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Co-inoculation Effects of *Rhizobium sullae* and *Pseudomonas* sp. on Growth, Antioxidant Status, and Expression Pattern of Genes Associated with Heavy Metal Tolerance and Accumulation of Cadmium in *Sulla coronaria*

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Abstract

Recently, phytoremediation assisted by soil bacteria has emerged as a potential tool to clean up the metal-contaminated/polluted environment. Three plant-growth-promoting bacteria (PGPBs): *Rhizobium sullae*, *Pseudomonas fluorescens*, and *Pseudomonas* sp. were found to tolerate cadmium (Cd) stress. *Sulla coronaria* inoculated with these PGPBs, and grown under different Cd concentrations (0, 100, and 200 µM), showed increases in dry biomass and proline content. Notable increases in different gas-exchange characteristics such as photosynthesis rate (A), transpiration rate (E), and water-use efficiency (WUE), as well as increases in nitrogen (N) and Cd accumulations were also recorded in inoculated plants compared to non-inoculated Cd stressed plants. The activities of antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPOX), catalase (CAT), and ascorbate peroxidase (APX) in *S. coronaria* roots increased under Cd stress after PGPB co-inoculation, suggesting that these PGPB species could be used for amelioration of stress tolerance in *S. coronaria*. The expression patterns of *ScPCS*, *ScMT*, *ScF*-box, *ScGR*, and *ScGST* in roots of *S. coronaria* indicated that these genes are differentially expressed under Cd treatments, suggesting their possible roles in Cd and heavy metal stress responses. The results indicate that co-inoculation with *R. sullae* and *Pseudomonas* sp. could alleviate Cd toxicity in *S. coronaria*. In the present study, the obtained data suggest that the application of PGPBs could be a promising strategy for enhancing the phytostabilization efficiency of Cd-contaminated soils.

Keywords Antioxidant activities · Cadmium · Gene expression patterns · Heavy metal stress · PGPB · RT-qPCR · *Sulla coronaria*

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DAS	Days after sowing
DW	Dry weight
EDTA	Ethylene diamine tetra-acetic
FAAS	Flame atomic absorption spectrophotometer
FW	Fresh weight
IAA	Indole acetic acid
ICP–MS	Inductively coupled plasma/mass spectrometry
GSH	Gluthatione
GSSG	Gluthatione disulfure
GPOX	Guaiacol peroxidase
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
PGPB	Plant-growth-promoting bacteria
PGPR	Plant-growth-promoting rhizobacteria
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
RDW	Root dry weight
ROS	Reactive oxygen species
SDW	Shoot dry weight
SOD	Superoxide dismutase
TBA	Thiobarbituric acid

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TCA Trichloroacetic acid
YEM Yeast extract medium

Introduction

Cadmium (Cd) is an important environmental pollutant, which is toxic to the organisms and its high mobility causes significant hazard to the both, flora and fauna (Gómez and Marino 2015). Plant roots take up Cd and transport it to aboveground biomass inducing phytotoxic impact, such as growth reduction and chlorosis (Oves et al. 2016).

Gallego et al. (2013) affirmed a strong inhibition of photosynthesis and consequently the reduction of plant growth caused by heavy metals.

It is well known that heavy metals produce a high concentration of reactive oxygen species (ROS) triggering oxidative damage. Heyno et al. (2008) reported that cadmium stimulates the production of ROS in the mitochondrial electron-transfer chain (Yi et al. 2010) leading to the inhibition of photoactivation of photosystem II (PSII) through reduction of electron transfer (Sigfridsson et al. 2004) and therefore affect plant metabolism processes such as nutrient uptake, protein synthesis, and turnover and enzymatic activities (Monteiro et al. 2009).

To overcome ROS, plants developed robust enzymatic antioxidant defense systems such as superoxide dismutase (SOD), peroxidases (POX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) (Pandey and Singh 2012).

Glutathione S-transferases (GSTs) represent a large and important group of enzymes found in organisms playing important roles in plant growth and development and involved in detoxification of environmental pollutants (Hoque et al. 2010).

Interestingly, metal-chelators (*metallothionein 2b* and *3*), antioxidation-related genes (*APX*) and metal transporter (*ZIP*, *Nramp*, and *CDF* families) genes are expressed at higher levels in the Zn/Cd hyperaccumulator *Arabidopsis halleri* (Chiang et al. 2006). These authors suggest that the high expression of genes related to heavy metal tolerance and accumulation might be associated with the extreme tolerance to oxidative stress generated by high levels of Zn and Cd in *A. halleri*. Therefore, while determining the molecular mechanisms and genetic basis of metal accumulation, their roles in the major processes involved in phytoremediation might be an important aspect for understanding key mechanisms of response and tolerance to heavy metal-induced plant stress as agents for the phytoremediation of contaminated sites.

The F-box protein family (F-box) is one of the largest gene families in plants that regulate several key biological processes including protein degradation, plant growth, and development, responses to biotic and abiotic stresses, embryogenesis, hormonal responses, and senescence (Jia et al. 2013). Plant metallothioneins (MTs) constitute heterogeneous superfamily of low molecular weight peptides, cysteine-rich, and metal-binding proteins, which play crucial roles in osmotic stresses, and hormone treatment. Interestingly, MTs play important roles in the detoxification of heavy metal ions, and metal transport adjustment, and they are often cited as useful biomarkers for toxic metal stress (Ahemad 2014). In tomato, Kisa et al. (2017) found that MTs' transcripts are regulated by Cu and Pb, and the expression pattern depended on MTs' isoforms and tissue types.

Phytochelatin synthase (PCS) has been identified in a large number of plant species, which catalyzes the biosynthesis of phytochelatin from glutathione. Ovečka and Takáč (2013) and Guo et al. (2013) revealed that the biosynthesis of phytochelatins (PCs) is essential for the detoxification of nonessential metals and metalloids such as cadmium and arsenic in plants and a variety of other organisms.

A great interest is laid upon plant-associated bacteria due to their potential use in phytoremediation.

Moreover, phytoremediation potential depended on the interactions of soil, heavy metals, bacteria, and plants. Indeed, phytoremediation strategies with appropriate heavy metal-adapted rhizobacteria have been highly used for cleaning up toxic metals from soil (Hao et al. 2014). Recently, phytoremediation assisted by soil bacteria, including rhizobia and endophytes, known as plant-growth-promoting bacteria (PGPBs) represents a growing area of research, since they improve plant growth and illustrate mechanisms for plant metal accumulation/translocation in plants (Gómez and Marino 2015).

