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Research Article

Zinc Oxide Nanoparticles Mitigate Toxic Effects of Cadmium Heavy Metal in Chilli (*Capsicum annuum* L.)

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Abstract: Heavy metals contaminated soils and water sources are one of the major global causes of inhibition of plant growth and productivity. Different strategies are being employed to overcome the challenging issue to increase plant yield requirements to fulfil the needs of future generations. The objective of the present study was to observe the effects of spray (foliar) of green synthesized ZnO nanoparticles (100 ppm), alone and its interaction in conjugation with Cd (Cd+ZnO-NPs) 100 ppm of both on the growth and biochemical activities of the target plant, *i.e.,* two chilli varieties. After two weeks of transplant, treatments *viz.,* Control (T1), ZnO nanoparticles 100 ppm (T2), Cd 100 ppm (T3), and ZnO nanoparticles 100 ppm + Cd 100 ppm (T4) were given for six weeks and different parameters of growth and biochemical analysis were made. Results have shown that 100 ppm foliar spray of ZnO-NPs has significantly increasing effects on root and shoot growth of chilli plants in alone (ZnO nanoparticles) and Combined (ZnO nanoparticles +Cd heavy metal) treatments mitigating toxic effects of Cd stress. A similar increase in values of total carbohydrates, soluble proteins, free amino acids, and photosynthetic pigments were observed mostly in a combination of Cd+ZnO-NPs treatment showing remediation properties of ZnO nanoparticles against Cd stress in chilli plant. In conclusion, it may be suggested that 100 ppm ZnO-NPs foliar spray can have an increasing effect on the growth parameters of the plants under stressful conditions of Cd heavy metal.

Keywords: Cd, Chilli, Interactions, Mitigation, ZnO-NPs

1. INTRODUCTION

The development of tolerance against several abiotic stresses including heavy metals, salinity, high temperature, drought, etc. is the worldwide need to improve the productivity of food with limited available resources. For this purpose, the use of minimum active mineral fertilizer can be helpful to overcome the toxic effect of heavy metals on plants. Different agricultural approaches have been employed to reshape modern agriculture [1].

Heavy metal toxicity in soil and water is correlated to a significant increase in the production of reactive oxygen species (ROS) such as superoxide, free radicals (O-), hydroxyl free radicals (OH-), and singlet oxygen (O_2). Which can cause oxidative stress within plants cell [2-4]. In the soil, heavy metals are classified into two types. The first category includes vital micro-nutrients which are required for proper plant growth, such as Fe, Mn, Zn, Cu, and Mg, while the second includes non-essential elements with uncertain biological and physiological roles, such as Cd, Cr, Pb, As, Co, and Hg, etc. [5-8]. Heavy metal deposition is of significant concern for environmental and nutritional purposes [9, 10].

Cadmium (Cd) is supposed to be one of the highly contagious non-essential trace metals due to its rapid transportation [11], water solubility, prompt absorption into the crop roots system, and transportation to the shoots [12]. Furthermore, it can translocate through the food chains and causes serious threats to humans, animals, and plants [13]. As a result, reducing Cd absorption and retention in crops is crucial in ensuring food safety [14, 15].

Nanotechnology is one of the most arising multi-disciplinary technologies with an

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encouraging agriculture method in the present era. Recent advances in nanotechnology have impacted agriculture and renewable energy [16]. Term nanotechnology has been used for the materials, systems, and processes which function at a hundred nanometer (nm) or less scale. Nanoparticles (NPs) consist of particles with very tiny dimensions particles and a comparatively large surface area due to distinctive characteristics such as a high ratio of surface area to volume, which advances their reactivity and putative biological activity [17]. Because of their unique properties and novel features, Nanoparticles have been extensively utilized in many areas of daily routine such as energy production, cosmetics, drug carriers in catalysts, and environmental energy [18].

The rapid development of nanotechnology, and encouraging studies depicted that metal oxide NPs could have significant potential to inhibit various heavy metal (Pb, Cu, and Cd, etc.) uptake and their accumulation in crops [19, 20]. Among this effect of zinc oxide NPs, titanium oxides NPs, Fe_3O_4 NPs, SiO_2 NPs, and Ag NPs, etc. has been studied by different workers [21-26]. Most of the workers have studied the impact of nanoparticles on heavy metals generally focusing on root application, whereas foliar applications studies are very little. For an instant, it is reported that foliar amendment of zinc oxide nanoparticles has inhibitory effect on Cd uptake and reduction of toxicity in maize. Furthermore, having the potential to improve plants' growth parameters, antioxidant, biochemical, and photosynthetic pigments activities found in the growth of plants under heavy metal Cd stress [27].

