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Article

Stress Ameliorative Effects of Indole Acetic Acid on *Hordeum vulgare* L. Seedlings Subjected to Zinc Toxicity

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Abstract: The heavy metals present in the environment accumulate in the plants and affect their productivity and yield. By entering the food chain, metals cause several serious health problems in human beings as well as in other organisms. Indole acetic acid (IAA) is known to act as a signaling molecule between symbiotic association of metal accumulating plants and plant growth promoting rhizobacteria (PGPR). Present study demonstrated a protective role of IAA against surplus Zinc (Zn)-induced toxicity to *Hordeum vulgare* seedlings. Elevated Zn concentrations suppressed the plant growth, caused a reduction in leaf relative water contents (RWC) and elevated free proline and non-protein thiols (NPT) accumulation. Zinc treatment also led to enhanced lipid peroxidation (MDA contents) as well as the activity of ascorbate peroxidase (APX), showing the involvement of antioxidative defense mechanism to reduce Zn induced toxicity. IAA oxidase activity was also observed to increase due to Zn treatment. IAA pretreatment of *H. vulgare* caryopsis could partly revert the Zn-induced toxicity in seedlings.

Keywords: Heavy metals; phytohormone; lipid peroxidation; non-protein thiols (NPT); relative water contents (RWC); free proline; ascorbate peroxidase (APX); IAA oxidase

1 Introduction

In recent years, due to industrialization, ecosystems are exposed to a variety of pollutants with the risk of environmental pollution and human issues [1,2]. Pollution of biosphere by toxic metals has accelerated dramatically since the beginning of industrial revolution [3]. Following their release from the various anthropogenic or natural sources heavy metals [HMs] a class of toxic metals accumulate in plants and other living organisms and thus enter the food chain; ultimately, the human health is at risk. Heavy metals are defined as elements having density greater than 5 $g \cdot cm^{-3}$. In contrast to the organic pollutants, which can undergo biodegradation, HMs are non-biodegradable. They can be easily adsorbed by soil particles and remain within the ecosystem for a long period [4]. Although, some of them are important as micronutrients e.g., Fe, Mn, Mo, Zn, Ni, Cu, Cr and are essential in trace amounts for cell metabolism. In contrast, there are some heavy metals that are toxic for plants and microorganisms e.g., Hg, Ag, Cd, Pb and U [5].

Heavy metals of biological significance can be divided into two groups namely, redox active and redox inactive metals. Autoxidation of redox active metals such as Fe^{2+} or Cu^{2+} results in successive reduction of molecular O_2 to H_2O_2 and yield the intermediates $O_2^{\bullet}$, $HO^{\cdot}$, and H_2O_2 which are potentially toxic as compared to molecular O_2 [6]. Another mechanism by which HMs affects the cellular metabolism is displacing the essential metal from the active site of enzyme and then reducing its catalytic activity. Due to the above described interferences, the membrane functioning in plant cells is influenced

by heavy metals at the level of H^+ ATPase activity [7]. Disturbed water relations [8] and seed germination inhibition are also consequences of heavy metal toxicity in plant cells.

Plants possess a range of mechanisms at cellular level that are involved in detoxification and in turn tolerance to heavy metals. Plants exposed to toxic heavy metal (HM) concentrations have been reported to produce free proline in high amounts [9]. Free proline serves as a compatible osmolyte and help in osmotolerance as HMs are known to deteriorate plant water relations. Since oxidative stress is involved in HM toxicity development, the role of antioxidants in reduction of HM toxicity is reasonable. The antioxidative defense systems include both non-enzymatic and enzymatic components. Major antioxidant metabolites are ascorbate (vitamin C), reduced glutathione (GSH), α-tocopherol (vitamin E) and carotenoids, polyamines and flavonoids. The enzymatic antioxidants include catalase, ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase, superoxide dismutase [10]. HM tolerance, among other features, could be ascribed to the activity to efficiently manage cellular ROS through antioxidative defense [6]. Root exudates have a variety of roles including that of metal chelators that may enhance the uptake of certain HMs [11]. Potential ligands include certain amino acids and organic acids and two classes of peptides, the phytochelatins and metallothioneins [12]. The phytochelatins (PCs) are a family of metal complexing peptides. PCs are synthesized non translationally using glutathione as a substrate by PC synthase [13]. i.e., activated in the presence of metal ions.

It is interesting that PGPR include a diverse group of free-living soil bacteria that can improve host plant growth and development in heavy metal contaminated soils by mitigating the toxic effects of heavy metals on the plants [14]. The foregoing information concerning PGPR implies a role for phytohormones specifically auxins, in amelioration of HM toxic effects. It has long been known that IAA in low concentrations is one of the best plant growth stimulators particularly in terms of enhanced development of plant root system [15]. Auxin synthesizing rhizobacteria are the most well studied phytohormone producers [4]. Among PGPR species, *Azospirillum* is one of the best studied IAA producers [16]. Addition of IAA to soil in a way like soil inoculation with some PGPR producing this auxin resulted in an increased, non-selective uptake of several elements (Zn, Fe, Mg, Ca, K and P) in plant roots [17].

In view of the available literature, it is of interest to examine in this study whether the application of exogenous IAA would influence the response of barley (*Hordeum vulgare*) to the supply of Zn. Though, Zn^{2+} is essential micronutrient for plant, but if accumulated in excess in plant tissues it causes alternation in vital growth processes. When Zn supply is large, Zn toxicity can rapidly be induced in nontolerant plants, inhibition of root elongation being a very sensitive parameter [18]. The specific objectives of present study were to monitor the effect of elevated Zn concentration on seedling growth relative water content, free proline accumulation, lipid peroxidation, phytochelatin accumulation, ascorbate peroxidase and IAA oxidase activity in barley seedlings. It also aimed to examine the influence of exogenous IAA on Zn mediated HM toxicity.

