Environmental Science and Pollution Research (2024) 31:27465–27484
https://doi.org/10.1007/s11356-024-32874-1

RESEARCH ARTICLE

Exploiting the role of plant growth promoting rhizobacteria in reducing heavy metal toxicity of pepper (*Capsicum annuum* L.)

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Received: 7 December 2023 / Accepted: 8 March 2024 / Published online: 21 March 2024

Abstract

Microorganisms are cost-effective and eco-friendly alternative methods for removing heavy metals (HM) from contaminated agricultural soils. Therefore, this study aims to identify and characterize HM-tolerant (HMT) plant growth-promoting rhizobacteria (PGPR) isolated from industry-contaminated soils to determine their impact as bioremediators on HM-stressed pepper plants. Four isolates [*Pseudomonas azotoformans* (*Pa*), *Serratia rubidaea* (*Sr*), *Paenibacillus pabuli* (*Pp*) and *Bacillus velezensis* (*Bv*)] were identified based on their remarkable levels of HM tolerance *in vitro*. Field studies were conducted to evaluate the growth promotion and tolerance to HM toxicity of pepper plants grown in HM-polluted soils. Plants exposed to HM stress showed improved growth, physio-biochemistry, and antioxidant defense system components when treated with any of the individual isolates, in contrast to the control group that did not receive PGPR. The combined treatment of the tested HMT PGPR was, however, relatively superior to other treatments. Compared to no or single PGPR treatment, the consortia (*Pa+Sr+Pp+Bv*) increased the photosynthetic pigment contents, relative water content, and membrane stability index but lowered the electrolyte leakage and contents of malondialdehyde and hydrogen peroxide by suppressing the (non) enzymatic antioxidants in plant tissues. In pepper, Cd, Cu, Pb, and Ni contents decreased by 88.0-88.5, 63.8-66.5, 66.2-67.0, and 90.2-90.9% in leaves, and 87.2-88.1, 69.4-70.0%, 80.0-81.3, and 92.3%% in fruits, respectively. Thus, these PGPR are highly effective at immobilizing HM and reducing translocation *in planta*. These findings indicate that the application of HMT PGPR could be a promising "bioremediation" strategy to enhance growth and productivity of crops cultivated in soils contaminated with HM for sustainable agricultural practices.

Keywords bioremediation · contaminated soil · eco-friendly procedures, environmental impact, heavy metals · plant growth promoting rhizobacteria · yield

Introduction

Pepper (*Capsicum annuum* L.) is an economically important crop that is cultivated for its nutritional values, bioactive compounds, antioxidant properties and natural colors (Santosh 2013). Like other vegetables in Egypt, pepper is mostly grown in soils originally polluted with heavy metals (HM) near industrial areas, or from disposal of wastes. These soils are less resilient and crops that grow in them exhibit reduced productivity (Gall et al. 2015; Alengebawy et al. 2021). Land degradation is also well-documented to have an impact on global food security and environmental quality (Alengebawy et al. 2021).

Although some essential HM, such as copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), silver (Ag), and zinc (Zn), play important roles in different biological systems, they are generally toxic to living organisms at high

Responsible Editor: Diane Purchase

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quantities (Rahman et al. 2022). On the other hand toxic, non-essential HM, including cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), and arsenic (As), can be harmful, even in small amounts (Rahman et al. 2022). Many pollution sources, such as chemical fertilizers, atmospheric precipitation, sewage sludge and agricultural industry, adversely increase HM concentrations in farmland soils and contribute to the accumulation of contaminants in edible plant parts (Kumari and Mishra 2021; Cheng et al. 2023). As a result, this affects not just plant productivity, but also human health and soil biodiversity (Manzoor et al. 2022).

In response to HM stress, cellular compartments activate signaling pathways and induce transcriptional programs to promote HM stress tolerance and establish homeostasis (Li et al. 2023). Oxidative stress is caused by the imbalance between production and accumulation of reactive oxygen species (ROS) in plants cells/tissues (Kohli et al. 2017; Sarkar et al. 2018). Depending on their concentration in plants, ROS play a crucial, dual role in plant biology (Hasanuzzaman et al. 2023). At high concentration, ROS cause damage to carbohydrates, proteins, lipids and DNA (Ahmad et al. 2009). At low/intermediate level, ROS act; however, as secondary messengers or signaling molecules to mediate plant responses to biotic and abiotic stresses and to remodel plant growth and development (Devireddy et al. 2021*)*.

Therefore, plants employ a sophisticated antioxidant defense system to protect themselves from oxidative damage by scavenging ROS (Sofy et al. 2020). For instance, the plant enzymes, catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase (GR) can catalyze reactions that break down ROS into less harmful molecules (Bhaduri and Fulekar 2012). The non-enzymatic antioxidants, such as ascorbic acid (AsA), carotenoids (Car), α-tocopherol (TOC), glutathione (GSH), and proline (Pro), play pivotal roles in plant acclimatization during abiotic stress *i.e.,* HM (Bhaduri and Fulekar 2012).

In soil, HM reduce water potential and compete with essential nutrients, making them scarce for the uptake by root; thus, contributing to drought-like symptoms and osmotic stress (Ghori et al. 2019). In addition, high levels of HM in the apoplasm of leaves disrupt water balance in plants, causing cells to lose water (cell dehydration) (El-Saadony et al. 2021; Khan et al. 2021). HM stress is also linked with decreased chlorophyll (Chl) contents, stomata closure, and increased damage in photosynthetic attributes. Moreover, environmental risks of excessive HM accumulation in soils substantially impact microbial populations and their soil activities (Li et al. 2020).

Plant growth-promoting rhizobacteria (PGPR), play a pivotal role in mitigating HM accumulation in plants through multifaceted mechanisms (Desoky et al. 2020; Ashry et al. 2022; Patil et al. 2023). PGPR can solubilize insoluble HM in the rhizosphere, reducing their availability for plant absorption (Oubohssaine et al. 2022; Khoso et al. 2024). This process involves the secretion of organic acids, siderophores, and chelating agents that bind to HM, rendering them less toxic. Additionally, PGPR enables plants to acquire essential nutrients, mainly nitrogen (N), phosphorus (P), potassium (K), and Fe, while inhibiting the absorption of HM (Bhat et al. 2023).

Furthermore, these beneficial bacteria induce systemic resistance (ISR) in plants against HM stress by activating various defense mechanisms, including production of phytochelatins, and antioxidant enzymes (Yu et al. 2022). PGPR can also promote growth and extension of the root systems in plants to enhance their ability to exclude and detoxify HM (Mantelin et al. 2006). For example, PGPR can alleviate/reduce HM stress in plants by producing salicylic acid, exopolysaccharides (EPS), biosurfactants, Pro, and siderophores, and chelating different metal ions (El-Meihy et al. 2019; Nazli et al. 2021). Overall, the intricate interactions between PGPR and plants demonstrate their potential as environmentally friendly biotechnological tools for reducing HM accumulation and improving plant health in contaminated environments (Oubohssaine et al. 2022; Patil et al. 2023).

Microbial biostimulant technology (or bioremediation) has become a sustainable strategy to remove, degrade or immobilize toxic elements (*i.e.,* HM) from contaminated soils (Desoky et al. (2020). *Nitrosomonas*, *Mycobacterium, Flavobacterium*, *Bacillus*, *Pseudomonas*, *Xanthobacter* and *Penicillium* are microorganisms that can degrade a variety of HM (Elnahal et al. 2022; Yin et al. 2023). *Paenibacillus* sp. is an endophyte isolated from *Tridax procumbens* roots that can grow in soils containing high concentrations of Zn, Pb, Cu, and Ar (Wu et al. 2021). Desoky et al. (2020) have also reported that the bacterial species, *Bacillus, Paenibacillus* and *Pseudomonas*, are considered potential bioremediators.

Several studies have demonstrated that PGPR can improve the defense mechanisms of plants growing under HM stress conditions (Eltahawy et al. 2022). As far as we know, no prior research has been performed to determine the effect of *Pseudomonas azotoformans* (*Pa*)*, Serratia rubidaea* (*Sr*)*, Paenibacillus pabuli* (*Pp*)*,* or *Bacillus velezensis* (*Bv*), as bioremediators on pepper plants cultivated in HM-polluted soils.

We hypothesized that the combined application (*Pa+Sr+Pp+Bv*) would boost the growth and HM stress tolerance in peppers, compared to the single or double applications of PGPR. In the present investigation, we aimed to (i) isolate HM-tolerant (HMT) PGPR capable of tolerating HM from contaminated soils; and (ii) determine the response of pepper plants (growth and yield attributes) to soil inoculation with the most promising PGPR consortia of isolates in the field. In the current investigation we also evaluated

the physio-biochemistry, components of antioxidant defense system, and HM accumulation in the tissues of pepper plants in response to PGPR.

Overall, this research can paved the way for developing new strategies to improve crop yields and HM bioaccumulation of pepper plants using *Pa+Sr+Pp+Bv* under field conditions.

Materials and methods

Collection of soil samples

In the current study, the HM contaminated soil samples were collected from the top 20 cm of six industrial regions in Abu-Zabal City, Qalyubia governorate, Egypt (30° 14′ 58″ N; 31° 21′ 16″ E) in sterile polythene bags. Soil samples were quickly transported to the Microbiology Laboratory, Faculty of Agriculture, Zagazig University, Zagazig, Egypt. The concentrations of HM in soil samples were determined by using AAnalyst 400 atomic absorption spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA).

Preliminary screening of HMT bacteria

Ten grams of homogenized soil samples were mixed into 90 mL sterilized peptone water (LB; Lab M Limited, Lancashire, UK). All flasks were kept on a gyratory shaker (Model G76, New Brunswick Scientific, NJ, USA) at 150 rpm at 30°C for 1 h. The flasks were left to settle for 30 more min and dilutions (10^{-2}–10^{-7}) were made using sterilized water.