The red Chilli (*Capsicum annuum* L.) is a valuable cash crop of the family Solanaceae with worldwide distribution and consumption. We hypothesized that zinc oxide (ZnO) NPs application may have amelioration potential to protect the plant from Cd toxicity, and may improve growth, biochemical and physiological attributes of the plants. The aims of the present study were to explore the responses of plants with ZnO nanoparticles and Cd heavy metal in alone form and their interaction of zinc oxide NPs with Cd heavy metal in combined form simultaneously in two varieties of chilli. The results may be explored to overcome Cd toxicity in the target plant. Keeping in view, the combined exposure of zinc oxide NPs and Cd was studied with a novel strategy to reduce Cd accumulation and mitigation its toxicity in the chilli varieties.

2. MATERIALS AND METHODS

2.1. Characterization and Synthesis of Zn Oxide NPs

Zn oxide NPs were synthesized by green synthesis method as described by Lee [28] with slight modifications, using leaves extract of green tea (*Camellia sinensis*). 25 gm fine powder of washed and air-dried green tea leaves was added to 500 ml of de-ionized water and was heated at 80 °C for 120 minutes using the water bath. The dark green coloured extract obtained was twice time filtered twice through filter paper Whatman No. 2. Green tea extract (60 ml) was mixed with 140 ml of 0.2 M solution of Zinc acetate dihydrate (Sigma).

The reacted solution of green tea extract and Zn acetate dihydrate was dried at 60 °C for overnight in an oven. Zn oxide nanoparticles were finally calcinated at 100 °C for 60 minutes. The resultant powdery Zn Oxide nanoparticles were depicted by XRD (X-Ray Diffraction), FT-IR (Fourier Transform Infra-Red Spectrophotometer), SEM (Scanning Electron Microscopy) and (EDX) Energy Dispersive X-Ray analysis.

2.2. Experimental Setup of Chilli Varieties

Experiments were conducted in botanical garden of the University of Gujrat (UOG), Pakistan. There are 2 varieties of chilli (Var. 1 hot pepper upward and Var. 2 hot pepper downward) were selected for the current study. Seeds of both varieties were collected from the local market and sown in labelled 2 × 2' sized beds containing well-dried loam soil mixed with animal manure. Seedlings of uniform size were transplanted into pots when plants were 6 inches in length. After two weeks of transplantation, four treatments were given such as Control (T1), ZnO nanoparticles 100 ppm (T2), Cd 100 ppm (T3), and Cd 100 ppm + ZnO 100 ppm (T4) having five replicates of each treatment. ZnO nanoparticles were utilized to plants by foliar spray whereas heavy metal treatments were given through root solution. After six weeks of treatments, physiological and biochemical attributes were studied. One plant of each replicate was harvested

for growth parameters (dry and fresh weight of root and shoot) measurements.

2.3. Chlorophyll Contents

Chlorophyll contents (Chlorophyll 'a', Chlorophyll 'b', and Chlorophyll 'Total') were calculated by the method described by Witham *et al.* [29]. Fresh leaf (0.5 g) material was ground in 20 ml of 80 % acetone for the preparation of plant samples for chlorophyll content estimation. The sample absorbance was observed at 645, 653, and 663 nm wavelength by using a spectrophotometer (UV 1100). The chlorophyll contents were calculated using the following formulas.

Chl. a (mg/g) =
[12.7(D 663) – 2.69 (D 645) × V/1000 × 1/W]

Chl. b (mg/g) =
[20.9(D 645) – 4.68 (D 663) × V/1000 × 1/W]

Ch. Total (mg/g) =
[20.2(D 645) – 8.02(D645) × V/1000 × 1/W]

2.4. Estimation of Soluble Protein

Soluble proteins were analyzed by following the Bradford dye method as described by Bradford. [30]. Fresh leaves (0.2 g) were standardized in 4 ml of 50 mM phosphate buffer to maintain the pH of the buffer around about 7.5. The extract samples were prepared according to the protocol applied by Bradford and BSA (Bovin serum albumin) was treated for standard curve formation. Bradford reagent (dye) was used for color formation in the samples. Absorbance of the samples was calculated at the level of 595 nm by using a spectrophotometer (UV 1100). TSP (Total soluble proteins) was detected by using the given formula.

