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Exogenously applied putrescine ameliorates zinc toxicity in oilseed rape (*Brassica napus* L.) by modulating the antioxidant system

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Abstract

Objective: The external application of putrescine (Put) may overcome the adverse impact of different abiotic stresses. The current study assessed the effect of Put on the growth and antioxidative responses of oilseed rape (*Brassica napus* L.) seedlings under excess levels of zinc (Zn).

Methods: The experiment was carried out as a completely randomized design with four treatments and four replications in a Hoagland nutrient solution containing supplemental concentrations of 20 μ M ZnCl₂ (Zn treatment), 0.2 mM Put (Put treatment), 20 μ M ZnCl₂ + 0.2 mM Put (Put + Zn), and a control treatment without extra Zn and Put.

Results: The excess Zn led to the reduction of plant growth and pigment content. The content of total phenols, flavonoids, and flavonols was not significantly changed except that of the flavonols in the roots which was enhanced under Zn toxicity. Likewise, phenylalanine ammonia-lyase (PAL) and antioxidant enzyme activity and also the content of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) was increased upon exposure to excess Zn. In contrast, Put improved the growth and pigment content of oilseed rape under Zn stress. Put also enhanced the flavonoids and flavonols content and induced the activity of PAL and polyphenol oxidase under Zn toxicity. However, H₂O₂, MDA, superoxide dismutase, and peroxidase in oilseed rape exposed to Put declined under Zn excess.

Conclusion: In conclusion, Put alleviated the negative impact of Zn toxicity partially due to the minimized endogenous levels of Zn in the root, the reduction of the H₂O₂ and MDA content, and modulation of the antioxidant system.

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Introduction

Zinc (Zn) has a role in maintaining growth, development, physiological performance, and productivity, and is also involved in intracellular processes such as protein synthesis, carbohydrate metabolism, phosphate metabolism, regulation of gene expression and ribosome structure integrity, hormone regulation, and maintenance of CO₂ concentration in mesophylls (Broadley *et al.* 2007; Gai *et al.* 2017; Ghassemi Golezani *et al.* 2022, 2023). The Zn level in soils may be strongly enhanced due to an increase in industrial and mining activities, excessive application of fertilizers, and chemical toxins (Kabata-Pendias 2011; Yang *et al.* 2011). Exposing the plant to high levels of Zn (300-400 µg g⁻¹ DW) leads to growth inhibition, deficiencies of some elements in plants, lipid peroxidation, loss of membrane activity, reduction of chlorophyll content, and disruption of antioxidant systems (Darinka 2018; Kaur and Garg 2021). The reactive oxygen species (ROS) are highly reactive and cause enzymatic activity reduction, protein oxidation, DNA/RNA damage, membrane lipid peroxidation, metabolic imbalances, and finally cell death (Mansoor *et al.* 2022).

According to Stuver *et al.* (2014), Chinese cabbage (*Brassica pekinensis*) was susceptible to elevated Zn toxicity at ≥ 5 µM which resulted in a decrease in growth, pigment content, and concentrations of metals such as Mn and Ca, and disturbed the sulfur metabolism. The Zn toxicity resulted in a decrease in plant growth, chlorophylls, and elements' content including phosphorus, copper, iron, manganese, and magnesium, and induced oxidative stress in oilseed rape plants (Wang *et al.* 2009). The impact of Zn on the seed oil content and its fatty acid composition differs based on the oilseed rape cultivars and Zn concentration. According to Manaf *et al.* (2019), the content of seed oil composition including oleic acid, linoleic acid, and erucic acid decreased under Zn toxicity in various oilseed rape cultivars.

Biologically active compounds like polyamines are potential agents in counteracting the harmful effects of ROS in plants (Shi and Chan 2014). Polyamines stabilize anionic macromolecules in the cell due to their polycationic nature and play important roles in cellular activities such as cell division and differentiation, gene expression, transcriptional regulation and translation, signal transduction, and homeostasis (Ramazan *et al.* 2023; Tripathi *et al.* 2023). Put is one of the most abundant polyamines in plants which acts as an osmolyte in different osmotic stresses, a transporter molecule between root-shoot as a cation or a nitrogen-containing molecule, and as a cryoprotectant at low temperatures (Cui *et al.* 2020). Put is involved in ATP production in chloroplasts and protection of the photosynthetic membrane especially the PSII structure (Ioannidis and Kotzabasis 2007). Also, Put may bind to the negative domain of the cell membrane due to its structure as a diamine, thereby,

possibly protecting proteins from oxidative damage by ROS (Rady and Hemida 2015). Put may induce plant antioxidant defense systems to eliminate free radicals, and overall stimulate the cellular defense strategy against oxidative stress caused by heavy metals. Foliar application of Put on oilseed rape under flooding conditions resulted in increased plant height, number of leaves, pigment content, and antioxidative enzyme activity (Harirforoush *et al.* 2019). Put improved chromium (Cr^{6+}) stress by increasing in the content of Cr^{6+} in the vacuoles of the roots in *B. juncea* cultivars. However, this effect was cultivar specific. In addition, Put improved seed number and weight in oilseed rape contaminated with Cr^{6+} (Jahan *et al.* 2021). Likewise, Put increased the seed oil (oleic acid) and glucosinolates contents of oilseed rape under drought stress. Put alleviated relative water content, chlorophyll, carotenoids, and soluble proteins of the leaf in oilseed rape (Ullah *et al.* 2012). In a field experiment, the application of Put increased number of seeds per plant and seed oil content of oilseed rape under water deficiency stress (Ghassemi-Golezani *et al.* 2019; Mabudi Bilasvar *et al.* 2022). Furthermore, Put decreased oleic acid and increased linoleic and linolenic acids of the seeds of oilseed rape and as a result improved the unsaturation index of oil seeds (Mabudi Bilasvar *et al.* 2022).

