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Nano-Hydroxyapatite and ZnO-NPs Mitigate Pb Stress in Maize

Bushra Ahmed Alhammad [1], Awais Ahmad [2] and Mahmoud F. Seleiman [2,3,*]

[1] Biology Department, College of Science and Humanity Studies, Prince Sattam Bin Abdulaziz University, Al Kharj, Riyadh 11942, Saudi Arabia
[2] Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia
[3] Department of Crop Sciences, Faculty of Agriculture, Menoufia University, Shibin El-Kom 32514, Egypt
* Correspondence: mseleiman@ksu.edu.sa

Abstract: Heavy metals (HMs) stress, particularly lead (Pb) stress, is one of the most hazardous environmental stresses that can negatively affect plants' growth, yield, and quality. Therefore, the effects of zinc oxide nanoparticles (ZnO-NPs; 50 mg L^{-1}), nano-hydroxyapatite (HP-NPs; 50 mg kg^{-1}), and their combination on growth, physiological, and yield traits of maize grown in soil contaminated with Pb (i.e., 100 mg kg^{-1}) were investigated. The results showed that Pb stress significantly reduced plant leaf area by 50.9% at 40 days after sowing (DAS), 55.5% at 70 DAS, and 54.2% at 100 DAS in comparison to the unstressed plants (control). However, the combined application of ZnO-NPs (50 mg L^{-1}) + HP-NPs (50 mg kg^{-1}) reduced the adverse effects of Pb on plant growth in terms of increasing leaf area by 117.6% in plants grown in Pb-contaminated soil (100 mg kg^{-1}). Similarly, the combined application of ZnO-NPs + HP-NPs resulted in increments in the total chlorophyll content by 47.1%, photosynthesis rate by 255.1%, and stomatal conductance by 380% in comparison to that obtained from maize stressed with Pb. On the other hand, antioxidants such as sodium dismutase (SOD; 87.1%), peroxidase (POX; 90.8%), and catalase (CAT; 146%), and proline content (116%) were significantly increased as a result of Pb stress compared to unstressed plants. Moreover, N, P, K, and Zn contents in the whole plant grown under Pb stress were decreased by 38.7%, 69.9%, 46.8%, and 82.1%, respectively, compared to those obtained from the control. Whereas the combined treatment of ZnO-NPs (50 mg L^{-1}) + HP-NPs (50 mg kg^{-1}) resulted in increased uptake of plant nutrients and, consequently, the highest values of ear weight, grain yield, and harvest index were obtained. Furthermore, the combined application of HP-NPs + ZnO-NPs in contaminated soil reduced Pb uptake in plant biomass by 77.6% and grains by 90.21% in plants exposed to Pb stress. In conclusion, the combined application of ZnO-NPs and HP-NPs significantly improved growth, physiological traits, antioxidants, and yield as well as elemental uptake of maize grown under Pb stress.

Keywords: Pb; ZnO-NPs; HP-NPs; maize productivity; elemental analysis

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1. Introduction

Abiotic stresses such as drought, heat, salinity, and heavy metals (HMs) stress can adversely affect the growth, development, yield, and quality of strategic crops [1–3]. HMs such as Pb (lead), Cu (copper), Co (cobalt), Ni (nickel), Cd (cadmium), and As (arsenic) have for long been accumulated in soils due to human activities such as sewage disposal, unreasonable mining, continuous application of agrochemicals, and industrial waste. There are more than 10 million contaminated sites worldwide, and nearly 50% of them are contaminated with HMs [4,5]. HMs are non-biodegradable, and thus there is a need for new agricultural practices or technologies to remove them or mitigate their negative effects on the environment, microorganisms, animals, and human health [6]. Some HMs are immobile, while other HMs are termed mobile and can be absorbed by plant roots' cells via metal transporters or through diffusion and endocytosis [7,8]. However, some HMs such as Zn and Cu are essential as micronutrients and are needed in very small quantities

as they act as cofactors for different enzymatic activities, while other HMs such as Pb, As, and Cd do not have a beneficial role in growth and development of plants and are toxic if they exceed the maximum limits [9]. Interestingly, plants are natural bio-accumulators and can uptake and collect HMs from contaminated soils, although such HMs may not be necessary for their growth and productivity [8,10].

HMs stress can negatively affect proteins and the activity of enzymes, as well as can interfere with substitution reactions of essential metal ions with biomolecules. Such a reaction can disrupt the integrity of the membranes and result in the alteration of basic metabolic reactions, for example, homeostasis, respiration, and photosynthesis [11]. Moreover, HMs stress can enhance productivity of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and superoxide radicals (O^{-2}). ROS can cause oxidative stress, which can cause lipid peroxidation [12,13].

Lead (Pb) is considered one of the most dangerous HMs that can contaminate the soil as a result of natural weather processes, mining, and smelting [14,15]. Once Pb enters the plant cell, it changes the hormonal balance, cell membrane integrity, and permeability, inhibits enzymes containing the sulfhydryl group, disturbs mineral nutrition, and reduces the water content to create reactive oxygen species [16,17]. Therefore, high levels of Pb in the soil can significantly affect plant health in terms of reducing root length, leaf chlorosis, and stunted growth [14]. Pb can cause oxidative stress in plants by disturbing the antioxidant defense system and through the overproduction of ROS. Plants can tolerate the toxicity of Pb depending on the production of antioxidant enzymes that can support scavenging ROS as well as on the buildup of osmoprotectants such as soluble sugars, proline, and glycine betaine [18,19]. Plants grown in Pb-stressed soil can have negatively affected photosynthetic pathways since Pb can disturb the ultrastructure of chloroplasts and prevent the production of essential photosynthetic pigments such as chlorophyll, carotenoids, and plastoquinone [10,16]. Moreover, Pb can disrupt the Calvin cycle and block the electron transport chain, as well as cause a shortage of CO_2 by closing stomatal pores on leaves [16].

In recent decades, attention has been given to develop different strategies for mitigating HMs toxicity in crops' cultivation to meet the global demands for food, feed, and fiber [20–22]. The development of nanoscience can provide a new direction for the advancement of soil remediation [23]. Nanomaterials are innovative and can be used for environmental remediation that can slowly degrade highly toxic compounds including HMs in contaminated soils [24,25]. Nanomaterials such as nanoparticles (NPs) can play a vital role to mitigate the negative impact of HMs absorption, uptake, and translocation effectively due to their large surface area [26,27]. NPs can be used to alleviate the toxicity of heavy metals in plants [26,28] and consequently can improve seed germination and photosynthetic systems [29–31].