TSP (mg/g FW (Fresh Weight) =
Reading of sample × Volume of Sample × Dilution factor / Weight of fresh tissue × 1000

2.5. Estimation of Free Amino Acids

Free amino acids of the samples were estimated by the method followed by Lowry [31]. Plant samples of 1 ml were extracted for estimation of soluble proteins and kept in a test tube and put in 1 ml of ninhydrin solution 2 % and 10 % pyridine and were mixed into all test tubes and the mixture was heated for 30 minutes in a water bath. The absorbance of the coloured solutions was observed at 570 nm by using the spectrophotometer (UV-1100). However, free amino acids were determined with the help of the given formula.

TFAC (mg/g FW (Fresh Weight) =
Reading of sample × Volume of Sample × Dilution factor / Weight of fresh tissue × 1000

2.6. Total Carbohydrates

Total carbohydrates were estimated as described by Ashwell [32]. A well-ground dried sample of 0.2 g was taken in a test tube mixed with 10 ml of 6 N HCl and was kept overnight using an electric shaker for complete digestion of the sample. Anthrone solution of 10 ml was kept in a test tube and added 1 ml of the sample solution in a test tube and was well shaken. Each test tube was heated for 12 minutes in a water bath. The samples were cooled down, and absorbance was estimated at 625 nm wavelength by using a spectrophotometer (UV-1100). Total carbohydrates of the samples were calculated with the help of following the given formula.

Total Carbohydrate (mg/g of plant dry weight) =
Conc. of glucose solution/absorbance of glucose × absorbance of the sample

2.7. Statistical Analysis

The data of the present study was analyzed and subjected to ANOVA based on experimental design followed by Gomez and Gomez [33]. The follow-up of the Analysis of Variance included the LSD 0.05. DMRT tests were also applied to compare the means of the treatment.

3. RESULTS AND DISCUSSION

3.1. Characterization of Nanoparticles

The nanoparticles of zinc oxide were characterized to confirm the biocompatibility, solubility, and suitability of NPs for application in biological science. XRD, SEM, FT-IR, and EDX analysis were performed for the characterization of NPs (Fig. 1).

3.2. XRD Analysis

The size of ZnO-NPs was confirmed by XRD spectroscopy. For this XRD spectroscopy. Zn-Ka radiation at a wavelength of 26 °A and a current of 30 mA was used. Power of 40 KV was used for this analysis. Characterization peaks of NPs were according to the Miller indices. For the calculation of the size of nanoparticles, the Debye Scherrer formula was used.

$$D = \frac{0.9\lambda}{\beta cos\theta}$$

Where λ= wavelength of X-ray, β= full-width half maximum in radiance, θ= Bragg's diffraction angle. The value of D was calculated at 16 nm.

The calculated average size of Zn oxide NPs was 15 to 40 nm and as shown in Figure 1. It shows spectra of XRD analysis of ZnO nanoparticles after preparation and calcination of ZnO at 100° C for complete water removal and attaining higher crystallinity of the nanoparticles. Diffraction planes (100), (101), (102), (103), (110), and (112) with corresponding peaks are obtained. The hexagonal structure of ZnO NPs is confirmed by this spectroscopy.

3.3. FT-IR Spectrophotometry

Fourier Transform Infra-Red Spectrophotometry of powdered ZnO-NPs sample was performed to analyze the interfaces for the purpose of knowing the availability of various functional groups as shown in Table 1.

In the IR spectrum obtained from green tea, the band at 3394 cm^{-1} shows stretching vibration of the hydroxyl (OH) group present in alcohols, water, and phenols. Other bands obtained at 2926, 2864, 1627, 1741 and 1037 cm^{-1} show different types of stretches confirming the presence of a variety of functional groups present in amino acids, polyphenols, proteins, polysaccharides, and other biomolecules as shown in Table 1. The reduction of these biomolecules and their stabilization role is clear in the ZnO-NPs from the absorption bands of such biomolecules.

ZnO-NPs showing two new peaks at 457 cm^{-1} and 682 cm^{-1} wavelengths may be concluded as characteristic peaks of Zinc oxide NPs. A higher percentage of phenolic groups are responsible for reduction ability and higher amino acids; amide linkages in proteins may have a crucial role in the stabilization process of ZnO-NPs (Figure 1).

3.4. Growth Attributes

Results in Figure 2 have shown a significant difference effect in both chilli varieties. An increase in fresh shoot and root weights of both chilli varieties

Table 1. Identification of functional groups present on the surface of Nanoparticles (ZnO) with the help of FTIR analysis.

