



PGPR-mediated alleviation of arsenic and cadmium toxicity in rice (*Oryza sativa* L.) through regulation of rhizosphere Microbiome and oxidative stress biomarkers

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Abstract

Heavy metal contamination, particularly by arsenic (As) and cadmium (Cd), severely impairs rice (*Oryza sativa* L.) growth, physiological performance, and food safety. This study evaluated the effectiveness of plant growth-promoting rhizobacteria (PGPR), including *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens*, in mitigating As and Cd toxicity in *O. sativa* under a controlled pot experiment. Plants exposed to individual As and Cd stress at 0, 100, and 200 mg kg⁻¹ exhibited marked reductions in plant growth, photosynthetic pigments, gas exchange parameters, antioxidant balance, nutrient uptake, and rhizosphere microbial diversity, along with pronounced increases in oxidative stress indicators. Conversely, *R. leguminosarum*, *B. subtilis*, and *S. marcescens* inoculation significantly improved plant growth and biomass, enhanced photosynthetic efficiency and gas exchange traits, strengthened antioxidant defense systems, and improved mineral nutrition. PGPR application reduced malondialdehyde and hydrogen peroxide levels, enhanced the ascorbate–glutathione cycle and proline metabolism, and limited As and Cd accumulation in plant tissues, resulting in reduced health risk indices. In addition, rhizosphere microbial diversity increased substantially, indicating improved soil microbial stability under metal stress. RT-qPCR-based gene expression analysis further supported PGPR-mediated enhancement of stress-responsive antioxidant and detoxification pathways. Overall, PGPR particularly *B. subtilis* proved effective in restoring physiological balance, reducing metal toxicity, and improving rhizosphere health. These findings highlight PGPR as a sustainable and eco-friendly strategy for improving rice performance and food safety in heavy metal-contaminated soils.

Keywords Phytoprotection · Rhizosphere · Detoxification · Antioxidants · Biostimulation · Metal toxicity

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Introduction

Heavy metal contamination of agricultural soils has emerged as a serious environmental and food safety concern due to its persistence and toxicity in plant systems (Alengebawy et al. 2021)(Sarraf et al. 2022). Among toxic metals, arsenic (As) and cadmium (Cd) are particularly hazardous because they interfere with essential physiological and metabolic processes, resulting in growth inhibition, oxidative damage, and yield reduction (Ali et al. 2022; Zulfiqar et al. 2022). In rice, arsenic disrupts photosynthesis, nutrient uptake, and cellular redox balance, ultimately impairing biomass accumulation and grain quality (Sun et al. 2021; Jalil et al. 2023). Cadmium similarly induces excessive reactive oxygen species production, damages cellular membranes, and suppresses enzymatic activities, leading to severe developmental constraints (Xue et al. 2023; Zhao et al. 2025). Given that rice is a staple crop for more than half of the global population and is highly prone to metal accumulation due to flooded cultivation practices, mitigating As and Cd toxicity is critical for ensuring food security and human health (Liu et al. 2019; Uddin et al. 2021). Therefore, improving rice growth and stress tolerance under metal-contaminated conditions is essential, and biological strategies such as PGPR inoculation offer promising, eco-friendly solutions.

Plant growth-promoting rhizobacteria (PGPR) represent an eco-friendly and sustainable approach for alleviating heavy metal stress in crops. These beneficial soil microbes enhance plant performance through multiple mechanisms, including improved nutrient availability, modulation of phytohormonal balance, reinforcement of antioxidant defense systems, and reduction of metal uptake and translocation (He et al. 2024; Chen et al. 2025). Several PGPR species like *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* have been reported to improve plant tolerance to metal stress by maintaining redox homeostasis and supporting physiological stability under adverse conditions. These PGPR have attracted attention due to their proven ability to promote plant growth, enhance stress resilience, and improve soil-plant interactions under contaminated environments (Khan and Singh 2021; Hasanuzzaman et al. 2022; Ilyas et al. 2022; Goyal and Habtewold 2023; Oware-sat et al. 2023; Kulkova et al. 2024). Therefore, investigating the effectiveness of these PGPR in alleviating heavy metal toxicity could provide important insights for developing viable microbial strategies to sustain agricultural productivity under metal-contaminated conditions.

Despite growing evidence supporting the role of PGPR in mitigating individual metal stresses, studies integrating their effects on plant physiology, molecular regulation, rhizosphere ecology, and food safety under dual As and Cd stress are scarce. Therefore, the present study aimed to

evaluate the comparative efficacy of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* in improving rice growth, photosynthetic performance, and antioxidant defense under combined As and Cd toxicity. In addition, the study investigates PGPR-mediated modulation of molecular responses related to stress tolerance, alterations in rhizosphere microbial diversity, and associated human health risk indices linked to metal accumulation in *O. sativa* tissues. By integrating plant performance, microbial interactions, and food safety assessment, this work addresses a critical knowledge gap and provides comprehensive insights into the potential of PGPR-based strategies for sustainable *O. sativa* production in metal-contaminated soils.

Materials and methods

Experimental setup and treatments

A controlled pot experiment was conducted under greenhouse conditions at the Department of Biotechnology, College of Science, Taif University, P.O. Box 11,099, Taif 21,944, Saudi Arabia. Pots were placed under glass house environment where they received natural sunlight, day/night humidity (60/70%) and day/night temperature (24/12°C), respectively. “*Oryza sativa* L. cv. IRRI-6” rice cultivar was used as a test plant and *O. sativa* seeds were taken from the Taif University. Before sowing, the seeds were carefully washed and sterilized in 0.1% HgCl₂ solution for 1 min and then washed three times with distilled water. Uncontaminated soil, obtained from the research field of Taif University, was air dried and passed through a 2-mm sieve. The initial physio-chemical properties of natural soil are provided in Supplementary Table S1. After contamination of soil with arsenic using sodium arsenate (Na₂HAsO₄·7 H₂O) and cadmium using cadmium chloride (CdCl₂·H₂O) (Direk et al. 2024), pots (30-cm-tall * 40-cm-wide) were filled with 10 kg of amended soil and undergone at four different cycles of water equilibrated for two months and then air. Arsenic was applied in the pentavalent form [As(V)] as sodium arsenate, and possible in situ redox transformations were not separately quantified but were considered within the natural soil redox dynamics under greenhouse conditions. After As and Cd supplementation, the soil pH was measured using a digital pH meter (soil: water ratio, 1:2.5) and remained within the neutral to slightly alkaline range (pH 7.3±0.2) throughout the experimental period. The pots used in this study were rotated regularly in order to avoid environmental effects on the plants. The total duration of experimental treatments was two months under controlled conditions. The experiment was laid out in a completely randomized design (CRD) with each treatment having 4 replicates of it.

No waterlogging was observed during the experiment, and intercultural operations were carried out as required. The plants were collected 60 days after sowing seeds for subsequent analysis. The detailed incubation process with plant growth-promoting rhizobacteria is described below. Analytical-grade chemicals were used throughout this study, all of which were acquired from Saudi Basic Industries Corporation, Saudi Arabia. A detailed schematic presentation of the entire methodology is provided in Fig. 1.