Rajkumar et al. (2012) found that PGPBs, may alleviate metal phytotoxicity and stimulate plant growth indirectly by the induction of defense mechanisms against phytopathogens, and/or directly through the solubilization of mineral nutrients (nitrogen, phosphate, potassium, iron, etc.), production of plant-growth-promoting substances (phytohormones), and secretion of specific enzymes (1-aminocyclopropane-1-carboxylate deaminase).

The symbioses between PGPBs and legumes have been widely proposed as effective bioinoculants and for heavy metal phytoremediation (Pajuelo et al. 2008; Dary et al. 2010). Results reported by Harzalli Jebara et al. (2015a, b) indicate that co-inoculation with Pb-resistant PGPB is a beneficial approach for decreasing Pb uptake and improving growth and yield of lentil (*Lens culinaris* L.) cultivated in soils contaminated with this heavy metal.

On the other hand, copper uptake was significantly inhibited in faba bean (*Vicia faba* L.) co-inoculated by Cu-resistant PGPB (Fatnassi et al. 2013, 2015).

Sulla coronaria constitutes an important genetic resource contributing to pastoral production, particularly in semiarid regions including Tunisia.

In this study, we focused on the use of *S. coronaria* which is a predominant leguminous in Mediterranean area, well adapted to marginal and drought-prone environments, versatility, high-protein forage crop, and its moderate level of condensed tannins beneficial to ruminant production (Ruisi et al. 2011). A previous study suggested that co-inoculation of *S. coronaria* with plant-growth-promoting rhizobacteria (PGPBs), including *Pseudomonas* and *Rhizobium sullae* increased production of plant-growth-promoting substances such as indole acetic acid and siderophores, and consequently improved tolerance of this species to Cd stress (Chiboub et al. 2018).

The main objectives of the current study were to investigate the physiological and molecular mechanisms of Cd tolerance in *S. coronaria* treated with different levels of Cd. The expression patterns of five specific genes (*PCS*, *MT*, *F*-box, *GR*, and *GST*) and putative functions in response to Cd stress were investigated. The effects of heavy metals generating antioxidative defense systems (SOD, CAT, APX, and GPX) were studied in the roots of *S. coronaria* grown hydroponically.

Materials and Methods

Plant Material, Growth, and Experimental Design

Sulla coronaria Bikra 21 is a Tunisian-registered variety since 2005, characterized by its drought and salt tolerance. Performance analysis of Bikra 21 showed its ability to adapt to different climates and soils. It is important to note that operating techniques of *S. coronaria* afford it the status of a cleaning plant (Slim et al. 2012).

Seeds of Bikra 21 were surface sterilized with diluted solution of mercuric chloride ($HgCl_2$, 0.1%) for 1 min, followed by 70% ethanol for 30 s and finally rinsed five times with sterile distilled water. Seeds were sown and germinated on perlite at 28 °C in continuous darkness; germinated seeds were separated into two parts: the controls and inoculated seeds. The first were irrigated by sterile water, whereas the inoculated seeds were supplied with the inoculums. Co-inoculation was performed with 10 mL of an aseptic suspension with the consortium formed by strains I3: *R. sullae*, I8: *R. sullae*, I9: *Pseudomonas fluorescens*, and I10: *Pseudomonas* sp. All these strains were isolated from nodules of *S. coronaria* cultivated in moderately contaminated Tunisian soil (Ghezela) and selected for their nodulation efficiency, cadmium tolerance, and PGP characteristics (Chiboub et al. 2018). Strains were grown in an Erlenmeyer flask containing Yeast Extract-Mannitol (YEM) medium (Vincent 1970) on a rotating incubator under continuous stirring at 200 rpm for 3–5 days at 28 °C to obtain a final concentration of 10^9 colony-forming units (CFU) mL^{-1}. For the co-inoculation treatment, the bacterial cultures of strains grown individually forming each consortium were mixed and added to seeds in perlite.

Seven days after sowing, seedlings were moved in tank of 5 L containing nutritive solution (Vadez et al. 1996). For the inoculated plants, a second inoculation was completed by adding equal volumes (1 mL) of inoculums. For the non-inoculated control, an equal volume of sterile nutrient solution was added.

At flowering stage (60 days after sown), *S. coronaria* was exposed to two Cd concentrations (100 and 200 µM of $CdCl_2$) for 30 days; then 90-day-old plants were harvested; a part of them were dried at 65 °C for 48 h to determine the shoot and root dry weights (SDW and RDW); and another part of the harvested plants were immediately frozen in liquid nitrogen, and stored at − 80 °C for further analytical analysis.

Determination of Cd Contents

Cadmium contents in root and shoot tissues were determined and analyzed by digestion of dried plant samples (20 mg) with a mixture of nitric acid and perchloric acid (HNO_3/$HClO_4$, 4/1 v/v). The mixture was heated at 100 °C for 2 h until dryness and then diluted in 25 mL HNO_3. The mixture was filtered through Whatman filter paper, and the metal concentration was determined by Flame Atomic Absorption Spectrophotometer (FAAS).

Physiological and Biochemical Analysis

Measurements of Photosynthetic Gas Exchange

Net photosynthesis (A), transpiration rate (E), and internal CO_2 concentration (Ci) were determined at flowering stage using Portable Photosynthesis System (LCpro+, UK). Measurements were done at 10 a.m., under atmospheric CO_2 and full sunlight. Water-use efficiency (WUE) was calculated by dividing net photosynthetic rate by transpiration rate.

Proline Content

Free proline content was determined spectrophotometrically using the ninhydrin method described by Bates et al. (1973). Root samples (0.5 g) were mixed with 5 mL of 3% sulfosalicylic acid using mortar and pestle. After centrifugation, the supernatant was mixed in a 1:1:1 ratio with ninhydrin acid and glacial acetic, heated at 100 °C for 30 min, and finally allowed to cool. Afterward, 6 mL of toluene was added and then transferred to a separating funnel. After thorough

mixing, the chromophore-containing toluene was separated, and its absorbance was measured at 520 nm in spectrophotometer against blank toluene. Concentration of proline was estimated by referring to a standard curve made from known concentrations of proline.