The HM salt stock solution used in the current study is composed of $HgCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $MnCl_2$, $K_2Cr_2O_7$, $NiCl_2$, $AgNO_3$, $ZnSO_4$, and $(CH_3COO)_2Pb$ (300 mg L^{-1} each). The HM salt stock solution was prepared in distilled water, filter-sterilized, and stored in sterile flasks in the dark at 4°C for 24 h (Kobya et al. 2005).

Aliquots (0.2 mL) from each dilution were spread onto Luria Bertani (LB; Lab M) agar plates supplemented with different concentrations of HM solutions (50, 100, 150, 250, and 300 mg L^{-1}). Four plates were made for each dilution, and the plates were air dried and incubated for 3 days in the dark at 28±2°C. Colonies were counted and expressed as $\log_{10}$ colony forming units (cfu) g dry weight (DW) $soil^{-1}$. All obtained bacteria were purified and stored on nutrient agar plates (Lab M). The purification and selection of colonies with a variety of morphologies was made possible through streaking on nutrient agar plates.

All the obtained HMT bacterial isolates were tentatively identified by colony morphology and by their cultural characteristics such as color, and the production of diffusible pigments. Biochemical tests were performed as described in Bergey's Manual of Systematic of Bacteriology (Logan and De Vos 2009). Bacterial isolates were further identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany; Bille et al. 2012).

Determination of minimum inhibitory concentration (MIC) of bacterial isolates to HM

To determine the bacterial tolerance to HM, LB broth supplemented with different HM concentrations (0, 150, 100, 150, 250, and 300 mg L^{-1}) was inoculated with 2 mL (10^8 cfu mL^{-1}) of each bacterial isolate. The Erlenmeyer flasks were incubated for 4 days in the dark at 28°C on an orbital shaker incubator (New Brunswick Scientific) at 250 rpm. To determine the MIC, (the lowest concentration used to inhibit the growth of a bacterial culture) of HM, the optical density (OD600) of each bacterial culture was measured (Kowalska-Krochmal and Dudek-Wicher 2021).

Field experiments

Field experiments were carried out on a private farm with HM contaminated soil in Abu-Zabal City, Qalyubia governorate, Egypt in two growing seasons (March-July) using pepper in 2022 (SI) and 2023 (SII). Experimental conditions were as the following: Day/night length=12-13/11-12 h; temperature=25±3°C day, and 15±2°C night; precipitation=5-10 mm, and relative humidity=65.4-72.2%. Before each season, soil samples were randomly collected from the research sites and analyzed according to previous protocols (Black 1958; Jackson 1958). The physical and chemical properties of the tested soil in both seasons are presented in Table S1.

Pepper seeds (*C. annuum* cv. Top Star) obtained from Sacata Company (Cairo, Egypt) were used for the field experiment. Seeds were sown in growth trays, and seedlings were transplanted after 45 days to plots (3.0×3.50 m=10.5 m^2), 60 cm-wide between the rows and 15–20 cm spaces between seedlings. Before planting, all plots were fertilized with a standardized fertilizer of 100 kg potassium sulfate (K_2O, 50%), 200 kg calcium superphosphate (P_2O_5, 15.5%), and 250 kg ha^{-1} of ammonium nitrate (NH_4NO_3). The empirical design of our study was a completely randomized plot for 12 treatments, each with three replicates.

The following treatments were applied on pepper plants: (1) pepper plants cultivated in non-inoculated soil (control; C); (2) pepper plants cultivated in soil inoculated with the single HMT isolate *Pseudomonas azotoformans* (*Pa*); (3) pepper plants cultivated in soil inoculated with the single HMT isolate *Serratia rubidaea* (*Sr*); (4) pepper plants cultivated in soil inoculated with the single HMT isolate *Paenibacillus pabuli* (*Pp*); (5) pepper plants cultivated in soil inoculated with the single HMT isolate *Bacillus velezensis*

(*Bv*); (6) pepper plants cultivated in soil inoculated with the double HMT isolates *Pa*+*Sr*; (7) pepper plants cultivated in soil inoculated with the double HMT isolates *Pa*+*Pp*; (8) pepper plants cultivated in soil inoculated with the double HMT isolates *Pa*+*Bv*; (9) pepper plants cultivated in soil inoculated with the double HMT isolates *Sr*+*Pp*; (10) pepper plants cultivated in soil inoculated with the double HMT isolates *Sr*+*Bv*, (11) pepper plants cultivated in soil inoculated with the double HMT isolates *Pp*+*Bv*; and (12) pepper plants cultivated in soil inoculated with the quadruple HMT isolates *Pa*+*Sr*+*Pp*+*Bv*.

Application of the selected HMT bacteria

The above bacterial isolates were cultivated on LB media to obtain a concentration of 10^9 cfu L^{-1} (Duxbury 1981). The selected bacteria (*Pa*, *Sr*, *Pp* and *Bv*) were applied to the soil in irrigation water at a rate of 10 L ha^{-1} in 3 equal doses at 20, 35, and 50 days after transplantation (DAT). The selected four bacteria were administered during the final 10 min of drip irrigation for each application.

Effect of bacteria on the growth and productivity of pepper plants

At 60 DAT, nine pepper plant samples were chosen randomly and plucked from the two outside rows of each experimental plot to measure stem length (SL; cm), shoot dry weight (SDW; g), number of leaves $plant^{-1}$, and leaf area (LA; cm^2 $plant^{-1}$). At the marketable stage, 50 pepper fruits $plot^{-1}$ were harvested to determine the number of fruits $plant^{-1}$, and yield (tons ha^{-1}).

Determination of the physicochemical properties of pepper plants

To evaluate the extent of photosynthetic pigments, leaf samples were extracted using acetone as described previously (Fadeel 1962). The OD was recorded at 480 nm (Chl a), 645 nm (Chl b), and 663 nm (Car) using a spectrophotometer (Beckman 640D, Brea, California, USA) to determine the contents of pigments [mg g^{-1} leaf fresh weight (FW)].

Leaf net photosynthetic rate (Pn), rate of transpiration (Tr), and stomatal conductance (gs) were measured using a portable photosynthesis system (LF6400XTR, LI-COR, Lincoln, NE, USA). To avoid potential stomatal closure around noon time, measurements were taken from the second wholly expanded leaf of the treated-plant at 9:00-11:00 AM.

We also estimated the relative water content (RWC) as previously described (Barrs and Weatherley 1962). The FW of a mixed sample of one leaf $plant^{-1}$ was determined. Leaves of the same sample were soaked in distilled water for 3 h, and the turgid weight (TW) was recorded. Leaf tissues were dried at 80°C for 24 h to measure their dry weight (DW). RWC was calculated according to the following formula:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

To calculate the membrane stability index (MSI), two sets of fresh leaf (0.2 g) were homogenized in 10-mL of double-distilled water: Group I was heated in a water bath at 40°C for 30 min and the electrical conductivity (EC) bridge (C1) was measured; while, Group II was heated at 100°C for 10 min in a boiling water bath to evaluate EC (C2) according to the method of Premachandra et al. (1990) and Oyenike et al. (2019). MSI was calculated using the following formula:

$$MSI\ (\%) = 1 - \frac{C1}{C2} \times 100$$

Twenty leaf discs (each 0.5 cm) were homogenized in ½ mL of distilled water to determine the electrolyte leakage (EL). To determine ECa, ECb and ECc, the solution was exposed to 25°C, 45-55°C for 30 min, and 100°C for 10 min, respectively. To calculate EL, the following formula was used (Hniličková et al. (2019):

$$\%EL = \frac{ECb - ECa}{ECc} \times 100$$

Fresh leaf samples (100 mg) were homogenized in Na-phosphate buffer to evaluate the content of malondialdehyde (MDA; µmol g^{-1}). The suspension was centrifuged at 20,000 × g for 25 min at 4°C (Heath and Packer 1968). The OD_{532} and OD_{600} of the supernatant were used to compensate the nonspecific turbidity.

H_2O_2 content (µmol g^{-1}) was measured according to the method described by Liu et al. (2000). Extract of leaves was diluted with sulfuric acid 1M, followed by the addition of NH_3 and titanium reagent. The OD at 415 nm was determined (Mukherjee and Choudhuri 1983). A standard curve was prepared using known concentrations of H_2O_2, and it was used for the calculations. The results were reported as l mol H_2O_2 g^{-1} FW.

The superoxide ($O_2^{\bullet-}$) content was evaluated by immersing leaf pieces for 1 h in 10 mM K-phosphate buffer (pH 7.4), nitro blue tetrazolium chloride (NBT);(0.05%), and sodium azide (10 mM). The mixture was boiled at 85°C for 15 min and cooled; followed by recording OD readings at 580 nm (Kubiś 2008).

Determination of non-enzymatic antioxidant compounds of pepper plants

The amount of Pro (µmol g^{-1} of leaf DW) was determined using standard L-Pro calibration curve (Bates et al. 1973).

Total soluble sugars (TSS) were extracted from leaves using 96% (v/v) ethanol. The extract was then mixed with a reagent containing 150 mL of sulfuric acid in 100 mL of 72% ethanol, boiled in a water bath for 10 min, and cooled. The mixture was quantified at 632 nm using spectrophotometer (Bausch and Lomb, Rochester, New York, USA) as recommended by Irigoyen et al. (1992).

The content of α-TOC (μmol g^{-1}) in dried leaves was determined using the high-performance liquid chromatography (HPLC) system (Waters Corporation HPLC, Milford, MA, USA) combined with a UV detector (1.5 mL min^{-1} flow rate at 292 nm) and a mobile phase (94 mL methanol, and 6 mL water) (Konings et al. 1996; Ching and Mohamed 2001). About 20 mg of butylated hydroxyl toluene was dissolved to 900 mL of the extraction solvent (800 mL n-hexane + 100 mL of ethyl acetate). α-TOC (0.5 mg 100 mL^{-1} n-hexane) was used to generate the standard and dilution 0.02-0.2 mg mL^{-1} solutions.