Inoculation with *R. leguminosarum*, *B. subtilis*, and *S. marcescens*

The bacterial strains *Rhizobium leguminosarum* RL-TU1 (GenBank accession no. OR999011), *Bacillus subtilis* BS-TU2 (OR999012), and *Serratia marcescens* SM-TU3 (OR999013) were selected based on their complementary plant growth-promoting traits, documented tolerance to metal-stressed environments, and reported relevance to cereal and rice rhizosphere systems. The strains represent functionally diverse PGPR groups with established roles in nutrient acquisition, redox regulation, and stress mitigation under adverse soil conditions. Each strain was cultured separately in Luria–Bertani (LB) broth at 28 °C for 24 h under continuous shaking to ensure optimal growth. After incubation, cultures were centrifuged to collect bacterial

cells, which were resuspended in sterile distilled water to obtain a uniform suspension with a final concentration of 10^8 CFU mL⁻¹ (Ballesteros-Gutiérrez et al. 2023; Liu et al. 2024; Fatima et al. 2025). Prior to sowing, *Oryza sativa* L. seeds were soaked in the respective bacterial suspensions for 2 h to promote effective root surface association. In addition, PGPR treatments were applied via soil drenching at a volume of 30 mL per pot using bacterial suspensions at concentrations of 5 ppm and 10 ppm according to the experimental design. To support sustained rhizosphere establishment of the inoculated strains, soil drenching was repeated at 15-day intervals throughout the experimental period. For non-PGPR treatments, a mock inoculum was prepared following the same protocol without bacterial cells, and sterile distilled water was applied at identical volumes and intervals to ensure consistent experimental conditions.

Plant harvesting and data collection

After 2 months, the remaining four plants were uprooted and washed gently with the help of distilled water to eliminate the aerial dust and deposition. Functional leaf in each treatment was picked at a rapid growth stage during 09:00 and 10:30. The sampled leaves were washed with distilled water, immediately placed in liquid nitrogen, and stored in a freezer at -80 °C for further analysis. All of the harvested

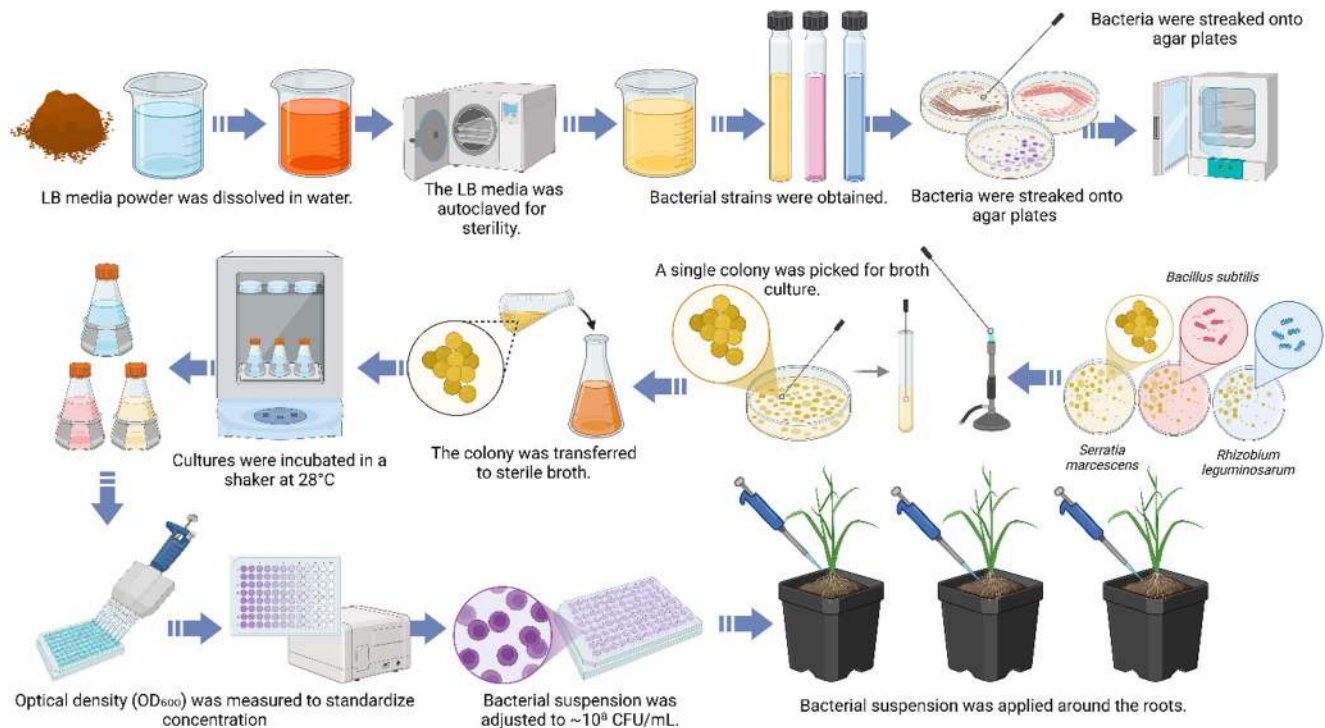


Fig. 1 Schematic representation of bacterial strain preparation and inoculation process used in the experiment. LB media was prepared, sterilized, and inoculated with *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* strains. After incubation at 28 °C,

a single colony was transferred to broth culture, and bacterial growth was standardized by measuring optical density (OD₆₀₀). The bacterial suspension (~10⁸ CFU mL⁻¹) was then applied around the root zone of rice (*Oryza sativa* L.) plants

plants were divided into two parts (i.e., roots and shoots) to study different physio-biochemical traits. Leaves from each treatment group were picked for chlorophyll, carotenoid, oxidative stress, and antioxidants analysis. Root and shoot lengths were measured straightway after the harvesting using a measuring scale and digital weighting balance to measure fresh biomass. Roots were uprooted and immersed in 20-mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) for 15–20 min to remove As and Cd adhered to the root surfaces. Then, roots were washed thrice with distilled water and, finally, once with de-ionized water, and dried for further analysis. The different parts of the plant (i.e., roots and shoots) were oven-dehydrated at 65 °C for 72 h for As and Cd determination, and the total plant dry weight was also measured.

Determination of photosynthetic pigments and gas exchange characteristics

Leaves were collected for the determination of chlorophyll and carotenoid contents. For chlorophylls, 0.1 g of fresh leaf sample was extracted with 8 mL of 95% acetone for 24 h at 4 °C in the dark. The absorbance was measured by a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan) at 646.6, 663.6, and 450 nm. Chlorophyll content was calculated by the standard method of (Arnon 1949; Anas et al. 2024a, b, c).

Net photosynthesis (P_n), leaf stomatal conductance (G_s), transpiration rate (T_s), and intercellular carbon dioxide concentration (C_i) were measured from four different plants in each treatment group. Measurements were conducted between 11:30 and 13:30 on days with a clear sky. Rates of leaf P_n , G_s , T_s , and C_i were measured with a LI-COR gas-exchange system (LI-6400; LI-COR Biosciences, Lincoln, NE, USA) with a red-blue LED light source on the leaf chamber. In the LI-COR cuvette, CO_2 concentration was set as 380 mmol mol^{-1} and LED light intensity was set at 1000 $\text{mmol m}^{-2} \text{s}^{-1}$, which was the average saturation intensity for photosynthesis in *O. sativa* (Austin 1990).

Determination of oxidative stress indicators

Markers of oxidative stress were evaluated to determine the extent of cellular damage under metal stress. Leaf tissues were homogenized in an ice-cold phosphate buffer, and lipid peroxidation and reactive oxygen species levels were determined spectrophotometrically following standard colorimetric assays. Values were expressed relative to fresh tissue weight and used as indicators of oxidative damage (Heath and Packer 1968; Jana and Choudhuri 1981).

Determination of antioxidant enzyme activities and relative gene expression

To assess the antioxidant response, enzyme extracts were prepared from frozen leaf samples using phosphate buffer under chilled conditions. Antioxidant activity was then measured through colorimetric enzyme assays, and absorbance was read at characteristic wavelengths corresponding to each reaction system. The results were calculated and normalized against total protein content (Nakano and Asada 1981; Aebi 1984; Chen and Pan 1996; Sakharov and Ardila 1999). Non-enzymatic antioxidants including total phenolics, flavonoids, anthocyanins, and ascorbic acid were extracted from fresh or frozen tissues using appropriate solvents. Quantification was carried out using well-established colorimetric methods involving reagents such as Folin–Ciocalteu or aluminum chloride, and the values were recorded using a UV-visible spectrophotometer. Standard calibration curves were used for quantification and the results were expressed per gram of fresh tissue (Bates et al. 1973; Anas et al. 2024a, b, c).

