



# Zinc oxide nanocatalyst mediates cadmium and lead toxicity tolerance mechanism by differential regulation of photosynthetic machinery and antioxidant enzymes level in cotton seedlings

N. Priyanka<sup>a</sup>, N. Geetha<sup>b</sup>, T. Manish<sup>c</sup>, S.V. Sahi<sup>d</sup>, P. Venkatachalam<sup>a,\*</sup>

<sup>a</sup> Plant Genetic Engineering and Molecular Biology Lab, Department of Biotechnology, Periyar University, Periyar Palkalai Nagar, Salem, 636 011, Tamil Nadu, India

<sup>b</sup> Department of Botany, Bharathiar University, Coimbatore, 624 046, Tamil Nadu, India

<sup>c</sup> National Botanical Research Institute (NBRI), CSIR, Lucknow, 226001, UP, India

<sup>d</sup> Department of Biological Sciences, University of the Sciences, Philadelphia, PA, 19104-4495, USA

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## ABSTRACT

Cadmium (Cd) and Lead (Pb) heavy metal pollution induced toxicity severely affects the plant growth and yield of various agricultural crops worldwide. The present study discuss the prime role of phycocomolecules coated zinc oxide nanoparticles (ZnONPs) application on development of heavy metal tolerance mechanism in cotton (*Gossypium hirsutum* L.) seedlings better than exposed to Cd and Pb treatments alone. Co-exposure of ZnONPs along with heavy metal treatments significantly promoted the shoot, and root growth as well as biomass compared to control, while it was down-regulated in Cd and Pb exposed seedlings. The intervention of ZnONPs had up-regulated the level of chlorophyll *a*, *b* and carotenoid contents in leaves grown under Cd and Pb treatments than the untreated control. Similarly, the level of total soluble protein and malondialdehyde (MDA-lipid peroxidation) contents was significantly increased in the co-presence of ZnONPs along with Cd and Pb treatments over their respective control. Accumulation of antioxidant defense enzymes viz., superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) was up-regulated significantly in seedlings upon co-exposure of ZnONPs with Cd and Pb treatments. Random amplified polymorphic DNA (RAPD) fingerprinting analysis exhibited no genomic changes/alterations in seedlings by co-existence of ZnONPs with heavy metals. Overall, the present results indicate that the addition of ZnONPs with Cd and Pb ion exposure protects cotton seedlings by alleviating heavy metal induced phytotoxicity and promoted physiochemical characteristics via differential regulation of photosynthetic machinery as well as antioxidative defense mechanisms in cotton seedlings. Results strongly suggest that phycocomolecule coated ZnO nanoparticles could be effectively used as nanofertilizer to cultivate agronomically important crops in heavy metal polluted soil in the future.

## 1. Introduction

Nowadays, the heavy metal pollution of crop cultivating area has increased worldwide and most widely in developing nations because of vigorous agricultural practices to meet future food demand [1]. Exposure of crop plants to various heavy metals, namely, cadmium (Cd), lead (Pb), chromium (Cr), arsenic (As), and mercury (Hg) is considerably affecting the agricultural crop yield and they are identified as the greatest toxic elements found in the environment [2]. Lead has been widely consumed in processing industries including electroplating, paint and dyes, explosive manufacturing, and batteries [3]. Pb is highly bio-accessible and responsive form of metal ion in the environment [4].

Cadmium solubility in water is regulated by various factors such as pH and type of compounds etc. Cadmium is released into aqueous ecosystems and soil as wastes of industrial plants as well as urban sewages [5]. Among various heavy metals used in the industries, Cd and Pb have been identified as most predominant heavy metals available in the environment due to its detrimental impacts on agricultural land, crop productivity, and also affect human as well as animal health severely via food chain [6,7].

Though different remediation technologies have been demonstrated to remove or clean up toxic heavy metals from polluted environment, nanoremediation is not fully explored due to limited information available. At present, nanobiotechnology is considered as modern tool

\* Corresponding author.

E-mail address: [venkat@periyaruniversity.ac.in](mailto:venkat@periyaruniversity.ac.in) (V. P.).

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which is applied in the field of agriculture as nanofertilizers for enhancement of crop productivity [8,9]. Nanomaterials can be used for the environmental remediation and it is being considered as an innovative technology that could be applied for the remediation of resistant or slowly degradable highly toxic compounds including metals from the polluted environment [10]. It has been reported that nanomaterials can play key role in heavy metal absorption, uptake and translocation effectively, because of their surface area [11]. The nanoremediation method has certain advantages such as eco-friendly, safe, simple, nontoxic for the rapid cleanup of contaminated area [12]. In this context, nanomaterials have tremendous potential to remediate the heavy metal polluted environment. Due to tremendous potential, nanotechnology is highly attracted towards effective removal of pollutants for environmental remediation in recent years. Zinc oxide has been widely used for removal of organic pollutants from the contaminated site. Among the nanomaterials, ZnONPs have been predominantly used in nanopesticides and nanofertilizers, Phycomolecule coated ZnONPs can promote plant growth depending upon the dose, duration of exposure and plant genotypes [8,13]. Cost effective zinc oxide is a suitable alternative to  $\text{TiO}_2$  [14]. ZnO nanoparticle is one of Zn derivative used in commercial fertilizers. Most of the earlier studies have been carried out to understand the phytotoxicity of ZnONPs at lower doses on seed germination, growth of seedlings of various plant species [8,15,16].

Cotton (*Gossypium hirsutum* L.) is one of the main sources of fiber-producing commercial cash crops all over the world [17]. In order to grow economically important crops in polluted agricultural land, it is a prerequisite to develop innovative tool using nanotechnology for enhanced crop productivity via alleviating heavy metal induced phytotoxicity by modulation of antioxidant defense mechanisms in plants. This study aimed to investigate the potential role of ZnO nanoparticles on alleviation of heavy metal induced phytotoxicity in plants. Cotton seedlings are normally less tolerant species to Cd and Pb heavy metal toxicity. Therefore, it is hypothesized that phycomolecule loaded ZnONPs supplemented with Cd and Pb in the hydroponic medium may improve the seedlings growth by alleviating metal induced phytotoxicity via differential regulation of photosynthetic machinery and antioxidative defense enzyme activity. In order to test the hypothesis, cotton seedlings were co-exposed to phycomolecule coated ZnONPs with Cd and Pb heavy metals in the hydroponic system. The present investigation is mainly focused: a) to determine the seedlings growth, biomass and the level of photosynthetic pigment contents, b) to estimate the total soluble protein, MDA contents and antioxidative enzyme activity and c) to identify the genotoxicity in cotton seedlings co-exposed ZnONPs with Cd and Pb heavy metals.