Ascorbic acid (AsA), and GSH concentrations were measured using the methods previously described by Maruta and Ishikawa (2022) and Tsiasioti and Tzanavaras (2023), respectively.

Estimation of antioxidants activities of pepper plants

For enzyme extractions, 1 g FW of leaves were homogenized in 10 mL of phosphate buffer (pH 7.0) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), and urea. The homogenate was centrifuged at 15,000 × g for 15 min under chilling conditions to obtain a supernatant referred to as the enzyme extract.

CAT activity was measured according to He et al. (2020) with some modifications. The enzyme extract (0.1 mL) was added to 0.5 mL of H_2O_2, and 2.4 mL of phosphate buffer (pH 5.0). Using Bausch and Lomb spectrophotometer, the solution was measured trice (one min each) at OD_{240}. The increase in absorbance of 0.01 U min^{-1} equals one enzymatic unit. The acquired values were estimated as U mL^{-1} min^{-1} DW using the following formula:

$$CAT\ activity = \frac{Af - As \times reaction\ vol}{time\ interval \times enzyme\ vol}$$

where, Af, final absorbance; As, initial absorbance; reaction volume=3; time interval=3; enzyme volume=0.1 mL.

The activity of POD was assessed at OD_{470} as recommended by Hussein et al. (2019). Leaves (1 g FW) was homogenized in 10 mL of phosphate buffer (pH 7.0), 0.5 mM EDTA, and polyvinyl pyrrolidone (PVP) for 10 min, and centrifuged at 15000 × g for 15 min at 4°C). The enzyme extract (0.1 mL) was added to 2.2 mL of guaiacol in phosphate buffer (3%), and incubated for 30 min, followed by 0.5 mL addition of H_2O_2. Using a spectrophotometer, absorbance at 470 nm was measured. The increase in absorbance of 0.01 min^{-1} equals one enzymatic unit. The obtained results (U mL^{-1} min^{-1} DW) were based on the calculations according to the following equation:

$$POD\ activity = \frac{Af - As \times reaction\ vol}{time\ interval \times enzyme\ vol}$$

Where, Af, final absorbance; As, initial absorbance; reaction volume=3; time interval=3; enzyme volume=0.1 mL.

APX was tested colorimetrically according to Faize et al. (2013). The test was conducted at 25°C in a 1-cm light path cuvette containing a reaction mixture of 1500 μL phosphate buffer (pH 7.0), 20 μl EDTA, 1000 μL sodium ascorbate, and 20 μL enzyme extract. After mixing, 480 μL H_2O_2 was added to commence the reaction, and the decrease relative to the blank (water) at OD_{290} was continuously measured for 2 min.

The SOD activity was assessed at 560 nm (He et al. (2020). The enzyme extract (0.1 mL) was added to 1 mL phosphate buffer (pH 5.0), 1 mL distilled water, 0.3 mL methionine (22 μM), and 0.1 mL NBT (20 μM). The vial was then exposed to UV-light for 15 min before 100 mL of 0.6 M riboflavin was added (as a substrate). One unit of SOD refers to the quantity of enzyme necessary to prevent the decrease of NBT by 50%. The OD at 470 nm was measured trice (one min each) using Bausch and Lomb spectrophotometer. The increase in absorbance of 0.01 U min^{-1} equals one enzymatic unit. The obtained results (U mL^{-1} min^{-1} DW) were calculated using this equation:

$$SOD\ activity = \frac{Af - As \times reaction\ vol}{time\ interval \times enzyme\ vol}$$

Where, Af, final absorbance; As, initial absorbance; reaction volume = 3; time interval = 3; enzyme volume = 0.1 mL.

Following the monitoring of NADPH oxidation, the activity of GR was evaluated by detecting three absorbance readings at 340 nm (Rao et al. 1996).

Determination of HM accumulation in leaves and fruits of pepper

The dried powdered leaf samples were weighed to estimate the HM content using the AAnalyst 400 atomic absorption spectrophotometer (Perkin Elmer) as recommended by Khan et al. (2015). Briefly, the upper completely extended leaves and fruits were burned at 500°C for 12 h and the ash was dissolved in HNO_3 (3.3%, v/v). The levels of Cd, Cu, Pb, and Ni were measured using the inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, Inc. Belrose NSW, Australia).

All HM concentrations in plants were compared to the certified Cd, Cu, Pb, and Ni concentrations in various reference plant materials received from the National Institute of Standards and Technology (NIST; Gaithersburg, USA).

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) in SAS Software version 9 (SAS Institute Inc., NC, USA). Mean values of treatments were subjected to pairwise comparisons using the Least Significant Difference (LSD) test at $P<0.05$ to determine the significant differences among treatments.

Results and discussions

Isolation and identification of HMT bacteria

Twenty bacterial strains were isolated from the HM-contaminated soils collected from Abu-Zabal city, Egypt, and were given a code according to their tolerance to HM (Table S2). These isolates were further screened *in vitro* for their tolerance to HM on LB medium supplemented with different concentrations of HM solutions (50, 100, 150, 250, and 300 mg L^{-1}).

All obtained 20 isolates grew on LB media when supplemented with 50 or 100 mg L^{-1} of HM. The current study showed that only 13, and 8 isolates grew on media containing 150 and 250 mg L^{-1} of HM, respectively. Only four isolates (HMT21, HMT40, HMT52 and HMT75) tolerated the high concentration of 300 mg HM L^{-1} and were considered highly HMT isolates and were elected for identification, and subsequent experiments.

Although researchers are dependent on the molecular identification of bacterial isolates, others have routinely relied on MALDI-TOF MS using ribosomal proteins for the identification (Ashfaq et al. 2022). Based on their ribosomal protein mass signal, particular strain can be compared to a publicly bacterial reference library (Topić Popović et al. 2023).

Under the microscope, HMT21, and HMT75 were identified as Gram-positive, motile, long rod, and spore-forming bacteria, suggesting that both bacteria might belong to *Bacillus* species. Based on the biochemical tests in Beregy's manual, isolate HMT21 was tentatively identified as *Pp* due to its high similarity to *Paenibacillus pabuli* DSM 3036T. Isolate HMT75, on the other hand, showed high similarity (99%) to *Bacillus velezensis* DSM 33864, and was tentatively identified as *Bv*.

The other two isolates (HMT40, and HMT52) were Gram-negative bacteria. Isolate HMT40 was a rod, motile bacterium with a single polar flagellum belonging to *Pseudomonas* species, whilst isolate HMT52 was red in color, rod-shaped, and non-spore-forming *Serratia* species. Isolates HMT40 (*Pa*), and HMT52 (*Sr*) were 99% similar to *Pseudomonas azotoformans* DSM 18862T, and *Serratia rubidaea* DSM 4480, respectively.

MIC of HMT bacterial isolates

The efficacy of the four isolates measured by MIC was assessed. The HMT40 isolate (*Pa*) had MIC values of 13.34, 16.24, 11.89, 20.01, and 18.27 against Pb, Zn, Cd, Cu and Cr, respectively (Fig. 1), suggesting that *Pa* is highly tolerant to HM. The isolate HMT21 (*Pp*) had less MIC values than those in *Pa*., thus indicating that *Pp* is the second HMT isolate (Fig. 1).

HMT52 (*Sr*) and HMT75 (*Bv*) were mostly comparable in their MIC for Pb, Cd, Ni, Ag and Hg; however, *Sr* had higher MIC values than *Bv* for Zn, Cu and Cr (Fig. 1). Both isolates were less tolerant to HM than *Pa* or *Pp* (Fig. 1). *Pa* (HMT40) was the strongest HMT isolate among all other tested bacterial strains. This was followed by *Pp*, and finally *Sr* and *Bv* (Fig. 1).

Similarly, Desoky et al. (2020) have previously investigated the sensitivity of *Bacillus* species isolated from soil samples to determine the levels of HM-tolerance of *Bacillus* strains to Pb, Zn and Cu. The identified isolates were also examined for their plant growth-promoting (PGP) properties and bioremediation abilities. The method that measures MIC in the current investigation can be used to determine HM-tolerance in bacteria. For instance, *Enterococcus mundtii* has been identified due to its high tolerance to soils contaminated with HM using this method (Efe 2020).

Effect of soil inoculation with HMT bacteria on growth and productivity of pepper plants grown in HM-contaminated soil

Toxicant pollution is a major environmental hazard that has caused serious concerns to human health and agricultural productivity (Priyanka et al. 2021; Górski et al. 2023). In the current study, HM stress in the two growth seasons SI and SII significantly ($P<0.05$) lowered SL, number of leaves, LA, SDW, number of pepper fruits, and yield (Table 1). Our findings are consistent with others (Lamhamdi et al. 2013; Taie et al. 2019), who found that soil contaminated with HM led to reduced growth and biomass in wheat and spinach, respectively, and that plants were less resilient to HM stress. HM accumulation in shoots is likely a consequence of the root uptake from the soil, and subsequently translocated to shoots in excess quantities, leading to impaired physio-chemical processes, reduced growth and development, nutrient imbalance and increased cellular oxidative damage in plants (Tang et al. 2023). HM toxicity minimizes mitotic division of meristematic

