Alleviation of zinc induced oxidative stress by polyamines in *Plantago ovata* Forsk

Paulami Pramanick
University of Calcutta
Anindita Chakraborty
UGC-DAE CSR: University Grants Commission Department of Atomic Energy Consortium for Scientific Research
Sarmistha Sen Raychaudhuri (sarmistha_rc@rediffmail.com)
University of Calcutta

Research Article

Keywords: Metallothionein, Putrescine, Spermidine, Spermine, Zinc, Oxidative stress

Posted Date: April 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-335842/v1

Abstract

Zinc causes toxicity to the plants in an excess concentration and it is manifested by chlorosis, rolling of leaf margins, and disruption of membrane integrity. The heavy metal stress also triggers the stimulation of enzymatic and non-enzymatic antioxidant systems. Polyamines are naturally occurring, secondary metabolites, protecting plants from heavy metal-induced stress. Plants also up-regulate the mRNA expression of *Metallothionein* in response to heavy metal-induced oxidative stress.

The alteration in *Metallothionein* type *2* (*PoMT2*) expression of a medicinally important herb *Plantago ovata* in presence of polyamines like Putrescine, Spermidine, and Spermine in addition to $ZnSO_4.H_2O$ by the semi-quantitative and the quantitative methods have been demonstrated in the present study. We have observed reductions in the expression of the *Metallothionein* type 2 gene in the presence of the aforementioned polyamines which implies their protective and antioxidant properties to fight against the zinc induced stress.

1 mM *Put* has been more efficient in increasing the total chlorophyll content (compared to 2 mM *Put*) by about 36% each in 1000 µM $ZnSO_4$ treated *P. ovata* seedlings. Spermidine also enhanced chlorophyll content. 2 mM *Put* and 0.5 mM *Spm* have shown even better efficiencies in increasing the total antioxidant and DPPH radical scavenging activities.

The lipid peroxidation has been found to decrease in *Put* and *Spm* supplemented samples by up to about 47% in both cases. Significant reductions in lipid peroxidation and down-regulation of *PoMT2* gene expression indicate the roles of polyamines in partially alleviating Zn-induced oxidative damage.

Introduction

Abiotic stresses are deemed responsible for the reduction in global crop yield which may account for as high as 70% reductions (Liu et al. 2015; Rouphael et al. 2016; Singh et al. 2016). Among the various abiotic stresses, heavy metals are enlisted as the priority pollutants by the United States Environmental Protection Agency (Morkunas et al. 2018). Heavy metals are classified as essential and non-essential. Non-essential heavy metals like Pb, Cr, Cd, Hg, and As are toxic even at low concentrations.

Essential heavy metals (Cu, Zn, Fe, etc.), are required by plants for cellular metabolic and physiological processes. However, zinc, like other essential heavy metals, causes severe oxidative stress, senescence and is detrimental in an excessive concentration in plants (Sunitha et al. 2014; Feigl et al. 2015). Heavy metals cause oxidative damage by producing Reactive Oxygen Species (ROS) and free radicals in plants. In a study made by our group in *Plantago ovata*, the manifestation of Zn stress is indicated by the stunted growth, chlorosis, reduced chlorophyll, and carotenoid content, and increased lipid peroxidation (Pramanick et al. 2017).

However, plants have adapted various strategies to acclimatize and combat these inclement conditions. Polyamines are the secondary metabolites produced by all plants in response to oxidative stress which

includes heavy metal-induced stress. Polyamines being polycationic compounds, bind to the negatively charged phospholipids, and proteins of the membrane and thus protect them from losing structural integrity (Calzadilla et al. 2014).

The three most abundant polyamines in plants are Putrescine (*Put*), Spermidine (*Spd*), and Spermine (*Spm*). They have specific functions to perform. Putrescine is known to play a vital role in response to abiotic stress, and maintains the water status of a leaf during drought stress by increasing the proline content (Pál et al. 2015). According to some reports, the levels of *Put* could be restored only with the exogenous supplementation of *Put* (Takahashi and Kakehi 2010; Pál et al. 2015). Putrescine aids in stress tolerance by activating the antioxidant system, regulating abscisic acid, and activating Flavonoid synthesis by inducing Phenylalanine ammonia lyase (Takahashi and Kakehi 2010). Spermidine is known to function in plant growth, and development. Mutations of *Spermidine synthases* result in defective embryo development in the heart stage of *Arabidopsis* (Imai et al. 2004). Spermine has a varied role in mitigating oxidative stress. It is known to combat the oxidative stress caused due to free radical generation and scavenges the same in the nucleus.

Lovaas (1997) reported that polyamines are known to form complexes with metal cations like Cu^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+} and are also able to inhibit metal-catalyzed oxidations or prevent singlet oxygen ($^1O_2$) or hydroxyl (·OH) radical formation. The chelation and metal-polyamine-complex formations are directly proportional to the number of nitrogen-groups present in the polyamine and also the polyamine chain length (Lovaas 1997). Lomozik and Wojciechowska (1989) reported that *Put* forms a seven-membered chelate ring structure with Cu^{2+}. This *Put*-Cu^{2+} chelate is not thermodynamically very stable. The higher polyamines like *Spm* and *Spd* form metal-complexes much easily than *Put* given the higher nitrogen content in them. *Spm* is also known to form a similar seven-membered chelate ring (Lomozik and Wojciechowska, 1989).

The precise role of polyamines in ameliorating heavy metal-induced stress may also be traced back to its function in inducing elevated levels of glutathione (GSH) in plants and subsequent production and accumulation of Phytochelatins both of which are potent metal chelators (Hasanuzzaman et al. 2019).

Sengupta et al., (2013) has reported that high doses (50 Gy and 100 Gy) of gamma radiation on imbibed seeds of *Vigna radiata* resulted in the production of high levels of endogenous polyamine.

When it comes to heavy metal stress in plants, the first two molecular entities that happen to come in our mind are Metallothioneins and Phytochelatins, because of their metal-chelating and detoxification properties (Cobbett and Goldsbrough, 2002). Plants undergo hormesis in response to oxidative stress. Metallothionein (stress-responsive protein) biosynthesis is one such hormetic activity (Morkunas et al. 2018). It showed up-regulation of its mRNA in response to diverse forms of stresses like exposure to an excess of heavy metals, drought, cold, heat shock, radiation, salinity- among the abiotic stresses (Cobbett and Goldsbrough, 2002; Ghoshal et al. 2013; Moulick et al. 2013).

In the present study, we have determined how plants cope with zinc induced oxidative stress. Metallothioneins bind heavy-metal ions with mercaptide bonds, while polyamines function in ameliorating the harmful effects of the same in a similar manner. The objective of the present study is to decipher the roles of two important entities- polyamines (Putrescine, Spermidine and Spermine) and *Metallothionein* type *2* (expression) under zinc stress in *Plantago ovata* Forsk which has not been reported earlier. *P. ovata* Forsk is a medicinally and commercially vital herb, cultivated in certain districts of Gujarat and Rajasthan, India. The plant is commonly called psyllium. *P. ovata* seeds are very interesting because of their husks with huge water-absorbing capacity are used to treat patients with constipation, irritable bowel syndrome, diabetes, and diarrhoea.

Material And Methods

Plant materials, growth conditions and stress treatment:

P. ovata seeds (cultivar HI-5) are procured from Madhya Pradesh, India. They are surface sterilized using 10% (v/v) Sodium hypochlorite (NaOCl) for 20 minutes and then washed extensively with autoclaved deionized water to remove traces of chlorine. The sterilized seeds are transferred to autoclaved agar-sucrose media (pH-7.6-7.8) composed of 3% sucrose (w/v) [Sisco Research Laboratory (SRL), Mumbai, India], and 0.9% agar-agar (w/v) [SRL, Mumbai, India] contained in plant tissue culture capped glass jars (250 mL). The growth conditions used for maintaining the cultures are- 1. Temperature: 22–25ºC, 2. Relative humidity: 55–60% and 3. Illumination: 1500 Lux for 16 hour duration of photoperiods in a plant tissue culture laboratory. In the case of Zinc Sulphate ($ZnSO_4.7H_2O$) [Merck, India] treatment, 25, 40, and 50 µL solutions of 1M $ZnSO_4.7H_2O$ are added to 50 mL of agar-sucrose medium so that the final concentration comes to 500, 800, and 1000 µM, respectively before sterilization by autoclaving. The lethal dose 50 (LD_{50}) of $ZnSO_4.7H_2O$ in *P. ovata* determined earlier is 1000 µM (Pramanick et al. 2017). In addition to $ZnSO_4.7H_2O$, the media are supplemented with 1 and 2 mM Putrescine Dihydrochloride (*Put*) [Himedia Laboratories, Mumbai, India], 0.5 mM Spermidine (*Spd*) [Himedia Laboratories, Mumbai, India] and 0.5 mM Spermine (*Spm*) [Himedia Laboratories, Mumbai, India]. The *Put*, *Spd*, and *Spm* solutions are filtered using the 0.2 µm acrodisc syringe filter [Acrodisc® 25 mm Syringe Filter, Pall Life Sciences, U.S.A] and then added to the autoclaved media (when the media was still liquid; temperature of media- 65˚-70˚C). Media without $ZnSO_4.7H_2O$ treatment and polyamines supplementations are treated as control. The $ZnSO_4.7H_2O$ treatment and polyamine supplementations are carried out for 7 days to observe the biochemical and *MT2* expressional changes.

