Plant Physiology and Biochemistry 143 (2019) 129–141

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Crosstalk among polyamines, phytohormones, hydrogen peroxide, and phenylethanoid glycosides responses in *Scrophularia striata* to Cd stress

Elham Sadat Beshamgan, Mohsen Sharifi*, Fatemeh Zarinkamar

Center of Excellence in Medicinal Plant Metabolites, Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Keywords:
Acteoside
Cd stress
Echinacoside
Phenylethanoid glycosides
Polyamine
Scrophularia striata

ABSTRACT

Plants respond to Cadmium (Cd) as a hazardous heavy metal through various mechanisms depending on their available metabolite resources. In this research, the physiological and signaling pathways mediating the responses to Cd stress in *Scrophularia striata* seedlings were characterized after they were exposed to different Cd concentrations at different time periods. The results showed that the polyamines (PAs), Abscisic acid (ABA) and hydrogen peroxide (H_2O_2) contents were significantly enhanced at 48 h. Moreover, the enzyme activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) as regulator enzymes in the phenylpropanoid pathway was increased, related to the reinforcement of phenolic compounds such as phenylethanoid glycosides (as a special compound of this plant). This metabolic profiling indicates that the signal transduction of Cd stress increased the activity of different enzymes (PAL and TAL) by regulating the PAs metabolism, the modulation of ABA, and the H_2O_2 content. As a result, it caused the accumulation of phenolic compounds, especially echinacoside and acteoside, both of which are required to improve the response of Cd stress in *S. striata*.

1. Introduction

Heavy metals (HMs) that have high stability, including Cadmium (Cd), pollute different biological systems. The plant roots absorb Cd from the soil, transfer it to the above-ground parts, and it accumulates in all parts of plants (Smolders, 2001). This element is non-essential and non-toxic, but in high concentrations, it can negatively change plant growth and many ranges of physiological, morphological, biochemical and metabolic processes by generating high quantities of reactive oxygen species (ROS), having direct interaction with key enzymes, substituting indispensable metal ions from special binding sites, deactivating the antioxidant enzyme system including catalase (CAT), guaiacol peroxidases (GPOX), and superoxide dismutase (SOD) (which are needed for the reduction of free radicals), and discharging antioxidant compounds with low molecular weights (Gupta et al., 2017; Emamverdian et al., 2015; Karcz and Kurtyka, 2007; Sandalio et al., 2001).

Plants manage a complicated organization of homeostatic mechanisms for confronting at surplus Cd (Clement, 2006; Meharg, 1994). The plant species may use more than one mechanism for facing under Cd stress, including: 1) constraint of absorption, 2) stimulation of enzymatic and non-enzymatic antioxidant systems, 3) use of different phytohormones that play significant roles in the signaling pathways at the responses of HMs stress, and 4) different modifications in the content of the main compounds, including polyamines (PAs), amino acids, phenolic compounds, and signaling molecules like hydrogen peroxide (H_2O_2) (Bücker-Neto et al., 2017; Sytar et al., 2018; Hossain et al., 2012). PAs are organic cations with low molecular weights which may operate as free radical scavengers or metal chelators (directly or indirectly) and also operate in inhibiting Cd uptake or its entry into cells in the responses at HMs stress (Bors et al., 1989; Cona et al., 2006; Lomozik et al., 2005). Phenolic compounds such as flavonoids and phenylethanoid glycosides (PhGs) operate as powerful antioxidants in biological systems (Kamalipourazad et al., 2016). PhGs are a group of water-soluble phenolic compounds with significant biological properties. The most important PhG compounds include echinacoside and acteoside which are distributed broadly in the plant kingdom such as *Cistanches Herba*, *Rehmannia glutinosa* var. purpurea (Orobanchaceae), and *Scrophularia striata Boiss* (Scrophulariaceae) (Alipieva et al.,2014; Murthy et al., 2014; Monsef-Esfahani et al., 2010; Khanpour-Ardestani et al., 2015; Ahmadi-Sakha et al., 2016).

Scrophularia striata Boiss, known as Tashne Dari by natives of Iran, is important in terms of the different aspects of plant physiology. Previous reports have indicated that *S. striata* operates as a resistant plant under various stresses, including methyl jasmonate, chitosan, and water deficit (Kamalipourazad et al., 2016; Sadeghnezhad et al., 2016; Falahi

---

* Corresponding author.
E-mail addresses: es.beshamgan@modares.ac.ir (E.S. Beshamgan), msharifi@modares.ac.ir (M. Sharifi).

https://doi.org/10.1016/j.plaphy.2019.08.028
Received 28 February 2019; Received in revised form 29 August 2019; Accepted 29 August 2019

Abbreviation

ABA	Abscisic acid
BRs	Brassinosteroids
Cad	Cadaverine
Cd	Cadmium
$CdCl_2$	cadmium chloride
CAT	Catalase
GR	Growth rate
DW	Dry weight
FW	Fresh weight
GPOX	Guaiacol peroxidase
HMs	Heavy metals
HCA	Hierarchical cluster analysis
IAAs	Indole 3 Acetic Acid
MDA	Malondialdehyde
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
H_2O_2	hydrogen peroxide
PhGs	Phenylethanoid glycosides
PAs	Polyamines
PCA	Principle component analysis
Put	Putrescine
PAL	Phenylalanine ammonia-lyase
ROS	Reactive oxygen species
RDIR	Relative dry weight increase rate
SA	Salicylic acid
SL	Shoot length
Spd	Spermidine
Spm	Spermine
SOD	Superoxide dismutase
TAL	Tyrosine ammonia-lyase

et al., 2018a, b). Because the metabolic mechanisms and the signaling pathways in the responses of *S. striata* to Cd stress have not been scrutinized, this study aimed to identify the signaling pathways and the metabolic responses that this plant applies to confront at Cd stress. It would be interesting to specify the role of PAs and ABA in the biosynthesis of PhGs and the signaling pathways at the responses of *S. striata*. This comprehensive examination could improve our understanding of the synchronous signaling pathways involved in the alleviation of heavy metal stress.

2. Materials and methods

2.1. Chemicals

spermidine, spermine, putrescine, cadaverine, abscisic acid, brassinosteroids, indole 3 acetic acid, cinnamic acid, caffeic acid, coumaric acid, salicylic acid, ferulic acid, daidzein, myricetin, kaempferol, naringinine, catechin, luteolin, diosmin, genistin, echinacoside, acteoside, and proline were all purchased from Sigma Aldrich (Taufkirchen, Germany). These compounds were used as received without any further purification.

2.2. Growth conditions

S. striata seeds were gathered from Ilam province, Iran. The seeds were immersed in running water for 48 h. Their surface was disinfected by 2% sodium hypochlorite solution for 20 min, and the disinfected seeds were washed with distilled water. Then they were immersed in 500 mg L^{-1} gibberellin for 24 h. For germination, the seeds were placed in pots containing water-soaked perlite for 43 days. Similar seedlings were moved to plastic receptacles (1.5 dm^3) containing the nutritional solution of Hoagland (pH 6.8) and located in the growth room with a cycle of 16 h light (220 µmol photons m^{-2} s^{-1}), air humidity of 60–80%, and a temperature of 30/25 °C day/night for four weeks.

2.3. Experimental design

This experiment was carried out in two separate steps. First, 73-day-old seedlings were subjected to the nutritional solution of Hoagland supplemented with cadmium chloride ($CdCl_2$) at concentrations of 0, 50, 200, and 400 µM. After three days (72 h), the plants were harvested for further analysis. Based on the data obtained from this step (Table 1), the Cd concentration of 200 µM was selected for the second step of experiments. Similar seedlings grown in a nutritional solution of Hoagland with 200 µM of $CdCl_2$ were collected after 12, 24, 48, 72 h and washed with distilled water. At each time step, the shoots were disconnected, frozen, and kept at −80 °C for further analysis.

2.4. Determination of growth parameters

All samples with similar growth rates were randomly selected. Six repeats per each treatment were conducted to determine shoot length (SL) (Liu et al., 2004; Zafari et al., 2017).

The growth rate (GR) was quantified by the following equation:

$$GR\ (\%) = \frac{(W_a - W_b)}{W_b} \times 100 \tag{1}$$

Where W_a is the dry weight of shoots after 48 h or 72 h of Cd treatment, and W_b is the dry weight of shoots after 0 h Cd treatment (control). The parameter of relative dry weight increase rate (RDIR) in various treatments was calculated using Equation (2) (Liu et al., 2004; Zafari et al., 2017):

$$RDIR\ (\%) = \frac{(GR_t - GR_c)}{GR_c} \times 100 \tag{2}$$

Where GR_t and GR_c show the growth rate of the treated and control plants, respectively.

Table 1

Accumulation of Cd, activity of antioxidant enzymes, content of PhGs, and content of proline in *S. striata* shoots at different Cd concentrations. Values within each column are mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

CdCl2 (µM)	Cd accumulation	SOD	CAT	GPOX	PAL	TAL	ECH	ACT	Proline
	mg kg^{-1} DW	U mg $protein^{-1}$ min^{-1}	µmol H_2O_2 mg^{-1} protein min^{-1}	µmol guaiacol mg-^{1} protein min^{-1}	µmol cinnamic acid mg^{-1} protein min^{-1}	µmol coumaric acid mg^{-1} protein min^{-1}	µg g^{-1} FW	µg g^{-1} FW	mg g^{-1} DW
0	0.01 ± 0^a	2.7 ± 0.6^a	18.3 ± 1.9a	15.2 ± 2.3^a	2.4 ± 0.07^a	0.991 ± 0.07^a	60.3 ± 2.2^a	34.8 ± 0.2a	13.5 ± 1.7^a
50	39.3 ± 0.1^d	4.1 ± 0.8^a	23.3 ± 1.9^b	23.1 ± 3.6^b	2.8 ± 0.06^b	1.3 ± 0.1^b	81.9 ± 1.5^c	79.0 ± 1.4^c	14.1 ± 2.2^a
200	58.4 ± 0. 2^c	5.8 ± 0.6^b	34.4 ± 1.9^c	32.2 ± 1.3^c	3.5 ± 0.13^c	2.1 ± 0.1^c	107.6 ± 1.2^d	93.1 ± 1.4d	20.9 ± 1.6^b
400	55.1 ± 0.01^c	5.7 ± 0.8^b	36.4 ± 1.2^c	36.3 ± 2.8^c	2.6 ± 0.04^b	1.2 ± 0.04^b	77.5 ± 2.0^b	52.9 ± 0.5b	19.5 ± 2.2^b

2.5. Determination of Cd accumulation

The dried shoots (1 g) were incinerated for 6 h at 500 °C and the powdered ashes of the samples were dissolved in 0.1 M HCl. In the next step, the Cd concentrations in the samples were assayed by spectrophotometry of atomic absorption (Shimadzu AA- 6709) based on the method of Camacho- Cristóbal and Gonzólez-Fontes (2002).

2.6. Determination of H_2O_2 and MDA contents

To determine the H_2O_2 and malondialdehyde contents (MDA), the fresh shoots (0.5 g) were extracted in 4 mL trichloroacetic acid (TCA; 0.1% W/V) and then centrifuged at 12,000 rpm for 10 min at 4 °C. H_2O_2 content was determined spectrophotometrically at 390 nm according to the method of Velikova et al. (2000) and then quantified using a calibration curve method.