## 2. Materials and methods

### 2.1. Plant materials and treatments

Cotton seeds were germinated and grown in soilrite. Seven-day-old seedlings were carefully collected from the soilrite and the roots were carefully washed with distilled water and then they were used for hydroponic experimental setup. Five seedlings were kept in each plastic container filled with 500 mL of Hoagland nutrient solution [18] and allowed to adapt for one week before subject to heavy metal treatment. Each container was connected with aerator to provide enough oxygen to the growing seedlings. Nanoparticles were synthesized as described previously elsewhere [19]. All the chemicals used for preparation of reagents in this experiment were analytical grade with 99.9 % purity.

In order to study the effect of nanoparticles towards modulation of heavy metal toxicity in cotton seedlings, initial screening experiments were carried out with different doses of ZnONPs (0–200  $\text{mg l}^{-1}$ ), Cd (0–15  $\text{mg l}^{-1}$ ) and Pb (0–250  $\text{mg l}^{-1}$ ) and identified the optimum concentration of 75  $\text{mg l}^{-1}$  ZnONPs for stimulation of the maximum seedlings growth. Also, the optimum dose for reduction of cotton seedlings growth recorded was 12.5  $\text{mg l}^{-1}$  and 150  $\text{mg l}^{-1}$  for Cd and Pb

respectively. Based on the screening results, the optimum dose of 75  $\text{mg l}^{-1}$  ZnONPs was used along with 12.5  $\text{mg l}^{-1}$  of Cd and 150  $\text{mg l}^{-1}$  of Pb in the hydroponic medium. The treatments used were 1) ZnONPs (75  $\text{mg l}^{-1}$ ), 2)  $\text{CdSO}_4$  (12.5  $\text{mg l}^{-1}$ ), 3)  $\text{Pb}(\text{NO}_3)_2$  (150  $\text{mg l}^{-1}$ ), 4) ZnONPs (75  $\text{mg l}^{-1}$ ) +  $\text{CdSO}_4$  (12.5  $\text{mg l}^{-1}$ ) and 5) ZnONPs (75  $\text{mg l}^{-1}$ ) +  $\text{Pb}(\text{NO}_3)_2$  (150  $\text{mg l}^{-1}$ ), while seedlings grown without ZnONPs and heavy metal served as control. For each treatment, five seedlings were maintained in triplicates. After 21 days of treatment, the seedlings were collected and shoot and root samples were stored at  $-80^\circ\text{C}$  for further analysis. Initially seedlings growth rate was measured and seedlings were separated into shoot and root samples and used for detection of biomass, photosynthetic pigments level, protein as well as MDA contents, antioxidative enzyme activity and genotoxic effects.

### 2.2. Determination of seedlings growth and biomass

The length of the shoot and root of each plant was measured using scale and expressed in centimeter. For biomass determination, seedlings were separated into roots and shoots and fresh weight (FW) was instantly measured and expressed in milligram. The samples were dried in a hot air oven for 48 h at  $65^\circ\text{C}$  for determination of dry weight (DW) and expressed in milligram.

### 2.3. Estimation of photosynthetic pigment contents

The photosynthetic pigment contents level was measured according to the method described by Arnon [20]. Briefly fresh leaves (100 mg) were collected and ground with 5 mL of 80 % (v/v) ice-cold acetone (analytical grade) and the extract was transferred into fresh centrifuge tubes and spun at 5000 rpm for 5 min. The supernatant was collected into fresh tube and the pellet was re-extracted with 2.5 mL of 80 % (v/v) ice-cold acetone, and it was repeated twice. The supernatant was used for quantification of photosynthetic pigment contents and 80 % (v/v) ice-cold acetone served as blank solution for calibration of UV vis spectrophotometer before reading absorbance at specific OD. Chlorophyll *a*, *b* and carotenoid contents were estimated using absorbance at 663, 645 and 470 nm, respectively, using a double-beam UV-vis spectrophotometer (spectrophotometer UV-1800, Shimadzu Tokyo, Japan). The chlorophyll *a*, *b* and carotenoids level was calculated using the formulas proposed by Lichtenthaler [21] and expressed in  $\text{mg g}^{-1}$  FW.

$$\text{Chl } a = 12.25 \times A_{663} - 2.79 \times A_{645} \quad (1)$$

$$\text{Chl } b = 21.50 \times A_{645} - 5.10 \times A_{663} \quad (2)$$

$$\text{Car} = (1000 \times A_{470} - 1.82 \times \text{Chl } a - 85.02 \times \text{Chl } b) / 198 \quad (3)$$

### 2.4. Quantification of MDA content (lipid peroxidation)

The amount of malondialdehyde (MDA) content in the root and leaf tissues of treated plants was determined as per the method described by Davenport et al. [22]. Fresh leaf tissue (200 mg) was homogenized with 2 mL of 5% (w/v) trichloroacetic acid (TCA) in an ice bath using a mortar and pestle and the extract was transferred into fresh microfuge tube and centrifuged at 10,000 rpm for 10 min. The supernatant was mixed with equal volume of 0.67 % (w/v) thiobarbituric acid (TBA) and the reaction mixture was incubated in boiling water bath for 30 min, then cooled and centrifuged as above, while equal volume of 5% TCA (without plant extract) and 0.67 % TBA reaction mixture was served as blank for reading absorbance. The absorbance was measured at 450, 532, 600 nm (denoted as  $A_{450}$ ,  $A_{532}$  and  $A_{600}$ , respectively). The MDA content ( $C_{\text{MDA}}$ ) was calculated using the following formula [23],

$$C_{\text{MDA}} = [6.45 \times (A_{532} - A_{600}) - (0.56 \times A_{450}) \times V_t/W], \text{ where } V_t = 0.0021; W = 0.2 \text{ g.} \quad (4)$$

## 2.5. Determination of soluble protein content

The total soluble protein contents from shoot and root tissues were determined according to the protocol developed by Bradford [24]. Leaf tissue (100 mg) was weighed and homogenized with 1 mL of 0.1 M Tris–HCl buffer (pH 7.0) using ice-cold mortar and pestle. The crude extract was centrifuged at 10,000 rpm for 10 min at 4 °C. Subsequently, the supernatant was carefully transferred into a sterile microfuge tube and used for the estimation of total soluble protein contents. Bovine serum albumin (BSA) was served as the standard and expressed in mg g l<sup>-1</sup> FW.