The main purpose of the current study was to investigate the potential of exogenous Put to diminish the harmful effects of Zn on oilseed rape (*B. napus* L.) through the evaluation of plant growth, photosynthetic pigments, antioxidant compounds, and enzymes.

Materials and Methods

Growth conditions and plant harvesting

Seeds of oilseed rape (*B. napus* var. Hyola-50) were prepared by Oilseeds Cultivation Development Company, Sari, Mazandaran province, Iran. The experiment was done at the Mazandaran University in 2022. The sterilized seeds were germinated in sterilized perlites for 10 days. Then, the seedlings were grown in a 25% Hoagland's nutrient solution (Hoagland and Arnon 1938). The seedlings were exposed hydroponically to Zn and Put at concentrations of 20 μM and 0.2 mM, respectively for 20 days. The experiment was carried out as a completely randomized design with four treatments. The treatments included the supplemental concentrations of 20 μM ZnCl_2 (Zn treatment), 0.2 mM Put (1,4-Diaminobutane with CAS number 0000110601, Sigma-Aldrich; Put treatment), 20 μM ZnCl_2 + 0.2 mM Put (Put + Zn), and a control treatment without extra ZnCl_2 and Put. The experiment was performed in a controlled growth chamber (24 and 19 $^{\circ}\text{C} \pm 2^{\circ}\text{C}$ day and night, respectively, 75% rh, and photoperiod of 14:10 h, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ by using LED). The nutrient solution was aerated through an air pump and refreshed after 10 days. After 20 days, the plants were harvested. After washing the roots, the shoots and roots were separated, and their fresh weight was

measured. To determine dry weight and Zn content, the oven-dried plant materials were used. To measure the total phenols, flavonoids, and flavonols, the plant materials were shade-dried at 30 °C. Frozen plant materials were used for measuring pigments, soluble proteins, malondialdehyde (MDA), hydrogen peroxide (H₂O₂), enzymes' activity, and detection of zymogram patterns.

Determination of Zn content

Oven-dried powdered plant materials were digested with sulphuric acid-perchloric acid (1:7, v/v) and nitric acid. Then, the residue was mixed with distilled water and heated for 30 min. To determine Zn content, the absorption of the filtered solution was measured by using the atomic absorption device at 213.9 nm (David 1958).

Assay of photosynthetic pigments

After extraction of frozen plant samples with acetone, the supernatant was centrifuged (Hermle, Z 206 A, Germany) at 30000 g for 20 min. Then, the absorbance was recorded using a spectrophotometer (Spectrum, sp-2100, China) at 663, 647, and 470 nm (Lichtenthaler 1987).

MDA assay

Fresh plant samples were extracted by 1% trichloroacetic acid (0.5 g 2 ml⁻¹). Then, the extracts were incubated in a water bath (Memmert GmbH & Co. KG, Germany) at 95 °C for 30 min. After centrifugation (15,000 rpm, 10 min), the MDA content was measured by a spectrophotometer at 532, 600, and 450 nm (Heath and Packer 1968).

H₂O₂ assay

After the extraction of fresh plant samples by trichloroacetic acid (10 g ml⁻¹), the extract was homogenized with phosphate buffer (10 mM, pH= 7) and potassium iodide (1 M). The reaction was kept in the dark for 1 h. Then, the absorbance of the reaction was recorded at 390 nm by using a spectrophotometer (Velikova *et al.* 2000).

Total phenols, flavonoids, and flavonols

Air-dried powdered plant materials were extracted with 70% methanol (Thygesen *et al.* 2007). The content of total phenols was determined by the Folin-Ciocalteu's reagent method, and the calibration curve of Gallic acid (GAE) was used to calculate the content of total phenols (Singleton *et al.*, 1999). The content of flavonoids and flavonols was measured by the Akkol method and the

calibration curve of Quercetin (QE) was used to calculate the content of flavonoids and flavonols (Akkol *et al.* 2008).

Enzyme activity and protein content

To prepare protein and enzyme extract, the frozen plant materials were extracted with potassium phosphate buffer (100 mM, pH 7.5) by the Bradford (Bradford 1976) method.

The activity of total superoxide dismutase (SOD, EC 1.15.1.1) was determined by the method of Beauchamp and Fridovich (1971). The activity of manganese/iron superoxide dismutase (Mn/Fe-SOD) isoenzyme was determined by inhibiting the activity of copper/zinc superoxide dismutase (Cu/Zn-SOD) by using potassium cyanide (3 mM; Fridovich 1975; Almansa *et al.* 1989).

The activity of peroxidase (POD, EC 1.11.1.7) was determined by measuring the oxidation of guaiacol in the reaction mixture containing enzyme extract, H₂O₂, guaiacol, and potassium phosphate buffer at 470 nm (Nakano and Asada 1981).

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activity was assayed by following the production of trans-cinnamic acid from L-phenylalanine at 260 nm in the reaction mixture containing enzyme extract, potassium phosphate buffer, and L-phenylalanine (Nagarathna *et al.* 1993).

To determine the activity of polyphenol oxidase (PPO, EC 1.10.3.1), the absorbance of the reaction solution containing enzyme extract, potassium phosphate buffer, and pyrocatechol was recorded at 420 nm (Hu-zhe 2005).