For molecular assessment, total RNA was extracted from frozen leaf tissues using a commercial RNA isolation kit according to the manufacturer's protocol. The extracted RNA was reverse-transcribed into cDNA, and expression levels of target genes associated with antioxidant responses and metal tolerance were analyzed using quantitative real-time PCR (qRT-PCR). Gene expression levels were normalized against a housekeeping gene and quantified using the $2^{-\Delta\Delta\text{Ct}}$ method (Sirhindi et al. 2016) and amplifications are presented in Table S2. Primer specificity and amplification efficiency were validated prior to expression analysis, and all primers showed efficiencies between 90% and 105%, meeting the requirements for the $2^{-\Delta\Delta\text{Ct}}$ method.

Determination of nutrient contents and Cu and as uptake

To determine the accumulation of As and Cd, oven-dried shoot and root samples were digested using a mixture of nitric acid and perchloric acid. The digested solution was filtered and analyzed using an atomic absorption spectrophotometer (Riaz et al. 2022). Metal concentrations were expressed as mg kg^{-1} dry weight of plant tissue (Amacher 1996; Anas et al. 2023). Essential nutrients such as calcium, magnesium, phosphorus, and iron were quantified from acid-digested plant material. Dried tissue samples were subjected to wet digestion, and the resulting extracts were analyzed using atomic absorption spectroscopy or colorimetric methods depending on the element. The concentration of each nutrient was expressed on a dry weight basis (Turner and Rust 1971).

Biochemical assay of GSH and AsA in plant tissues

Leaf tissues were homogenized under cold conditions in a suitable extraction buffer to isolate redox-related metabolites. The levels of ascorbate, dehydroascorbate, GSH and GSSG were determined using established spectrophotometric techniques. All values were calculated using standard calibration curves and expressed per unit fresh weight (Singh et al. 2015; Anas et al. 2024a, b, c).

Measuring proline and cell wall component fractionation

Proline content was measured using the acid ninhydrin method where leaf tissues were homogenized in sulfo-salicylic acid and reacted with ninhydrin reagent. Absorbance was measured at 520 nm, and values were quantified using a proline standard curve. Enzymes related to proline metabolism were assayed using crude enzyme extracts and spectrophotometric protocols under controlled temperature conditions (Bates et al. 1973). To analyze cellular components related to structural defense, cell wall materials contents were measured from dried and ground plant material. Fractionation was carried out through sequential extraction steps using acidic and basic solvents, followed by colorimetric quantification. Values were expressed as a percentage of dry biomass, indicating the structural adaptation of cell walls under stress (Yang et al. 2011).

Rhizosphere microbiome, health risk, RT-qPCR-based gene expression responses

To examine the microbial diversity in the rhizosphere, soil closely attached to rice roots was collected and processed for metagenomic DNA extraction using a soil-specific DNA isolation kit under sterile conditions. Microbial community assessment focused on alpha-diversity metrics to evaluate functionally relevant rhizosphere responses to metal stress and PGPR inoculation. Targeted amplification of bacterial 16 S rRNA (V3–V4) and fungal ITS1 regions was conducted using region-specific primers, followed by high-throughput sequencing on an Illumina MiSeq platform. For bacterial community analysis, the 16 S rRNA V3–V4 region was amplified using universal primers 341 F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), while fungal ITS1 regions were amplified using ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATG C-3'). These primers are widely used for microbial community profiling (Toju et al. 2012; Klindworth et al. 2013). Raw sequence data were analyzed using the QIIME2 pipeline to construct operational taxonomic units, and microbial

community richness and diversity were quantified using alpha diversity indices, including Chao1 and Shannon (Vlachos et al. 1996). To evaluate the dietary exposure risk of As and Cd, the health risk index (HRI) was calculated using standard toxicological parameters. The daily intake of metals (DIM) was estimated using the formula $DIM = (C \times IR) / BW$, where C represents the metal concentration in edible rice tissues ($mg\ kg^{-1}$), IR denotes the average daily rice consumption rate ($0.345\ kg\ person^{-1}\ day^{-1}$), and BW represents the average adult body weight (70 kg). The HRI was calculated as the ratio of DIM to the oral reference dose (RfD). The RfD values used for As and Cd were 0.0003 and $0.001\ mg\ kg^{-1}\ day^{-1}$, respectively, following USEPA guidelines as reported by (Rosser 1993). For protein profiling, leaf tissues were finely powdered in liquid nitrogen, and total soluble proteins were extracted using a buffer containing Tris-HCl, sucrose, and β -mercaptoethanol in a phenol phase. Protein precipitation was carried out using cold acetone supplemented with ammonium acetate, and total protein content was quantified using the Bradford assay. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was employed to resolve protein banding patterns, which were subsequently analyzed using image densitometry. The analysis focused on comparative changes in protein band intensity across treatments rather than on protein identification. Prominent bands within the molecular weight range of approximately 50–70 kDa were evaluated as indicative stress-responsive protein fractions. Relative band intensities were quantified to assess treatment-induced changes in overall protein expression patterns.

Sequence analysis, taxonomic classification, and data submission

Raw 16 S rRNA (V3–V4) and ITS1 sequencing reads obtained from the rhizosphere samples were processed using the QIIME2 platform (version 2023.2) (Bolyen et al. 2019). Low-quality bases and sequencing adapters were trimmed, and chimeric sequences were removed using the DADA2 plugin (Callahan et al. 2016). High-quality reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the VSEARCH algorithm (Rognes et al. 2016). Taxonomic assignment of bacterial reads was performed against the SILVA v138 reference database (Quast et al. 2012), while fungal ITS sequences were classified using the UNITE v8.3 database (Nilsson et al. 2019). Alpha-diversity indices, including Chao1, Shannon, and Simpson, were computed within QIIME2 to assess microbial richness and diversity under different treatment conditions.

Statistical analysis

The experiment was conducted using a completely randomized design with four biological replicates per treatment. All quantitative data were statistically analyzed using Statistix 8.1 software by applying analysis of variance (ANOVA) to evaluate treatment effects. Mean comparisons were performed using the least significant difference (LSD) test at a 5% probability level ($P \leq 0.05$). Results are presented as means $\pm$ standard error (SE). To ensure biological relevance, emphasis in the Results section was placed on consistent and treatment-responsive trends rather than marginal numerical differences. Parameters showing minor variations without clear treatment-related patterns were interpreted cautiously and not overemphasized. Graphical visualization of key responses was performed using OriginPro 2019 to facilitate interpretation of treatment effects. This statistical approach has been widely used in controlled pot experiments to assess physiological, biochemical, and molecular responses under stress conditions.

Results

Plant growth and photosynthetic pigments

In the present study, various growth parameters and photosynthetic pigments and also the gas exchange parameters in rice (*Oryza sativa* L.) under individual arsenic (As) and cadmium (Cd) stress with the application of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* were measured. Growth and biomasses of *O. sativa* are presented in Fig. 2, while gas exchange attributes are presented in Fig. 3. According to the given results, As and Cd caused a significant toxicity in *O. sativa* and decreases the root length, shoot length, shoot dry weight, root dry weight, chlorophyll-a, chlorophyll b, chlorophyll a/b, carotenoid, net photosynthesis, stomatal conductance, transpiration rate and intercellular CO₂. However, application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* caused increase in root length, shoot length, shoot dry weight, root dry weight, chlorophyll-a, chlorophyll b, chlorophyll a/b, carotenoid, net photosynthesis, stomatal conductance, transpiration rate and intercellular CO₂ compared to the plants which were grown in the control. The application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens*, when applied to the plants which were not treated with individual As and Cd, there was a significant increase in root length, shoot length, shoot dry weight, root dry weight, chlorophyll-a, chlorophyll b, chlorophyll a/b, carotenoid, net photosynthesis, stomatal conductance, transpiration rate and intercellular CO₂ was observed in the plants compared to those which were not

treated with the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens*.