Lipid peroxidation due to Cd stress was determined by measuring the formation of MDA based on the thiobarbituric acid method using a UV/vis spectrophotometer at 532 and 600 nm (Stewart and bewley, 1980). The MDA content was measured using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.7. Determination of proline content

To determine proline content, the shoots (0.5 g) were extracted within sulphosalicylic acid (3%) and centrifuged at 3000 rpm for 10 min. The proline content was determined spectrophotometrically at 520 nm according to the method of Bates et al. (1973) and quantified by a calibration curve method using proline as the standard.

2.8. Determination of enzyme activities

To determine enzymatic activities, the fresh shoots (0.5 g) were extracted within 2 mL potassium phosphate buffer pH 7 containing 1 mM Dithiothreitol in ice. The mixture was centrifuged at 12,000 rpm and at 4 °C for 20 min. The supernatant was used immediately for determining enzymatic activities. The protein content was measured according to the method of Bradford (1976).

The activity of SOD was determined spectrophotometrically at 560 nm according to the method of Giannopolitis and Ries (1977). The activity of CAT was assayed by a spectrophotometer at 240 nm according to the method of Cakmak and Marschner (1992). The activity of GPOX was measured according to the guaiacol oxidation at 470 nm (Lin and Wang, 2002). The PAL activity was assayed according to the produced quantity of cinnamic acid at 290 nm (Wang et al., 2006; Hodgins, 1971). The activity of TAL was assayed according to the produced quantity of p-coumaric acid at 320 nm (Wang et al., 2006; Hodgins, 1971).

2.9. Determination of amino acids

The shoot tissue (0.2 g) was powdered in liquid N_2, extracted within 80% ethanol (2 mL) (v/v), and centrifuged at 10,000 rpm for 10 min. Then, the supernatant was dried and the debris was dissolved in 1 mL of water (Di Martino et al., 2003). The Biermann et al. (2013) method was used to identify amino acids by HPLC (Agilent Technologies 1260 Infinity, USA). The peaks of samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥99%) by fluorometric detector (FLD HP 1100) which was done with excitation and emission wavelength of 230 nm and 455 nm, respectively.

2.10. Determination of PAs

The shoot tissue (0.2 g) was homogenized in 3 mL perchloric acid (5% v/v) and centrifuged at 20,000 rpm and 4 °C for 30 min. Supernatant and pellet were utilized to assay the free and conjugated PAs, respectively (Sharma and Rajam, 1995). Finally, the analyses were performed by HPLC (Agilent Technologies 1260 infinity) as described by Falahi et al. (2018b). Different PAs were identified with a detector of UV dual-array at 254 nm. The peaks of samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥99%).

2.11. Determination of phytohormones

Shoot tissue (0.5 g) was homogenized within 3 mL of absolute methanol and left overnight at 4 °C. Then the extract was concentrated by evaporation and filtered (0.22 μm) (Razifah et al., 2014). Finally, the analyses were performed by HPLC (Knauer, Germany) as described by Delavar et al. (2017). The peaks of the samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥98%) at 220 nm with an ultraviolet detector (PDA, Berlin, Germany).

2.12. Determination of phenolic compounds

2.12.1. Determination of phenolic acids

Shoot tissue (0.5 g) was extracted within 4 mL methanol. The extract was then dried at 35 °C under vacuum, dissolved within 4 mL acetonitrile, washed three times by 3 mL hexane, and dried. The debris was dissolved again by 0.5 mL absolute methanol (Owen et al., 2003). Finally, the analyses were performed by HPLC (Agilent Technologies 1260 Infinity, USA) as described by Falahi et al. (2018b). The peaks of the samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥97%) at 280 and 340 nm with an ultraviolet detector (PDA, Berlin, Germany).

2.12.2. Determination of flavonoids

Shoot tissue (0.5 g) was homogenized by 40% methanol (1.5 mL) including 0.5% acetic acid. Thereafter, the extracts were shaken overnight. The mix was centrifuged at 13,000 rpm for 12 min (Keinänen et al., 2001). Finally, analyses were performed by HPLC (Agilent Technologies 1260 Infinity, USA) as described by Gudej and Tomczyk (2004). The peaks of the samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥98%) at 254, 280, 300, and 350 nm with an ultraviolet detector (PDA, Berlin, Germany).

2.12.3. Determination of phenylethanoid glycosides

Shoot tissue (0.5 g) was extracted within methanol (5 mL) and centrifuged at 10,000 rpm for 20 min. The supernatant was dried, dissolved in 0.5 mL methanol, and centrifuged at 13,000 rpm for 20 min (Khanpour-Ardestani et al., 2015). Finally, analyses were performed by HPLC (Agilent Technologies 1260 Infinity, USA) using the method of Vertuani et al. (2011). The differentiation of PhGs was performed with a C18 column as the stationary phase (Perfectsil Target ODS-3 (5 μm); 250 × 4.6 mm; MZ Analysenthecnik, Mainz, Germany). The rate of flow was 1 mL min^{-1} using a gradient between mobile phases A and B, which were 0.04% deionized water containing phosphoric acid and acetonitrile, respectively. Different PhGs were identified at 330 nm by an ultraviolet detector (PDA, Berlin, Germany). The peaks of the samples were determined by adjusting their retention times with the original standards (Sigma Aldrich, ≥98%).

2.13. Statistical analysis and the relation between analyses

For the mean comparison of data, SPSS software version 24 was used to perform the variance analysis. The Duncan's new multiple-range test was used to determine the significant differences among the samples ($p < 0.05$). For principal component analysis (PCA) and correlation analysis, the algorithms set on the web-based MetaboAnalyst (http://www.metaboanalyst.ca) and metabolite data from this study were used. PCA was used to investigate the credibility of this experiment. Correlation analyses, including PatternHunter and Hierarchical Cluster Analysis (HCA), were operated based on the Pearson correlation coefficient. PatternHunter was used to show the correlation between the echinacoside as PhGs and different members of the signaling pathways, including H_2O_2, phytohormones, and PAs; HCA analyses were performed for metabolite classification.

3. Results

3.1. Growth parameters decreased while cadmium accumulation increased in S.striata under cadmium

In the first step of the experiment, the Cd accumulation in shoots showed positive linear relevance with different Cd concentrations in the Hoagland solutions up to 200 μM (Table 1). The results showed that the Cd accumulation of 28.38, 44.94, 55.7, and 58.33 mg kg^{-1} DW in the seedling shoots grown under 200 μM $CdCl_2$ for 12, 24, 48, and 72 h, respectively, indicating that the Cd accumulation increased linearly to 48 h but remained constant after three days (72 h) of treatment (Fig. 1).

The effects of Cd on the growth indices, such as RDIR and SL, after 48 h and 72 h treatments were showed in Fig. 2. The Cd treatment resulted in about 20% inhibition of RDIR and about 10% reduction in SL after 48 h. There was no significant difference in the RDIR and SL amounts either at 48 h or 72 h.

3.2. Cd caused oxidative stress while enhanced the antioxidant enzyme activity

The amount of H_2O_2 increased 0.94-, 1.20-, 1.34-, and 1.33-fold at 12-h, 24-h, 48-h, and 72-h treatments, respectively, compared with the control (Fig. 3A). The MDA production is a cytotoxic product of lipid peroxidation, which is produced under stress conditions. The MDA content was positively related at all times, but it had lower enhancement than the 48-h and 72-h treatments (Fig. 3B). The increased proline content was showed in Cd concentration at all times in Tables 1 and 2.

The different concentrations of Cd significantly increased the SOD, CAT, and GPOX of the enzyme activities. No significant difference in the activity of any of the three enzymes was determined in 200 μM and 400 μM concentrations of Cd (Table 1). The SOD activity in the treated seedlings was significantly increased compared with the controls at all times and the same trend was achieved in the enhanced activities of GPOX and CAT. The amount of the increased activities of SOD, CAT, and GPOX peaked at 48 h (Fig. 3C–E).

3.3. S.striata modulates the levels of polyamines and phyohormones under cadmium stress

The results indicated that in the treated shoots, free-PAs (including Spd, Put, and Cad) were significantly compressed at 48 h (Fig. 4C, E, and G), whereas conjugated PAs (including Spd, Put, and Cad) were compressed at 72 h of Cd treatment (Fig. 4D, F, and H). Cd reduced the levels of free and conjugated Spm compared with their controls (Fig. 4A–B). The changed free and conjugated PAs (except Spm) were positively correlated with the increased content of the phenolic compound, such as PhGs (Fig. 10B–C).

HPLC analysis indicated that the endogenous content of various phytohormones changed in the Cd treatment at different times. Although the endogenous content of IAA decreased significantly to 40% of the control, after 48 h of the treatment, ABA was significantly increased, and the BR content was not changed in any of the times (Fig. 5). IAA was negatively related to echinacoside (r = −0.901) and acteoside (r = −0.776), and ABA, was positively related to echinacoside (r = 0.849) and acteoside (r = 0.751) (Fig. 10B, Table 4).

3.4. Cadmium stimulates the phenylpropanoid pathway for the end product biosynthesis in different routs

PAL and TAL are regulator enzymes of phenylpropanoid pathway which the rate of changes in the activity of these enzymes showed in Table 1 and Fig. 6 under various Cd concentrations at different times ($p \le 0.05$). The highest activities of PAL and TAL enzymes were 1.67- and 1.3-fold higher compared with the control in 200 μM Cd at 48 h.

HPLC analysis showed a significant increase in salicylic acids, p-coumaric acid, and ferulic acid in all treatment times (Fig. 7A–C), whereas caffeic acid and cinnamic acid contents were decreased in all times except 12 h and 24 h of treatment compared with their controls (Fig. 7D–E). HPLC analysis indicated changes in the echinacoside and acteoside contents under different concentrations of Cd and at different times in the shoots of *S. striata*. Generally, the levels of echinacoside and acteoside were higher at 48 h and 72 h of treatment compared with their controls at 200 μM Cd (Table 1 and Fig. 8).

The correlation analysis shows that PhGs (echinacoside and acteoside, respectively) have a positive correlation with salicylic acid (r = 0.792, r = 0.696), p-coumaric acid (r = 0.79,218, r = 0.796), and ferulic acid (r = 0.7921, r = 0.696) and a negative correlation with caffeic acid (r = −0.702, r = −0.703) and cinnamic acid (r = −0.755, r = −0.718) (Fig. 10C). It was also realized that acteoside and echinacoside are positively related to the enzyme activity of, respectively, PAL (r = 0.964, r = 0.786) and TAL (r = 0.903, r = 0.783) (Table 4).