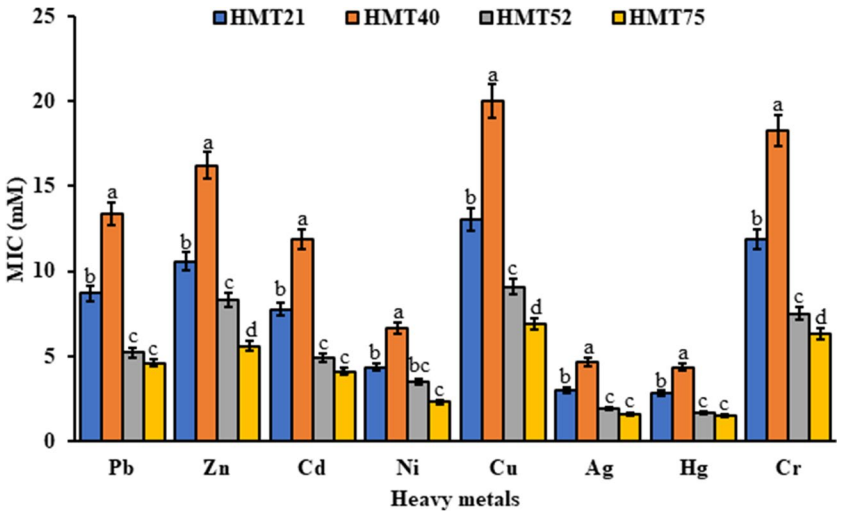

Fig. 1 Minimum inhibitory concentration of the tested bacterial isolates to HM. Data are means (n=9) ± standard error of the mean. Values of different letters indicate significant differences among isolates to the particular HM according to the least significant difference (LSD) test at $P \leq 0.05$. MIC, minimum inhibitory concentration; HM, heavy metal; Pb, lead; Zn, zinc; Cd, cadmium; Ni, nickel; Cu, copper; Ag, silver; Hg, mercury; Cr, chromium; HMT, heavy metal tolerant; HMT21, *Paenibacillus pabuli* (*Pp*); HMT40, *Pseudomonas azotoformans* (*Pa*); HMT52, *Serratia rubidaea* (*Sr*); HMT75, *Bacillus velezensis* (*Bv*)

cells in roots and shoots, resulting in decreased length, dry mass, and yield (Rady et al. 2021).

In the current study we determined the effect of the soil inoculation of the four HMT bacterial strains on pepper plants grown in HM-contaminated soil in the field. Soil application with any of the HMT PGPR isolates either individually or in combination significantly (P<0.05) increased all parameters of growth and yield compared to control plants that were grown in non PGPR-inoculated soil (Table 1). On the other hand, the combined applications of any two tested bacterial strains to the soil significantly (P<0.05) increased all growth and yield parameters of pepper compared to control or individual application of isolates (Table 1).

The quadruple treatment (*Pa+Sr+Pp+Bv*) showed the highest increase in SL (94.9-98.1%), number of leaves (129-130%), LA (198.9-199.6%), SDW (184-185%), number of fruits (314-321%), and fruit yield (318-331%) compared to the control treatment during the two seasons. PGPR have the ability to enhance the productivity and HM tolerance of crop plants (Busnelli et al. 2021). Our findings were corroborated by Bhuyan et al. (2022), who found that PGPR can aid maize (*Zea mays*) plant growth while reducing HM levels in the soil. Furthermore, enhancement of plant growth and mitigation of HM stress by PGPR are associated with reducing the endogenous levels of HM *in planta* (Sun et al. 2023).

Impact of soil inoculation with HMT bacteria on photosynthetic pigment contents and photosynthetic efficiency of pepper cultivated in HM-contaminated soil

Under HM stress conditions, pepper plants grown in soils without the supplementation of any PGPR (control) showed decreased contents of Chl a and b, and Car, causing a reduction in Pn, Tr and gs (Table 2). Individual or dual applications of bacterial strains to the HM-polluted soil significantly (P<0.05) increased the levels of photosynthetic pigments and enhanced photosynthetic efficiency, compared to plants cultivated in non-PGPR inoculated soils (Table 2). The combined treatment of *Pa+Sr+Pp+Bv* on pepper plants showed the best results with an increment of 129, and 125% in Chl a, 75, and 78% in Chl b, 29, and 25% in Car, 86, and 85% in Pn, 88, and 86% in Tr, and 96, and 88% in gs in 2021/2022 and 2022/2023 seasons, respectively, compared to the control (Table 2). Similar trends were also found in rice plants inoculated with *Burkholderia* sp., which decreased Cd translocation in tissues and elevated the photosynthetic efficiency in plants (Dong et al. 2016). Furthermore, the contents of Chl and Car increased when rice plants were inoculated with *Pseudomonas putida* and *Chollera vulgaris* upon As treatment (Srivastava et al. 2018).

Table 1 Effect of soil inoculation with the tested bacterial isolates on growth and productivity of pepper (*Capsicum annuum* L.) plants grown in HM-contaminated saline soil

Treatment	SL (cm)	Number of leaves plant^{-1}	LA (cm^2 plant^{-1})	SDW (g)	Number of fruits plant^{-1}	FY (tons ha^{-1})
SI						
C	32.6±1.2f	28.6±1.3g	543.5±11h	4.32±0.12h	1.45±0.09g	12.7±0.66h
Pa	51.5±2.5cd	48.3±1.9d	2437±15e	8.47±0.19e	3.01±0.12de	24.6±1.2e
Sr	48.4±2.3d	42.5±2.1e	1312±13f	7.88±0.21f	2.68±0.11e	22.2±1.3ef
Pp	44.1±2.8d	33.5±1.6f	828.2±15g	7.18±0.25f	2.25±0.13ef	20.2±1.4f
Bv	38.7±1.9e	31.8±1.3fg	666.5±16j	6.65±0.18g	1.86±0.07f	18.6±0.85g
Pa+Sr	61.5±3.6b	63.2±2.9ab	4448±18ab	11.4±0.36b	5.59±0.21ab	52.8±2.2a
Pa+Pp	59.8±3.2b	62.2±3.4b	4301±13b	11.2±0.45bc	5.17±0.25b	48.5±2.6b
Pa+Bv	59.5±3.5b	60.9±3.9bc	4226±12b	10.9±0.52c	4.78±0.29bc	48.5±2.9b
Sr+Pp	57.5±3.6bc	60.6±3.4bc	4005±15c	10.3±0.48c	4.36±0.32c	44.9±2.7c
Sr+Bv	55.3±2.8c	56.3±3.2c	3263±13d	9.66±0.36d	3.94±0.14cd	32.6±1.8d
Pp+Bv	54.8±2.4c	53.6±3.1cd	2946±17de	9.11±0.24d	3.55±0.21d	30.3±1.6d
Pa+Sr+Pp+Bv	64.6±3.9a	65.9±3.5a	4689±19a	12.3±0.85a	6.11±0.33a	54.8±2.6a
SII						
C	33.6±1.6g	28.9±2.2h	554.8±12i	4.35±0.21g	1.49±0.08h	13.2±0.74h
Pa	52.4±1.9d	48.7±2.3e	2438±22f	8.55±0.28d	3.08±0.11f	25.0±1.8e
Sr	49.3±2.4e	42.9±2.5f	1314±23g	7.98±0.31e	2.71±0.14fg	22.5±1.6ef
Pp	45.0±2.5e	33.9±2.8g	829.3±25h	7.27±0.18e	2.29±0.13g	20.3±1.1f
Bv	39.6±1.4f	32.2±3.1g	754.8±27h	6.76±0.19f	1.89±0.14f	18.7±1.2g
Pa+Sr	62.4±3.4b	63.6±3.9b	4449±29ab	11.5±0.54b	5.61±0.21b	53.3±3.6a
Pa+Pp	60.7±3.6b	62.6±4.2b	4302±25b	11.3±0.55b	5.19±0.23c	49.0±2.9b
Pa+Bv	60.4±3.8b	61.3±4.1bc	4227±26b	10.9±0.66bc	4.81±0.22cd	47.6±2.7b
Sr+Pp	58.4±2.9c	61.0±4.9bc	4006±29c	10.5±0.45bc	4.39±0.24d	45.4±2.6c
Sr+Bv	56.2±2.5c	56.7±3.6c	3265±27d	9.74±0.32c	3.99±0.2e	33.1±1.4d
Pp+Bv	55.7±2.4cd	54.0±3.5d	2947±19e	9.19±0.36c	3.59±0.23ef	30.8±1.8d
Pa+Sr+Pp+Bv	65.5±2.9a	66.3±3.9a	4691±16a	12.4±0.47a	6.17±0.36a	55.2±3.2a

Data are means (n=9) ± SE. Values with the same letters indicate no significant differences (P>0.05) according to the Least Significant Difference (LSD) test ($P \leq 0.05$)

HM, heavy metal, *SI/II*, season I/II, *SL*, stem length, *LA*, leaf area, *SDW*, shoot dry weight, *FY*, fruit yield, *C*, control (no bacteria), *Pa, Pseudomonas azotoformans, Sr Serratia rubidaea, Pp Paenibacillus pabuli*, *Bv Bacillus velezensis*

Our results were also in agreement with other studies indicating that plant photosynthesis is restrained when exposed to HM stress. This may occur directly by affecting the key enzymes of chlorophyll biosynthesis (Madhu and Sadagopan 2020), water-oxidizing complex of PSII (Dutta and Pal 2019), decreasing RuBPCase activity in leaves (Cuypers et al. 2023), causing damages to electron transfer and disturbance in photosynthesis (Chen et al. 2021), or preventing the absorption of essential elements from the soil for the formation of photosynthetic pigments (Guo et al. 2019). Deficiency of Fe, stomatal closure and reduction in gs (Chen et al. 2020), alteration cell size and stomatal density in the epidermis of leaves (ur Rehman et al. 2020) are among indirect causes of photosynthesis disruption. Normally, Fe deficiency in plants increases under HM stress conditions, leading to chlorosis (Burd et al. 1998). PGPR tend to bind to Fe and form siderophore complexes to provide plants with Fe.

PGPR can bind directly with HM to reduce their bioavailability and toxicity (Thiem et al. 2018; Patil et al. 2023). Moreover, these plant-associated bacteria may boost the plant defense system when exposed to HM stress. For example, PGPR may confer HM stress tolerance by mediating ISR and/or enhancing phytohormones (Yu et al. 2022; Patil et al. 2023). They can regulate metal transporter genes upon Cd exposure to reduce its accumulation in corn (Wang et al. 2016).