Electrophoresis

Electrophoretic separation of isoenzymes was performed using the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Aghajanzadeh *et al.* 2021).

To detect the SOD isoenzymes, after loading the enzyme extract into the gel and performing the electrophoresis (Paya Pajooresh Pars, EPS.30, Iran), the gel was shaken using a shaker (Fan Azma Gostar, Mod 1:TM52A, Iran) in a reaction mixture containing sodium phosphate buffer (4 mM, pH 7.8), EDTA (1 mM), riboflavin (0.05 mM), NBT (0.1 mM), and TEMED (0.3%). To identify Mn/Fe-SOD isoenzyme, the gel was stained separately in the presence or absence of 3 mM potassium cyanide (an inhibitor of Cu/Zn SOD). Then, the gel was shacked into the same reaction mixture in light for 60 min (Beauchamp and Fridovich 1971).

To detect better the isoenzyme pattern of POD, the proteins of the enzyme extract were concentrated by the ammonium sulfate method and dialyzed. Then, the enzyme extract was loaded into the gel. After electrophoresis, the gel was stained in sodium acetate buffer (50 mM) with pH 4.5,

containing benzidine (2 mM), and 200 ml of 3 mM H₂O₂ to visualize the POD isoenzymes (Lin and Kao 2001; Aghajanzadeh and Jazayeri 2018).

To detect PPO isoenzymes, the enzyme extract was loaded into the gel and subsequently electrophoresis was run. Then, the gel was stained in potassium phosphate buffer (100 mM) with pH 8 containing catechol (50 mM) to detect PPO isoenzymes (Vanloon 1971).

Statistical analysis

A one-way analysis of variance was performed to compare the treatments at $p \leq 0.01$ and subsequent Tukey's post-hoc test. Data on growth characteristics and pigment content (chlorophyll a, b, and carotenoids) were obtained from four biological replicates in each experiment and four plants in each replicate. Data on the content of MDA, H₂O₂, total phenols, flavonoids, flavonols, proteins, Zn, and activity of enzymes were obtained from four biological replicates with 10 plants in each replicate. To perform the statistical analyses and draw the graphs, the SPSS software, version 20, and the Graph Pad Prism software (San Diego, CA, USA) were used, respectively.

Results

Growth traits and pigment content

The results showed that the fresh and dry weight of shoots was reduced by 50% under excess Zn exposure. However, the Put treatment alleviated the negative effect of Zn and led to the increase of the fresh and dry weight of the shoots by 1.89 and 1.75-fold, respectively (Table 1). Similarly, Zn treatment decreased both the fresh and dry weight of roots by 52 and 43%, respectively. However, the application of exogenous Put in plants exposed to Zn stress resulted in an increase in the fresh and dry weight of the roots by 1.79 and 1.60-fold, respectively (Table 1).

The excess Zn decreased the content of chlorophyll a, b, and carotenoids by 39%, 33%, and 49%, respectively (Table 1). However, the treated plants with external Put led to an increase in the content of chlorophyll a, b, and carotenoids up to 1.74, 2.56, and 1.50-fold, respectively under excess Zn conditions (Table 1).

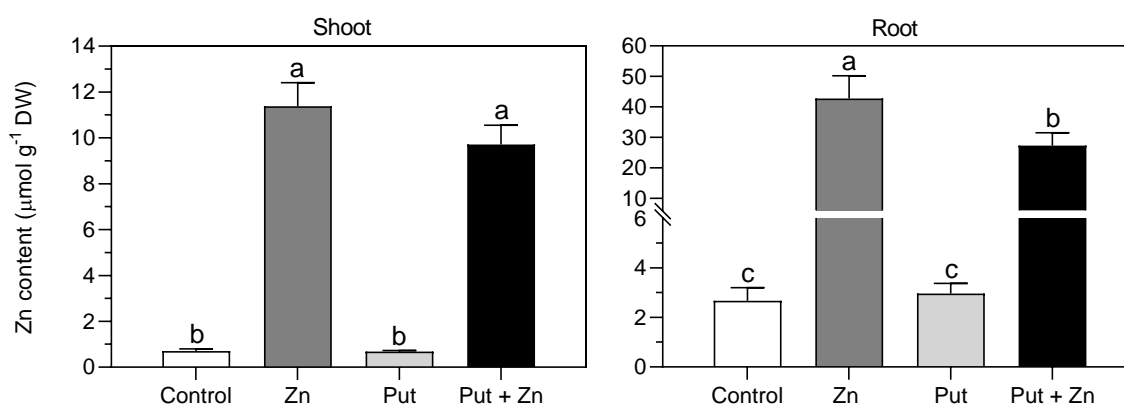
Zn content

The Zn content in shoots and roots increased by almost 16-fold under Zn stress (Figure 1). However, exposing the Zn-stressed plants to Put resulted in a decrease in the Zn content by 36% in the roots, but that of the shoots was not significantly changed (Figure 1).

Table 1. Fresh weight and dry weight of shoots and roots and the content of pigments of oilseed rape exposed to Zn, Put (Put), and Put + Zn.

