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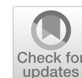

Hydrogen peroxide supplementation alleviates the deleterious effects of cadmium on photosynthetic pigments and oxidative stress and improves growth, yield and pods quality of pea (*Pisum sativum* L.) plants

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Abstract

The effects of foliar applied H_2O_2 on chlorophyll, carotenoids, the non-enzymatic defense system (ascorbic acid), malondialdehyde (MDA) hydrogen peroxide (H_2O_2) and growth were assessed in roots and shoots of pea (*Pisum sativum* L.) plants exposed to excess cadmium. In addition, we evaluated the influences of H_2O_2 spraying on proline, soluble sugars and soluble proteins contents. Excessive cadmium treatment caused reduction in the growth parameters (dry mass, pods and seeds dry weights), chlorophyll and carotenoids contents, roots total free amino acids, roots soluble sugars as well as shoots and roots soluble proteins levels but increased total free amino acids and soluble sugars contents in shoots. Concentrations of hydrogen peroxide and MDA was enhanced under Cd treatment. The foliar treatment of H_2O_2 alleviated the detrimental effects generated under Cd treatment that represented as increment in pea growth. H_2O_2 spraying increased photosynthetic pigments, growth characteristics, soluble proteins, and ascorbic acid contents comparing to the control sets not receiving H_2O_2. Similarly, a higher up-regulation was detected in proline contents of Cd + H_2O_2 set than Cd group ones at 0.25 mM Cd. Contrarily, malondialdehyde (MDA), soluble sugars and total free amino acids contents of Cd + H_2O_2 set revealed a lower decrease than Cd group ones especially in roots. The results demonstrated that H_2O_2 treatment could inverse the harmful effects of cadmium on growth, through inducing the non-enzymatic defense system (ascorbate), proline accumulation, maintenance of chlorophyll in pea leaves and lowering the intensity of H_2O_2 and lipid peroxidation (MDA).

Keywords Antioxidant · Ascorbic acid · Chlorophyll · Malondialdehyde · Proline

Abbreviations

AsA Ascorbic acid
Chl Chlorophyll
LP Lipid peroxidation
GSH Glutathione
MDA Malondialdehyde
ROS Reactive oxygen species
SS Soluble sugars
SP Soluble proteins
TAA Total free amino acids
TCA Trichloroacetic acid

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Introduction

Naturally, plants are subjected to many adverse environmental circumstances like abiotic and biotic stresses. Trace element stress is of great interest which has remarkable harmful effects on crop growth and productivity (Gill 2014). The increased agricultural dependence on sewage wastewater irrigation, chemical fertilizers and rapid development of industry have increased amount of toxic metals in agricultural soils resulting in detrimental effects on soil–plant environment system (Gadallah and Sayed 2014; Jali et al. 2016).

Cadmium is a poisonous metal and is regarded with a major environmental concern to the agricultural system. The divalent cation (Cd^{2+}) is almost exclusively present in

fertilized soils and accompanied with inorganic and organic complexes. Many studies have now found that the free Cd^{2+} ion or Cd complexes by inorganic ligands is the predominant Cd species exist in the soil solution of most soils. Furthermore, Cd is constantly, cumulated in soil, throughout anthropogenic and natural resources, for example, weathered cadmium-rich rocks, smelting, mining, the application of sewage sludge or excessive use of phosphate fertilizers, and metal contaminated water for crop irrigation (Alloway 2013; Hooda 2010; Kabta-Pendias 2011).

Cd is absorbed rapidly via plant roots and accumulates in plants to concentrations that could potentially cause animal toxicity. Cadmium uptake by plants is affected by array of variables—plant cultivar, pH of soil, salinity, mineralogy and organic matter content, cation interchange ability and concentrations of other nutrients, especially N, P and Zn (Burzynski et al. 2005; Clemens et al. 2013; Migocka et al. 2011; Plaza et al. 2015; Verbruggen et al. 2013). Cadmium solubility is greatest in acidic soil. The toxicity of Cd due to its high solubility and mobility within the ecosystem (Groppa et al. 2012; Jali et al. 2016) affects plant growth, induces necrosis and chlorophyll degradation and changes nutrient absorption, protein metabolism, carbon fixation and membrane functioning (Abdallah et al. 2015; Ahmad et al. 2015; Jali et al. 2016; Khan et al. 2013; Shah et al. 2017; Singh and Prasad 2014). Furthermore, cadmium enhances the activeness of antioxidant enzymes (Peng et al. 2017) and has high affinity towards the sulfhydryl groups of enzymes (Mendoza-Cozatl et al. 2005). Ultimately, cadmium prompts oxidative stress through its elevated affinity for carboxyl, SH and amine groups of the proteins (DalCorso et al. 2008).

Hydrogen peroxide is a paramount cellular molecule, performs numerous functions in metabolism, development and constancy of aerobes (Bienert et al. 2006). It's generation increased is due to various stress conditions (Neill et al. 2002). Hydrogen peroxide acts as an essential reactive oxygen species in signal transmission paths which activates plant defences against different imposed ecological stresses (Xu et al. 2011). In plants, H_2O_2 is the mostly stable ROS and can regulate vital metabolic pathways, as defence, development as well as, acclimation (Ślesak et al. 2007) and guard cell signaling (Song et al. 2014).

H_2O_2 is relatively stable molecule, more diffusible through membranes, considered as a long distance signal component (Vranová et al. 2002), can trigger Ca^{2+} influxes, protein alterations and gene expression (Bienert et al. 2006).

