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The endophyte Stenotrophomonas maltophilia EPS modulates endogenous antioxidant defense in safflower (Carthamus tinctorius L.) under cadmium stress

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Abstract

Cadmium (Cd) pollution in agricultural soils induces oxidative stress in plants that in turn is the foremost limiting factor for agricultural productivity. In past few decades, metal binding ability of microbes is of great interest as an emerging environmentally friendly technology that can be exploited to alleviate metal stress in plants. Considering these, in the present study an endophytic bacterium strain EPS has been isolated from the roots of common bean. The 16S rRNA sequence revealed its identity closely similar to *Stenotrophomonas maltophilia*. The strain showed tolerance to Cd stress up to 200 mg L^{-1} Cd^{2+}. The inoculation of strain EPS in safflower seeds significantly enhanced the antioxidant defense of plants under Cd-stress conditions throught increasing the levels of antioxidant molecules like phenolics, flavonoids and carotenoids as well as improving the activities of the antioxidative enzymes including guaiacol peroxidase (POX), ascorbate peroxidase (APX) and superoxide dismutase (SOD). The output of this study is that strain EPS inoculation mitigates Cd-induced oxidative damage and consequently strain EPS may be beneficial, especially in Cd-contaminated crop fields.

Introduction

Over wide world, heavy metals accumulation in agricultural soils is a serious problem threats crop production (He et al. 2015; Rizwan et al. 2016a). Heavy metals are among the most main causes of environmental stresses. Overaccumulation of heavy metals in the soils causes dangerous phytotoxicity that can store in plants and easy transmit through the food chain resulting in negatively impact on human and animal health (Fryzova et al. 2017). Hyperlevels of heavy metals alter normal plant functions and metabolism causing repression of vital processes such as photosynthesis, respiration, and enzymatic activities (Hossain et al. 2012). On the other hand, high levels of heavy metals can induce excess generation of reactive oxygen species (ROS) as well as cytotoxic compounds, leading to oxidative stress via demolishing the equilibrium between prooxidants and antioxidants within the plant cells (Zengin and Munzuroglu 2005; Hossain et al. 2012; Sytar et al. 2013). This results in cellular damage as well as decreasing plant productivity (Raja et al. 2017).

Cadmium (Cd) is non-essential element for living organisms, and it is highly toxic to plants and animals even at very low concentrations (Dai et al. 2012). Cadmium mainly originates from industrial processes and phosphate fertilizers, releases into agricultural lands and has long biological half-life (Gill et al. 2013). In plants, the exposure to cadmium induces numerous hazards physiological and growth changes as well as oxidative stress by generating ROS, that react with lipids, proteins, pigments and nucleic acids in the plant cell, leading to cellular damage and consequently decreasing productivity (Romero-Puertas et al. 2004). Furthermore, cadmium also can transfer into human via food chain and can result in kidney, bone and lung diseases (Bernard 2008).

Traditional remediation techniques for heavy metal-contaminated soils are expensive and destructive to environment (Meagher 2000). Therefore, scientists and engineers intensify their efforts to find cost effective and safe technologies (Boyajian and Carreira 1997; Wasay et al. 1998). Most of plant

associated microorganisms are metal resistant, whose application in heavy metal contaminated soils can improve metal immobilization in soils and plant biomass (Ma et al. 2011; Ma et al. 2016).

Despite that the applications of some potential bacterial strains to remediate soils contaminated with heavy metals have been reported, it is urgent to search a new microbial resources that can be used efficiently in heavy metals remediation (Tirry et al. 2018).

Safflower (*Carthamus tinctorius* L.) is herbaceous annual plant belongs to family Asteraceae. It is cultivated from prehistoric times throughout many areas with temperate climates over the world including southern Asia, China, India, Iran and Egypt (Dordas and Sioulas 2008; Weiss 2000). Safflower is commercially used for vegetable oil extraction, as well as in the traditional medicine for the treatment of rheumatism, paralysis, vitiligo, psoriasis and mouth ulcers (Delshad et al. 2018). Moreover, it has numerous pharmacological activities i.e, antioxidant, analgesic, anti-inflammatory and antidiabetic activities (Asgarpanah and Kazemivash 2013).

It has been reported that safflower plants can accumulate high levels of Cd in their roots and leaves (Shi et al. 2010; Namdjoyan et al. 2011). Although, some scientific data exists on the antioxidant defense mechanisms in response to cadmium stress in safflower cultivars (Namdjoyan et al. 2011), to our knowledge, there is no study dealing with alleviation of Cd- induced oxidative stress in safflower by using bacteria. Therefore, the present work was designed to investigate the potentiality of the endophytic bacterium *Stenotrophomonas maltophilia* strain EPS to alleviate Cd-induced oxidative stress in safflower plants.