Among nanomaterials, phycomolecule-coated ZnO-NPs can promote plant growth [32]. The application of ZnO-NPs can protect plants seedlings under Cd and Pb stress by alleviating HMs-induced phytotoxicity and promoting physiochemical activities through differential regulation of photosynthesis and antioxidative defense mechanisms in plant seedlings [32]. ZnO-NPs are excellent sorbents and adsorbents for HMs contaminants and can become probable candidates for the removal of most of the toxic metal ions, such as Pb^{2+} in water or soil solutions [33,34]. Previous studies conducted by Karn et al. [35] and Le et al. [36] revealed that ZnO-NPs have high removal (>85%) efficiency of Pb metal ions from wastewater in the form of metal oxidation/reduction and adsorption mechanisms. Zinc is well known for its role as a cofactor for sodium oxide dismutase (SOD) catalyzing the dismutation of superoxide radicals (O^{2-}) to O^2 and H_2O_2, thus playing a major role in alleviating the oxidative stress during the presence of HMs. Therefore, ZnO-NPs' application in plants can reduce the oxidative stress and help the plants in quenching ROS produced during abiotic stress [28,37]. ZnO-NPs hinder HMs uptake and accumulation of metals, thus curtailing the expression of stress-induced genes [38]. Furthermore, NPs accumulate

in the plants' cell wall and make HMs ions unavailable by assembling complexes [39], thereby impeding the transportation of HMs in plants and reducing their biological activity.

The uptake, concentration, accumulation, distribution, and balance of mineral ions play an essential role in maintaining plant growth; however, HMs disturb the minerals balance in the roots and leaves of plants. In this case, ZnO-NPs help plants' uptake of some nutrients (Zn, Mn, Cu) in the roots, thus improving the growth under HMs stress [40]. HMs lower the protein metabolism in plants [41] while ZnO-NPs can enhance the uptake and total protein–nitrogen content in plants [42]. Zinc ions from ZnO-NPs enhance cell stability by directly influencing nutrition uptake and transpiration and increase the resistance level of plants against HMs [43]. Moreover, Zn^{2+} acts as an essential component of many biochemical processes in plants, such as chlorophyll biosynthesis and carbohydrate and nitrogen metabolism [44]. Under HMs stress, ZnO-NPs can enhance the nitrate reductase activity of seeds, increase glycolytic metabolism and cell wall synthesis during germination, and increase root growth [42], which results in vigorous seedlings. However, the excessive application of ZnO-NPs upon bioaccumulation shows phytotoxicity, but the toxic ranges vary as per plant species and soil chemistry. For example, Shen et al. [45] investigated the effects of different levels of ZnO-NPs on the growth of *Brassica chinensis* and reported that 100 mg ZnO-NPs kg^{-1} of soil significantly improved plant growth and nutrients uptake, whereas 1000 mg ZnO-NPs kg^{-1} was reported to be toxic for human health risks. Moreover, Youssef and Elamawi [46] reported that the application of 25 mg ZnO-NPs L^{-1} improved seed germination and seedling growth of *Vicia faba* L., while higher applications (i.e., 100–200 mg ZnO-NPs L^{-1}) showed toxic effects. Nano-hydroxyapatite (NHAP), as phosphate mineral (phosphate), is an important material for the immobilization of HMs due to its nano size, high stability under reducing and oxidizing conditions, increased surface area, and high sorption capacity [47–50]. For example, the application of NHAP can reduce Cd concentrations by 17.4% in potato tubers in comparison to those obtained from untreated plants [51]. The application of NHAP can reduce the water- and acid-soluble, exchangeable, and reducible fractions of Pb [46], and consequently can reduce its mobility and bioavailability in soil [52]. NHAP can mitigate the negative effects of Pb on plant growth by enhancing soil pH and reducing the solubility and mobility of HMs [52]. Wang et al. [53] reported that NHAP can be an effective remediator in HM-contaminated soil, and it was reported to enhance the chlorophyll content and antioxidant system of plants grown in Cd-contaminated soil [54].

Maize (*Zea mays* L.) is one of the world's preeminent cereal crops for food, feed, and bioenergy usage [55–57]. Globally, the cultivated area with maize for grain purposes was 197 million hectares in 2021 [58]. In developed countries, maize is primarily used as a livestock feed crop with varied roles as an industrial and energy crop [58]. Thus, maize plays a diverse role in the global agricultural food system and food/nutrition security [59]. It belongs to C4 plants and is considered moderately sensitive to HMs stresses. However, maize productivity can be significantly affected when grown under HMs stress [60,61].

Therefore, the objective of the study was to investigate the integrative effects of NP-NPs and ZnO-NPs on physiological and biochemical traits, productivity, and elemental analysis of maize grown in Pb-stressed soil. Our hypothesis is that the application of NH-NPs, ZnO-NPs, and their combinations could mitigate the negative effect of Pb and enhance the growth and productivity of maize.

2. Materials and Methods

2.1. Plant Materials and Treatments

The pot study was conducted to investigate the integrative effects of NP-NPs, ZnO-NPs, and their combinations on physiological and biochemical traits, productivity, and elemental analysis of maize grown in Pb-stressed soil. The characteristics of ZnO-NPs were 99.9% purity, 55 m^2 g^{-1} specific surface area, 16–35 nm particle size, and 5.6 g cm^{-3} density, while the characteristics of HP-NPs were 96.0% purity, and 60 nm particle size (Sigma-Aldrich, Burlington, MA, USA). The study included eight treatments as follows:

control (zero Pb + zero NPs), NH-NPs (i.e., 50 mg kg^{-1}), ZnO-NPs (i.e., 50 mg L^{-1}), NH-NPs + ZnO-NPs, Pb (i.e., 100 mg kg^{-1}), NH-NPs + Pb, ZnO-NPs + Pb, and ZnO-NPs + NH-NPs + Pb. The treatments were incorporated and well mixed with soil in each pot prior to sowing process, except ZnO-NPs that were applied at 30 and 60 DAS as exogenous application, and ZnO-NPs treatment were applied based on the level of 1000 L ha^{-1} (i.e., about 60 mL per pot).

The design of experiment was completely randomized in a factorial design, and the number of replications was six. A weight of 10 kg of soil was added to each pot. Roughly, 6 maize grains were sown at 5 cm depth in each pot. At 12 DAS, three healthy seedlings were kept in each pot. All agricultural practices were applied according to the recommended regulations. Uniform irrigation was applied to different pots when water was needed to avoid the water deficit. The source of the irrigation water was groundwater and its analysis was as follows: pH 7.21, EC 3.04 dS m^{-1}, Ca^{2+} 203.17 mg L^{-1}, Mg^{2+} 131.01 mg L^{-1}, Na^{+} 286.27 mg L^{-1}, K^{+} 18.94 mg L^{-1}, and Cl^{-} 354.41 mg L^{-1}.