The specific doses of $ZnSO_4.7H_2O$ and polyamines (*Put*, *Spd*, and *Spm*) used in the experimental design are given in Table 1:

Table 1
The dosages of $ZnSO_4.7H_2O$ and polyamines (*Put*, *Spd* and *Spm*) that are used for performing the biochemical assays, Reverse Transcription PCR and Real Time (q) PCR

Concentrations of Polyamines	Doses used in experimental design
1 mM Putrescine (Put**)**	Control (0 mM *Put* + 0 µM $ZnSO_4.7H_2O$), (1 mM *Put* + 0 µM $ZnSO_4.7H_2O$), (1 mM *Put* + 500 µM $ZnSO_4.7H_2O$), (1 mM *Put* + 800 µM $ZnSO_4.7H_2O$), (1 mM *Put* + 1000 µM $ZnSO_4.7H_2O$)
2 mM Putrescine (Put**)**	Control (0 mM *Put* + 0 µM $ZnSO_4.7H_2O$), (2 mM *Put* + 0 µM $ZnSO_4.7H_2O$), (2 mM *Put* + 500 µM $ZnSO_4.7H_2O$), (2 mM *Put* + 800 µM $ZnSO_4.7H_2O$), (2 mM *Put* + 1000 µM $ZnSO_4.7H_2O$)
0.5 mM Spermidine (Spd**)**	Control (0 mM *Spd* + 0 µM $ZnSO_4.7H_2O$), (0.5 mM *Spd* + 0 µM $ZnSO_4.7H_2O$), (0.5 mM *Spd* + 500 µM $ZnSO_4.7H_2O$), (0.5 mM *Spd* + 800 µM $ZnSO_4.7H_2O$), (0.5 mM *Spd* + 1000 µM $ZnSO_4.7H_2O$)
0.5 mM Spermine (Spm**)**	Control (0 mM *Spm* + 0 µM $ZnSO_4.7H_2O$), (0.5 mM *Spm* + 0 µM $ZnSO_4.7H_2O$), (0.5 mM *Spm* + 500 µM $ZnSO_4.7H_2O$), (0.5 mM *Spm* + 800 µM $ZnSO_4.7H_2O$), (0.5 mM *Spm* + 1000 µM $ZnSO_4.7H_2O$)

The experiments have been designed using 1 and 2 mM Putrescine but only 0.5 mM each of Spermidine and Spermine as higher doses caused shrinkage in *P. ovata* seedlings.

Preparation of Plant Extracts:

The plant extracts are prepared following the methods of Brolis et al., (1998). 100 mg of 7 days old untreated, $ZnSO_4.7H_2O$ treated, and *Put*, *Spd*, and *Spm* supplemented seedlings of *P. ovata* are homogenized with 1 mL of 50% aqueous ethanolic solution [HPLC grade, Merck, Germany]. The homogenized mixtures are subjected to ultrasonication [Sonics Vibra Cell VC300, Connecticut, U.S.A] in ice for about 20 minutes. The ultrasonicated samples are then centrifuged at 10,000 *g* for 5 minutes. Then the supernatants are collected and kept at -20°C and further used as 50% ethanolic plant extracts for the determination of total antioxidant content and DPPH radical scavenging activity.

Estimation of Chlorophyll and Carotenoid Contents:

Total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid content are determined following the method developed by Sestak et al. (1971) and Lichtenthaler (1987). 100 mg each of untreated, $ZnSO_4$ treated and polyamines supplemented shoot and leaf tissues of *P. ovata* are crushed and homogenized in 5mL of 100% ice-cold acetone [SRL, Mumbai, India] in a mortar. Then the homogenate is centrifuged at 7728 *g* for 10 minutes at 4° C in an ultracentrifuge [HERMLE Z383 K, Hermle Machine Company]. Then the clear supernatant is taken in a glass cuvette and spectrophotometric absorbance is measured in the wavelengths of 662, 663, 645, 646, and 470 nm using UV-1800 SHIMADZU UV Spectrophotometer.

$$\text{Chlorophyll a } (\mu g/\text{ mL}) = 11.24\ (A_{662}) - 2.04\ (A_{645})$$

$$\text{Chlorophyll b } (\mu g/\text{ mL}) = 20.13\ (A_{645}) - 4.19\ (A_{662})$$

$$\text{Total chlorophyll } (\mu g/\text{ mL}) = 7.15\ (A_{663}) + 18.71(A_{646})$$

$$\text{Carotenoids (Carotene + Xanthophylls) } (\mu g/\text{ mL}) = [1000\ (A_{470}) - 1.90 \text{ chl a} - 63.14 \text{ chl b}] / 214$$

Estimation of Total Antioxidant Activity:

To determine the total antioxidant content of the plant extracts of *P. ovata*, the phosphomolybdenum assay performed by Prieto et al. (1999) is followed. Appropriately, 0.3 mL each of the 50% aqueous ethanolic extracts of untreated control, $ZnSO_4.7H_2O$ treated and polyamines supplemented 7 days old seedlings are added to 3 mL of Phosphomolybdenum buffer. Then the reaction mixtures are incubated at 95°C for 90 minutes in a water bath. Then the absorbances are measured at a wavelength (λ_{max}) of 695 nm. The observation of the total antioxidant activity is expressed in terms of milligrams (mg) of ascorbic acid equivalents (AAE) per gram (g) FW tissue. An ascorbic acid [Sigma Aldrich] calibration curve is plotted to calculate the concentration of antioxidants present in the ethanolic plant extracts.

Estimation of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay:

The DPPH radical scavenging assay is performed by the method followed by Brand-Williams et al., 1995. The reaction mixtures are prepared by adding 0.15 mL of each of the 50% ethanolic extracted samples of *P. ovata* to 2.85 mL of the working stock of DPPH solution. The reaction mixtures are allowed an hour of incubation in dark at room temperature. Then the spectrophotometric reading of each of the samples is measured at a wavelength (λ_{max}) of 517 nm.

The percentage radical scavenging activity is measured using the following formula:

$$[(A_{control} - A_{sample}) / A_{control}] \text{ X } 100\%$$

Where $A_{control}$ is the absorbance of the DPPH reagent without any 50% ethanolic extracted samples and A_{sample} is the absorbance of the DPPH reagent with each of the 50% ethanolic extracted samples.

Estimation of Oxidative damages:

The Lipid peroxidation assay is performed following the method of Issawi et al (2018). The amount of malondialdehyde (MDA) produced is indicative of the amount of membrane lipid oxidized. 7 days old *P.*

ovata seedlings of untreated control, $ZnSO_4$ treatment and polyamines supplementation are homogenized in 0.1% (w/v) Trichloroacetic acid (TCA) [SRL] solution, in a mortar. Then the crushed samples are centrifuged at 17,388 *g* for 10 min. 1 mL of supernatant from each sample is added to 4 mL of TCA-Thiobarbituric acid (TBA) solution composed of 0.5% (w/v) TBA [SRL] and 20% (w/v) TCA. The mixture is heated at 100º C in a hot water bath for about 30 min. and then immediately cooled on ice to stop the reaction. The absorbances are measured at 532 nm against a blank containing TBA-TCA solution. The degree of Lipid peroxidation is expressed as MDA equivalents in micromoles per litre per gram FW. The extinction coefficient of MDA is 155 $mmolL^{-1}cm^{-1}$.

Total RNA Extraction from P. ovata Seedlings:

The total RNA is extracted from 100 mg shoot tissues of each type of *P. ovata* seedlings using the PureLink RNA Mini kit [PureLink RNA Mini kit, Ambion by Life Technologies, New Delhi, India]. After completion of extraction, the Total RNA samples are stored at -80ºC.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR):

To determine the expression level of *MT2* gene in untreated control and $ZnSO_4.7H_2O$ treated seedlings (exogenously supplemented with 1 and 2 mM *Put*, 0.5 mM *Spd*, and 0.5 mM *Spm*) of *P. ovata*, RT-PCR [QIAGEN One-Step RT- PCR kit, QIAGEN, New Delhi, India] is carried out using gene-specific primers (Table 2). The RT-PCR reaction mixtures contained 2 µg/50 µL of total RNA. The reaction conditions are shown in Table 3.