Materials And Methods

Isolation and identification of endophytic bacteria

Healthy fresh roots of common bean plants (*Vigna unguiculata* L.) were collected in sterile plastic bags from Aswan University greenhouse. Immediately nodules were surface- sterilized using 70 % ethanol (30 sec) followed by 5 % sodium hypochlorite (3 min) and then washed three times with sterilized distilled water (Vincent 1970). Under aseptic conditions, nodules were crushed in a test tube contained one mL of sterilized saline solution. Loopful of the obtained suspension was streaked on the surface of tryptic soy agar and nutrient agar plates. Plates were incubated at 37 ^{o}C for 72 h for the appearance of colonies.

The ribosomal (16S rRNA) gene of the selected strain was amplified using 27F and 1492R primers (Frank et al. 2008) in Applied Biotechnology lab at Ismailia, Egypt. PCR product was sent to SolGent Co., Ltd., South Korea for sequencing. Then, the similarity of the obtained sequence was evaluated based on BLAST outputs using NCBI reference sequence database. Neighbor-joining phylogenetic tree of the strain was constructed using MEGA X 10.1.7 software (Kumar et al. 2018).

Cd tolerance of the strain

Maximum tolerable concentration (MTC) of cadmium by the strain was determined according to the method of Vashishth and Khanna (2015), with slight modification. Briefly, 10 mL of yeast extract-mannitol broth (YMB) in glass tubes was supplemented with different concentrations of $CdCl_2$ i.e., 0 (control), 50, 100, 150, 200, 250 and 300 mg L^{-1}. 10 mL of YMB without $CdCl_2$ was used as control. Tubes were inoculated with 1 mL of inoculum (10^7 CFUmL^{-1}), and incubated for 48 h at 28 ^oC and 150 rpm. The optical density (OD) was measured at 600 nm. The highest concentration of cadmium ($CdCl_2$) that allowed visible bacterial growth after 48 h of incubation was considered as the maximum tolerable concentration (MTC).

Evaluation of Cd- adsorption potential of the strain

The ability of the whole culture of the present strain (cells and supernatant) for adsorping cadmium was evaluated using the method of Du et al. (2016 b). 100 mL of the whole culture broth contained 50 and 100 mg L^{-1} of $CdCl_2$ was shaken at 120 rpm and 28 ^oC for 24 h. Cells were then removed by centrifugation. Concentration of the residual, non-adsorbed metal ion in the solution was estimated by atomic absorption spectrophotometer (Thermo Scientific™ iCE™ 3000). Experiment was performed in triplicate. The adsorption efficiency (%) was calculated according to the following formula:

$$\text{Adsorption efficiency } (\%) = \frac{[\text{Cdi}-\text{Cde}]}{\text{Cdi}} \times 100$$

where Cdi and Cde are the concentration of initial and equilibrium Cd ion in the solution (mgL^{-1}) respectively.

Seed inoculation and pot experiment

Seeds of safflower (cv. Giza-1) were obtained from Faculty of Agriculture and Natural Resources, Aswan University. Seeds were surface sterilized with 70 % ethanol for 3 min, rinsed three times with sterilized distilled water. Seeds thereafter were soaked in a freshly prepared bacterial suspension (1×10^8 CFU mL^{-1}) for 1 h, and left to dry before sowing. Seeds used for control were soaked in sterilized distilled water.

Seeds were sown in pots containing an autoclaved mixture of clay and sand (1:1 w/w), with maintaining field capacity at 90 %. Pots were kept under normal climatic conditions. After three weeks of sowing, five homogenous plants in each pot were subjected to three Cd treatments including 0 (control), 50 and 100 mg L^{-1} of $CdCl_2$. After three weeks of cadmium exposure, healthy expanded leaf samples were collected, frozen and then used for measuring the defensive non-enzymatic and enzymatic antioxidant activities. The experiment was repeated twice.

Estimation of hydrogen peroxide (H_2O_2) content

To evaluate the H_2O_2 content of the leaves, the method of Velikova et al. (2000) was followed. One gram of fresh leaves was homogenated in 10 mL trichloroacetic acid (0.1 %) using a mortar and pestle, and

then centrifuged. To 0.5 mL of the supernatant, 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI were added. The mixture was vortexed, and the absorbance was read at 390 nm. A calibration curve of different concentrations (µmol) of 30 % (v/v) H_2O_2 was used as standard.