Oxidative stress and response of antioxidants

In the present study different oxidative stress biomarkers i.e., malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) were measured from *O. sativa* as presented in Fig. 6. According to the results, it was observed that the individual As and Cd caused a significant increase in MDA and H₂O₂ content in the plants compared to the control. Although the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* decreases the MDA and H₂O₂ content in *O. sativa*. We have also noticed that the individual application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* also decreases MDA and H₂O₂ content, when plants were grown without the contamination of As and Cd in the soil.

Different enzymatic antioxidants i.e., superoxidase dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT) and non-enzymatic compounds i.e., phenolic, anthocyanin, flavonoids, ascorbic acid and flavonoid and also their relevant gene expression i.e., SOD, POD, CAT and APX were also measured from the leaves of *O. sativa*. The results regarding the enzymatic antioxidants and their relevant gene expression are presented in Figs. 4 and 5 respectively, and the results regarding the non-enzymatic compounds are presented in Fig. 6. According to the results, we have noticed that the individual As and Cd stress in the soil significantly increases the enzymatic antioxidants i.e., SOD, POD, CAT and APX and their relevant gene expression and also the non-enzymatic compounds i.e., phenolic, anthocyanin, flavonoids, ascorbic acid and flavonoid compared to the plants grown in the control treatment. Present findings also showed that the alone application of either *R. leguminosarum*, *B. subtilis*, and *S. marcescens* also increases the activity of SOD, POD, CAT and APX and their relevant gene expression and also the non-enzymatic compounds i.e., phenolic, anthocyanin, flavonoids, ascorbic acid and flavonoid compared to the plants which were not treatment with the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* in individual As and Cd stressed soil and also the normal soil i.e., which was not spiked with individual As and Cd.

Nutrients and metal uptake

The assimilation of nutrients in shoots of *O. sativa* was examined in this study, particularly under the influence of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* in As and Cd-contaminated soil. Figure 6 illustrates the nutrient profiles in the shoots of *O. sativa* subjected to *R. leguminosarum*, *B. subtilis*, and *S. marcescens* and As and Cd toxicity.

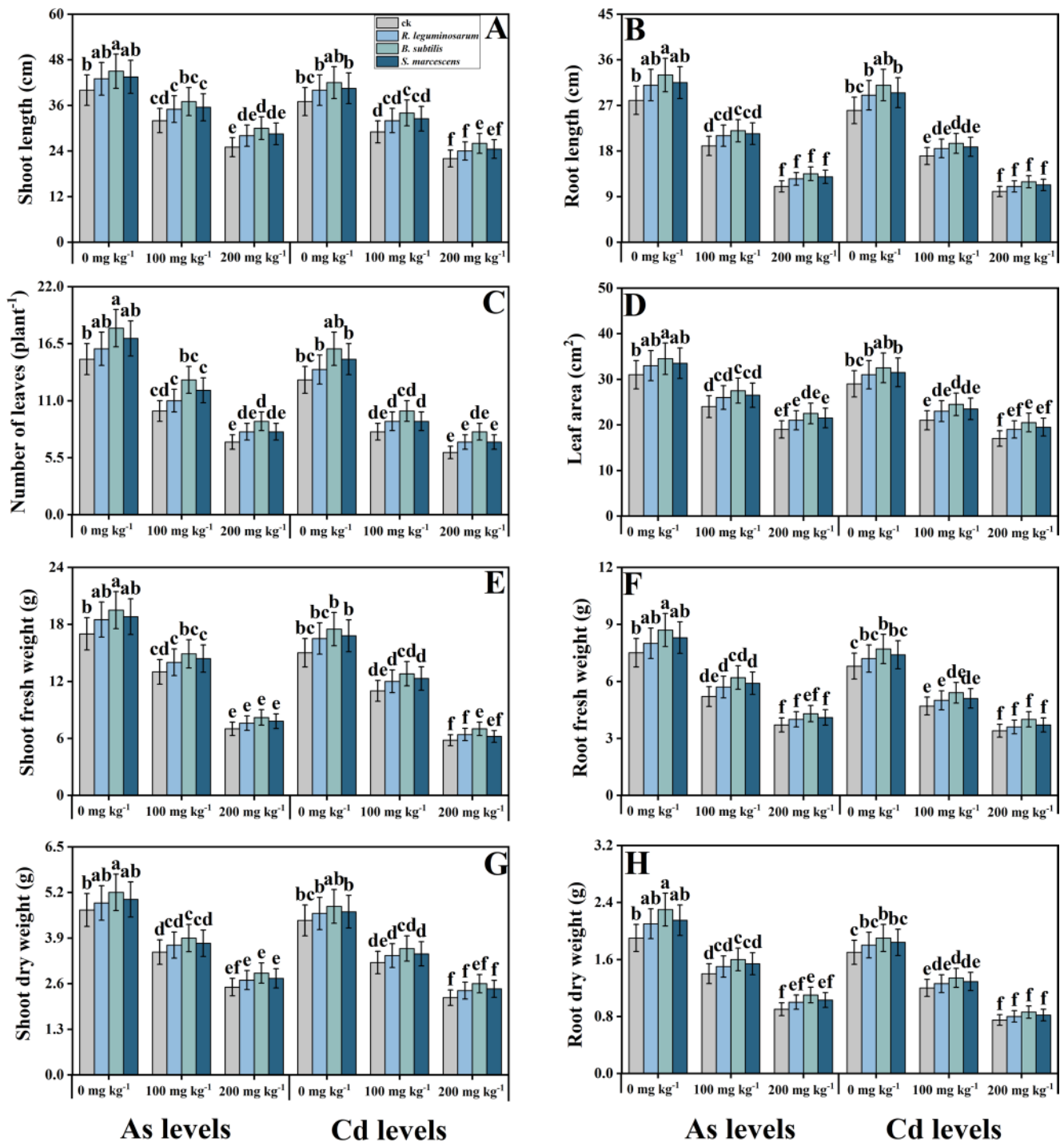


Fig. 2 Effects of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* on growth and biomass attributes of rice (*Oryza sativa* L.) under arsenic (As) and cadmium (Cd) stress. Panels show (A) shoot length, (B) root length, (C) number of leaves, (D) leaf area, (E) shoot fresh weight, (F) root fresh weight, (G) shoot dry weight,

and (H) root dry weight under different As and Cd levels. Bars represent mean $\pm$ SE ($n=3$). Different lowercase letters above the bars indicate significant differences among treatments at $P \leq 0.05$ according to Tukey's HSD test

The results showed that essential nutrients (Ca^{2+} , Mg^{2+} , Fe^{2+} and P) were significantly reduced in plants grown in individual As and Cd-contaminated soil compared to those grown in soil without As and Cd. Notably, the application

of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* significantly contributed to the increased uptake of these essential nutrients (Ca^{2+} , Mg^{2+} , Fe^{2+} and P) in As and Cd-affected soil, as depicted in Fig. 6. Furthermore, the endogenous As