Shoot	Treatment			
	Control	Zn (20 μ M)	Put (0.2 mM)	Put + Zn
Fresh weight (gr)	0.71 \pm 0.02a	0.35 \pm 0.02b	0.63 \pm 0.08a	0.66 \pm 0.04a
Dry weight (gr)	0.048 \pm 0.002a	0.024 \pm 0.001c	0.044 \pm 0.003ab	0.042 \pm 0.002b
Chlorophyll a (mg g ⁻¹ FW)	1.52 \pm 0.15a	0.92 \pm 0.09b	1.75 \pm 0.09a	1.60 \pm 0.17a
Chlorophyll b (mg g ⁻¹ FW)	0.54 \pm 0.05c	0.36 \pm 0.02d	0.68 \pm 0.07b	0.92 \pm 0.05a
Carotenoids (mg g ⁻¹ FW)	0.43 \pm 0.03a	0.22 \pm 0.02c	0.44 \pm 0.01a	0.33 \pm 0.01b
Root				
Fresh weight (gr)	0.029 \pm 0.0002a	0.014 \pm 0.001c	0.027 \pm 0.0024ab	0.025 \pm 0.001b
Dry weight (gr)	0.0035 \pm 0.0003a	0.0020 \pm 0.0002c	0.0030 \pm 0.0001b	0.0032 \pm 0.0002b

Treatment means with different letters in each row are statistically significant based on Duncan's multiple range test.

**Figure 1.** The Zn content in oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters in each trait are statistically significant based on Duncan's multiple range test.

H₂O₂ and MDA concentration

In the plants exposed to exceeded Zn, H₂O₂ concentration was enhanced by almost 40 and 37% in shoots and roots, respectively. While, treating the oilseed rape plants, subjected to Zn, with Put, significantly alleviated the H₂O₂ concentration at the level of the control plant in both shoots and roots (Figure 2a). Similarly, the MDA content increased in both shoots and roots of the plants exposed to Zn by 1.4 and 1.2-fold, respectively (Figure 2b). However, the external application of Put in plants exposed to Zn stress led to a significant decrease of MDA by 33 and 23% in shoots and roots, respectively as compared to those of the plants exposed to Zn (Figure 2b).

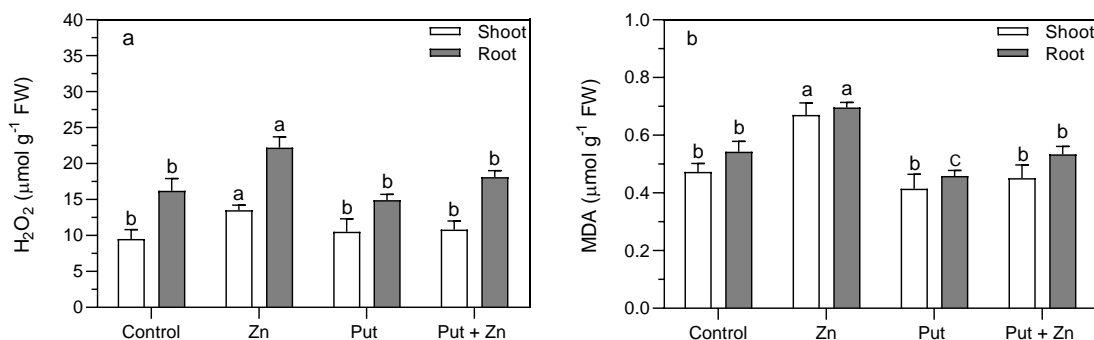


Figure 2. The content of H₂O₂ and MDA (µmol g⁻¹ FW) in shoots and roots of oilseed rape exposed to Zn (20 µM), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters in each figure are statistically significant based on Duncan's multiple range test

Total phenols, flavonoids, and flavonols content

The results showed that either Zn or Put along with Zn did not influence the content of total phenols in both shoots and roots (Figure 3a).

The flavonoid content was not significantly changed in the shoots of oilseed rape exposed to excess Zn, while it was enhanced by 1.4-fold upon exposure to Put under excess Zn. Furthermore, the flavonoid content was not significantly changed in the roots of the oilseed rape treated with excess Zn with or without Put (Figure 3b).

The flavonols' content in the oilseed rape exposed to Zn was enhanced by 23 and 29% in the shoots and roots, respectively, but the increase in the shoots was not significant. In addition, the external application of Put enhanced the flavonols' content under Zn stress in the shoots and roots by 25 and 20%, respectively (Figure 3c).

The activity of the PAL enzyme

The results showed that the activity of the PAL enzyme was not significantly changed in the shoots but enhanced in the roots by almost 43% under Zn excess (Figure 4). However, the exogenous application of Put in the plants exposed to Zn stress significantly enhanced the activity of PAL in the shoots and roots by 1.8 and 1.2-fold, respectively (Figure 4).

Activity and isozyme pattern of antioxidant enzymes

It was revealed that under Zn toxicity, the activity of total SOD in shoots and roots was increased by 20 and 75% over the control, respectively (Figure 5a). However, Put almost modulated the enzyme

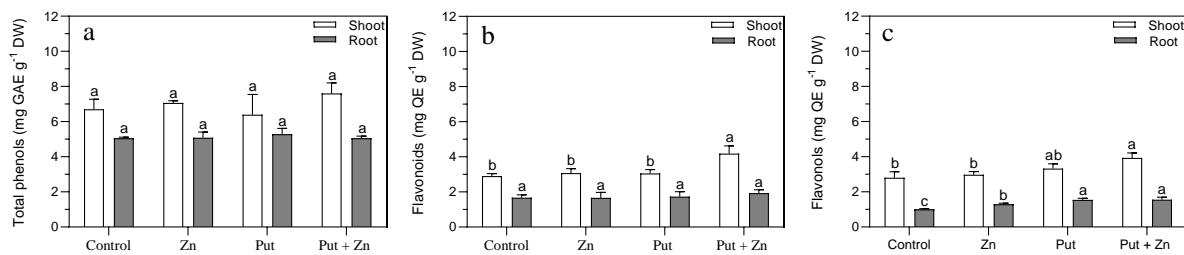


Figure 3. The content of phenols, flavonoids, and flavonols in shoots and roots of oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters in each figure are statistically significant based on Duncan's multiple range test.