Table 2 Effect of soil inoculation with the tested bacterial isolates on photosynthetic pigment contents and photosynthetic efficiency of pepper (*Capsicum annuum* L.) plants grown on HM-contaminated saline soil

Treatment	Chl a (mg g^{-1} FW)	Chl b	Total carotenoids	Pn (μmol CO_2 m^{-2} s^{-1})	Tr (mmol H_2O m^{-2} s^{-1})	gs
SI						
C	0.71±0.04g	0.468±0.03h	0.251±0.01g	7.18±0.22i	3.82±0.21j	0.333±0.01j
Pa	1.13±0.09e	0.538±0.04ef	0.282±0.01ef	9.84±0.25e	5.24±0.25g	0.453±0.02g
Sr	0.95±0.02f	0.517±0.03f	0.278±0.02f	9.18±0.36f	4.46±0.32h	0.403±0.03h
Pp	0.91±0.07f	0.493±0.02g	0.269±0.02fg	8.91±0.38g	4.34±0.35h	0.376±0.02i
Bv	0.87±0.03f	0.478±0.02gh	0.262±0.01k	8.25±0.42h	4.13±0.36i	0.366±0.01i
Pa+Sr	1.52±0.08ab	0.800±0.06ab	0.321±0.02ab	13.2±0.66a	6.97±0.38b	0.636±0.04ab
Pa+Pp	1.46±0.06b	0.752±0.05b	0.316±0.02b	13.0±0.74ab	6.87±0.54bc	0.623±0.05b
Pa+Bv	1.36±0.08c	0.737±0.06bc	0.312±0.01bc	12.8±0.85b	6.75±0.63c	0.586±0.04c
Sr+Pp	1.34±0.09c	0.707±0.05c	0.310±0.02c	11.3±0.96c	6.15±0.68d	0.546±0.03d
Sr+Bv	1.33±0.04cd	0.662±0.04d	0.302±0.01d	10.9±0.99dc	5.81±0.58e	0.516±0.04e
Pp+Bv	1.23±0.05d	0.556±0.04e	0.290±0.01e	10.3±0.98d	5.50±0.54f	0.486±0.03f
Pa+Sr+Pp+Bv	1.63±0.08a	0.819±0.07a	0.326±0.01a	13.4±0.89a	7.20±0.85a	0.653±0.06a
SII						
C	0.74±0.06g	0.488±0.02g	0.291±0.01f	7.29±0.45h	3.90±0.22j	0.363±0.02j
Pa	1.16±0.09e	0.588±0.04de	0.322±0.02d	9.95±0.52e	5.32±0.25g	0.483±0.03g
Sr	0.98±0.05f	0.567±0.03e	0.318±0.01de	9.29±0.58f	4.54±0.36h	0.433±0.03h
Pp	0.95±0.06f	0.543±0.05ef	0.309±0.01e	9.02±0.69fg	4.42±0.39h	0.406±0.02i
Bv	0.91±0.04f	0.528±0.04f	0.302±0.02e	8.36±0.85g	4.21±0.45i	0.396±0.01i
Pa+Sr	1.57±0.11b	0.850±0.07ab	0.361±0.01ab	13.3±0.78ab	7.05±0.52b	0.666±0.05ab
Pa+Pp	1.50±0.12b	0.802±0.08b	0.356±0.02b	13.1±0.98b	6.95±0.69bc	0.653±0.04b
Pa+Bv	1.40±0.011c	0.787±0.06bc	0.352±0.01bc	12.9±0.95b	6.83±0.66c	0.616±0.05c
Sr+Pp	1.37±0.09c	0.757±0.06c	0.350±0.02bc	11.4±0.96c	6.23±0.85d	0.576±0.03d
Sr+Bv	1.36±0.08cd	0.712±0.05cd	0.342±0.01c	11.0±0.65cd	5.89±0.54e	0.546±0.04e
Pp+Bv	1.26±0.08d	0.606±0.06d	0.330±0.02cd	10.4±0.85d	5.58±0.47f	0.516±0.03f
Pa+Sr+Pp+Bv	1.67±0.13a	0.869±0.07a	0.366±0.01a	13.5±0.74a	7.28±0.96a	0.683±0.05a

Data are means (n=9) ± SE. Values with the same letters within each column indicate no significant differences according to the Least Significant Difference (LSD) test ($P \leq 0.05$)

HM, heavy metal, *SI/II*, season I/II, *Chl*, chlorophyll, *Pn*, net photosynthetic rate, *Tr*, transpiration rate, *gs*, stomatal conductance, *C*, control (no bacteria), *Pa, Pseudomonas azotoformans, Sr Serratia rubidaea, Pp Paenibacillus pabuli*, *Bv Bacillus velezensis*

Impact of soil inoculation with HMT bacteria on pepper leaf integrity, oxidative stress indicators, and oxidative damage in HM-contaminated soil

PGPR colonizing the rhizosphere or internally inhabiting plant roots as endophytes enhance plant growth by solubilizing and assisting nutrient acquisition (El-Tarabily et al. 2021; Kaur and Pandove 2023), producing plant growth regulators (PGRs, El-Tarabily et al. 2020; Mathew et al. 2020; Shaffique et al. 2023), acting as biocontrol agents (Alwahshi et al. 2022; Nagrale et al. 2023), and producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (El-Tarabily et al. 2019; Shahid et al. 2023). Under HM stress, PGPR with ACC-deaminase activity has the potential to lower ethylene production in plants by cleaving ACC into α-ketobutyrate and ammonia (Misra and Chauhan 2020). *Bacillus anthracis PM21* enhanced growth of the legume tree, sesban (*Sesbania sesban*), by the production of ACC-deaminase, IAA and EPS under HM stress conditions (Ali et al. 2021). The application of *Bacillus gibsonii* PM11 and *Bacillus xiamenensis* PM14 to flax (*Linum usitatissimum*) resulted not only in increased tolerance to HM but also enhanced plant PGP traits (IAA, siderophores, EPS and ACC deaminase) (Sarkar et al. 2018; Zainab et al. 2020).

Production of organic acid or acidification by Zn-solubilizing PGPR is a major mechanism used to sequester Zn cations and decrease the pH of nearby soils (Alexander 1997; Singh et al. 2023). In addition, the anions can chelate Zn and enhance its solubility (Jones and Darrah 1994). *Pseudomonas*, *Bacillus*, and *Serratia* species have been reported as Zn-solubilizing PGPR to increase Zn uptake and nutrition in corn, soybean, wheat, and rice (Vaid et al. 2014; Pawar et al. 2015; Kamran et al. 2017). These reports confirm that PGPR isolates may have one or several mechanism(s) to

alleviate HM toxicity, such as metal-detoxification, biosorption, bioaccumulation, bioleaching, bioexclusion, metal-solubilization, acidification, protonation, chelation, and metal-immobilization (Sorour et al. 2022).

In the current investigation, our objective was to figure out the ecophysiological mechanisms that underlie the interactions between plants and microorganisms in the presence of natural HM pollution. In general, HM toxicity in plants leads to notable damage to the cell membranes, resulting in decreased RWC, EL and MSI and elevated levels of H_2O_2, MDA, and $O_2^{\bullet-}$ in the leaves of pepper plants (Rady et al. 2023). These damages can be intensified as the concentrations of HM increase. Pepper plants (control) grown in soils contaminated with HM without the supplementation of any PGPR showed decreased RWC and MSI but increased levels of EL, MDA, H_2O_2, and $O_2^{\bullet-}$ (Table 3). Our results align with those reported by Rady et al. (2023) that the detrimental impacts could be attributed to ROS production. Thus, the harmful effect of ROS may interact with lipids, proteins and nucleic acids to trigger lipid peroxidation, impair membrane integrity, deactivate enzymes, disrupt the structure of membrane transporters, and consequently affect cell viability and function (Jung et al. 2021).

Application of the HMT PGPR showed completely opposite patterns in the abovementioned attributes. The combined application of *Pa+Sr+Pp+Bv* increased RWC in pepper plants by about 52%, and MSI by 39% (Table 3). The same treatment decreased EL by almost 55%, MDA by 72%, H_2O_2 by 71%, and $O_2^{\bullet-}$ by 56% compared to control plants in the two growing seasons of pepper. According to Rahman et al. (2022), our data showed that antioxidant defense mechanisms resulted from PGPR might have contributed to the mitigation of HM stress in plants.

Table 3 Effect of soil inoculation with the tested bacterial on leaf integrity, oxidative stress markers and oxidative damage of pepper (*Capsicum annuum* L.) plants grown on HM-contaminated saline soil