2 Materials and Methodologies

2.1 Chemicals and Seed Source

Seeds of barley (*Hordeum vulgare L.*) cultivar BHS-169 were obtained from Indian Agricultural Research Institute substation, Tutikandi, Shimla, India (31°05'40.6"N, 77°09'23.8"E).

Table 1: List of Chemicals used

S. No.	Name of chemical	Origion	%age Purity
1.	$ZnSO_4 \cdot 7H_2O$	Loba	98
2.	IAA	Himedia	Extra pure
3.	Sulphosalicylic acid (SSA)	Loba	99
4.	Ninhydrin	Loba	Extra pure
5.	Glacial acetic acid	Loba	99.7
6.	Toluene	Loba	99.5

7.	HCI	Loba	Extra pure
8.	EDTA	Loba	99.4
9.	Na-phosphate	Loba	99

Information regarding the origin and percentage purity of chemicals used is as per the description written on their containers.

2.2 Seed Germination

In the first set of experiments, Zn toxicity to barley seedlings was ascertained. For this, seeds of uniform size were selected and imbibed for 16 h in different concentrations of zinc (0, 0.5, 1.0, 1.5, 2.0 mM) (Zn supplied as heptahydrate form $ZnSO_4$). 20 imbibed seeds were transferred for germination to each Petri plate (having diameter r = 9 cm) lined with triple layers of filter paper made wet with 7 ml of respective concentrations of Zn (0-2 mM). The Petri plates were placed in plant growth chamber for germination and seedling growth for 6 d at 25 ± 2°C (day, 14 h) and 18 ± 2°C (night, 10 h). After 6 days, 10 uniform seedlings were selected from each Petri plate after that, root length, shoot length and seedling fresh weight were measured. On the basis of this experiment, 1.5 and 2.0 mM Zn concentrations were chosen for all subsequent experiments. In the subsequent experiments, seeds of uniform size were selected and imbibed with 0, 1, 10, 100 µM IAA for 16 h. Then imbibed seeds were treated with 0, 1.5, 2.0 mM Zn by soaking them in respective Zn solutions (heptahydrate form $ZnSO_4$) for 8 h. After that, the seeds were thoroughly washed with distilled water and then 20 seeds were transferred to each Petri plate for germination. Petri plates were lined with three layers of filter paper made wet with 7 ml of respective concentrations of Zn (0, 1.5, 2.0 mM). After 6 days, seedlings growth was measured in terms of root length, shoot length and seedling fresh weight.

2.3 RWC (Relative Water Content) Measurement

Leaves of 6-d-old barley seedlings treated with Zn/lAA, as described above, were subjected to the measurement of RWC. The leaf fresh weight, turgid weight (after keeping with H_2O for 4 h) and dry weight (after keeping for 70°C for 48 h) were recorded and RWC calculated according to Beadle et al. [19].

$$RWC\ (\%) = \left(\frac{W_f - W_d}{W_t - W_d}\right) * 100 \quad (1)$$

W_f = fresh weight; W_t = turgid weight; W_d = dry weight.

2.4 Determination of Proline Content

Free proline was estimated spectrophotometrically following the method of Bates et al. [20]. The 250 mg plant tissue [root and shoot, separately] was homogenized with 5 ml of 3% sulphosalicylic acid (SSA). The homogenate was centrifuged in Eltek cooling centrifuge MP 400 R at 9986 g for 10 min. 1 ml of supernatant was reacted with 1 ml freshly prepared ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) and 1 ml of glacial acetic acid in a test tube and then kept in a boiling water bath at 100°C for 1 h. The reaction was terminated in an ice bath and then shifted to room temperature. Thereafter, the reaction mixture was extracted with 2 ml toluene, mixed vigorously with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from aqueous phase and absorbance read at 520 nm using toluene as blank. The proline concentration was determined from the calibration curve.

2.5 Estimation of Non-Protein Thiols (NPTs)

The non-protein thiol content was estimated according to Noctor and Foyer [21]. Approximately, 100 mg plant tissue [root and shoot, separately]) was homogenized with 1 ml extraction buffer (0.1 N HCI; I mM EDTA). The homogenate was centrifuged at 7618 g in Eltek cooling centrifuge MP 400 R for 10 min. The supernatant served as the extract. The assay typically contained 800 µl of assay buffer (120 mM Na-phosphate (pH 7.8) and 6 mM EDTA), 100 µl 6 mM dithiobis nitrobenzoic acid (DTNB,

Ellman's reagent) and 100 µl extract. The absorbance was recorded at 412 nm and NPTs quantified from a calibration curve prepared with reduced glutathione (GSH).

2.6 Measurement of Lipid Peroxidation

Lipid peroxidation was estimated from accumulated malondialdehyde (MDA) following the method given by Dhindsa et al. [22]. In brief, the plant tissue (200 mg) [1 day and 3 day old barley seedlings, separately] was homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 7618 g in Eltek cooling centrifuge MP 400 R for 5 min and supernatant collected. 1 ml of supernatant was reacted with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was then heated at 95°C for 30 min and rapidly cooled in an ice bath. Absorbance was read at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. MDA contents were determined using the extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$ [23].

2.7 Ascorbate Peroxidase Assay

Ascorbate peroxidase (APX) activity was estimated according to Hossain and Asada [24]. The plant tissue [root and shoot, separately] (100 mg) was homogenized with 1 ml 100 mM HEPES-NaOH buffer (pH 7.6) containing 5 mM ascorbate (ascorbate prepared and stored in amber reagent bottle). The homogenate was centrifuged at 7618 g in Eltek cooling centrifuge MP 400 R for 5 min (at 4°C). The supernatant serves as enzyme extract. The reaction mixture contained 50 µl 5 mM ascorbate in water, 1 ml 50 mM HEPES-NaOH (pH 7.6) buffer, 50 µl enzyme extract and 100 µl 3 mM H_2O_2. Following the addition of substrate, change in A290 was monitored. The enzyme activity was determined using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$ for ascorbate.