Wavenumber (cm^{-1})	Structural Formula	Functional Groups Name
900-665	N-H wag	1°, 2° amino
1300-1150	C-H wag, (-Ch-X)	Alkyl halides
1320-1000	C-O stretch	Alcohols, carboxylic acids, esters, ether
1470-1450	C-H bend	Alkane
1500-1400	C-C stretch in-ring form	Aromatics
1600-1585	C-C stretch in-ring form	Aromatics
1710-1665	C=O stretch	α, β-unsaturated esters aldehydes, ketones
1760-1690	C=O stretch	Carboxylic acids
1760-1690	C=O stretch	Carbonyls
3100-3000	C-H stretch	Aromatics
3330-3270	-C≡C-H: C-H	Alkynes (terminal)
3300-2500	O-H stretch	Carboxylic acids
3400-3250	N-H stretch	1°, 2° amino
3500-3200	O-H stretch, H-bonded	Alcohols, Phenols

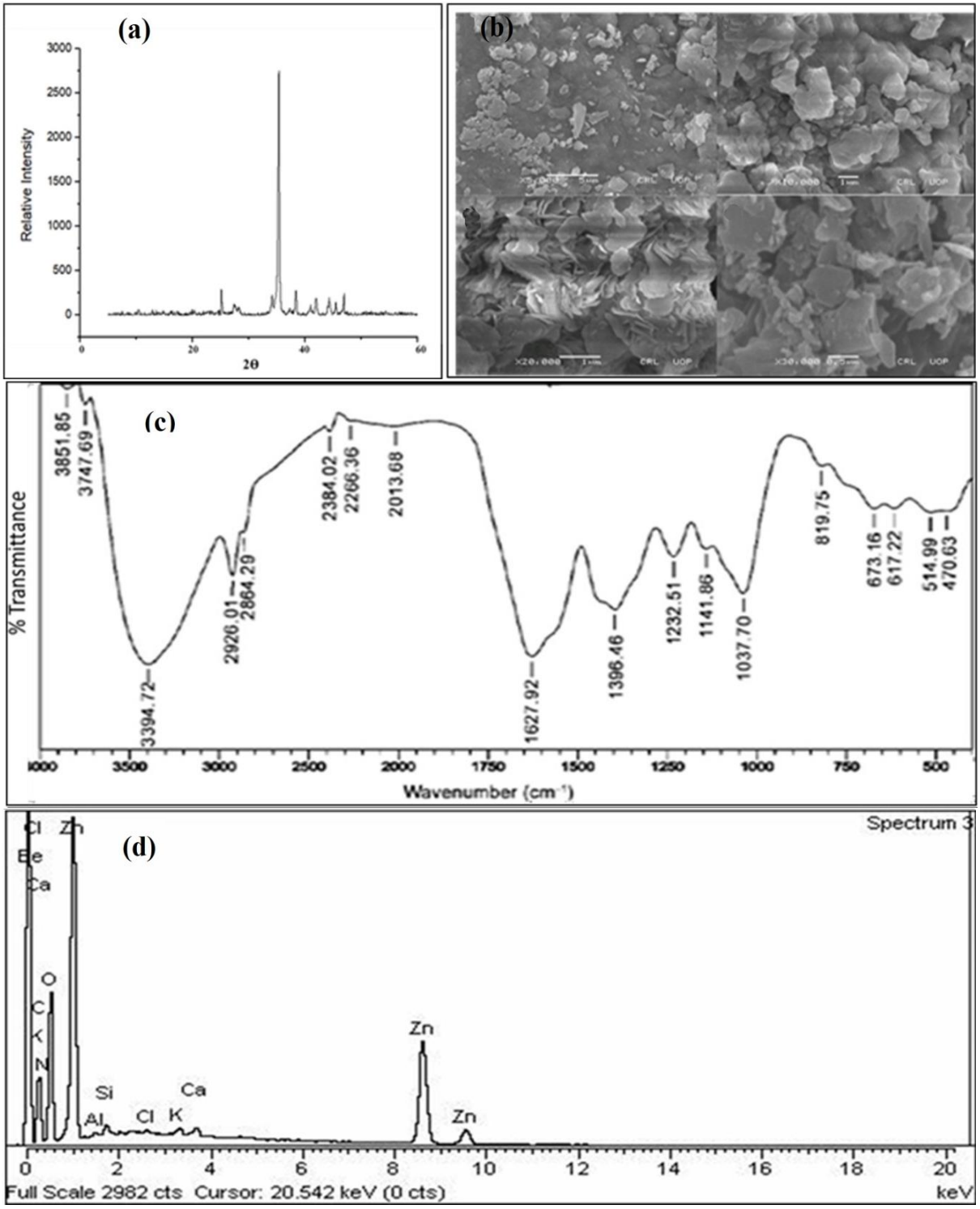

Fig. 1. Representation of vertical farming system (Source: [5])