Table 2
The sequences of the gene-specific primers of *MT2* and β- *actin* of *P. ovata*.

Name of gene	Forward primer sequence	Reverse primer sequence
PoMT2	5'ATGTCTTGCTGCAACGGAAACT3'	5'CTATTTGCAATTGCATGGATTG3'
β-actin	5'ATCATGAAGTGTGATGTTGA3'	5'ACCTTAATCTTCATGCTGCT3'

Table 3: The Reverse Transcription PCR conditions of *MT2* gene in *P. ovata*.

Name of gene	*PoMT2*		
Reverse Transcription	Temperature (˚C)	**50**	
	Duration	**30 min.**	
Initial Denaturation	Temperature (˚C)	**95**	
	Duration	**15 min.**	
Denaturation	Temperature (˚C)	**94**	
	Duration	**1 min.**	**25 cycles**
Annealing	Temperature (˚C)	**55**	
	Duration	**30 sec.**	
Extension	Temperature (˚C)	**72**	
	Duration	**1min. 30 sec.**	
Final Extension	Temperature (˚C)	**72**	
	Duration	**10 min.**	

Real-Time- Polymerase Chain Reaction (qPCR):

Total RNAs extracted from untreated, polyamine treated, and (polyamine + $ZnSO_4.7H_2O$) treated *P. ovata* seedlings are used for synthesizing the first-strand cDNA using RNA to cDNA kit [Invitrogen, Grand Island, New York, USA]. 1 µg/µL of cDNA is used in the reaction mixture as templates for performing real-time PCR. 2X POWER SYBR Green master mix [Applied Biosystems, Foster City, USA] is used for carrying out the qPCR reactions. β-*actin* gene is the endogenous control. Each of the reactions in this real-time PCR experiment is carried out in triplicates using Applied Biosystems Step One Plus Real-Time PCR system [Applied Biosystems, Foster City, USA]. The relative gene expression of the *PoMT2* gene is calculated by the $2^{-\Delta\Delta C}{}_T$ method according to Livak and Schmittgen (2001) (Livak and Schmittgen, 2001).

Statistical Analysis:

All the experimental results are represented as mean ± standard error of the mean (SEM) except for the Real-time PCR data represented as mean ± standard deviation (SD) shown in Figs. 7(a) (i), 7(a) (ii), 7(b) and 7 (c). The data are analyzed by calculating the variance by Two-way ANOVA using KyPlot (version 2.0) and Bonferroni post-tests are performed using Graphpad Prism software version 6.0. Graphs are prepared using Microsoft Excel 2007.

Result

Chlorophyll and Carotenoid Content

As depicted in Fig. 1(a), the total chlorophyll contents in 500, 800, and 1000 μM $ZnSO_4$ treated *P. ovata* got enhanced by 51.92%, 36.54%, and 2.09% respectively, compared to untreated control seedlings. This increase (though found decreasing with increasing doses of $ZnSO_4$) is evidence of the role of Zn in chlorophyll biosynthesis (Pramanick et al. 2017). 1mM *Put* supplementation increased the total chlorophyll content in 0, 800, and 1000 μM $ZnSO_4$ treated seedlings further by 8.2%, 4.48%, and 36.3% respectively, compared to polyamine untreated seedlings of *P. ovata*. 2mM *Put* could enhance the total chlorophyll content of 1000 μM $ZnSO_4$ treated samples only by 10.79%.

0.5 mM *Spd* increased the total chlorophyll content in 0, 500, and 1000 μM $ZnSO_4$ treated *P. ovata* seedlings further by 19.4%, 15.65%, and 13.36% respectively, compared to polyamine untreated samples as depicted in Fig. 1(a).

With 0.5 mM *Spm* treatment we observed gradual and statistically significant reductions in the total chlorophyll level (Fig. 1(a)).

Put supplementation reversed the effect of 1000 μM $ZnSO_4.7H_2O$ on *P. ovata* seedlings as we observe from Fig. 1(b). The carotenoid contents of 1000 μM $ZnSO_4.7H_2O$ treated plants got enhanced from (0.132 ± 0.0014) mg/g FW to (0.169 ± 0.0003) and (0.148 ± 0.0002) mg/g FW when supplemented with 1 and 2 mM *Put*, respectively.

Figure 1(b) shows that 0.5 mM *Spd* has somewhat tried to take up the levels of carotenoid, especially in 500 μM $ZnSO_4$ treated *P. ovata* seedlings but, 0.5 mM *Spm* resulted in significant reductions in the carotenoid content of *P. ovata* (Fig. 1(b)).

Total Antioxidant Activity

500, 800, and 1000 μM $ZnSO_4$ treatment showed a dose-dependent increase in the total antioxidant activity in *P. ovata*. Exogenous supplementation of 1 and 2 mM *Put* enhanced the total antioxidant contents of (1 mM *Put* + 0 μM $ZnSO_4.7H_2O$) and (2 mM *Put* + 0 μM $ZnSO_4.7H_2O$) treated *P. ovata* ethanolic plant extracts to (7263.67 ± 77.88) and (8139.33 ± 68.19) μg Ascorbic acid equivalent (AAE)/g FW from (5481.67 ± 49.38) μg AAE/g FW of the untreated control. 2 mM *Put* increased the total antioxidant activities in 0, 500, and 800 μM $ZnSO_4$ treated samples further by 48.49%, 38.31%, and 13.48%, respectively. The graphical representation in Fig. 2 depicts that 2 mM *Put* supplementation has been more effective than 1 mM *Put*, in increasing the total antioxidant content of the ethanolic plant extracts of *P. ovata.*

0.5 mM *Spd* supplementation showed reduced total antioxidant activity of the ethanolic plant extracts in *P. ovata*.

0.5 mM *Spm*, when added exogenously to the media, showed an increment in the total antioxidant levels up to 52.49% in 500 µM $ZnSO_4.7H_2O$ treated samples compared to polyamine untreated seedlings. On further increasing the dose of $ZnSO_4.7H_2O$, a decrease in the total antioxidant levels is observed (Fig. 2).

DPPH Radical Scavenging Activity

Exogenous addition of 1 mM *Put* to culture media significantly enhanced the DPPH free radical scavenging activity up to (89.14 ± 0.07) % in 1000 µM $ZnSO_4.7H_2O$ treated samples.

0.5 mM *Spd* also enhanced the DPPH radical scavenging activity of 1000 µM $ZnSO_4.7H_2O$ treated ethanolic *P. ovata* extracts to (89.565 ± 0.60) % (Fig. 3).

0.5 mM *Spm*, on the other hand, could increase the radical scavenging activity up to 800 µM $ZnSO_4.7H_2O$ treated samples with a maximum of percentage radical scavenging activity of (89.875 ± 1.89) % in Zn untreated extracts (Fig. 3).

Oxidative damages: MDA content

Lipid peroxidation is one of the most important parameters to study in the case of abiotic stresses in plants. 1 mM *Put* supplementation resulted in about 0.36 fold decrease in malondialdehyde (MDA) production in (1 mM *Put* + 0 µM $ZnSO_4.7H_2O$) *P. ovata* seedlings. Although 1 mM *Put* could not reduce the MDA contents significantly in 500, 800, and 1000 µM $ZnSO_4.7H_2O$ treated plants, the dose of 2 mM *Put* was potent enough in reducing the MDA concentrations. Exogenous addition of 2 mM *Put* decreased the MDA concentrations of 0, 500, 800, and 1000 µM $ZnSO_4.7H_2O$ treated *P. ovata* seedlings by 0.4, 0.47, 0.39, and 0.4 folds, respectively compared to the polyamine untreated samples (Fig. 4).

Like 1 mM *Put*, 0.5 mM *Spd* was also not potent enough in reducing the rate of membrane lipid peroxidation as depicted in Fig. 4.

However, the exogenous supplementation of 0.5 mM *Spm* in the media helped in reducing the rate of lipid peroxidation efficiently. It decreased the MDA contents in 0, 500, 800, and 1000 µM Zn treated *P. ovata* plants by 0.14, 0.45, 0.47, and 0.43 folds, respectively compared to the polyamine untreated seedlings (Fig. 4).