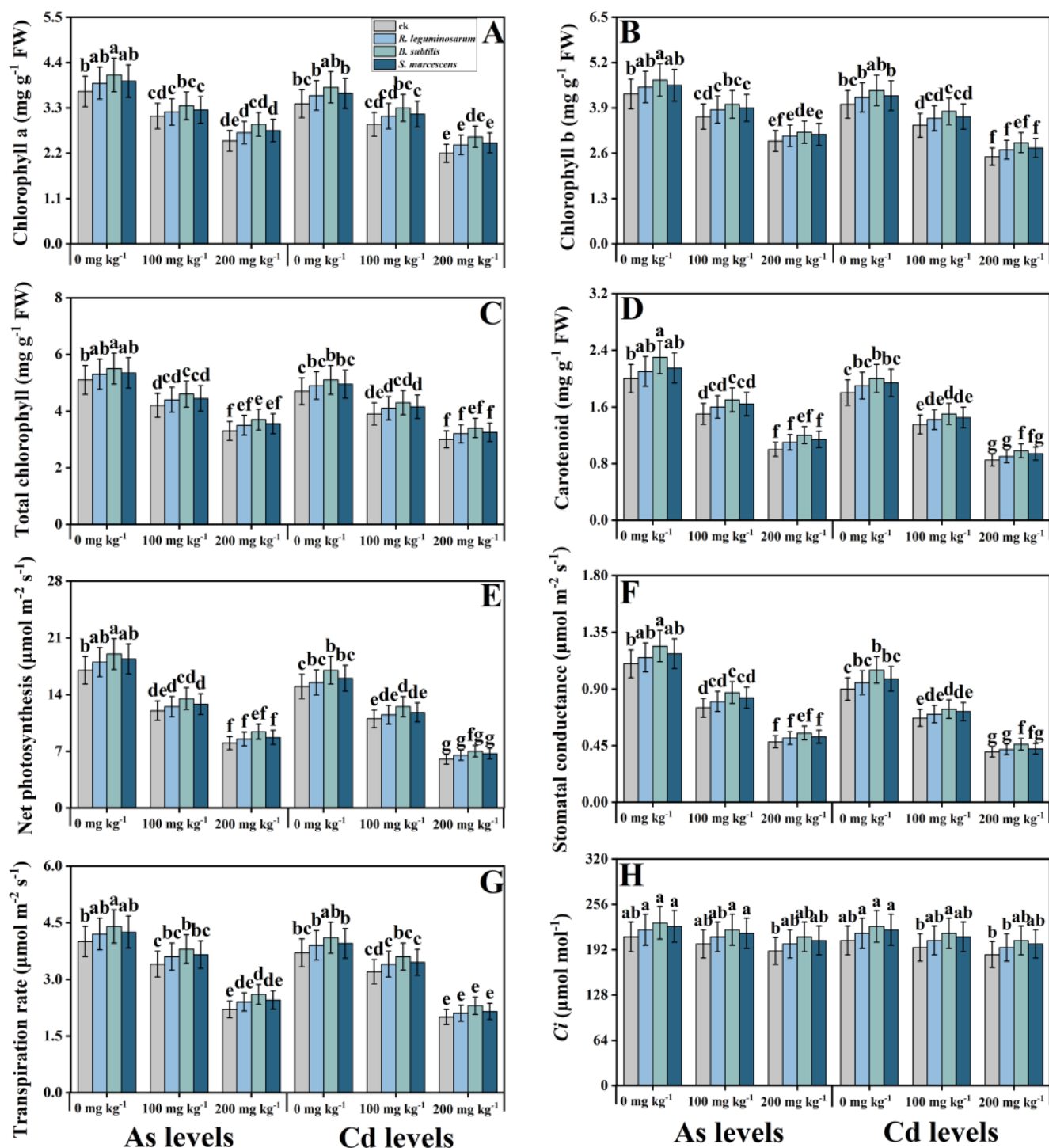


Fig. 3 Effects of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* on photosynthetic pigments and gas exchange attributes of *O. sativa* under As and Cd stress. Panels represent (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) carotenoid content, (E) net photosynthesis, (F) stomatal conductance, (G) transpiration rate, and (H)

intercellular CO₂ concentration (Ci) under different As and Cd levels. Bars indicate mean ± SE (*n* = 3). Different lowercase letters above the bars denote significant differences among treatments at *P* ≤ 0.05 according to Tukey's HSD test

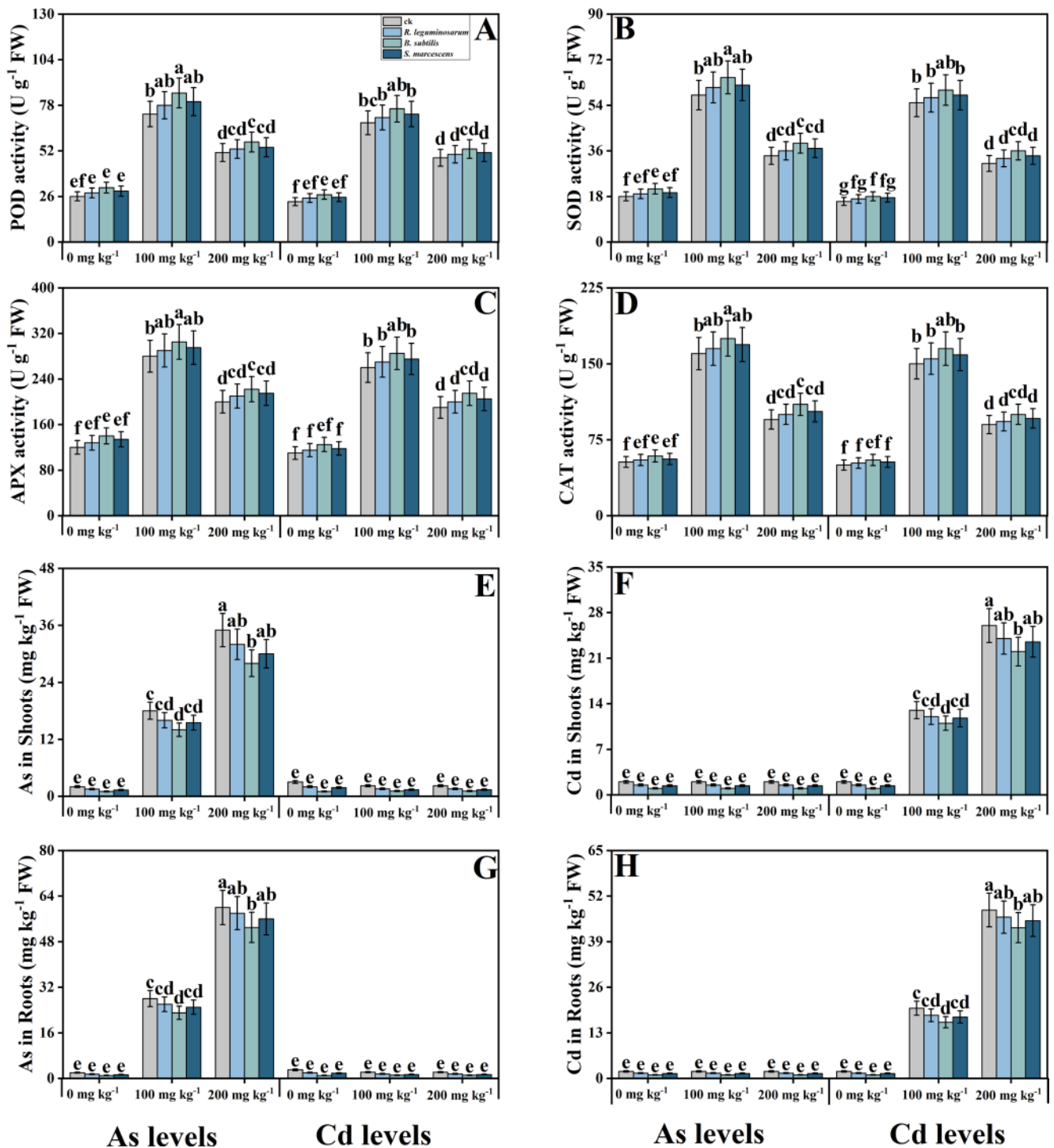


Fig. 4 Effect of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* on antioxidant enzyme activities and heavy metal accumulation in rice (*Oryza sativa* L.) plants under arsenic (As) and cadmium (Cd) stress. (A) Peroxidase (POD) activity, (B) Superoxide dismutase (SOD) activity, (C) Ascorbate peroxidase (APX) activity,