Assessments of non-enzymatic antioxidants

Total phenolics

The Folin-Ciocalteu assay described by Singleton et al. (1999) was followed to determine the total phenolic compounds in the leaves extracts. Absorbance was read at 700 nm, and the content of total phenolics was expressed as mg gallic acid equivalents per gram of fresh weight using gallic acid as a reference.

Total flavonoids

Aluminum chloride method according to Chang et al. (2002) was used for quantifying the total contents of flavonoids of the extracts. The absorbance was recorded at wavelength 510 nm. The concentration of flavonoids was calculated from quercetin calibration curve as mg quercetin equivalents per gram of fresh weight.

Total carotenoids

Pigments were extracted from fresh leaves and their contents were estimated as described by Lichtenthaler and Wellburn (1983). One gram of fresh leaves was macerated in 80 % acetone, the supernatant was filtered and makeup to 50 mL with the solvent. The total contents of chlorophylls a (Chl a), chlorophylls b (Chl b) and carotenoids were measured by reading the absorbance at wavelengths 646, 663 and 440.5 nm respectively. The content of each pigment was calculated in mg per gram of fresh weight using the following equations:

Chl a (mg g f.wt.$^{-1}$) = $(12.21\times A_{663}) - (2.81\times A_{646})$

Chl b (mg g f.wt.$^{-1}$) = $(20.13\times A_{646}) – (5.03\times A_{663})$

Carotenoids (mg g f.wt.$^{-1}$) = $(4.69\times A_{440.5}) - 0.268\times$ (Chla + Chlb)

Total antioxidant capacity

Total antioxidant capacity of the ethanolic extracts of the leaves was measured per gram of fresh weight as mg ascorbic acid equivalents using ascorbic acid standard curve, according to phosphomolybdnum assay (Prieto et al. 1999).

Assessments of enzymatic antioxidants

Antioxidant enzymes were extracted from fresh leaves according to Cavalcanti et al. (2004) with slight modification. One gram of fresh leaves was homogenized using a mortar in 10 mL of extraction buffer containing 0.2 M of potassium phosphate buffer (pH 7.2), 0.1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride as proteinase inhibitor. The homogenate was filtered. The obtained filtrate was used for enzymatic assays.

Catalase (CAT) activity

Catalase activity was estimated by the method of Kato and Shimizu (1987). To 3 mL of the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2, 100 μl of enzymatic extract was added. The decrease in H_2O_2 was followed as decline in optical density at 240 nm. Catalase activity was calculated with the extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1}), and expressed as 1μmol of H_2O_2 decomposed per minute under assay conditions.

Guaiacol peroxidase (POX) activity

The activity of guaiacol peroxidase enzyme was determined following the method of Kim and Yoo (1996). Briefly, the reaction mixture contained 0.2 mL of enzyme extract, 0.8 mL of phosphate buffer (0.2 M, pH 7.2), 1 mL of guaiacol (15 mM) and 1 mL of hydrogen peroxide (3 mM) was incubated for 10 min at 30 °C. Reaction was terminated using 0.5 mL of H_2SO_4 (5 %), and the absorbance was read at 470 nm. POX activity was calculated using the extinction coefficient of oxidation product (tetraguaiacol), (ε470= 26.6 mM cm^{-1}) as follow:

U/mL = [Change in absorbance min^{-1}× Reaction mixture volume (mL) × Dilution factor]/ [ε470× Enzyme extract volume (mL)]

Ascorbate peroxidase (APX) activity

Ascorbate peroxidase activity was evaluated according to Senthilkumar et al. (2021). To 0.8 mL of a reaction mixture contained potassium phosphate buffer (50 mM), ascorbic acid (0.5 mM), H_2O_2 (1.0 mM) and EDTA (0.1 mM), 0.2 mL of the enzyme extract was added. After 30 s the decrease in absorbance at 290 nm was followed up to 60 s with an interval of 15 s. One unit of enzyme activity was expressed as the amount of enzyme required to oxidize 1 μmoL of ascorbic acid per minute with absorbance coefficient 2.8 mM cm at 290 nm.

Superoxide dismutase (SOD) activity

Superoxide dismutase activity was estimated according to Van Rossun et al. (1997). Three mL of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 50 μM nitroblue tetrazolium (NBT), 10 μM riboflavin, 12 mM L-methionine and 100 μl of crude extract. Tubes contained the same reaction mixture without enzyme extract used as control. The tubes were placed under two 15 W fluorescent lamps for 15 min to start the reaction. The absorbance was

recorded at 560 nm. One unit of SOD activity was defined as the amount of enzyme which reduced the absorbance to 50 % compared with the control.

Statistical analysis

Experimental data were compared using one-way analysis of variance (ANOVA) with Tukey's HSD test. Values were expressed as means ± standard errors (SEs) of three biological replicates from two independent experiments. Differences were considered significant if $p \leq 0.05$.