2.8 IAA Oxidase Assay

Approximately, 200 mg plant tissues [root and shoot, separately] was homogenized with Tris-HCl buffer (0.2 M) (pH = 6.8). The homogenate was centrifuged at 7618 g in Eltek cooling centrifuge MP 400 R for 5 min. Supernatant served as an enzyme extract. The reaction mixture contained 1 ml of enzyme extract, 0.25 ml IAA (200 µg/ml) and 2.5 ml Tris-HCl buffer. To 1 ml of above reaction mixture added 2 ml of Sarkowski reagent (2% v/v 0.5 M $FeCl_3$ in 35% $HClO_4$). Reaction mixture was incubated for 30 minutes at 25°C. Absorbance was read at 525 nm as t = 0 min. Another 1 ml of reaction mixture was incubated for 50 min at 25°C after which, 2 ml of Sarkowski reagent was added and kept for 30 min. and absorbance read. Change in absorbance was calculated by subtracting absorbance at t = 50 from absorbance at t = 0 minutes. Amount of IAA degraded was measured from the calibration curve.

2.9 Protein Estimation

Protein in the enzyme extract (used for APX and IAA oxidase) was estimated following the method of Lowry et al. [25]. 0.5 ml enzyme extract was precipitated with 1 ml chilled trichloroacetic acid (10%) for 3 h at 2°C and then centrifuged. Then pellet was dissolved in 1 ml 0.1 N NaOH. To this 5 ml $CuSO_4$ reagent ($CuSO_4$ reagent was prepared by mixing reagent A (2% Na_2CO_3 in 0.1 N NaOH) with reagent C (1% sodium potassium tartarate) and reagent B (0.5 % $CuSO_4 \cdot 5H_2O$) in order A: C: B in ratio 50 ml: 0.25 ml: 0.25 ml before assay) was added and kept at 25°C for 10 min. Thereafter, 1.0 ml phenol reagent (1 N Folin-Ciocalteau) was added and the reaction mixture was kept at 30°C for 30 min. The absorbance was read at 660 nm. Protein contents were determined with the help of calibration curve prepared with BSA.

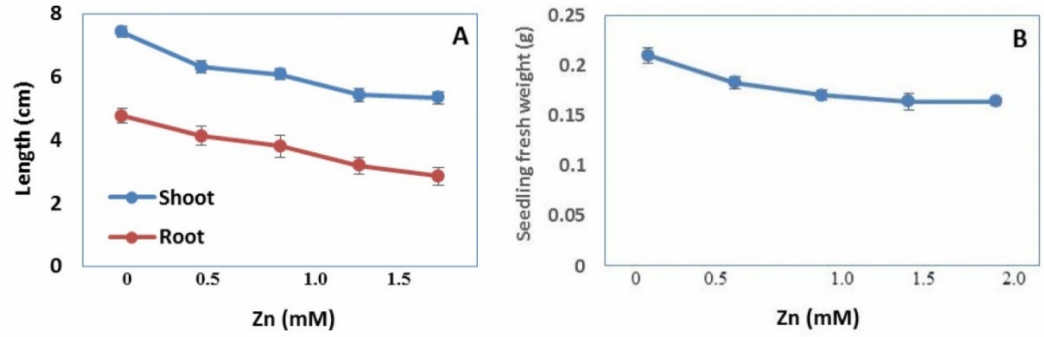
2.10 Statistics

All experiments were performed in triplicate and repeated at least twice. Data are represented as arithmetic mean ± S.E.M. To test the statistically significant difference between the treatments, one way-analysis of variance (ANOVA) was carried out followed by post hoc Tukey's test using SPSS 16 software. For all the analyses, a *p* value less than 0.05 were considered statistically significant.

3 Results

3.1 Effects of Zn on Seedling Growth

Zn suppressed the root and shoot length as well as seedling fresh weight in a concentration-dependent manner (Fig. 1(A)). Thus, root length was 87, 80, 67, and 60% of control at 0.5, 1.0, 1.5 and 2 mM Zn, respectively. Similarly, shoot length was 85, 82, 73, and 72% of control at 0.5, 1.0, 1.5 and 2.0 mM Zn, respectively The suppression of root and shoot elongation was reflected in decreased seedling fresh (Fig. 1(B)) weight which was 87, 80, 78, and 78% of control at 0.5, 1.0, 1.5 and 2.0 mM Zn, respectively.



S. No.	Parameter	Treatment		Mean	SE ±
1.	Root length	Zn (mM)	Control	4.75 [a]	0.23
			0.5	4.13 [a]	0.3
			1.0	3.8 [b]	0.35
			1.5	3.18 [b]	0.27
			2.0	2.85 [c]	0.28
2.	Shoot length	Zn (mM)	Control	7.42 [a]	0.17
			0.5	6.31 [ab]	0.2
			1.0	6.08 [ab]	0.18
			1.5	5.42 [b]	0.22
			2.0	5.34 [b]	0.19
3.	Seedling fresh weight	Zn (mM)	Control	0.21 [a]	0.008
			0.5	0.183 [b]	0.006
			1.0	0.17 [b]	0.005
			1.5	0.164 [c]	0.008
			2.0	0.164 [c]	0.004