During the last decades, the acclimatory role of this component in plant has progressively become an interested fact. External H_2O_2 applications concurrently stimulated multi-tolerance mechanism towards cold, heat, drought and salinity stresses in *Zea mays* seedlings (Gong et al. 2001). AzevedoNeto et al. (2005) and Uchida et al. (2002) explained that addition of H_2O_2 to nutrient solution inducts acclimatization to salt stress in maize and rice seedlings. Also, Ismail et al. (2015) reported that H_2O_2 have regulatory impacts on plant growth, evolution and nutritional value of fruits. Hossain et al. (2015) indicated that H_2O_2 pretreatment improves abiotic oxidative stress acclimation. On *Brassica napus*, the hydrogen peroxide pretreatment mitigates cadmium stimulated oxidative stress damage (Hasanuzzaman et al. 2017). Khan et al.(2017) established that the seedlings of *Brassica* subjected to water-deficient condition that were supplied with H_2O_2 and Ca^{2+} recovered from chlorosis, overcoming water loss in plant, and the plants were able to grow normally. The exogenous H_2O_2 application has been accompanied by an increasing in its endogenous production (Terzi et al. 2014).

Considerable scientists confirmed the detrimental impacts of Cd on the outgrowth of plants, however; publications' concerning the ameliorating effects of H_2O_2 in cadmium-stressed plants is scarce. In addition, responses of plants to H_2O_2 foliar application and Cd stress are not still recognizable. Our study was performed with the assumption that H_2O_2 application able to modulate the adverse influence of cadmium stress on pea growth. Therefore, we investigated effects of foliar H_2O_2 spraying on improving cadmium stress tolerance of *Pisum sativum* and whatever the protecting effect was associated with some metabolic regulation in the shoots and roots tissues.

Materials and methods

Growth conditions and treatments

Seeds of *Pisum sativum* L. (cultivar Master B) were achieved from the Agricultural Research Center, Giza, Egypt. Plants was cultivated in plastic pots holding 4 kg of clean and air dry soil (clay/sand 2:1) in the experimental greenhouse in normal field conditions of humidity, temperature, light, and day/night pattern at Botany and Microbiology Department, Faculty of Science, Assiut University (Egypt). Extract of this soil records an electrical conductivity (EC) and pH as 0.876 mS cm^{-1} and 7.83, respectively. Three plants in each pot were allowed to grow for 5 weeks; water content of the soil kept at field capacity. Plants irrigated twice with 500 cm^3 full strength nutrient solution (Down and Hellmers 1975). The stock nutrient solution composited (mM) of: KNO_3 100; $Ca(NO_3)_2$ 100; $MgSO_4 \cdot 7H_2O$ 100; $NH_4H_2PO_4$ 100; KCl 50; H_3BO_3 25; $MnSO_4 \cdot H_2O$; $ZnSO_4 \cdot 7H_2O_2$; $CuSO_4 \cdot 5H_2O$ 0.5; H_2MoO_4 0.5 and Fe·EDTA 20 mM.

Various treatments applied for pot experiments were classified to control (C), Cd stress (Cd) and, Cd stress combined with H_2O_2 ($Cd + H_2O_2$). Five-week-old plants were irrigated with (900 ml/pot) 0.00, 0.125, 0.250, 0.500 and 1 mM $CdCl_2 \cdot 2.5\ H_2O$. Soil was irrigated 5 times at 3 day intervals

with these solutions. Cadmium solution was applied without nutrient solution. After 2 weeks of Cd supplying one set of the plants (0.0, 0.125, 0.250, 0.500 and 1 mM $CdCl_2 \cdot 2.5 H_2O$) was foliar sprayed with distilled water, the second set was foliar sprayed with 1 m M H_2O_2 solution, and the third set was foliar sprayed with 2 m M H_2O_2 solution. Foliar applications were done three times at 3 day intervals. H_2O_2 solutions were prepared from stock solution (1 M/100 ml distilled water). All treatments took place at the same time (at the end of the day). The concentration of Cd and H_2O_2 were chosen from the preliminary results. Randomly, five replicates were allocated to each treatment combination at each application. Seven days following preceding foliar (three times at 3 days intervals) H_2O_2 applications, the plants were analyzed.

Determination of soil electric conductivity (EC) and pH value

Electric conductivity (EC) of the soil was measured employing conductivity meter (model 4310 JEN WAY), as stated by the methods from Jackson (1967). Soil water extracts (1:5) was prepared by shaking 40 g of dry soil with 200 ml distilled water for 2 h, then filtrated to obtain a clear filtrate. Soil reaction of the filtrate was measured using electric pH-meter (model pH-206, Lutron).

Determination of photosynthetic pigments

Chlorophylls (a and b) and carotenoids were extricated from fresh leaves (0.25 g in 10 ml 95% ethyl alcohol) and absorbance readings measured spectrophotometrically (Unico UV-21 00, Unico, USA). The absorption was measured at 645 nm (Chl a), 663 nm (Chl b) and 470 nm (carotenoids). Chlorophylls and carotenoids concentrations (as mg g^{-1} FW) were estimated using equations as cited by Wellburn (1994).

Hydrogen peroxide (H_2O_2) determination

H_2O_2 content was determined by crushing 0.5 g fresh tissues of plants with 5 ml of trichloroacetic acid (TCA 0.1%) and centrifuged at 12,000×*g* for 15 min at 4 °C. To 0.5 ml of the supernatant, 0.5 ml of 10 mM potassium phosphate buffer (pH = 7.0) and 1 ml of 1 M KI were added. Absorbance was measured at 390 nm (Unico UV-21 00, Unico, USA). Concentration of H_2O_2 estimated as μmol g^{-1} FW (Velikova et al. 2000).

Determination of malondialdehyde (MDA)

A lipid peroxidation level was assessed by determination of malondialdehyde (MadhavaRao and Sresty 2000). 0.2 g fresh tissues sample of plants was crushed in 5 ml 0.1% TCA and centrifuged at 10,000×*g* for 5 min. 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added to 1 ml of the supernatant aliquot. Mix was incubated at 95 °C for 15 min and immediately cooled. The non-specific absorbance of the supernatant at 600 nm was deducted from the maximal absorbance at 532 nm utilizing spectrophotometer (Unico UV-21 00, Unico, USA). The concentration (μmol g^{-1} FW) of malonydialdhyde was recorded using ($\epsilon = 155\ mM^{-1}\ cm^{-1}$).