3.5. Flavonoid profiles in S. striata responses under cadmium

This paper reports the natural diversity of flavonoids and changes in the levels of these compounds at Cd stress in *S. striata*, as determined by HPLC. Increased levels of catechin (6.86%), luteolin (57.14%), kaempferol (30.89%), genistin (18.63%), diosmin (22.99%), daidzein (24.37%), and myricetin (15.13%) were determined at all treatment times, especially at 48 h (Table 3). The naringenin content was partly increased at 12 h and 24 h of treatment and then reduced significantly to 37.93% of the control after 48 h of treatment (Table 3). As shown in Fig. 10C, there were positive correlations between the key enzymes of phenylpropanoids (PAL and TAL, respectively) and different flavonoids, including catechin (r = 0.928, r = 0.896), luteolin (r = 0.911, re = 0.886), kaempferol (r = 0.796, r = 0.804), genistin (r = 0.879, r = 0.734), diosmin (r = 0.816, r = 0.751), daidzein (r = 0.693, r = 0.690), and myricetin (r = 0.954, r = 0.829) and a negative correlation with naringenin (r = −698, r = −0.684).

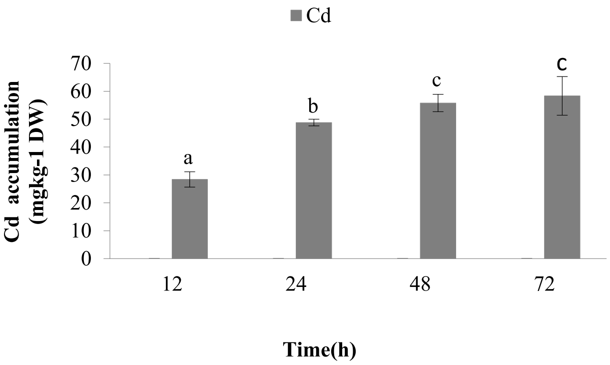

Fig. 1. Accumulation of Cd in the shoots of *S. striata* grown under 200 μM Cd. Data are mean ± SD of three replicates. Significant differences at $p \le 0.05$ are denoted by different letters.

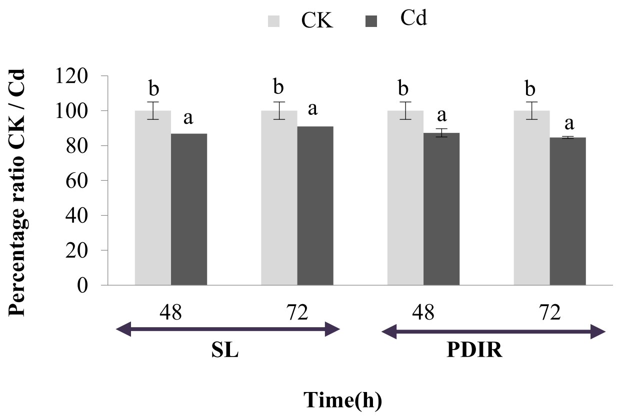

Fig. 2. Modification of RDIR and SL in *S. striata* exposed to Cd at time course. Data is reported as the mean ± SD of three replicates. Significant differences at $p \leq 0.05$ are denoted by different letters.

3.6. Influence of time on amino acid profile after S.striata exposure to cadmium

In sum, the changed levels of free amino acids in *S. striata* under Cd was indicated in Fig. 9 and Table 2. Generally, the highest quantitative modifications in the content of free amino acids were obtained at 48 h of treatment. The results indicate that the contents of Phe, Tyr (precursors of phenolic compounds), Arg, Met (precursors of polyamines), and Glu (precursors of Arg, Gln, Gly, and Pro) increased significantly at 12 h and 24 h of treatment, whereas their contents decreased significantly at 48 h and 72 h of Cd treatments compared with the control. The levels of Ala, Gly-Thr, Leu, Ile, His, and Asp increased significantly at all. Val was not changed at 12 h and 24 h, but it was increased at 48 h and 72 h times (Table 2). The correlations between all of the measured amino acids and other metabolites are showed in Fig. 10C.

3.7. Classification of the examined metabolites

The patterns of the different metabolites which were examined using PCA showed a strong pattern in the subdivision of metabolites. Principal components 1 and 2 were calculated as 61.4% and 26.9%, respectively, of the total variance in the collection of data, indicating an obvious separation based on seedling type at 200 μM $CdCl_2$. Generally,

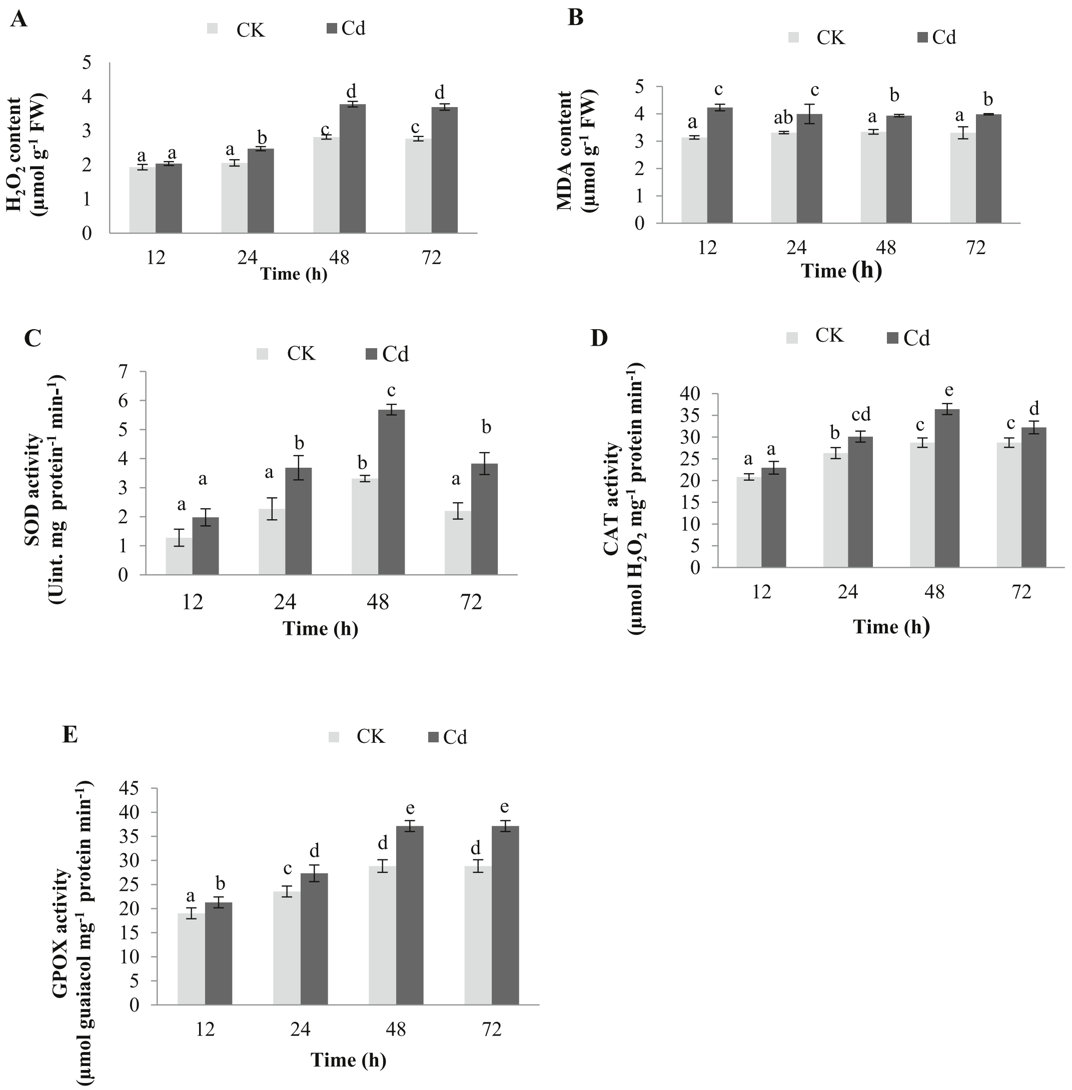

Fig. 3. H_2O_2 content (A), level of MDA (B), and enzymatic activities of SOD (C), CAT (D), and GPOX € in *S. striata* exposed to Cd at time course. Data is reported as the mean ± SD of three replicates. Significant differences at $p \leq 0.05$ are denoted by different letters.

Table 2

Accumulation of amino acids in *S. striata* exposed to Cd at time course; the values within each column are mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

Amino acid (nmolg^{-1}FW)	CK				Cd			
	12h	24h	48h	72h	12h	24h	48h	72h
Phenyl-alanine(Phe)	17.27 ± 0.38^{b}	16.94 ± 0.03^{b}	17.83 ± 0.08^{b}	17.84 ± 0.04^{b}	24.66 ± 0.03^{c}	25.78 ± 5.89^{c}	10.98 ± 0.03^{a}	10.85 ± 0.08^{a}
Tyrosine(Tyr)	13.63 ± 0.43^{a}	15.61 ± 0.76^{b}	19.88 ± 0.09^{c}	19.89 ± 0.04^{c}	19.57 ± 0.73^{c}	23.25 ± 1.06^{d}	15.58 ± 0.03^{b}	13.92 ± 0.09^{a}
Aspartic acid(Asp)	108.07 ± 1.46^{a}	113.03 ± 2.23^{ab}	117.22 ± 6.8^{b}	126.07 ± 0.26^{c}	119.12 ± 4.4^{b}	138.16 ± 11.8^{d}	142.39 ± 6.66^{d}	141.55 ± 6.37^{d}
Glutamic acid(Glu)	69.07 ± 1.7^{c}	72.55 ± 1.5^{d}	79.01 ± 0.37^{e}	79.04 ± 0.17^{e}	77.77 ± 2.89^{e}	92.4 ± 4.21^{f}	61.93 ± 0.13^{b}	55.33 ± 0.34^{a}
Arginine(Arg)	25.4 ± 0.78^{a}	25.73 ± 0.67^{a}	34.45 ± 1.39^{c}	35.28 ± 0.08^{c}	32.49 ± 0.63^{b}	37.91 ± 1.84^{d}	30.44 ± 1.67^{b}	24.69 ± 0.15^{a}
Methionine(Met)	32.08 ± 0.78^{a}	35.41 ± 0.67^{a}	41.1 ± 1.4c	42.52 ± 0.9^{cd}	39.9 ± 1.11^{bc}	44.6 ± 1.84^{d}	38.02 ± 6.13^{b}	31.37 ± 0.15^{a}
Alanine(Ala)	49.06 ± 0.2^{a}	49.68 ± 1.19^{a}	53.34 ± 1.27^{b}	51.92 ± 0.85^{b}	52.34 ± 0.66^{b}	52.6 ± 1.28^{b}	58.17 ± 0.61^{c}	66.33 ± 0.04^{d}
Valine(Val)	26.17 ± 0.35^{b}	25.24 ± 3.26^{b}	21.55 ± 3.1^{a}	23.94 ± 0.5^{a}	26.42 ± 1.03^{b}	27.07 ± 2.51^{b}	27.14 ± 1.08^{b}	28.41 ± 0.07^{c}
Serine(Ser)	104.19 ± 2.85^{a}	106.67 ± 3.8^{a}	128.47 ± 2.88^{b}	109.75 ± 3.53^{a}	104.36 ± 3.79^{a}	114.06 ± 7.6^{ab}	116.72 ± 2.6^{ab}	101.91 ± 0.11^{a}
Histidine(His)	251.93 ± 5.63^{a}	280.23 ± 11.6^{b}	326.14 ± 6.85^{d}	309.04 ± 8.44^{c}	309.29 ± 4.62^{c}	332.61 ± 0.98^{d}	389.49 ± 1.19^{e}	402.27 ± 0.55^{e}
Glycine_Threonine (Gly/Thr)	18.29 ± 0.68^{a}	17.36 ± 0.34^{a}	18.03 ± 1.05^{a}	24.12 ± 1.09^{c}	21.86 ± 0.22^{b}	25.82 ± 1.82^{c}	28.01 ± 1.02^{d}	29.11 ± 1.74^{d}
Leucine(Ile)	10.31 ± 0.35^{b}	11.25 ± 0.27^{c}	11.8 ± 0.08^{cd}	9.45 ± 0.16^{a}	11.98 ± 0.2^{cd}	12.03 ± 0.06^{d}	13.34 ± 0.34^{e}	11.5 ± 0.07^{bc}
Isoleucine(Leu)	13.28 ± 0.96^{a}	16.96 ± 0.33^{b}	17.58 ± 1.03^{b}	18.16 ± 0.04^{b}	17.87 ± 0.66^{b}	21.23 ± 1.78^{c}	25.36 ± 1^{d}	32.62 ± 1.19^{e}
Proline(Pro)	22.81 ± 2.05^{a}	25.7 ± 3.51^{a}	32.21 ± 2.28^{b}	33.65 ± 2.43^{b}	34.3 ± 1.57^{b}	42.25 ± 2.47^{c}	59.12 ± 4.56^{d}	54.46 ± 1.91^{d}