(D) Catalase (CAT) activity, (E) Arsenic content in shoots, (F) Cadmium content in shoots, (G) Arsenic content in roots, and (H) Cadmium content in roots. Values represent mean ± SE ($n=3$). Bars followed by different letters indicate significant differences at $P \leq 0.05$ according to Tukey's HSD test

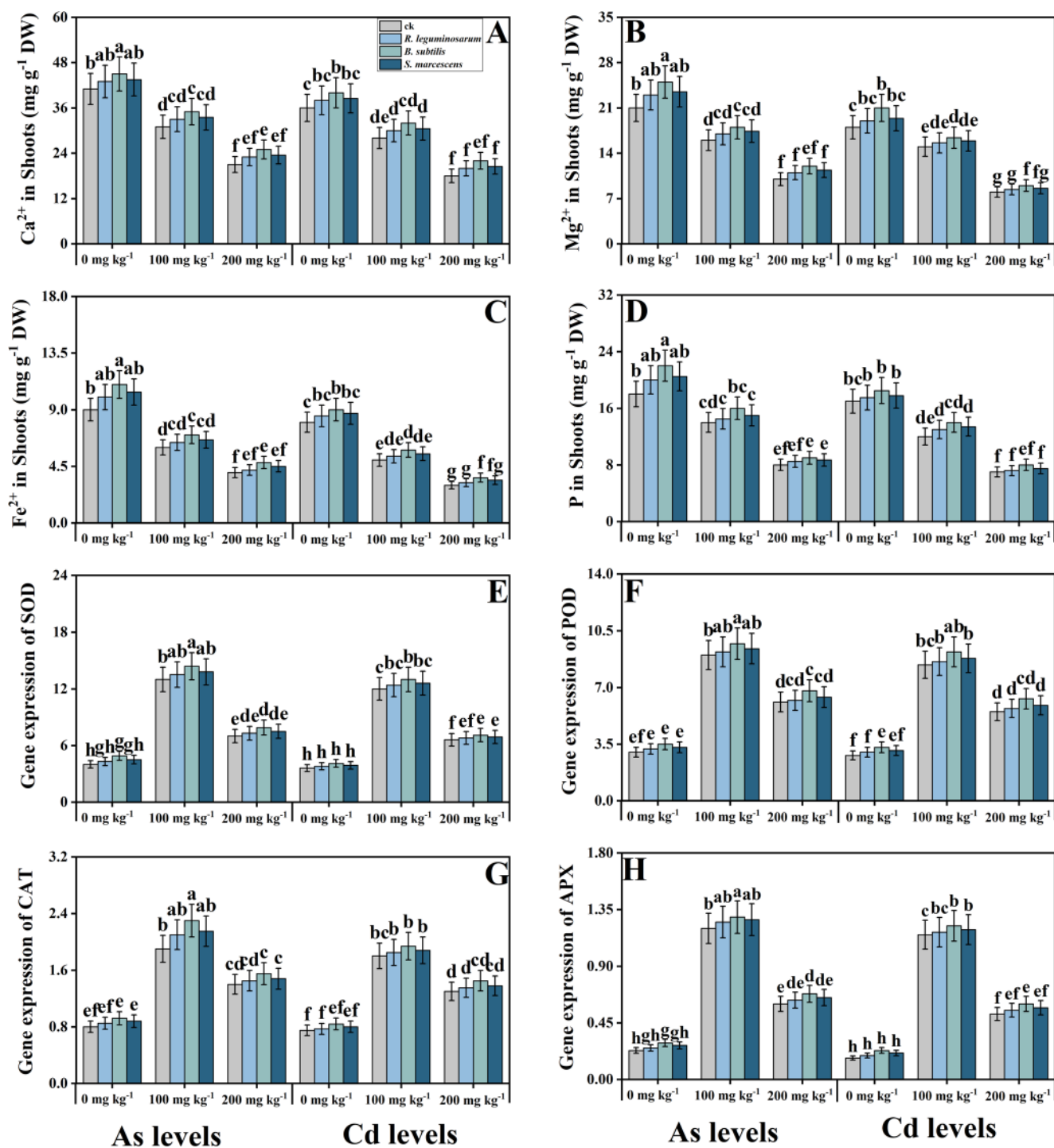


Fig. 5 Effect of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* on nutrient uptake and gene expression of antioxidant enzymes in *O. sativa* under As and Cd stress. (A) Calcium (Ca^{2+}), (B) Magnesium (Mg^{2+}), (C) Iron (Fe^{2+}), (D) Phosphorus (P), (E) Superoxide dismutase

(SOD), (F) Peroxidase (POD), (G) Catalase (CAT), and (H) Ascorbate peroxidase (APX). Values represent mean $\pm$ SE ($n=3$). Bars followed by different letters indicate significant differences at $P \leq 0.05$ according to Tukey's HSD test

and Cd, concentrations in both roots and shoots of *O. sativa* treated with *R. leguminosarum*, *B. subtilis*, and *S. marcescens* and grown in As and Cd-toxic soil are presented in Fig. 4. A significant increase in endogenous both As and

Cd levels was observed in both plant parts under As and Cd-contaminated conditions compared to the plants grown in natural soil. *O. sativa* cultivated in As and Cd-free soil showed very low As and Cd concentrations in both aerial