Determination of ascorbic acid content

Ascorbic acid concentration (μmol g^{-1} FW) assayed as designated by Mukherjee and Choudhuri (1983) through mingling 2 mol l^{-1} Folin-Ciocalteu reagent and 10% TCA with 20% of fresh tissue homogenate. After 10 min of centrifugation, the blue colour established in the supernatant was measured at 760 nm (Unico UV-21 00, Unico, USA). Ascorbic acid concentration was determined from a standard curve using different concentrations of ascorbic acid.

Determination of soluble carbohydrates and nitrogen metabolites

Contents of soluble sugars, total free amino acids and soluble proteins were recorded spectrophotometrically in hot water plant extract of both root and shoot tissues.

Content of soluble sugars was measured using phenol-sulfuric acid procedure of Dubois et al. (1956). One ml of 5% (v/v) phenol followed by 5 ml of sulfuric acid was appended, respectively, to a known volume of plant sample. The previous mixture was stirred and cooled in room temperature for 15 min. Absorbance was registered at 490 nm. Calibration curve using glucose was constructed.

Amino acids and soluble protein contents were determined utilizing the ninhydrin reagent (Lee and Takahashi 1966) and folin–phenol reagent (Lowry et al. 1951) procedures. Calibration curves using glycine and bovine serum albumin as standard was, respectively, constructed.

Proline content determination

Proline extracted from plant fresh tissue samples, its concentration was recorded following the methods of Bates et al. (1973). Fresh tissue samples were powdered in 3% sulphosalicylic acid; centrifuged at 3000×*g* for 20 min. The supernatant reacted with glacial acetic acid, ninhydrin reagent, boiled for 1 h and cooled. The developed colour was detached in toluene stratum and the absorbance estimated at 520 nm spectrophotometrically (Unico UV-21 00, Unico, USA). Proline was stated as μmol g^{-1} FW.

Statistics

Analysis of variance (ANOVA) with post hoc Duncan (1955) Multiple Comparison test was performed applying SPSS of Windows (Ver. 13.0, SPSS Inc., USA). Significance concerning the means among control and treatments were estimated using probability level $p < 0.05$. The values of H_2O_2 treatment were compared with those of Cd at each Cd level.

The relative role of each factor on the entire influence of treatment combination was calculated from the coefficient of determination (η^2).

$$\eta^2 = \frac{\text{Sum of squares due to the factor}}{\text{Total sum of squares due to the treatment combination}}$$

Experimental results

Growth

Results in Table 1 indicated that increased cadmium concentrations lowered shoots and roots biomass. Pods and seeds dry weights decreased progressively with rising cadmium concentrations. Number of seeds showed significant decrease with increasing Cd concentrations. The H_2O_2 foliar spray increased the yield characteristics in the Cd-stressful plants. Effectively it increases dry weight of shoots, roots, seeds and pods.

Photosynthetic pigments

Content of chlorophylls (a and b) was declined gradually with rising cadmium concentrations (Table 2). Cadmium at

Table 1 Effects of hydrogen peroxide (H_2O_2 mM) on shoots, roots and seeds dry weight (g) and number of seeds of *Pisum sativum* L. plants grown under different concentrations of cadmium (Cd)

Parameter	Cd (mM)	H_2O_2 (mM)		
		Control	(1 mM)	(2 mM)
Shoots dry weight	0.000	2.739 ± 0.192 c.A	2.717 ± 0.156 cde.A	3.154 ± 0.154 e.B
	0.125	2.590 ± 0.125 b.A	2.370 ± 0.400 bc.A	2.580 ± 0.100 cd.A
	0.250	2.500 ± 0.227 c.A	2.236 ± 0.041 abc.A	2.433 ± 0.182 bc.A
	0.500	2.413 ± 0.067 bc.A	3.077 ± 0.078 de.B	2.429 ± 0.076 abc.A
	1.000	1.145 ± 0.078 a.A	1.805 ± 0.014 a.B	2.030 ± 0.047 ab.C
Roots dry weight	0.000	0.929 ± 0.108 c.B	0.655 ± 0.032 ab.A	0.890 ± 0.054 bc.AB
	0.125	0.473 ± 0.060 bc.A	0.693 ± 0.243 ab.A	0.756 ± 0.107 abc.A
	0.250	0.447 ± 0.034 bc.A	0.526 ± 0.114 a.A	0.509 ± 0.098 a.A
	0.500	0.403 ± 0.045 b.A	1.075 ± 0.047 c.B	0.617 ± 0.072 a.A
	1.000	0.340 ± 0.036 a.A	0.418 ± 0.029 a.A	0.484 ± 0.070 a.A
Seeds dry weight	0.000	1.120 ± 0.454 b.A	1.233 ± 0.203 bc.A	1.580 ± 0.163 c.A
	0.125	0.953 ± 0.104 b.A	1.257 ± 0.273 bc.A	0.890 ± 0.090 ab.A
	0.250	0.803 ± 0.168 b.A	1.150 ± 0.067 bc.A	1.123 ± 0.023 abc.A
	0.500	0.530 ± 0.116 b.A	1.133 ± 0.098 abc.A	1.190 ± 0.060 bc.A
	1.000	0.084 ± 0.030 a.A	0.785 ± 0.286 ab.B	0.600 ± 0.144 a.AB
Fruit dry mass	0.000	2.190 ± 0.571 b.A	2.237 ± 0.206 cd.A	2.620 ± 0.150 d.A
	0.125	1.727 ± 0.158 b.A	1.970 ± 0.354 abc.A	1.613 ± 0.093 ab.A
	0.250	1.500 ± 0.175 b.A	1.907 ± 0.135 abc.A	1.920 ± 0.064 abc.A
	0.500	1.2 87 ± 0.155 b.A	2.053 ± 0.105 bcd.A	1.847 ± 0.171 abc.A
	1.000	0.377 ± 0.026 a.A	1.380 ± 0.271 a.B	1.370 ± 0.012 a.B
Number of seeds	0.000	24.333 ± 0.882 d.B	11.667 ± 1.856 b.A	11.333 ± 0.882 ab.A
	0.125	18.000 ± 1.000 b.B	09.000 ± 1.528 ab.A	09.000 ± 0.577 ab.A
	0.250	17.000 ± 0.001 bc.B	09.667 ± 0.333 ab.A	11.333 ± 2.028 ab.A
	0.500	15.667 ± 0.667 c.B	11.667 ± 0.882 b.A	09.000 ± 1.155 ab.A
	1.000	08.500 ± 0.289 a.A	09.500 ± 1.443 ab.A	07.000 ± 1.155 a.A

