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A Functional Trade-Off Between Chromium Phytoremediation and Silymarin Preservation Driven by Distinct Pseudomonas Strains in Silybum marianum L.

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Research Article

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A Functional Trade-Off Between Chromium Phytoremediation and Silymarin Preservation Modulated by *Pseudomonas* Strains in *Silybum marianum* L.

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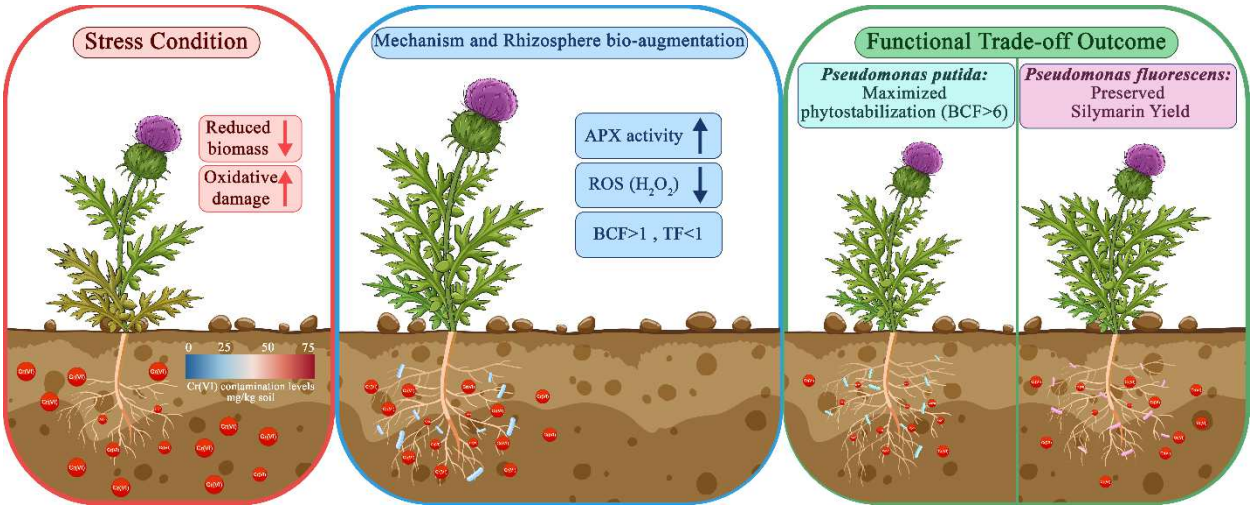
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Graphical Abstract



Abstract

Background and Aims Soil contamination with Cr (VI) disrupts essential plant physiological processes. This study evaluated the phytoremediation potential of milk thistle (*Silybum marianum* L.) inoculated with plant growth-promoting bacteria (PGPB), *Pseudomonas putida* and *Pseudomonas fluorescens*, across varying Cr (VI) levels (0–75 mg kg^{-1}). **Methods** Plants were grown in Cr-contaminated soils with or without bacterial inoculation. Biomass, physiological traits, silymarin content, antioxidant enzyme activities, and Cr accumulation were evaluated to assess phytoremediation efficiency. **Results** Cr (VI) significantly reduced plant biomass, membrane stability, photosynthetic pigments, and silymarin content. Uninoculated plants accumulated H_2O_2, indicating insufficient endogenous defense. However, PGPB inoculation markedly alleviated oxidative stress; decreased guaiacol peroxidase activity coupled with a 4-fold increase in ascorbate peroxidase activity and reduced H_2O_2 levels suggested an optimized ROS detoxification pathway. *S. marianum* displayed a phytostabilization strategy (bioconcentration factor > 1, translocation factor < 1), which *P. putida* enhanced by reducing soil Cr concentrations by 70–89%. Crucially, a functional trade-off emerged: *P. putida* maximized phytoremediation efficiency, whereas *P. fluorescens* preserved silymarin yield across all Cr levels. **Conclusions** Combining efficient soil decontamination with the preservation of high-value medicinal biomass, this integrated plant–microbe system provides a sustainable strategy for managing Cr (VI)-contaminated lands.