Assays of Antioxidant Enzymes

For antioxidant enzyme assays, 1 g of frozen root samples was ground to a fine powder in liquid nitrogen and homogenized in an ice-cooled mortar with 50 mg polyvinyl-pyrrolidone (PVP), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM dithiothreitol, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 50 mM potassium phosphate buffer (pH 7.8). Only for the APX assay, the buffer was supplemented with 5 mM ascorbate (Gogorcena et al. 1997). Homogenate was centrifuged at 14000 g for 20 min, and the supernatant of the crude extract was used immediately for enzyme assays. The total protein contents were determined according to Bradford (1976) method using bovine serum albumin as a standard. All procedures were carried out at 4 °C.

Superoxide Dismutase (SOD) (E C, 1.15.1.1)

SOD activity was determined spectrophotometrically according to the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm. The assay reaction mixture consisted of 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 10 mM methionine, 2.7 µM riboflavine, and 75 µM NBT. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C (Yu and Rengel 1999).

Ascorbate Peroxidase (APX) (EC, 1.11.1.11)

APX activity was measured by monitoring the disappearance of ascorbate (0.5 mM) at 290 nm during 1 min ($\varepsilon = 2.8\ \mathrm{mM^{-1}\ cm^{-1}}$). The reaction mixture contained 0.2 mM H_2O_2, 50 mM phosphate buffer (pH 7.0) (Amako et al. 1994).

Guaiacol Peroxidase (GPOX) (EC, 1.11.1.7)

GPOX activity was determined by monitoring the formation of tetraguaiacol from guaiacol (9 mM) at 470 nm for 1 min ($\varepsilon = 26.6\ \mathrm{mM^{-1}cm^{-1}}$) in the presence of H_2O_2 (19 mM) added for starting the reaction (Anderson et al. 1995).

Catalase (CAT) (EC, 1.11.1.6)

CAT activity was assayed by monitoring the decline of absorbance at 240 nm caused by the decomposition of H_2O_2 (10 mM) during 3 min ($\varepsilon = 36\ \mathrm{mM^{-1}\ cm^{-1}}$), and the activity was measured according to the method of Aebi (1984.

Total RNA Extraction and First Strand cDNA Synthesis

Total RNA from root tissues of *S. coronaria* was isolated following a modified CTAB method (Chang et al. 1993). 700 µL of extraction buffer (100 mM Tris–HCl; pH 8; 25 mM EDTA; pH 8; 2 M NaCl; 2% CTAB; 2% PVP) and 10 µL of β-mercaptoethanol were added to 200 mg of ground plant material. The mixture was incubated at 65 °C for 30 min and homogenized by vortexing 3–4 times during incubation. Then, an equal volume of chloroform/isoamyl alcohol (24:1) was added, vortexed, and centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant of each sample was collected and transferred to a new tube containing 1/4 volume of LiCl (10 M) and incubated overnight at 4 °C for total RNA precipitation. Afterward, the samples were centrifuged at 13,800 rpm for 30 min at 4 °C. The pellets were dissolved in 200 µL of DEPC-treated water, followed by adding of 600 µL of absolute ethanol and 100 µL of 3 M NaAc (pH 5.2) and further incubated for 30 min at − 80 °C. The total RNA was recovered by centrifugation at 13,800 rpm for 30 min at 4 °C. The RNA pellets were washed with 100% ethanol (600 µL), centrifuged for 5 min at 13,800 rpm, air dried, and finally dissolved into 50 µL of DEPC-treated water. The RNA concentration was determined spectrophotometrically using UV-2700 (Shimadzu, Tokyo, Japan), and its integrity was assessed by electrophoresis in 1.2% agarose gels. To eliminate any possible contamination with genomic DNA, the RNA samples were treated, prior to cDNA synthesis, with 1 µL DNase I, RNase-free (1 U μL^{-1}) (Biomatik; Wilmington, Delaware, USA) for 30 min at 37 °C. First-strand cDNA was synthesized from 5 µg of total RNA using 200 U Turbo-I reverse transcriptase (Biomatik; Wilmington, Delaware, USA) according to the manufacturers' instructions.

Primer Design and PCR Confirmation

Specific primer pairs were designed to recognize conserved regions which were predetermined using alignment of F-box, MTs, PCS, GR, and GST genes sequences from model legume plants including *Medicago truncaluta* and *Glycine max* that are available at the GenBank. The alignment for these identified genes was performed by ClustalW. Each primer pair for the selected genes was designed by Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/) (Table 1).

In order to check the specificity and the appropriate amplification conditions of the primers, they were initially tested by conventional PCR reaction using cDNA as a template. Semiquantitative PCR of the studied genes (*ScF-box,*

Table 1 Primers used for semiquantitative RT-PCR analysis

Primers	Sequences	Tm (°C)
ScFboxF	5′-ACCACCTCTGAGACCCTTGT-3′	57
ScFboxR	5′-TGCAGCCATCATTCTCAGCA-3′	
ScMetF	5′-CATTCGGTGTAACGGTTGCC-3′	58
ScMetR	5′-CCTTCGAGATAGCCCAACCC-3′	
ScPCSF	5′-CCGGAGCAAAAGTTGAAGCC-3′	59
ScPCSR	5′-GTTTGTTTGAGGGCAGCTCG-3′	
ScGSTF	5′-AAGAGCCTTGGCACGTTTCT-3′	56
ScGSTR	5′-AGCGTCTTCTGTTTCCTCAA 3′	
ScGRF	5′-CTGGAAGTGGTGGTGTTCGT-3′	57
ScGRR	5′-AATGACACACGTTCCACCGA-3′	
Mt18SF	5′-GGCCGTTCTTAGTTGGTGGA-3′	58
Mt18SR	5′-AAGCGGCCATAGTCCCTCTA-3′	

ScMTs, ScPCS, ScGR, and *ScGST*) was performed in 20-µL reaction mixtures. The PCR conditions were modified according to the primer properties. PCR amplification was performed for all genes using the following program: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 56–59 °C (depending on the primers used, see Table 1), and 1 min at 72 °C. An elongation step at 72 °C for 5 min was conducted for the last cycle. PCR reactions were performed in an Applied Biosystems® 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The assays were repeated three times. PCR products (15 µL) were electrophoresed on 1.5% (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer.