Treatment	RWC (%)	MSI	EL	MDA (μM g^{-1} FW)	H_2O_2	$O_2^{\bullet-}$
SI						
C	58.7±3.2k	52.4±2.2g	16.3±1.2a	3.54±0.12a	17.6±1.3a	0.88±0.06a
Pa	69.5±3.6g	60.5±2.6e	11.3±1.1d	2.64±0.11c	11.9±1.2e	0.64±0.04e
Sr	67.1±38h	59.7±2.9e	11.9±1.3d	2.80±0.16c	13.1±1.1d	0.68±0.05d
Pp	62.4±3.6i	57.2±2.7f	13.2±1.4c	3.15±0.14b	14.4±1.4c	0.74±0.06c
Bv	60.5±3.7j	56.5±2.5f	14.7±1.6b	3.32±0.19b	15.5±1.2b	0.77±0.04b
Pa+Sr	88.2±4.1ab	71.7±3.1b	7.43±0.65ij	1.12±0.11fg	5.46±0.33hi	0.41±0.03jk
Pa+Pp	85.3±4.5b	69.8±3.6c	7.57±0.55i	1.29±0.13f	5.83±0.36h	0.42±0.03j
Pa+Bv	83.2±4.6c	69.3±3.2c	7.98±0.56h	1.38±0.14f	6.46±0.45g	0.48±0.02i
Sr+Pp	81.5±4.9d	68.3±3.9cd	8.14±0.74g	1.54±0.12e	6.90±0.52g	0.51±0.04h
Sr+Bv	78.6±4.1e	66.8±3.7d	8.74±0.65f	1.62±0.13e	8.03±0.65f	0.56±0.04g
Pp+Bv	75.0±3.6f	65.6±3.6d	9.53±0.85e	1.99±0.13d	8.50±0.68f	0.61±0.06f
Pa+Sr+Pp+Bv	89.7±4.9a	73.3±3.8a	7.31±0.65j	1.00±0.09g	5.03±0.36i	0.39±0.01k
SII						
C	59.3±2.5k	52.7±2.9g	15.8±1.3a	3.49±0.14a	17.5±1.6a	0.85±0.07a
Pa	70.1±3.6g	60.7±2.6d	10.9±1.2d	2.75±0.16d	11.8±0.66e	0.61±0.05d
Sr	67.7±3.8h	60.0±2.8d	11.4±1.2d	3.10±0.15c	13.0±0.89d	0.65±0.05d
Pp	63.0±4.1i	57.5±3.1e	12.8±1.3c	3.27±0.14b	14.3±1.3c	0.71±0.04c
Bv	61.1±3.9j	52.7±3.2f	14.2±1.5b	1.07±0.12k	15.4±0.98b	0.74±0.06b
Pa+Sr	88.7±4.9ab	72.1±3.9ab	7.03±0.44h	1.24±0.14j	5.37±0.25hi	0.37±0.01jk
Pa+Pp	85.9±3.8b	70.2±3.7b	7.07±0.48h	1.33±0.11i	5.74±0.32h	0.39±0.02j
Pa+Bv	83.7±4.7c	69.7±4.1b	7.48±0.49gh	1.49±0.13h	6.37±0.54g	0.45±0.04i
Sr+Pp	82.1±4.6d	68.5±4.6bc	7.74±0.36g	1.71±0.17g	6.81±0.41g	0.48±0.03h
Sr+Bv	79.2±4.1e	67.0±4.5c	8.34±0.65f	1.94±0.15f	7.94±0.69f	0.53±0.02g
Pp+Bv	75.6±3.9f	65.8±4.1cd	9.13±0.85e	2.60±0.16e	8.41±0.75f	0.58±0.03f
Pa+Sr+Pp+Bv	90.3±4.8a	73.7±4.9a	6.81±0.41i	0.95±0.08l	4.94±0.39i	0.36±0.01k

Data are means (n=9) ± SE. Values with the same letters within each column indicate no significant differences according to the Least Significant Difference (LSD) test ($P \leq 0.05$)

HM, heavy metal, *SI/II*, season I/II, *RWC*, relative water content, *MSI*, membrane stability index, *EL*, electrolyte leakage, *MDA*, malondialdehyde, *FW* fresh weight; H_2O_2, hydrogen peroxide, $O_2^{\bullet-}$, superoxide radical, *C*, control (no bacteria), *Pa, Pseudomonas azotoformans, Sr Serratia rubidaea, Pp Paenibacillus pabuli*, *Bv Bacillus velezensis*

The HM stress-induced EL is usually accompanied with ROS accumulation (Demidchik et al. 2014). The present study observed a significant increase (up to 51%) in membrane EL under HM stress in the control treatment (Table 3). The high levels of EL might be attributed to the increased production of ROS (Din et al. 2020). In radish plants, 18% EL was released after inoculation of PGPR under HM stress conditions (Ahmad et al. 2018). The findings of our study presented evidence that the PGPR strains under investigation are capable of coping with the effects of HM stress, presumably through the enhancement of MSI.

In the current investigation, inoculating soil containing pepper seedlings with individual or consortia of PGPR significantly reduced MDA content (Table 3) compared to their non-inoculated soils. Similarly, under HM stress conditions, Ahmad et al. (2018) showed that radish plants inoculated with PGPR had lower MDA levels. In HM-stressed plants, MDA production is often linked with membrane lipid peroxidation caused by oxidative stress (Biswas and Mano 2021). The MDA content reacts with amino groups, resulting in the degradation of proteins and macromolecules, as well as peroxidation of lipids, leading to necrosis (Islam et al. 2016).

Effect of soil inoculation with HMT bacteria on leaf osmo-protection and antioxidant activities in HM-stressed pepper plants

Pepper plants cultivated in soils lacking HMT bacteria exhibited higher levels of antioxidant enzymes and osmoprotectants than pepper plants grown in soils supplied with HMT bacteria (Table 4). The decreased profiles of oxidative stress and/or ROS-scavenging enzymes were previously remarked by Sapre et al. (2018), Alexander et al. (2020), and Shahid et al. (2021).

Table 4 Effect of soil inoculation with the tested bacterial isolates on osmoprotector and antioxidant contents of pepper (*Capsicum annuum* L.) plants grown on HM-contaminated saline soil

Treatment	Free Pro (μmol g^{-1} DW)	TSS (mg g^{-1} DW)	α-TOC (μmol g^{-1} DW)	AsA (μmol g^{-1} FW)	GSH
SI					
C	37.0±2.2a	28.4±1.5a	3.53±0.11a	2.30±0.11a	1.15±0.09a
Pa	34.8±2.3bc	26.1±1.6b	2.97±0.12d	2.08±0.13c	1.04±0.06c
Sr	35.2±2.1b	26.4±1.4b	3.02±0.13d	2.12±0.15b	1.06±0.07b
Pp	35.8±2.5ab	27.3±1.7ab	3.17±0.15c	2.17±0.18b	1.09±0.08b
Bv	36.4±2.6ab	28.0±1.7a	3.37±0.17b	2.23±0.19ab	1.12±0.09a
Pa+Sr	28.1±1.6fg	20.7±1.2e	1.69±0.08ij	0.97±0.05f	0.48±0.01fg
Pa+Pp	29.2±1.4f	21.5±1.1e	1.73±0.09i	1.01±0.08e	0.51±0.04f
Pa+Bv	31.6±2.1e	23.1±1.3d	1.79±0.11h	1.08±0.07e	0.54±0.03f
Sr+Pp	32.3±2.3de	23.8±1.4d	2.62±0.12g	1.82±0.06de	0.91±0.08e
Sr+Bv	33.5±2.4d	25.1±1.5c	2.74±0.14f	1.92±0.09d	0.96±0.07d
Pp+Bv	33.9±2.5c	25.6±1.6bc	2.82±0.13e	1.99±0.16cd	0.99±0.06d
Pa+Sr+Pp+Bv	25.0±1.3g	18.6±1.2f	1.66±0.12j	0.92±0.08g	0.46±0.03g
SII					
C	36.6±1.9a	28.1±1.8a	3.59±0.13a	2.37±0.15a	1.22±0.08a
Pa	34.4±1.8c	25.8±1.5b	3.08±0.12bc	2.14±0.16b	1.07±0.06bc
Sr	34.8±1.6bc	26.2±1.6b	3.23±0.11b	2.18±0.17b	1.08±0.09b
Pp	35.4±1.7b	27.1±1.7ab	3.43±0.14ab	2.23±0.16b	1.12±0.07b
Bv	36.0±1.5ab	27.7±1.6a	1.75±0.09e	2.29±0.15ab	1.16±0.08ab
Pa+Sr	27.7±1.4h	20.5±1.1de	1.79±0.11e	1.02±0.09de	0.49±0.03de
Pa+Pp	28.9±1.8g	21.3±1.2d	1.85±0.12e	1.06±0.08d	0.53±0.04d
Pa+Bv	31.2±2.2fg	22.8±1.3cd	2.68±0.14d	1.12±0.07d	0.57±0.05d
Sr+Pp	31.9±2.3f	23.6±1.3c	2.80±0.13c	1.85±0.09cd	0.94±0.07cd
Sr+Bv	33.1±2.8e	24.9±1.5bc	2.88±0.18c	1.96±0.08c	0.98±0.06c
Pp+Bv	33.5±2.7d	25.4±1.4b	3.03±0.17bc	2.03±0.11c	1.02±0.08c
Pa+Sr+Pp+Bv	24.6±1.6i	18.3±1.1e	1.72±0.09f	0.97±0.07e	0.44±0.03e

Data are means ($n = 9$) ± SE. Values with the same letters within each column indicate no significant differences according to the the Least Significant Difference (LSD) test ($P \leq 0.05$)

HM, heavy metal, *SI/II*, season I/II, *Pro*, proline, *TSS*, total soluble sugar, *DW/FW*, dry/fresh weight, α-*Toc*, α-tocopherol, *AsA*, ascorbic acid, *GSH*, glutathione, *C*, control (no bacteria), *Pa, Pseudomonas azotoformans, Sr Serratia rubidaea, Pp Paenibacillus pabuli*, *Bv Bacillus velezensis*