Keywords:

Milk thistle; Hexavalent chromium; Phytostabilization; Silymarin; Antioxidant defense system

Introduction

Soil contamination by heavy metals resulting from anthropogenic and industrial activities has emerged as one of the most critical environmental challenges in recent decades, increasingly

threatening global food security, public health, and ecosystem stability (Briffa et al., 2020; Liu et al., 2018). Among various heavy metals, chromium (Cr) has become a ubiquitous and hazardous soil pollutant due to its extensive application in electroplating, leather tanning, textile manufacturing, and steel production, combined with the uncontrolled discharge of industrial effluents (Abreu et al., 2014; Pati et al., 2014). The hexavalent form of chromium [Cr (VI)] exhibits significantly higher toxicity compared to its other oxidation states. Owing to its high mobility, environmental persistence, and strong oxidizing capacity, Cr (VI) readily permeates plant tissues, inducing severe growth inhibition, chlorophyll degradation, enzymatic dysfunction, and profound oxidative stress (Mishra et al., 2021; Singh et al., 2020).

Although conventional physical and chemical remediation techniques offer partial efficacy, they are frequently cost-prohibitive, labor-intensive, and prone to generating hazardous secondary wastes (Ojuederie & Babalola, 2017). Conversely, phytoremediation has gained substantial attention as a green, sustainable, and cost-effective eco-technology. This approach harnesses the innate capacity of plants to absorb, translocate, and sequester environmental contaminants; however, its overall efficiency heavily relies on selecting suitable plant species and managing rhizosphere dynamics (Jabeen et al., 2009; Thakur et al., 2020). Milk thistle (*Silybum marianum* L.), a member of the Asteraceae family, represents a promising candidate for phytoremediation owing to its high biomass yield, robust adaptability to abiotic stress, and rich profile of endogenous antioxidant compounds, including silymarin, flavonoids, and phenolic acids (Anjitha et al., 2021; Ledda et al., 2013). From an economic standpoint, silymarin is a high-value pharmaceutical compound; maintaining its biosynthesis under heavy metal stress creates a dual-benefit system that pairs soil decontamination with the production of valuable bioactive metabolites, thereby enhancing commercial feasibility.

Simultaneously, plant growth-promoting bacteria (PGPB) can substantially enhance phytoremediation performance through diverse biochemical mechanisms. *Pseudomonas putida* and *Pseudomonas fluorescens* are among the most thoroughly characterized PGPB strains capable of mediating the bio reduction of toxic Cr(VI) to the less soluble and less toxic Cr(III), producing siderophores, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and augmenting essential mineral nutrient bioavailability(Ackerley et al., 2004; Liu et al., 2021; Syed et al., 2023). Nevertheless, the synergistic influence of inoculating these bacterial

strains on the physiological performance of *S. marianum* under Cr stress particularly regarding the preservation of silymarin biosynthesis alongside quantitative phytoremediation metrics remains systematically unexplored.

To bridge this knowledge gap, the present study systematically investigates the comparative and synergistic efficacy of *Pseudomonas putida* and *Pseudomonas fluorescens* in optimizing the trade-off between chromium phytostabilization and the preservation of high-value secondary metabolites (silymarin) in *Silybum marianum*.

Materials and Methods

Plant material and experimental design

Seeds of milk thistle (*Silybum marianum* L.) were obtained from the Gorgan region, located in northeastern Iran and southeastern of the Caspian Sea. The Gorgan plant population has been reported to contain the highest silymarin content among Iranian milk thistle populations (Amjadi et al., 2025). Seeds were surface-sterilized and sown in trays containing sterile peat moss. Twenty-one-day-old seedlings were transferred to plastic pots filled with a pre-autoclaved mixture of garden soil and washed sand (2:1 v/v). Plants were grown under day/night temperatures of 25/18 °C and a 16/8 h light/dark photoperiod.