was seen in treatment having a combination of 100 ppm Cd+ZnO NPs as compared to all other treatments. Shoot dry biomass production was significantly decreased in chilli variety 1 in the treatment of alone ZnO-NPs and alone Cd whereas both varieties were successful in maintaining it in the combined treatment of ZnO + Cd. An increase in root dry biomass production was calculated in two varieties in all treatments as compared to control but only significant in variety 1. Results of dry biomass production of root and shoot in chilli. Chilli varieties have shown that foliar amendment

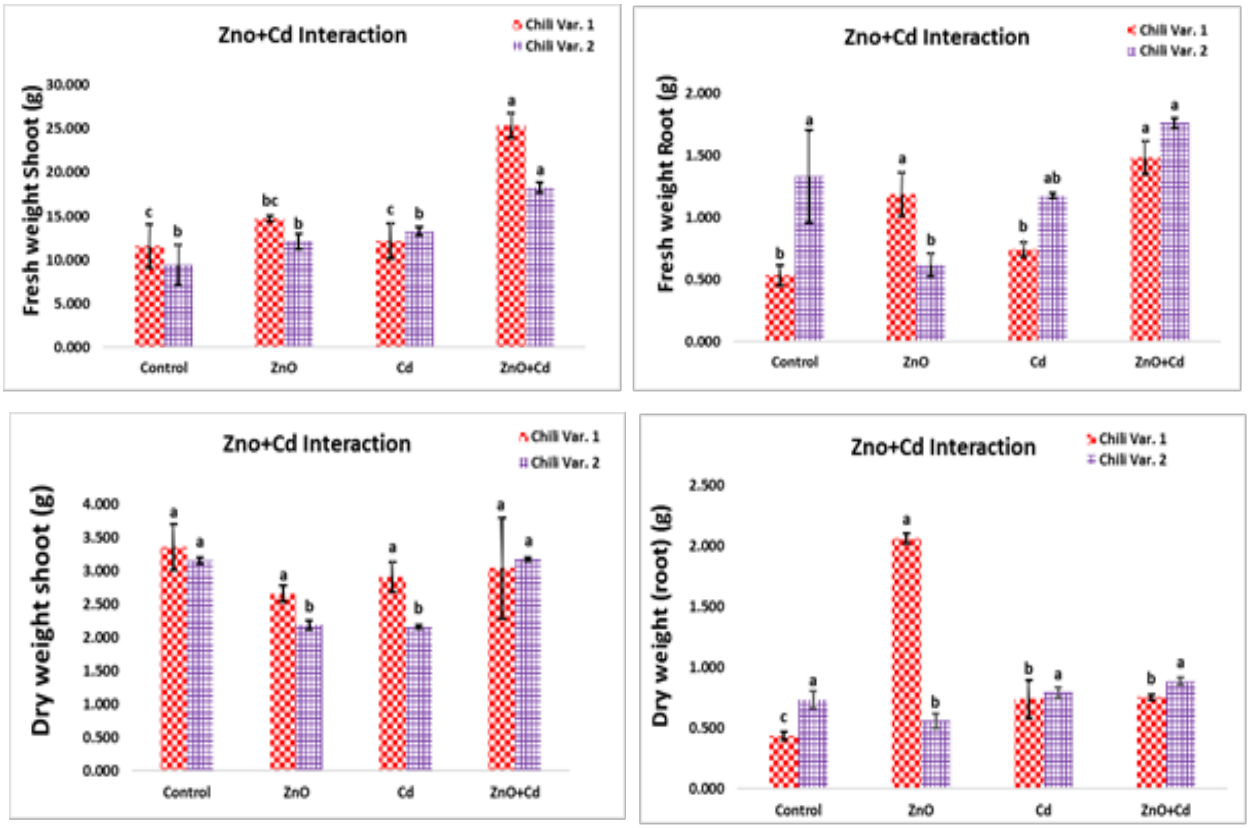

Fig. 2. Fresh and dry, shoot and root weights of chilli varieties in response to different treatments.

of 100 ppm of ZnO-NPs in combination with 100 ppm of Cd heavy metal treatment found a maximum effect in both varieties of chilli. Such increased dry biomass may be due to the potential of ZnO-NPs to alleviate the toxic effects of Cd heavy metal on the growth of chilli plants. These findings confirm the results of earlier workers who documented the positive role of ZnO nanoparticles in the mitigation of Cd heavy metal toxicity and enhancement of growth [34-38].