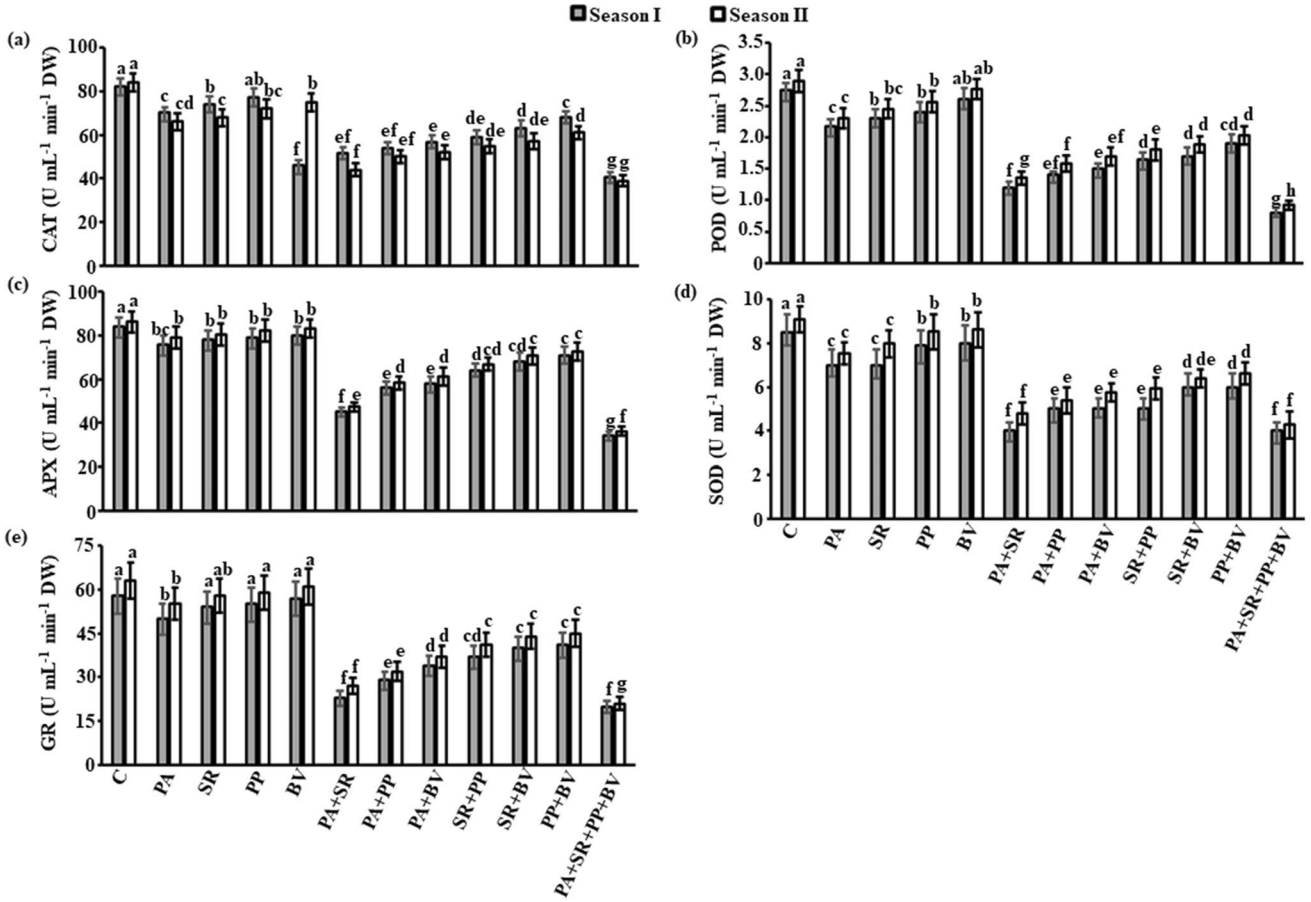

Fig. 2 Effect of soil inoculation with HM-tolerant PGPR on antioxidant enzymes of pepper plants under HM stress inoculated with or without *Pa*, *Sr*, *Pp* and *Bv* in Season I and II. Data are means (n=3) ± SE. Values of different letters indicate significant differences among individual or consortia of isolates in a particular season according to the least significant difference (LSD) test at $P \leq 0.05$. HM, heavy metal; (**a**) catalase (CAT); (**b**) peroxidase (POD); (**c**) ascorbate peroxidase (APX); (**d**) superoxide dismutase (SOD); (**e**) glutathione reductase (GR), *Pseudomonas azotoformans* (*Pa*); *Serratia rubidaea* (*Sr*); *Paenibacillus pabuli* (*Pp*); and *Bacillus velezensis* (*Bv*)

The osmo-protection and antioxidant activities in control pepper plats under HM toxicity without the supplementation of bacteria, significantly ($P<0.05$) increased in leaves (Table 4; Fig 2). In contrast, PGPR application significantly ($P<0.05$) decreased free Pro, TSS, α-TOC contents, and the non-enzymatic AsA and GSH contents (Table 4). Furthermore the application of PGPR significantly ($P<0.05$) decreased the enzymatic antioxidant activities (CAT, POD, APX, SOD and GR; Fig. 2) in HM-stressed-plants.

This reduction in the current study was relatively superior in the combined treatment of the four tested PGPR. This was evident with the reduced levels of free Pro (32.4-32.7%), TSS (34.8-35.5%), α-Toc (52.1-52.9%), AsA (59-60%), and GSH (60.0-63.9%) in the consortia of the four isolates in both seasons (Table 4). CAT, POD, APX, SOD, and GR were also reduced by approximately 50, 70, 57, 54% and 67%, respectively, in pepper plants stressed with HM and cultivated in soils inoculated with the PGPR, compared to plants grown in non-PGPR inoculated soils (control; Fig. 2).

Our results are in accordance with Kang et al. (2014) who reported that the activities of CAT, POD, and polyphenol oxidase in cucumber (*Cucumis sativus*) grown in soils inoculated with *Burkholdera cepacia* SE4, *Promicromonospora* sp. SE188, and *Acinetobacter calcoaceticus* SE370 were found to be lower than cucumber grown in soils not inoculated with these bacteria in response to salt and osmotic stress. Similarly, our results are in agreement with Manaf and Zayed (2015) who also found that SOD activity and Pro content in cowpea plants treated with mycorrhizae or *Pseudomonas fluorescence* alone were lower than those in untreated plants. It was thought that the negative consequences of excessive HM caused plants to lose the ability to control their metabolites.

The reduction in Pro and antioxidant enzymes seen in maize plants treated with *Bacillus* in HM contaminated soil may have been brought about by the development of extracellular polysaccharides (EPS) and biofilm on the surface of the roots (Misra and Chauhan 2020). This prevented the

plants from absorbing excessive levels of HM, hence reducing the negative impacts of hazardous ions on the plants. Furthermore, reduced levels of enzymatic and non-enzymatic antioxidants, as well as osmoregulators, were found in the oat tissues of *Klebsiella* treated plants, suggesting that they did not perceive stress as strongly as untreated plants under salinity stress conditions (Sapre et al. 2018). It was also reported that the increased levels of α-TOC in spinach plants grown in soils not inoculated with heavy metal–resistant bacteria can be attributed to its role as a component of the defense mechanisms to combat oxidative stress (Eltahawy et al. 2022).

In the current study, pepper plants cultivated in soils without HMT bacteria exhibited higher levels of AsA than pepper plants grown in soils supplied with HMT bacteria (Table 4). The incorporation of HMT PGPR in the HM polluted-soil inhibited the enzymatic activities in pepper plants (Table 4). It is well known that the endogenous production of AsA and GR increased the ROS-scavenging and HM-chelating capabilities in response to abiotic stresses (Desoky et al. 2019). In general, these components can help plants build complex antioxidant defense mechanisms against the oxidative stress generated by HM to attenuate and repair the ROS-induced damage (Rady et al. 2021). Moreover, AsA is a powerful antioxidant that helps in the protection from ROS damage (Kakan et al. 2021). APX uses AsA to oxidize MDA to dehydroascorbate (DHA); thus, reducing the effect of ROS (McClean and Davison 2022). AsA may also play a role as the "terminal antioxidant", leading to low redox potential of radicals (Rodríguez-Ruiz et al. 2019).

AsA synthesis from hexose phosphate and its effect on plant defense against the photo-oxidative stress suggest that there may be links between AsA and photosynthesis (Mukarram et al. 2021). In cucumber plants cultivated in HM-contaminated soils, the application of AsA increased plant tolerance to HM by improving their photosynthetic efficiency, growth, and antioxidant activities (Ghosh et al. 2022). This was also associated with reduced levels of HM in roots and leaves. AsA can maintain normal metabolic activities in stressed plants through osmotic modulation and cellular compatibility as suggested by Ghosh et al. (2022).

Table 4 shows that pepper plants cultivated in soils deficient in HMT bacteria had far greater levels of Pro than pepper plants grown in soils supplemented with HMT bacteria. Our results coincide with Alam et al. (2019) who repropted that the accumulation of Pro in tissues is one of the most important adaptations in plants to HM stress. Pro content restores the integrity of cell membranes and increases the enzymes present in AsA–GSH cycle in response to HM stress in tobacco (Khatun et al. 2020). As an efficient antioxidant defense component, Pro might also be involved in enzymatic and non-enzymatic antioxidant activities. It has been reported that Pro can decrease HM toxicity by eliminating toxins from ROS and boosting ASA and GSH levels, as well as enzymatic activities to induce gene expression machinery and antioxidant defense (Ghosh et al. 2022). In addition, Pro can react with Cd^{+2} ions in plants to form non-toxic compounds (Sharma and Devi 2010). It can also help protect plant cell membranes from damages caused by HM or EL (Aman Shamil 2022).

The increased enzymatic activity can alleviate the oxidative damage induced by HM (Desoky et al. 2019). ROS overproduction is a frequent response to various stimuli, including HM. In response to HM, the enzymatic (SOD, CAT, APX, POD, and GR) and the non-enzymatic antioxidants (Pro, α-TOC, and GSH) regulate ROS levels in plant tissues (Tripthi et al. 2020). The present investigation is the first study showing that HMT isolates of *Pa*, *Sr*, *Pp* and *Bv* had the ability to decrease enzymatic and non-enzymatic antioxidant activities in pepper plants under HM stress conditions.

Effect of soil inoculation with HMT bacteria on HM contaminants in plant tissues in response to HM stress

There was a significant ($P>0.05$) difference in the accumulation of Cd, Cu, Pb and Ni in the leaves and fruits of pepper plants cultivated in a soil contaminated with HM (Table 5). The rate of accumulation of these elements in the fruits was higher in control treatment (8.52-8.57, 47.0-47.8, 26.8-27.4, 12.5-12.7 mg kg^{-1} for Cd, Cu, Pb, and Ni, respectively) in both SI and SII seasons. Except of Cu, the concentrations of the other HM (Cd, Pb, and Ni) were higher than the accepted (0.8-1.0 mg kg^{-1}; Luo et al. (2019). All single or combined treatments of PGPR significantly reduced HM concentrations relative to the control group (Table 5).

