FOLIAR APPLICATION OF ZINC OXIDE NANOPARTICLES AMELIORATED THE ADVERSE EFFECTS OF COBALT TOXICITY IN *RAPHANUS SATIVUS* BY REGULATING GROWTH, PHOTOSYNTHESIS AND ANTIOXIDANT DEFENSE MACHINERY

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ABSTRACT: Heavy metal toxicity exert oxidative stress in plants by generating reactive oxygen species (ROS), which in turn cause damage to proteins, lipids, nucleic acids, and disrupt the structural integrity of mitochondria and chloroplasts. In this study, the potential of zinc oxide nanoparticles (ZnO NPs) for alleviation of cobalt (Co) toxicity (100 mg kg^{-1}) in radish plants was explored. Foliar application of ZnO NPs (20, 40, and 80 mg L^{-1}) significantly enhanced the growth attributes and photosynthetic pigments of *Raphanus sativus* during both stressed and non-stressed conditions. Under normal conditions, the ZnO NPs (80 mg L^{-1}) increased the root and shoot fresh biomass, carotenoids, chlorophyll *a*, and chlorophyll *b* levels by 49%, 56%, 29%, 45%, and 61%, respectively than nontreated control. Nevertheless, Co toxicity remarkably reduced growth parameters and photosynthetic attributes while enhanced the levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), antioxidant enzymes, phenol, proline and flavonoids in *R. sativus*. Whereas, foliar application of ZnO NPs (80 mg L^{-1}), enhanced the quantities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), proline, phenol and flavonoids by 23%, 42%, 34%, 29%, 25% and 39% respectively in *R. sativus* subjected to Co stress. Additionally, ZnO NPs (80 mg L^{-1}) significantly decreased the MDA, H_2O_2, and Co uptake in the roots and shoots of *R. sativus*. The outcomes of the present study demonstrate the promising potential of ZnO NPs as an eco-friendly approach for sustainable cultivation of vegetables in cobalt-contaminated soils.

Keywords: *Metal toxicity, Lipid peroxidation, Oxidative stress, Phytostabilization, Radish, Zn-based nanoparticles.*

INTRODUCTION

Heavy metal pollution has become an alarming issue in developing countries posing serious threat to soil fertility and agricultural production. Heavy metals (HMs) retain an atomic number higher than 20 and a density greater than 5 g cm^{-3}. The prevalence of heavy metals has increased significantly due to human induced activities such as mining, industrial discharges, excessive fertilizer use and urban runoff (Raychaudhuri *et al*. 2021). The buildup of these toxic heavy metals in soils and streams puts human health and biodiversity at risk as they may reach the food chain. (Chakraborty *et al*., 2022). HMs exposure results in oxidative stress in plants, disrupts photosynthesis and nutrient uptake, and prevents roots and shoots growth by producing excessive reactive oxygen species (ROS), that injure membranes and cellular components (Mahey *et al*., 2020).

Cobalt is a brittle, shiny, greyish-silver metal. Co is hard, durable, and has a polish surface. Cobalt (Co), with an atomic mass of 58.93 amu, is considered a component of vitamin B12 and aids in nitrogen fixation in legume plants. Higher dose of Co induce toxicity in crops that leads to oxidative stress, disrupting mitochondrial and chloroplast function. In humans, cobalt exposure is associated with cardiomyopathy, thyroid disorders, and cancer, underscoring the importance of managing soil contamination (Uddin *et al*., 2020). Conventional physical and chemical methods, including soil excavation, leaching, vitrification, and chemical immobilization, are often costly, labor-intensive, and potentially harmful due to their non-selective mechanisms and secondary pollution (Ali *et al*., 2018). The metal's availability to plants and their toxicity levels limit the application of phytoremediation, which employs plants to extract or stabilize toxins. Nevertheless, phytoremediation is affordable and eco-friendly alternative for decontamination of Co infested soil. (Petavratzi *et al*., 2019).

Zinc oxide nano-particles (ZnO NPs) are less than 100 nm in size, have revealed a lot of promise in reducing the heavy metal toxicity in vegetables. Zinc is a necessary micronutrient that influences enzyme activity, protein metabolism, and DNA synthesis. By supporting antioxidant enzymes like catalase and superoxide dismutase, which reduce ROS and maintain cellular integrity, ZnO NPs have enormous capability to mitigate the hazards of toxic metals in crops. Additionally, by binding to toxic ions in the rhizosphere, ZnO NPs can restrict their uptake (Broadley *et al*., 2007). However, their effectiveness varies with dosage; low concentrations encourage plant growth and stress tolerance, while high concentrations of ZnO NPs can result in oxidative damage and genotoxicity. Hence, careful dose selection and application methods of ZnO NPs are essential for sustainable cultivation of vegetables under toxic metal stress regimes (Salam *et al*., 2022).

Raphanus sativus, is an important vegetable that provides vital essential nutrients to humans (Sharma *et al*., 2025). *R. sativus* is an edible root crop and may be easily raised in greenhouse or field conditions. Research on radish is often useful in broader agricultural contexts because the findings can often be generalized to other root vegetables. The main purpose of the current trial was (i) To investigate the phytotoxic outcomes of cobalt on growth, physiological, biochemical, and oxidative stress-related parameters of *R. sativus*. (ii) To decipher the potential of ZnO NPs for imparting cobalt stress resilience in *R. sativus*. (iii) Unravelling the capability of ZnO NPs for immobilization of Co in the roots and shoots of radish. The present findings will support the non-toxic and environment-friendly use of nanotechnology for the cultivation of vegetables in cobalt-contaminated soils.

METHODOLOGY

A. Pot Experiment: A pot experiment was carried out at the Greenhouse of the College of Earth and Environmental Science (CEES), University of the Punjab (PU), Lahore (31.5204°North, 74.3587°East). Cobalt chloride ($CoCl_2$) was sourced from the CEES chemical store, Punjab University, Lahore. The Co stress was amended in soil keeping the dose at 100 mg kg^{-1} soil and placed in glasshouse of CEES for two weeks for homogenous mixing and aging of Co in soil (Jayakumar *et al*., 2007). The field capacity of Co-amended soil was maintained by watering at regular intervals during aging time span. The experiment included two treatment types: one with cobalt (Cobalt, Co+ZnO-NPs at 20, 40, and 80 mg L^{-1}) and one without cobalt (Control, ZnO-NPs at 20, 40, and 80 mg L^{-1}). The seeds (8-10) sown in pots containing 1.5 kg soil on November 3, 2024, and afterwards thinned to three seedlings per pot. ZnO NPs were foliarly applied twice, first dose 15 days after seedling emergence and second dose 30 days later (Semida *et al*., 2021). Radish seeds were purchased from local market. After two months, plants were harvested, and growth parameters were recorded.