The experiment was conducted as a factorial arrangement in a completely randomized design with three replications in the research greenhouse of the Faculty of Science, Ferdowsi University of Mashhad. The experimental factors included three levels of bacterial inoculation (no inoculation, *Pseudomonas putida* KT2440, and *Pseudomonas fluorescens* PFG5) and four levels of soil contamination with hexavalent chromium (0, 25, 50, and 75 mg Cr (VI) kg^{-1} soil).

Preparation of bacterial and chromium treatments

The strains *P. putida* KT2440 and *P. fluorescens* PFG5 were obtained from the microbial culture collection of the Microbiology Laboratory. Bacteria were cultured in Nutrient Broth for 14 h at 27 °C with shaking at 120 rpm. The bacterial suspension was prepared after adjusting turbidity to the equivalent of a 1 McFarland standard (approximately 10^8 CFU mL^{-1}). One week after transplanting the seedlings into pots, 10 mL of the bacterial suspension was injected at a depth of 3 cm below

the soil surface around the roots of each plant. Two months after sowing, chromium contamination was applied via irrigation with potassium dichromate ($K_2Cr_2O_7$) solution at levels of 0, 25, 50, and 75 mg Cr kg^{-1} soil. Subsequent irrigations were performed based on the water-holding capacity of the pots, without drainage of gravitational water.

Harvest and measurement of morphological traits

Shoots and roots were harvested separately 120 days after sowing. Fresh weights of shoots and roots were measured immediately after harvest, and dry weights were recorded after oven-drying at 60 °C to a constant weight. Rhizosphere soil and roots were collected separately and transferred to the laboratory for microbial counting analyses.

Microbial population counts

To determine the *Pseudomonas* population in the rhizosphere and roots, a serial dilution method with plating on King's B agar containing streptomycin (50 µg mL^{-1}) was used (Schreiter et al., 2014). One gram of rhizosphere soil or root was suspended in 9 mL of sterile phosphate-buffered saline, and after preparing appropriate dilutions, plates were incubated at 27 °C for 16 h. Results were reported as Log_{10} CFU g^{-1} (Cappuccino & Sherman, 2005).

Physiological measurements

Relative water content (RWC) of leaves was measured according to Bian & Jiang (2009). The membrane stability index (MSI) was determined by measuring electrolyte leakage at 40 and 100 °C following the method of Sairam & Saxena (2000). The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were calculated using the method of Lichtenthaler (1987) after extraction with 80% acetone and reading absorbance at 470, 647, and 664 nm.

$$RWC = ((FW - DW)/ (TW - DW)) \times 100$$

$$MSI = [1 - (C1/C2)] \times 100$$

$$\text{Chl a (mg } g^{-1} \text{ FW)} = [(12.25 \times A664) - (2.79 \times A647)] \times (V/W)$$

Chl b (mg g^{-1} FW) = [(21.21 × *A647*) − (5.1 × *A664*)] × (*V*/*W*)

Chl Total (mg g^{-1} FW) = *Chl a* + *Chl b*

Carotenoid (mg g^{-1} FW) = [((1000 × *A470*) − (1.8 × *Chl a*) − (85.02 × *Chl b*))/198] × (*V*/*W*)

Biochemical measurements

Protein was extracted from leaf tissue using phosphate buffer (100 mM, pH = 6.0) containing EDTA, DTT, and PVP, and the soluble protein concentration was determined by the Bradford (1976) method. The activities of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) were measured according to Chance & Maehly (1955) and Nakano & Asada (1981), respectively, using molar extinction coefficients of ε = 26.6 mM^{-1} cm^{-1} and ε = 2.8 mM^{-1} cm^{-1}, and expressed as U mg^{-1} protein. H_2O_2 content was measured by the Sagisaka (1976) method using potassium iodide as the reagent and reading absorbance at 390 nm.

