanoparticles absorbed at the foliar level of *H. annuus* immobilized Pb and Cd through metal oxidation-reduction processes, managing to improve the height of plants and roots (Pérez-Hernández et al., 2024).

Regarding root length (Figure 2 c), the greatest length corresponded to the NP treatment (16 cm), followed by U+NP (12 cm) and U+B (12 cm), while the lowest value corresponded to the control soil (9 cm). It is important to note that an extension of the experiment that is not part of this report, resulted in a root length of 60.5 cm after 75 days of cultivation for the NP treatment, followed by B+NP (57 cm).

The fresh weight of both the shoot and the root showed a significant difference (p < 0.05) in the treatment with B+ZnNP-SCM, which reached the maximum weight in the shoots after 30 days (12.4 g) (see Figure 2 b). The other treatments did not show differences between them. The extension of the experiment towards 75 days recorded a maximum weight for ZnNP-SCM (78.44 g) unlike the control soil (31.6 g). Regarding the root at 30 days, the B+NP treatment achieved the maximum root weight (4.3 g) unlike the control soil (0.5 g) (see Figure 2 d).

The application of ZnNP-SCN at the foliar level, history has shown good results in the application of ZnNP in general. Although ZnNP has been applied in different concentrations, an application of 10 mg/L in *Brassica nigra* generated the elimination of DPPH oxidant radicals, and increased non-enzymatic antioxidant molecules (Zafar et al., 2023). In the case of *S. rebaudiana*, free radicals have been eliminated and excessive oxidative stress has been avoided, improving germination. However, the application of doses higher than 1000 mg/L has been observed for serious phytotoxic effects (Jampílek & Kráľová, 2021). Pérez-Hernández et al. (2024) applied ZnO NPs in *H. annuss* and achieved plant growth 25 days after emergence; on the contrary, the interaction between nanoparticles and heavy metals negatively affected the length of the root. In other words, the results of the process depend on the actual specific conditions of the environment, including the levels of the contaminant present in the soil.

Regarding the dose used of ZnNP-SCM, it does not imply phytotoxicity, although there is controversy among researchers, we suggest that this will depend on the specific physicochemical properties of each soil, and the microbial community present in the soil, the cultivation period, among others.

The tolerance index was observed after 30 days, the range varied from 32.8% (B+NP) to 95.2% (U+B). It is important to highlight the applications of U+NP and NP, since these were also significant (IT of 87.5 and 71.2% respectively). This demonstrated a significant difference in tolerance for each treatment. The TI values surpassed other tests where the same plant was used to treat soils with 1000 mgPb/kg (TI < 80%) (Chauhan et al. 2020). The presence of ZnNP-SCM or the combination with urea and the urea+biochar form are capable of promoting the increase in the TI of *Helianthus annuss*, inducing to stabilize the synthesis and protect the enzymatic proteins by mitigating their denaturation at the cytoplasmic level (Yadav, 2020). ZnNP-SCMs, of nanometer size (1–100 nm), entered through the shoot epidermis, acted as biostimulants and maintained cellular integrity by activating the gene expression of tolerance to environmental stress (Kolenčík et al., 2020).

It is important to note that the crops did not show symptoms of toxicity such as chlorosis, yellowing of the leaves and oxidation of the stem; unlike other research that shows that the presence of Pb and Cd metals has inhibitory effects on plant development (Chauhan et al., 2020). However, in our research, the growth of *H. annuus* was reduced in the control soil that received no treatment compared to most treatments. The growth of the roots was much more notable than the length obtained in the aerial part. Cultures with U+NP and U+B flowered first, after 60 days. Table 3, shows the application of one-way ANOVA for statistical analysis to explain plant development and the decrease in Cd and Pb content in treated Muqui soils.