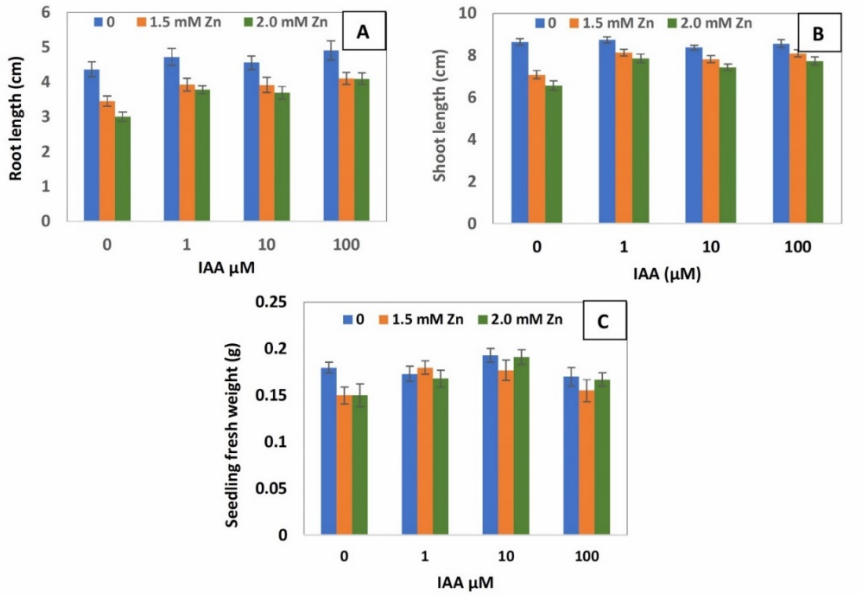
Figure 1: Effect of Zn on seedling growth of *H. vulgare*: A. Root, shoot length; B. Seedling freshweight. Data after 6 days of treatment. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$. (n = 20).

3.2 Interactive Effects of Zn and IAA on Seedling Growth

Root length in barley seedlings was inhibited by Zn in concentration dependent manner; a 21 and 31% inhibition were observed at 1.5 and 2.0 mM Zn, respectively. The magnitude of Zn-induced suppression of root elongation was, however, reduced when seeds were pretreated with IAA (1-100 µM) (Fig. 2(A)). For example, in case of IAA (1 µM) pretreated seeds Zn- induced inhibition was 17 and 20% at 1.5 and 2.0 mM Zn, respectively. Likewise, at 10 µM IAA, Zn induced an inhibition of 14 and 19% at 1.5 and 2.0 mM concentration, respectively.

Shoot length of barley seedling was reduced due to Zn treatment; 18 and 24% inhibition was observed at 1.5 and 2.0 mM Zn, respectively. Due to pretreatment with IAA, magnitude of Zn induced inhibition was reduced (Fig. 2(B)). For example, at 1 µM IAA, Zn induced inhibition was only 7 and 10% of control at 1.5 and 2.0 mM Zn, respectively. Similar reversal of Zn effect was observed at 10 and 100 µM IAA with varying magnitudes.

Like root and shoot elongation, Zn treatment led to reduction of barley seedling fresh weight; a 17% reduction was observed at 1.5 and 2.0 mM Zn. Zn-induced suppression of seedling fresh weight was reversed almost completely by IAA at 1 and 10 µM (Fig. 2(C)). The magnitude of reversal was lower at 100 µM IAA.



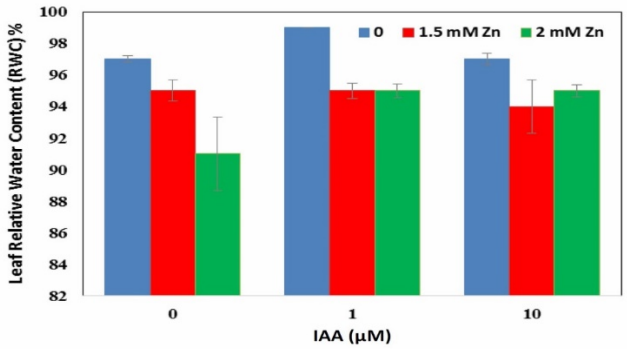
S. No.	Parameter	Treatment		Mean	SE ±
1.	Root length	Control		4.36 [a]	0.22
		Zn (mM)	IAA (µM)		
		1.5	0	3.45 [c]	0.15
		1.5	1	3.92 [b]	0.19
		1.5	10	3.91 [b]	0.22
		1.5	100	4.1 [a]	0.17
		Control		4.36 [a]	0.22
		Zn (mM)	IAA (µM)		
		2.0	0	3.0 [c]	0.14
		2.0	1	3.78 [b]	0.12
		2.0	10	3.69 [b]	0.18
		2.0	100	4.09 [a]	0.16
2.	Shoot length	Control		8.63 [a]	0.15
		Zn (mM)	IAA (µM)		
		1.5	0	7.08 [b]	0.2
		1.5	1	8.12 [a]	0.17
		1.5	10	7.82 [b]	0.16
		1.5	100	8.09 [a]	0.18

		Control		8.63 [a]	0.15
		Zn (mM)	IAA (µM)		
		2.0	0	6.56 [c]	0.22
		2.0	1	7.86 [b]	0.2
		2.0	10	7.43 [b]	0.16
		2.0	100	7.73 [b]	0.19
3.	Seedling fresh weight	Control		0.18 [a]	0.006
		Zn (mM)	IAA (µM)		
		1.5	0	0.15 [ab]	0.009
		1.5	1	0.18 [a]	0.007
		1.5	10	0.177 [a]	0.011
		1.5	100	0.155 [ab]	0.012
		Control		0.18 [a]	0.006
		Zn (mM)	IAA (µM)		
		2.0	0	0.15 [c]	0.012
		2.0	1	0.168 [b]	0.009
		2.0	10	0.191 [a]	0.008
		2.0	100	0.167 [b]	0.007

Figure 2: Influence of IAA pretreatment on Zn induced suppression of root length (A), shoot length (B) and seedling fresh weight (C) of *H. vulgare* seedlings. Data after 6 days of treatment. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 24)

3.3 Relative Water Content

Due to the treatment with Zn, the leaf RWC of *H. vulgare* seedlings was decreased. The decrease was marginal at 1.5 mM Zn but marked at 2.0 mM Zn (Fig. 3). The RWC reduced due to Zn (2.0 mM) was improved in the presence of IAA from 91 to 95%.