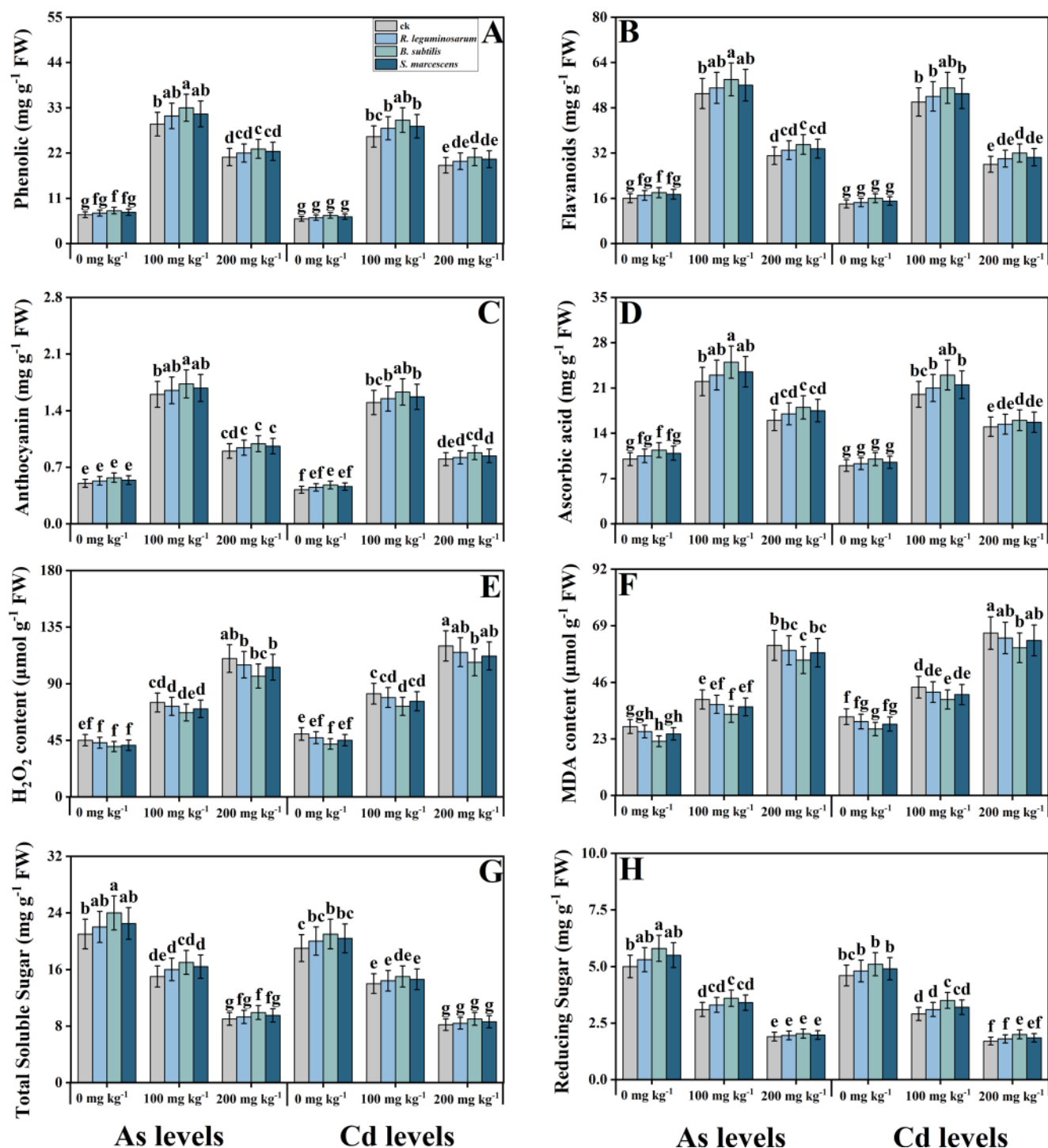


Fig. 6 Effect of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* on non-enzymatic antioxidants and oxidative stress biomarkers in *O. sativa* under As and Cd stress. (A) Total phenolic content, (B) Flavonoid content, (C) Anthocyanin content, (D) Ascorbic acid, (E) Hydro-

gen peroxide (H₂O₂) content, (F) Malondialdehyde (MDA) content, (G) Total soluble sugars, and (H) Reducing sugars. Values represent mean $\pm$ SE ($n=3$). Bars followed by different letters indicate significant differences at $P \leq 0.05$ according to Tukey's HSD test

and underground parts. However, the external application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* resulted in a significant reduction of As and Cd content in the seed primed plants compared to those that were not primed with *R. leguminosarum*, *B. subtilis*, and *S. marcescens*.

AsA-GSH cycle, proline metabolism and cellular fractionation

In the present study the proline metabolism such as proline, pyrroline-5-carboxylate, pyrroline-5-carboxylate reductase and pyrroline-5-carboxylate dehydrogenase were also measured from the *O. sativa* tissue and results showed that the individual As and Cd toxicity induced a significant decrease in the proline, pyrroline-5-carboxylate, pyrroline-5-carboxylate reductase and pyrroline-5-carboxylate dehydrogenase compared to the control (Fig. S1). However, the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* induced a significant increase in the content of proline, pyrroline-5-carboxylate, pyrroline-5-carboxylate reductase and pyrroline-5-carboxylate dehydrogenase compared to those plants which were grown without the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens*.

AsA-GSH cycle including the contents of glutathione, ascorbate, glutathione disulfide, dehydroascorbic acid was also measured and we have noticed that the individual As and Cd toxicity significantly decreases the content of glutathione, ascorbate and dehydroascorbic acid while increases the content of glutathione disulfide from the tissues of the *O. sativa* (Fig. S1). We have also noticed that the plants which were not contaminated with the As and Cd and applied with the individual application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* showed a significant increase in the content of glutathione, ascorbate, dehydroascorbic acid and glutathione disulfide when compared to the plants which were not applied with the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens*.

Cellular compartments fractionation i.e., pectin methylesterase activity, uronic acid, hemicellulose I, hemicellulose II, cellulose and pectin methylesterase were also determined from the *O. sativa* and presented as Fig. S2. Results from the present study showed that the individual As and Cd stress causes a significant increase in the pectin methylesterase activity, uronic acid, hemicellulose I, hemicellulose II, cellulose and pectin methylesterase when compared to the control. In addition, the plants grown without the concentration of As and Cd in the soil decreases the pectin methylesterase activity, uronic acid, hemicellulose I, hemicellulose II, cellulose and pectin methylesterase compared to those which were not applied with the application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens*. However, application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* to the

plants which were spiked with the As and Cd concentration, further increases the content of pectin methylesterase activity, uronic acid, hemicellulose I, hemicellulose II, cellulose and pectin methylesterase.

Rhizosphere microbiome, health risk, RT-qPCR-based gene expression responses

In the present study, rhizosphere microbial diversity, community structure, health risk indices, and molecular responses were evaluated in *O. sativa* plants under combined As and Cd stress. The results revealed that As and Cd stress markedly reduced bacterial and fungal richness and diversity compared to the control, as indicated by lower Chao1 and Shannon indices (Table 1). However, inoculation with *R. leguminosarum*, *B. subtilis*, and *S. marcescens* significantly improved both bacterial and fungal diversity under metal stress conditions. In addition to microbiome responses, As and Cd stress significantly increased the health risk index (HRI) values for both As and Cd in *O. sativa* tissues compared to the control. The application of *R. leguminosarum*, *B. subtilis*, and *S. marcescens* effectively reduced HRI values, indicating a decreased potential dietary risk associated with metal accumulation in *R. leguminosarum*, *B. subtilis*, and *S. marcescens* -treated plants. Protein banding analysis revealed that As and Cd stress altered overall protein expression patterns in *O. sativa* leaves, as evidenced by reduced intensity of several prominent protein bands compared to the control. In contrast, inoculation with *R. leguminosarum*, *B. subtilis*, and *S. marcescens* partially restored the intensity of these protein bands, indicating an improvement in protein expression stability under metal stress conditions. These changes reflect treatment-induced modulation of protein profiles rather than specific protein regulation. Consistent with these observations, RT-qPCR-based gene expression analysis showed that As and Cd stress downregulated key antioxidant defense genes, while inoculation with *R. leguminosarum*, *B. subtilis*, and *S. marcescens* significantly enhanced their expression, indicating improved molecular stress responsiveness in *O. sativa* under As and Cd stress.

Discussion

The present study demonstrates that inoculation with *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* effectively mitigated the adverse effects of arsenic (As) and cadmium (Cd) stress in rice (*Oryza sativa* L.) through interconnected physiological, biochemical, microbial, and molecular mechanisms. Rather than acting through a single pathway, PGPR-mediated stress alleviation appeared to result from coordinated modulation of rhizosphere microbial

Table 1 Effects of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* inoculation on rhizosphere Microbiome diversity, health risk indices, RT-qPCR-based gene expression responses in rice plants under as and cd stress