in this study, PCA showed that Cd affected significantly the total variance in the collection of data (Fig. 10A). The profile of PatternHunter showed that echinacoside as a PhG was positively correlated with different PAs, ABA, and H_2O_2 (Fig. 10B). Fig. 10C shows a high clarity illustration from an HCA heat map colored according to the Pearson correlation coefficient for each metabolite (red and blue display enhanced and reduced quantity, respectively). The HCA indicates a strong correlation between the primary and secondary metabolites in the seedlings treated with Cd. The HCA heat map is comprised of six main clusters (1–6) that show a positive correlation among PAs (except Spm), PhGs, and three phenolic acids (salicylic acid, p-coumaric acid, and ferulic acid) and a negative correlation among three groups, including two phenolic acids (cinnamic acid and caffeic acid) and PhGs, the aromatic amino acids and PhGs, and other amino acids (Met, Arg, and Glu) and PAs (Fig. 10C).

4. Discussion

Cd forms ROS which leads to oxidative stress, damage to cellular membranes, and changes in the activity of various enzymes such as auxin oxidase. Vitti et al. (2014) reported that the IAA level decreased with increases in the activity of auxin oxidase in *Arabidopsis thaliana* shoots under HMs stress. The current results demonstrated that in treated *S. striata* shoots, the increased auxin oxidase activity may reduce IAA levels, which reduce the growth indices (Fig. 5B.). In return, plants are equipped with different metabolites and the signaling pathways to counteract Cd toxicity (Rodziewicz et al., 2014).

Various studies have reported that the metabolism of PAs shifts toward catabolism to prevent the harmful effects of high PA content in stressed plants (Pála et al., 2017; Gupta et al., 2016). In the current study, the decreased level of Spm and the increased levels of Put and Spd indicate that *S. striata* has made catabolism more active, avoiding the damage of high PA levels to confront of Cd stress. Also, it was shown that the increased PA contents at 48 h and 72 h are accompanied by a decrease in Glu, Arg, and Met levels as their precursors (Fig. 4 and Table 2), which is consistent with the results of other research (Falahi et al., 2018b; Li et al., 2016; Liu et al., 2011). These results highlight that the changes in PA levels, metabolism direction, and the level of their primary precursors can be the first adaptive responses in *S. striata* subjected to Cd stress. These results can be compared with other research findings (Alcázar et al., 2006; Groppa et al., 2007; Zafari et al., 2017).

Previous studies have determined the complication of the relevance among PAs and the regulation of phytohormones biosynthesis. Anwar et al. (2015) stated that Put is positively related to the ABA biosynthesis. The roles of Spd are quite opposite from the Put and Spm down-regulated biosynthesis of ABA. According to HPLC analyses in the current study, the decreased level of Spm and the increased levels of Put and Spd may cause the increased contents of ABA in stressed *S. striata* (Fig. 5).

PAs and phytohormones play clear, dual roles in the modifications of metabolism and signaling pathways in stressed plants. Several studies have shown that ABA induces the increased enzyme activity in polyamine oxidase which participates in the catabolism of PAs and the production of H_2O_2. Generally, there is a bi-directional regular mechanism, so that the amounts of different polyamines regulate the modulation of hormones as well as the amounts of ABA regulate the metabolism direction and the level of polyamines in *S. striata* under Cd. This result is consistent with other reports (Pál et al., 2018).

One of the most important paths of H_2O_2 production is the oxidation pathway of PAs in plants. Diao et al. (2017) reported that different stresses cause the increase in cellular PAs that increase the level of ABA, the induction of the PAs catabolism direction, and the increased levels of H_2O_2 in *Lycopersicon esculentum*. This process is performed to modulate the metabolism of polyamines and the H_2O_2 content for the signaling pathway of resistance responses in plants (Wu et al., 2018; Iannone et al., 2013; Guo et al., 2014). It seems that one of the mechanisms that *S. striata* applies to deal with Cd stress is the increase of ABA, the direction regulation of PAs metabolism, and the regulation of H_2O_2 content. Generally, the production of H_2O_2 is an extraordinarily important process, because the level of H_2O_2 can determine the fate of the plants toward death or tolerance by different mechanisms (Kamalipourazad et al., 2016). Some researchers have reported that H_2O_2 operates as a signal molecule in the signal transduction and causes deep alterations in the activity of key enzymes in the phenylpropanoid pathway (PAL and TAL), different metabolite levels, and the activity of different antioxidant enzymes to be recruited as mechanisms for confronting under Cd (Jiang et al., 2012; Kovacik et al., 2010).

The PAL and TAL enzymes as checkpoints of the phenylpropanoid pathway regulate the accumulation of phenolic compounds. The activity of these enzymes is regulated by the ABA signaling pathway and H_2O_2 induced by HMs (Jamalian et al., 2013; Jiang and Joyce, 2003). The current findings showed that the increase in PAL activity was 2.10 times higher than TAL activity when the seedlings of *S. striata* were subjected to Cd. These results prove that the PAL enzyme is more active than TAL for phenolic compound accumulation in *S. striata* (Fig. 6). Hamayun et al. (2017) reported that plants can alleviate the harmful effects of Cd stress by increasing the levels of flavonoids such as daidzein and genistein. This result is consistent with the current results. On the other hand, Falahi et al. (2018b) reported that increasing PhGs

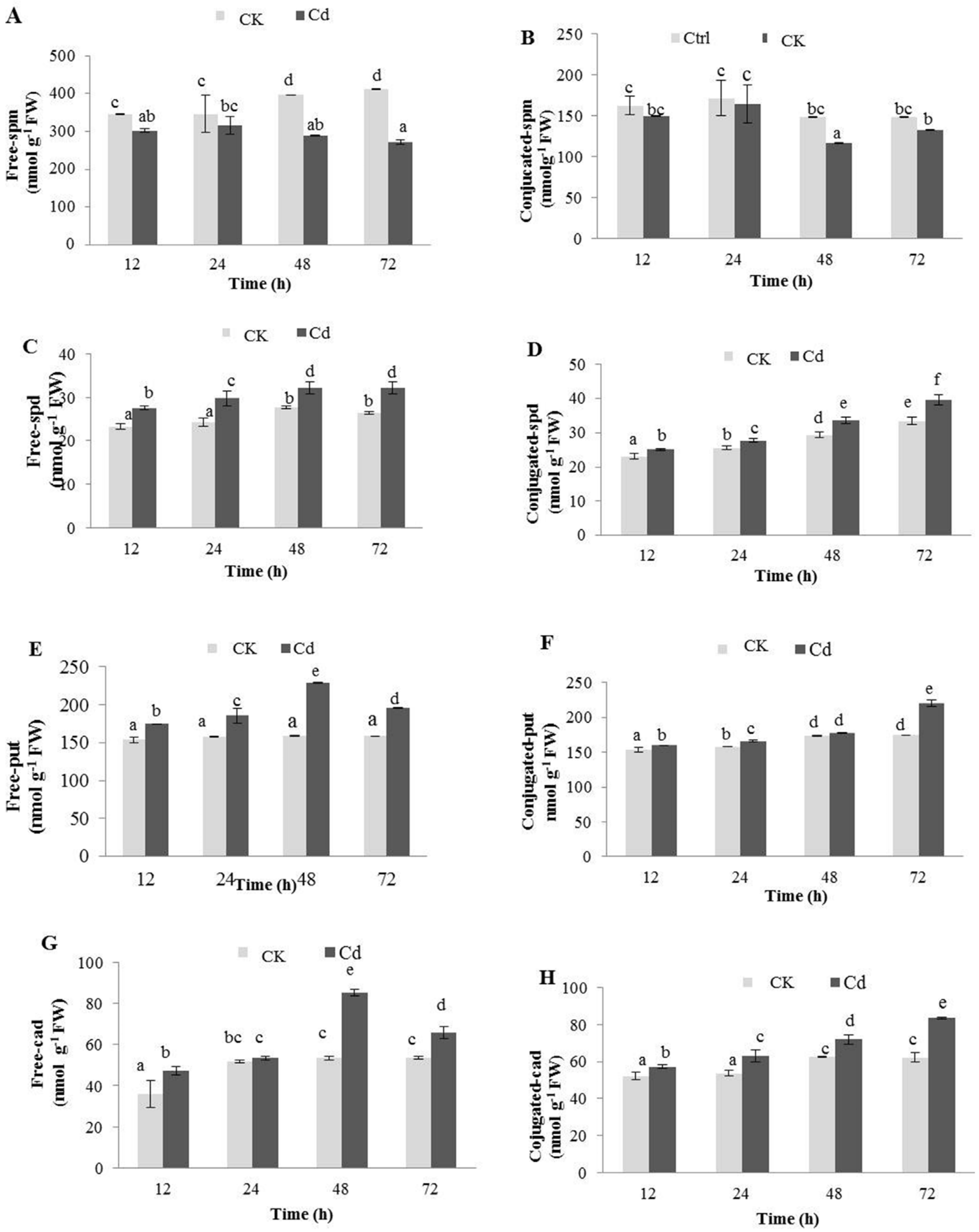

Fig. 4. Accumulation of free and conjugated PAs in *S. striata* exposed to Cd at time course: Spm (A, B), Spd (C, D), Put (E, F), and Cad (G, H) in *S. striata*. Data is reported as mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

contents as the specific phenolic compound is one of the important resistance responses of *S. striata* to water stress, and that is also consistent with the current results. Data in this study showed the route of biosynthesis of phenolic compounds shifts toward the synthesis of fewer flavonoids or more PhGs in the treated shoots of *S. striata*. Thus, it can be suggested that PhGs are more active than flavonoids in the responses of *S. striata* shoots to Cd. These results are accompanied by decreased levels of aromatic amino acids, such as Phe and Tyr. These topics highlight that Phe and Tyr are used for the biosynthesis of phenolic compounds in *S. striata* under HM stress (Table 2 and Fig. 10C).