Data of photosynthetic pigments of chilli varieties has shown an increase in chlorophyll a, b, and total values. This improvement of photosynthetic pigments may be the result of inhibition of Cd uptake in plants. Zinc (ZnO-NPs) being an important constituent of different enzymes and precursor metabolites involved in the biosynthetic machinery of photosynthetic pigments. This improvement is matching with the findings [39-42].

3.5. Biochemical Attributes

Results for chlorophyll contents are shown in Figure 3. It is clear that foliar spray of ZnO nanoparticles has a considerably decreasing effect on chlorophyll 'a', chlorophyll 'b', and chlorophyll 'Total' in chilli variety 1. Whereas both chilli varieties maintained their all-photosynthetic pigments with treatments of Cd alone and a combination of Cd+ZnO-NPs like that of control.

3.6. Total Carbohydrates of Root and Shoot

Results for carbohydrates of shoot and root are represented in Figure 4. It has revealed that ZnO nanoparticles and Cd alone treatments have no significant effect on root and shoot carbohydrates contents of both varieties as compared to control, except ZnO + Cd combination treatment in which significant increase of shoot and root carbohydrates content was observed. Data of photosynthetic pigments of chilli varieties has shown an increase in chlorophyll a, b, and total values. This improvement of photosynthetic pigments may be the result of inhibition of ROS production due to a reduction in Cd in plants. Zinc (ZnO-NPs) being an important constituent of different enzymes and precursor metabolites involved in the biosynthetic machinery

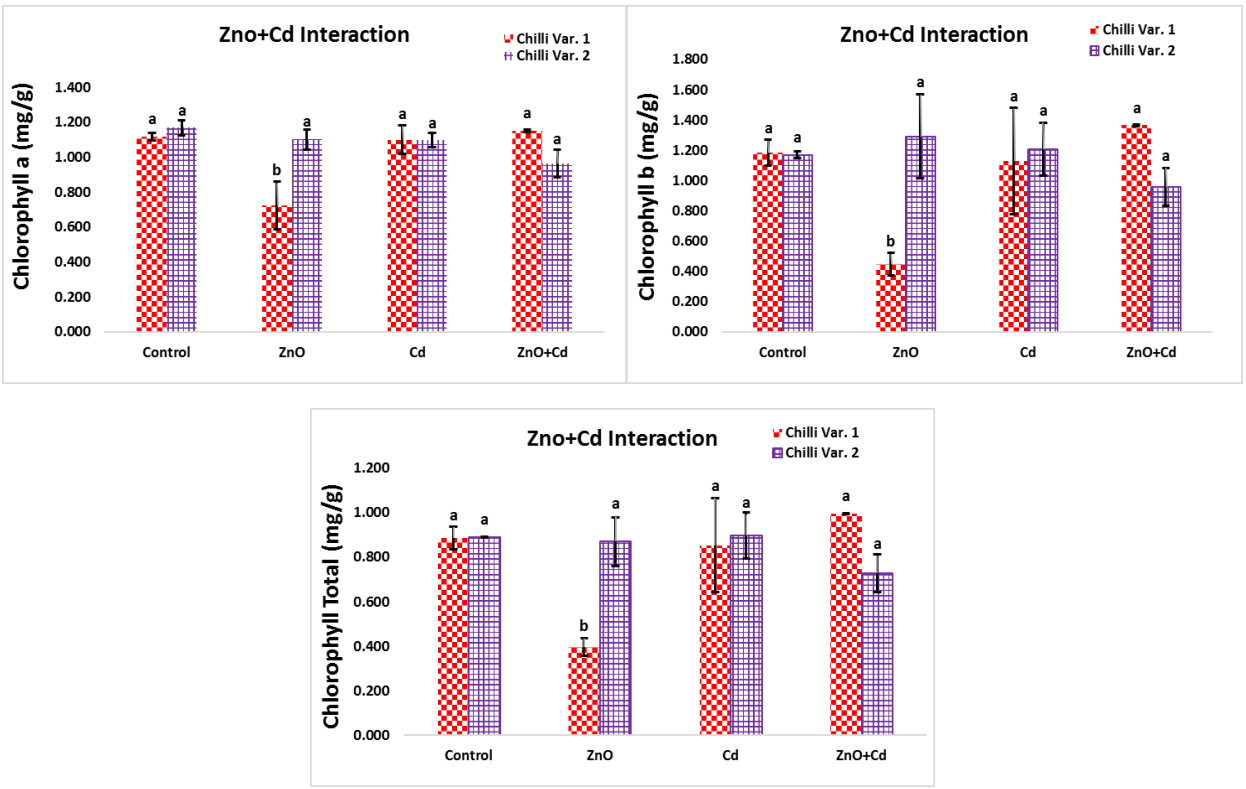

Fig. 3. Chlorophyll contents of chilli varieties in response to different treatments.

of photosynthetic pigments. This improvement is matching with the findings [39-42].