B. Assessment of photosynthetic traits of *R. sativus* subjected to Co stress: Assessment of chlorophyll and carotenoids content was carried out in Radish seedlings grown under Co stressed and non stress circumtances. Mature leaves (0.5g) were crused in liquid nitrtogen and extarcted with 80% ethanol. Crushed samples were centrifuged to obtain supernatant. The absorbance of spectrophotometer was observed at 663nm, 645nmm and 480 nm for estimating Chl. *a, b* and carotenoids contents (Arnon, 1949).

C. Evaluation of flavonoid content of *R. sativus* exposed to Co stress: Aluminum chloride colorimetric method was used to measure the flavonoid content as described previously by Chang *et al*. (2002). Plant extracts were homogenized with deionized water, sodium nitrite and allowed to stand for 6 mins. Afterwards aluminum chloride was added to the reaction mixture followed by sodium hydroxide addition. Then we recorded the absorbance at 510nm.

D. Assessment of phenolic contents in *R. sativus* exposed to Co stress: For determination of total phenolic contents, one gram plant material was crushed in 3mL of 80% methanol (Pavel *et al*., 2006). After crushed material's centrifugation, 1 mL of clear plant extract was mixed with 5 mL of deionized water, 1 mL of 5% Folin reagent and 1 mL of 20% sodium carbonate. For 30 mins, the reaction mixture was heated at 40°C using water bath. The optical density was recorded at 765 nm.

E. Determination of proline level of *R. sativus* subjected to Co toxicity: Proline contents in leaf sample *R. sativus* s of was quantified using protocol described by Bates *et al* (1973). Briefly, 1 g plant tissue was crushed in sulfosalicylic acid (3%) and then filtered. The resuting filtrate was added to the solution of glacial acetic acid and acid ninhydrin, then incubated at 100 °C in water bath for 1 hour. Afterwards icebath was used to terminate the reaction. Toluene was used to extract the chromophore. By using spectrophotometer, the absorbance of toluene layer was measured at 520nm.

F. Estimation of antioxidant enzymes (SOD, CAT, POD) of *R. sativus* cultivated in Co stressed soil: Enzyme extract was prepared by crushing 0.5 g plant sample in 50 mM phosphate buffer containing 1% polyvinylpolypyrrolidone (PVP) and 0.2 mM EDTA using a pre chilled pestle and mortar. The homogenate was centrifuged at 12,000×*g* for 20 mins at 4 °C. The resulting supernatant was collected and stored for enzymatic activity assays. Catalase activity was quantified using the method of Aebi (1974) by monitoring the consumption of H_2O_2 for 1 min at 240nm. Peroxidase activity was quantified using the protocol described by Egley *et al*. (1983) by measuring the rate of guaiacol oxidation. Superoxide dismutase activity was analyzed at 560 nm spectrophotometrically, based on the amount of the enzyme required to inhibit 50% nitroblue tetrazolium (NBT) photochemical reduction (Beauchamp and Fridovich, 1971).

G. Estimation of malondialdehyde and hydrogen peroxide in *R. sativus* exposed to Co stress: The effects of ZnO nanoparticles on oxidative damage in Radish plant exposed to cobalt stress were assessed on the basis of malondialdehyde (MDA) concentration and hydrogen peroxide (H_2O_2). MDA content was examined according to the protocol described by Heath and Packer (1968) while, absorbance was measured at 450nm, 532nm and 600nm spectrophotometrically. By using Velikova at al (2000) method, Hydrogen peroxide (H_2O_2) concentration in leaf samples was determined . Absorbance of the mixture was observed at 390nm.

H. Analysis of Cobalt content in the root and shoot of *R. sativus*: Cobalt (Co) content was analyzed by digesting 1 gram of finely ground, dried plant material with 5 mL of perchloric acid ($HClO_4$) and 10 mL of nitric acid (HNO). The mixture was transferred to a digestion flask and kept on a hot plate at 150 °C till getting a clear solution with its volume reduced to about 5 mL. After cooling, the solution was filtered using whatman filter paper and diluted to a total volume of 50 mL with distilled water (Wolf, 1982). The cobalt content in the final solution was measured using an atomic absorption spectrophotometer (AAS).

The plant's tolerance to cobalt was assessed by calculating the Metal Tolerance Index (MTI) using the following equation:

$$MTI = \frac{\text{Mass (g) of Treated Plant}}{\text{Mass of Control Plant}} \times 100$$

The translocation was analyzed as described by (Daraz *et al*., 2023).

$$TF = \frac{\text{Cobalt contents in the shoot}}{\text{Cobalt contents in root}}$$

The bio-concentration factor was evaluated by:

$$BCF = \frac{\text{Co contents in Shoot}}{\text{Co contents in Soil}}$$

I. Statistical analysis: During current study the treatments were arranged as per completely randomized design (CRD) randomized layout, with three replications. Data handling and interpretation was performed using DSAASTAT software. Each replication also included 3 individual plants. By using a one-way analysis of variance (ANOVA), statistical comparisons were made and significance among treatments was determined using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. Pearson correlation and principal component analysis were performed using Origin software (2018).

RESULTS

A. **Effect of ZnO NPs on growth traits of *R. sativus* subjected to Co toxicity:** When grown in contaminated soil, radish plant experiences stress. This is evident from the relative growth characteristics such as the shoot length (SL), root length (RL), shoot fresh weight (shoot FW), root fresh weight (root FW), root dry weight (root DW), and dry biomass of shoot (shoot DW) shown in table1. The analysis of plant growth parameters relative to the control (C) reveals significant variations due to different treatments. The *R. sativus* root length increased by 35%, 26% and 17% in unstressed soil during ZnO NP 80, 40, 20 mgL^{-1} concentration supply, compared to related control. The Co stress reduced root length by 36% thant that of control. Radish cultivated in Co-contaminated soil exhibits a 15%, 24%, and 30% increase in root length, respectively, when treated with ZnO NP 20, 40, and 80 mg L^{-1} in comparison to the relevant control. This indicates that the zinc treatment promotes root development, which may lead to better absorption of nutrients and water. The ZnO NP 80 mg L^{-1} led with a 50% increase in number of leaves of radish grown under non-stress conditions, a 40% and 25% increase by ZnO NP 40 mg L^{-1} and ZnO NP 20 mg L^{-1} respectivel. More leaves indicate the enhanced photosynthetic capacity and production of energy in unstressed control of radish. Co stress significantly reduced the number of leaves in *R. sativus* by 34% compared to control. Less leaves indicate the limited photosynthetic activity. When the zinc treatment (20, 40 and 80 mg L^{-1}) applied together with the stress, the number of leaves of radish boosted to 20%, 43%, 50% respectively (Table 1). Analogous trend was showed in the number of roots of ZnO NPs supplied *R. sativus* under Co stressed conditions.