## 2.6. Measurement of antioxidative enzyme activity

To detect the antioxidative enzyme activity, all the reagents were prepared using analytical grade chemicals with 99.9 % purity. Fresh leaf and root tissues (100 mg) from each treatment were separately homogenized in a pre-chilled mortar and pestle under ice-cold conditions with 1.0 mL of ice-cold extraction buffer, containing 50 mM phosphate buffer (pH 7.5) and 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was collected carefully into fresh tubes and used for determination of antioxidative enzymes activity.

The superoxide dismutase (SOD; EC 1.15.1.1) enzyme activity was quantified by measuring its capacity to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) [25]. The reaction mixture (3 mL) consists of 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2.25 mM NBT, 60 µM riboflavin and enzyme extract and mixed thoroughly, exposed to light irradiation (40 W light) for 15 min. Buffer with enzyme extract was kept under dark conditions served as blank, while buffer with no enzyme extract was kept in the light served as control. The absorbance was read at 560 nm against a blank using UV–vis spectrophotometer. NBT reduction in the light was recorded in the presence and absence of enzyme extract. One unit of SOD activity was the amount of enzyme required for 50 % reduction in color and was expressed in units of the enzyme (mg g<sup>-1</sup> FW).

The catalase (CAT; EC 1.11.36) enzyme activity was determined by measuring the decomposition of hydrogen peroxide [26]. The enzyme extract was aliquoted into the reaction mixture (3 mL) containing 100 mM phosphate buffer (pH 7.0) and 75 mM H<sub>2</sub>O<sub>2</sub> and reaction buffer without enzyme extract served as blank. The decrease of the absorbance at 240 nm was recorded. Enzyme activity was calculated using an extinction coefficient of 39.04/mM/cm. One unit of CAT activity was defined as the amount required for decomposing 1 µmol of hydrogen peroxide per minute under assay conditions and expressed in units of the enzyme (mg g<sup>-1</sup> FW).

The peroxidase (POX; EC 1.11.1.7) enzyme activity was estimated as per the method described [27]. The enzyme extract was transferred to the reaction mixture (3 mL) contained 100 mM potassium phosphate buffer (pH 6.1), 96 mM guaiacol, and 12 mM H<sub>2</sub>O<sub>2</sub>, and enzyme extract was used for measurement of enzyme activity while reaction mixture with no enzyme extract was used as blank. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm using UV–vis spectrophotometer. The enzyme activity was calculated using the extinction coefficient of 25.5 mM<sup>-1</sup> cm<sup>-1</sup>. One unit of enzyme was the amount necessary to decompose 1 µmol of hydrogen peroxide per minute under assay conditions and expressed in units of the enzyme (mg g<sup>-1</sup> FW).

Ascorbate peroxidase (APX; EC1.11.1.11) enzyme activity was detected as described by Nakano and Asada [28]. Fresh root and shoot samples (50 mg) from control and treated seedlings were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The extract was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was used for measurement of enzyme activity. Reaction mixture consists of 50 mM potassium phosphate buffer (pH 7.0), 1 mM

EDTA, 0.5 mM sodium ascorbate, 0.1 mM H<sub>2</sub>O<sub>2</sub> and enzyme extract and buffer without enzyme extract served as blank. The decrease in absorbance was measured at 290 nm. The enzyme activity was calculated by using an extinction coefficient of 2.8 mM<sup>-1</sup> cm<sup>-1</sup>. One unit of enzyme activity is defined as 1 nmol ascorbate oxidized min<sup>-1</sup> under assay conditions and expressed in units of the enzyme (mg g<sup>-1</sup> FW).

## 2.7. Genomic DNA isolation and RAPD analysis

Total genomic DNA was extracted from leaf samples by CTAB method [29]. Briefly, fresh leaf samples (100 mg) from ZnONPs and heavy metal treated along with ZnONPs plants (control, ZnONPs, Cd, Pb, Cd + ZnONPs, Pb + ZnONPs) were selected and used for DNA extraction. The washed leaf samples were homogenized with 1 mL of 2 × CTAB buffer (2% (w/v) hexadecyltriethyl-ammonium bromide, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris–HCl (8.0), 1% (w/v) polyvinyl pyrrolidone (PVP), 1% (v/v) 2-mercaptoethanol and transferred into fresh centrifuge tubes. The DNA extract was incubated in water bath at 65 °C for 30 min and centrifuged at 8000 rpm for 10 min. The aqueous phase was carefully transferred into fresh tubes and re-extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) as described above. The supernatant was collected carefully without disturbing bottom phase and re-extracted with equal volume of chloroform and isoamyl alcohol (24:1). After centrifugation, aqueous phase was transferred into fresh tubes and added 0.6 vol of ice-cold isopropyl alcohol (100 %) to the supernatant and stored at -20 °C for 20 min for DNA precipitation and centrifuged as described above. The DNA pellet was carefully washed with 70 % (v/v) ethanol, air dried and dissolved in TE buffer and used for RAPD-PCR analysis. DNA amplification reactions were carried out according to the method of Williams et al. [30]. PCR amplification was performed in 20 µL reaction volume, which contained 1 × PCR buffer [100 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>], 1.5 mM dNTPs, 0.5 U Taq DNA polymerase enzyme and 25 ng template DNA and 25 nM of random oligo primer (Operon Technologies Inc., CA, USA). The DNA amplification was carried out in a Thermal cycler (Gradient Master Cycler, USA). The PCR amplification profile consisted of initial denaturation at 94 °C for 4 min, followed by 40 cycles denaturation at 94 °C for 1 min, annealing at 37 °C for 1.5 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. On completion of the PCR cycles, DNA amplicons were analyzed on agarose gel electrophoresis. The reproducibility of the amplified products was repeated twice for each experiment.