Results And Discussion

Metal toxicity and stress in plants triggering the excessive accumulation of ROS in mitochondria, chloroplast, and peroxisomes (Kochian et al. 2004), resulting in imbalance between the generation of ROS and antioxidant defense systems, that in turn causes oxidative stress to plants (Gupta et al. 2013). Oxidative stress disturbs physiological and metabolic processes of the plants leading to a limitation in plant growth, crop production and yield, and consequently causes massive agricultural loss (Tran and Popova 2013). Recently, plant root-associated bacteria are globally used for the amelioration of crop performance to encounter heavy metal contamination in agricultural soils (Mitra et al. 2018b; He et al. 2020; Ghosh et al. 2022).

Identification of isolated strain

The selected strain was coded as EPS. The NCBI- BLAST analysis of strain EPS sequence showed closely similarity with percent identity of 100 % to *Stenotrophomonas maltophilia* strain IAM 12423 (MN240936) (Fig.1). The 16S rRNA gene sequence of strain EPS was deposited to NCBI GenBank with accession number (OK584766).

Cd tolerance by the strain

Cd concentration was significantly (f-value= 20.759; p-value= 0.00001) effected on the growth of the strain. It was observed that the strain could grow until 200 mg mL^{-1} Cd^{2+}, above this concentration its growth was dramatically declined.

Cd- adsorption potential of the strain

The Cd- adsorption efficiency by strain EPS was measured for the whole culture (cells and supernatant containing EPS). It was observed that the Cd- adsorption efficiency was 95.42 % and 89.96 % in 50 and 100 mg L^{-1} Cd-supplemented culture media by strain EPS. Our findings are in agreement with Liaquat et al. (2020) investigation who reported that *Stenotrophomonas maltophilia* has remarkable Cd- adsorption potential under varying concentrations.

Bacterial inoculation and antioxidant defense of safflower under Cd-stress

Levels of antioxidants within the plant cell tend to fluctuate at cadmium exposure (Ali et al. 2019). The interaction between plants and microorganisms at biochemical, physiological and molecular levels largely directs plant responses toward abiotic stresses (Farrar et al. 2014; Meena et al. 2017). This crucial aspect considered as an interest gateway for scientists to search novel cost-effective and eco-friendly methods to alleviate the abiotic stresses in field grown plants. The application of bacteria to mitigate stress-induced negative impact in plants and their role to make plants tougher toward abiotic stresses have been documented (Panlada et al. 2013; Nadeem et al. 2014; Kaushal and Wani 2016; Rizvi and Khan 2018; Ghosh et al. 2022). In this study, the efect of *S. maltophilia* EPS inoculation on the antioxidant defense of safflower plants (*Carthamus tinctorius* L.) exposed to different levels of Cd was investigated.

Hydrogen peroxide (H_2O_2) content

As a result of many stresses, the cellular concentration of superoxide radicals increases, which are subsequently converted to hydrogen peroxide by mitochondrial manganese superoxide dismutase (Huseynova et al. 2015). Hydrogen peroxide is one of the major contributors causing oxidative damage to plant cell, leading to inhibition of plant growth and development, or to death (Hung et al. 2005; Hossain et al. 2015). In the current work, it was found that the inoculation of safflower with *S. maltophilia* EPS significantly reduced the accumulation of H_2O_2 in the plant leaves under all tested Cd concentrations compared with control plants (Fig.2).

Non-enzymatic antioxidants levels

The non-enzymatic antioxidants like phenolic compounds, flavonoids, ascorbate as well as carotenoids considered as the half of the antioxidant machinery of the plant cell (Das and Roychoudhury 2014). They play a vital role in the plant cell through protecting the cell components from oxidative damage as well as improving plant growth and development via modifying cellular processes such as mitosis, cell elongation, senescence and cell death (de Pinto and De Gara 2004). In the present study, the total phenolics significantly increased (f = 9.11; p = 0.0129) with increasing Cd concentration in strain EPS-inoculated plants comparing to control plants (Fig. 3 a). Phenolics are better and more efficient antioxidant due to the presence of hydroxyl ions in their structure that can chelate metal ions, trap active oxygen species as well as inhibit lipid peroxidation (Michalak 2006; Ali et al. 2019).

Flavonoids are secondary antioxidants with variable phenolic structures that act as reactive oxygen species (ROS) scavengers (Fini et al. 2011; Das and Roychoudhury 2014). Significantly, the inoculation with strain EPS enhanced the total flavonoids content at all the tested Cd concentrations (Fig. 3 b). The content of total flavonoids was increased in inoculated safflower plants by 38.9 and 49.4 % over the control plants at Cd concentrations of 50 and 100 mg L^{-1} respectively.