The foliar application with ZnO NP 80 mg L^{-1}, 40 mg L^{-1}, 20 mg L^{-1} boosted the fresh weight of root of radish by 59%, 44%, and 28% correspondingly as related to the concerned control. Increased root biomass accelerated the capability of plant to absorb H_2O and nutrients. The Co stress caused reduction in root fresh weight of *R. sativus* by 39% than that of untreated control. The dose of ZnO NP 20 mg L^{-1}, 40 mg L^{-1}, 80 mg L^{-1} enhanced the fresh weight (root) of radish grown in Co contaminated soil by 31%, 50%, and 61% respectively with respect to relevant control. A similar trend was followed for the shoot fresh weight in ZnO NPs assisted radish under Co stress (Table 1).

The ZnO NP at the concentration of 80 mg L^{-1}, 40 mg L^{-1}, 20 mg L^{-1} showed an increase of 64%, 50%, and 28% increment in root dry weight of radish plant under unstressed conditions. Larger root dry weight indicates the presence of complex root biomass and structural integrity. Co stress caused the reduction in root dry weight of radish by 56% as compared to the untreated control. Low root dry biomass showed the presence of low root biomass. The exogenous supply of ZnO NP (20, 40, 80) mg L^{-1} increase the root dry mass by 34%, 64%, and 73% respectively in *R. sativus* grown in soil amended with Co in comparison with relevant control. A similar trend was followed for the shoot dry weight in ZnO NPs assisted radish plant during Co stressed environment (Table 1).

Table 1: Influence of ZnO-NPs on the growth features of *R. sativus* grown in Co-contaminated soil

Treatments	Growth characteristics						
	Root Length	Shoot Length	Leaves	Root FW	Shoot FW	Root DW	Shoot DW
	cm	cm		g	g	g	g
C	$5.40±0.26^{abcd}$	$14±0.7^{cde}$	$6±1^{cd}$	$0.47±0.02^{f}$	$8.18±0.38^{d}$	$0.18±0.01^{f}$	$2.76±0.1^{d}$
ZnO NP 20	$6.00±0.29^{abc}$	$15±0.74^{c}$	$8±1.5^{bc}$	$0.65±0.03^{d}$	$9.84±0.48^{c}$	$0.25±0.01^{d}$	$3.21±0.1^{c}$
ZnO NP 40	$6.50±0.31^{ab}$	$17±0.84^{b}$	$10±1.5^{ab}$	$0.83±0.03^{b}$	$12.5±0.60^{b}$	$0.36±0.01^{b}$	$4.58±0.2^{b}$
ZnO NP 80	$7.30±0.36^{a}$	$22±1.07^{a}$	$12±1.5^{a}$	$1.12±0.05^{a}$	$18.2±0.85^{a}$	$0.49±0.02^{a}$	$6.23±0.3^{a}$
Co	$3.50±0.13^{d}$	$10.7±0.53^{f}$	$4±0.5^{e}$	$0.29±0.01^{g}$	$4.58±0.21^{f}$	$0.08±0.03^{g}$	$1.56±0.1^{e}$
Co+ ZnO NP 20	$4.09±0.16^{cd}$	$12.6±0.63^{e}$	$5±1^{de}$	$0.42±0.02^{g}$	$6.10±0.29^{e}$	$0.12±0.01^{g}$	$2.07±0.1^{e}$
Co+ ZnO NP 40	$4.6±0.19^{bcd}$	$13.4±0.66^{de}$	$7±1^{cd}$	$0.58±0.02^{e}$	$9.45±0.45^{c}$	$0.22±0.01^{e}$	$2.85±0.1^{d}$
Co+ ZnO NP 80	$5.01±0.23^{abcd}$	$14.3±0.71^{cd}$	$8±1^{bc}$	$0.73±0.03^{c}$	$12.2±0.60^{b}$	$0.29±0.01^{c}$	$3.14±0.1^{c}$

The values are the three replicates' means ± SD. According to DMRT at p≤ 0.05, different letters in a column, indicate significant difference among treatments. ZnO-NPs 20, 40, 80 mg L^{-1}: Zinc oxide nanoparticles at concentrations of 20, 40, and 80 mg L^{-1}; C: Untreated Control; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80 mg L^{-1}. Cobalt and ZnO nanoparticles added to soil at concentrations of 20, 40, and 80 mg L^{-1}; DW refers to dry weight; FW stands for fresh weight.

B. **Influence of ZnO NPS on chlorophyll and carotenoid contents of *R. sativus* exposed to Co toxicity:** The ZnO NP at the conc. of 20 mg L^{-1}, 40 mg L^{-1} and 80 mg L^{-1} showed an enhancement in the chlorophyll a content by 29%, 32% and 25% respectively in radish under without Co stress in contrast with related control. Higher the levels of chlorophyll a, enhanced the capacity of photosynthesis of plants. Co stress reduced the Chlorophyll a content of radish by 35% with respect to control. Reduction in the Chlorophyll a showed the limited photosynthetic capacity of radish. The ZnO NP 20 mg L^{-1}, 40 mg L^{-1}, and 80 mg L^{-1} application enhanced the total chlorophyll content by 11%, 20%, and 29%, respectively in radish under Co stress compared to relevant control. A similar pattern was observed in the amount of chlorophyll b in ZnO NPs sprayed radish plants cultivated in soil polluted with cobalt (Table 2).

Results exhibited that ZnO NP 20 mg L^{-1}, 40 mg L^{-1} and 80mg L^{-1} increased the level of chlorophyll b by 21%, 25% and 28% respectively in radish under no Co stress corresponding to the unstressed control. Higher total chlorophyll indicates the plant better health and photosynthesis capacity. Co stress lowered the total chlorophyll b content of radish by 40% as linked to the un-polluted control. Reduction in the Chlorophyll b showed the poor plant health and growth. The dose of ZnO NP 20, 40, 80 mg L^{-1} boosted the total chlorophyll b content of radish grown in Co contaminated soil by 15%, 31% and 40% respectively with respect to related control (Table 2).

The exogenous ZnO NP (80, 40, and 20) mg L^{-1} supply boosted the radish's total carotenoids content by 61%, 52%, and 35%, respectively under no toxic scenario. In comparison to the untreated control, the total carotenoid concentration of radish was 61% lower after co-stress. The foliar doses of ZnO NP 20 mg L^{-1}, 40 mg L^{-1}, and 80 mg L^{-1} escalated the total carotenoid contents by 50%, 66%, and 75% respectively, in radish exposed to Co toxicity in contrast with relevant control (Table 2).