## 2.8. Statistical analysis

Each experiment was performed in triplicates and data were recorded. Data analysis was performed by using one way ANOVA and differences among treatments were computed taking p ≤ 0.05 as significant level and Duncan's multiple ranges tests were conducted for pair analysis between treatments.

## 3. Results

### 3.1. Impact of co-exposure of ZnONPs on physiological changes in cotton seedlings grown under Cd and Pb stress

Cotton seedlings were exposed to ZnONPs, Cd and Pb heavy metals alone as well as in combination with ZnONPs treatment for 21 days. After treatment, the seedlings were collected and used for measurement of shoot and root lengths, as well as fresh and dry weights to assess the influence of ZnONPs on growth parameters of cotton seedlings grown under Cd and Pb heavy metal induced toxicity. The results were depicted in Table 1. The seedlings growth rate was slightly increased by the supplementation of ZnONPs over control. The heavy metal exposure showed decreased rate of shoot and root growth of 25.7 % and 27.3 % for Cd and 23.2 % and 20.1 % for Pb, respectively. Interestingly, the

**Table 1**

Effect of ZnONPs on growth of cotton seedlings exposed to Cd and Pb phytotoxicity.

Treatments Doses (mg/L)	Length(cm)	
	Shoot	Root
Control	13.053 ± 0.588 <sup>a</sup>	8.276 ± 0.509 <sup>b</sup>
ZnONPs	13.367 ± 0.487 <sup>a</sup>	8.385 ± 0.735 <sup>a</sup>
Cd	9.693 ± 0.387 <sup>c</sup>	5.850 ± 0.345 <sup>bc</sup>
Pb	9.991 ± 0.242 <sup>b</sup>	6.618 ± 0.426 <sup>cd</sup>
Cd + ZnONPs	13.414 ± 0.518 <sup>a</sup>	7.933 ± 0.286 <sup>cd</sup>
Pb + ZnONPs	10.68 ± 0.268 <sup>b</sup>	6.9112 ± 0.374 <sup>d</sup>

\*Data are means ± SEM. Mean values within same column followed by different letter show significant difference at P < 0.05 significance level according to the Duncan's multiple ranges test.

shoot length was slightly increased in Cd + ZnONPs treated seedlings over control while, the shoot and root length decrease recorded was 17.8 % and 15.8 % in Pb + ZnONPs treatment than the control. Similarly, the biomass was also slightly increased (FW is 1.057 mg and 0.096 mg & DW is 0.157 mg and 0.019 mg for shoot and root tissues respectively) in ZnONPs treated seedlings compared to the respective control (Table 2). Both the fresh and dry weights of shoot and root tissues were found to be significantly decreased in Cd and Pb alone treated seedlings over control, however, the biomass level was increased in seedlings exposed to Cd + ZnONPs and Pb + ZnONPs combination treatments. Results indicate that addition of ZnONPs exposure showed improved growth parameters over control and ZnONPs and Cd as well as Pb combination treatments exhibited increased biomass rate than heavy metal treatment alone. The possible way for enhanced seedlings growth and biomass could be due the co-presence of growth promoting phycomolecules capped ZnO nanoparticles.

**3.2. Effect of ZnONPs supplementation on biochemical changes in cotton seedlings grown under Cd and Pb stress**

**3.2.1. Measurement of Photosynthetic pigment contents level**

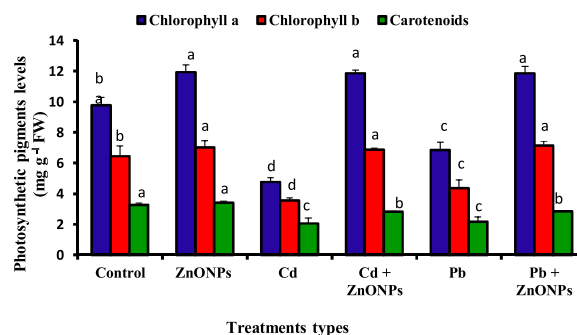
The influence of different types of treatments such as ZnONPs, Cd, Pb, Cd + ZnONPs and Pb + ZnONPs on photosynthetic parameters was investigated. The level of photosynthetic pigment contents was measured spectrophotometrically and the results are presented in Fig. 1. Among the treatments, the level of chlorophyll a content was increased by 126.7 % in Cd + ZnONPs treatment followed by Pb + ZnONPs and ZnONPs exposure whereas, the maximum rate of chlorophyll b content recorded was 111.3 % in Pb + ZnONPs followed by ZnONPs and Cd + ZnONPs treated cotton seedlings over control. The carotenoid contents level was increased in ZnONPs treated seedlings but, the rate was

**Table 2**

Influence of ZnONPs on biomass of cotton seedlings exposed to Cd and Pb phytotoxicity.

Treatments	Fresh biomass (mg)		Dry biomass (mg)	
	Shoot	Root	Shoot	Root
Control	1.043 ± 0.069 <sup>a</sup>	0.130 ± 0.020 <sup>c</sup>	0.090 ± 0.003 <sup>a</sup>	0.016 ± 0.004 <sup>d</sup>
ZnONPs	1.057 ± 0.102 <sup>a</sup>	0.157 ± 0.018 <sup>a</sup>	0.096 ± 0.003 <sup>a</sup>	0.019 ± 0.003 <sup>c</sup>
Cd	0.513 ± 0.085 <sup>d</sup>	0.117 ± 0.026 <sup>d</sup>	0.052 ± 0.004 <sup>d</sup>	0.018 ± 0.004 <sup>c</sup>
Pb	0.687 ± 0.058 <sup>c</sup>	0.110 ± 0.025 <sup>d</sup>	0.073 ± 0.006 <sup>c</sup>	0.016 ± 0.004 <sup>d</sup>
Cd + ZnONPs	0.697 ± 0.058 <sup>c</sup>	0.143 ± 0.009 <sup>b</sup>	0.074 ± 0.003 <sup>c</sup>	0.026 ± 0.002 <sup>a</sup>
Pb + ZnONPs	0.753 ± 0.116 <sup>b</sup>	0.147 ± 0.024 <sup>b</sup>	0.080 ± 0.004 <sup>b</sup>	0.028 ± 0.006 <sup>a</sup>

\*Data are means ± SEM. Mean values within same column followed by different letter show significant difference at P < 0.05 significance level according to the Duncan's multiple ranges test.