Treatment	Bacterial Richness (Chao1)	Bacterial Diversity (Shannon index)	Fungal Richness (Chao1)	Fungal Diversity (Shannon index)	Health Risk Index (HRI for As)	Health Risk Index (HRI for Cd)	Differential Protein Expression (%)	Antioxidant-related Gene Expression (fold change)	Metal Transporter Gene Expression (fold change)	Detoxification-related Gene Expression (fold change)
Control	50.0±1.1 F	96.1±4.9 B	78.6±4.3 C	67.9±1.8 B	32.5±1.7 B	32.5±1.7 B	24.6±2.2 A	89.3±3.1 A	68.1±2.7 A	76.6±2.2 C
As stress	68.9±3.4 F	31.2±1.7 E	43.4±1.3 B	49.3±4.8 B	56.5±4.9 C	82.8±4.2 B	36.0±2.2 A	61.1±1.4 E	67.4±3.7 D	23.7±2.8 B
Cd stress	29.8±4.9 A	59.6±4.1 D	22.8±4.8 E	92.7±4.6 D	40.7±3.4 A	73.0±4.7 F	44.9±1.4 F	61.6±1.8 D	63.7±1.2 C	34.8±2.3 D
As + Cd stress	51.1±4.1 B	41.7±1.8 B	86.3±1.0 F	48.5±4.3 C	42.5±3.8 A	63.4±3.9 B	31.3±4.1 F	84.2±1.3 E	26.0±2.4 F	99.0±1.5 B
As + <i>R. leguminosarum</i>	89.0±1.5 B	69.9±3.9 F	46.5±4.0 A	25.1±3.2 D	44.9±4.1 B	46.0±3.0 F	78.4±3.1 F	71.0±2.7 C	91.0±1.1 D	57.8±1.4 E
Cd + <i>R. leguminosarum</i>	22.5±2.2 A	70.9±1.6 E	45.1±4.7 D	60.7±4.2 D	92.6±3.5 D	39.9±4.5 E	52.8±4.2 D	80.4±1.7 F	38.3±4.6 E	26.2±3.2 D
As + Cd + <i>R. leguminosarum</i>	84.6±2.7 F	91.7±1.9 C	45.4±1.5 D	28.8±2.4 E	38.2±4.8 B	54.2±2.3 D	85.4±3.1 B	88.9±3.8 F	20.6±2.5 C	60.9±4.9 A
As + <i>B. subtilis</i>	49.4±3.7 E	40.1±2.0 D	59.8±1.6 B	44.1±3.0 B	42.8±4.9 E	23.0±2.0 B	68.8±3.7 E	60.2±4.0 F	24.1±2.0 A	42.3±3.9 D
Cd + <i>B. subtilis</i>	47.3±1.4 E	70.6±1.1 A	70.7±3.0 B	62.9±1.9 B	27.2±3.6 A	86.8±1.7 B	45.7±3.8 F	34.9±2.5 F	23.3±4.7 A	67.3±1.6 E
As + Cd + <i>B. subtilis</i>	71.4±3.2 E	29.1±4.6 A	94.0±4.6 E	90.2±3.5 E	40.6±2.4 F	72.8±2.4 E	85.4±3.9 C	64.4±4.6 D	62.4±4.5 B	39.3±4.1 C
As + <i>S. marcescens</i>	72.6±4.2 F	65.5±3.0 D	32.9±3.6 A	91.9±1.9 E	68.5±3.8 F	20.7±1.9 D	28.1±2.3 E	73.1±4.0 A	20.4±3.6 F	32.9±4.4 F
Cd + <i>S. marcescens</i>	95.2±2.2 F	96.3±2.5 E	93.2±4.4 B	49.4±3.0 D	41.2±1.8 B	39.5±3.9 B	97.8±2.1 E	51.4±1.1 D	91.4±3.6 A	70.5±1.7 F
As + Cd + <i>S. marcescens</i>				49.6±2.3 B	21.2±1.7 F	94.3±3.2 E	54.3±4.7 D	97.3±3.8 B	97.1±3.3 D	88.2±1.4 B

Values represent the mean±standard deviation (n=4). Different letters (A–F) within a column indicate significant differences among treatments according to the least significant difference (LSD) test at $P \leq 0.05$, where 'A' represents the highest and 'F' the lowest values

structure, antioxidant defense systems, nutrient acquisition, and restriction of metal translocation (Yang et al. 2021; Liu et al. 2022; Alshididi et al. 2024; Anas et al. 2025). One of the key mechanisms underlying stress mitigation was the restoration of rhizosphere microbial diversity under metal stress (Goyal and Habtewold 2023; Zhou et al. 2023; Ai et al. 2025). Heavy metals are known to disrupt native soil microbial communities, thereby impairing nutrient cycling and root–microbe interactions. In this study, *R. leguminosarum*, *B. subtilis*, and *S. marcescens* inoculation significantly improved bacterial and fungal diversity, indicating re-establishment of a functionally active rhizosphere (Zheng et al. 2022; Balti et al. 2025; Wang et al. 2025). Such microbial restructuring likely enhanced nutrient solubilization and availability, which in turn supported improved plant growth and photosynthetic performance (Singh et al. 2022; Song et al. 2024). Similar links between rhizosphere microbial diversity and nutrient uptake efficiency under metal stress have been reported previously.

Improved antioxidant defense constituted another central mechanism contributing to stress tolerance. Arsenic and cadmium stress markedly elevated oxidative damage, as reflected by increased hydrogen peroxide and malondialdehyde levels (Asgher et al. 2021; Arshed and Saleem 2024; Ozoani et al. 2024), whereas PGPR application consistently reduced these oxidative stress markers (Sharma et al. 2022) (Sarraf et al. 2024). This reduction was associated with enhanced activities and gene expression of antioxidant enzymes, as well as improved functioning of the AsA–GSH cycle, indicating strengthened redox homeostasis (Nivetha et al. 2021; Alhaj Hamoud et al. 2025). By limiting lipid peroxidation and preserving membrane integrity, enhanced antioxidant capacity likely reduced metal mobility within plant tissues, thereby restricting excessive translocation of As and Cd from roots to shoots (Goncharuk and Zagorskina 2023) (Shomali et al. 2023). *R. leguminosarum*, *B. subtilis*, and *S. marcescens*-mediated modulation of nutrient uptake and metal exclusion further contributed to stress alleviation. Inoculated plants exhibited improved acquisition of essential nutrients while simultaneously limiting As and Cd accumulation in tissues (Syed et al. 2023; Danish et al. 2025). This dual effect suggests that PGPR may influence root ion selectivity and rhizosphere chemistry, favoring essential elements over toxic metals (Gupta et al. 2024) (Bouzroud et al. 2025). Strengthening of cell wall components, including pectin and cellulose fractions, may also have contributed to metal immobilization at the root interface, thereby reducing internal metal transport and associated toxicity.

At the molecular level, comparative protein banding patterns in SDS–PAGE and RT–qPCR–based gene expression analyses indicated enhanced stress adaptation in *R. leguminosarum*, *B. subtilis*, and *S. marcescens*-inoculated plants,

consistent with previous reports that plant growth-promoting rhizobacteria can induce changes in stress-responsive gene expression and antioxidant enzyme profiles under abiotic stress condition (Abdelkrim et al. 2023; Saleem et al. 2024). Metal stress altered overall protein expression patterns and suppressed the expression of antioxidant-related genes (Singh et al. 2025), whereas PGPR inoculation partially restored protein band intensity and enhanced expression of key stress-responsive genes (El-Ballat et al. 2023). These molecular responses, although targeted rather than genome-wide, align with improved physiological performance and antioxidant capacity, suggesting coordinated regulation of stress tolerance pathways (Saleem et al. 2022, 2025). Collectively, the findings indicate that *R. leguminosarum*, *B. subtilis*, and *S. marcescens* mitigate As and Cd stress through integrated mechanisms involving rhizosphere microbiome restructuring, enhanced antioxidant defense, improved nutrient uptake, and restricted metal translocation. This multifaceted mode of action underscores the potential of PGPR-based strategies as sustainable tools for improving *O. sativa* productivity and food safety in metal-contaminated soils.

Conclusion

The outcomes of the current study demonstrate that toxic levels of arsenic (As) and cadmium (Cd) markedly impaired plant growth and biomass, photosynthetic pigments, gas exchange traits, antioxidant machinery, mineral uptake, the ascorbate–glutathione (AsA–GSH) cycle, proline metabolism, and rhizosphere microbial diversity in rice (*Oryza sativa* L.). In addition, As and Cd stress enhanced oxidative stress indicators, root exudations, and As/Cd accumulation in plant organs. Conversely, the individual application of *Rhizobium leguminosarum*, *Bacillus subtilis*, and *Serratia marcescens* alleviated metal-induced toxicity by improving plant growth and biomass, reducing reactive oxygen species (ROS) production, maintaining essential mineral levels, strengthening the AsA–GSH cycle, enhancing rhizosphere microbial diversity, and improving photosynthetic, physiological, and biochemical attributes. While the present findings are derived from controlled pot conditions, they highlight the potential of PGPR-based interventions for mitigating heavy metal stress in *O. sativa*. Future research should focus on long-term field-scale validation and explore interactions among root exudations, metal stress, PGPR or silicon fertigation regimes, and nutrient mobility across different crops. Such studies will provide deeper mechanistic insights and support the development of sustainable strategies to improve crop resilience under heavy-metal stress.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Consent to publish Written consent was sought from each author to publish the manuscript.

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