Reverse Transcription- Polymerase Chain Reaction- Densitometric Analysis

$ZnSO_4.7H_2O$ treatment resulted in an enhancement of the expression of the *Metallothionein* type *2* gene in *P. ovata*. It showed 1.38, 1.39, and 1.76 normalized fold increases in the 500, 800, and 1000 µM $ZnSO_4.7H_2O$ treated samples of *P. ovata*. 1 and 2 mM *Put* supplementation resulted in statistically

significant reductions in the *MT2* gene expressions. The densitometric analysis shows that 1 mM *Put* decreased the expression levels of *MT2* from (1 ± 0.00) to (0.57 ± 0.0045) in Zn untreated control samples and (1.38 ± 0.0289) to (0.66 ± 0.0062), (1.39 ± 0.0239) to (0.57 ± 0.0060) and (1.764 ± 0.0286) to (0.64 ± 0.0046) in 500, 800, and 1000 µM $ZnSO_4.7H_2O$ treated samples of *P. ovata*, respectively. Similarly, 2 mM *Put* also aided in reducing the *MT2* gene expression by 0.44, 0.64, 0.69, and 0.905 folds in Zn untreated and treated *P. ovata* shoot tissues (Fig. 5). Again, 1mM *Put* has been more efficient in decreasing the expression of *PoMT2*.

The exogenously added 0.5 mM *Spd* also reduced the relative expression of *MT2* mRNA in *P. ovata* by 0.405 fold in 1000 µM $ZnSO_4.7H_2O$ treatment (Fig. 5).

The nutrient media supplementation with 0.5 mM *Spm*, could rather, more efficiently (than *Spd*) decreased the expression of *MT2* mRNA in *P. ovata*. It reduced the expression of *PoMT2* from (1 ± 0.00), (1.38 ± 0.0289), (1.39 ± 0.0239), and (1.764 ± 0.0286) to (0.55 ± 0.0077), (0.95 ± 0.0097), (0.88 ± 0.0086), and (1.05 ± 0.0087), respectively, in $ZnSO_4.7H_2O$ untreated and $ZnSO_4.7H_2O$ treated *P. ovata* shoot tissues (Fig. 5).

Real Time PCR (qPCR)

Exogenous supplementation of 1mM and2mM *Put* showed up to 0.26 and 0.675 fold decreases in the *MT2* expression, respectively, in *P. ovata* (Figs. 6(a)(i) and 6(a)(ii)).

0.5mM *Spd* and 0.5mM *Spm* supplementations showed up to 0.42 and 0.36 fold decreases in the relative gene expression of *MT2*, respectively, in *P. ovata* (Figs. 6(b) ad 6(c)).

Statistical Analysis

Results of two-way Analysis of Variance (ANOVA) indicate significant changes in the mean values and interactions between the two independent variables $ZnSO_4.7H_2O$ and Polyamines concentration (*Put*, *Spd*, and *Spm*) in all the cases of biochemical and molecular biological analyses. At 5% level of significance ($\alpha = 0.05$), we obtained $F(cal) > F(0.05)$ which holds true for all the three polyamines. We, therefore, reject the null hypothesis (which says, there are no significant changes in the mean values and that there is no interaction between the two independent variables).

Discussion

Polyamines are often associated with plant growth, DNA and RNA stability, membrane stability, stress resistance, and survival of plant as a whole (Marco et al. 2012; Pál et al. 2015). The effect of exogenous addition of polyamines to alleviate stress depends on various factors like plant species, plant cultivars, duration of given stress, and the dosage of the stress, the tissue type, and also on the way of exogenous polyamine application, that is, through nutrient media supplementation or foliar spray (Soudek et al. 2016).

According to a report *Brassica juncea* plants exposed to Cd stress showed decrease in the chlorophyll content. Foliar spray of *B. juncea* with *Put* enhanced the chlorophyll a content whereas, that with *Spd* resulted in an increase of both chlorophyll a and chlorophyll b content (Kumar et al. 2014).Wang et al. (2003) also showed that Hg^{2+} and Cr^{6+} treatment decreased the chlorophyll content in amaranth leaves which was later reversed by exogenous *Spd* addition. In the present study, we have also observed that exogenously supplemented *Put* and *Spd* could alleviate the effect of $ZnSO_4$ to some extent and result in an increment of the chlorophyll and carotenoid content in *P. ovata*.

In a study by Howladar et al., (2018), endogenous *Spd* and *Spm*, which also enhanced GSH content conferred Cd tolerance in wheat plants (Howladar et al. 2018). Polyamines can act as antioxidants due to their dual binding properties with anions and cations (Groppa et al. 2007; Cicatelli et al. 2010). Polyamines bind to cations, thereby preventing the formation of ROS (Groppa et al. 2007; Lovaas 1997; Lomozik and Wojciechowska, 1989). As explained by Lovaas, the antioxidant properties of polyamines are attributed by the interaction between the positively charged polyamines and the positively charged metal ions which generate a Coulombic repulsive force and this repulsion is neutralized when negatively charged biomolecules (phospholipids, DNA, and RNA) adsorb polyamines on to them (Lovaas 1997).

Various reports say that polyamines like *Put*, *Spd*, and *Spm* are potent scavengers of free radicals like the superoxide ($O_2^{\cdot -}$), hydroxyl (·OH), alkoxyl (RO·), peroxyl (ROO·) radicals, singlet oxygen ($^1O_2$), and ozone (O_3) (Lovaas 1997; Groppa and Benavides 2008; Marco et al. 2012). In this study, we observe an overall increment in the total antioxidant activity with 2 mM *Put* and 0.5 mM *Spm* supplementation. *Spd*, on the other hand, reduced the total antioxidant activity in *P. ovata* which is in congruence with the results of Mandal et al., (2013) (Mandal et al. 2013). *Salvia natans* treated with H_2O_2 showed reduced SOD, GPX, APX, and GR activities with exogenous *Put* supplementation. *Spm* also reduced the APOX activity of wheat leaves by 31% but restored the GR activity to the levels of control when added before Cd and Cu (Groppa et al. 2007). According to some reports, exogenous *Spm* completely restored the GR and SOD activities which were otherwise compromised under Cd^{2+} and Cu^{2+} treatment in sunflower plants (Chen et al. 2019).

The fact that polyamines act as free radical scavengers is well established. Polyamines and specifically *Spm* have the potential to protect DNA from free radical-induced oxidative damage (Groppa et al. 2007; Marco et al. 2012). Therefore, the observation of increased DPPH radical scavenging activity by *Put*, *Spd*, and *Spm* in *P. ovata* is evident in their function as antioxidants. According to the reports of Sengupta and Raychaudhuri (2017), exogenously added *Put* significantly increased the free radical scavenging activity of gamma-irradiated *Vigna radiata* seedlings in comparison to *Put*- untreated seedlings (Sengupta and Raychaudhuri, 2017). Das and Adak, (2015) have found out that 2 mM *Spd* caused about 24.91% decrease in DPPH radical scavenging activity in *Marsilea minuta* L. treated with 200 µM $CdCl_2$ (Das and Adak, 2015). This is again in agreement with our results, where *Spd* initially showed a decrease in the DPPH radical scavenging activity in *P. ovata* as well.

Membrane lipid peroxidation is one of the important markers of oxidative stress induced by various abiotic sources. Zn exposure also led to an enhanced rate of lipid peroxidation in *P. ovata* (Pramanick et al. 2017). Foliar spray of *Put* and *Spd* decreased the MDA concentration in Cd treated *B. juncea* plants (Kumar et al. 2014). According to the reports of Groppa et al., (2001 and 2007) *Spm* efficiently reduced the TBARS produced during membrane lipid peroxidation in sunflower leaf disk and wheat leaves when exposed to both Cd and Cu (Groppa et al. 2001; Groppa et al. 2007). Putrescine supplementation also reduced the MDA levels in H_2O_2 treated *S. natans* significantly (Mandal et al. 2013). Cucumber seedlings under hypoxia stress treated with exogenous *Spd* resulted in decreased malondialdehyde production (Chen et al. 2019). All these results are on par with our observation of reductions in the lipid peroxidation by *Put*, *Spd*, and *Spm* in Zn treated *P. ovata* seedlings.

This finding of reduced MDA production by *Put*, *Spd*, and *Spm* supplemented *P. ovata* seedlings can be correlated with the reduced expression of the *MT2* gene. The *Metallothionein* being a stress-responsive gene is induced by heavy metal exposure causing its up-regulation. However, on supplementing the nutrient media with *Put*, *Spd*, and *Spm* we obtained a down-regulation and decreased expression of the *PoMT2* gene. Metallothioneins are a group of cysteine-rich, low molecular weight proteins that aid in essential metal homeostasis, detoxification of excess heavy metals, and protection from oxidative damage induced by them. They are well-known for their superoxide and hydroxyl radical scavenging activities (Ruttkay-Nedecky et al. 2013). In a previous study by our group, *MT2* gene expression studied by qPCR technique showed an enhancement by 4.57 and 5.78 folds in 500 and 800 µM Zn treated *P. ovata* samples (Pramanick et al. 2017). According to Choudhary et al., (2012), 7 days old radish seedlings pre-treated with 1 mM *Spd* showed a 3.5 fold decrease in the relative gene expression of *RsMT1C* (*Metallothionein type 1* gene) and is similar to our findings (Choudhary et al. 2012).