**Fig. 1.** Effect of zinc oxide nanoparticles (ZnONPs) co-exposed with Cd and Pb metals on photosynthetic pigments namely chlorophyll a, chlorophyll b and carotenoid contents from cotton seedlings. Data are means ± SEM. Bars followed by different letter(s) are statistically significant at P < 0.05 level according to the Duncan's multiple ranges test.

slightly decreased in all other treatments compared to control. However, the photosynthetic pigment contents level was significantly decreased by Cd and Pb heavy metal induced toxicity in cotton seedlings than the respective control.

**3.2.2. Estimation of total soluble protein contents level**

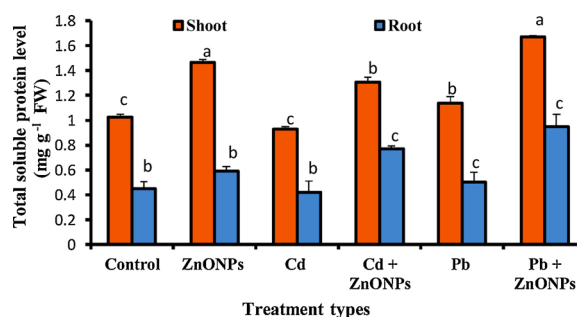
The application of ZnONPs on total soluble protein contents level in cotton seedlings grown under Cd and Pb heavy metal stress was described in Fig. 2. Results show that the level of total soluble protein content was increased to 131.4 %; 118.1 %; 163.7 % and 115.5 %; 155.5 %; 211.1 % in shoot and root tissues collected from the seedlings grown under ZnONPs; Cd + ZnONPs; Pb + ZnONPs treatments, respectively than the control. Interestingly, a two-fold increase in protein content level was observed in seedlings exposed to Pb + ZnONPs treatment. It is quite evident that the protein content level was significantly decreased in seedlings exposed to both heavy metals (Cd and Pb) in the absence of ZnONPs compared to their respective controls.

**3.2.3. Quantification of MDA content level**

The MDA content was estimated in cotton seedlings exposed to five treatments namely ZnONPs, Cd, Pb, Cd + ZnONPs and PbZnONPs for 21 days and the data is presented in Fig. 3. Result clearly indicated that the level of MDA content was significantly increased to 193.6 % and 177 % in Cd and Pb heavy metal treated plants respectively, compared to untreated control. In is interesting to mention that the level of MDA content was decreased significantly in seedlings exposed to ZnONPs, Cd + ZnONPs; Pb + ZnONPs treatments over untreated control.

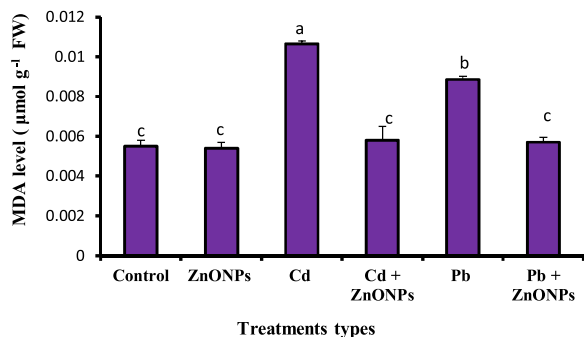
**3.2.4. Measurement of antioxidative enzyme activity**

Antioxidant enzymes including SOD, CAT, POX and APOX are mainly involved in plants to overcome adverse conditions under biotic



**Fig. 2.** Impact of zinc oxide nanoparticles (ZnONPs) co-exposed with Cd and Pb metals on total protein contents from cotton seedlings. Data are means ± SEM. Bars followed by different letter(s) are statistically significant at P < 0.05 level according to the Duncan's multiple ranges test.





**Fig. 3.** Effect of zinc oxide nanoparticles (ZnONPs) co-exposed with Cd and Pb metals on MDA content from cotton seedlings. Data are means ± SEM. Bars followed by different letter(s) are statistically significant at  $P < 0.05$  level according to the Duncan’s multiple ranges test.