3.7. Soluble Proteins and Free Amino Acids

The soluble proteins and free amino acids of two varieties of chilli are shown in Figure 5. Results have shown that 100 ppm of Cd treatment has a significantly decreasing effect on soluble proteins of chilli variety 2 and free amino acids of both varieties, whereas the increased value of soluble proteins was observed in chilli variety 1 under the same treatment. A significant increase in soluble proteins was observed with ZnO + Cd combined treatment whereas the increased value of free amino acids was noted both in ZnO alone and ZnO + Cd combined treatments.

Data for analysis of variance (ANOVA) for growth and biochemical attributes are shown in Table 2. In growth attributes, almost all parameters including shoot and root, fresh and dry weight showed highly significant differences in both varieties among parameters except the dry and fresh

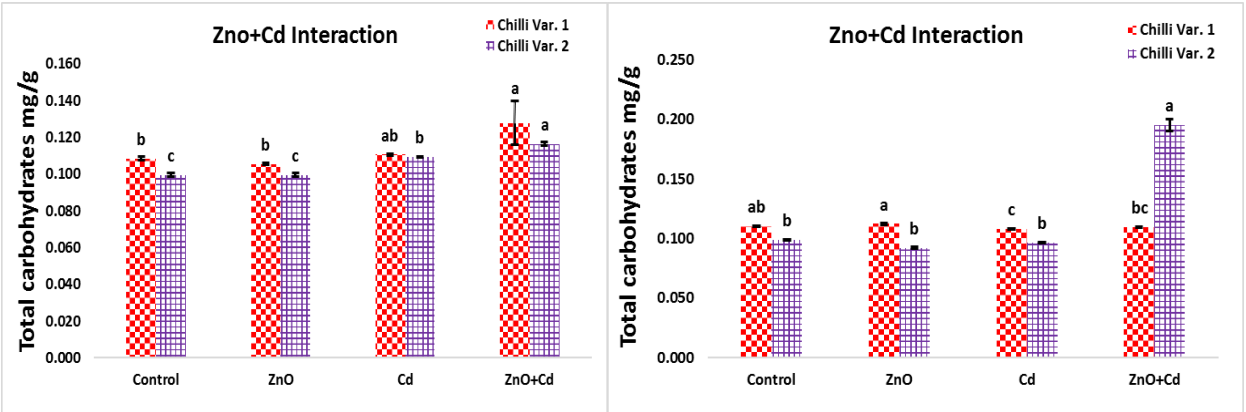

Fig. 4. Total carbohydrate contents of chilli varieties in response to different treatments

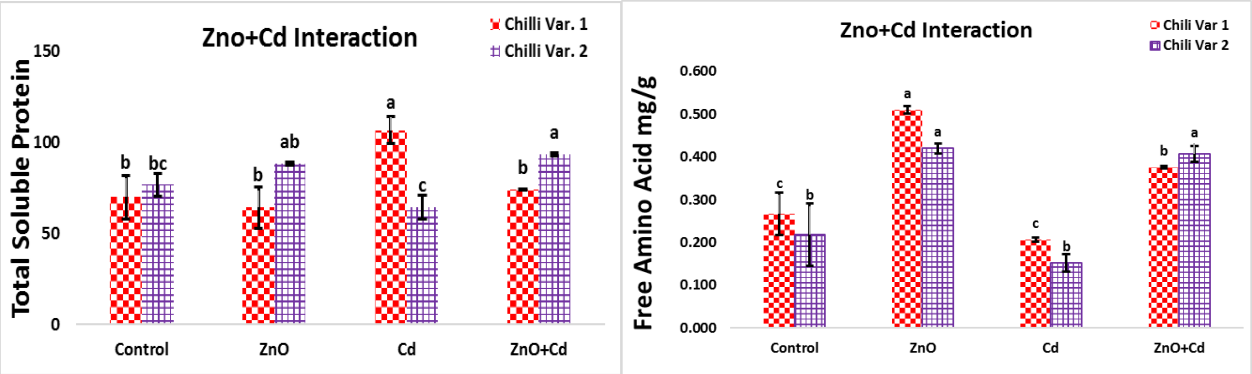

Fig. 5. Soluble protein and Free Amino Acid of chilli varieties in response to different treatments.