Carotenoids are lipophilic antioxidants in the plant plastids. They prevent oxidative damage and protect photosynthetic apparatus via detoxifying multiple forms of ROS (McElroy and Kopsell 2009). It was observed that the content of carotenoids of control plants subjected to Cd treatments was remarkably

decreased (Fig.3 c). This agreed with the findings of Shi et al. (2010) who reported that Cd exposure resulted in a decrease of carotenoids contents of safflower plants. This may be attributed to Cd-induced decrease of the photosynthetic rate (Mobin and Khan 2007; Shi et al. 2010). In contrast to the control plants, the contents of carotenoids of inoculated plants were significantly increased (f = 12.375; p= 0.0055) at all tested Cd levels (Fig.3 c). Thus the inoculation with strain EPS significantly improved the quantities of carotenoids antioxidants of safflower plants under Cd stress.

In the present study, it was found that the total antioxidant capacity of the inoculated plants was increased by 78.1 and 34 % over the control plants at 50 and 100 mg L^{-1} Cd respectively (Fig.3 d). Total antioxidant capacity was strongly correlated with total phenolics and total flavonoids contents. The positive correlations between total phenolics, total flavonoids and antioxidant activities were reported by other researchers (Gouveia and Castilho 2011; Contreras-Calderón et al. 2011; Aryal et al. 2019; Santos and MagalhÃes 2020; Butkeviciute et al. 2022).

Enzymatic antioxidants levels

Plants possess multiple antioxidative enzymes including catalase (CAT), guaiacol peroxidase (POX), Ascorbate peroxidase (APX) and superoxide dismutase (SOD) that alleviate oxidative stress and maintain redox homeostasis through catalyting the transformation of ROS into stable nontoxic molecules (Sáez and Están-Capell 2014). In the current study, no significant difference (f = 1.7256; p = 0.218) was observed in CAT activity between inoculated and control plants (Fig.4 a). On the other hand, the inoculation with strain EPS significantly ($p < 0.05$) enhanced the activities of POX, APX and SOD at all the tested Cd levels (Fig. 4 b, c, d). POX activity upon strain EPS inoculation was found to be increased by 20.6 % to 29.6 % under Cd stress compared with the control plants. The activities of APX and SOD of safflower plants were improved upon strain EPS inoculation by 40.5 to 109.9 % and 96.9 to 124.6 % over the control plants under Cd stress respectively.

Conclusion

The output of the present work is that the inoculation of strain EPS into safflower seeds protects plants from Cd-induced oxidative stress through adsorption as well as increasing the levels of antioxidant compounds and enhancing the activities of antioxidant enzymes. This study provides an eco-friendly and safety method for alleviating Cd-stress in plants that can guarantee safe agricultural productivity in Cd-contaminated fields.

Declarations

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CRediT authorship contribution statement

Noura Sh. A. Hagaggi: Conceptualization, Methodology, Software, Formal analysis, Writing- Original draft preparation. **Usama M. Abdul- Raouf:** Methodology, Investigation, Supervision, writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