According to Cicatelli et al., (2010), Arbuscular-mycorrhizal-fungal (AMF) association has resulted in up-regulation of both *MT* genes (*PaMT1*, *PaMT2*, and *PaMT3*) as well as polyamine biosynthetic genes *ADC* and *SPDS* in *Populus alba* clone AL35 grown in Cu and Zn polluted soil (Cicatelli et al. 2010). Although a direct relationship between *MT2* gene expression and exogenous supplementation of polyamines in nutrient media is not very well elucidated till date, the findings by Cicatelli et al., (2010) and many more gives an insight into how exogenous polyamines can regulate the expression levels of *MT* genes in plants and that *MT* transcriptional expression and action of exogenous polyamines are correlated (Cicatelli et al. 2010). According to Li et al., exogenous *Spd* supply enhanced *MT* gene expression in white clover under both well-watered and drought conditions but the same with dicyclohexylamine (DCHA), a secondary amine, resulted in a down-regulation of two leaf-tissue specific *MT* gene expression in *Trifolium repens* (white clover) supplied with 0.05 mM *Spd* (Li et al. 2016).

Therefore, in this study we hypothesized the ameliorating effects of exogenous Putrescine, Spermidine, and Spermine when applied against oxidative stress induced by $ZnSO_4.7H_2O$ in *P. ovata*. From the results obtained we can infer that the total antioxidant activity, the free radical scavenging activity, and chlorophyll and carotenoid content get enhanced. On the other hand, the simultaneous down-regulations of malondialdehyde production and the *Metallothionein Type 2* expression are key symbols of alleviation

of Zn induced oxidative stress. We, therefore, propose that excess Zn causes stress which induces the enhanced transcription of the *MT2* gene in *P. ovata*. When confronted with doses of Putrescine, Spermidine, and Spermine against zinc stress, the *Metallothionein Type 2* shows a reduced expression due to amelioration of stress by the polyamines.

Declarations

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Material

The experiments were performed in replicates and experimental data were generated in our laboratory by us.

Competing Interests

There are no competing interests.

Funding

The authors would like to express their sincere gratitude to the UGC- DAE Consortium for Scientific Research, Kolkata Centre, West Bengal, India (UGC- DAECSR- KC/CRS/13/RB- 01/0808) for financial support.

Author's Contributions

SSRC designed and supervised the research work. PP and SSRC conducted the experiments. AC helped with writing the manuscript. PP and SSRC analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Acknowledgement

The authors would like to show sincere gratitude to UGC-CAS and UGC-DAE Consortium for Scientific Research, Kolkata Centre for their financial support.

References

Brand-Williams W, Cuvelier ME, Berset C, (1995) Use of a free radical method to evaluate antioxidant activity, Lebenson Wiss Technol, 28: 25–30.

Brolis M, Gabetta B, Fuzzati N, Pace R., Panzeri F, Peterlongo F, (1998) Identification by high performance liquid chromatography–diode array detection–mass spectrometry and quantification by high performance liquid chromatography–UV absorbance detection of active constituents of *Hypericum perforatum*, J Chromatogr, 825: 9-16.

Calzadilla PI, Gazquez A, Maiale SJ, Ruiz OA, Bernardia MA, (2014) Polyamines as indicators and modulators in the abiotic stress in plants, In: Anjum NA, Gill SS, Gill R (ed) *Plant adaptation to environmental change: significance of amino acids and their derivatives*, CAB International, Wallingford, UK, pp 109-128.

Chen D, Shao Q, Yin L, Younis A, Zheng B, (2019) Polyamine function in plants: Metabolism, regulation on development, and roles in abiotic stress responses, Front Plant Sci, 9: 1945, doi: 10.3389/fpls.2018.01945.

Choudhary SP, Oral HV, Bhardwaj R, Yu JQ, Tran LSP, (2012) Interaction of brassinosteroids and polyamines enhances copper stress tolerance in *Raphanus sativus*, J Exp Bot, 63 (15): 5659- 5675.

Cicatelli A, Lingua G, Todeschini V, Biondi S, Torregiani P, Castiglione S, (2010) Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and polyamine biosynthetic gene expression, Ann Bot, 106: 791- 802.

Cobbett C and Goldsbrough P, (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis, Ann Rev Plant Biol, 53:159–182.

Das K and Adak MK, (2015) Polyamine mediated anti-oxidation activity facilitates of *Marsilea minuta* L. under Cd toxicity, Plant Gene Trait, 6 (8): 1-13.

Feigl G, Lehotai N, Molnar A, Ordog A, Rodriguez- Ruiz M, Palma Jose M, Corpus Francisco J, Erdei L, Kolbert Z, (2015) Zinc induces distinct changes in the metabolism of reactive oxygen and nitrogen species (ROS and RNS) in the roots of two *Brassica* species with different sensitivity to zinc stress, Ann Bot, 116: 613–625.

Ghoshal N, Talapatra S, Moulick A, Chakraborty A, Raychaudhuri SS, (2013) Alterations in transcriptome and proteome on metallothioneins following oxidative stress induced by sub lethal doses of cadmium and gamma rays in *Plantago ovata*, Int J Radiat Biol, 89(7): 571- 582.

Groppa MD and Benavides MP, (2008) Polyamines and abiotic stress: recent advances, Amino Acids, 34: 34- 45.

Groppa MD, Tomaro ML, Benavides MP, (2001) Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs, Plant Sci, 161: 481–488.

Groppa MD, Tomaro ML, Benavides MD, (2007) Polyamines and heavy metal stress: the antioxidant behavior of Spermine in cadmium- and copper-treated wheat leaves, BioMetals, 20: 185- 195.

Gupta K, Sengupta A, Chakraborty M, Gupta B, (2016) Hydrogen peroxides and polyamines act as double edged swords in plant abiotic stress responses, Front Plant Sci, 7: 1343, doi: 10.3389/fpls.2016.01343.

Hasanuzzaman M, Haifa ASA, Parvin K, Borhanuddin Bhuyan MHM, Tanveer M, Mohsin SM, Nahar K, Soliman MH, Mahmud JA, Fujita M, (2019) Polyamine action under metal/ metalloid stress: Regulation of Biosynthesis, Metabolism and Molecular Interactions, Int J Mol Sci, 20: 3215. doi:10.3390/ijms20133215.

Howladar SM, Al-Robai SA, Al-Zahrani FS, Howladar MM, Aldhebiani AY, (2018) Silicon and its application method effects on modulation of cadmium stress responses in *Triticum aestivum* (L.) through improving the antioxidative defense system and polyamine gene expression. Ecotox Environ Safe, 159: 143–152.

Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M, Saji H, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Komeda Y, Takahashi T, (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*, Plant Physiol, 135: 1565–1573.

Issawi M, Guillaumot D, Sol V, and Riou C, (2018) Responses of an adventitious fast-growing plant to photodynamic stress: comparative study of anionic and cationic porphyrin effect on *Arabidopsis thaliana*, Physiol Plant, 162(3): 379-390.

Kumar P, Gogia N, and Tiwari B, (2014) Plants defensive response against Cadmium toxicity stress: A review, Journal of the Kalash Science, 2(1):75-82.

Li Z, Zhang Y, Zhang X, Peng Y, Merewitz E, Ma X, Huang L, Yan Y, (2016) The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover, Environ Exp Bot, 124: 22- 38.

Lichtenthaler HK, (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Method Enzymol, 148:350–382.

Liu JH, Wang W, Wu H, Gong X, Moriguchi T, (2015) Polyamines function in stress tolerance: from synthesis to regulation, Front Plant Sci, 6: 827, doi: 10.3389/fpls.2015.00827.

Livak KJ and Schmittgen TD, (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods, 25:402–408.

Lomozik L and Wojciechowska A, (1989) Complexation of Putrescine with Copper (II), Zinc (II), Lead (II) and Magnesium (II) in aqueous solution, Polyhedron, 8(22): 2645- 2648.

Lovaas E, (1997) Antioxidant and Metal- Chelating Effects of Polyamines, Adv Pharmacol, 38: 119- 149.

Mandal C, Ghosh N, Adak MK, Dey N, (2013) Interaction of polyamine on oxidative stress induced by exogenously applied hydrogen peroxide in *Salvinia natans* Linn, Theor Exp Plant Phys, 25 (3): 203-212.