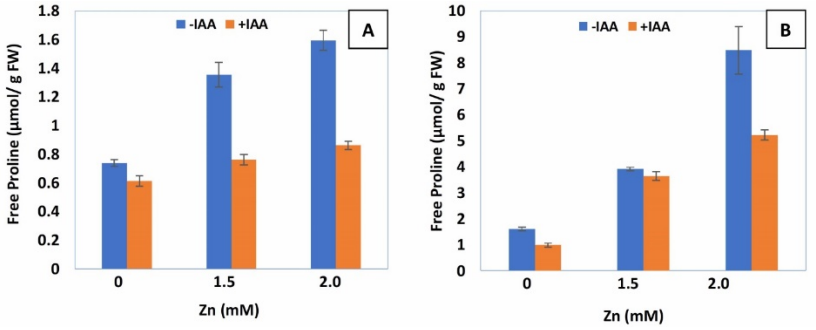


S. No.	Parameter	Treatment		Mean	SE ±
1.	RWC	Control		97 [a]	0.2
		Zn (mM)	IAA (µM)		
		1.5	0	95 [a]	0.67
		1.5	1	95 [a]	0.49
		1.5	10	94 [a]	1.69
		Control		97 [a]	0.2

	Zn (mM)	IAA (µM)		
	2.0	0	91 [a]	2.32
	2.0	1	95 [a]	0.45
	2.0	10	95 [a]	0.37

Figure 3: Interactive effects of Zn and IAA (µM) on leaf Relative Water Content (RWC) of 6 days old *H. vulgare* seedlings. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 6)

3.4 Free Proline Content



S. No.	Parameter	Treatment		Mean	SE ±
1.	Root	Control		0.0737 [a]	0.024
		Zn (mM)	IAA (µM)		
		1.5	0	1.354 [b]	0.085
		1.5	10	0.76 [c]	0.035
		Control		0.194 [a]	0.08
		Zn (mM)	IAA (µM)		
		2.0	0	1.594 [b]	0.069
		2.0	10	0.861 [bc]	0.0281
2.	Shoot	Control		1.59 [a]	0.0791
		Zn (mM)	IAA (µM)		
		1.5	0	3.9 [b]	0.161
		1.5	10	3.63 [b]	0.0698
		Control		1.59 [a]	0.0791
		Zn (mM)	IAA (µM)		
		2.0	0	8.47 [b]	0.192
		2.0	10	5.2125 [c]	0.92

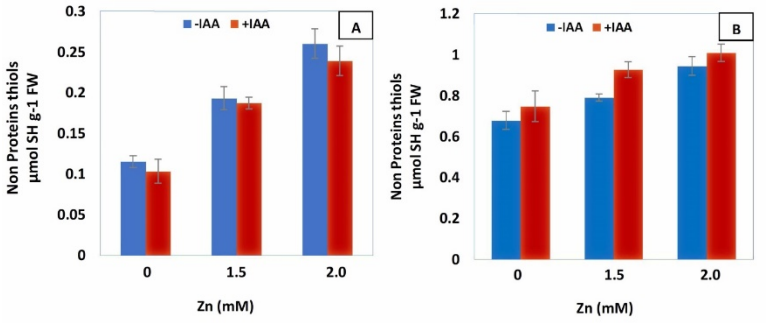
Figure 4: Interactive effects of Zn and IAA (10 µM) on free proline contents in roots (A) and shoots (B) of 6 d old *H. vulgare* seedlings. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 6)

Free proline contents were measured both in the root and shoot tissues of *H. vulgare* seedlings. In general, proline contents were greater in shoots than in roots. Due to Zn treatment, free proline contents increased substantially in both the organs in a concentration-dependent manner. An organ-specific difference in the magnitude of Zn induced free proline accumulation was observed (Figs. 4(A) and 4(B)). A 2.46 and 5.33 fold increase in shoot proline content was observed at 1.5 and 2.0 mM Zn, respectively. In contrast, increase in proline content was of lower magnitude in case of roots. Thus, a 1.83 and 2.16 fold

increase was observed at 1.5 and 2.0 mM Zn, respectively. IAA pretreatment was generally associated with a reduced degree of Zn-induced proline accumulation in roots. Thus, Zn (2.0 mM) induced proline increase was reduced from 2.46 to 1.41 fold in root tissues due to 10 µM IAA. IAA did not affect the Zn induced proline change in shoot tissues (Figs. 4(A) and 4(B)).

3.5 Non Protein Thiol (NPT) Content

Upon exposure to heavy metals plants synthesize phytochelatins (Pcs). The contents of non-protein thiols (NPTs) were measured as an index of phytochelatins in Zn-treated barley seedlings. The NPT contents were increased both in roots and shoots of barley seedlings in response to Zn treatment. But increase in the roots was more than in shoots. In shoots which were not pretreated with IAA, NPT contents were measured to be 1.17 and 1.40 fold of control at 1.5 and 2.0 mM Zn, respectively. In case of 10 µM IAA pretreated seedlings, NPT contents were observed to be slightly higher i.e., 1.24 and 1.35 fold of control (Fig. 5(A)). In roots NPT contents were observed to be 1.68 and 2.26 fold of control at 1.5 and 2.0 mM Zn, respectively in non IAA pretreated seedlings and 1.82 and 2.32 fold of control for 10 µM IAA pretreated ones (Figs. 5(B)).