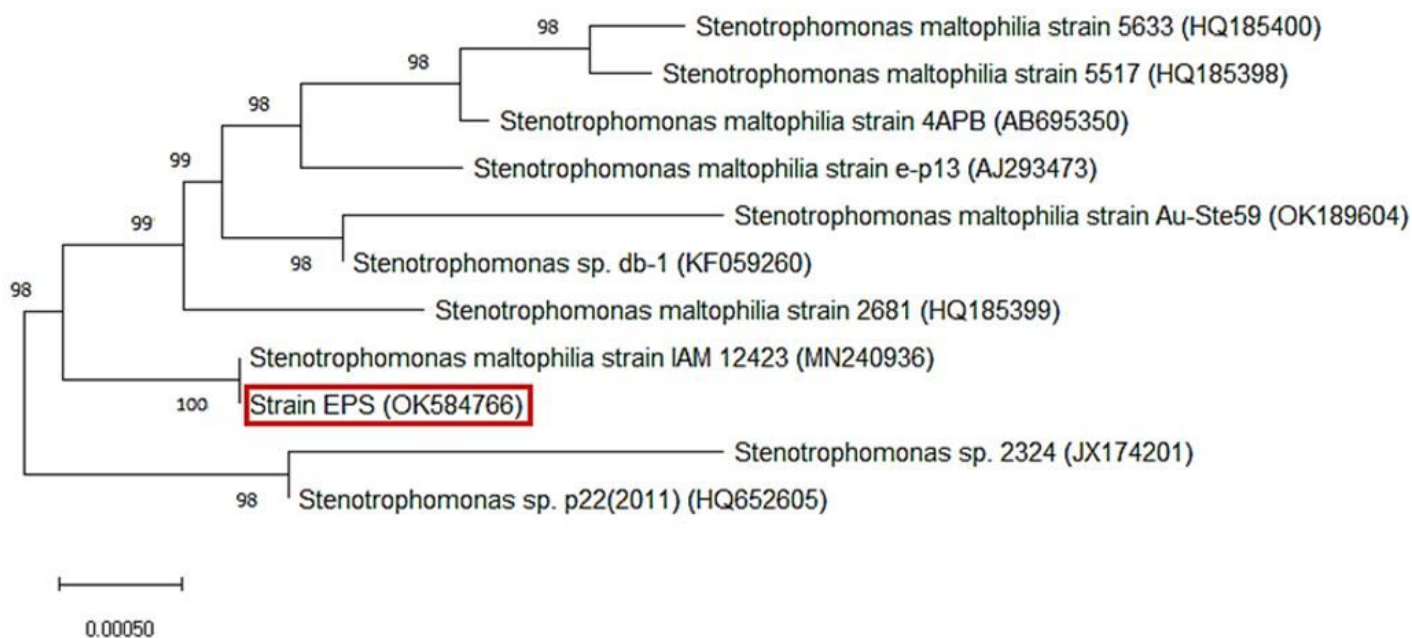

Figure 1

Neighbor-joining phylogenetic tree with 1000 bootstrap replication using MEGA X10.1.7 software displaying the relationship between strain EPS and the closely related members of genus *Stenotrophomonas* derived from NCBI reference sequence database.