Phenolic compounds and silymarin

Extraction from dried leaf powder was performed with 80% methanol (1:10 w/v, 24 h, 25 °C). Total phenol, total flavonoid, and phenolic acid contents were determined according to Velioglu et al. (1998), Chang et al. (2002), and Matkowski et al. (2008), respectively, and reported as gallic acid equivalents (mg GAE g^{-1} DW), quercetin equivalents (mg QE g^{-1} DW), and caffeic acid equivalents (mg CAE g^{-1} DW). The silymarin percentage, expressed as silybin, was determined by a spectrophotometric method based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) reagent at 490 nm with an extinction coefficient of A1%1cm = 537 (Hasanloo et al., 2005; Taghvaei et al., 2023). Antioxidant activity was also measured and expressed as IC_{50} using the DPPH free radical scavenging assay (Akowuah et al., 2005).

SM (%)=(*A*×10×*V1*×*V2*×100)/(537×100×*M*)

Measurement of Cr and phytoremediation indices

The concentration of Cr in leaves, roots and soil was measured by ICP-OES after acid digestion with aqua regia (HCl:HNO_3, 3:1) and reported as mg kg^{-1} (El Hosry et al., 2023). phytoremediation indices, including the bioconcentration factor (BCF = C_{root}/C_{soil}), translocation factor (TF =

C_{shoot}/C_{root}), and bioaccumulation factor ($BF = C_{shoot}/C_{soil}$), were calculated according to McGrath & Zhao (2003).

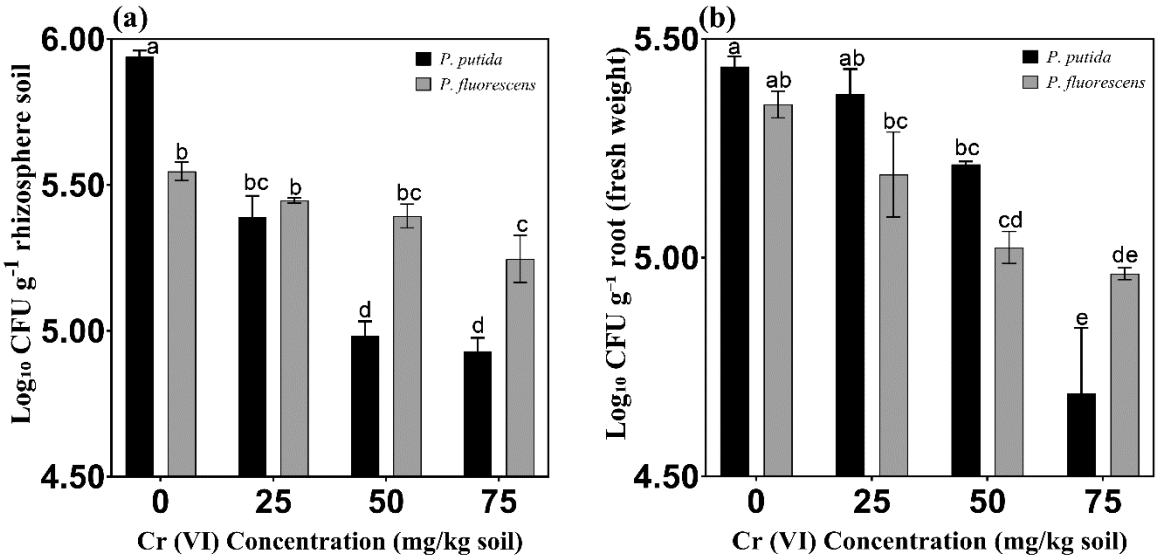
Statistical analysis

Analysis of variance and comparison of means were performed using Duncan's multiple range test at a 5% probability level with SPSS software version 27. The main effects and the two-way interaction (bacteria × chromium) were evaluated for all traits. all figures were created with GraphPad Prism version 10.6.1.