Gene Expression Analysis by Quantitative Real-Time RT-PCR

RT-qPCR was carried out in an optical 96-well plate with a 7300 Real-Time PCR System (Applied Biosystems, USA). For each sample, 50 ng of cDNA was used in the reaction mixture in a final volume of 25 µL. Reactions were performed using 12.5 µL Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Biomatik; Wilmington, Delaware, USA), 1 µL of each primer at 10 µM (Table 1), and 10.5 µL ddH_2O. The following thermal cycling condition was used for all amplifications: initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. After 40 cycles, the specificity of the amplifications was analyzed through the dissociation curve profiles. *Mt18SF* and *Mt18R* primers were used as a control to normalize the samples. Relative quantitation was performed according to the comparative $2^{-\Delta Ct}$ method as described previously by Schmittgen and Livak (2008).

Statistical Analysis

Data are means ± standard deviations (SD) of three independent biological replicates. Differences among treatments were assessed using a one-way Analysis of Variance (ANOVA, $P < 0.05$), followed by LSD test in the SPSS software.

Results

Effect of Cd Treatments and Inoculation on Growth of *Sulla coronaria*

The inoculation of *S. coronaria* with the bacterial consortium contained four bacterial strains (two *R. sullae, P. fluorescens* and *Pseudomonas* sp.) increased the production of shoots and roots biomass (2.75 and twofold, respectively) (Table 2). Nodulation of *S. coronaria* was markedly increased. Indeed, the average number of nodules per plant increased to 45 under symbiotic condition. Moreover, nitrogen content increased in shoots and roots by 65 and 158%, respectively (Table 2).

On the other hand, Cd treatment induces severe reduction in the non-inoculated plants either in SDW by 69 and 80% or in RDW by 61% and 69% under 100 and 200 µM, respectively. Nitrogen content was inhibited only by 200 µM either in shoots or in roots by 28 and 27%.

Table 2 Effects of Cd treatment and co-inoculation on nitrogen content, nodule number, shoot and root dry weights of *S. coronaria*

	Cd treatment (µM)	Number of nodules	Shoot dry weight (SDW) g plant^{-1}	Root dry weight (RDW)	Nitrogen content	
					Leaves	Roots
Non-inoculated	0	nd	3.05 ± 0.40^{b}	1.39 ± 0.33^{b}	0.95 ± 0.11^{c}	0.96 ± 0.10^{c}
	100	nd	0.93 ± 0.02^{de}	0.54 ± 0.05^{c}	0.9 ± 0.08^{c}	0.90 ± 0.20^{c}
	200	nd	0.61 ± 0.08^{ef}	0.43 ± 0.06^{cd}	0.68 ± 0.14^{d}	0.70 ± 0.12^{d}
Inoculated	0	45^{a}	8.26 ± 0.36^{a}	2.79 ± 0.35^{a}	1.57 ± 0.27^{a}	2.48 ± 0.07^{a}
	100	28^{b}	2.34 ± 0.35^{c}	1.45 ± 0.09^{b}	1.48 ± 0.21^{a}	2.41 ± 0.03^{a}
	200	25^{b}	1.19 ± 0.11^{d}	0.47 ± 0.11^{cd}	1.31 ± 0.04^{b}	2.21 ± 0.12^{b}

Plants were treated at the flowering stage with different concentrations of Cd (0, 100, and 200 µM) for 30 days. Values represent average measurements ± SD of three replicates. Different letters indicate a significant difference ($P < 0.05$) according to the LSD test

However, SDW and RDW of inoculated and Cd-treated *S. coronaria* were greater than non-inoculated and Cd-treated plants. Analysis of results showed that inoculation of Cd-treated plants enhanced SDW by 152 and 95% under100 and 200 µM Cd, whereas it enhanced significantly RDW only under 100 µM Cd by 169% (Table 2).

Similar results were also detected for N content. Inoculated and Cd-treated *S. coronaria* plants exhibited significantly enhanced N content in shoots by 64 and 93% and in roots by 168 and 216% under 100 and 200 µM Cd. Moreover, a decrease close to 38 and 44% was observed in nodules' number at 100 and 200 µM Cd concentrations (Table 2).

Experiments showed that inoculation of *S. coronaria* with the bacterial consortium significantly improved plant-growth parameters, whereas Cd treatments mainly at 200 µM reduced them significantly.

Effects of Cd Treatments and Co-inoculation on Leaf Gas Exchange

Significant effects of Cd treatment on the net photosynthesis rate, transpiration rate, and water-use efficiency were observed on both inoculated and non-inoculated plants.

Analysis of results showed that inoculation ameliorated the net photosynthesis rate by 137%, elsewhere transpiration rate and water-use efficiency were significantly enhanced by 25% (Table 3). With 100 and 200 µM Cd concentration, net photosynthesis rate was inhibited by 72 and 77%, respectively. Similarly, transpiration rate was reduced by 52% and 46%. Water-use efficiency changed in response to 100 and 200 µM Cd treatments by up to 61% and 71%, respectively.

The inoculation of the Cd-treated plants ameliorated significantly photosynthetic parameters, and thus, net photosynthesis rates achieved 107 and 136%, transpiration rates gained by 31 and 18%, and water-use efficiencies regained 55 and 100% under 100 and 200 µM Cd treatments, respectively.

Effects of Cd Treatments and Co-inoculation on Cadmium Uptake in *Sulla coronaria*

Results regarding inoculation effect on Cd uptakes in *S. coronaria* treated by the two Cd concentrations are presented in Table 4. This experiment showed that under the non-inoculated condition, *S. coronaria* accumulated Cd in the roots (27.06 ± 1.04 µg g^{-1}) more than the shoots essentially under 200 µM Cd. Inoculation enhanced Cd uptake essentially by 200 µM Cd treatment in roots by 57% and slowly in shoots by 15%.

Effects of Cd Treatments and Inoculation on Proline Content

The production of proline in inoculated plants was considerably ($P < 0.05$) improved in control and in plants treated with the both Cd levels (100 and 200 µM). Furthermore, the proline amounts in inoculated and non-inoculated plants increased with the rise of Cd concentrations; thus, results showed that inoculation enhanced proline content in the roots by 40 and 6.6 times, whereas in the shoots proline content elevated to about 11 and five times in plants treated by 100 and 200 µM Cd, respectively (Table 5).

Antioxidant Enzyme Activities in *Sulla coronaria* Plants Exposed to Different Cd Applications

The changes in antioxidant enzyme activities were determined in roots of inoculated and non-inoculated *S. coronaria* submitted to two Cd concentrations (100 and 200 µM Cd) (Table 6). SOD is a key enzyme in the regulation of intracellular concentrations of ROS; it converts superoxide radicals into H_2O_2. GPOX, APX, and CAT convert H_2O_2 to water.