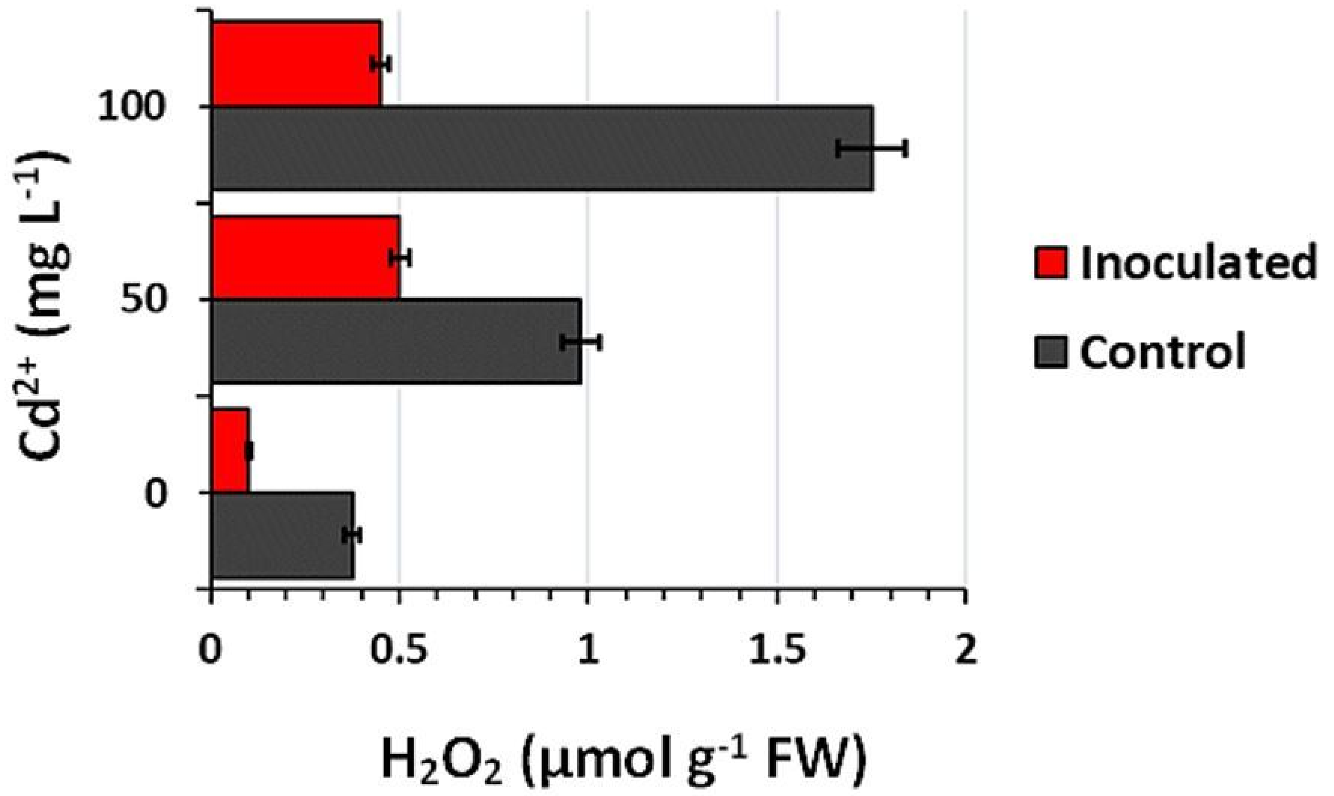

Figure 2

Differences in hydrogen peroxide (H_2O_2) content between inoculated and control safflower plants at different Cd-concentrations.

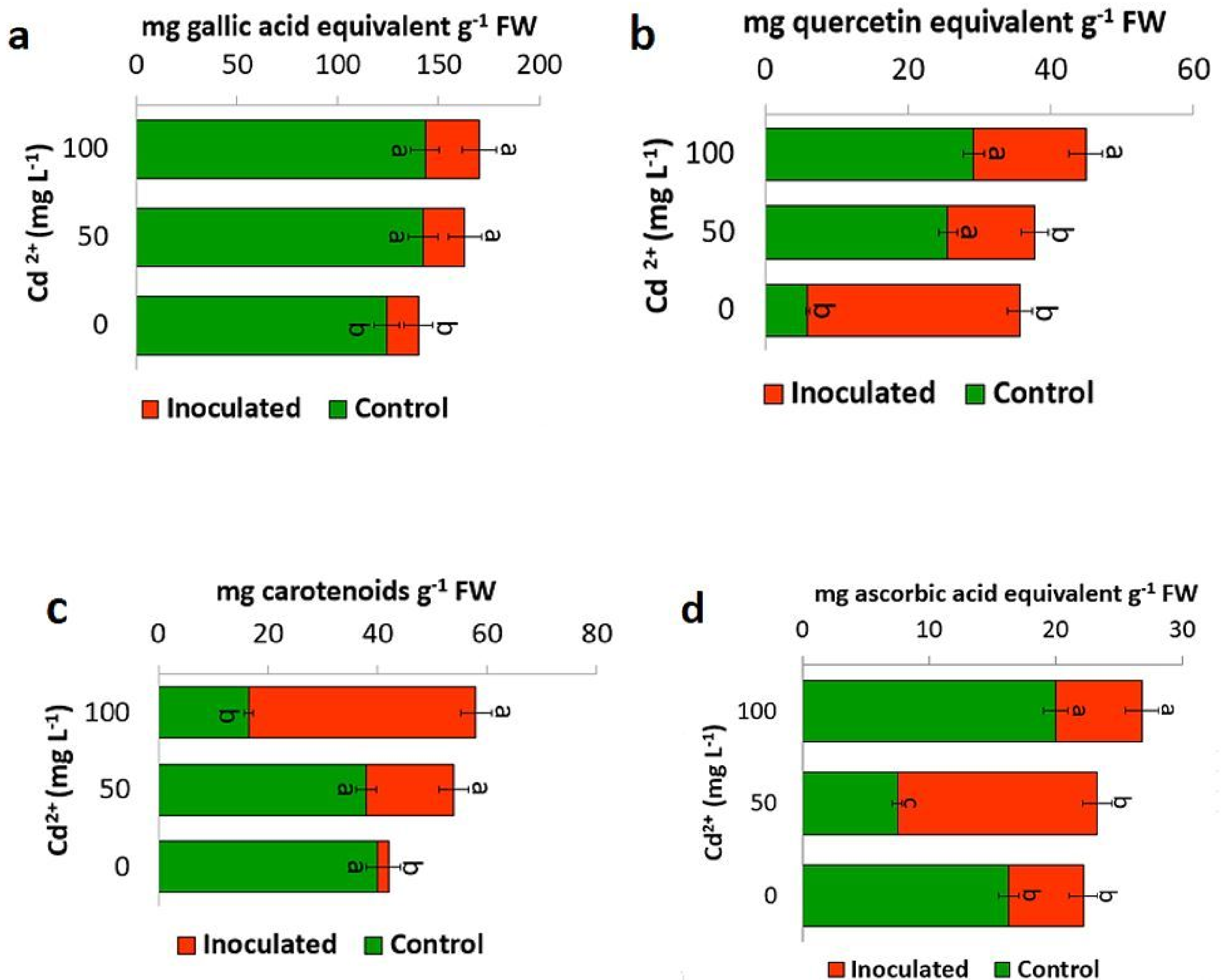

Figure 3

Variation of (a) total phenolics, (b) flavonoids , (c) Carotenoids and (d) total antioxidant capacity (mg/g FW) under different Cd-concentrations. Data are means ± SEs of three independent replicates (n = 3). Different letters indicate significant differences ($p \leq 0.05$) between inoculated and control plants according to Tukey's HSD test.