The numbers are Means ± SD of three replicates. Different letters in a column indicate significant difference among the treatments according to DMRT at p≤ 0.05. Chl. a: Chlorophyll a; Chl b: Chlorophyll b; Total Chl: Total Chlorophyll; ZnO-NPs 20, 40, 80: Zinc oxide nanoparticles at concentrations of 20, 40, and 80 mg L^{-1}; C: Untreated Control; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80: Cobalt and zinc oxide nanoparticles added to soil at concentrations of 20, 40, and 80 mg L^{-1}.

C. **Impact of ZnO NPS on catalase (CAT), peroxidase (POD) and super oxide dismutase (SOD) levels of *R. sativus* exposed to Co stress:** In unstressed condition, ZnO NP 80, 40 and 20 mg L^{-1} showed the elevated SOD concentration by 24%, 17% and 12% respectively that that of untreated control. The Co stress increased the SOD level by 36% than that of untreated control. The doses of ZnO NP 20, 40, 80 mg L^{-1} increased SOD concentration of radish in Co contaminated soil by 10%, 19%, and 24% respectively in contrast with relevant control (Figure 1).

Table 2: Consequences of Zinc oxide nanoparticles on Chlorophyll *a*, Chlorophyll *b*, Total Chlorophyll, and Carotenoid contents of *R. sativus* grown in Cobalt contaminated soil.

Treatment	Biochemical attributes			
	Chl. *a*	Chl. *b*	Total Chl.	Carotenoids
	mg/g FW	mg/g FW	mg/g FW	mg/g FW
C	1.99± 0.09^{c}	0.97±0.04^{e}	2.96 ±0.7	0.32±0.01^{f}
ZnO NP 20	2.17±0.1^{b}	1.28±0.06^{c}	3.45±0.6	0.46±0.02^{d}
ZnO NPs 40	2.26±0.1^{ab}	1.48±0.07^{b}	3.74±0.5	0.75±0.03^{b}
ZnO-NP 80	2.35±0.1^{a}	1.76±0.08^{a}	4.1±0.4	0.82±0.03^{a}
Co	1.307±0.06^{e}	0.49±0.02^{f}	1.79±0.5	0.12±0.01^{h}
Co+ ZnO NP20	1.46±0.07^{e}	0.63±0.03^{f}	2.09±0.5	0.247±0.01^{g}
Co+ ZnO NP40	1.62±0.08^{e}	0.95±0.04^{e}	2.57±0.4	0.36±0.01^{e}
Co+ ZnO NP80	1.84±0.08^{d}	1.12±0.05^{d}	2.96±0.5	0.485±0.02^{c}

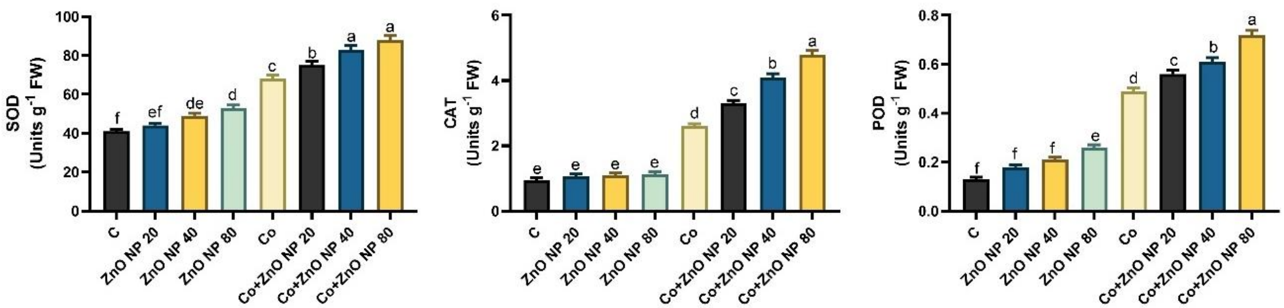

Figure 1: Effects of Zinc Oxide nanoparticles on superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) levels of *R. sativus* under Co stress. Different letters on the bars show significant difference among treatments according to DMRT at *p*≤ 0.05. C: Control; ZnO-NPs 80, 20, 40: Zinc oxide nanoparticles at 80, 20 and 40 mg L^{-1}; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80 mg L^{-1}: Soil amended by Cobalt and Zinc oxide nanoparticles at 20, 40, 80 mg L^{-1}.

In unstressed conditions, ZnO NP 80, 40 and 20 mg L^{-1} showed 16%, 13% and 10% increment respectively in the CAT levels of radish. The Co increased the CAT level by 64% compared to the control (Figure 1). The concentration of ZnO NP 20, 40, 80 mg L^{-1} increased CAT level in Co contaminated soil by 22%, 37%, and 46% respectively when compared with corresponding control (Figure 1).

During the unstressed condition, ZnO NPs slightly accelerated the levels of POD in radish. The Co stress increased the POD level in radish by 73%, compared with untreated control. The ZnO NP 20, 40, and 80 mg L^{-1} increased the POD concentration in radish raised in Co contaminated soil by 15%, 22% and 34 % respectively with respect to related stress control (Figure 1).

D. **Affluence of ZnO NPS on phenol, flavonoids and proline quantities of *R. sativus* subjected to Co stress:** Zinc oxide treatments (ZnO NPs 80, 40, 20) mg L^{-1} showed 25%, 18% and 10% upregulation in the phenolic content of radish during unstressed circumstances. The treatment Co+ ZnO NPs 20 mg L^{-1} showed 9% increase in phenolic content of radish as compared to the concerned control. The Co+ ZnO NP 40 mg L^{-1} and Co+ ZnO NP 80 mg L^{-1} treatments also exhibited the upsurge of 18% and 28% in phenol contents respectively as compared to relevant Co control (Table 3). Higher phenolic content enables plant to improve antioxidant activity and stress tolerance. The Co+ ZnO NP 20, 40, 80 mg L^{-1} treatments showed 21%, 28% and 41% increase respectively, in flavonoid contents of radish plant as compared to the concerned control. Increased flavonoid content enhances the plants defense mechanisms against stress (Table 3).

The Zn NPs minimally increased the proline contents of radish during non toxic conditions. Co stress significantly escalated the proline level in radish by 59% as compared to untreated control. Whereas, ZnO NPs (20, 40, 80 mg L^{-1}) exogenous supply further augmented the amounts of proline by 12%, 19% and 25% respectively in radish exposed to Co toxicity in contrast with corresponding toxic control (Table 3).

The numbers are Means±SD of three replicates. Different letters in a column indicate significant difference among treatments according to DMRT at *p*≤ 0.05: ZnO-NPs 20, 40, 80: Zinc oxide nanoparticles at concentrations of 20, 40, and 80 mg L^{-1}; C: Untreated Control; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80: Cobalt and zinc oxide nanoparticles added to soil at concentrations of 20, 40, and 80 mg L^{-1}.