Under the control condition, the co-inoculation of *S. coronaria* enhanced SOD, APX, and CAT activity by 5, 7,

Table 3 Effects of Cd treatment and co-inoculation on net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol m^{-2} s^{-1}), and water-use efficiency (WUE, µmol CO_2 mol^{-1} H_2O) of *S. coronaria* after 30 days of Cd treatments

Treatment	Inoculation	A	E	WUE
Controls	–	0.95 ± 0.15^{b}	5.08 ± 0.34^{b}	0.28 ± 0.04^{a}
	+	2.25 ± 0.30^{a}	6.35 ± 0.41^{a}	0.35 ± 0.06^{a}
100 µM Cd	–	0.27 ± 0.05^{d}	2.45 ± 0.74^{c}	0.11 ± 0.01^{c}
	+	0.56 ± 0.04^{c}	3.21 ± 0.16^{c}	0.17 ± 0.01^{b}
200 µM Cd	–	0.22 ± 0.04^{d}	2.72 ± 0.54^{c}	0.08 ± 0.01^{d}
	+	0.52 ± 0.06^{c}	3.21 ± 0.27^{c}	0.16 ± 0.01^{b}

Values represent average measurements ± SD of three replicates. Different letters indicate a significant difference ($P < 0.05$) according to the LSD test

Table 4 Cd uptakes in *S. coronaria* shoots and roots

	Treatment	Cd accumulation (µg g^{-1}DW)	
	Cd µM	Shoots	Roots
Non-inoculated	0 (Control)	0.30 ± 0.09^{d}	0.48 ± 0.07^{d}
	100	16.5 ± 1.80^{a}	19.5 ± 2.70^{c}
	200	2.67 ± 0.26^{c}	27.06 ± 1.04^{b}
Inoculated	0	0.24 ± 0.10^{d}	0.56 ± 0.12^{d}
	100	18.4 ± 1.10^{a}	21.8 ± 1.30^{c}
	200	3.06 ± 0.06^{b}	42.61 ± 2.15^{a}

Values are presented as mean ± SD for the ten replicates. Different letters indicate a significant difference ($P < 0.05$) according to the LSD test

Table 5 Effects of Cd treatment and co-inoculation on proline content of *S. coronaria* roots

Treatment	Inoculation	Proline (mg g^{-1} MF)
Controls		
Shoots	–	1.04 ± 1.08^{h}
	+	11.64 ± 1.50^{e}
Roots	–	2.42 ± 1.80^{h}
	+	9.82 ± 2.19^{e}
100 µM Cd		
Shoots	–	5.68 ± 0.64^{f}
	+	63.42 ± 5.24^{b}
Roots	–	1.56 ± 0.23^{h}
	+	64.25 ± 6.33^{b}
200 µM Cd		
Shoots	–	4.22 ± 0.54^{g}
	+	20.50 ± 6.33^{d}
Roots	–	39.53 ± 2.63^{c}
	+	259.66 ± 0.14^{a}

Values are presented as mean ± SD for the ten replicates. Different letters indicate a significant difference ($P < 0.05$) according to the LSD test

and 4 times, respectively, compared with the non-inoculated, whereas GPOX was inhibited by 28%.

In the non-inoculated plants, the applications of 100 and 200 µM enhanced significantly roots SOD activity by four and five times, respectively, compared to the controls; co-inoculation of plants treated by 100 µM Cd enhanced this activity by 157%, whereas the exposure to 200 µM Cd had no significant effect (Table 6).

In the non-inoculated plants, the treatment of plants by 100 µM Cd induced GPOX activity by 167%, and its value was the highest and reached 41.42 ± 2.14 mM H_2O_2 min^{-1} μg^{-1} protein; in contrast, the treatment of 200 µM inhibited it by 48%. Co-inoculation of plants treated by 100 µM Cd inhibited GPOX activity by 21%; however, its value remains high (32.8 ± 1.98 mM H_2O_2 min^{-1} μg^{-1} protein), and the exposure of inoculated plants to 200 µM Cd enhanced this activity by 240% (Table 6).

The addition of 100 µM Cd in the non-inoculated plants enhanced APX activity by five times, the same result was found in the case of 200 µM Cd treatment, whereas, the co-inoculation inhibited APX activity by 21% essentially after the application of 100 µM Cd.

Similarly, CAT activity increased by six times in plants subjected to either 100 or 200 µM Cd. Conversely, co-inoculation of 100 µM Cd-treated plants inhibited CAT activity by 25% and had no significant effect when they are submitted to 200 µM Cd.

Effects of Cadmium Treatments and Co-inoculation on Metal Transporter Gene

The putative role of inoculation with bacterial consortium on regulation of metal transporters genes in *S. coronaria* under Cd treatments was assessed by quantitative real-time reverse transcription PCR (Fig. 1).

Results indicated that the inoculation and Cd-treatments influenced the expression of studied genes (*ScPCS*, *ScMT*, *ScF*-box, *ScGR* and *ScGST*). Indeed, the inoculation with the bacterial consortium substantially reduced the expression of the studied genes in 100 and 200 µM Cd compared to control. Plants inoculated with the bacterial consortium recorder lowest *ScF*-box transcripts followed by plants subjected to 100 µM Cd, plants inoculated and treated by100 µM Cd and plants inoculated and treated by 200 µM Cd. By contrast plants treated only by 200 µM Cd recorded the maximum relative expression among other treatments (Fig. 1a), likewise in *ScPCS* gene similar results were observed (Fig. 1b). The expression pattern of root *ScMT* was different to *ScF*-box and *ScPCS*. In fact, root *ScMT* expression level was significantly decreased either by addition of 100 or 200 µM Cd. Moreover, the bacterial consortium inoculation substantially reduced the *ScMT* expression either in 100 or 200 µM Cd treatments (Fig. 1c). The *S. coronaria* plants exposed to 100 and 200 µM

Table 6 Specific antioxidant enzyme activities: superoxide dismutase SOD (U SOD μg^{-1} protein), guaiacol peroxidase GPOX (mM H_2O_2 min^{-1} μg^{-1} protein), ascorbate peroxidase APX (mM ascorbate min^{-1} μg^{-1} protein), and catalase CAT (mM H_2O_2 min^{-1} μg^{-1} protein) of *S. coronaria* roots control and inoculated plants