Cadmium stress causes secondary stresses, such as oxidative and water deficiency stresses, in plants, which use various mechanisms to deal with these stresses. Various studies have shown that phenolic compounds as antioxidant compounds in the roots of *Panax ginseng*, the shoots of *Prosopis*, and *Phaseolus vulgaris* improve responses in oxidative stress produced by HMs (Zafari et al., 2016; Singh et al., 2008; Ali et al., 2006; Diaz et al., 2001). Sabine et al. (2004) stated that phenolic compounds such as flavonoids and PhGs have hydroxyl groups in their structure which operate as electron or hydrogen donors with antioxidant enzymes (SOD, CAT, and GPOX). In this study, it seems that the activity of these enzymes was regulated by phenolic compounds such as PhGs and flavonoids, and CAT activity increased more than GPOX activity, displaying that CAT might be more accountable for the modulation of H_2O_2 than GPOX in high concentrations of Cd. Yang et al. (2009) indicated that PhGs operate directly as scavengers of hydroxyl free radicals and ROS which cause oxidative stress. The ROS scavenger potency of acteoside is greater than that of echinacoside. It seems that *S. striata* specifically produces more acteoside to avoid the dangerous

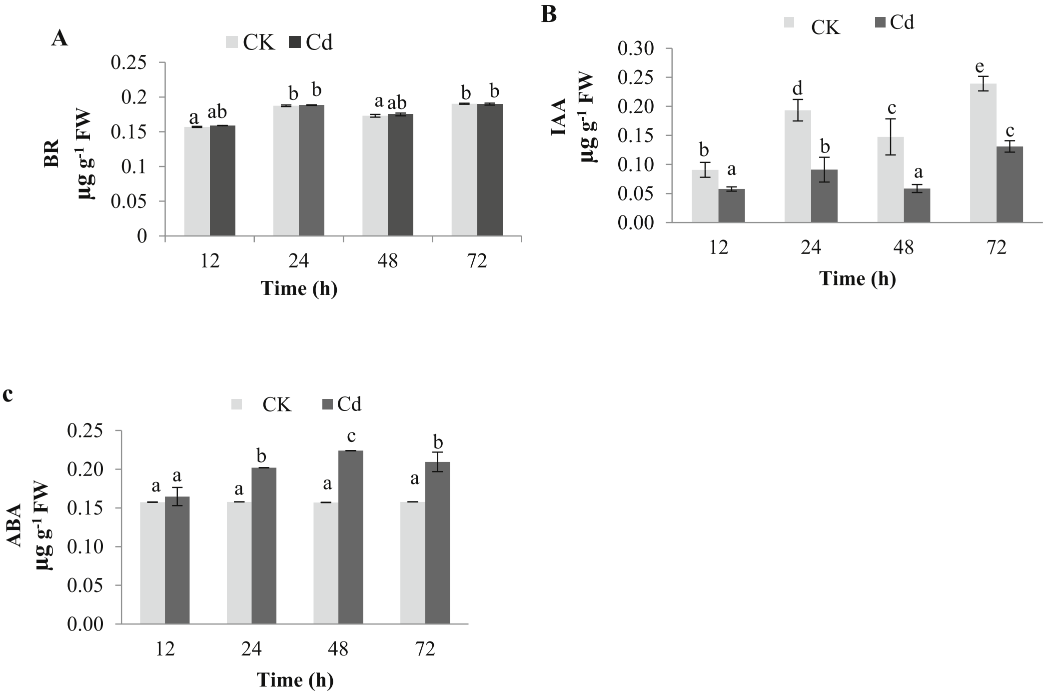

Fig. 5. Contents of phytohormones in *S. striata* exposed to Cd at time course: BRs (A), IAA (B), ABA (C), in *S. striata*. Data is reported as mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \le 0.05$.

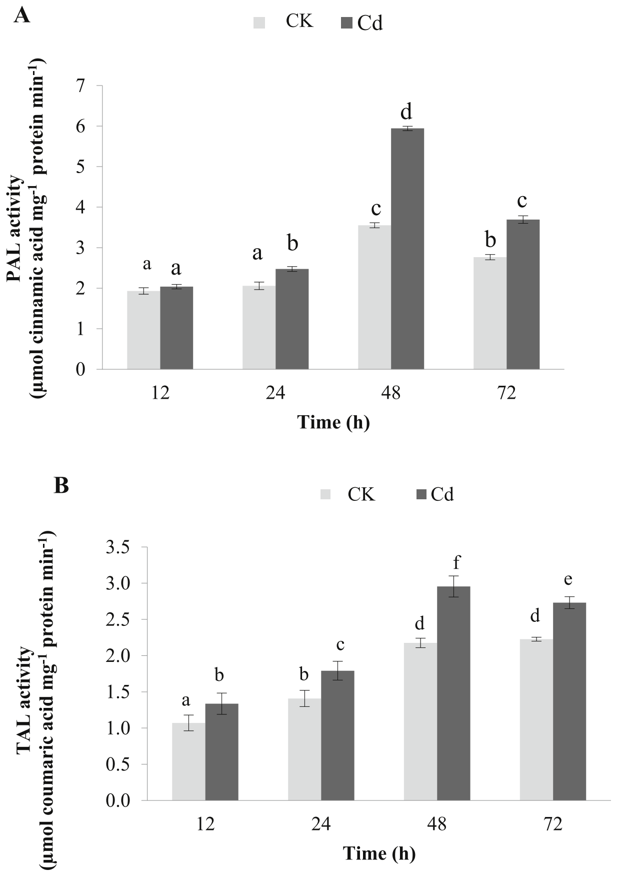

Fig. 6. Enzyme activities of PAL (A) and TAL (B) in *S. striata* exposed to Cd at time course. Data is reported as mean ± SD of three replicates. Different letters on the data show statistically significant differences at $p \le 0.05$.

effects of free radicals and ROS derived from cadmium stress. Barceló and Poschenrieder (1990) stated that HMs induce events which cause water deficiency stress in plants. On the other hand, Falahi et al. (2018b) reported that PhGs such as echinacoside operate as osmotic regulators to maintain water homeostasis; their results are consistent with the current results. Generally, *S. striata* produces PhGs specifically to cope with secondary stresses such as oxidative and water deficiency stresses induced by cadmium stress.

In short, changes in the accumulation of amino acids as precursors of proteins and secondary metabolites and as compatible solutes are the main compatibility responses to avoid the toxicity of Cd in plants. The current results indicated that proline accumulated in the treated shoots of *S. striata* and may recruit as a ROS scavenger and NADP$^+$ regenerator to improve the damages caused by Cd stress; that is consistent with other reports (Soares et al., 2018; Sharmila et al., 2017; Hayat et al., 2012; Signorelli et al., 2014: Gubrelay et al., 2013; Signorelli, 2016). Sharma and Dietz (2006) stated that Asp could operate as ligands for Cd, Pb, and Zn, and an asparagine-HMs complex may alleviate HMs stress; this is consistent with the current results. Zafari et al. (2016) suggested that the reason for the reduced Glu content in *prosopis* treated with Pb is that it feeds into the greater synthesis of Gln, Arg, and Pro, and this agrees with the current results. Ahsan et al. (2012) (reported that the enhancement of Gly may participate in the biosynthesis of phytochelatin as the main biomarker in HMs stress, and *S. striata* may use this mechanism under Cd stress. The changed level of Ser could also be attributed to the biosynthesis of Gly and Cys during the term of the stress (Gallardo et al., 2003; Seki et al., 2007). L-Histidine as an amino acid contributes to the antioxidant activity by playing a substantial role as an ROS scavenger and an inhibitor of lipid peroxidation in the stress alleviation of plants. Therefore, the increased Ser and L-Histidine levels caused by Cd in the treated seedlings can be an important mechanism against stress (Table 2). Generally, changing the amino acid anabolism by Cd is necessary for confronting *S. striata* shoots under cd; this result is consistent with those of previous studies (Zafari et al., 2016).

The PCA of data clearly indicated that there were significant differences among the plants treated by Cd and the control (Fig. 10A). Sun et al. (2017) and Zafari et al. (2017) revealed a positive correlation

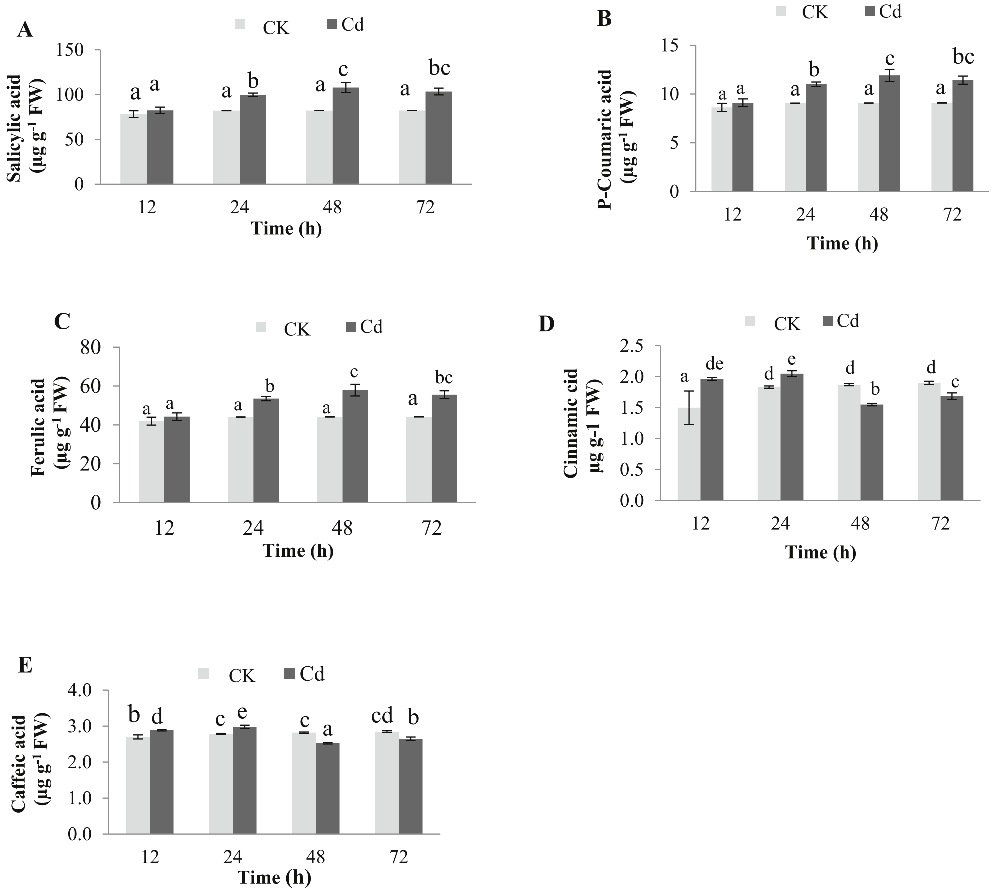

Fig. 7. Contents of phenolic acids in *S. striata* exposed to Cd at time course: salicylic acid (A), caffeic acid (B), p-coumaric acid (C), cinnamic acid (D), and ferulic acid (E) in *S. striata*. Data is reported as mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