Marco F, Alcazar R, Altabella T, Carrasco P, Gill SS, Tuteja N, Tiburcio AF, (2012) Polyamines in developing stress- resistant crops, *Improving crop resistance to abiotic stress*, first edition, 623- 635, DO - 10.1002/9783527632930.ch27.

Morkunas I, Wozniak A, Mai VC, Rucinka- Sobkowiak R, Jeandat P, (2018) The role of heavy metals in plant response to biotic stress, Molecules, 23: 2320, doi: 10.3390/molecules23092320.

Moulick A, Raychaudhuri SS, Talapatra S, Ghoshal N, Mukhopadhyay D, (2013) Molecular cloning, modeling and characterization of type 2 metallothionein from *Plantago ovata* Forsk, Sequencing, doi:10.1155/2013/756983.

Pál M, Szalai G, Janda T, (2015) Polyamines are important in abiotic stress signaling, Plant Sci, 237: 16-23.

Pramanick P, Chakraborty A, Raychaudhuri SS, (2017) Phenotypic and biochemical alterations in relation to *MT2* gene expression in Plantago ovata Forsk under zinc stress, Biometals, 30 (2): 171- 184.

Prieto P, Pineda M, Aguilar M, (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum complex: specific application to the determination of vitamin E, Anal Biochem, 269: 337–341.

Rouphael Y, Colla G, Bernardo L, Kane D, Trevisan M, Lucini L, (2016) Zinc excess triggered polyamines accumulation in Lettuce Root Metabolome, As Compared to Osmotic Stress under High Salinity, Front Plant Sci, 7: 842, doi: 10.3389/fpls.2016.00842.

Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T, Stiborova M, Adam V, Kizek R, (2013) The role of metallothionein in oxidative stress, Int J Mol Sci, 14: 6044–6066.

Sengupta M, Chakraborty A, Raychaudhuri SS, (2013) Ionizing radiation induced changes in phenotype, photosynthetic pigments and free polyamine levels in Vigna radiata (L.) Wilczek, Appl Radiat Isot, 75: 44-49.

Sengupta M and Raychaudhuri SS, (2017) Partial alleviation of oxidative stress induced by gamma irradiation in *Vigna radiata* by polyamine treatment, *Int J Radiat Biol*, 93(8), 803- 817.

Sestak Z, Catsky J, Jarvis PG, (1971) Plant photosynthetic production: manual of methods. The Hague: Dr. W Junk.

Singh S, Parihar P, Singh R, Singh VP, Prasad SM, (2016) Heavy metal tolerance in plants: Role of transcriptomics, Proteomics, Metabolomics, and Ionomics, Front Life Sci, 6: 1143, doi: 10.3389/fpls.2015.01143.

Soudek P, Ursu M, Petrova S, Vanek T, (2016) Improving crop tolerance to heavy metal stress by polyamine application, Food Chem, 213: 223- 229.

Sunitha MSL, Prashant S, Anil Kumar S, Rao S, Narasu ML, Kavi Kishor PB, (2014) Cellular and molecular mechanisms of heavy metal tolerance in plants: a brief overview of transgenic plants overexpressing phytochelatin synthase and metallothionein genes, Plant Cell Biotechnol Mol Biol, 14 (1 & 2): 33- 48.

Takahashi T and Kakehi JI, (2010) Polyamines: ubiquitous polycations with unique roles in growth and stress responses, Ann Bot, 105: 1- 6.

Urano K, Hobo T, Shinozaki K, (2005) Arabidopsis ADC genes involved in polyamine biosynthesis are essential for seed development, FEBS Lett, 579: 1557–1564.

Wang X, Shi G, Xu Q, (2003) Toxic effects of Cr6+ on *Nymphoides peltatum* mitigated by exogenous polyamine, Acta Sci Circumst, 23: 689–693. doi: 10.3321/j.issn:0253-2468.2003.05.024.

Figures

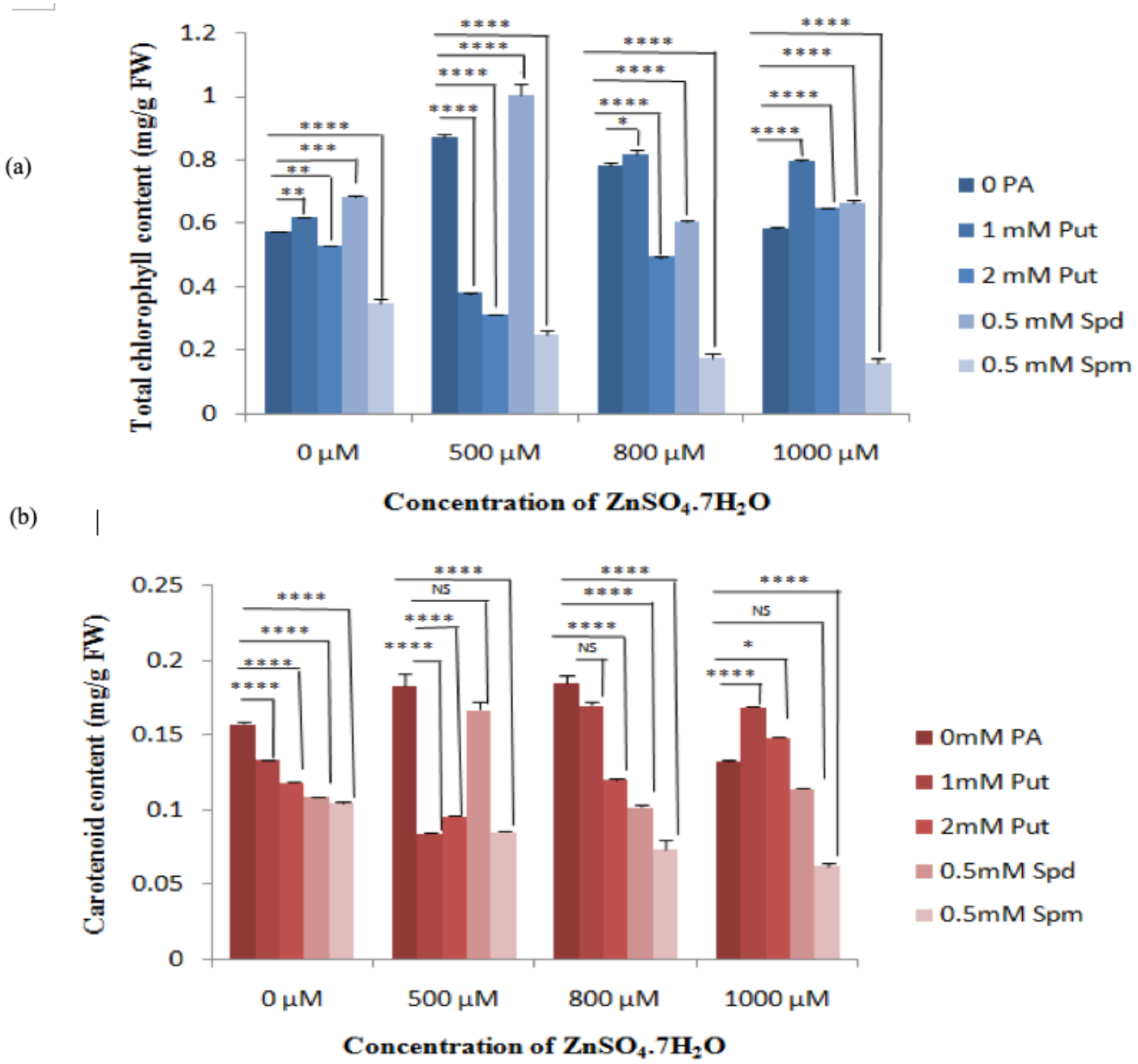


Figure 1

Total chlorophyll, and (b) carotenoid contents of 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence of any polyamines and the presence of 1 and 2 mM Putrescine, 0.5 mM Spermidine and 0.5 mM Spermine. The data are represented as mean ± standard error of mean (SEM) (n= 3). **** indicates (p<= 0.0001), *** indicates (p<= 0.001), ** indicates (p<= 0.01) and * indicates (p<= 0.05), N.S. indicates not significant (p>0.05)