	Inoculation	SOD	APX	GPOX	CAT
Controls	–	0.14 ± 0.01^{d}	0.80 ± 0.12^{d}	15.49 ± 0.20^{d}	0.12 ± 0.01^{c}
	+	0.64 ± 0.05^{c}	5.95 ± 0.89^{a}	11.22 ± 0.16^{e}	0.53 ± 0.07^{b}
100 µM Cd	–	0.58 ± 0.07^{c}	4.24 ± 0.08^{b}	41.42 ± 2.14^{a}	0.67 ± 0.05^{a}
	+	1.49 ± 0.24^{a}	3.37 ± 0.45^{c}	32.8 ± 1.98^{b}	0.50 ± 0.04^{b}
200 µM Cd	–	0.78 ± 0.10^{b}	4.16 ± 0.17^{b}	8.04 ± 0.44^{f}	0.75 ± 0.01^{a}
	+	0.58 ± 0.04^{c}	4.6 ± 0.10^{b}	27.32 ± 15^{c}	0.70 ± 0.04^{a}

Plants were treated at flowering stage with two types of treatments: 100 and 200 µM Cd, and harvested after 30 days. Values are presented as mean ± SD for the three replicates in three independents pots. Different letters indicate a significant difference ($P < 0.05$) according to the LSD test

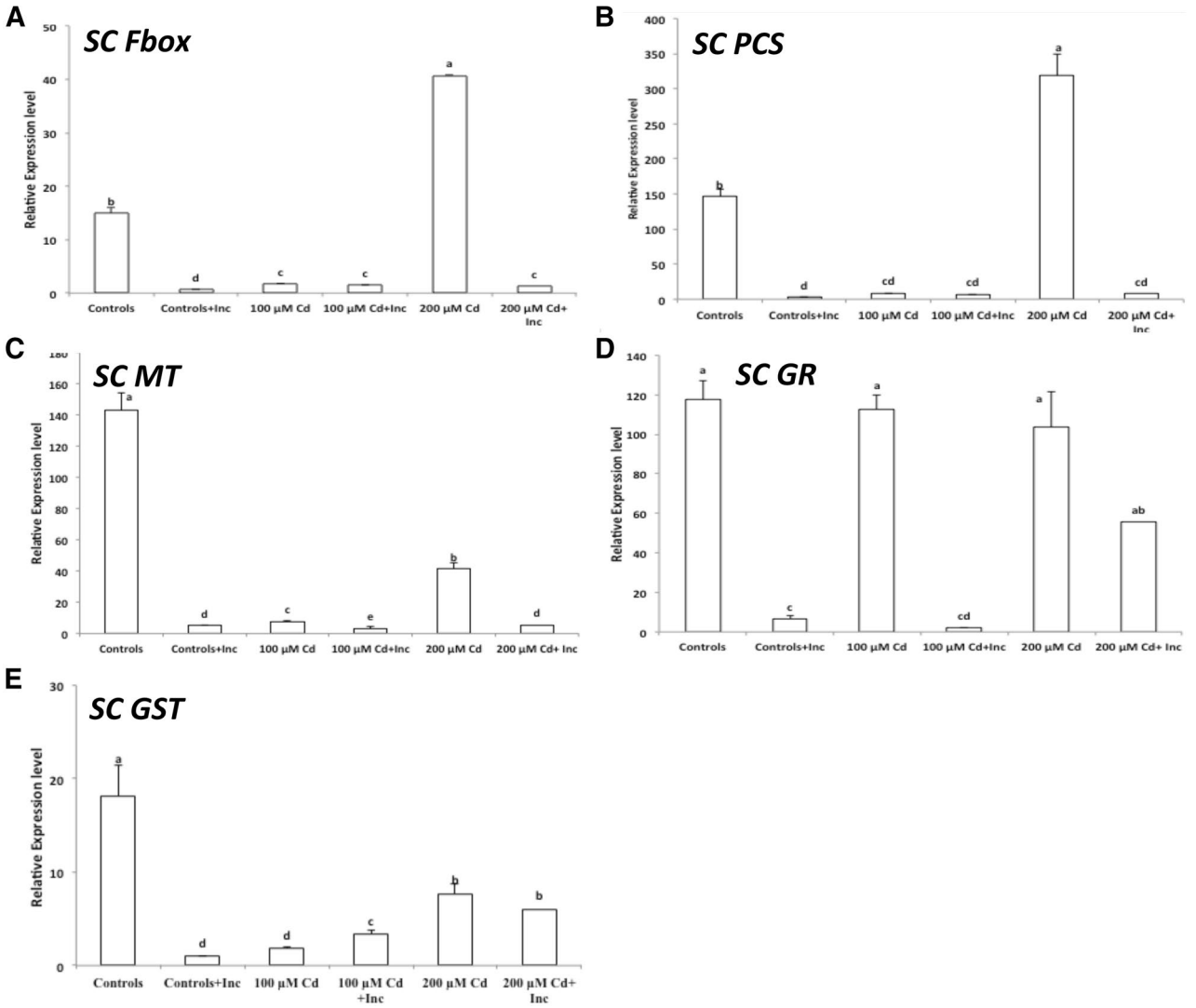

Fig. 1 Expression patterns of *ScPCS*, *ScMT*, *ScF-box*, *ScGR,* and *ScGST* in root of *S. coronaria* inoculated or non-inoculated with the bacterial consortium and treated with different Cd concentrations. Vertical bars mean the error of three replicates. Different letters denote significant differences (Tukey's HSD, $P < 0.05$)

Cd showed high *ScGR* mRNA accumulation, similarly to control condition followed by inoculated and 200 µM Cd-treated plants, while the lowest level of *ScGR* transcripts were revealed in plants only inoculated by the bacterial consortium and plants inoculated and treated with 100 µM Cd (Fig. 1d).

Thus, the RNA levels of *ScGST* were similar to that reported by *ScMT,* essentially in the plant roots of non-inoculated and treated with 200 µM Cd (Fig. 1e).