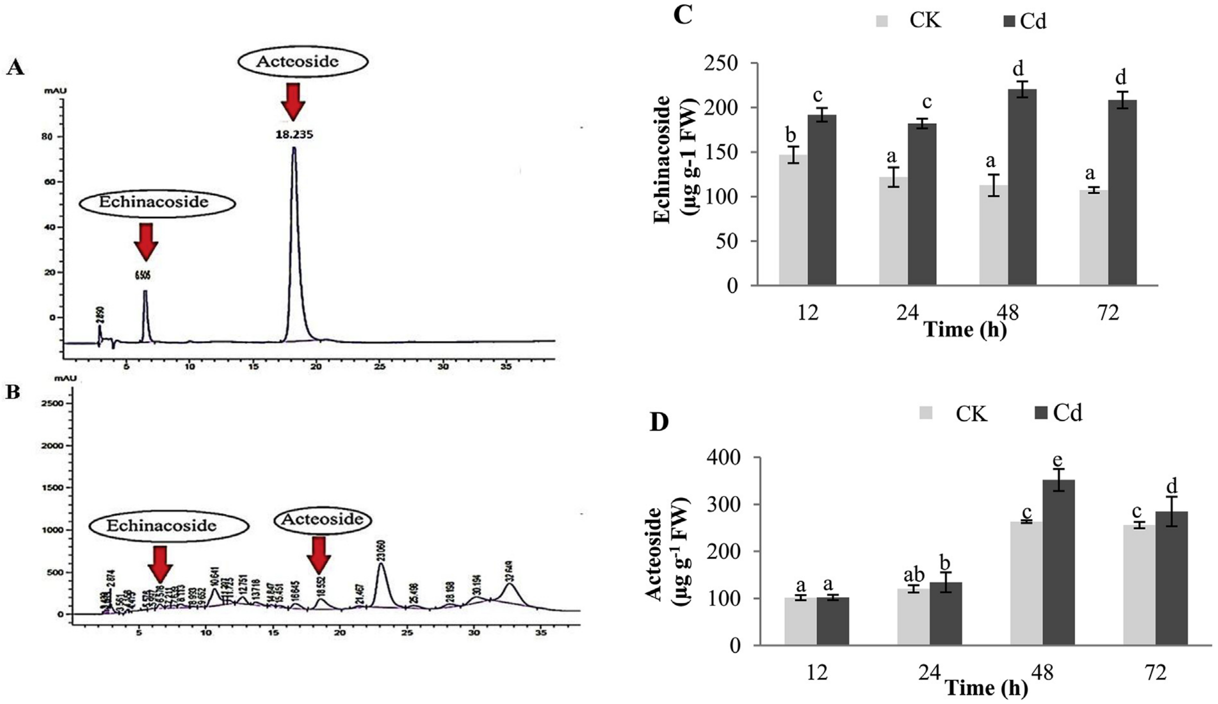

Fig. 8. HPLC chromatogram of phenylethanoid glycosides: (A) standard; (B) shoot samples of *S. striata*, (C) level of echinacoside, and (D) level of acteoside in *S. striata* exposed to Cd at time course. Data is reported as mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

Table 3

Different flavonoid contents of S. striata shoots under different concentrations of Cd. The values within each column are mean ± SD of three replicates. Different superscript letters on the data show statistically significant differences at $p \leq 0.05$.

Flavonoids (μgg^{-1} FW)	CK				Cd			
	12h	24h	48h	72h	12h	24h	48h	72h
Catechin	7.7 ± 0.2^a	8.2 ± 0.2^a	9.5 ± 0.3^b	9.5 ± 0.42^b	8.07 ± 0.34^a	9.11 ± 0.39^b	10.6 ± 0.65^c	10.2 ± 0.14^c
Luteolin	0.11 ± 0.02^a	0.13 ± 0.01^a	0.15 ± 0.01^a	0.17 ± 0.02	0.26 ± 0.06^b	0.25 ± 0.04^b	0.35 ± 0.01^c	0.29 ± 0.02d^b
Kampferol	1.68 ± 0.18^a	1.96 ± 0.05^b	2.08 ± 0.05^b	2.16 ± 0.08	2.06 ± 0.2^b	2.61 ± 0.14^c	3.01 ± 0.17^d	2.47 ± 0.04^c
Genistein	11.9 ± 2.5^a	22.5 ± 0.7^c	23.4 ± 1.3^c	21.3 ± 0.3^c	17.3 ± 1.02^b	22.8 ± 1.61^c	29.3 ± 0.76^d	30.5 ± 1.15^d
Diosmin	16.1 ± 0.57^a	27.9 ± 0.17^b	28.2 ± 0.17^b	27.2 ± 2^b	18.01 ± 0.22	31.01 ± 1.47^c	36.2 ± 0.19^d	31.85 ± 3.23^c
Daidzein	10.17 ± 0.89	10.19 ± 0.07	10.35 ± 0.07	10.46 ± 0.1	12.15 ± 2.5^b	12.81 ± 2.5^b	13.62 ± 2.6^c	13.6 ± 4.8^c
Myricetin	12.3 ± 0.26^b	12.7 ± 0.08^b	12.9 ± 0.08^b	11.3 ± 0.18	13.2 ± 0.1^c	13.4 ± 0.1^c	15.2 ± 1.33^e	14.13 ± 0.23^d
Naringenin	1.01 ± 0.21^a	1.04 ± 0.06^a	1.45 ± 0.07^b	1.3 ± 0.04^b	1.7 ± 0.05^c	1.83 ± 0.05^c	0.9 ± 0.07^a	1.21 ± 0.04^b

14.13 ± 0.23^d.

Table 4

Pearson coefficient shows linear correlations among PhGs, different enzymes, H_2O_2, and ABA during the period of Cd treatment in *S. striata* shoots.

	Echinacoside	ABA	SA	MDA	Acteoside	H_2o_2 Content	PAL Activity
ABA	0.849**						
SA	0.792*	0.992**					
MDA	0.160	0.559	0.652				
Acteoside	0.230	0.751*	0.796*	0.470			
H_2o_2 Content	0.796*	0.777*	0.807*	0.549	0.938**		
PAL Activity	0.786*	0.709*	0.757*	0.621	0.964**	0.982**	
TAL activity	0.783*	0.715*	0.735*	0.426	0.903**	0.893**	0.888**

Levels of significance are: **P B 0.01, *P B 0.05.

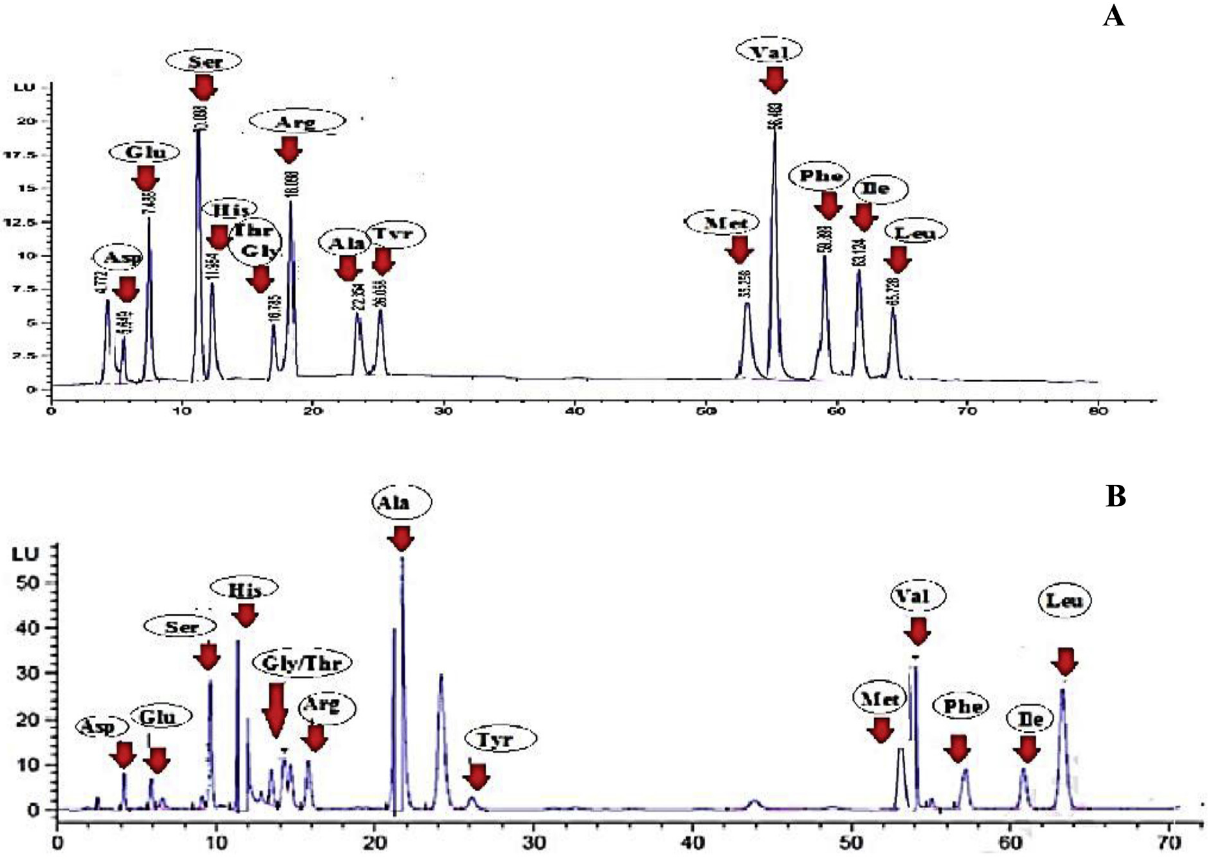

Fig. 9. HPLC chromatogram of amino acids: (A) standard; (B) *S. striata* shoots.