Table 3. Analysis of variance of the lengths and weights of the aerial part and roots of *H. annuus* and the content of heavy metals in soils, during the 30-day Phytoremediation process.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| *Origin of variations* | *SS* | *DF* | *MS* | *F* | *p* | *Origin of variations* | *SS* | *DF* | *MS* | *F* | *p* |
| ***Shoot Length (cm)*** |  |  |  |  | ***K*** |  |  |  |  |  |
| BG | 180 | 6 | 30 | 0.076 | 0.998 | BG | 3840.7 | 5 | 768.1 | 0.27 | 0.9197 |
| WG | 13767.2 | 35 | 393.3 |  |  | WG | 33875.4 | 12 | 2822.9 |  |  |
| Total | 13947.3 | 41 |  |  |  | Total | 37716.2 | 17 |  |  |  |
| ***Root length*** |  |  |  |  |  | ***Cd*** |  |  |  |  |  |
| BG | 248.1 | 6 | 41.3 | 0.162 | 0.985 | BG | 0.25 | 5 | 0.05 | 0.23 | 0.9437 |
| WG | 8923.4 | 35 | 254.9 |  |  | WG | 2.7 | 12 | 0.22 |  |  |
| Total | 9171.6 | 41 |  |  |  | Total | 2.96 | 17 |  |  |  |
| ***Shoot weigth*** |  |  |  |  |  | ***Pb*** |  |  |  |  |  |
| BG | 2440.4 | 7 | 348.6 | 0.542 | 0.798 | BG | 1629 | 5 | 325.8 | 0.07 | 0.9958 |
| WG | 25750 | 40 | 643.7 |  |  | WG | 56210 | 12 | 4684.1 |  |  |
| Total | 28190.4 | 47 |  |  |  | Total | 57839.1 | 17 |  |  |  |
| ***Root weight*** |  |  |  |  |  |  |  |  |  |  |  |
| BG | 52.6 | 6 | 8.7 | 0.051 | 0.999 |  |  |  |  |  |  |
| WG | 6067.5 | 35 | 173.3 |  |  |  |  |  |  |  |  |
| Total | 6120.1 | 41 |   |   |   |   |   |   |   |   |   |

BG = Between groups; WG = Within the groups

*Helianthus annuus* L. is a hyperaccumulator plant, it is generally applied in the process of heavy metal phytoremediation due to its high efficiency (Kolenčík et al., 2020). The ANOVA shows that there was no statistically significant difference between the five treatments applied to the plant-soil system; under the combinations NP, B+NP, U+NP, U+B+NP, U+B with the exception of the control soil. The doses of Cd and Pb did not mortally degrade the plants, despite their toxicity, plant growth occurred, the tolerance of the plants was demonstrated by a high TI for the NP, U+NP and U+B treatments. There was no significant difference between the treatments in terms of the variation in the final concentrations of metals in the soil, but the removal was discrete for Pb (10.8 to 12.21%) and for Cd (6.4 to 25.2 %). Unlike other authors, during the 30 days of cultivation there was no discoloration or visual oxidation of the plants, as well as chlorosis and leaf necrosis. It is known that Cd is the most toxic element for plants and reduced the biomass of different species. On the other hand, in the shoots the roots gained weight and responded to the absorption of heavy metals in the plant, in a similar way to that recorded by Mallarino-Miranda et al. (2022) who applied *H annuss* to remove Cd and Pb. It is important to mention that the H. annuuss biomass harvested post experiment will be used for new studies on the generation of fuel for burning from contaminated biomass. Likewise, we consider the recommendations to include organic acids in future studies to increase the mobility of the heavy metals studied.

**Conclusion**

The application of ZnO nanoparticles stabilized with *Schinus Molle* extract (NPZnO-SM) used as an amendment to the leaf part of *Helianthus annuus* at the beginning of the phytoremediation process of soils contaminated with Pb and Cd was evaluated. Only Cd showed a differentiated behaviour. Between the U+NP and U+NP+B treatments, the first achieved lower Cd removal but greater plant shoot length, contrary to the second case. The pH was a relevant factor that limited the availability of metals in the soil. No significant differences were shown between treatments except the control. Foliar uptake of the NPZnO-SM bio stimulant maintained cellular integrity and increased plant size, roots, and fresh biomass. No signs of toxicity were shown in the plants; the tolerance indices were high, demonstrating that the application of NPZnO-SM is an eco-friendly alternative that improves the phytoremediation process.

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