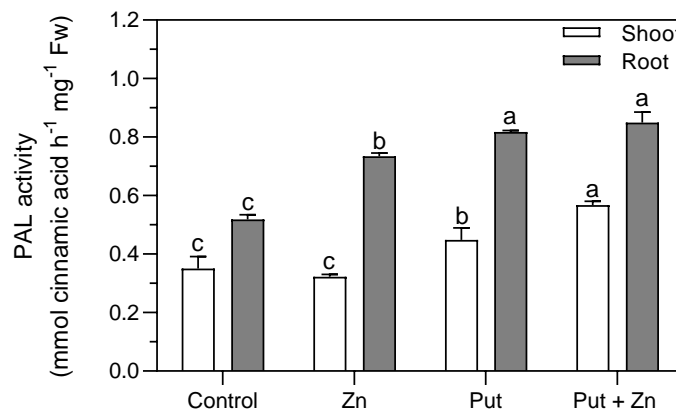


Figure 4. Activity of phenylalanine ammonia-lyase (PAL) enzyme in shoots and roots of oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters are statistically significant based on Duncan's multiple range test.

activity and decreased SOD activity by 26 and 46% in shoots and roots of oilseed rape exposed to excess Zn (Figure 5a).

The isoenzyme pattern of SOD showed two bands in the shoots which were identified as Mn/Fe-SOD and Cu/Zn SOD. In addition, five bands were detected in the roots including one band as Mn/Fe-SOD and four bands (a, b, c, and d) as Cu/Zn SOD isoenzymes (Figure 5b). An almost similar expression pattern was found under Zn stress for both types of isoenzymes. The activity of Mn/Fe-SOD in the both shoots and roots of plants exposed to Zn enhanced 1.3 and 2-fold, respectively. Application of exogenous Put in plants exposed to Zn led to a reduction of activity of Mn/Fe-SOD by 60 and 46% in shoots and roots, respectively (Figure 5a). Similarly, the activity staining of Mn/Fe-SOD in gel enhanced under Zn stress but, Put alleviated its intensity in both shoots and roots under Zn excess (Figure 5b).

The activity of Cu/Zn SOD was calculated based on the subtraction of Mn/Fe-SOD from the total was increased by 1.4 and 2.8-fold in shoots and roots, respectively. However, exogenous Put reduced

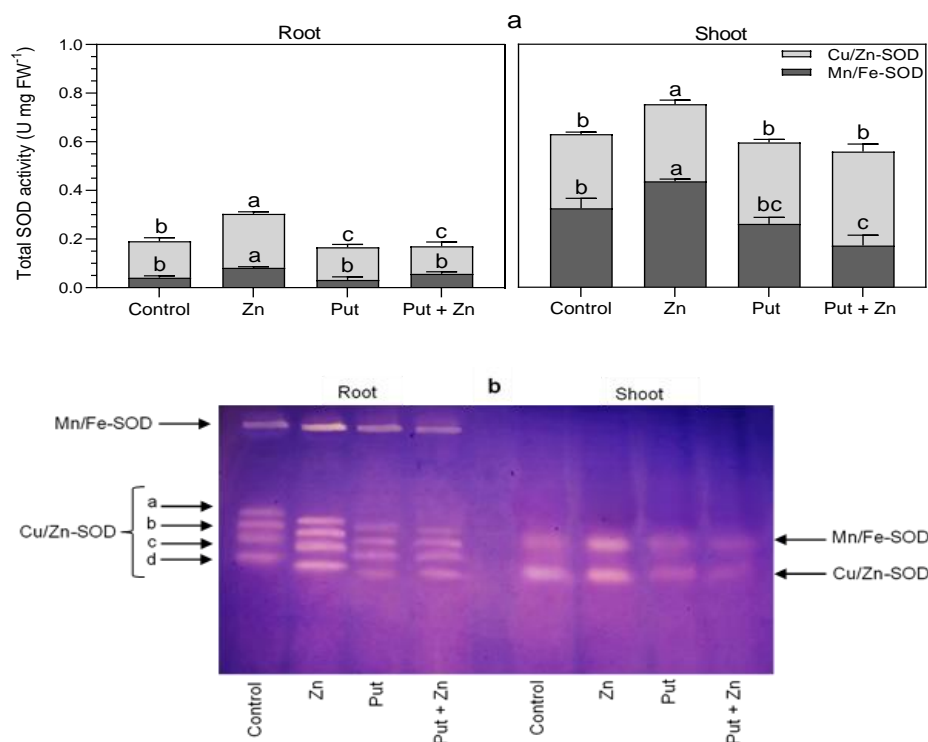


Figure 5. Activity of superoxide dismutase (SOD)(a) and isoenzymes pattern of SOD (b) in shoots and roots of oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters in each figure are statistically significant based on Duncan's multiple range test.

the activity of Cu/Zn SOD by 50% in shoots and 70% in roots in plants exposed to Zn toxicity (Figure 5a). The isoenzyme pattern of Cu/Zn SOD showed that the intensity of all isoenzyme bands in both shoots and roots was significantly enhanced under Zn treatment but, their intensity in shoots and roots declined under the influence of Put treatment (Figure 5b).