Values are averages (± SD) of 5 replicates. Different letters indicate significantly differences among diverse treatments at $p \leq 0.05$. The values in the first column were compared vertically to indicate the effect of increasing Cd concentrations. The values in columns 2 and 3 were compared horizontally with those in the first column at each Cd level to indicate the effect of H_2O_2

Lower and upper case letters (a–e and A–C) indicated significant differences among means in both columns and rows, respectively

Table 2 Effects of hydrogen peroxide (H_2O_2 mM) in chlorophylls (Chl a and b) and carotenoids amounts (mg g^{-1} FW) of *Pisum sativum* L. plants grown under different concentrations of cadmium (Cd)

Parameter	Cd (mM)	H_2O_2 (mM)		
		Control	(1 mM)	(2 mM)
Chl a	0.000	1.635 ± 0.022 c.A	1.536 ± 0.113 b.A	1.397 ± 0.041 ab.A
	0.125	1.563 ± 0.050 bc.AB	1.881 ± 0.186 c.B	1.664 ± 0.051 ab.A
	0.250	1.397 ± 0.104 b.A	1.447 ± 0.092 ab.A	1.544 ± 0.052 b.A
	0.500	1.115 ± 0.056 b.B	1.452 ± 0.021 ab.B	1.249 ± 0.017 a.A
	1.000	0.937 ± 0.002 a.A	1.345 ± 0.040 ab.B	1.448 ± 0.030 a.B
Chl b	0.000	0.278 ± 0.005 b.B	0.260 ± 0.010 a.AB	0.241 ± 0.014 a.A
	0.125	0.253 ± 0.010 ab.A	0.366 ± 0.083 b.A	0.261 ± 0.010 a.A
	0.250	0.237 ± 0.025 ab.A	0.224 ± 0.009 a.A	0.267 ± 0.005 a.A
	0.500	0.220 ± 0.019 ab.A	0.236 ± 0.013 a.A	0.247 ± 0.006 a.A
	1.000	0.200 ± 0.011 a.A	0.224 ± 0.006 a.A	0.225 ± 0.008 a.A
Carotenoids	0.000	1.089 ± 0.012 c.B	1.047 ± 0.061 b.B	0.633 ± 0.064 a.A
	0.125	1.268 ± 0.073 d.B	1.179 ± 0.026 b.B	0.735 ± 0.031 a.A
	0.250	0.653 ± 0.061 b.A	0.648 ± 0.070 a.A	1.094 ± 0.055 b.B
	0.500	0.635 ± 0.035 b.A	0.734 ± 0.020 a.A	0.690 ± 0.056 a.A
	1.000	0.417 ± 0.059 a.A	0.628 ± 0.007 a.B	0.605 ± 0.016 a.B

Values are averages (± SD) of 5 replicates. Different letters indicate significant differences among diverse treatments at $p \leq 0.05$. The values in first column were compared vertically to indicate the effect of increasing Cd concentrations. The values in columns 2 and 3 were compared horizontally with those in the first column at each Cd level to indicate the effect of H_2O_2

Lower and upper case letters (a–c and A–B) indicated significant differences among means in both columns and rows, respectively

the concentrations 0.500 and 1 mM diminished considerably the content of the carotenoids in comparison with control.

However, the H_2O_2 foliar treatment could decrease the negative influence of imposed Cd on photosynthetic pigments. Supplementation with H_2O_2 increased Chlorophylls a, b and carotenoids content at Cd-stressed plants over cadmium concentration range from 0.250 to 1 mM Cd but decreased its contents in Cd-unstressed (0 mM) and low stressed plants (0.125 mM Cd).

Soluble sugars

Cadmium stress enhanced soluble sugars content in shoots but reduced the contents in roots (Table 3). Treatment with H_2O_2 reduced the contents of soluble sugars in roots of Cd-stressed and unstressed plants. Shoots showed similar response at Cd concentration of 0.250 and 0.500 mM Cd and opposite response was found in unstressed (0 Cd) and highly stressed (1 mM Cd) plants. On the other hand H_2O_2 foliar application increased soluble sugars content in shoots, especially at higher Cd concentrations (0.5 and 1.00 mM).

Soluble proteins and total free amino acids

Soluble proteins amounts in roots and shoots decreased progressively during increasing cadmium concentration (Table 3). Total free amino acids content showed a similar response in roots but opposite trend was found in shoots where shoots of Cd-stressed plant had higher total amino acids contents than plants without Cd. Foliar spraying with H_2O_2 increased soluble proteins content in shoots of Cd unstressed and stressed plants. In roots, the same response was noticed at higher Cd concentrations (0.500 and 1 mM Cd), however, in Cd free plants or those received low Cd concentrations (0–0.250 mM Cd) soluble proteins was lower than those not supplemented with H_2O_2.

In the existence or deficiency of Cd, amino acids contents in each of shoots or roots showed low concentrations in plant sprayed with H_2O_2 (Cd-unstressed and Cd stressed plants at 0.500 mM were exceptions).

Ascorbic acid, hydrogen peroxide and MDA

Data in Table 4 revealed cumulative amounts of ascorbic acid and as well as peroxidized lipids in roots and shoots of growing pea plants in response to Cd exposure. Lower concentration of Cd^{2+}, decreased hydrogen peroxide content in shoots, whereas the higher Cd levels (0.500 and 1 mM cadmium) increased the content. In roots, hydrogen peroxide content increased gradually with increasing cadmium concentration.