Discussion

The uptake and accumulation of heavy metals by plants using phytoremediation technology seem to be a prosperous way to clean up heavy metal-contaminated environment. Indeed, phytostimulation approach is an emerging technology, and it recently has gained more and more attention (Imam 2017). Several previous studies reported that heavy metals caused a significant decline in photosynthesis and consequently reduction in plant growth, biomass, and yield (Mourato et al. 2015). Similarly, in the present study, the increasing concentration of Cd under the hydroponic condition reduced plant growth, nodulation, and nitrogen content; therefore, the inoculation of *S. coronaria* ameliorated plant-growth parameters in spite of Cd treatment. These results were confirmed in the study of Chiboub et al. (2018) indicating that *S. coronaria* cultivated hydroponically and treated by 100 µM Cd for 7 days or 50 µM for 30 days, showed amelioration in plant growth by inoculation. Comparable results have also

been documented in other inoculated legumes submitted to other heavy metals contamination such as in maize cultivated in Pb polluted soil and inoculated by *Rhizobium* and *Azatobacter* which showed highly significant increase in dry biomass (Hadi and Bano 2010).

A consortium of bacteria combining *Enterobacter cloacae*, *Rhizobium* sp. CCNWSX0481, *Pseudomonas* sp. 2(2010), and *Rhizobium leguminosarum* bv. *viciae* exhibited a positive influence on faba bean plant growth and seed yield, through increasing fresh shoot and fresh root weights in moderate copper-contaminated soils (Fatnassi et al. 2014). The inoculation of lentil grown hydroponically with inoculums formed by efficient and Pb-resistant bacteria including *Agrobacterium tumefaciens*, *Rahnella aquatilis*, *Pseudomonas*, and *Rhizobium* sp. enhanced plant biomass, which suggests a key role in phytostabilization of Pb-contaminated soils (Harzalli Jebara et al. 2015b).

In this experiment, we suggest that co-inoculations by *Rhizobium* and *Pseudomonas* increased the excretion of nod-gene by seedling roots of *S. coronaria* which could be associated with increases in the production of lateral roots, root hair density, and in root hair branching (Figure supplementary material), and consequently RDW.

Significant increases in soybean nodule number, shoot dry weight, and yield after combined inoculation with *Rhizobium* and *Azospirillum* were found by Jabbar and Saud (2012). According to Iruthayathas et al. (1983), this may suggest that *Rhizobium* and *Pseudomonas* bacteria have some compounds, which encourage new hair formation and then infection.

The nitrogen content in plants varied with treatments, and the lowest amount was recorded in leaf and root of the non-inoculated plants compared to inoculated ones.

At low concentration of 100 µM Cd, the heavy metal reduced RDW but did not affect nitrogen level, and plant root continued nitrogen absorption which forms complex ligands with organic compounds such as protein, inducing inactivation of vital enzymes and reducing plant growth (Louis 2009). Under severe Cd contamination (200 µM), the toxic effect of the heavy metal was more pronounced, and the transporters of membrane were more affected; in fact, it has been demonstrated that Cd exposure of *S. coronaria* enhanced MDA content (Chiboub et al. 2018).

Increases in nitrogen uptake in Cd-treated and inoculated plants may be due to better mineral nutrition of the *S. coronaria*, which is beneficiated by *Rhizobium* nodule formation and symbiotic nitrogen fixation activities (Burdman et al. 1997); in addition, the PGPRs used as inoculum have adapting mechanisms to the metal contaminant and qualified by its PGP traits such as IAA and siderephore production, P-solubization capacity, nitrogen fixation, and nodulation (Chiboub et al. 2016). Generally under heavy metal stress, PGPB developed many strategies to avoid toxicity and improve yield by supplying growth regulators (Ahemad 2014).

Photosynthesis parameters are often useful to assess the metal stress in plants. In the present study, lower WUE in plants with 100 and 200 µM Cd concentrations can be interpreted as a failure of plants to improve their water regime. These data are in agreement with those of other studies; according to Carson et al. (1975), simultaneous inhibition of photosynthesis and transpiration in maize by Cd stress decreased the WUE that could be associated with changes in stomatal function. Dong et al. (2005) found that seedlings of tomato responded to Cd stress with the decreasing photosynthetic rate and growth reduction. Indeed, stomatal conductance (gs) and intracellular CO_2 concentration (Ci) were significantly reduced. Moreover, morphological and physiological modifications were reported in tomato seedlings after treatment with 1, 5, and 10 (µmol L^{-1} Cd). According to Becerril et al. (1989), excess Cd induces disorders in plant–water relation, such as reduced water uptake, which can be partly explained by root growth inhibition, translocation, and transpiration.

It is reported that accumulation of heavy metals in vegetables is influenced by many factors such as climate, plant developmental stage, concentration of heavy metals, and nature of soil (Broadley et al. 2007). Cadmium accumulated in plant organs of *S. coronaria* were lower than those of *Sedum alfredii* hence that is proposed as a new Zn/Cd hyperaccumulator (Xiong et al. 2004). In the present study, Cd content in plants was more significant in roots and it is more translocated to the shoots by the addition of 200 µM Cd in culture medium. The inoculated *S. coronaria* treated by 200 µM Cd increased Cd up take essentially in roots suggesting that the removal of Cd metal from the nutrient solution was due to action of phytostabilization mechanism rather than phytoextraction mechanism.

It has been shown that proline plays a key role in stabilization and maintenance of cell turgor, and its accumulation is considered as a stress indicator in plants under abiotic stress (Shamsul et al. 2012).

In this study, inoculation increased significantly proline accumulation in *S. coronaria* plants under Cd stress compared to non-inoculated plants. The accumulation of proline in leaves and roots under Cd stress provides possibly protection by maintaining the water balance (Schat et al. 1997), scavenging hydroxyl radicals (Smirnoff et al. 1989), chelating heavy metals in the cytoplasm (Farago and Mullen 1979), and protecting the plasma membrane from Cd toxicity. The increase in proline content under cadmium stress, and essentially after inoculation, suggests the important role of the consortium in enhancing *S. coronaria* Cd tolerance.