In addition, urea (i.e., N source) and DAP (di-ammonium phosphate) were applied twice during the growth of maize in the current experiment.

The soil was collected from the field and sieved through 2 mm prior to its usage in the current investigation. The physio-chemical properties of soil were analyzed using the standard methods [62]. The soil was sandy loam with 56.5% sand, 28.5% silt, and 15.0% clay. In addition, the pH, EC, and OM of soil were 7.73, 3.34 dS m^{-1}, and 0.53%, respectively. Moreover, the soil contained 3.02 g N kg^{-1}, 1.46 g P kg^{-1}, 0.11 mg K kg^{-1}, 39.7 mg Mn kg^{-1}, 10.0 mg Cu kg^{-1}, 3.6 mg Cd kg^{-1}, 5.9 mg Pb kg^{-1}, and 11.1 mg Zn kg^{-1}.

2.2. Measurements

2.2.1. Leaf Area

At 40, 70, and 100 DAS, leaf area $plant^{-1}$ (cm^2) was measured by LI-COR (LI-3000C, Portable Leaf Area Meter, LI-COR Inc., Lincoln, NE, USA).

2.2.2. Total Chlorophyll (SPAD)

The total chlorophyll was recorded from fully expanded maize leaves at 75 DAS using SPAD (SPAD-502, Minolta Sensing Ltd., Osaka, Japan).

2.2.3. Gas Exchange Traits

Gas exchange traits such as net photosynthetic rate and stomatal conductance were measured at 70 DAS between 8.00 and 12.00 a.m. on a fully expanded leaf using a portable photosynthesis system (LI-6400 XT, Li-Cor, Lincoln, NE, USA). The portable photosynthesis system was calibrated using a leaf area of 6 cm^2 with a controlled CO_2 flux at a concentration of 400 µmol CO_2 mol^{-1} air. In addition, the photon flux density (PPFD) was fixed to be 1500 µmol m^{-2} s^{-1} with a blue-red LED light source (6400-02B LED) and a controlled leaf temperature (30 °C).

2.2.4. Photosynthetic Pigments

About 500 mg of fresh maize leaves were ground with a pestle and mortar in liquid nitrogen (N). The samples were treated with 80% acetone, and the mixture was centrifuged at 20 °C for 5 min at 4500× *g*. A spectrophotometer (Model 6305, Jenway, Staffordshire, UK) was used for reading the filtrate absorbance at wavelengths of 665 nm to evaluate carotenoids [63].

2.2.5. Proline

Roughly 100 mg of maize leaf materials was mixed with 10 mL of 3% sulfosalicylic acid. Then, the extraction was inserted in tubes and shaken for 60 min. Afterward, the extraction samples were filtered and analyzed as explained by Bates et al. [64] to calculate proline content.

2.2.6. Malonaldehyde (MDA)

About 500 mg of maize leaf material was mixed with 2.5 mL of 0.1% trichloroacetic acid. The mixture was centrifuged at 4000× *g* rpm for 30 min at 4 °C, and MDA was determined as described by Cakmak and Horst [65].

2.2.7. Antioxidant Enzymes

For enzyme extraction, 500 mg of maize leaves material was extracted with 50 mM potassium phosphate buffer (ph = 7.5). Then, 1 mM EDTA + 1 mM PMSF and 5% PVPP were mixed with the extraction solution. Thereafter, samples were centrifuged at 14,000× *g* rpm for 10 min at 4 °C, and supernatants were collected for antioxidant enzymes analysis. The antioxidant enzymes were expressed in mg of protein. The superoxide dismutase (SOD, EC 1.15.1.1) activity was determined via the capability of inhibiting nitrotetrazolium blue (NBT) photoreduction; catalase (CAT, EC 1.11.1.6) activity was measured via H_2O_2 consumption at 240 nm for 3 min, with an extinction coefficient of 36 mM^{-1} cm^{-1}. In addition, the peroxidase (POX, EC 1.11.1.7) was estimated through the oxidation of guaiacol at 470 nm, with a 26.6 mM^{-1} cm^{-1} extinction coefficient. The activities of the three enzymes were determined using the described method by Garcıa-Limones et al. [66].

2.2.8. Elemental Analysis

Total N content: Each maize sample (200 mg) from whole plant biomass was weighted and a different process of the Dumas combustion method was followed. Vario MAX CN (Elementar Analysensysteme GmbH, Hanau, Germany) was used to determine total N content. For analyzing macro and trace elements, a random plant sample was taken from each replicate to represent the whole plant biomass for grinding. About 200 mg of each sample was weighed and placed in a Teflon tube with HNO_3 to be digested. The digested samples were filtered, and the filtered solution was used for analyzing P, K, Zn, and Pb by ICP-Optical Emission Spectrometry (iCAP 6200, Thermo Fisher Scientific Inc., Cambridge, UK).

2.2.9. Yield Components

At harvest (i.e., physiological maturity; 112 DAS), the weight of ear (g), grain yield per plant, and harvest index (HI) were analyzed from each plot unit. HI was calculated as follows:

$$\mathrm{HI} = \frac{Grains\ dry\ weight}{Biological\ dry\ yield} \times 100$$

2.3. Statistical Analysis

Raw data obtained from the effects of different NPs treatments on different traits of maize grown in Pb-stressed soil were subjected to analysis of variance (ANOVA) using PASW statistics 21.0 (IBM Inc., Chicago, IL, USA). Means of different treatments were compared using Tukey's multiple range test at a significant difference of $p \leq 0.05$.

3. Results

The results showed that leaf area of maize was significantly decreased by 50.9% when plants were grown in soil stressed with 100 mg Pb kg^{-1} DW soil compared to unstressed plants at 40 DAS (Table 1). However, in soil contaminated with Pb, the application of HP-NPs and ZnO-NPs significantly increased leaf area of maize by 42.2% and 59.5% compared to untreated plants, respectively. Furthermore, the highest increase (+103.3%) in leaf area was recorded from maize grown in soil treated with the combined application of HP-NPs (50 mg kg^{-1}) + ZnO-NPs (50 mg L^{-1}) in soil contaminated with Pb. At 70 DAS, maize grown in soil contaminated with 100 mg Pb kg^{-1} DW soil showed a reduction in leaf area by 55.5% compared to unstressed plants; however, combined application of HP-NPs + ZnO-NPs mitigated the adverse effects of Pb and increased leaf area of maize by 113.5% compared to stressed plants with Pb without the application of NPs (Table 1).