Foliar supplementation of H_2O_2 increased ascorbic acid content in the shoots. In roots low (1 mM) H_2O_2 concentration decreased ascorbic acid content but the

Table 3 Effects of H_2O_2 (mM) on soluble metabolites amounts (mg g^{-1} DW) in both shoots and roots of *Pisum sativum* L. plants grown under different concentrations of cadmium (Cd)

Parameter	Cd (mM)	H_2O_2 (mM)		
		Control	(1 mM)	(2 mM)
Total amino acids				
Shoots	0.000	10.030 ± 0.368 a.A	12.793 ± 0.477 abc.B	12.593 ± 0.711 abc.B
	0.125	13.327 ± 0.437 b.B	12.113 ± 0.657 ab.AB	11.147 ± 0.230 a.A
	0.250	15.210 ± 0.694 c.A	13.003 ± 1.593 abc.A	12.403 ± 0.725 abc.A
	0.500	13.650 ± 0.679 bc.B	10.590 ± 0.439 a.A	14.767 ± 0.734 c.B
	1.000	14.133 ± 0.120 bc.B	14.530 ± 0.119 bc.B	11.080 ± 0.897 a.A
Roots	0.000	09.140 ± 0.452 ab.B	007.94 ± 0.187 cde.B	4.943 ± 0.368 ab.A
	0.125	10.450 ± 0.069 b.C	004.173 ± 0.354 a.A	8.620 ± 0.261 e.B
	0.250	07.980 ± 0.452 a.B	004.897 ± 0.446 ab.A	7.123 ± 0.596 cd.B
	0.500	07.963 ± 0.511 a.B	005.660 ± 0.116 b.A	7.043 ± 0.631 de.B
	1.000	07.797 ± 0.498 a.A	006.777 ± 0.197 c.A	7.067 ± 0.247 cd.A
Soluble proteins				
Shoots	0.000	192.667 ± 1.530 c.A	201.833 ± 6.479 d.A	193.833 ± 1.004 cd.A
	0.125	185.500 ± 2.136 b.A	194.500 ± 4.407 cd.B	185.367 ± 0.867 bc.B
	0.250	178.000 ± 2.074 a.A	193.600 ± 1.955 cd.B	180.067 ± 1.593 b.A
	0.500	162.300 ± 2.211 a.A	189.700 ± 4.246 bc.A	183.200 ± 2.714 bc.A
	1.000	155.333 ± 1.559 b.A	168.933 ± 1.683 a.B	160.233 ± 5.626 a.AB
Roots	0.000	72.700 ± 0.681 bc.B	67.967 ± 1.325 e.A	64.867 ± 0.960 cde.A
	0.125	68.300 ± 2.423 c.C	45.367 ± 3.697 a.A	60.937 ± 1.002 bcd.B
	0.250	65.400 ± 0.625 a.B	56.680 ± 2.491 b.A	59.897 ± 2.050 bc.AB
	0.500	52.633 ± 1.317 bc.B	65.617 ± 1.475 cde.A	66.613 ± 1.882 de.A
	1.000	49.900 ± 1.305 b.A	69.180 ± 0.233 e.B	67.253 ± 0.835 e.B
Soluble sugars				
Shoots	0.000	35.890 ± 2.080 a.A	41.117 ± 0.410 bcd.B	45.637 ± 0.909 e.B
	0.125	41.573 ± 0.929 b.B	44.097 ± 1.999 de.B	35.917 ± 0.489 a.A
	0.250	42.553 ± 0.947 b.A	40.987 ± 1.600 bce.A	39.330 ± 0.861 abc.A
	0.500	54.767 ± 1.060 a.B	41.690 ± 1.828 bcd.A	79.993 ± 0.502 ab.C
	1.000	36.877 ± 1.411 ab.A	43.110 ± 1.132 cde.B	41.130 ± 0.627 bcd.B
Roots	0.000	33.450 ± 0.690 c.B	24.267 ± 0.258 cd.A	24.360 ± 0.767 cd.A
	0.125	29.883 ± 0.476 b.C	20.703 ± 0.288 a.A	24.963 ± 0.138 d.B
	0.250	26.727 ± 0.402 a.C	21.780 ± 0.711 ab.A	24.767 ± 0.119 d.B
	0.500	27.117 ± 0.463 a.A	25.713 ± 0.824 de.A	25.857 ± 0.685 de.A
	1.000	29.417 ± 0.384 b.C	22.713 ± 0.735 bc.A	26.897 ± 0.552 e.B

Values are averages (± SD) of 5 replicates. Different letters indicate significant differences among diverse treatments at $p \leq 0.05$. The values in the first column were compared vertically to indicate the effect of increasing Cd concentrations. The values in columns 2 and 3 were compared horizontally with those in first column at each Cd level to indicate the effect of H_2O_2

Lower and upper case letters (a–e and A–C) indicated significant differences among means in both columns and rows, respectively

concentration of 2 mM H_2O_2 induced slightly increase in ascorbic acid content in Cd-supplied plants. Two used H_2O_2 concentrations decreased malondialdehyde (M DA) content in roots and shoots of Cd-untreated as well as treated plants, except for shoot in Cd untreated plants. In shoots, MDA content was increased with elevation of Cd concentration (compared to control). H_2O_2 application (1 mM and 2 mM) decreased the concentration of MDA contents especially at high Cd concentration (compared to their controls at each H_2O_2 treatment). Furthermore, the content of MDA was suppressed in roots treated with H_2O_2 (1 mM and 2 mM) compared to their controls (at each Cd level).

Hydrogen peroxide content (Table 4) enhanced as a result of H_2O_2 treatment within roots of plants without Cd as well as in Cd applied plants. In shoots, its content increased at Cd concentrations 0, 0.125 and 0.250 m M Cd and down-regulated at higher Cd levels.