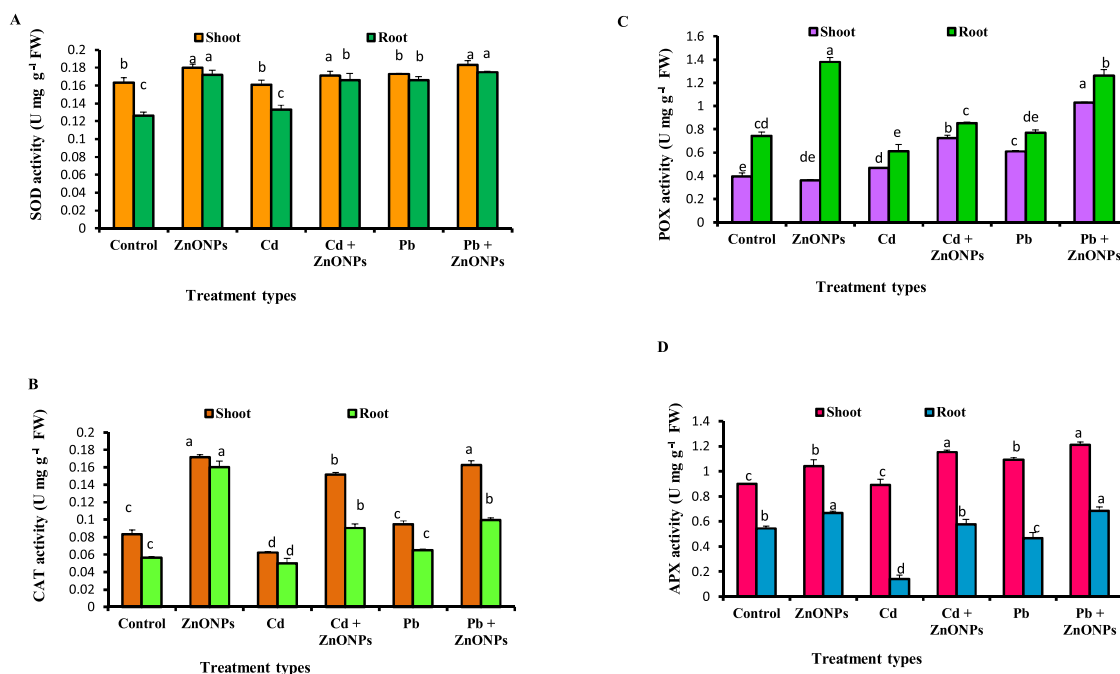
and abiotic stress. Results on antioxidative enzymes level in cotton seedlings exposed to various treatments are depicted in Fig. 4. When seedlings were exposed to ZnONPs, the SOD activity was slightly increased in both shoots and root tissues than the untreated control. Similarly, an increased SOD activity was recorded in Cd + ZnONPs and Pb + ZnONPs combination treatments compared to their respective control seedlings (Fig. 4A). Interestingly, the SOD activity was significantly increased in root tissues than shoot tissues of cotton seedlings exposed to ZnONPs alone, Cd + ZnONPs and Pb + ZnONPs combination treatments over control. The effect of Cd and Pb heavy metals alone and/or in combination with ZnONPs treatment on CAT activity is presented in Fig. 4B. The CAT activity was found to be decreased significantly in Cd and Pb heavy metal treated shoot and root tissues over control. Notably, the CAT activity was increased significantly ( $p < 0.05$  %) by 182.0 & 121.1 % and 195.2 & 180.1 % in shoot & root tissues against Cd + ZnONPs and Pb + ZnONPs combination treatments, respectively, compared to the untreated control (Fig. 4B). It is interesting to note that, the CAT activity was significantly increased by 138.5 and 285.7 % for shoot and root tissues, respectively in ZnONPs treatment than the

untreated control.

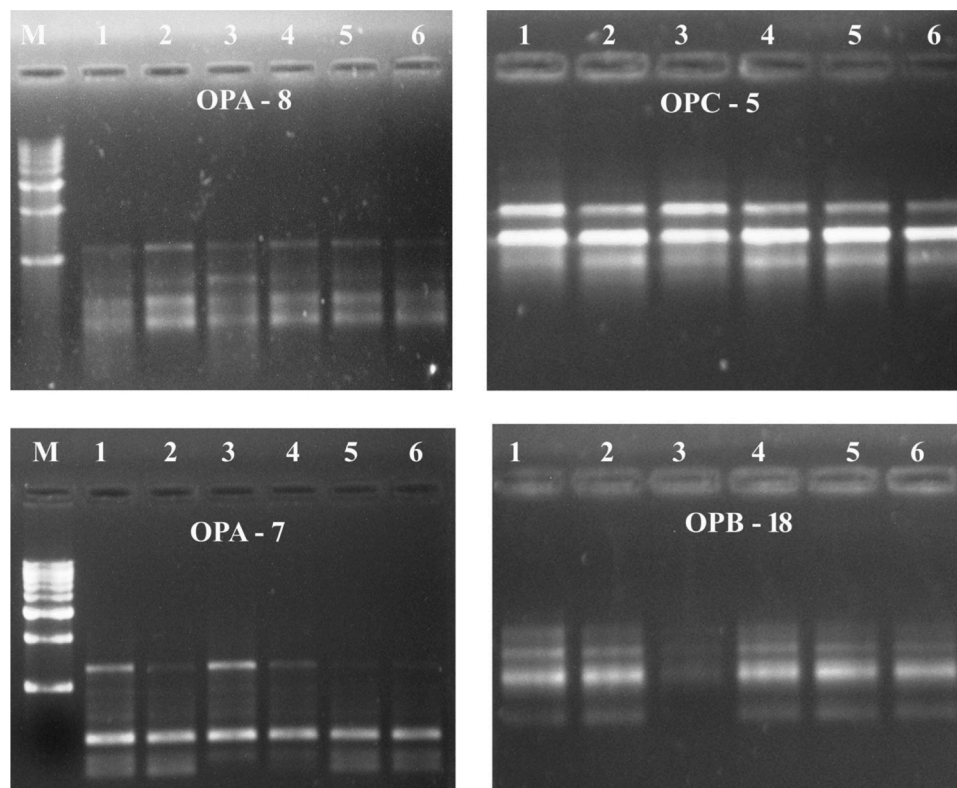
The POX activity of the cotton seedlings was elevated after exposure to ZnONPs, Cd + ZnONPs and Pb + ZnONPs and results are illustrated in Fig. 4C. Cd and Pb heavy metal treatments were found to be significantly decreased the POX activities in cotton seedlings when compared with ZnONPs combination treatments. The increased POX activity was found to be high in root tissues than the shoot tissues. The POX activity was significantly enhanced by 183.7 & 114.7 and 307.6 & 137.9 in shoot & root tissues exposed to Cd + ZnONPs and Pb + ZnONPs combination treatments, respectively, over untreated control (Fig.4C). The effect of various treatments on ascorbate peroxidase (APX) activity in cotton seedlings is displayed in Fig. 4D. The APX activity was increased by 115.7 % & 123.5 % in shoot and root tissues exposed to ZnONPs than the control. However, an increased APX activity was recorded by 129.2 % & 107 % and 135.9 % & 125.9 % in shoot & root tissues of seedlings exposed to Cd + ZnONPs and Pb + ZnONPs combination treatments over untreated control.

### 3.3. Identification of genotoxicity

In order to detect the genotoxicity of heavy metal treatments on cotton seedlings, the DNA was extracted from leaves and used for RAPD-PCR analysis. Among the 80 random decamer primers used, 10 primers showed clear DNA fingerprinting patterns in PCR, but, scorable DNA bands with intensity changes were noticed with 4 primers (OPA-08, OPC-5, OPA-7 and OPB-18). Interestingly, the PCR amplicons generated by OPA-08 primer showed 3 DNA bands in all samples except Cd treated sample in which one additional band was appeared (Fig. 5). About three DNA bands appeared in the RAPD pattern generated by OPA-05 primer, but, the intensity of amplified DNA bands was slightly decreased in all samples compared to the untreated control. With OPA-07 primer, two bands were present in all samples but the intensity was decreased in heavy metal and nanoparticle combination treatments. It is noteworthy to point out that OPB-18 primer exhibited 4 DNA bands in all samples except Cd treated seedlings. There were 2 bands amplified with weaker intensity compare with other samples.