The results revealed that POD activity enhanced under Zn toxicity in shoots and roots by almost 2.3 and 1.4-fold, respectively (Figure 6a). However, exogenous application of Put in plants exposed to 20 μ M Zn led to a decrease in POD activity by 41% in the roots but that of the shoots remained unaffected (Figure 6a).

The isoenzyme pattern of POD in the roots revealed five bands a, b, c, d, and e. Although the protein was concentrated, no bands were visible on the zymogram with the shoots' extract. The intensity of bands b and e in the roots of oilseed rape was increased upon exposure to Zn toxicity while the application of exogenous Put reduced the intensity of bands a, b, c, and e under Zn toxicity (Figure 6b).

The activity of the PPO enzyme decreased by almost 70% in the shoots of plants exposed to Zn toxicity while it was enhanced by 20% in the roots (Figure 7a). The isoenzyme pattern of PPO in the shoots showed two bands of a and b and in roots, four bands including a, b, c, and d. The intensity of

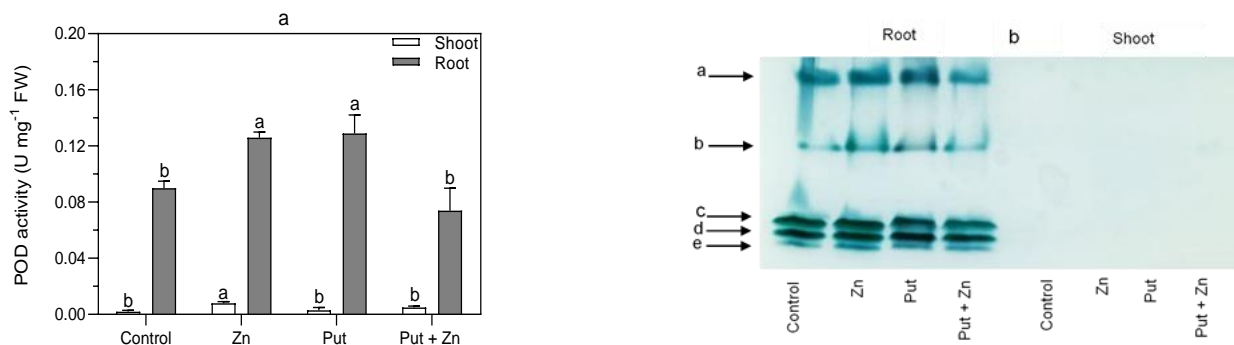


Figure 6. Activity of peroxidase (POD) (a) and isoenzymes pattern of POD (b) in shoots and roots of oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters are statistically significant based on Duncan's multiple range test.

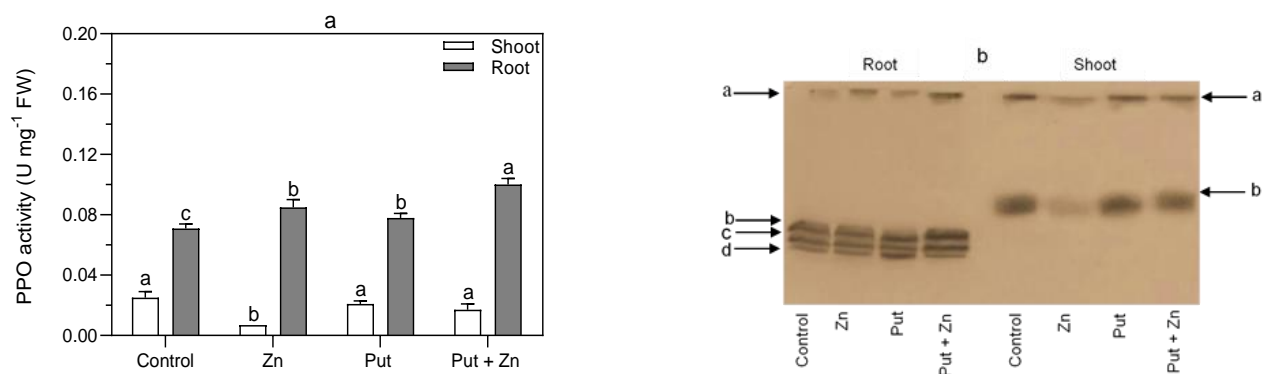


Figure 7. Activity of polyphenol oxidase (PPO) and isoenzymes pattern of PPO (b) in shoots and roots of oilseed rape exposed to Zn (20 μ M), Put (Put) (0.2 mM), and Put + Zn. Treatment means with different letters are statistically significant based on Duncan's multiple range test.

both bands a and b in the shoots decreased upon exposure of the plants to Zn toxicity. In the roots, the intensity of band a enhanced but that of the bands b, c, and d was hardly changed (Figure 7b). Exogenous treatment of Put increased the activity of PPO by 2.5 and 1.2-fold in the shoots and roots under Zn toxicity, respectively (Figure 7a). The isoenzyme pattern of PPO showed that the application of exogenous Put enhanced the intensity of both isoenzymes' bands a and b in the shoots and the intensity of bands a, b, and c in the roots of plants exposed to Zn toxicity while the intensity of the band d was hardly changed (Figure 7b).