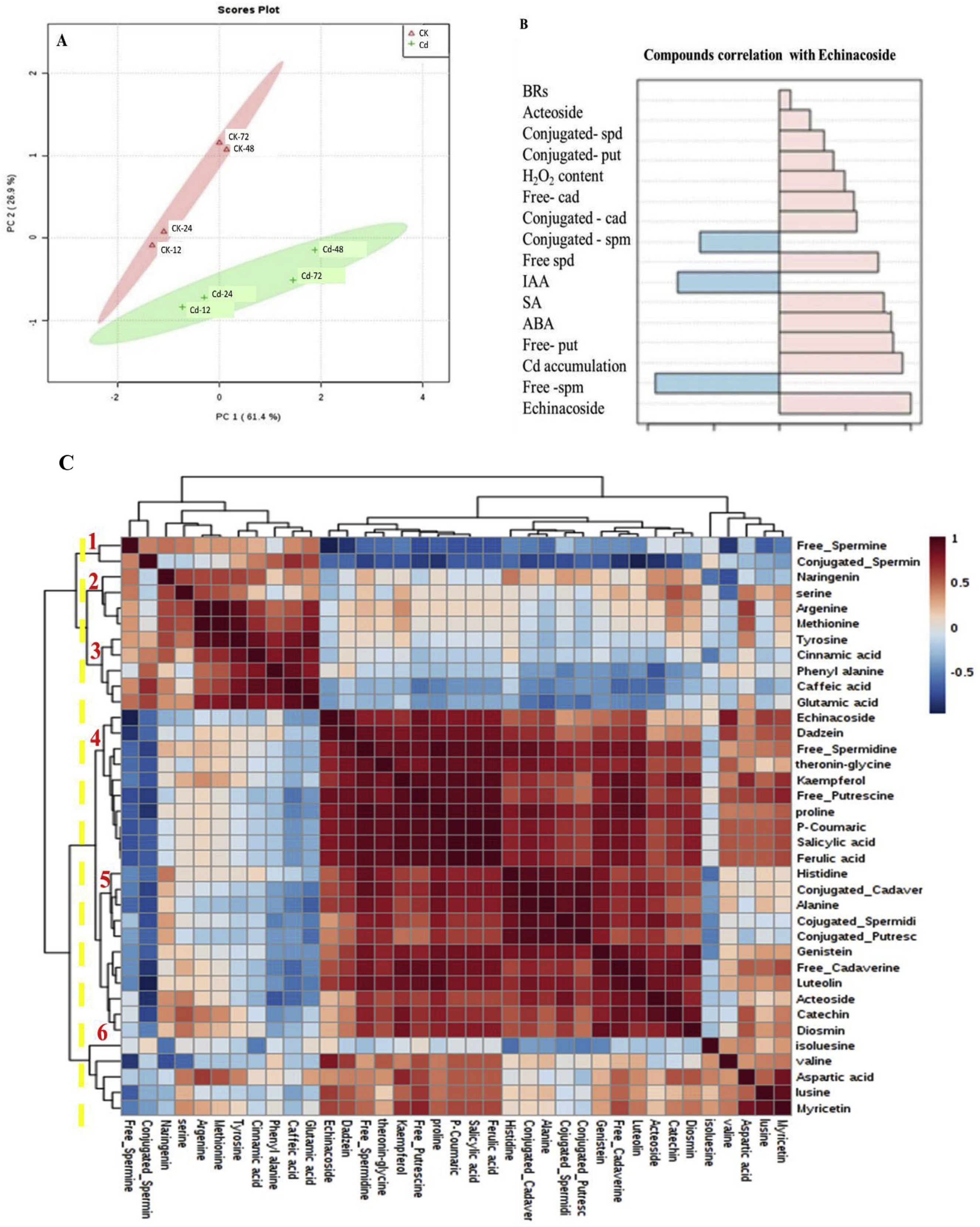

Fig. 10. (A) Score plots of principal component analysis (PCA) show clear segregation of two components. The classification of different metabolites measured in *S. strata* treated with Cd. (B) PatternHunter analysis with Pearson correlation coefficients show that echinacoside are correlated with different compounds of signaling pathways such as PAs, phytohormones, H_2O_2, and PhGs. (C) Hierarchical cluster analysis (HCA) with the Pearson correlation coefficient. Colors in the matrix boxes show the quantification and orientation of the correlations: blue and red demonstrate potent negative and positive correlations, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

between phenolic compounds, ABA, and PAs under abiotic stress. In this research, the PatternHunter profile showed that the echinacoside content as PhGs had a negative correlation with Spm and IAA, while it had a positive correlation with other polyamines and ABA (Fig. 10B). The results of HCA clusters 3, 4, and 5 shown in Fig. 10C and Table 4 indicated that Cd changes the PAs metabolism direction by regulating the levels of PAs, the level of ABA, and the H_2O_2 content. Finally, the collection of these changes increases the PAL and TAL activity, enhances the PhGs (acteoside and echinacoside) and phenolic acids (ferulic acid, salicylic acid and p-coumaric acid), reduces other phenolic acids (caffeic acid and cinnamic acid) and some amino acids like Phe, Tyr, Arg, Met, and Glu as the precursors, respectively, for the biosynthesis of PhGs and PAs to improve Cd stress. Therefore, it seems that *S. striata* can use a complex metabolic network and different signaling pathways to confront Cd stress.

5. Conclusion

S. striata uses a complicated metabolic network to deal with damage caused by Cd stress. It is activated by perceiving HMs through signaling molecules, such as various phytohormones and H_2O_2. The present study revealed that Cd caused changes in the metabolism of PAs that may interfere with the biosynthesis of ABA, H_2O_2 and signaling pathways. These signaling pathways cause fluctuations in amino acids and the accumulation of phenolic compounds, especially PhGs that inhibit lipid peroxidation or operate as antioxidants and osmotic regulators in the face of conflicting demands. A significant challenge for the future would be to identify the connection between these metabolites and the signal molecules in the plants subjected to various stresses.

Author's contribution

This research paper was accomplished in collaboration among all authors. Elham Sadat Beshamgan performed the experiments, analyzed, and interpreted the experimental data and wrote the manuscript. Mohsen Sharifi designed and supervised the study. Fatemeh Zarinkamar assisted us in manuscript evaluation.

Acknowledgments

The authors give their sincere thanks to Tarbiat Modares University (Tehran, Iran) for providing the financial support. The manuscript editing by Native English Edit (United Kingdom) and Hamyarapply Research Institute (Tehran, Iran) is greatly acknowledged.

References

Ahsan, N., Nakamura, T., Komatsu, S., 2012. Differential responses of microsomal proteins and metabolites in two contrasting Cd (Cd)-accumulating soybean cultivars under Cd stress. Amino Acids 42, 317–327.

Ahmadi-Sakha, S., Sharifi, M., Niknam, V., 2016. Bioproduction of phenylethanoid glycosides by plant cell culture of *Scrophularia striata* Boiss from shake-flasks to bioreactor. Plant Cell Tissue Organ 124, 275–281.

Alcázar, R.1., Marco, F., Cuevas, J.C.0, Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F., Altabella, T., 2006. Involvement of polyamines in plant response to abiotic stress. Biotechnol. Lett. 28, 1867–1876.

Ali, M.B., Singh, N., Shohael, A.M., Hahn, E.J., Paek, K.Y., 2006. Phenolic metabolism and lignin synthesis in root suspension cultures of *Panax ginseng* in response to copper stress. Plant Sci. 171, 147–154.

Alipieva, K., Korkina, L.G., Orhan, I.K., Georgiev, M.I., 2014. Verbascoside — a review of its occurrence, (bio) synthesis and pharmacological significance. Biotechnol. Adv. 32, 1065–1076.

Anwar, R., Mattoo, A.K., Handa, A.K., 2015. Polyamine interactions with plant hormones: crosstalk at several levels. Polyamines 267–302.

Barceló, J., Poschenrieder, Ch, 1990. Plant water relations as: affected by heavy metals a review. J. Plant Nutr. 13 (1), 1–37.

Bates, L.S., Waldren, S.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207.

Biermann, M., Bardl, B., Vollstädt, S., Linnemann, J., Knüpfer, U., Seidel, G., Horn, U., 2013. Simultaneous analysis of the non-canonical amino acids norleucine and nor valine in biopharmaceutical-related fermentation processes by a new ultra-high performance liquid chromatography approach. Amino Acids 44, 1225–1231.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bors, W., Langebartels, C., Michel, C., Sandermannjr, H., 1989. Polyamines as radical scavengers and protectants against ozone damage. Phytochemistry 28, 1589–1595.

Bücker-Neto, L., Paiva, A.L.S., Machado, R.D., Arenhart, R.A., MargisPinheiro, M., 2017. Interactions between plant hormones and HMs responses. Genet. Mol. Biol. 40, 373–386.

Camacho- Cristóbal, J.J., Gonzólez-Fontes, A., 2002. Relationship between the organic-nitrogen status and the nitrate content in leaves of wild-type and mutant tobacco plants. J. Plant Nutr. Soil Sci. 165, 347–351.

Cakmak, I., Marschner, H., 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 98, 1222–1227.

Clement, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88, 1707–1719.

Cona, A., Rea, G., Angelini, R., Federico, R., Tavladoraki, P., 2006. Functions of amine oxidases in plant development and defence. Trends Plant Sci. 11, 80–88.

Diao, Q., Song, Y., Shi, D., Qi, H., 2017. Interaction of polyamines, abscisic acid, nitric oxide, and hydrogen peroxide under chilling stress in tomato (*Lycopersicon esculentum Mill.*) seedlings. Front. Plant Sci. 8, 203.

Delavar, K., Ghanati, F., Zare-Maivan, H., Behmanesh, M., 2017. Effects of silicon on the growth of maize seedlings under normal, aluminum, and salinity stress conditions. J. Plant Nutr. 40 (10), 1475–1484.

Di Martino, C., Delfine, S., Pizzuto, R., Loreto, F., Fuggi, A., 2003. Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress. New Phytol. 158, 455–463.

Diaz, J., Bernal, A., Pomar, F., Merino, F., 2001. Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum anuum L*) seedlings in response to copper stress and its relation to lignification. Plant Sci. 161, 179–188.

Emamverdian, A., Ding, Y., Mokhberdoran, F., Xie, Y., 2015. Heavy metal stress and some mechanisms of plant defense response. Sci. World J. 1–18 2015.

Falahi, H., Sharifi, M., zare mayvan, H., Ahmadian chashmi, N., 2018a. Phenylethanoid glycosides accumulation in roots of *Scrophularia striata*as a response to water stress. Environ. Exp. Bot. 147, 13–21.

Falahi, H., Sharifi, M., Ahmadian chashmi, N., zare mayvan, H., 2018b. Water stress alleviation by polyamines and phenolic compounds in *Scrophularia striata* is mediated by NO and H_2O_2. Plant Physiol. Biochem. 130, 139–147.

Gallardo, F., Fu, J., Jing, Z.P., Kirby, E.G., Cánovas, F.M., 2003. Genetic modification of amino acid metabolism in woody plants. Plant Physiol. Biochem. 41, 587–594.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases: I. occurrence in higher plants. Plant Physiol. 59, 309–314.

Groppa, M.D., Tomaro, M.L., Benavides, M.P., 2007. Polyamines and heavy metal stress: the antioxidant behavior of spermine in Cd- and copper-treated Wheat leaves. Biometals 20, 185–195.

Gubrelay, U., Singh, R.K., Kaur, R., Sharma, R., 2013. Effect of heavy metal Cd on some physiological and biochemical parameters of Barley (*Hordeum vulgare L*). Intl. J. Agric. Crop Sci. 5 (22), 2743–2751.

Gupta, D.K., Penea, L.B., Romero-Puertas, M.C., Hernandez, A., Inouhe, M., Sandalio, L.M., 2017. NADPH oxidases differentially regulate ROS metabolism and nutrient uptake under Cd toxicity. Plant Cell Environ. 40 (4), 509–526.

Gupta, K., Sengupta, A., Chakraborty, M., Gupta, B., 2016. Hydrogen peroxide and polyamines act as double edged swords in plant abiotic stress responses. Front. Plant Sci. 7, 1343.

Gudej, J., Tomczyk, M., 2004. Determination of flavonoids, tannins and ellagic acid in leaves from *Rubus L.* species. Arch Pharm. Res. (Seoul) 27, 1114–1119.

Guo, H., Dang, X., Dong, J., 2014. Hydrogen peroxide and nitric oxide are involved in salicylic acid-induced salvianolic acid B production in *Salvia miltiorrhiza* cell cultures. Molecules 19, 5913–5924.