Results

Effect of Cr stress on bacterial colonization in the rhizosphere and root

The rhizosphere microbial population was significantly influenced by Cr concentration, bacterial strain, and their interaction ($P < 0.05$). Overall, the two strains exhibited comparable root colonization capacity; however, their response patterns to Cr stress were distinct. Under non-stressed conditions, *P. putida* showed the highest colonization capacity in both the rhizosphere (5.94 Log_{10} CFU g^{-1}) and the root (5.44 Log_{10} CFU g^{-1}), significantly exceeding that of *P. fluorescens*. With increasing Cr concentration, however, this advantage was reversed. At moderate and high Cr levels (50 and 75 mg kg^{-1}), the decline in *P. putida* populations in the rhizosphere was markedly greater than that of *P. fluorescens*, such that at the highest stress level, the rhizosphere population of *P. fluorescens* (5.25 Log_{10} CFU g^{-1}) was significantly higher than that of *P. putida* (4.93 Log_{10} CFU g^{-1}). A comparable pattern was observed in the root, where at 75 mg kg^{-1} Cr, the population of *P. fluorescens* (4.96 Log_{10} CFU g^{-1}) significantly exceeded that of *P. putida* (4.69 Log_{10} CFU g^{-1}) (Fig. 1). In general, increasing Cr concentration progressively reduced the populations of both strains in the rhizosphere and root; nevertheless, *P. fluorescens* maintained greater colonization stability under moderate and severe stress. This differential stability under Cr stress likely reflects differences in Cr tolerance mechanisms between the two strains and may have important implications for phytostablization efficiency under severely contaminated conditions.



195

196 **Fig. 1.** (a) Rhizospheric and (b) root colonization of *P. putida and P. fluorescens* (Log_{10} CFU g^{-1})
197 in *S. marianum* across four Cr(VI) levels. Values are means ± SE (n=3). Different letters above
198 bars indicate statistically significant differences among treatments based on Duncan's multiple
199 range test ($P \leq 0.05$).

200 **Effect of Cr stress and bacterial inoculation on morphological and physiological traits**

201 **Plant biomass**

202 Increasing Cr concentration caused a progressive and significant decline in plant biomass ($P <$
203 0.05), such that at the highest stress level (75 mg kg^{-1}), bacterial inoculation exerted a positive and
204 significant effect on biomass at all stress levels; however, the relative efficiency of the two strains
205 shifted distinctly with stress intensity. Under non-stressed conditions, the shoot dry weight of
206 plants inoculated with *Pseudomonas fluorescens* was 43.1% higher than that of the control. The
207 most striking result was observed at 50 mg kg^{-1} Cr, where the shoot dry weight of plants inoculated
208 with *Pseudomonas putida* increased by 101% relative to the control (Fig.2a). At the highest stress
209 level, root dry weight in the *P. putida* treatment was 168% higher than that of the control (Fig. 2b).
210 Overall, these results reveal a shift in strain superiority along the stress gradient, with *P.*
211 *fluorescens* performing more effectively in the absence of stress or under mild stress, whereas *P.*
212 *putida* was more efficient in sustaining plant biomass under moderate to severe stress.