By far, the consortia of PGPR treatment performed the best among all other treatments, by decreasing Cd content (88.0-88.5% in leaves, and 87.2-88.1% in fruits), Cu (63.8-66.5% in leaves, and 69.4-70.0% in fruits), Pb (66.2-67.0% in leaves, and 80.0-81.3% in fruits), and Ni (90.2-90.9% in leaves, and 92.3% in fruits) (Table 5).

In response to HM stress, plant cells are unable to maintain lower levels of Cd^{+2} or Pb^{+2} through effective detoxification mechanisms; thus, resulting in oxidative damage to different cellular constituents (Desoky et al. 2019), and reduction in plant growth (Table 1). The plants employ adaptive mechanisms to reduce HM absorption and translocation towards their roots and stems in response to stressful conditions (Abdul 2010). In the current investigation, soil inoculation with HMT PGPR led to a decrease in HM accumulation in leaves and fruits of pepper (Table 5). Our results are in accordance with Rizvi and Khan (2018) who showed that inoculation of corn plants with *Azotobacter chroococcum* resulted in a reduction in Cu and Pb accumulation in plant tissues. This was most likely due to

Table 5 Effect of soil inoculation with the tested bacterial isolates on HM accumulation in leaves and fruits of pepper (*Capsicum annuum* L.) plants grown on HM-contaminated saline soil

Treatment	Cd		Cu		Pb		Ni	
	Leaf	Fruit	Leaf	Fruit	Leaf	Fruit	Leaf	Fruit
	(mg kg^{-1})							
SI								
C	12.1±0.69a	8.57±0.65a	66.7±3.2a	47.8±2.5a	37.0±2.6a	27.4±2.5a	17.8±0.85a	12.7±0.89a
Pa	5.07±0.45cd	2.97±0.22d	41.4±2.5d	26.5±1.9c	26.5±2.8c	17.2±1.6c	9.51±0.66c	7.83±0.54c
Sr	5.60±0.52c	3.22±0.24cd	45.7±2.6c	28.6±1.6b	27.4±2.1c	17.6±1.4c	10.6±0.98bc	8.55±0.63c
Pp	6.09±0.63bc	3.57±0.23c	48.6±2.8bc	29.2±2.3b	28.6±2.3b	18.2±1.8b	11.5±0.89b	9.22±0.85b
Bv	6.77±0.66b	4.59±0.36b	52.5±2.4b	30.7±3.1b	30.6±2.9b	18.7±1.7b	12.4±089b	9.66±0.66b
Pa+Sr	1.56±0.12g	1.23±0.11k	25.8±2.3g	15.4±1.4ef	13.6±1.1fg	7.48±0.62j	2.35±021f	1.95±0.09g
Pa+Pp	1.97±0.11f	1.43±0.12g	26.2±1.2g	16.7±1.2e	14.8±1.4f	8.36±0.55f	3.94±0.22e	2.90±0.11f
Pa+Bv	2.07±0.18ef	1.70±0.14fg	29.7±1.6f	17.9±1.8de	16.5±1.5e	9.80±0.69f	4.39±0.25de	3.62±0.13e
Sr+Pp	2.66±0.19e	1.85±0.12f	30.5±2.1f	19.4±1.6d	17.4±1.3e	12.6±0.85e	5.96±0.36d	4.25±0.15e
Sr+Bv	2.97±0.21e	1.99±0.13e	31.3±2.2f	20.4±1.7d	18.5±1.8e	14.2±0.99d	6.66±0.56d	5.45±0.25de
Pp+Bv	4.87±0.66d	2.07±0.21e	36.6±2.6e	25.4±1.9c	24.2±1.9d	15.5±0.85d	8.35±0.75cd	6.40±0.85d
Pa+Sr+Pp+Bv	1.45±0.11g	1.09±0.09h	24.1±1.2h	14.6±1.1f	12.5±1.2g	5.47±0.36g	1.74±0.08g	0.97±0.07h
SII								
C	12.02±0.69a	8.52±0.54a	67.2±4.2a	47.0±2.1a	36.4±2.1a	26.8±1.9a	17.2±0.74a	12.5±0.85a
Pa	5.03±0.45c	2.91±0.25c	41.1±3.2c	26.3±2.2bc	26.2±2.2c	17.0±0.98b	9.43±0.89c	7.78±0.88b
Sr	5.50±0.52b	3.16±0.23bc	45.3±3.2bc	28.1±19b	27.1±2.3bc	13.2±0.85c	10.4±0.66b	8.48±0.68b
Pp	6.04±0.63b	3.50±0.24b	48.2±3.2b	28.8±1.5b	28.1±2.4b	17.9±0.96b	11.5±0.95b	9.16±0.57b
Bv	6.73±0.66b	4.53±0.25b	52.1±3.9b	30.5±2.9b	30.3±2.6b	18.5±0.97b	12.4±0.85b	9.61±0.85b
Pa+Sr	1.51±0.12ef	1.15±0.11ef	25.4±1.9f	15.1±1.1ef	13.3±1.2fg	7.22±0.65g	2.29±0.11f	1.88±0.09de
Pa+Pp	1.93±0.11e	1.37±0.12e	25.7±2.1f	16.3±1.3e	14.5±1.6f	8.15±0.52fg	3.88±0.18e	2.85±0.14d
Pa+Bv	2.02±0.18e	1.63±0.14de	29.3±2.2e	17.6±1.8de	16.3±1.2ef	9.59±0.85f	4.33±0.25e	3.55±0.25d
Sr+Pp	2.60±0.19d	1.78±0.16d	30.0±1.8e	19.1±1.4d	17.2±1.5e	12.4±0.81e	5.90±0.32d	4.21±0.32c
Sr+Bv	2.92±0.21d	1.90±0.16d	30.8±2.7e	20.0±2.3d	18.2±1.2e	13.9±0.66de	6.61±0.33d	5.40±0.42c
Pp+Bv	4.87±0.66c	2.00±0.18cd	35.9±2.8d	25.1±2.6c	23.8±1.6d	15.3±0.58d	8.27±0.54c	6.35±0.63c
Pa+Sr+Pp+Bv	1.40±0.11f	1.01±0.08f	22.5±1.7g	14.1±1.5f	12.0±1.3g	4.99±0.32h	1.55±0.07f	0.90±0.08e

Data are means (n=9) ±SE. Values with the same letters within each column indicate no significant differences according to the Least Significant Difference (LSD) test. ($P \leq 0.05$)

HM, heavy metal, *SI/II*, season I/II, *Cd*, cadium, *Cu*, copper, *Pb*, lead, *Ni*, nickel, *C*, control (no bacteria), *Pa, Pseudomonas azotoformans, Sr Serratia rubidaea, Pp Paenibacillus pabuli*, *Bv Bacillus velezensis*

the release of different metabolites, protons, and exudates that act as metal chelators and limit Pb immobilization. Consistent with the findings of the present study, *Bacillus megaterium* and *Exiguobacterium*, which colonized the root surfaces of *Vigna radiata*, respectively, demonstrated decreased Ni and As translocation (Rajkumar et al. 2013; Pandey and Bhatt 2016).

At the molecular level, PGPR can induce biotransformation of As in wheat plants, through the upregulation of *arsC*, *aioA*, and *arsM* genes (Gu et al. 2017). In addition, metal homeostasis in plants is predominantly governed by transporters, which are bioavailable in relation to the metals (Noor et al. 2022). Although Cd contamination induced the expression of metal transporters in plant tissues, PGPR reduced the transcript levels (Chen et al. 2017). Metal chelators, which are typically composed of sulfhydryl (-SH) groups, immobilize metals through effective binding.

The current research also aligns with the investigations conducted by Mahmoud et al. (2021) regarding *Scenedemus bijugatu*-maize interaction under Cu stress. Furthermore, Cu accumulation regulates the levels of glutathione, thereby promoting metal binding and metal sequestration (Nagalakshmi and Prasad 2001). Inoculation with *P. putida* into rice plants exposed to As toxicity resulted in increased concentrations of glutathione, non-protein thiols, and phytochelatins (Awasthi et al. 2018), thus promoting the biosynthesis of metal-chelating compounds.

In general, PGPR-mediated resilience to interacting effects of HM stress in plants is increasingly acknowledged as a potent strategy for stress mitigation (Oubohssaine et al. 2022; Patil

et al. 2023). At the top of our list of priorities is the investigation of potential future directions that focus on clarifying the mechanism that is responsible for reducing the toxicity of HM in pepper plants by the PGPR that was tested.

Conclusion

Land deterioration and metal pollution pose substantial problems to global food security. The current study demonstrated that bioremediation using PGPR was a viable method for reducing damage in pepper plants grown under HM stress conditions. This was associated with significant decreases in Cd, Cu, Pb, and Ni absorption and/or translocation to higher parts of plants. Our findings showed that inoculating soil containing pepper seedlings with bacterial strains (*Pa*, *Sr*, *Pp*, and *Bv*) promotes HM assimilation. The deployment of HMT PGPR consortia promotes food security and sustainable agricultural production. In this context, the application of stress tolerant PGRP in an appropriate manner for the purpose of remediating contaminated soils of HM could make a significant contribution to the achievement of this goal.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-024-32874-1.

Author contributions **ME-S:** Conceptualization, data curation, investigation, methodology, and writing – original draft; **E-SD:** Data curation, and methodology; **KE-T:** Conceptualization, design, funding acquisition, investigation, methodology, writing – original draft, and writing – review & editing; **SAQ:** Conceptualization, data curation, funding acquisition, investigation, methodology, writing – original draft, and writing – review & editing; **AS:** Conceptualization, design, and formal analysis. All authors read and approved the final version.

Funding This project was supported by Khalifa Center for Biotechnology and Genetic Engineering-UAEU (Grant number: 31R286), Abu Dhabi Award for Research Excellence-Department of Education and Knowledge (Grant number: 21S105), and UAEU program of Advanced Research (Grant number: 12S169).

Data availability Data will be made available on request.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to for publication Not applicable.

Competing interest The authors have no competing interests to declare that are relevant to the content of this article.

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