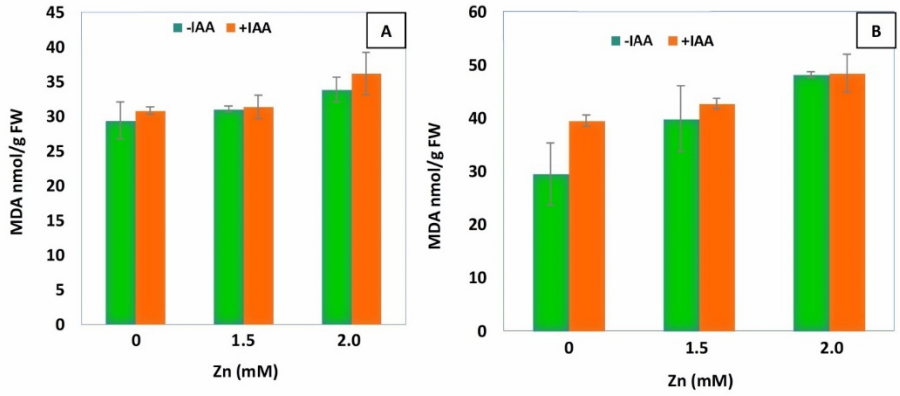


S. No.	Parameter	Treatment		Mean	SE ±
1.	Root	Control		0.115 [a]	0.007
		Zn (mM)	IAA (µM)		
		1.5	0	0.193 [b]	0.014
		1.5	10	0.187 [b]	0.007
		Control		0.115 [a]	0.007
		Zn (mM)	IAA (µM)		
		2.0	0	0.26 [b]	0.018
		2.0	10	0.239 [b]	0.018
2.	Shoot	Control		0.677 [a]	0.044
		Zn (mM)	IAA (µM)		
		1.5	0	0.789 [b]	0.018
		1.5	10	0.926 [c]	0.039
		Control		0.677 [a]	0.044
		Zn (mM)	IAA (µM)		
		2.0	0	0.943 [b]	0.045
		2.0	10	1.009 [c]	0.042

Figure 5: Interactive effects of Zn and IAA (10 µM) on non-protein thiol contents in shoots (A) and roots (B) of *H. vulgare* seedlings. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 6)

3.6 Lipid Peroxidation

Heavy metals are known to induce the intracellular production of reactive oxygen species (ROS) that initiate lipid peroxidation. Malondialdehyde (MDA), an indicator of lipid peroxidation, is often used to assess oxidative stress. In response to Zn treatment, MDA contents generally increased in barley seedlings in a concentration and time-dependent manner. After 1 day of treatment, MDA contents of barley seedlings were 106 and 115% of control at 1.5 and 2.0 mM Zn, respectively (Fig. 6(A)). After 3 days, they increased to 135 and 164% of control at 1.5 and 2.0 mM Zn, respectively. 10 µM IAA slightly enhanced the MDA contents but did not change much the lipid peroxidation extent due to Zn (Fig. 6(B)).

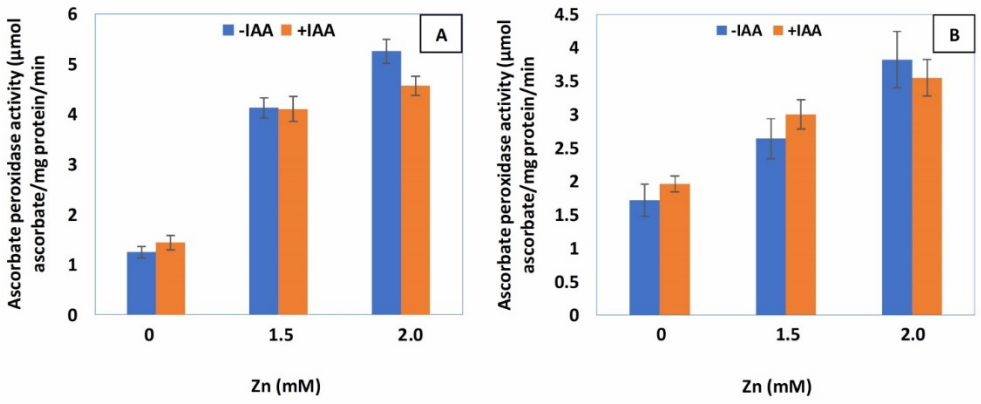


S. No.	Parameter	Treatment		Mean	SE ±
1.	1st Day	Control		29.4 [a]	2.644
		Zn (mM)	IAA (µM)		
		1.5	0	31.04 [ab]	0.427
		1.5	10	31.37 [ab]	1.692
		Control		29.4 [a]	2.644
		Zn (mM)	IAA (µM)		
		2.0	0	33.87 [b]	1.813
		2.0	10	36.17 [b]	3.04
2.	3rd Day	Control		29.44 [a]	5.849
		Zn (mM)	IAA (µM)		
		1.5	0	39.8 [b]	6.2
		1.5	10	42.67 [c]	0.967
		Control		29.44 [a]	5.849
		Zn (mM)	IAA (µM)		
		2.0	0	48.1 [b]	0.531
		2.0	10	48.37 [b]	3.533

Figure 6: Interactive effects of Zn and IAA (10 µM) on lipid peroxidation contents in *H. vulgare* seedlings after 1(A) and 3 (B) days of treatment. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 3)

3.7 Ascorbate Peroxidase Activity

The activity of APX, an antioxidative enzyme responsible for H_2O_2 degradation, was substantially enhanced in root as well as shoot tissues in response to the Zn treatment. In shoot, APX activity was 3.31 and 4.22 fold relative to control at 1.5 and 2.0 mM Zn, respectively. In roots, these values were 1.55 and 2.22 fold as compared to control at 1.5 and 2.0 mM Zn, respectively. In case of IAA pretreatment, APX activity was observed to be 2.80 and 3.13 fold of control for shoot and 1.54 and 1.80 fold of control for root at 1.5 and 2.0 mM Zn, respectively (Figs. 7(A) and 7(B)). Thus, IAA slightly weakened the Zn induced stimulation of activity. IAA alone was slightly enhanced APX activity.