**Fig. 4.** Effect of zinc oxide nanoparticles (ZnONPs) co-exposed with Cd and Pb metals on activities of various antioxidative enzymes: **4A)** superoxide dismutase (SOD); **4B)** catalase (CAT); **4C)** peroxidase (POX) and **4D)** ascorbate peroxidase (APX) from cotton seedlings. Data are means ± SEM. Bars followed by different letter (s) are statistically significant at  $P < 0.05$  level according to the Duncan’s multiple ranges test.



**Fig. 5.** Effect of zinc oxide nanoparticles (ZnONPs) co-exposed with Cd and Pb metals on RAPD banding patterns generated with different random oligonucleotide primers (OPA-8, OPC-5, OPA-7 and OPB-18) from cotton seedlings. Lanes M-Molecular size marker and 1 to 6- treatments types viz., control, ZnONPs, Cd, Cd + ZnONPs, Pb, Pb + ZnONPs.

#### 4. Discussion

The major focus of this investigation is to provide evidence that phycocompound coated ZnONPs alleviate Cd and Pb induced phytotoxicity by modulation of physiochemical mechanisms in cotton seedling. Heavy metals including Cd and Pb are considered to be strong toxicants to crop plants even at lower doses [1,13]. Accumulation of heavy metals in crop plants indicates various adsorption mechanisms on ZnONPs and earlier report shows that the uptake of Cd and Pb ions in the presence of ZnO nanoparticles can efficiently modify the accumulation level in plants due to the co-existence of both heavy metals and ZnONPs [13,31,32]. In this study, decreased growth rate and biomass were noticed in seedlings exposed to heavy metal treatment than the unexposed control. Due to heavy metal induced oxidative stress, absorption and transport of water molecules and nutrients might affect initially and it can alter physiological characteristics such as growth rate as well as biomass in heavy metal exposed seedlings over untreated control. It is interesting to note that the co-presence of ZnO nanoparticles with Cd and Pb, the shoot and root lengths were significantly increased in cotton seedlings in the present study. In addition, the plant growth tolerance index and biomass rate was also enhanced by ZnO nanoparticle amended treatments (Cd + ZnONPs and Pb + ZnONPs) compared to the Cd and Pb metal treatment alone. These results strongly suggest that ZnO nanoparticles might play a significant role in promoting plant growth characteristics as well as biomass by enhancing the heavy metal stress tolerance potential in cotton seedlings. Our results are consistent with a recent report of Hussain et al. [33] that the application of FeNPs enhanced the biomass in wheat plants under Cd stress. It has been suggested that heavy metals (Cd and Pb) exposure associated reduction in seedlings growth and biomass might be due to the alterations of various physiochemical mechanisms in plant cells including water deficit, photosynthetic machinery, and antioxidative defense system [13,34,35]. Raliya et al. [36] reported that co-presence of ZnONPs showed significant increase in

shoot and root growth as well as biomass rate in *Solanum lycopersicum*. It has been shown that application of silicon nanoparticles had alleviated the Pb induced phytotoxicity and enhanced growth rate as well as biomass in rice seedlings [37]. The enhanced rate of seedlings growth and biomass (Fresh and Dry Weights) with the co-presence of ZnONPs strongly suggests that phycocompound loaded ZnO nanoparticles are playing significant role for enhancing the seedlings growth characteristics via regulation of Cd and Pb metal tolerance potential by reduction of oxidative stress. Earlier, Venkatachalam et al. [38] demonstrated the positive growth promoting role of phycocompound coated ZnONPs along with P supplementation in cotton seedlings.

Photosynthetic machinery is being considered as one of the essential factors for detection of heavy metal oxidative stress induced toxicity in seedlings as bioindicators. Chlorophyll pigment contents are very important biological compounds for photosynthesis in plants. In the present investigation, the co-occurrence of phycocompound loaded ZnONPs, the level of chlorophyll *a*, *b* and carotenoid contents was significantly increased in plant leaves grown hydroponically under Cd and Pb toxicity. It has been documented that heavy metal induced phytotoxicity in leaf photosynthesis might be severely decreased either by degradation of chlorophyll pigments or reduction of its biosynthesis in cells [1]. Earlier, it has been reported that photosynthetic pigment contents were significantly increased in cotton seedlings grown hydroponically in co-presence of ZnONPs with P supplementation [38]. Present results are consistent with recent reports of Hussain et al. and Sundaria et al. [33,39], who documented that the addition of iron nanoparticles had increased the chlorophyll contents level in wheat seedlings grown under Cd stress. Also, Sebastian et al. [10] reported increased rate of chlorophyll contents in rice seedlings grown under Cd stress alleviated by the co-existence of silver nanoparticles. The possible reason for production enhanced level of chlorophyll contents in leaves might be due to the occurrence of low oxidative stress in cotton seedlings grown with co-exposure of ZnONPs together with Cd and Pb heavy

metals as evidenced by the production of reduced concentration of MDA content level.

It has been documented that Cd and Pb induced phytotoxicity to seedlings can generate high oxidative stress due to the production of increased rate of ROS, which can modify the synthesis rate of macromolecules such as proteins, nucleic acids and lipids in cells [40]. The ameliorative role of co-exposed to Cd + ZnONPs on enhanced rate of total soluble protein content was observed in cotton plants. Similarly, Tripathi et al. [34] also recorded increased level of total protein content in pea seedlings exposed to Cd with addition of SiNPs. Occurrence of heavy metal induced oxidative stress can induce either more reactive oxygen species (ROS) or suppress the antioxidative enzyme production level in plant cells. Measurement of MDA content is directly exhibiting the rate of lipid peroxidation which can critically reflect the occurrence of oxidative stress level in cells. In the present study, Cd and Pb heavy metal treatment exhibited significantly higher concentration of MDA (lipid peroxidation) content in cotton seedlings than control associated with more damage to lipids as evidenced by the increased rate of lipid peroxidation results. However, MDA content was found to be low in seedlings exposed to co-presence of ZnONPs together with Cd and Pb heavy metals over untreated control. Therefore, the present results strongly suggest that co-presence of ZnONPs with Cd and Pb acted as potential molecule to prevent the cell membrane damage which was induced by heavy metal oxidative stress. Similarly, the positive role of TiO<sub>2</sub> nanoparticles in alleviating Cd-induced phytotoxicity by decreasing the MDA content level was reported in cowpea [1] and in rice [41].