Discussion

Heavy metal stress alters many metabolic processes in plants. In the current study, the growth of the oilseed rape was reduced upon exposure to 20 μ M Zn in the root environment (Table 1) which could be due to the interference of Zn with internal plant processes associated with the growth (Gai *et al.*

2017; Aghajanzadeh *et al.* 2020). Cell wall alterations like callose deposition or lignification may be responsible for the inhibition of plant growth under Zn toxicity (Feigl *et al.* 2015). The reduction of photosynthetic pigment content under Zn stress (Table 1) can also lead to plant growth reduction. The decrease in photosynthetic pigments' levels under Zn excess is probably due to the inhibition of the enzymes responsible for the production of chlorophyll such as δ -aminolevulinic acid dehydratase and protochlorophyll reductase (Mukhopadhyay *et al.* 2013) and/or stimulation of chlorophyllase which is involved in chlorophyll degradation (Drazkiewicz 1994).

Polyamines have strong biological activity to reduce the toxicity of heavy metals (Taie *et al.* 2019). Likewise, this could be monitored in the current study via the improved oilseed rape growth by application of exogenous Put under Zn toxicity (Table 1). The improvement of root growth by the application of Put could be partially due to the minimized endogenous levels of Zn under Zn toxicity (Table 1), however, that of the shoot was independent of Zn level because Put did not influence significantly the Zn content in the shoot (Figure 1). The mechanism behind the reduction of the Zn content in the root under Put treatment is unclear. It has been suggested that Put may alleviate Cu toxicity by modulation of Cu uptake and transportation (Chen *et al.* 2013). Mansour and Al-Mutawa (1999) proposed that Put has a significant role in the protein structure and stabilization of membrane integrity. The impact of polyamines in blocking cation channels and reducing the uptake and transportation of ions has been already indicated (Wang *et al.* 2007). In wheat plants, Put stimulates plant growth by an increase in the content of endogenous stimuli such as auxin, gibberellins, and cytokinin, along with a decrease in the content of inhibitors such as abscisic acid (El-Bassiouny *et al.* 2008). The current study showed that exogenous Put stimulated chlorophyll synthesis (Table 1) which can probably be followed by improving plant growth. The contents of chlorophyll biosynthesis precursors including δ -aminolevulinic acid and porphobilinogen were enhanced by exogenous Put in cucumber seedlings under salinity stress (Yuan *et al.* 2018). Put restrains the activity of the chlorophyllase and subsequently inhibits chlorophyll degradation under stress situations (Schindler *et al.* 1994). An increase in the absorption of magnesium (an element involved in chlorophyll structure) by Put may improve the synthesis of chlorophylls in the plant (El-Bassiouny *et al.* 2008).

Interestingly, in the current study, the chlorophyll b content was enhanced by Put under toxicity of Zn even more than that of chlorophyll b in the control plant. It may be concluded that Put enhanced the gene expression of chlorophyll a oxygenase (CAO), the enzyme responsible for the formation of chlorophyll b by oxygenation of chlorophyll a (Chen *et al.* 2010). It has been observed that an increase in the content of chlorophylls and subsequently photosynthetic capacity influenced the seed yield of oilseed rape. In addition, glucose as a carbon source and precursor of saturated and unsaturated long-

chain fatty acids is a photosynthetic product that affects seed oil quality (Wang *et al.* 2023). Therefore, improvement in chlorophyll content by Put may be associated with increased seed yield and quality of oilseed rape. Also, considering the possible negative effect of Zn on the composition of fatty acids in oilseed rape and due to the reduction of Zn by Put, it can be possible that the Put improves the quality of oil seed.

Due to the role of carotenoids in scavenging free radicals (Candan and Tarhan 2003), an increase in the content of carotenoids in oilseed rape exposed to Put (Table 1) may lead to preventing the harmful effects of Zn and improving plant growth.

The increase in the content of H₂O₂ and MDA in oilseed rape under Zn toxicity (Figure 2) can be a sign of oxidative stress which damages proteins, lipids, and DNA, and in the long term severely disturb the cell metabolism (Apel and Hirt 2004). The ROS levels in plant tissues are controlled by antioxidant defense systems (Rady and Hemida 2015). Among phenolic compounds, only flavonols' content was enhanced in the roots of oilseed rape under Zn toxicity (Figure 3). Phenolic compounds are not only involved in the process of free radical scavenging but also show a higher ability to chelate and transport toxic heavy metals into vacuoles in plants (Jiang *et al.* 2017). The PAL activity (a key enzyme responsible for the biosynthesis of phenolic compounds) was enhanced under Zn excess (Figure 4). The stimulatory effect of Put on increasing the activity of PAL in oilseed rape under Zn toxicity was in line with an increase in the content of flavonols (Figures 3 and 4). The positive impact of the external application of Put could be attributed to the stimulatory effect of Put on photosynthesis (Farooq *et al.* 2009) which acts as a main source for the production of phenolic compounds through the shikimic acid pathway (Taiz and Zeiger 2002). In addition, the positive effect of Put on the deamination of L-phenylalanine by the increase in the activity of PAL may lead to the production of different types of phenolic compounds (Alrawaiq and Abdullah 2014).