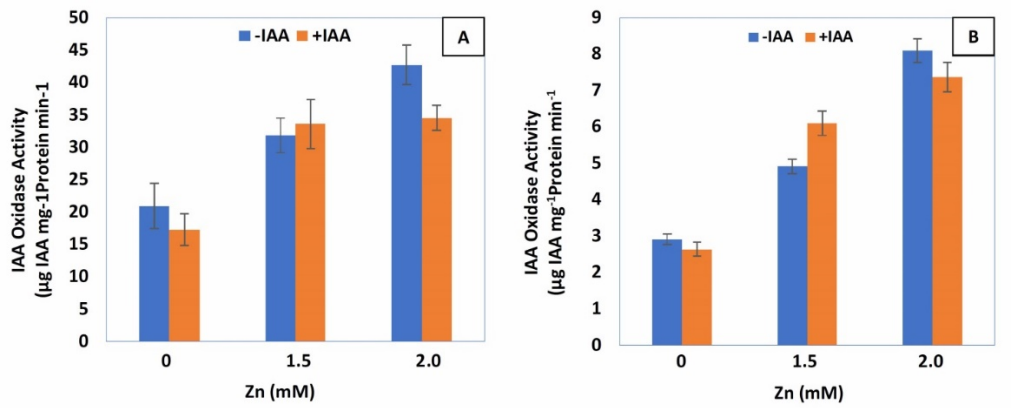


S. No.	Parameter	Treatment		Mean	SE ±
1.	Root	Control		1.72 [a]	0.24
		Zn (mM)	IAA (µM)		
		1.5	0	2.64 [b]	0.3
		1.5	10	3 [c]	0.22
		Control		1.72 [a]	0.24
		Zn (mM)	IAA (µM)		
		2.0	0	3.82 [b]	0.42
		2.0	10	3.55 [b]	0.27
2.	Shoot	Control		1.25 [a]	0.11
		Zn (mM)	IAA (µM)		
		1.5	0	4.13 [b]	0.2
		1.5	10	4.1 [b]	0.25
		Control		1.25 [a]	0.11
		Zn (mM)	IAA (µM)		
		2.0	0	5.25 [c]	0.24
		2.0	10	4.56 [bc]	0.19

Figure 7: Interactive effects of Zn and IAA (10 µM) on ascorbate peroxidase activity in Shoot (A) and Root (B) of 6 d old *H. vulgare* seedlings. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$. (n = 6).

3.8 IAA Oxidase

IAA oxidase activity with Zn treatment was observed to increase both in root and shoot. The activity of IAA oxidase was greater in roots as compared to shoots. In roots, IAA oxidase activity was 1.52 and 2.03 fold of control at 1.5 and 2.0 mM Zn, respectively. Due to pretreatment with IAA, the values were changed to 1.94 and 2.0 fold of control at 1.5 and 2.0 mM Zn, respectively (Fig. 8(A)). In case of shoot, IAA oxidase activity was 1.73 and 2.81 fold of control at 1.5 and 2.0 mM Zn, respectively. These values 2.36 and 2.80 fold of control when seeds were pretreated with 10 µM IAA (Fig. 8(B)). Thus, there was no definite influence of IAA pretreatment on Zn induced change in IAA oxidase activity.



S. No.	Parameter	Treatment		Mean	SE ±
1.	Root	Control		20.91 [a]	3.5
		Zn (mM)	IAA (µM)		
		1.5	0	31.82 [b]	2.7
		1.5	10	33.64 [b]	3.8
		Control		20.91 [a]	3.5
		Zn (mM)	IAA (µM)		
		2.0	0	42.73 [c]	3.1
		2.0	10	34.55 [bc]	1.9
2.	Shoot	Control		2.91 [a]	0.15
		Zn (mM)	IAA (µM)		
		1.5	0	4.91 [b]	0.2
		1.5	10	6.1 [bc]	0.34
		Control		2.91 [a]	0.15
		Zn (mM)	IAA (µM)		
		2.0	0	8.1 [bc]	0.32
		2.0	10	7.37 [b]	0.4

Figure 8: Interactive effects of Zn and IAA (10 µM) on IAA oxidase activity in roots (A) and shoots (B) of 6 d old *H. vulgare* seedlings. Data presented as mean ± SE and means with same letters are not significantly different from each other at $p < 0.05$ (n = 6).

4 Discussion

In this study, barley (*H. vulgare*) was chosen due to its economic importance and capacity to grow rapidly. Also, barley has been shown to accumulate high amounts of Zn. Zinc was taken as an example of HMs. An excess of Zn^{2+} has been reported to have a negative effect on mineral nutrition [26] and alteration in vital growth processes such as photosynthesis and chlorophyll biosynthesis and affect membrane integrity also [27]. Zn suppressed the *H. vulgare* seedling growth in terms of root/shoot length and seedling fresh weight. These observations are in conformity with the toxic effects of Zn reported in several crop plant species e.g., chickpea [28], Sunflower [29]. The observed toxic effects both in roots and shoots suggest that the uptake of Zn by roots was followed by its translocation to shoot.