Table 4 Effects of hydrogen peroxide (H_2O_2 mM) on proline, ascorbic acid, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents (µmol g^{-1} FW) of both shoots and roots in *Pisum sativum* L. plants grown in different concentrations of cadmium (Cd)

Parameter	Cd (mM)	H_2O_2 (mM)		
		Control	(1 mM)	(2 mM)
Proline				
Shoots	0.000	103.127 ± 5.049 bc.B	071.990 ± 4.260 a.A	071.787 ± 1.784 a.A
	0.125	085.997 ± 1.684 c.B	053.890 ± 1.594 a.A	087.020 ± 7.716 a.B
	0.250	083.093 ± 9.802 b.A	197.450 ± 0.531 c.B	146.223 ± 19.09 b.A
	0.500	080.487 ± 6.932 b.A	88.147 ± 10.728 a.A	109.107 ± 5.846 ab.A
	1.000	059.748 ± 3.207 a.A	257.716 ± 47.76 d.A	064.015 ± 9.033 a.A
Roots	0.000	21.680 ± 0.434 b.B	12.137 ± 1.542 b.A	015.167 ± 0.960 c.A
	0.125	19.497 ± 1.238 b.B	14.760 ± 0.092 c.A	014.794 ± 0.569 c.A
	0.250	18.467 ± 0.696 a.A	11.387 ± 0.988 bc.B	010.14 ± 0.2660 b.A
	0.500	17.757 ± 5.062 b.A	18.433 ± 1.152 d.A	012.58 ± 0.829 bc.A
	1.000	14.387 ± 1.674 ab.B	4.877 ± 1.206 a.A	022.397 ± 0.653 e.C
Ascorbic acid				
Shoots	0.000	0.868 ± 0.007 a.A	1.214 ± 0.359 cd.AB	1.303 ± 1.0630 d.B
	0.125	1.202 ± 0.029 ab.C	1.066 ± 1.000 abcd.B	0.929 ± 0.7700 ab.A
	0.250	1.033 ± 0.105 ab.A	1.025 ± 0.736 abc.A	1.117 ± 0.963 abcd.A
	0.500	0.909 ± 0.051 a.A	0.977 ± 0.585 abc.AB	1.199 ± 0.9100 bcd.B
	1.000	1.286 ± 0.193 b.B	0.962 ± 0.762 abc.AB	0.856 ± 0.7490 a.A
Roots	0.000	0.229 ± 0.014 a.A	0.335 ± 0.011 b.B	0.387 ± 0.00200 c.C
	0.125	0.398 ± 0.037 b.B	0.290 ± 0.004 a.A	0.446 ± 0.00700 d.B
	0.250	0.377 ± 0.061 b.A	0.348 ± 0.021 b.A	0.370 ± 0.00100 bc.A
	0.500	0.388 ± 0.021 b.B	0.293 ± 0.004 a.A	0.405 ± 0.02300 c.B
	1.000	0.382 ± 0.007 b.A	0.396 ± 0.017 c.A	0.395 ± 0.00900 c.A
MDA				
Shoots	0.000	52.093 ± 5.565 a.A	62.676 ± 0.910 ab.B	65.553 ± 1.2330 bc.B
	0.125	56.602 ± 3.068 a.A	73.650 ± 0.590 c.B	63.237 ± 1.4640 ab.A
	0.250	67.178 ± 3.769 a.A	63.097 ± 4.298 ab.A	63.537 ± 1.8110 c.A
	0.500	72.927 ± 1.082 a.A	57.917 ± 4.880 ab.A	65.103 ± 5.2600 bc.A
	1.000	86.603 ± 0.823 a.A	55.150 ± 1.646 a.A	56.743 ± 1.8080 ab.A
Roots	0.000	12.203 ± 0.479 a.AB	11.809 ± 0.309 ab.A	13.304 ± 0.1510 bc.B
	0.125	14.998 ± 0.575 a.B	10.242 ± 0.559 a.A	13.339 ± 0.4840 bc.B
	0.250	15.067 ± 0.408 a.A	13.558 ± 0.873 abc.A	13.408 ± 0.1380 bc.A
	0.500	19.743 ± 0.589 b.B	14.445 ± 0.988 d.A	15.077 ± 0.7570 cd.A
	1.000	20.609 ± 1.406 b.B	13.961 ± 0.753 cd.A	15.393 ± 0.1640 d.A
H_2O_2				
Shoots	0.000	51.563 ± 3.363 a.A	73.517 ± 2.553 d.B	48.243 ± 2.9750 ab.A
	0.125	46.060 ± 4.587 a.A	47.540 ± 1.386 ab.A	50.863 ± 2.1820 bc.A
	0.250	47.907 ± 0.970 a.A	55.035 ± 1.008 c.B	52.110 ± 1.7250 bc.A
	0.500	54.013 ± 11.678 a.A	49.367 ± 2.135 abc.A	48.486 ± 0.3040 ab.A
	1.000	79.847 ± 4.567 b.A	43.823 ± 3.069 a.A	47.680 ± 1.6450 ab.A
Roots	0.000	20.935 ± 0.601 a.A	25.437 ± 0.685 abc.B	23.297 ± 0.954 a.AB
	0.125	26.156 ± 0.780 c.A	26.852 ± 1.089 bc.A	23.610 ± 0.9260 a.A
	0.250	23.087 ± 0.576 ab.A	32.681 ± 0.592 d.C	25.65 ± 0.6820 bc.B
	0.500	23.459 ± 0.348 abc.A	25.212 ± 0.825 ab.A	35.453 ± 1.2170 e.B
	1.000	24.980 ± 1.450 bc.A	26.043 ± 0.551 abc.A	28.027 ± 0.6940 c.A

Values are averages (± SD) of 5 replicates. Different letters indicate significant differences among diverse treatments at $p \leq 0.05$. The values in the first column were compared vertically to indicate the effect of increasing Cd concentrations. The values in columns 2 and 3 were compared horizontally with those in the first column at each Cd level to indicate the effect of H_2O_2

Lower and upper case letters (a–e and A–C) indicated significant differences among means in both columns and rows, respectively

Proline

Proline content (Table 4) decreased gradually with increasing Cd concentration in both shoots and roots. The foliar supplementation with H_2O_2 increased proline contents in shoots of Cd treated plants over Cd concentration range from 0.250 to 1 mM. Opposite response was observed in roots at 0.125 and 0.25 mM Cd for both H_2O_2 concentrations used.