E. Influence of ZnO NPS on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents of *R. sativus* cultivated in Co amended soil: During the unstressed condition, ZnO NP 20 mg L^{-1} showed a decrease in the MDA concentration of 8%, followed by ZnO NP 40 mg L^{-1} with 13% decrease and ZnO NP 80 mg L^{-1} with a 16% decrease. The Co stress increased the MDA level by 65%, with reference to un-treated control. The foliar spray of ZnO-NP at varied levels such as 20, 40, and 80 mg L^{-1} decreased the MDA concentration of radish under Co stressed soil by 12%, 19% and 27% respectively than that of corresponding stress control (Figure 2).

Table 3: Consequences of Zinc oxide nanoparticles on Proline, Phenol and Flavonoids contents of *R. sativus* grown in Cobalt contaminated soil.

Treatment	Biochemical attributes		
	Proline	Phenol	Flavonoids
	µM/g FW	mg GAE/g DW	mg QE/ g DW
C	0.36±0.01f	22.4±1.1g	20±0.9f
ZnO NP 20	0.42±0.02f	24.8±1.2fg	23±1.1f
ZnO NPs 40	0.47±0.02f	27.1±1.3ef	28±1.3e
ZnO NP 80	0.54±0.02e	29.6±1.4e	32±1.5e
Co	0.86±0.04d	49.3±2.3d	54±2.6d
Co+ ZnO NP20	0.97±0.04c	53.7±2.5c	68±3.2bc
Co+ ZnO NP40	1.05±0.05b	59.5±2.8b	75±3.6b
Co+ ZnO NP80	1.14±0.05a	68.2±3.3a	89±4.4a

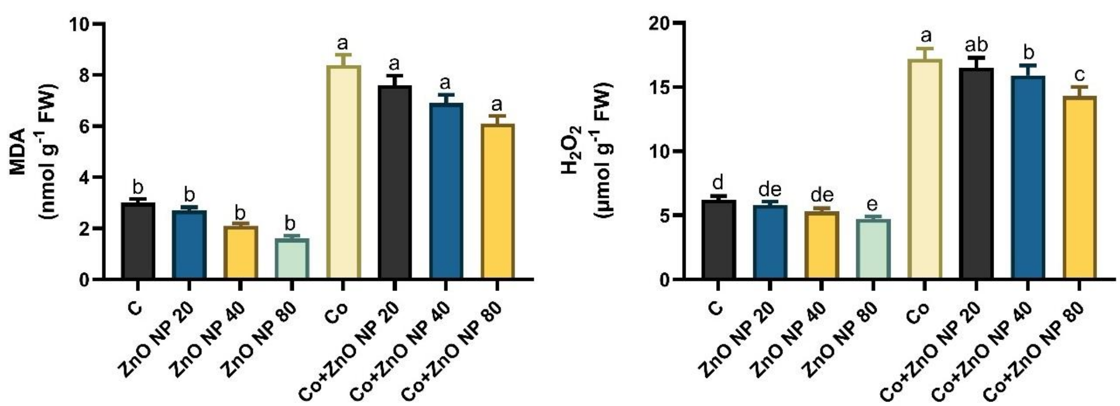

Figure 2: Effects of ZnO NPs on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents of *R. sativus* under Co stress. Different letters on the bars show significant difference among treatments according to DMRT at p≤ 0.05. C: Control; ZnO-NPs 80, 20, 40: Zinc oxide nanoparticles at 80, 20 and 40 mg L^{-1}; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80 mg L^{-1}: Soil amended by Cobalt and Zinc oxide nanoparticles at 20, 40, 80 mg L^{-1}.

During the non toxic regime, ZnO NP 20, 40, 80 mg L^{-1} showed a decrease in the H_2O_2 concentration by 7%, 15% and 25% respectively. The Co stress increased the MDA and H_2O_2 level of radish by 63% and 68% respectively relevant to the untreated control. The level of ZnO NP 20, 40, 80 mg L^{-1} decreased the H_2O_2 concentration in radish cultivated under Co contaminated soil by 9%, 17% and 23% respectively with respect to related stress control. Similarly ZnO NPs (20, 40, 80) lowered the MDA contents of radish by 8%, 15% and 27% respectively when compared with relevant Co control (Figure 2).

F. Effect of ZnO NPS on Co accretion in root and shoot tissues *R. sativus* under Co toxicity: Root and shoot showed a non-detectable content of cobalt in controlled or unstressed condition. The combined treatment Co+ ZnO NP 20 mg L^{-1} reduced the metal uptake in root of radish plant up to about 18% as compared to the relevant control. Similarly, Co+ ZnO NP 40 mg L^{-1} and Co+ ZnO NP 80 mg L^{-1} decreased the root metal uptake by 33% and 48% respectively (Table 4). The metal uptake in shoot also declined by 58% in Co+ ZnO NP 80 mg L^{-1} and about 37% and 22% in the Co+ ZnO NP 40 mg L^{-1} and Co+ ZnO NP 20 mg L^{-1} treatments respectively. The ZnO NPs (80 mg L^{-1}) foliar spray upgraded the radish tolerance index by index 149.1. under Co toxic regimes. The ZnO NPs declined the radish plant's translocation factor from 0.58 to 0.47, indicating that zinc oxide nanoparticles played crucial role for phytostabiliztion of Co.

Table 4: Effect of Zinc oxide nanoparticles on Co uptake in *R. sativus*

Treatment	Co uptake				
	Root Co	**Shoot Co**	**TF**	**BCF**	**MTI**
	mg/kg DW	mg/kg DW			
C	ND	ND	-	-	-
ZnO NP 20	ND	ND	-	-	120.2c
ZnO NP 40	ND	ND	-	-	152.8b
ZnO NP 80	ND	ND	-	-	223.2a
Co	67±3.29a	39±2.36a	0.58a	0.39a	55.9e
Co+ ZnO NP20	56±2.76b	31±01.72b	0.54a	0.31b	74.5d
Co+ ZnO NP40	47±2.08c	25±01.23c	0.53a	0.25c	115.5c
Co+ ZnO NP80	36±0.83d	17±0.79d	0.47b	0.17d	149.1b

Numerical value indicates the means ± standard deviation of three replicates. Different letters in a column indicate significant difference among treatments according to DMRT at $p \leq 0.05$: Un-treated Control; ZnO-NPs 20, 40, 80 mg L^{-1}: Zinc oxide nanoparticles at 20, 40 and 80 concentrations; Co: Cobalt (100 mg kg^{-1}); Co + ZnO-NP 20, 40, 80 mg L^{-1}: Soil amended by Cobalt and Zinc oxide nanoparticles at 20, 40, 80 concentrations. MTI: Metal Tolerance Index; BCF: Bio concentration Factor; TF: Translocation Factor, ND; Not detected.