Nevertheless, there could be other reasons responsible for the observed Zn toxicity. We observed a Zn-imposed deterioration of plant-water relations in barley seedlings as seen in lowered leaf RWC. However, this effect was restricted to high Zn concentration only. HMs including Zn has been reported to influence the plant water relations [30]. In order to produce such effects, HMs might influence the

stomatal functions as has been observed in *Vicia faba* [31]. In the latter study, heavy metal treatments caused reduced stomatal apertures and corresponding increased stomatal diffusive resistance (SDR).

In the present study, Zn treatment led to proline accumulation; magnitude being greater in shoot than in root tissues of *H. vulgare* seedlings. This organ-specific accumulation of proline suggests that Zn disturbed the water relations of barley seedlings particularly leaf tissues. Proline accumulation appears to be a general plant response to HMs stress as it has been demonstrated for several plant tissues e.g. *Brassica juncea* [32], *Brassica napus* L. [33], cauliflower [34]. In view of the established osmoprotective function of proline, it is quite likely that Zn-induced proline would be involved in appropriately adjusting the tissue water relations (RWC). Proline is a compatible osmolyte and accumulates as an osmoprotectant and protects the plants from osmotic stress and enzymes from denaturation by regulation of cytosolic pH and NAD $(P)^+$ / NAD (P) H ratio. Thus, proline might protect the plants from metal toxicity by chelating heavy metals in the cytoplasm [35].

Plant cells rely on cytoplasmic chelation of heavy metal ions for their detoxification. We observed Zn treatment of barley seedlings to result in increased levels of non-protein thiols (NPT) which reflect the biosynthesis of phytochelatins. Roots contained greater Pc contents than the shoots presumably due to Zn burden. Pcs have been shown to play a relatively insignificant role in detoxification of metal ions such as Zn and Ni in *Arabis paniculata* [36]. Zn is also an ineffective activator of PC synthase in vitro [35]. The fact that NPT levels increased due to Zn treatment in this study indicates that Pcs are involved in Zn detoxification even if it is to a lower extent. Plant exposure to heavy metals often results in a severe depletion of cellular GSH pool.

These findings apparently link the Zn toxicity to elevated levels of ROS that originate from membrane lipid peroxidation. The Zn induced enhanced MDA contents have been reported in various plant species such as wheat [37] and *Vicia faba* [38]. Univalent reduction of oxygen because of spin restrictions result in the formation of toxic active oxygen species (AOS), such as superoxide radicals (O_2^{-}) hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), alkoxyl radical ($RO^{\cdot}$) etc. [10]. Protonation of O_2^{-} can produce the hydroperoxyl radical ($H_2O^{\cdot}$) which can convert fatty acids to toxic lipids peroxide destroying biological membranes. As compared to Cu^{2+} and Fe^{3+} which were redox active elements, Zn^{2+} being a redox inactive element, enhanced the generation of ROS by inactivating the cellular antioxidative pool and by disturbing the cellular metabolic balance [39].

Due to Zn induced oxidative stress APX activity was observed to increase both in root and shoot tissues of *H. vulgare* seedlings. This is in conformity with earlier reports. For example, APX activity was observed to increase in rice plants under the toxicity of Pb [40]. Similarly, due to exposure to Zn, APX activity was observed to increase in *Pisum sativum* seedlings. This activity was more in case of shoot than in roots [41]. Enhanced APX activity in Zn- stressed tissues suggests the role of antioxidative defense in cellular management of Zn toxicity. The heavy metal toxicity is indeed strongly related with cellular redox imbalance [6].

Phytohormones play a role in plant responses to HM stress. In plants, IAA has recently been proposed to serve as a reciprocal signaling molecule between micro and macro partner of association between plant and PGPR [42]. In certain experimental set-ups, exogenous IAA or its precursor has been demonstrated to mimic the PGPR application. Inoculation of bacteria also enhanced the initiation of lateral roots which gives the plant a better assess to soil resources [14]. Similarly, heavy metal resistant strain, J62 having the characteristics of producing IAA, may indirectly promote the metal accumulation by increasing the plant biomass [43].

To get further insight into the interaction between IAA metabolism and Zn toxicity, IAA oxidase levels were measured. IAA oxidase activity was observed to increase due to Zn treatment in root and shoot tissues of barley seedlings. The activity of IAA oxidase was generally more in roots than in shoot tissues. Zn induced promotion of IAA oxidase activity was also observed in rice *(Oryza sativa*) seedlings [44]. Similar increase in IAA oxidase activity due to Cu and Cd has also been reported [45].

The Zn induced increase in IAA oxidase implies reduced endogenous IAA levels that might contribute to Zn toxicity. It seems that the endogenous levels of IAA in Zn-stressed seedlings remain in suboptimal range adversely affecting the processes necessary for growth and development of seedlings. This became further clear from the fact that exogenous application of IAA partially reverted the Zn effect obviously by maintaining the endogenous IAA levels close to the optimum range. There could be other explanations also for IAA-dependent protection against Zn-toxicity.

5 Conclusion

Zn treatment led to enhanced lipid peroxidation as well as the activity of ascorbate peroxidase, showing the involvement of antioxidative defense mechanism to reduce Zn induced toxicity. IAA oxidase activity was observed to increase due to Zn treatment. It seems that the endogenous levels of IAA in Zn-stressed seedlings remain in suboptimal range adversely affecting the processes necessary for growth and development of seedlings. In conclusion, the findings demonstrate that IAA could partly undo the Zn-induced toxicity in barley seedlings.

Conflicts of Interest: I hereby declare that I have been granted authorization by my co-authors to submit and correspond on behalf of myself and co-authors. The article submitted is an original work and has neither been submitted or published in any other peer-reviewed journal. I also declare that no conflict of interest.

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