In general, exposures of plants to heavy metals generate reactive oxygen species, which can lead to oxidative damage. To minimize the damaging effects of ROS, plants evolved

both nonenzymatic (such as vitamins C and E, glutathione, and β-carotene) and enzymatic antioxidant defenses including SOD, CAT, APX, and GPOX (Jebara et al. 2010; Becana et al. 2010; Wu et al. 2015). Several studies reported that PGPB could greatly improve plant growth and enhance tolerance toward heavy metals' stress (Ma et al. 2011; Wu et al. 2006), and the activation of the antioxidant capacity of PGPB in plants likely accounts for plant-growth improvement and the alleviation of Cu toxicity stress (Islam et al. 2015). In the present investigation, inoculation with the bacterial consortium caused significantly increased SOD, APX and CAT activities and significantly decreased the activity of GPOX in root tissues (Table 6). On the other hand, at 100 and 200 µM Cd, the activities of APX, GPX, SOD, and CAT in the roots of the inoculated and non-inoculated plants increased dramatically compared to the control. Moreover, the activities of GPOX and APX were always higher compared to those of CAT and SOD at both 100 and 200 µM Cd supply, suggesting that GPOX and APX activities seem to be more important to resist the oxidative stresses caused by Cd excess in *S. coronaria*. The increases in APX and GPOX activities in *S. coronaria* roots confirmed their role in the detoxification of H_2O_2, as previously reported by Chiboub et al. (2018). Significantly increased CAT activities at 100 and 200 µM Cd advocate its role in detoxification of H_2O_2 in *S. coronaria* roots. Our results are in agreement with previous findings observed by Chiboub et al. (2018) who reported that these enzyme activities were stimulated in the shoots and roots of *S. coronaria* subjected to increased Cd dose. Similar results were obtained in garden grass and strawberry plants (Gill et al. 2012; Muradoglu et al. 2015). Indeed, these authors reported augmentation in SOD, CAT, and APX activities in response to elevated Cd concentrations. In this study, the increases of CAT, GPOX, SOD, and APX activities in inoculated plants suggest that the consortium helped *S. coronaria* to relieve oxidative damage to biomolecules in Cd-contaminated soil.

F-box proteins are largely studied as they play a crucial role in plant tolerance to biotic and abiotic stresses (Gupta et al. 2015). Among 972 putative F-box proteins identified in the genome of *Medicago truncatula*, several were revealed to be expressed in response to heavy metals stress (Song et al. 2015). *ScF-box* was markedly upregulated in *S. coronaria* under 200 µM Cd treatments. However, *ScF*-box transcription was dramatically downregulated under the both 100 and 200 µM treatments in inoculated plants. Together, these results indicate that *ScF*-box exhibited a diverse expression pattern in response to Cd metal treatments and might suggest that *ScF*-box could be involved in responses to Cd stress in *S. coronaria*. In contrast to these, Chen et al. (2014) found that expression of *CaF*-box was downregulated in response to heavy metal stress, suggesting *CaF*-box may play a negative role in response to metal stresses.

In the present study, one *F*-box gene was chosen for quantitative real-time PCR analysis under Cd stress and inoculation with PGPB in roots of *S. coronaria*; however, the other *F*-box genes exist and must be studied in order to understand the mechanisms of gene expression regulation under Cd and inoculation conditions. More interestingly, a comparative genomics analysis of *S. coronaria F*-box protein genes family could be performed and in order to cover their putative regulatory roles in *S. coronaria* growth and development, and tolerance to heavy metal stress.

Phytochelatins are an important cadmium chelators which are synthesized enzymatically, by phytochelatin synthase (PCS) (Cobbett 2000). The expression of *NnPCS1* in leaves of *Nelumbo nucifera* was dramatically increased in response to Cd treatment (Liu et al. 2012). Exposed to 200 µM Cd, ScPCS protein levels increased significantly compared with control plants. A possible explanation may be that the Cd induced the transcription of *ScPCS* in the presence of 200 µM Cd. Indeed, Zhang et al. (2005) reported that the *AsPCS1* mRNA accumulation was induced in the presence of 200 µM Cd in the booth, roots, and leaves of *Allium sativum*. Moreover, *Arabidopsis* lines overexpressing *NnPCS1* accumulated more Cd compared to the wild-type (Liu et al. 2012). Interestingly, several studies suggested that an increase in phytochelatins production could contribute to improve accumulation of heavy metals and Cd tolerance (Zhu et al. 1999). The expression of *ScPCS* in this study can reveal putative key role in the response of *S. coronaria* to Cd stress.

Metallothioneins (MTs) are involved in heavy metals' detoxification and in the maintenance of intracellular metal ion homeostasis (Hamer 1986). In this study, we showed that the levels of *ScMT* expression in Cd-treated and inoculated or non-inoculated plants decreased following the treatment compared to control plants. This result was in agreement with Sereno et al. (2007) and Guo et al. (2013), who inferred that cadmium tolerance and accumulation in sugarcane might derive from other mechanisms. We suggest that other member(s) of metallothioneins might play a key role in Cd detoxification and homeostasis in *S. coronaria*.

Glutathione S-transferases (GSTs), encoded by a large family of multifunctional proteins, play a central role in detoxification processes and tolerance to oxidative stress. Indeed, glutathione peroxidase (GPX) pathway (including GPX, GR, and GST) is another process that converts H_2O_2 to H_2O in plants depending on glutathione (GSH). According to Adamis et al. (2004), GST may catalyze Cd complexation with GSH, which leads to alleviating Cd toxic effects and promoting Cd retention in plant roots. In the current study, *ScGST* was downregulated by Cd in inoculated and non-inoculated treatments. However, *ScGR* expression was significantly decreased in inoculated plants compared to control condition. The results indicate that the GPX pathway may

not be involved in the mechanism of ROS scavenging for *S. coronaria*.

Conclusions

The positive effect of *Rhizobium* inoculation with *Pseudomonas* was obvious in increasing growth, proline accumulation, net photosynthesis rate, water-use efficiency, and nitrogen concentration in *S. coronaria* plants grown at different Cd concentrations (0; 100 and 200 mM).

In general, the results showed that CAT, APX, SOD, and GPOX activities in inoculated plants were higher than those of non-inoculated plants, which probably may be a result of the increased capacity for ROS scavenging. The effects of inoculation on the expression of five metal transporter families (*ScPCS*, *ScMT*, *ScF-box*, *ScGR*, and *ScGST*) showed that these genes are differentially expressed in root tissues in response to different Cd treatment levels, suggesting that *ScPCS*, *ScMT*, *ScF-box*, *ScGR,* and *ScGST* might be involved in the response to Cd stresses in *S. coronaria*.

Taken together, the effects of symbiosis on physiological, biochemical, and molecular changes of *S. coronaria* plant suggest that *Rhizobium* inoculation with *Pseudomonas* could help host plant to cope with Cd toxicity. Therefore, combined inoculation seems to be substantially improving Cd toxicity alleviation in *S. coronaria*, which needs to be further studied in detail.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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