G. **Pearson Correlation and Principal Component Analysis:** The correlation matrix demonstrated the strong positive and strong negative correlations between the 21 parameters measured of radish plant under cobalt stress (Figure 3). The correlation heatmaps indicated that the growth attributes such as shoot length, root length, no.of leaves, shoot and root fresh/dry weight, were positively correlated with photosynthetic pigments such as carotenoids, chlorophyll a, chlorophyll b, total chlorophyll and. Whereas the growth attributes were negatively correlated with enzyme and non-enzyme antioxidant such as SOD, CAT, POD, proline content, phenol content and flavonoid content. In addition, MDA, H_2O_2 and cobalt content in shoot and root showed strong negative correlation with growth parameters of the radish plant.

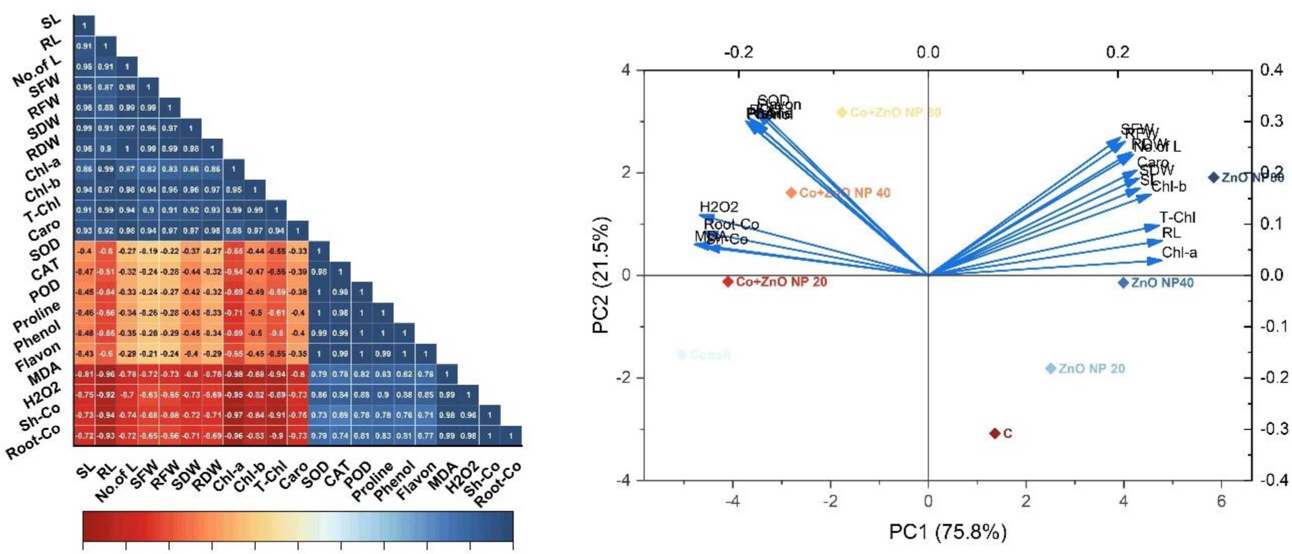

Figure 3: Correlation matrix and Principal component analysis (PCA) showing effect of ZnO NPs on the associations among parameters of *R. sativus* exposed to Co stress.

The PCA biplot suggested that the physiological state of the radish plants was remarkably altered under various treatments (Figure 3). The proximity of the distance between different concentration of zinc nanoparticle from the control treatment of both stress and non-stress condition indicates that various concentrations of ZnO-NPs had a notable impact on radish plant. In nonstress condition ZnO-NPs application accelerated growth attributes and photosynthetic pigments while under cobalt stress ZnO-NPs alleviated the stress by producing enzyme and non-enzymatic antioxidants to neutralize reactive oxygen species produced during cobalt toxicity.

DISCUSSION

Heavy metals are considered as hazardous substance and they pollute the environment. Because heavy metals are water-soluble and non-biodegradable, they can readily enter plants and have negative effects on plant defence system. Natural and anthropogenic activities are the main source of HM release into environment. Heavy metals affect plants in many ways, such as growth inhibition, decreased physiological and biological activity, and resistance to heavy metal detoxification (Bharti *et al*., 2022). Nanoparticles are crucial for lowering HMs toxicity and encouraging plant development. The ZnO NPs are assumed for regulating oxidative stress and structural alterations caused by Co in the radish plant. John *et al*., (2009), stated that varying amounts of Cd and Pb, lowered root growth, and the fresh biomass of *B. juncea*. This study also depicted more decline of root growth rather than shoot which may be associated with disruption of the activities of the proton pumps and restricted root elongation due to Cd toxicity. Similarly, our study showed that Co stress reduced the root length, shoot length, biomass production and photosynthetic activity. In contrast with the control, the shoot and root lengths of *Leucaena leucocephala* (white lead tree) increased when ZnONPs were applied (Venkatachalam *et al*., 2021). Similarly, when we applied zinc oxide nanoparticles to *R. sativus*, we observed a significant improvement in root and shoot length under Co stress and no stress situation. With the increasing concentration of ZnO NPs, the roots and shoots grew longer.

When we applied the concentration of ZnO NPs 20-80 mg L^{-1}, an progressive increase is the number of leaves was observed in comparison to the control. With the higher concentration of the nanoparticles, the number of leaves were also increased gradually and maximum increase was observed at the concentration of ZnO nanoparticles 80 mg L^{-1}. Similarly, in *Zea mays* subjected to Cd stress, foliar application of ZnO nanoparticles (25–600 ppm) resulted in a substantial increase of growth and biochemical parameters. ZnO-NP supplementation restored photosynthetic pigment levels, improved nutrient uptake (particularly magnesium, potassium, and zinc), and increased antioxidant enzyme activity. The improved nutrient status and oxidative stress tolerance may have contributed to better root and shoot development, ultimately enhancing plant productivity even under metal toxicity (Singh *et al*., 2025). Similarly, foliar application of Zinc oxide nanoparticles increased the root length as well as in the shoot length and biomass of radish under cobalt stress. Moreover, high dose of Zinc oxide nanoparticles 80 mg L^{-1} showed better growth and photosynthesis in radish during Co stress.