Table 1. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on leaf area (cm^2 $plant^{-1}$) of maize grown in soil contaminated with Pb.

Treatments	Traitst	Leaf Area (cm^2 $Plant^{-1}$)		
		40 DAS	**70 DAS**	**100 DAS**
Pb (0 mg kg^{-1})	0 NPs (Control)	2751 b	8259 b	7752 a
	HP-NPs (50 mg kg^{-1})	2894 a	8344 b	7756 a
	ZnO-NPs (50 mg L^{-1})	2938 a	8561 a	7866 a
	ZnO-NPs + HP-NPs	2953 a	8592 a	7884 a
Pb (100 mg kg^{-1})	0 NPs	1350 e	3677 f	3554 d
	HP-NPs (50 mg kg^{-1})	1927 d	6722 e	6311 c
	ZnO-NPs (50 mg kg^{-1})	2153 c	7125 d	6982 b
	ZnO-NPs + HP-NPs	2745 b	7850 c	7733 a
	$LSD_{0.05}$	75	105	145

LSD = least significant differences; DAS = days after sowing. Different letters within same column show significant differences at $p \leq 0.05$.

Maize was negatively affected in terms of total chlorophyll, net photosynthesis, and carotenoid pigments when grown in soil contaminated with Pb (Table 2). At 70 DAS, total chlorophyll content, net photosynthesis, and carotenoids pigments of maize grown in soil contaminated with Pb were reduced by 34.2, 70.1, and 45.3% in comparison to that obtained from non-contaminated soil (control), respectively. However, application of ZnO-NPs, HP-NPs, and/or their combination mitigated the adverse effects of Pb and enhanced total chlorophyll content, net photosynthesis, and carotenoid pigments. For instance, application of HP-NPs, ZnO-NPs and their combination in soil contaminated with Pb improved the total chlorophyll content by 26.6, 35.3, and 47.1% in comparison to that obtained from soil only contaminated with Pb, respectively. Furthermore, application of HP-NPs, ZnO-NPs and their combination in maize grown in Pb-contaminated soil enhanced photosynthesis rate by 148.6%, 191.6%, and 255.1% compared to that obtained from soil only contaminated with Pb, respectively. In Pb-contaminated soil, combination treatment of HP-NPs + ZnO-NPs enhanced carotenoid pigments by 78.8% in comparison to those obtained from only Pb-stressed soil.

Table 2. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on total chlorophyll (SPAD), photosynthesis (*Pn*), and stomatal conductance ($\underline{gs}$) of maize grown in soil contaminated with Pb.

Treatments	Traitst	Total Chlorophyll (SPAD)	*Pn* (μmol CO_2 m^{-2} s^{-1})	$\underline{gs}$ (mol H_2O m^{-2} s^{-1})
Pb (0 mg kg^{-1})	0 NPs (Control)	49.1 a	15.56 c	0.22 b
	HP-NPs (50 mg kg^{-1})	49.9 a	18.54 b	0.23 b
	ZnO-NPs (50 mg L^{-1})	50.4 a	20.54 a	0.29 a
	ZnO-NPs + HP-NPs	50.5 a	20.61 a	0.29 a
Pb (100 mg kg^{-1})	0 NPs	32.3 e	4.65 f	0.05 d
	HP-NPs (50 mg kg^{-1})	40.9 d	11.56 e	0.18 bc
	ZnO-NPs (50 mg kg^{-1})	43.7 c	13.56 d	0.21 b
	ZnO-NPs + HP-NPs	47.5 ab	16.51 c	0.24 b
	$LSD_{0.05}$	2.1	1.15	0.04

LSD = least significant differences. Different letters within same column show significant differences at $p \leq 0.05$.

A substantial reduction of 77.3% in stomatal conductance was recorded in maize grown in Pb-soil contaminated compared to those grown in uncontaminated soil (Table 2). Nevertheless, application of HP-NPs and ZnO-NPs decreased the adverse effects of Pb and enhanced the stomatal conductance by 260% and 320% compared to maize grown in only Pb-stressed soil, respectively. In addition, the combined application of HP-NPs + ZnO-NPs

resulted in highest increase (380%) in stomatal conductance in comparison to plants grown in only Pb-stressed soil.

Proline content in maize grown in soil contaminated with Pb was increased by 116% compared to the control (Table 3). However, application of HP-NPs, ZnO-NPs, and/or their combination reduced the proline content by 15.5, 31.1 and 48.3% compared to maize exposed to Pb stress, respectively. On the other hand, results showed that Pb stress increased the MDA by 143.1% in maize plants as compared to that obtained from control (Table 3). However, application of HP-NPs and ZnO-NPs significantly decreased MDA and the highest reduction (53.5%) was recorded when the combination of HP-NPs + ZnO-NPs was applied.

Table 3. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on proline, malonaldehyde (MDA), and carotenoids in maize grown in soil contaminated with lead (Pb).

Treatments / Traitst		Proline (mg g^{-1} DW)	MDA (μM g^{-1} FW)	Carotenoids (mg g^{-1} FW)
Pb (0 mg kg^{-1})	0 NPs (Control)	109.21 e	8.07 f	2.85 b
	HP-NPs (50 mg kg^{-1})	103.15 f	8.91 ef	2.82 b
	ZnO-NPs (50 mg L^{-1})	99.04 f	8.51 f	3.02 a
	ZnO-NPs + HP-NPs	97.58 f	8.56 f	3.08 a
Pb (100 mg kg^{-1})	0 NPs	235.87 a	19.62 a	1.56 e
	HP-NPs (50 mg kg^{-1})	199.25 b	12.58 b	2.15 d
	ZnO-NPs (50 mg kg^{-1})	162.58 c	11.85 c	2.39 c
	ZnO-NPs + HP-NPs	121.98 d	9.12 d	2.79 b
	LSD $_{0.05}$	4.52	0.46	0.07

LSD = least significant differences. Different letters within same column show significant differences at $p \leq 0.05$.

Enhancing antioxidants activity can control autoxidation by interrupting or by inhibiting the formation of free radicals and subsequently reducing oxidative stress, improving immune function, and increasing the longevity of pants under stressful conditions. In the current study, maize grown in Pb-stressed soil had higher activity of SOD (87.1%), POX (90.8%), and CAT (146%) than those obtained from maize grown in control (Table 4). The combined application of HP-NPs + ZnO-NPs reduced oxidative stress and, as a result, reduced the antioxidants activity by 42.3% for SOD, 43.8% for POX, and 55.2% for CAT in comparison to those obtained from plants grown in only Pb-stressed soil.