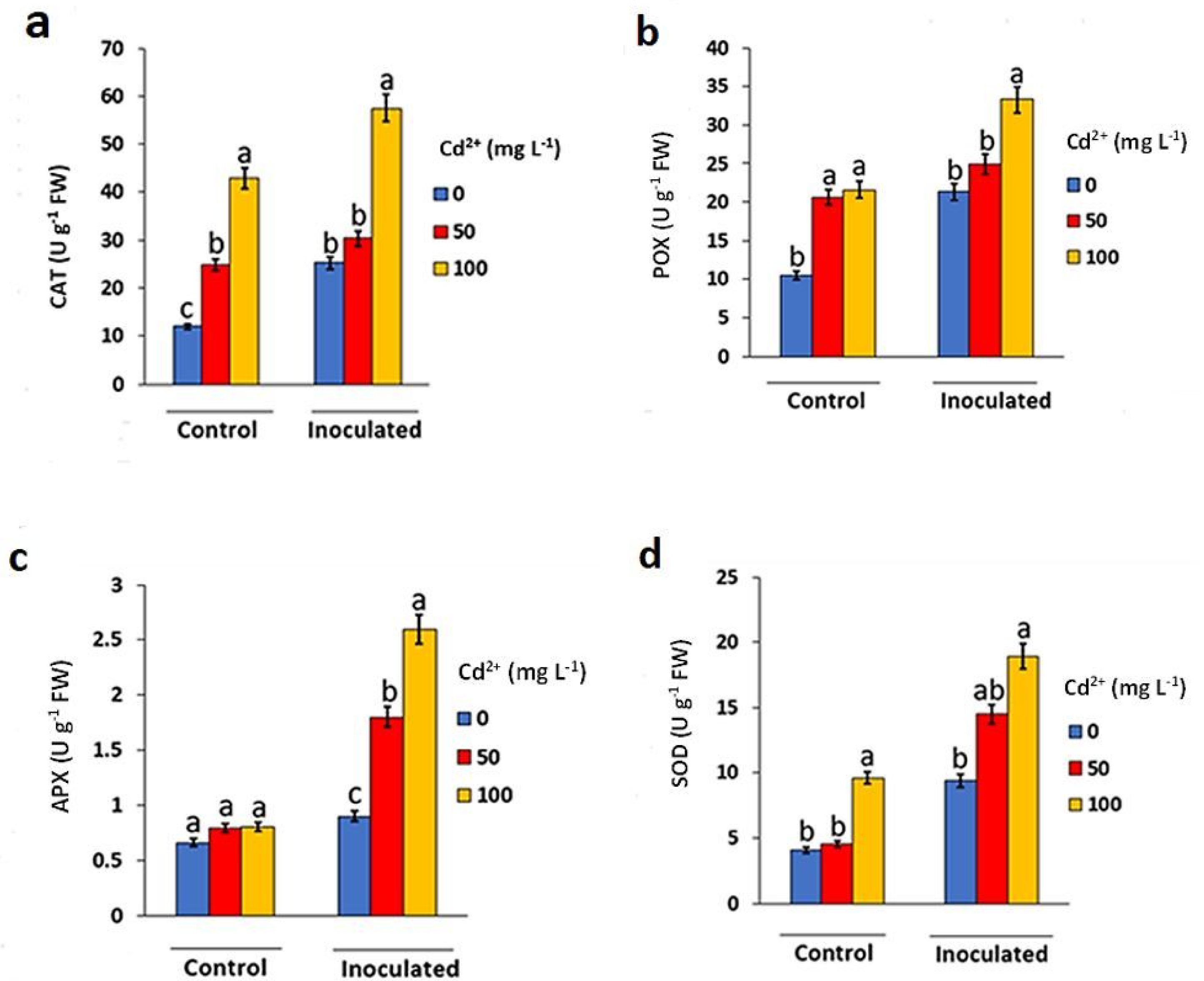

Figure 4

Antioxidant enzyme activities of (a) catalase (CAT), (b) guaiacol peroxidase (POX), (c) Ascorbate peroxidase (APX) and (d) superoxide dismutase (SOD) representing inoculated and control plants under different Cd-concentrations. Different letters indicate significant differences ($p \leq 0.05$) between inoculated and control plants according to Tukey's HSD test.