According to (Rizwan *et al*., 2021), nano-particles considerably increased carotenoid, Chl a, and Chl b in comparison with control. He revealed that nano particles of zinc oxides at 100 parts per million augmented the levels of carotenoid by 112%, chlorophyll a by 55%, and chlorophyll b by 133% respectively. In a similar fashion, when ZnO NP concentration increased, these photosynthetic parameters also increased in radish during toxic and non toxic regimes. While comparing our findings, the Co stressed group exhibited the lowest contents of chlorophyll a, carotenoids and chlorophyll b. Conversely, ZnO NPs at 20 mgL^{-1}, 40 mgL^{-1}, and 80 mgL^{-1} supply remarkably increased the levels of carotenoids, chlorophyll a, and chlorophyll b in radish under Co stress regimes. It is assumed that use of ZnO NPs increased the photosynthetic rate by improving the gas flow parameters and chlorophyll contents. All the photosynthetic parameters chlorophyll a, chlorophyll b, carotenoids were up surged in nanoparticle assisted crops. A study carried out by Rai-Kalal & Jajoo, (2021) demonstrates the impacts of seed priming with ZnO nanoparticles on wheat cultivar showed significant improvements in photosynthetic pigment content (chlorophyll *a*, *b*, and total chlorophyll). Fluorescence analysis 30 days after cultivation showed enhanced PSII photochemistry, increased absorption, trapping, and electron transport (ET) efficiencies, indicating enhanced primary photochemistry. These results imply ZnO-NP nano-priming enhances photosynthetic performance by boosting PSII photochemistry in toxic metal stressed plants which is response of reducing oxidative stress.

A study examining foliar application of oleylamine-coated Zn-NPs on *Solanum lycopersicum* (tomato), researchers found that a spray dosage of 15 mg L^{-1} significantly increased chlorophyll content. When application of the ZnO nanoparticles was carried out then all the photosynthetic contents showed an enhancement, as compare to the stress condition (Tryfon *et al*., 2023). In our study we observed that when the experiment was performed only with the Zinc oxide nanoparticles, then the elevation in photosynthetic parameters of the plants was observed as compare to the stress conditions. A recent experiment on *Zea mays L.* (maize) seedlings subjected to cadmium stress investigated the role of exogenously applied ZnO-NPs at 25 mg L^{-1} and 50 mg L^{-1} dosages for improvement of growth and photosynthesis process. In another study on *Capsicum chinense* (pepper) exposed to 50 μM $CdCl_2$ in hydroponic culture, foliar application of ZnO-NPs (15 mg L^{-1}, twice daily) substantially increased photosynthetic performance under Cd stress. Cadmium severely disrupted photosystem II function, gas exchange, chlorophyll content, and inhibited root activity and morphology. In contrast, ZnO-NP treatment along with the cadmium stress, restored chlorophyll levels, gas exchange traits, and maximum quantum yield of PSII (Tahira *et al*., 2025).

Hussain *et al*., (2024) demonstrated that wheat leaves subjected to various NP treatments had higher SOD and POD activity than the control. Higher concentrations of NPs applied compared to control showed a more notable increase in antioxidant enzyme activity of plants during stress regimes. The foliar application of ZnO NP at (100 ppm) enhanced SOD activity by way of 40% and POD activity in plants by 54% as compared to control. During present study, Cobalt induced stress in radish was observed as sudden increase in all of the above antioxidant enzymes. When higher concentrations of the nanoparticles, such as ZnO-NPs 80 mg L^{-1} and ZnO nanoparticles 40 mg L^{-1}, increment in SOD and POD values of radish was noticed during toxic circumatances. The SOD, POD, and CAT activities increased in nano assisted radish under Co stress as compared to control, showing that the treatment actively re-activated the antioxidant machinery. The improvement was attributed to the dual effect of zinc-oxide nanoprticles supplying Zn (a cofactor for many antioxidant enzymes) and essential micro nutrient. The study concluded that ZnO-NPs act as a synergistic antioxidant defense booster, capable of mitigating Co-induced oxidative stress far more effectively than conventional treatments. The combined effect of both the ZnO nanoparticles and the cobalt stress showed the improvement of POD, SOD, and CAT and a significant increase was observed when the highest concentration of Zin coxide nanoparticles was applied to radish under Co stress. It higher antioxidant enzymes levels of radish may helped it for quenching ROS generated under Co toxicity.

Present results depicted that the concentrations of H_2O_2 and MDA significantly increased in radish under Co stress. Similarly, in a controlled experiment on *Oryza sativa*, plants under cadmium stress represents a significant rise in oxidative stress biomarkers. When zinc oxide nanoparticles (ZnO NPs) were sprayed foliarly at 50 mg L^{-1}, there was a notable decline in malondialdehyde content and H_2O_2 levels compared to cadmium-only plants. The study revealed that ZnO NP application effectively suppressed lipid peroxidation and reduced ROS generation, as illustrated by the reduced H_2O_2 and MDA values (Faizan *et al*., 2021). In rice (*Oryza sativa*), exposure to cadmium (Cd) or copper (Cu) obviously augmented H_2O_2 synthesis in radicle tissues prior to expression of the ricMT (rice metallothionein) gene. Importantly, overexpression of ricMT in transgenic suspension cell lines substantially suppressed H_2O_2 formation under toxic metal stress and reduced cell death, showing that repression of H_2O_2 is associated with improved metal tolerance. While this study primarily focuses on H_2O_2, the interplay with antioxidative defenses (e.g metallothioneins acting as both metal chelators and ROS scavengers) underscores the role of H_2O_2 as both a signaling molecule and stress indicator under Cd/Cu toxicity (Zhang *et al*., 2017). In a similar way, ZnO NPs application significantly declined the MDA and H_2O_2 contents of radish exposed to Co stress conditions.

The study by Alsherif *et al*., (2022) investigated the effects of heavy metal contamination on stress biomarkers in *Amaranthus retroflexus L.* collected from heavy metals contaminated sites in Khulais, Saudi Arabia. Results showed that plants growing in contaminated soils exhibited a marked increase in hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels, with the highest accumulation observed in the roots due to their direct contact with metal-rich soil. Increased MDA content demonstrated significant lipid peroxidation and membrane damage, whereas elevated H_2O_2 levels suggested augmented synthesis ROS. The mechanism involves reinforcing ROS scavenging to prevent lipid peroxidation and stabilize membranes by detoxifying H_2O_2 before it accumulates. This breaks the oxidative chain reaction that leads to MDA synthesis. Regarding MDA, all treatments decreased its content under control condition while TiO_2 and ZnO NPs declined MDA content under Pb stress condition. Overall, TiO_2 + ZnO NP treatment was observed to be the optimal treatment in significantly lowering H_2O_2 and MDA content (Panahirad *et al*., 2024). Our study is solely related with this investigation that ZnO NPs supply reduced the level of MDA and H_2O_2 in radish under Co stress. Our study also aligns with the above research in the way that we applied the heavy metal stress such as of cobalt to the Radish plant there was an increase in the stress biomarkers such as both MDA and H_2O_2. But when we applied the Zinc oxide nanoparticles on the Co stressed radish plants then the results showed a surprisingly decline in the H_2O_2 and MDA amounts.