Table 4. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on antioxidant enzymes (superoxide dismutase (SOD), guaiacol peroxidase (POX), and catalase (CAT)) of maize grown in soil contaminated with lead (Pb).

Treatments / Traitst		SOD (unit g^{-1} FW)	POX (unit g^{-1} FW)	CAT (unit g^{-1} FW)
Pb (0 mg kg^{-1})	0 NPs (Control)	33.56 e	31.02 de	2.65 c
	HP-NPs (50 mg kg^{-1})	34.89 de	31.61 de	2.81 c
	ZnO-NPs (50 mg L^{-1})	33.97 e	30.36 e	2.75 c
	ZnO-NPs + HP-NPs	34.90 de	31.52 de	2.80 c
Pb (100 mg kg^{-1})	0 NPs	62.79 a	59.19 a	6.52 a
	HP-NPs (50 mg kg^{-1})	43.89 b	45.34 b	3.62 b
	ZnO-NPs (50 mg kg^{-1})	39.46 c	41.25 c	3.41 b
	ZnO-NPs + HP-NPs	36.21 de	33.27 d	2.92 c
	LSD $_{0.05}$	1.98	2.51	0.47

LSD = least significant differences. Different letters within same column show significant differences at $p \leq 0.05$.

N, P K, and Zn contents in whole plant biomass were significantly decreased due to Pb stress in the soil as compared to other treatments including the control, individual, and combinations of HP-NPs and ZnO-NPs treatments (Table 5). For instance, the stress of Pb reduced the content of N, P, K, and Zn by 38.7%, 69.9%, 46.9%, and 82.1% in comparison to that obtained from the control, respectively. However, there was an improvement in nutrients content when HP-NPs, ZnO-NPs, and their combinations were applied in Pb-contaminated soil. For example, the combined application of HP-NPs + ZnO-NPs to maize plants grown in soil contaminated with Pb resulted in an increment in content of N, P, K, and Zn by 58.8%, 217.4%, 73.4%, and 552.1%, respectively, compared to those only exposed to Pb stress. In contrast, maize grown in Pb-contaminated soil contained the highest Pb in plant biomass (13.47 mg kg^{-1}) and grains (5.11 mg kg^{-1}) in comparison to all other treatments. However, the combined application of HP-NPs + ZnO-NPs in contaminated soil reduced Pb uptake in plant biomass by 77.6% and grains by 90.21% in comparison with plants only exposed to Pb stress.

Table 5. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on nitrogen (N), phosphorus (P), potassium (K), zinc (Zn), and lead (Pb) in maize grown in soil contaminated with Pb.

Treatments	Traitst	N	P	K	Zn	Pb	Pb
		In Biomass (Above Ground)					In Grains
		(g kg^{-1} DM)				(mg kg^{-1} DM)	
Pb (0 mg kg^{-1})	0 NPs (Control)	16.39 a	1.53 a	7.84 a	40.23 c	0.00 d	0.00 d
	HP-NPs (50 mg kg^{-1})	16.52 a	1.75 a	7.55 a	42.09 c	0.02 d	0.00 d
	ZnO-NPs (50 mg L^{-1})	16.71 a	1.49 ab	7.56 a	51.21 a	0.05 d	0.00 d
	ZnO-NPs + HP-NPs	16.74 a	1.77 a	7.56 a	53.53 a	0.04 d	0.00 d
Pb (100 mg kg^{-1})	0 NPs	10.05 d	0.46 d	4.17 c	7.21 e	13.47 a	5.11 a
	HP-NPs (50 mg kg^{-1})	12.53 c	1.21 c	6.72 ab	35.40 d	7.25 b	1.74 b
	ZnO-NPs (50 mg kg^{-1})	12.99 c	1.07 c	6.99 a	45.96 b	6.26 b	1.57 b
	ZnO-NPs + HP-NPs	15.96 ab	1.46 ab	7.23 a	47.02 b	3.02 c	0.05 c
	$LSD_{0.05}$	0.62	0.23	0.95	2.05	1.21	0.24

LSD = least significant differences. Different letters within same column show significant differences at $p \leq 0.05$.

The ear weight, grain yield per ear, and harvest index of maize grown in Pb-stressed soil were significantly reduced by 41.1, 61.2, and 48.6% in comparison to those obtained from control, respectively (Figure 1). However, the application of the combined treatment of HP-NPs and ZnO-NPs mitigated the adverse effects of Pb and improved the ear weight, grain yield, and harvest index of maize plants. For instance, application of combined treatment of HP-NPs and ZnO-NPs in Pb-stressed soil (i.e., 100 mg kg^{-1}) resulted in a significant increase in ear weight, grain yield, and harvest index by 64.4%, 147%, and 88.9% in comparison to those obtained from only Pb-stressed soil, respectively.