Hamayun, M., Hussain, A., Khan, S.A., Kim, H., Khan, A.,M., Waqas, M., Irshad, M., Iqbal, A., Rehman, G., Jan, S., Lee, I., 2017. Gibberellins producing endophytic fungus *porostereum spadiceum* AGH786 rescues growth of salt affected soybean. Front. Microbiol. 8, 686. https://doi.org/10.3389/fmicb.2017.00686. Article.

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments: a review. Plant Signal. Behav. 7, 1456–1466.

Hossain, M.A., Piyatida, P., Jaime, A., Silva, T.D., Fujita, M., 2012. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. J. Bot. 37 2012.

Hodgins, D.S., 1971. Yeast phenylalanine ammonia-lyase. Purification, properties, and the identification of catalytically essential dehydroalanine. J. Biol. Chem. 246, 2977–2985.

Iannone, M.F., Rosales, E.P., Groppa, M.D., Benavides, M.P., 2013. H_2O_2 involvement in polyamine-induced cell death in tobacco leaf discs. J. Plant Growth Regul. 32, 745–757.

Jiang, Y., Cheng, F., Zhou, Y., Xia, X.J., Mao, W.H., Shi, K., 2012. Hydrogen peroxide functions as a secondary messenger for brassinosteroids-induced CO_2 assimilation and carbohydrate metabolism in *Cucumis sativus*. J. Zhejiang Univ. B 13, 811–823.

Jiang, Y., Joyce, D.C., 2003. ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. Plant Growth Regul. 39 (2), 171–174.

Jamalian, S., Gholami, M., Esna-Ashari, M., 2013. Abscisic acid-mediated leaf phenolic compounds, plant growth and yield is strawberry under different salt stress regimes. Theor. Exp. Plant Physiol. 25 (4), 291–299.

Kamalipourazad, M., Sharifi, M., Zare Maivan, H., Behmanesh, M., Ahmadian Chashmi, N., 2016. Induction of aromatic amino acids and phenylpropanoid compounds in *Scrophularia striata* Boiss. Cell culture in response to chitosan-induced oxidative stress. Plant Physiol. Biochem. 107, 374–384.

Karcz, W., Kurtyka, R., 2007. Effect of Cd on growth, proton extrusion and membrane potential in maize coleoptile segments. Biol. Plant. 51, 713–719.

Keinänen, M., Oldham, N.J., Baldwin, I.T., 2001. Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. J. Agric. Food Chem. 49, 3553–3558.

Khanpour-Ardestani, N., Sharifi, M., Behmanesh, M., 2015. Establishment of callus and cell suspension culture of *Scrophularia striata Boiss* an in vitro approach for acteoside production. Cytotechnology 67 (3), 475–485.

Kovacik, J., Klejdus, B., Hedbavny, J., Backor, M., 2010. Effect of copper and salicylic acid on phenolic metabolites and free amino acids in *Scenedesmus quadricauda* (Chlorophyceae). Plant Sci. 178, 307–311.

Li, S., Jin, H., Zhang, Q., 2016. The effect of exogenous spermidine concentration on polyamine metabolism and salt tolerance in *zoysiagrass (Zoysia japonica steud)* subjected to short-term salinity stress. Front. Plant Sci. 7 (1221), 1–13.

Lin, J.S., Wang, G.X., 2002. Doubled CO could improve the drought tolerance better is sensitive cultivars than in tolerant cultivars in spring wheat. Plant Sci. 163 (3), 627–637.

Liu, H.F., Dong, B.H., Zhang, Y.Y., Liu, Z.P., Liu, Y.L., 2004. Relationship between osmotic stress and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings. Plant Sci. 166, 1261–1267.

Liu, C., Zhao, L., Yu, G., 2011. The dominant glutamic acid metabolic flux to produce γ-amino butyric acid over proline in *Nicotiana tabacum* leaves under water stress relates to its significant role in antioxidant activity. J. Integr. Plant Biol. 53 (8), 608–618.

Lomozik, L., Gasowska, A., Bregier-Jarzebowska, R., Jastrzab, R., 2005. Coordination chemistry of polyamines and their interactions in ternary systems including metal ions, nucleosides and nucleotides. Coord. Chem. Rev. 249, 2335–2350.

Meharg, A.A., 1994. Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. Plant Cell Environ. 17 (9), 989–993.

Monsef-Esfahani, H.R., Hajiaghaee, R., Shahverdi, A.R., Khorramizadeh, M.R., Amini, M., 2010. Flavonoids, cinnamic acid and phenylpropanoid from aerial parts of *Scrophularia striata*. Pharm. Biol. 48, 333–336.

Murthy, H.N., Kim, Y.S., Park, S.Y., Paek, K.Y., 2014. Biotechnological production of caffeic acid derivatives from cell and organ cultures of *Echinacea* species. Appl. Microbiol. Biotechnol. 98 (18), 7707–7717.

Owen, R.W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B., Bartsch, H., 2003. Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined *olive drupes*. Food Chem. Toxicol. 41, 703–717.

Pál, M., Tajti, J., Szalai, G., Peeva, V., Végh, B., Janda, T., 2018. Interaction of polyamines, abscisic acid and proline under osmotic stress in the leaves of wheat. Plants. Sci. Rep. 8, 12839.

Pála, M., Csávásb, G., Szalaia, G., Tímeaa, O., Khalilc, R., Yordanovad, R., Gella, G., Birinyia, Z., Németha, E., T, Jandaa, T., 2017. Polyamines may influence phytochelatin synthesis during Cd stress in rice. J. Hazard Mater. 340, 272–280 HAZMAT 18708.

Razifah, M.R., Mamat, A.S., Adzemi, M.A., Nor-Shariah, S., 2014. Effect of seed cluster and gibberellic acid (GA3) on the gender development of *salacca edulis Reinw*. (Snake fruit) grown on BRIS soil. J. Biol. Agric. Healthc. 4 (9), 15–20.

Rodziewicz, P., Swarcewicz, B., Chmielewska, K., Wojakowska, A., Stobiecki, M., 2014. Influence of abiotic stresses on plant proteome and metabolome changes. Acta Physiol. Plant. 36, 1–19.

Sabine, G., Pierre-Henri, D., Oliver, T., Gerhard, W., Lucien, B., 2004. Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Mol. Biol. 54, 817–835.

Sadeghnezhad, E., Sharifi, M., Zare-Maivan, H., 2016. Profiling of acidic (amino and phenolic acids) and phenylpropanoids production in response to methyl jasmonate-induced oxidative stress in *Scrophularia striata*. Planta 244, 75–85.

Sandalio, L.M., Dalurzo, H.C., Gomez, M., Romero-Puertas, M.C., Del Rio, L.A., 2001. Cd-induced changes in the growth and oxidative metabolism of pea plants. J. Exp. Bot. 52, 2115–2126.

Seki, M., Umezawa, T., Urano, K., Shinozaki, K., 2007. Regulatory metabolic networks in drought stress responses. Curr. Opin. Plant Biol. 10, 296–302.

Sharma, R., Rajam, M.V., 1995. Spatial and temporal changes in endogenous polyamine levels associated with osmotic embryogenesis from different hypocotyls segments of eggplant (*Solanum melongena L.*). J. Plant Physiol. 146, 658–664.

Sharma, S.S., Dietz, K.J., 2006. The significance of amino acids and amino acid derived molecules in plant responses and adaptation to heavy metal stress. Exp. Bot. 57 (4), 711–726.

Sharmila, P., Kumari, P.K., Singh, K., Prasad, N.V.S.R.K., Pardha-Saradhi, P., 2017. Cd toxicity induced proline accumulation is coupled to iron depletion. Protoplasma 254, 763–770.

Singh, H.P., Batish, D.R., Kaur, G., Arora, K., Kohli, R.K., 2008. Nitric oxide (as sodium nitroprusside) supplementation ameliorates Cd toxicity in hydroponically grown wheat roots. Environ. Exp. Bot. 63, 158–167.

Signorelli, S., 2016. The fermentation analogy: a point of view for understanding the intriguing role of proline accumulation in stressed plants. Front. Plant Sci. 7, 1339.

Signorelli, S., Coitiño, E.L., Borsani, O., Monza, J., 2014. Molecular mechanisms for the reaction between OH radicals and proline: insights on the role as reactive oxygen species scavenger in plant stress. J. Phys. Chem. B 118, 37–47.

Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2018. Plants facing oxidative challenges - a little help from the antioxidant networks. Environ. Exp. Bot. 3659.

Smolders, E., 2001. Cd uptake by plants. Int. J. Occup. Med. Environ. Health 14 (2), 177–183.

Stewart, R.R.C., bewley, D., 1980. Lipid peroxidation associated with accelerated aging of *Soybean Axes*. Plant Physiol. 65, 245–248.

Sun, R., Cheng, G., Li1, Q., He, Y., Wang, Y., Lan, Y., Li1, S., Zhu1, Y., Song, W., Xiao-Di Cui1, X., Chen, W., Wang, Jun, 2017. Light-induced variation in phenolic compounds in cabernet sauvignon grapes (*Vitis vinifera L.*) involves extensive transcriptome reprogramming of biosynthetic enzymes, transcription factors, and phytohormonal regulators. Front. Plant Sci. 8, 547.

Sytar, O., Kumari, P., Yadav, S., Brestic, M., Rastogi, A., 2018. Phytohormone priming:regulator for heavy metal stress in plants. J. Plant Growth Regul. 38, 739–752 Published online.

Velikova, V., Yordanov, I., Edreva, A., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Plant Sci. 151, 59–66.

Vertuani, S., Beghelli, E., Scalambra, E., Malisardi, G., Copetti, S., Toso, R.D., Baldisserotto, A., Manfredini, S., 2011. Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. Molecules 16, 7068–7080.

Vitti, A., Nuzzaci, M., Scopa, A., Tataranni, G., Tamburrino, I., Sofo, A., 2014. Hormonal response and root architecture in *Arabidopsis thaliana* subjected to heavy metals. Int. J. Plant Biol. 5, 5226.

Wang, J.W., Zheng, L.P., Wu, J.Y., Tan, R.X., 2006. Involvement of nitric oxide in oxidative burst, phenylalanine ammonia-lyase activation and Taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. Nitric Oxide 15 (4), 351–358.

Wu, M., Luo, Q., Zhao, Y., Long, Y., Liu, S., Pan1, Y., 2018. Physiological and biochemical mechanisms preventing Cd Toxicity in the new hyperaccumulator *Abelmoschus manihot*. J. Plant Growth Regul. 37, 709–718.

Yang, J.H., Hu, J.P., Rena, K., Du, N.S., 2009. Structure-activity relationships of phenylethanoid glycosides in plants of *Cistanche salsa* on antioxidative activity. J. Chin. Med. Mater. 32, 1067–1069.

Zafari, S., Sharifi, M., Ahmadian chashmi, N., Mur, L., 2016. Modulation of Pb-induced stress in *Prosopis* shoots through an interconnected network of signaling molecules, phenolic compounds and amino acids. Plant Physiol. Biochem. 99, 11–20.

Zafari, S., Sharifi, M., Ahmadian Chashmi, N., 2017. Nitric oxide production shifts metabolic pathways toward lignification to alleviate Pb stress in *Prosopis farcta*. Environ. Exp. Bot. 141, 41–49.