A study conducted by Hussain *et al*., (2024) explained that when plants were exposed to Pb treatment, the phenolic levels of *Euphorbia helioscopia* L and *Parthenium hysterophorus* L were escalated. Similarly, we found that when the radish plant was exposed to Co treatment, its phenolic levels increased, in comparison to control. Increased phenolic content was associated with better stress tolerance and antioxidant activity. Neverthless, our study revealed that applying ZnO nanoparticles to radish plants exposed to Co stress further raised their phenolic contents which might have endowed plant resilience against Co induced toxicity. The ZnO NPs have been shown to modify phenolic metabolism in crops under toxic metal stress, strengthening antioxidant defense mechanisms. Harmonious to our findings, foliar ZnO NPs treatment decreased oxidative damage of Pb in *Brassica juncea* by improving growth and phenolic contents (Singh *et al*., 2025).

According to a plant study by Han *et al*. (2024), maize seedlings exposed to heavy metals exhibited a coordinated upshift of the flavonoid pathway: metabolomics verified a larger accumulation of several flavonoid subclasses, and transcription of important biosynthetic genes increased. According to the authors, this is an adaptive antioxidant response that stabilizes membranes and buffers reactive oxygen species caused by toxic heavy metals in plants. A number of flavonoids that were differentially accumulated were closely linked to metal stress signaling and redox protection. Similarly higher levels of flavonoids were noted in ZnO NPs assisted radish exposed to Co toxicity. Exogenous proline boosted tissue proline and greatly reduced Cd-induced growth inhibition in Indian mustard (Brassica juncea), although Cd stress increased endogenous proline. In accordance with its functions in redox buffering and metal sequestration/transport modulation, proline supplementation mechanistically decreased ROS overaccumulation, preserved photosynthetic pigments and cell survival, and even decreased tissue Cd concentration. This indicates that proline can be used to mitigate the phytotoxicity of heavy metals and that heavy metals raise the endogenous proline concentration as a stress signal. Simialrly increased levels of proline were recorded in ZnO-NPs supplied radish plants cultivated under Co stress regimes.

Researchers discovered that tomatoes accumulated more proline and flavonoids when they were subjected to Cd stress alone and when ZnO-NP was applied in addition. ZnO-NPs modulated flavonoid/phenolic/proline responses and enhanced photosynthetic ability and antioxidant enzyme activities (Sun *et al*., 2023). Significant metabolic alterations, including the induction of stress-related metabolites, were upregulated by Cd stress in plants. ZnO-NP foliar treatment reduced Cd accumulation in tissues, modified amino-acid metabolism (including proline-related pathways), and was associated with increases in protective metabolites. To conclude that ZnO-NPs reprogram primary and secondary metabolism to reduce Cd toxicity, part of that response is elevated proline and modulation of phenolic/flavonoid metabolism that contributes to antioxidative buffering (Sun *et al*., 2023b). Recent studies further support our findings on the role of ZnO nanoparticles for reducing Co uptake in root and shoot tissues of radish. Similarly, Xu *et al*. (2022) reported that cadmium exposure in tobacco caused significant increases in proline and disturbances in flavonoid metabolism, while the application of ZnO nanoparticles not only reduced cadmium accumulation but also restored metabolic balance, resulting in further enhancement of proline and flavonoid biosynthesis. Similarly, Hassan *et al*. (2024), reviewed evidence across several crops and concluded that ZnO nanoparticles consistently elevate proline and other osmolytes, along with flavonoid and phenolic antioxidants, thereby reinforcing both osmotic adjustment and redox homeostasis. These findings confirm that while heavy metals alone induce proline and flavonoid accumulation as stress responses, ZnO nanoparticle co-application amplifies these protective pathways and strengthens plant tolerance agaist toxic metal stress (Zou *et al*., 2022). Singh and colleagues investigated the application of green-synthesized ZnO nanoparticles on *Vigna radiata* (mung bean) under normal and stress conditions. Their results demonstrated that ZnO-NPs at moderate concentrations enhanced physiological parameters and biochemical traits, including a prominent increase in proline concentration and stimulation of phenolic and flavonoid accumulation in plants exposed to toxic metal stress.

When ZnO nanoparticle seed priming was tested in *Triticum aestivum* (wheat), treated seedlings accumulated greater proline concentrations and showed an increase of flavonoid production in comparison to unprimed controls (Pandya *et al*., 2024). These biochemical shifts were associated with improved germination and seedling vigor, indicating that ZnO-NPs can modulate osmolyte and antioxidant metabolite production as part of a growth-promoting effect, independent of external stress factors. In our study while performing the experiment, we examined that when the heavy metal stress such as Cobalt was applied to the Radish plant, there was a significant increase in proline and the flavonoid content in the plants. Similarly, when the combination of the stress and Zn oxide nanoparticles was applied the non-enzymatic parameters proline, phenol and flavonoids also increased.

The ZnO NPs exogenpus application reduced the Co translocation in root and shoot of radish. The ZnO NPs supply lowered the TF and BCF while improved metal tolerance index. The ZnO NPs may have activated the sysnthesis of roots exudates and phytochelatins in radish which may have immobilized the Co accretion in root and shoot tissues. Our findings regarding Co translocation and uptake in radsih are in congruent with Timilsina *et al*. (2023) who revealed that ZnO NPs application (100 mg L^{-1}) declined the Cd uptake in root and shoot tissues of lettuce. Hence, ZnO NPs application may be a suitable option for phytostabilization of toxic metal contaminated soil.

Conclusion: The practice of zinc oxide nanoparticles had a major progressive influence on the growth of *Raphanus sativus* plant species and markedly improved their photosynthetic efficiency and growth under normal as well as stressed conditions. The Co stress significantly decreased the growth parameters, photosynthesis activity, while enhanced the enzymatic and non-enzymatic antioxidants as well as lipid peroxidation in *Raphanus sativus*. However, ZnO NPs application lessen the Co induced oxidative stress in *R. sativus* by increasing the growth characteristics, photosynthetic traits, non-enzymatic and enzymatic antioxidants, while reducing lipid peroxidation and Co accumulation in root and shoot tissues. These biochemical responses in Co stressed *Raphanus sativus* indicate that ZnO NPs improve ROS-scavenging capacity and increase plant tolerance to cobalt induced stress by activation antioxidative defense machinery of plants. These findings recommended the effective role of ZnO nanoparticles in boosting vegetable crop resilience against Co toxicity. The current study also lays the groundwork for creating creative and sustainable methods for growing safe and nutritious crops in toxic metal contaminated soils by employing nanomaterials. Future research should focus on investigating the underlying mechanisms using state-of-the-art techniques such as proteomics, metabolomics, and gene expression analysis, which can demonstrate how ZnO NPs impact plant metabolism, stress-responsive genes, and protein function in plants to cope with toxic metal stress. The long-term safety and environmental impact of ZnO NPs must also be assessed, and these findings must be confirmed in real agricultural contexts in the shape of field trials.

Funding Statement: The author received no specific funding for this research.

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