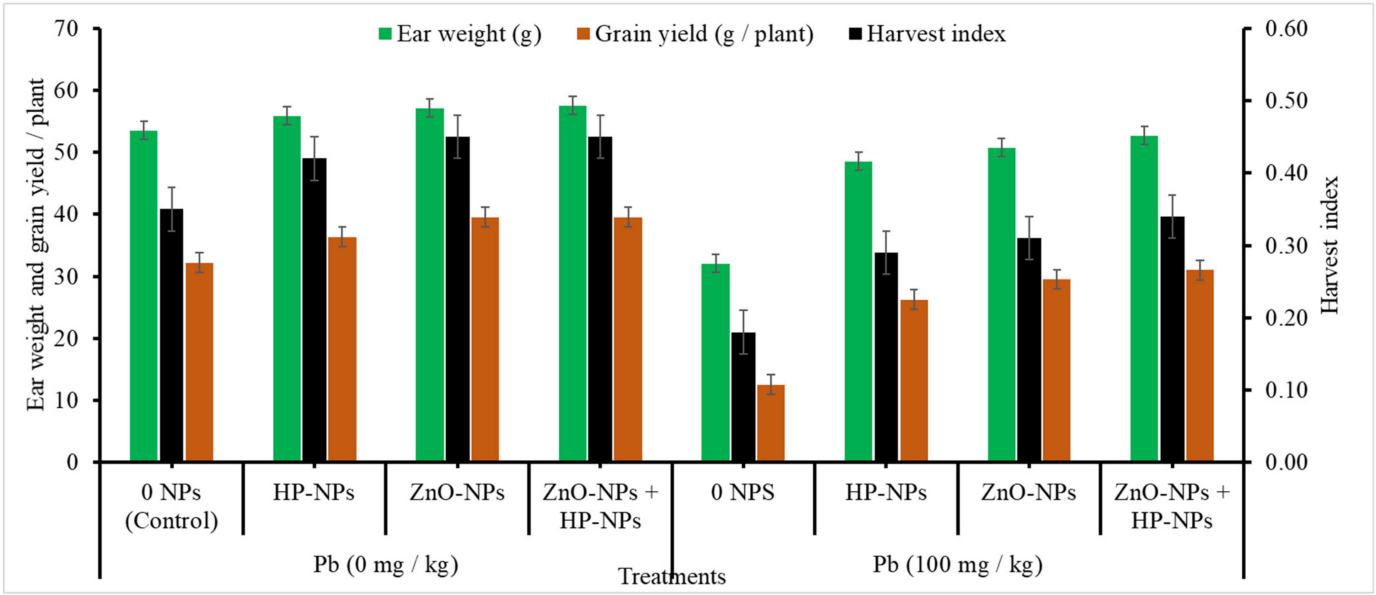

Figure 1. Effects of nano-hydroxyapatite NPs (NH NPs), zinc oxide NPs (ZnO-NPs), and their combinations on ear weight, grain yield, and harvest index of maize grown in soil contaminated with Pb. Bars = Least significant differences (LSD).

4. Discussion

(a) Plant growth and physiological performance

The current study showed that growth and physiological attributes such as leaf area (Table 1), total chlorophyll (SPAD values), rate of photosynthesis, and stomatal conductance (Table 2) were significantly reduced when maize plants were subjected to Pb stress as compared to non-stressed plants. Understandably, Pb, being a transition element, disrupts ionic balance at molecular level, which subsequently may distort cell ultrastructure, leading to impaired metabolic and physiological activities in plants. Sharma et al. [16] reported that Pb disrupted the biosynthesis of essential photosynthetic molecules such as chlorophyll, plastoquinone and carotenoids, in addition to chloroplast ultrastructures. According to Collin et al. [67], Pb-induced reduction in photosynthetic molecules is more related to its effect on nutrients uptake (i.e., Mg, Fe and Ca) and subsequently results in oxidative stress. However, the physiological impairment caused by Pb stress is more dependent upon cellular membrane integrity and permeability and water imbalance, which consequently results in disrupted cell cycle and growth [68]. High concentrations of Pb in plant leaves can disrupt photosynthesis by causing structural damage, disturbing chlorophyll synthesis, blocking electron transport, and closing stomata to reduce net CO_2 assimilation [69], and thus can adversely affect leaf development and leaf area of plants [68]. In addition, Pb can affect lipid composition in thylakoid membranes, chloroplast fine structure, grana stacks, and amount of stroma. Thus, it can decrease and/or inhibit PS-II. Moreover, Pb can affect the assimilation of CO_2 by affecting ribulose–bisphosphate carboxylase activity, which can result in a reduction in the utilization of ATP for CO_2 fixation. Therefore, a strong relationship exists between Pb and reduction in plant photosynthesis [16]. Pb can disrupt the electron transport chain and inhibit the photocatalytic activity of chlorophyll molecules and the Calvin cycle, resulting in excess of intercellular CO_2 which can lead to the closure of stomata [70,71]. The deleterious effects of Pb stress on plant growth, photosynthetic apparatus, and pigments were also reported in *Eichhornia crassipes* [72], *Robinia pseudoacacia* [73], *Coronopus didymus* [74], *Spinacia oleracea* and *Triticum aestivum* [75], *Vigna Radiata* [14], and *Jatropha curcas* [76].

In the current study, even though the application of nanoparticles (i.e., HP-NPs and ZnO-NPs) both individually as well as combined improved plant growth and physiological performance in maize as compared to control. However, the significant importance of HP-NPs and ZnO-NPs was clearly noted for leaf area, chlorophyll contents, rate of photosynthesis, and stomatal conductance in plants under Pb stress (Tables 1 and 2). The combination of HP-NPs and ZnO-NPs was followed by individual applications of ZnO-NPs and HP-NPs. Understandably, HP-NPs can reduce the movement and bioavailability of Pb in soil, and consequently can reduce the uptake of Pb by plant roots [48,52]. Furthermore, HP-NPs are effective remediators of HMs from the soil as well, as they can enhance the chlorophyll content and antioxidant system in plants [53,54]. Several studies have reported the efficacy of nano-hydroxyapatite (HP) to mitigate various HMs toxicity in plants such as *Lolium perenne* [48,52], *Solanum tuberosum* [51], and *Brassica chinensis* [54].

On the other hand, ZnO-NPs reduced Pb uptake and increased Zn uptake by maize grown in contaminated soil. The benefit of applying ZnO-NPs is that Zn can bind with Pb in the soil, and consequently can reduce its uptake by roots [39]. In addition, the application of ZnO-NPs can protect plants by reducing the phytotoxicity of Pb, increasing protein synthesis, enhancing activity of enzymes, and regulating photosynthetic machinery [28]. Therefore, it can result in better growth of maize grown in contaminated soil with metals stress. At the molecular level, Pb ions replace the central Mg^{2+} in the chlorophyll structure, thus impairing chlorophyll functionality [77,78]. However, NPs can increase the uptake of some elements that can restore chlorophyll molecules [79]. Furthermore, Pb can constrain chlorophyll formation by impairing the uptake of essential nutrients such as iron (Fe) and magnesium (Mg) by plants' roots [80,81]. Pb can damage the photosynthetic structures due to its affinity for protein's N- and S-ligands; thus, chlorophyll degradation can occur due to the increased chlorophyllase activity [17,82]. It is proposed that Pb can cause a strong dissociation of the oxygen-developing extrinsic polypeptide of PS-II and displacement of Cl, Mn, and Ca from the oxygen-evolving complex [83]. Likewise as our results, the role of ZnO-NPs in enhancing plants' tolerance against HMs stress was reported in several agricultural species, e.g., *Leucaena Leucocephala* [33], *Oryza Sativa* [28], etc.

The combined treatment of ZnO-NPs + HP-NPs increased the photosynthesis rate presumably by reducing the uptake of Pb and enhancing the efficiency of photosynthesis, as Zn is vital in the formation of photosynthetic pigments and can provide the energy through electron transport during light and dark reactions of photosynthesis [84].