Role of Cd and H_2O_2 in plant stress and their interactions

Statistical analysis present in Table 5 indicated that the effects of cadmium (Cd), hydrogen peroxide, (H_2O_2) and the interaction (Cd × H_2O_2) were significant for most variables tested as indicated by F values. Further analysis of data through computation of the coefficient of determination (η^2) that represented the proportional share of (Cd), (H_2O_2) and (Cd × H_2O_2) on the total influence of treatment combination (Table 5) signified to those: (1) Cadmium was predominant in affecting shoots and roots dry weights, fruit (pods) dry weight, shoot soluble proteins, Chl a and carotenoids. (2) Hydrogen peroxide had dominant effects on roots TAA, SP and SS and number of seeds. (3) The share of interaction (Cd × H_2O_2) was dominant in affecting shoots TAA and SS and shoots and roots proline, ascorbic acid, H_2O_2 and Chl b content. (4) Cd and Cd × H_2O_2 interaction had equal dominant share in affecting MAD contents in shoots and roots. (5) Cd, H_2O_2 and the interaction (Cd × H_2O_2) seem to play duality share in their subordinate influence.

Table 5 F and η^2 estimates for the effects of hydrogen peroxide (H_2O_2), cadmium (Cd) and their interactions (H_2O_2 × Cd) on leaves pigments (Chl a, Chl b, and Carotenoids), fruits dry weight, number of seeds and contents of dry weight, malondialdehyde (MDA), hydrogen peroxide (H_2O_2), ascorbic acid, TAA, SP, proline and SS of both shoots and roots of *Pisum sativum* L. plants

Parameter	Cd		H_2O_2		Cd × H_2O_2	
	F	η^2	F	η^2	F	η^2
Dry weight						
Shoots	070.819**	0.672	024.914**	0.118	06.313*	0.210
Roots	310.055**	0.560	069.801**	0.063	59.709**	0.377
TAA						
Shoots	005.031*	0.186	003.262	0.060	05.820*	0.754
Roots	004.858	0.047	106.940**	0.511	13.204**	0.442
Soluble proteins (SP)						
Shoots	071.690**	0.625	053.783**	0.234	04.618	0.141
Roots	029.250**	0.292	071.459**	0.356	10.097**	0.352
Soluble sugars						
Shoots	003.128	0.089	017.284**	0.246	06.665*	0.665
Roots	019.375**	0.103	254.363**	0.673	12.074**	0.224
Proline						
Shoots	058.199**	0.224	081.391**	0.156	26.402**	0.620
Roots	014.740*	0.292	011.360**	0.112	08.602**	0.596
MDA						
Shoots	008.941**	0.483	002.880	0.078	02.321	0.439
Roots	032.339**	0.422	071.324**	0.465	02.482	0.113
Ascorbic acid						
Shoots	000.8925	0.061	000.248	0.008	03.919	0.931
Roots	009.029**	0.217	023.533**	0.283	05.938*	0.500
H_2O_2						
Shoots	005.139**	0.148	005.197*	0.075	07.683*	0.777
Roots	020.068**	0.209	040.514**	0.211	15.951**	0.580
Chlorophyll a						
Leaf	021.691**	0.519	012.142**	0.138	04.340	0.343
Chlorophyll b						
Leaf	006.153*	0.379	004.551	0.140	02.231	0.481
Carotenoids						
Leaf	079.252**	0.512	007.880*	0.025	20.474**	0.463
Fruit dry weight	013.118**	0.726	002.262	0.063	01.086	0.211
Number of seeds	025.063**	0.282	092.779**	0.523	04.934	0.195

The marked levels with stars (p*and p**) indicated significant confidence of 1% and 5%, respectively

Discussion

Plants perform several mechanisms to compete against the adverse effects of pollution. These mechanisms may be enhanced by the addition of chemicals to plants (Gadallah 1995). Adaptation of *Pisum sativum* plants to toxic effect of Cd as expressed in various metabolic changes and growth improvement was enhanced by exogenously added H_2O_2. Generally, the H_2O_2 applied as foliar spray affected positively the growth and yield aspects in the cadmium suffered plants. This positive effect could be attributed to an increased in photosynthetic pigments.

Hydrogen peroxide treatment mitigates the injuries of the several abiotic stressors like cadmium stressor in rice (Bai et al. 2011; Hu et al. 2009). Guzel and Terzi (2013) reported that hydrogen peroxide increases water content, growth, mineral concentration, total sugar content, soluble protein and proline content compared to their relative (H_2O_2 free) sets in young maize plants.

In our work, H_2O_2 supplying increased photosynthetic pigments that permitted high photosynthetic activities and increased shoots dry matter content (Khandaker et al. 2012). In addition, the lower H_2O_2 accumulation induced by the H_2O_2 supplementation at higher Cd concentration is evidence that plants of *Pisum sativum* could regulate oxidative injuries generated by ROS in the photosynthetic apparatus and retain leaf gas exchange (Gondium et al. 2013). The prevented chlorophyll degradation due to H_2O_2 addition may be assigned to retain lower hydrogen peroxide content and higher leaf relative water content in leaves under abiotic stresses (Chakraborty et al. 2012; Gondium et al. 2013; Khan et al. 2017).

Though, accurate metabolism of the defensive action of H_2O_2 treatment at low doses against different stressors particularly Cd stress is still un-interpreted.

Generally, *Pisum sativum* plants supplemented with H_2O_2 had low contents of soluble sugars in their shoots and roots could be due to the enhancement of sugars utilization for the formation of new cells and tissues.

Data of present study indicated that cadmium-induced loss of soluble protein and stimulation of amino acids accumulation disappeared when the *Pisum sativum* plants were treated with H_2O_2 could be due to decrease oxidative stress of proteins by H_2O_2, retaining of the structure of proteins or/and an increased protein synthesis.