As Cd and Pb heavy metal toxicity can cause cellular damage in plants via generation of excess ROS, enhanced activity of antioxidative defense regulatory enzymes such as SOD, CAT, POX and APX will protect the stressed plant cells from the extensive oxidative damage. SOD serves as the first critical defense antioxidative enzyme to scavenge the excess ROS [42] and it regulates the generation of superoxide (O<sub>2</sub>) and POX is playing significant role in reduction of ROS accumulation in cells under heavy metal stress [43]. It is hypothesized that SOD enzyme activity was down-regulated in cotton seedlings due to more oxidative stress caused by Cd and Pb heavy metal treatments that might have not been enough to mop excess ROS as resulted by high concentration of MDA content (lipid peroxidation) over their controls. A decreased level of SOD enzyme activity might be due to the production of excess reactive oxygen species in Pb and Cd treated plant cells due to heavy metal induced cell toxicity. It is noteworthy to point out that increased level of SOD enzyme activity was noticed in seedlings grown under co-exposure of phycomolecule loaded ZnONPs along with Cd and Pb heavy metals over untreated control plants. Occurrence of increased SOD activity in cotton seedlings exposed to ZnONPs might be due to the generation of decreased level of ROS than the control. The present results are in agreement with other plant species including cowpea [1] and wheat [33]. CAT activity was also elevated under Cd and Pb stress but it was triggered by co-exposure of ZnONPs with heavy metals. Co-exposure of ZnONPs with Cd and Pb metal treatments promoted the CAT activity significantly in cotton seedlings than control. Both catalase and peroxidase antioxidative enzymes are considered as important ROS scavengers to protect plant cells from heavy metal induced oxidative stress. POX acts as principal scavenging enzyme for H<sub>2</sub>O<sub>2</sub> in plant cells exposed to abiotic stress. Increased rate of POX activity indicates that co-presence of ZnONPs together with Cd and Pb heavy metal ions might be involved in suppression of H<sub>2</sub>O<sub>2</sub> occurrence in cotton seedlings in the present study. Recently, Hussain et al. [33] reported the foliar application of FeNPs enhanced the POX enzyme activity in wheat plants exposed to Cd toxicity. Similarly, Cd and Pb heavy metal exposed cotton seedlings showed significant reduction in respect of APX activity in root tissues than control. In the present study, the co-application of ZnONPs along with heavy metals showed increased APX activity in both shoot and root tissues over their untreated control seedlings. A recent report was also confirmed the positive role of CdsNPs on accumulation of

antioxidants level in the living organism [44] Varmazyari) Most recently, Ogunkunle et al. [1] observed enhanced APX activity in cowpea leaves exposed to TiO<sub>2</sub> nanoparticles with Cd metal treatment. Overall, enhanced levels of SOD, CAT, POX and APX enzyme activities recorded with co-presence of ZnONPs with Cd and Pb exposed plants indicate their potential role for scavenging excess ROS by alleviation of heavy metal induced phytotoxicity in cotton seedlings. Similarly, it has been reported that chitosan polymer showed a protective role against Pb induced toxicity by increasing the level of antioxidative enzymes such as SOD, CAT, GPx in *Oreochromis mossambicus* [45] hilagar and Samuthirapandian, (2020). Also most recently, the protective role of lemon juice against lead induced toxicity was reported in living system [46]. We hypothesized that Cd and Pb ions could be effectively transported into cotton cells via zinc transporters.

RAPD-DNA fingerprinting analysis was carried out to assess the Cd and Pb heavy metal stress induced genotoxicity in cotton seedlings. As expected, a broad range of DNA changes/alterations was observed with PCR amplicons generated by OPA-07, OPA-08, and OPB-18 primers in Cd and Pb treated plants. However, the co-application of ZnONPs with Cd and Pb treatments did not show any distinct DNA alternations in cotton seedlings. Similarly, Mattiello et al. [47] reported that TiONPs exposure did not cause any variations in the RAPD-PCR fingerprinting profile of *Hordeum vulgare*. One of the possible reasons for not causing genomic changes in NPs treated cotton seedlings might be due to the presence of natural growth promoting compounds capped ZnO nanoparticles which may regulate the uptake of low concentration of Cd and Pb metal ions into growing plant cells.

## 5. Conclusion

In conclusion, the overall effect of phycomolecule loaded ZnONPs on physiological and biochemical characteristics are found to be positive for enhanced growth and biomass as alleviation of heavy metal induced phytotoxicity. The level of photosynthetic pigments, MDA and protein contents was enhanced in cotton leaves in the presence of ZnONPs intervention. Similarly, the activity of antioxidative enzymes such as SOD, CAT, POX and APOX that are involved in removal of excess ROS was increased significantly due to the co-exposure of ZnO nanoparticles. Further, co-presence of ZnONPs in combination with Cd and Pb metals did not show distinct genomic alterations in the RAPD banding pattern. Overall, these results strongly suggest that the application of ZnO nanoparticles intervention at suitable concentration might alleviate the Cd and Pb induced heavy metal toxicity by modulation of physicochemical characteristics via activation of differential regulation of antioxidant defense mechanisms in cotton seedlings.

## Author's contributions

NP, NG, PV conceived original project idea. NP, PV were involved in collection of samples, in vitro experimental work. NP, PV, NG, TM, SVS prepared the first draft copy with editorial review support from TM, SVS. All the authors read and approved the final manuscript copy.

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## Availability of data and materials

The data generated in the current study are available from the corresponding author on request.

## Ethics approval and consent to participate

Not applicable.



## Consent for publication

Not applicable.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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