(b) Stress indicators and antioxidant enzymatic activity

The proline and MDA contents are generally used as stress indicators in plants. Our results showed that the proline and MDA contents were significantly increased by the Pb stress in maize plants. Carotenoids are essential pigments and have a photoprotective function in plants (Table 3). The reduction in carotenoids content was recorded in maize plants exposed to Pb stress in the current study (Table 3). The significant reduction in the carotenoids content might be due to the reduction in the chlorophyll content since the uptake of essential nutrients (Fe, Mg, N) was reduced in maize grown in soil contaminated with Pb. Similarly, increased oxidative stress in chloroplasts due to Pb stress can severely reduce carotenoids content because carotenoids serve as antioxidants to scavenge or quench the free radicals in the chloroplast [53,85]. Similar findings were also reported in *Spinacia oleracea* and *Triticum aestivum* [75], *Jatropha curcas* [76], and *Gossypium herbaceum* [32].

Proline is a proteogenic amino acid that can act as a beneficial solute in plants, and its accumulation can increase in plants grown under stress conditions. Maize plants exposed to Pb stress had a high level of proline because it can contribute to stabilizing sub-cellular structures (e.g., membrane proteins), scavenges free radicals, and buffer cellular redox [86]. Thus, in response to HMs stress, plants can accumulate significant levels of proline, which can act as a heavy metal chelator, thereby alleviating heavy metals stress [87]. Proline can induce the formation of phytochelatins, which can chelate HMs, thereby decreasing their toxicity [88]. In the present study, the combined treatment of ZnO-NPs + HP-NPs resulted in a significant reduction in the uptake of Pb (Table 3), thus reducing plant stress, which resulted

in a lower proline production in plants as compared to control treatment. Moreover, MDA is the final product of lipids peroxidation and accumulates when plants suffer oxidative stress. Thus, the high level of MDA can cause an increment in the production of free radicals due to Pb-induced stress on maize plants. The application of ZnO-NPs + HP-NPs as a combination treatment alleviated the Pb toxicity by reducing its bioavailability and uptake. Such a result can cause less production of free radicals and consequently minimize the lipids membrane peroxidation and MDA content in plants. A number of recent studies have reported the effectiveness of nano-hydroxyapatite (HP) in immobilization of various HMs in contaminated soils and consequent reduction in antioxidant enzymatic activities in plants such as Pb in *Lolium perenne* [48,52] and Cd in *Triticum aestivum* [50], *Solanum tuberosum* [51], and *Brassica chinensis* [54]. Likewise, ZnO-NPs have also been reported as significant mitigator of HMs stresses in plants such as *Oryza sativa* [28] and *Leucaena leucocephala* [33].

There was a considerable increase in the antioxidants in maize plants when they were exposed to Pb stress in comparison to those obtained from other treatments [Table 4]. The increased level of antioxidants in plants can be due to the enhanced production of reactive oxygen species such as hydroxyl radical (-OH), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). Lipid peroxidation is an indicator of oxidative damage, and Pb ions can enhance lipid peroxidation, reduce the saturated fatty acids, and enhance the unsaturated fatty acids of the membrane [16,53]. Pb is not an oxide-reducing metal, such as Fe; thus it can lead to the production of ROS by enhancing the pro-oxidant status of cells [89]. In response to oxidative stress, plants have a comprehensive homeostatic mechanism that can serve to eliminate ROS. Increased POD activity in response to Pb stress can be associated with the release of POD localized in the cell walls due to de novo synthesis of enzymatic proteins [90]. Shu et al. [76] reported nearly similar results when *Jatropha curcas* L. seedlings were exposed to Pb (i.e., 0.5, 1, 2, and 3 mM kg^{-1}). An overproduction of ROS and higher activity of antioxidant enzymes were reported when *Coronopus didymus* was exposed to intensive Pb stress (i.e., 500, 900, 1800, and 2900 mg kg^{-1}) [74]. Lamhamdi et al. [75] reported high antioxidant activity in *Spinacia oleracea* and *Triticum aestivum* when exposed to Pb concentrations of 1.5, 3, and 15 mM.

The combination treatment of ZnO-NPs + HP-NPs were significantly effective in reducing the concentration of antioxidant enzymes (Table 4). It probably reduces the uptake of Pb and its accumulation in plant cells, which can result in lower expression of stress-induced genes [38] and lower the antioxidants production in plants compared to those obtained from soil contaminated with Pb. The individual applications of ZnO-NPs were found better than HP-NPs in reducing the SOD and POX contents in maize plants under Pb stress (Table 4). Akhtar et al. [42] reported that under HMs stress, ZnO-NPs can increase glycolytic metabolism and cell wall biosynthesis, which can result in better growth. Priyanka et al. [32] reported that ZnO-NPs in *Gossypium herbaceum* effectively mitigated Pb and Cd toxicity by regulating antioxidant enzymes. Similarly, in *Oryza sativa* the application of ZnO-NPs along with salicylic acid managed arsenic (As) toxicity by significantly improving redox status and the antioxidant defense mechanism at cellular level [28]. Nearly similar findings were also reported by Majeed et al. [34] which highlighted the role of ZnO-NPs in minimizing the activity of the antioxidant system by adsorbing and immobilization of HMs ions in the cell wall of plant cells. Furthermore, the HP-NPs have significantly been known for immobilization of HMs in soil and hence could lower the toxicity in the root zone. Recently, Jin et al. [52] reported that HP-NPs significantly managed plant growth in *Lolium perenne* grown in Pb-contaminated soil where HP-NPs minimized the bioavailability of Pb and hence lowered the antioxidant enzymatic activity in roots. Liu et al. [51] reported similar results for Cd in *Solanum tuberosum*, as well as Ding et al. [48] for Pb in *Lolium perenne* and Li and Huang [54] in *Brassica chinensis* for Cd.

(c) Mineral ions uptake

Maize plants exposed to Pb showed lower uptake of nutrients such as N, P, K, and Zn [Table 5]. The high concentration of Pb in soil can cause mineral imbalance in the soil solution and plant tissues. Previous studies have revealed that Pb can cause significant changes in nutrients content as well as the internal ratio of nutrients in plants [67]. In most cases, Pb can block the entry of cations such as K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{3+} and anions (NO_3^-) in plants' root systems [16]. Pb can induce disorder in the cell metabolism that can lead to changes in enzyme activity in the membrane structure. For example, efflux of K^+ from the root can occur due to the extreme sensitivity of K^+-ATPase and –SH groups of a cell membrane protein to Pb [16]. Pb can block the access of various nutrients from the absorption sites of the roots by changing levels of mineral elements in the roots. The study of Walker et al. [91] revealed that Pb decreased the uptake of K, Ca, Mg, Fe, N, and P in maize and cucumber (*Cucumis sativus* L.). Pb can reduce the N content in plants by reducing the nitrate reductase activity and N metabolism [92]. Similar findings were also reported by Lamhamdi et al. [75] in *Spinacia oleracea* and *Triticum aestivum*, Ashraf et al. [14] in *Vigna radiata*, and by Walker et al. [91] in *Zea mays*.