Supplementation of H_2O_2 increased proline contents in the tissues of cadmium stressed pea. Together, our data and results of Yang et al. (2009) showed that external H_2O_2 application caused a significant accumulation of proline in radicles and coleoptiles of maize seedlings. Proline accumulation recognized as a monitor of a biotic stress and regarded as essential protecting agent (Gadallah 1999). This increase in this amino acid content could be due to: (a) an increment in proline biosynthesis (Charest and Phan 1990), (b) a reduction in proline degradation, (c) the induction of a proline-producing enzyme and the inhibition of the catabolic enzyme proline oxidase (Nayyar 2003) and (d) a decrease in proline utilization.

These increment in proline content might possibly due to its several functions i.e. redox-regulation, osmo-regulation, protection against damage by ROS and metal chelation (Guzel and Terzi 2013). Hyat et al. (2013) reported that the foliar treatment of *Cicer arietinum* with proline caused the mitigation of the negative effects initiated by cadmium introduction. Accordingly, our data pointed to the inducement of ROS scavenging bio components e.g. proline in pea plants treated by hydrogen peroxide under cadmium stress.

Several studies showed that the addition of H_2O_2 at low doses might benefit plant resistance to heavy metal exposure (Bai et al. 2011; Gondim et al. 2010; Hu et al. 2009; Lin et al. 2004, 2012; Xu et al. 2011). The enhanced tolerance towards metallic stress might due to stimulated antioxidant defence mechanism following treatment by H_2O_2 in rice plants (Bai et al. 2011; Hu et al. 2009).

Amongst all undesirable effects stimulated by cadmium, malondialdehyde formation is the most detrimental as it can imply to cell membrane deterioration (Nazar et al. 2012). In this study malondialdehyde (MDA) significantly increased after treatment with Cd. Similar upward trend in MDA was indicated in cotton suffered from Cd toxicity (Khan et al. 2013). Conversely, H_2O_2 application induced downward regulation in MDA contents in shoots and roots in cadmium-treated *Pisum sativum* plants compared to their controls at each Cd level. The high content of malondialdehyde and increasing activity of antioxidant enzymes is an ideal detector in determining cadmium tolerance in *Fragaria x ananassa* plant (Muradoglu et al. 2015).

Endogenous hydrogen peroxide content raised in response to H_2O_2 treatment at roots of pea plants (without Cd as well as in Cd-exposed plants) and in shoots (in plants exposed to low Cd concentrations) that was in agreement with results of Xu et al. (2011). Terzi et al. (2014) found that endogenic hydrogen peroxide concentration lightly increased in hydrogen peroxide pretreated seedlings comparing to H_2O_2 free. This increase resulted from permeation of externally applied H_2O_2 to maize leaves. On the other hand, H_2O_2 application decreased H_2O_2 content at elevated Cd concentration (compared to their H_2O_2 sprayed controls at 1 mM and 2 mM) in pea shoot. Hasanuzzaman et al. (2017) and Hossain et al. (2015) noticed that hydrogen peroxide treatment mitigates Cd-induced oxidative stress via regulation of the antioxidant protective and glyoxalase mechanism in *Brassica napus* L. They concluded that the increment of both the

enzymatic and non-enzymatic antioxidants benefit in decrement the oxidative injury as cleared by reduced amounts of MDA as well as H_2O_2.

Exogenous application of H_2O_2 improved the content of important reactive oxygen species scavenge components, GSH, AsA as well as the antioxidant enzyme activities that stimulated ROS scavenge pathway (Hasanuzzaman et al. 2017; Hossain et al. 2015). Supplementation with 2 mM H_2O_2 increased the contents of ascorbic acid in highly Cd-treated *Pisum sativum* plants that are related directly to hydrogen peroxide scavenge metabolism (Ashraf 2009; Blokhina et al. 2003; Gill and Tuteja 2010). Xu et al. (2011) noticed that hydrogen peroxide stimulated up-regulation of ascorbic acid and metabolism related to aluminum acclimation in *Triticum aestivum* L. plants. According to Noctor and Foyer (1998), the ascorbate able to react directly reactive oxygen species hence stimulate oxidative defense contra various stressful conditions. Furthermore, it was observed that the ascorbic acid and the alterations in ascorbate redox status are instantly related to stress acclimation in different plant species (Wang et al. 2010; Xu et al. 2011).

The present data indicated significant interactions between cadmium stress and H_2O_2 and their effects on the variables examined as indicated by F values. So as, in natural habitats the plants not only respond to the environmental factors as separate factors, however, also affected by their interactions. At certain cases e.g. shoots TAA, shoots SS, shoots and roots proline content, ascorbic acid, H_2O_2 and leaf chlorophyll b content, the relative impact of the interaction between the single factors was dominant but the role of separate factors was subsidiary or the minor one, though even significant.

Conclusions

Results illustrated that treatment with H_2O_2 enables *Pisum sativum* plant to endure the injurious effect of cadmium, causing improvement in growth, seeds and pods quality. The cadmium resistance prompted by H_2O_2 foliar treatment is due to decrease in endogenous MDA and H_2O_2 contents at higher Cd levels and enhancement of the non-enzymatic defense system (ascorbate), accumulation of proline especially in shoot and maintenance of chlorophyll content in *Pisum sativum* leaves. These characteristics promote oxidative protection against Cd stress and allow *Pisum sativum* plants to sustain increment of metabolic rates in cadmium stressful condition and ameliorate the growth. Finally, it can be concluded that H_2O_2 treatment in sub-lethal doses can exert an ameliorative effect and helped *Pisum sativum* plants to grow successfully in the areas subjected to cadmium pollution, such as in mining or smelting area.

Author contribution statement SS and MG have a same contribution towards experiment design, attainment of data, analysis and performance data and prearranging of the manuscript. The ultimate manuscript was read and established via SS and MG.

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Compliance with ethical standards

Conflict interest The authors stressed that they have not any kind of conflict concerning interest.

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