In the present study, Zn toxicity caused a significant increase in the activity of total SOD which was due to an increase in both Mn/Fe-SOD and Cu/Zn SOD activities in the roots as well as shoots (Figure 5). These enzymes are present in mitochondria, chloroplast, peroxisome, and cell wall which convert superoxide to hydrogen peroxide and molecular oxygen (Apel and Hirt 2004). In addition, the isoenzymes pattern of SOD showed that the intensity of all isoenzyme bands of Mn/Fe-SOD and Cu/Zn SOD was enhanced under Zn toxicity and associated with the activity of both types of SOD enzymes in shoots and roots (Figure 5). The most important impact of Zn stress in plants is the production of oxidative stress due to the generation of a burst of superoxide, which severely disrupts the state of cell regeneration and the normal metabolism of the cell in plants (Apel and Hirt 2004). In general, the overproduction of superoxide radicals can act as a signal to accelerate the induction of

the SOD activity (Almeselmani *et al.* 2006). In the current study, an increase in the activity of SOD could explain the accumulation of H₂O₂ in Zn-stressed oilseed rape (Figures 2 and 5). Zn element as a cofactor may also accelerate the activity of the Cu/Zn SOD enzyme. POD as a primary H₂O₂-detoxifying enzyme exists in the chloroplasts, cytosol, cell membrane, and wall of plant cells (Apel and Hirt 2004). The present data exhibited the stimulation of POD which suggests its role in the constant detoxification of H₂O₂ in the oilseed rape seedlings under excess Zn (Figure 6). In addition, the strengths of two peroxidase isoenzyme bands (bands a and b) were correlated with the POD activity in the roots (Figure 6). The activity of the POD was enhanced under Zn stress in shoots too, however, its activity was too low compared to that of the POD in roots (Figure 6a). Similarly, the POD isoenzymes were hardly visible in the case of shoots despite protein concentration by ammonium sulfate (Figure 6b). PPO is involved in the phenylpropanoid pathway, which can lead to the production of phenylpropanoid derivatives (phenols and flavonoids) in the plant, thus helping to reduce oxidative stress (Ali *et al.* 2023). The PPO enzyme has also a role in lignin biosynthesis and cell wall thickness in response to environmental stresses, especially, heavy metals toxicity (Taranto *et al.* 2017). In the present study, Zn stress increased PPO activity in plant roots (Figure 7a) which was consistent with the increase in the density of the isoenzyme bands a, b, and c in the roots (Figure 7b). It seems that increased activity of PPO in response to toxic levels of Zn protects plants against oxidative damage and thus makes the plant resistant to stress. However, a reduction in the activity of this enzyme in the shoots may show an inactivation of the enzyme or damage by free radicals under Zn stress as was reported in grapes (Yang *et al.* 2011).

Put is supposed to be a modulator for maintaining cellular redox and able to alleviate the antioxidant pool through the regulation of the enzymatic antioxidant system (Ghosh and Adak 2016; Mohammadrezakhani *et al.* 2017). In the current study, Put relieved the activity of anti-oxidative enzymes including total SOD, Mn-SOD, Cu/Zn SOD, and POD under Zn stress. Additionally, enhancing the activity of the antioxidant enzymes in oilseed rape under Zn stress in response to Put treatment was correlated with the increase in the intensity of most of their isoenzymes. Similarly, the application of Put diminished the catalase activity under salinity stress such as drought (Ghosh and Adak 2016). Findings showed the activity of anti-oxidative enzymes such as SOD and POD was upregulated under different abiotic stresses (Shi *et al.* 2013; Hamid *et al.* 2018; Li *et al.* 2018; Farzane *et al.* 2020). It was suggested that the reaction of enzymatic antioxidants depends on different species and types of stresses. Various mechanisms were proposed to explain the increase in oxidative resistance attributed to polyamines. It was suggested that polyamines may act as antioxidants directly or regulate the anti-oxidation pathways in plants (Ghosh and Adak 2016). In the current study,

decreased SOD and POD activity in the presence of Put was associated with lower production of hydrogen peroxide, which subsequently could lead to a decrease in the activity of antioxidant enzymes (Hsu and Kao 2007). There is evidence that polyamine could conjugate with antioxidant molecules and remove ROS (Hussain *et al.* 2011). Polyamines, which are polycationic in nature, bind to the negative charge of the membrane, and therefore, by avoiding lipid peroxidation reactions in plants under various abiotic stresses, they prevent oxidative damage and moderate ROS and the production of free radicals (Zhao *et al.* 2021). Similarly, MDA as one of the products of lipid peroxidation in the cells was reduced in oilseed rape exposed to excess Zn when treated by exogenous Put (Figure 2b). Furthermore, the external application of Put led to the reduction of H₂O₂ in oilseed rape under Zn stress. Similarly, the content of MDA and H₂O₂ has been reduced in *B. rapa* treated with Put (Thiruvengadam and Chung 2015). These results indicated that the tolerance to Zn stress can be related to the inhibition of high ROS production. Put induced PPO activity which was consistent with an increase in the density of isoenzyme protein bands. It has been reported that Put stimulates PPO activity in spinach under salt stress, which may be due to the binding of polyamines to the enzyme and changing its structure (Öztürk and Demir 2003). Therefore, a further increase in the activity of the PPO enzyme by exogenous Put in oilseed rape exposed to excess Zn indicates a synergy between Put and this enzyme that acts as a protective mechanism.

Conclusion

Zn toxicity resulted in the reduction of plant growth and pigment content. In addition, the excess Zn caused oxidative damage due to the increase in the activity of antioxidative enzymes that subsequently resulted in the production of H₂O₂ and MDA. Put may alleviate the negative impact of Zn toxicity by minimizing the endogenous levels of Zn, reducing the content of H₂O₂ and MDA, and modulation of antioxidative enzymes' (PAL, PPO, SOD, and POD) activity. Following the reduction of oxidative damage plant growth was alleviated. Furthermore, Put improved the content of photosynthetic pigments that are considered as a satisfactory indicator of plant performance and production. Because chlorophylls are the basis of the photosynthesis process, the chlorophyll content is related to plant health and production.

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Conflict of Interest

The authors declare that they do not have any relevant financial or non-financial competing interests.

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