In the present study, the combined application of ZnO-NPs + HP-NPs, followed by ZnO-NPs and HP-NPs, can enhance the uptake of total protein–nitrogen content in plants (Table 5). It is presumed that the apoplastic translocation of Pb is involved in the transmembrane movement of thiol compounds and mineral ions [93]. Plants can overcome the HMs toxicity by limiting their mobility using glutathione, thiols, and cysteine-rich metal-binding polypeptides in root hairs [49]. It suggests that ZnO-NPs could possibly alleviate Pb toxicity by promoting the synthesis of thiol compounds. Thus, by speeding up thiol metabolism and lignin formation, ZnO-NPs can reduce the toxicity of HMs such as Pb and Cd by inhibiting the movement of HMs from roots to leaves [94,95]. Moreover, ZnO-NPs have the capability to increase phenylalanine ammonia lyase (PAL) activity which catalyzes the conversion of phenylalanine to lignin. Thus, higher lignification minimizes HMs entry to cytoplasm by enhancing the plastid barrier and reducing cell wall permeability [94,96]. Furthermore, lignin in the root cell wall anchors HMs by providing binding sites which can effectively prevent HMs translocation [97]. Since Pb uses Zn membrane transport proteins for membrane mobility and uptake, ZnO-NPs mitigate the impact of Pb toxicity by blocking the entry of Pb uptake [30]. The application of ZnO-NPs can support the plants by the uptake of Zn, Mn, and Cu in the roots; thus, it can increase the plant growth under HMs stress [40]. Similar results were also reported by Faizan et al. [28], Venkatachalam et al. [33], Majeed et al. [34], Gao et al. [94], and Emamverdian et al. [96] for various heavy metals toxicity in contaminated soils where the applications of ZnO-NPs effectively improved plant health by regulating nutrients uptake and osmotic balance.

The present study demonstrated that the application of HP-NPs in combination with ZnO-NPs decreased the uptake of Pb in maize. HP-NPs can significantly reduce the Pb mobility and availability in soil [15,48]. The immobilization of Pb by hydroxyapatite (HAp) is administered by two different mechanisms [98]: firstly, the dissolution of HAp followed by a phosphate reaction with dissolved Pb^{2+} with subsequent pure hydroxypyromorphite (HP) precipitation [99,100]; secondly, the exchange of Pb^{2+} with the Ca^{2+} on the Hap lattice [101,102]. Ding et al. [48] reported that HP-NPs have a larger surface area and increase the process of dissolution in soil solution, then release the phosphate ions. Phosphate ions and Pb^{2+} in the soil solution make lead phosphate—a compound of low solubility. Thus, the application of HP-NPs could alleviate the biotoxicity of Pb and lower its mobility in soil to ensure the healthy growth and development of plants [48]. According to Yan et al. [49], HP-NPs lower the Pb toxicity in contaminated soils by immobilization of Pb^{2+} ions rather than directly improving plant tolerance. Similarly, Feng et al. [50] called HP-NPs as "passive mitigators" of Cd stress in alkaline soils. Liu et al. [51], Jin et al. [52] and Li et al. [54] also supported this idea for *Solanum tuberosum, Lolium perenne*, and *Brassica chinensis*, respectively.

(d) Yield and yield attributes

In the current study, the ear weight, grain yield, and harvest index of maize were significantly reduced in soil contaminated with Pb (Figure 1). This might be due to the reduction in the uptake of nutrients such as Zn, Fe, N, and Mg as a result of Pb stress. Such stress from Pb can cause an imbalance of the mineral nutrients in the plant tissues and consequently can reduce the leaf area, ear weight, grain yield, and harvest index of maize. Ashraf et al. [78] reported that Pb toxicity in contaminated soils significantly affected grain yield, quality and production of *Oryza sativa*. Sofy et al. [60] highlighted the negative effects of Pb on maize's growth, yield, and grain quality. They added that Pb-induced decline in mineral uptake, photosynthetic pigments, growth, and biomass production resulted in consequent lower yield in Pb-stressed plants as compared to unstressed plants. Moreover, Hussain et al. [103] correlated Pb-induced reduction in quality and grain yield with deteriorated plant growth in *Oryza sativa*. However, the application of ZnO-NPs into soil contaminated with Pb might support maize to uptake some nutrients such as Zn, Mn, and Cu and improve the growth of maize grown under HMs stress [40]. Furthermore, the application of HP-NPs can mitigate the negative effects of Pb on plant growth by increasing the soil pH and decreasing the solubility and mobility of HMs [52]. Thus, the combined application of ZnO-NPs + HP-NPs resulted in an increment in the uptake of mineral nutrients by decreasing the uptake of Pb that can improve ear weight, grain yield, and harvest index of maize (Figure 1). The soil application of HP-NPs causes immobilization [49], minimizes bioavailability [48], reduces root uptake [52], and improves soil microbial activity [50] of HMs, which consequently alleviates oxidative stress in plants and enhances physiological performance, growth, and yield [99–101]. On the other hand, ZnO-NPs application in plants successfully mitigates HMs toxicity by regulating mineral uptake [33,34], improving photosynthetic processes [28], and enhancing physiological performance, ultimately resulting in better quality and higher yield [94,96].

5. Conclusions

The combined application of ZnO-NPs + HP-NPs was found to be the most suitable and best treatment in comparison to other treatments for improving the growth and increasing the yield of maize by mitigating Pb stress. The total chlorophyll, carotenoids, photosynthesis rate, and stomatal decrease in maize exposed to Pb stress were significantly reduced while the application of ZnO-NPs + HP-NPs as a combined treatment enhanced most of the growth, physiological, yield, and elemental profile of maize grown in soil contaminated with Pb. Similarly, the activity of antioxidants enzymes such as SOD, CAT, and POX and proline content were significantly increased due to Pb stress. Similarly, MDA content was higher in plants grown under Pb stress, while ZnO-NPs + HP-NPs as a combination application reduced the production of MDA by reducing the uptake of Pb. The uptake of nutrients such as N, P, K, and Zn was decreased in plants grown under Pb stress, while ZnO-NPs + HP-NPs increased the uptake of these nutrients in whole plant biomass. In conclusion, such results strongly suggest that the application of ZnO-NPs + HP-NPs as one combination treatment each at the rate of 50 mg kg^{-1} of soil can mitigate Pb stress or toxicity by modulation of physiochemical traits in maize plants.

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