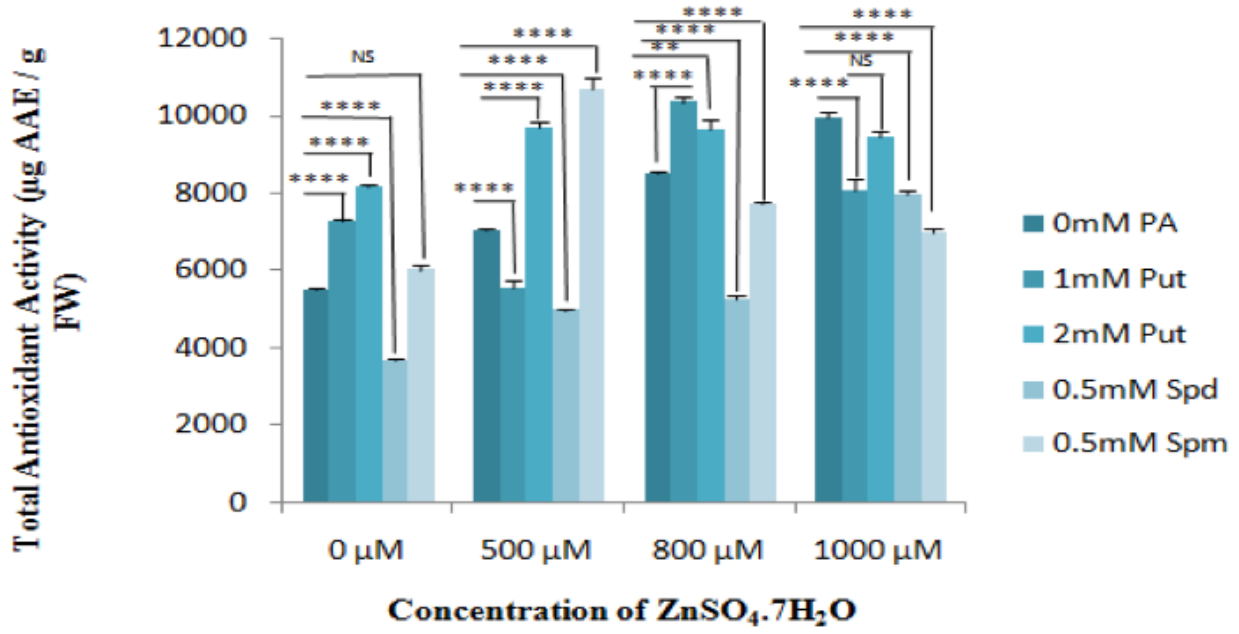


Figure 2

Total Antioxidant content of 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence of any polyamines and the presence of 1 and 2 mM Putrescine, 0.5 mM Spermidine and 0.5 mM Spermine. The data are represented as mean ± standard error of mean (SEM) (n= 3). **** indicates (p<= 0.0001), *** indicates (p<= 0.001), ** indicates (p<= 0.01) and * indicates (p<= 0.05), N.S. indicates not significant (p>0.05)

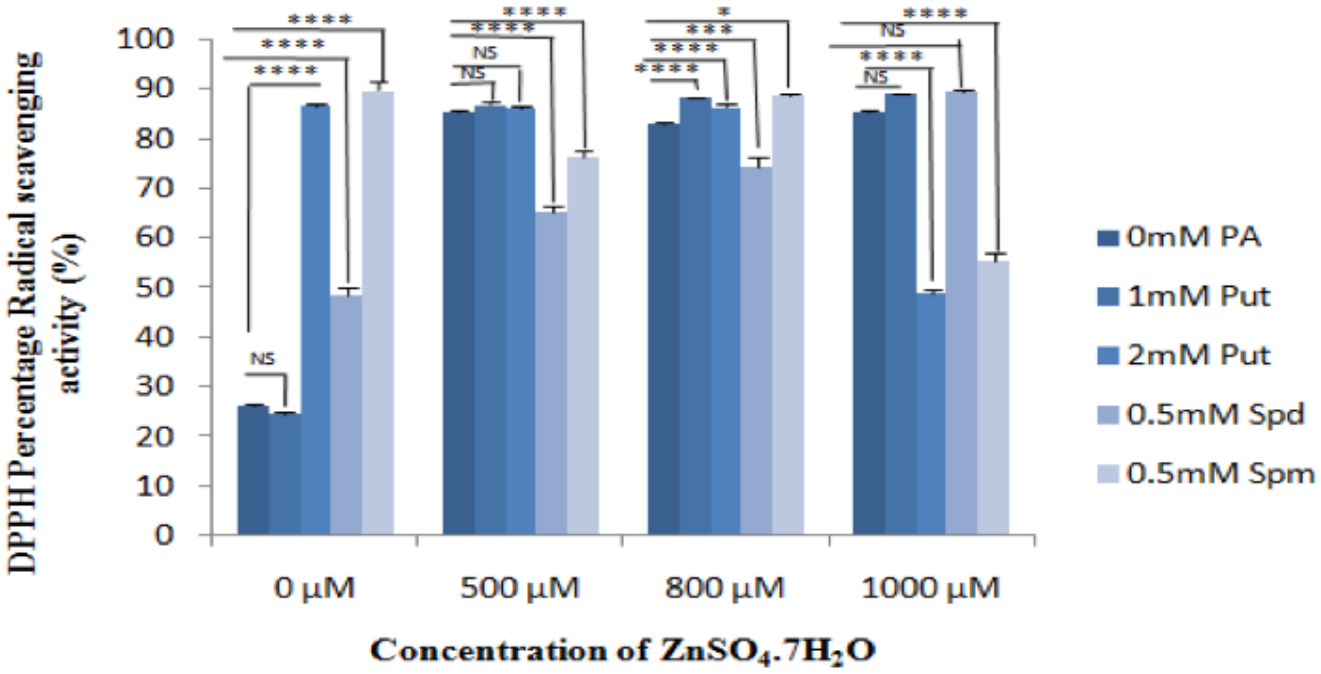


Figure 3

DPPH radical scavenging activity of 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence of any polyamines and the presence of 1 and 2 mM Putrescine, 0.5 mM Spermidine and 0.5 mM Spermine. The data are represented as mean ± standard error of mean (SEM) (n= 3). **** indicates (p<= 0.0001), *** indicates (p<= 0.001), ** indicates (p<= 0.01) and * indicates (p<= 0.05), N.S. indicates not significant (p>0.05)

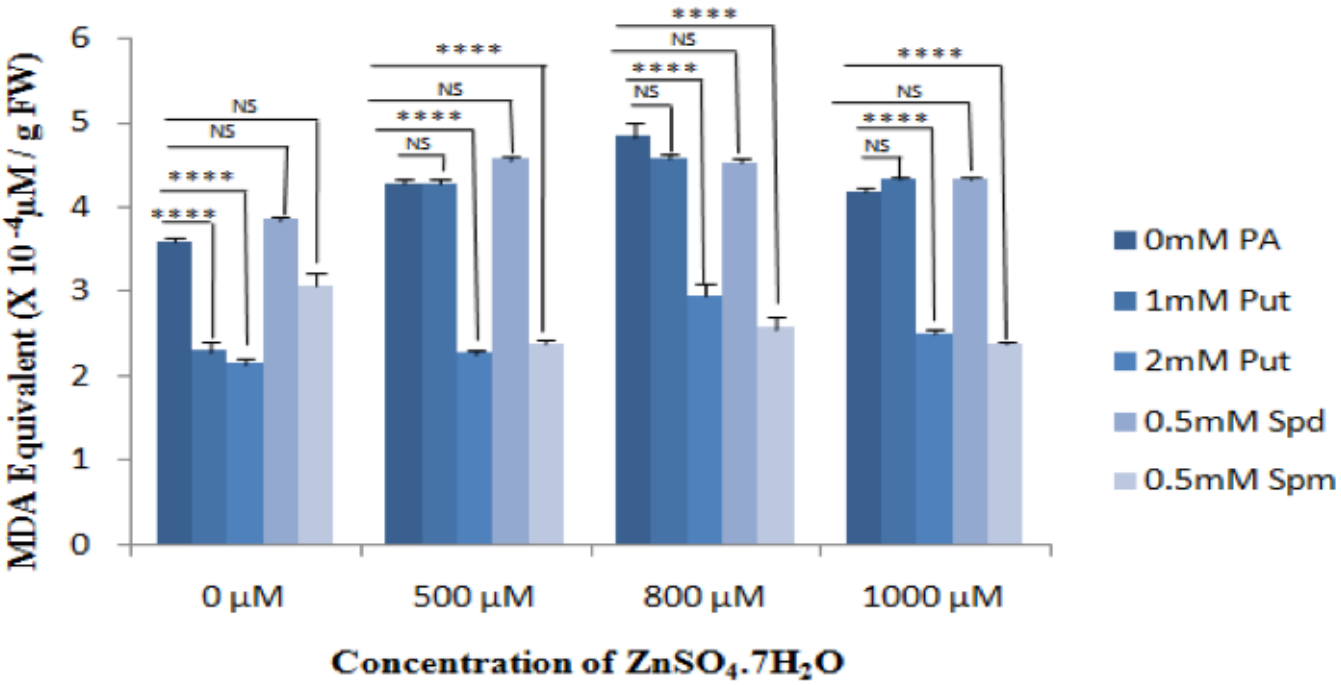


Figure 4

Lipid Peroxidation with respect to Malondialdehyde content of 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence of any polyamines and the presence of 1 and 2 mM Putrescine, 0.5 mM Spermidine and 0.5 mM Spermine. The data are represented as mean ± standard error of mean (SEM) (n= 3). **** indicates (p<= 0.0001), *** indicates (p<= 0.001), ** indicates (p<= 0.01) and * indicates (p<= 0.05), N.S. indicates not significant (p>0.05)