Table 2. F-values derived from ANOVA for growth and biochemical attributes.

Parameters	Varieties	F-ratio	P-Values	$LSD_{0.05}$
		Growth Attributes		
	Var. 1	0.442	0.7256ns	1.305
Dry Weight Shoot	Var. 2	154.18	0.0000***	0.143
	Var. 1	20.469	0.0000***	0.179
Dry Weight Root	Var. 2	6.583	0.0041**	0.159
	Var. 1	0.442	0.725ns	0.247
Fresh Weight Shoot	Var. 2	123.583	0.0001***	0.129
	Var. 1	12.51	0.0001***	0.362
Fresh Weight Root	Var. 2	6.022	0.0060**	0.577
		Biochemical Attributes		
	Var. 1	6.147	0.179*	0.263
Chlorophyll ‘a’	Var. 2	0.823	0.516 ns	0.283
	Var. 1	4.798	0.0338*	0.599
Chlorophyll ‘b’	Var. 2	0.668	0.5948ns	0.571
	Var. 1	5.717	0.0217*	0.36
Chlorophyll ‘Total’	Var. 2	0.328	0.5142ns	0.283
	Var. 1	2.993	0.0767ns	0.018
Carbohydrate (Shoot)	Var. 2	129.503	0.0000***	0.002
	Var. 1	11.135	0.0008***	0.001
Carbohydrate (Root)	Var. 2	405.566	0.0000***	0.007
	Var. 1	26.878	0.0000***	0.079
Free Amino Acid	Var. 2	11.67	0.0007***	0.121
	Var. 1	4.469	0.025*	27.855
Soluble Protein	Var. 2	8.629	0.0025**	13.589

*F= F-ratios were obtained from ANOVA tables, LSD=Least significant difference at P=0.05, NS=Non significance; *, **, ***, significant at 0.05, 0.01 and 0.001, respectively.*

weight of shoot showed non-significant differences in variety 1 among parameters. However, the results of biochemical parameters including Chlorophyll, a, b, Total, Carbohydrate shoot, and root, free amino acids, and soluble protein found significant differences among all biochemical parameters except chlorophyll a, b, Total in variety 1 and shoot carbohydrates and chlorophylls of variety 2 showed non-significant differences.

Data of results of free amino acids and soluble proteins has shown increased values for both parameters, but this increase is shown only in alone ZnO NPs and combined Cd+ZnO NPs treatments whereas decreased values of both attributes are depicted in alone Cd heavy metal treatment. Adjustment of different osmolytes in plant cells under stressful conditions may be the result of the production of various organic solutes including free amino acids, protein soluble, and carbohydrates, not necessarily all solutes but each plant may have a free choice of selection of any of osmolytes under stress. The increase in free amino acids and soluble proteins in our trial may be in accordance with the findings of Shallan *et al.* [45], who noted an increase in free amino acids and soluble protein values due to the activity of NPs in cotton plants under stress. Similar positive role of ZnO nanoparticles was given by Hussain *et al.* [46], who reported that ZnO-NPs increases the growth of Wheat plant by reducing electrolyte leakage, providing osmotic adjustment to the plants growing under Cd stress.

4. CONCLUSION

The present study concluded that increased uptake of Cd from the soil has an inhibitory effect on the root and shoot biomass production of plants resulting in decreased growth. Similar decreased values of total carbohydrates, chlorophyll contents, proteins soluble, and free amino acids were also observed showing toxicity of Cd heavy metal. Exogenous application of Zinc in the form of a spray (foliar) of ZnO nanoparticles has enhanced the growth of chilli plants. Same enhancements were also observed in values of total carbohydrates, chlorophyll contents, proteins soluble, and free amino acids both in alone and combination with Cd treatments. It may be established that ZnO-NPs may have the potential to increase the organic solutes of the plants by increasing free amino acids and proteins soluble providing osmotic adjustment in plants resulting in inhibition of Cd uptake. This defensive strategy has resulted in the alleviation of Cd toxicity with increased growth of plants. These findings may be helpful in developing the tolerance of Cd heavy metal in chilli plants growing in Cd-contaminated soils and water sources. Same investigation may be extended to other species of the same family like brinjal & tomato etc.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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