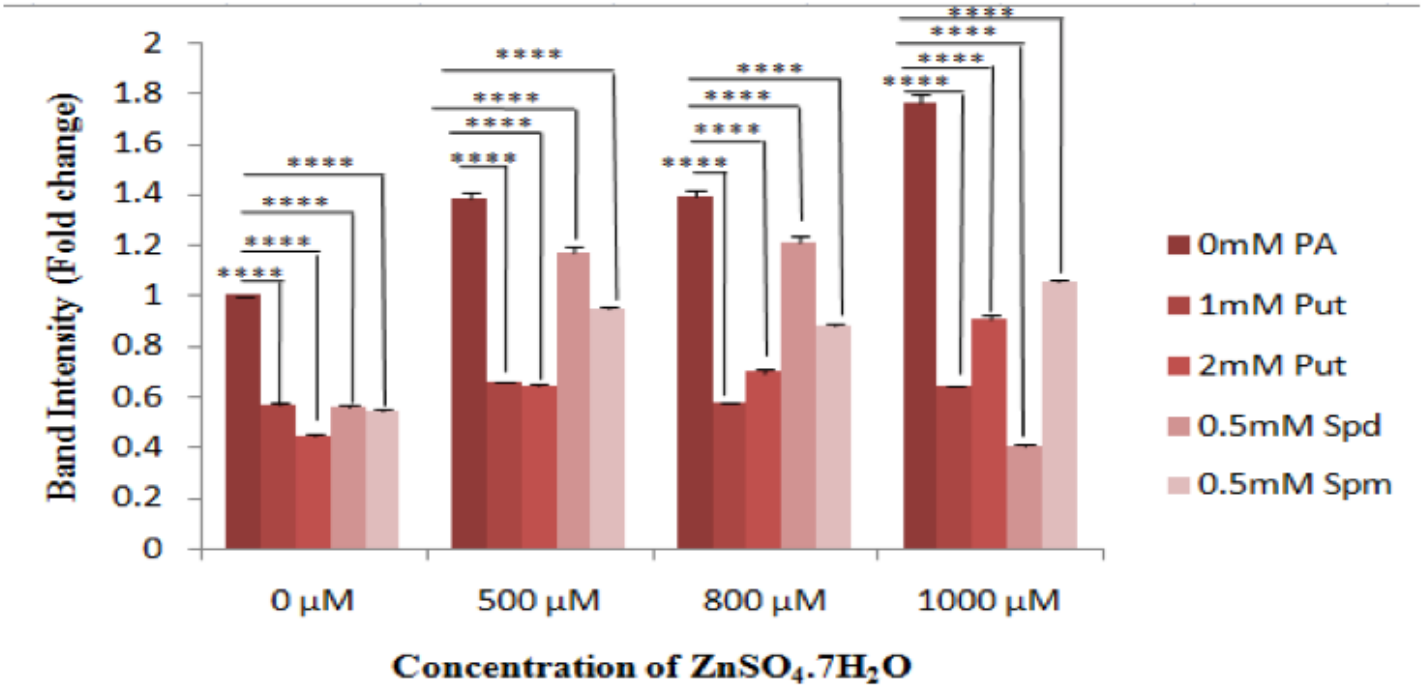
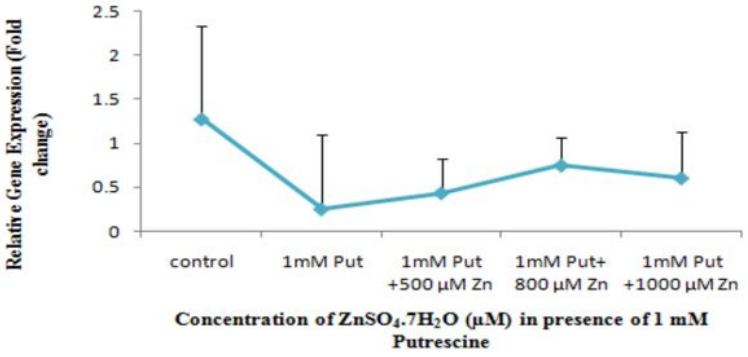


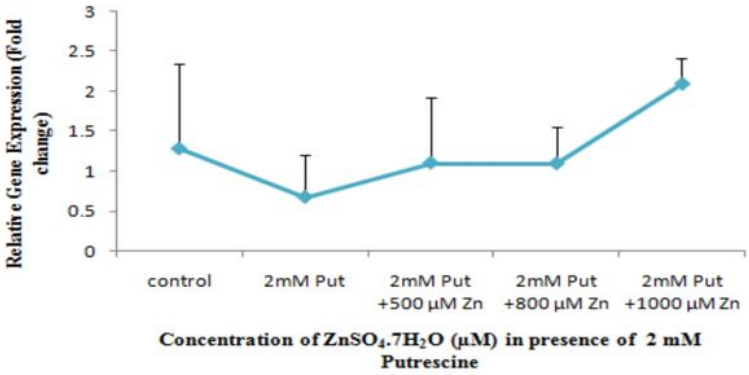
Figure 5

Densitometric analysis and the normalized fold change of MT2 gene in 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence of any polyamines and the presence of 1 and 2 mM Putrescine, 0.5 mM Spermidine and 0.5 mM Spermine. The data are represented as mean ± standard error of mean (SEM) (n= 3). **** indicates (p<= 0.0001), *** indicates (p<= 0.001), ** indicates (p<= 0.01) and * indicates (p<= 0.05), N.S. indicates not significant (p>0.05)

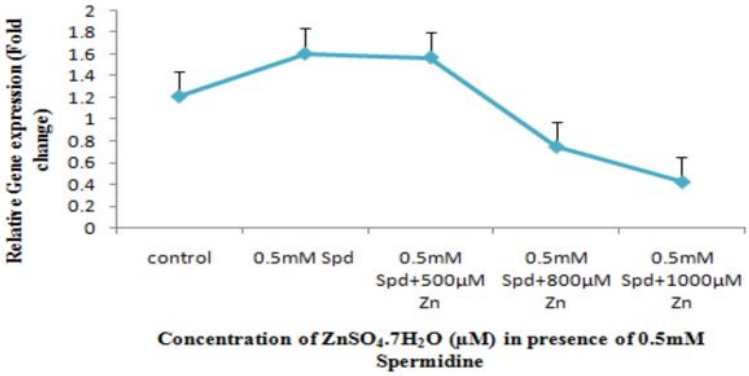
(a)(i)



(a)(ii)



(b)



(c)

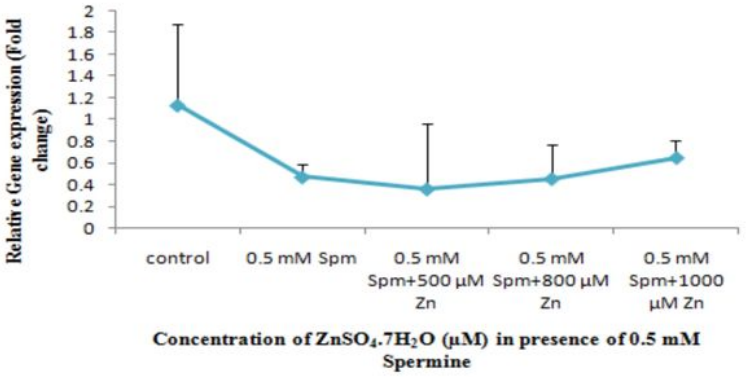


Figure 6

Real Time PCR (qPCR) Analysis and the normalized fold change of MT2 gene in 7 days old P. ovata with increasing doses of ZnSO4.7H2O in the absence and the presence of (a)(i) 1 mM Putrescine, (a)(ii) 2mM Putrescine, (b) 0.5 mM Spermidine, and(c) 0.5 mM Spermine. The data are represented as mean ± standard deviation (SD) (n= 3)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydocuments.docx