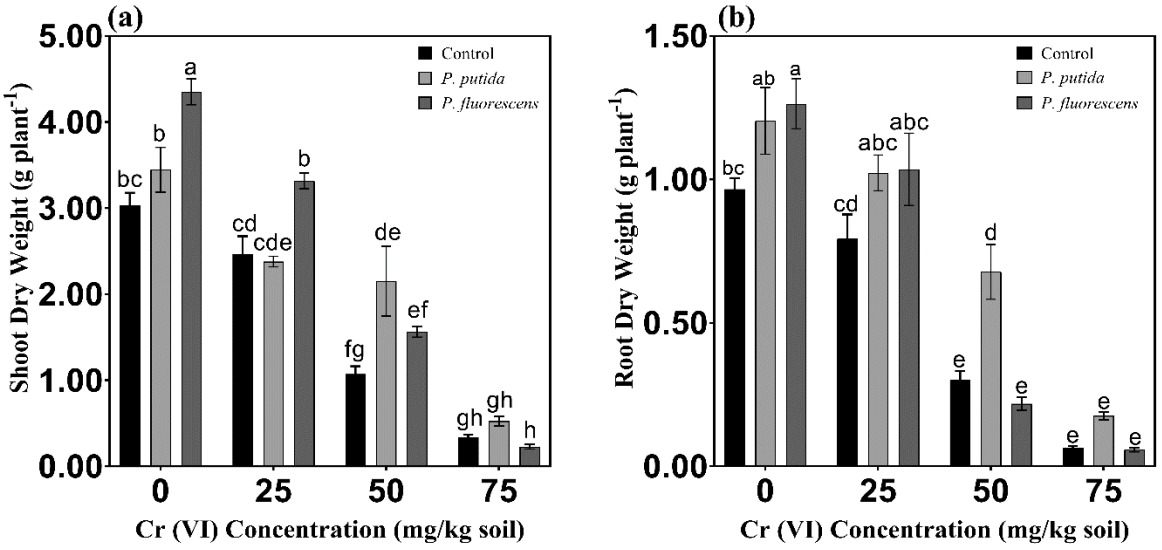


Fig. 2. Effect of Cr(VI) stress and PGPB inoculation on plant biomass in *S. marianum*. (a) shoot dry weight, (b) root dry weight, and (g plant^{-1}) across four Cr(VI). Values are means ± SE (n=3). Different letters above bars indicate statistically significant differences among treatments based on Duncan's multiple range test ($P \leq 0.05$).

Leaf relative water content and membrane stability

Both Cr concentration and bacterial inoculation had highly significant effects on RWC and MSI ($P < 0.001$), whereas the protective effect of PGPB remained uniform across all stress levels. This pattern contrasts with the biomass results, in which the interaction effect was significant and strain efficiency depended on stress intensity. Both traits declined with increasing Cr concentration, although the magnitude of the decline differed. RWC showed a relatively limited reduction and did not differ statistically between 50 and 75 mg kg^{-1}(Fig 3a). In contrast, MSI declined sharply and progressively, reaching 25.02% at the highest stress level (Fig. 3b). Bacterial inoculation significantly improved both traits relative to control plants. With respect to RWC, this increase reached 67% for the *P. putida* strain, while MSI improved by 41.7% compared with the control (Fig. 3).

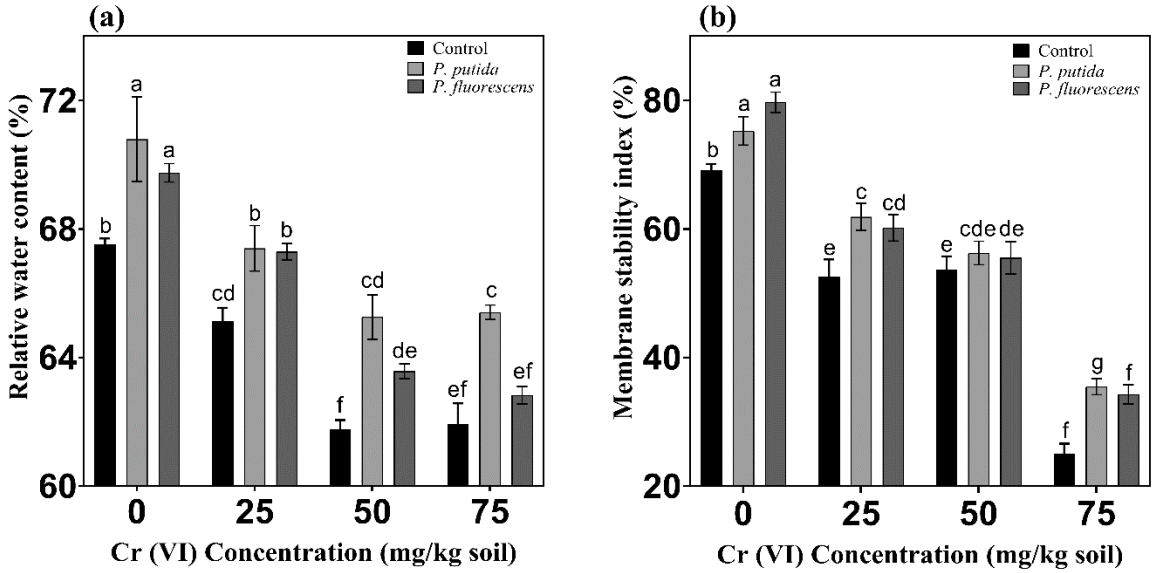
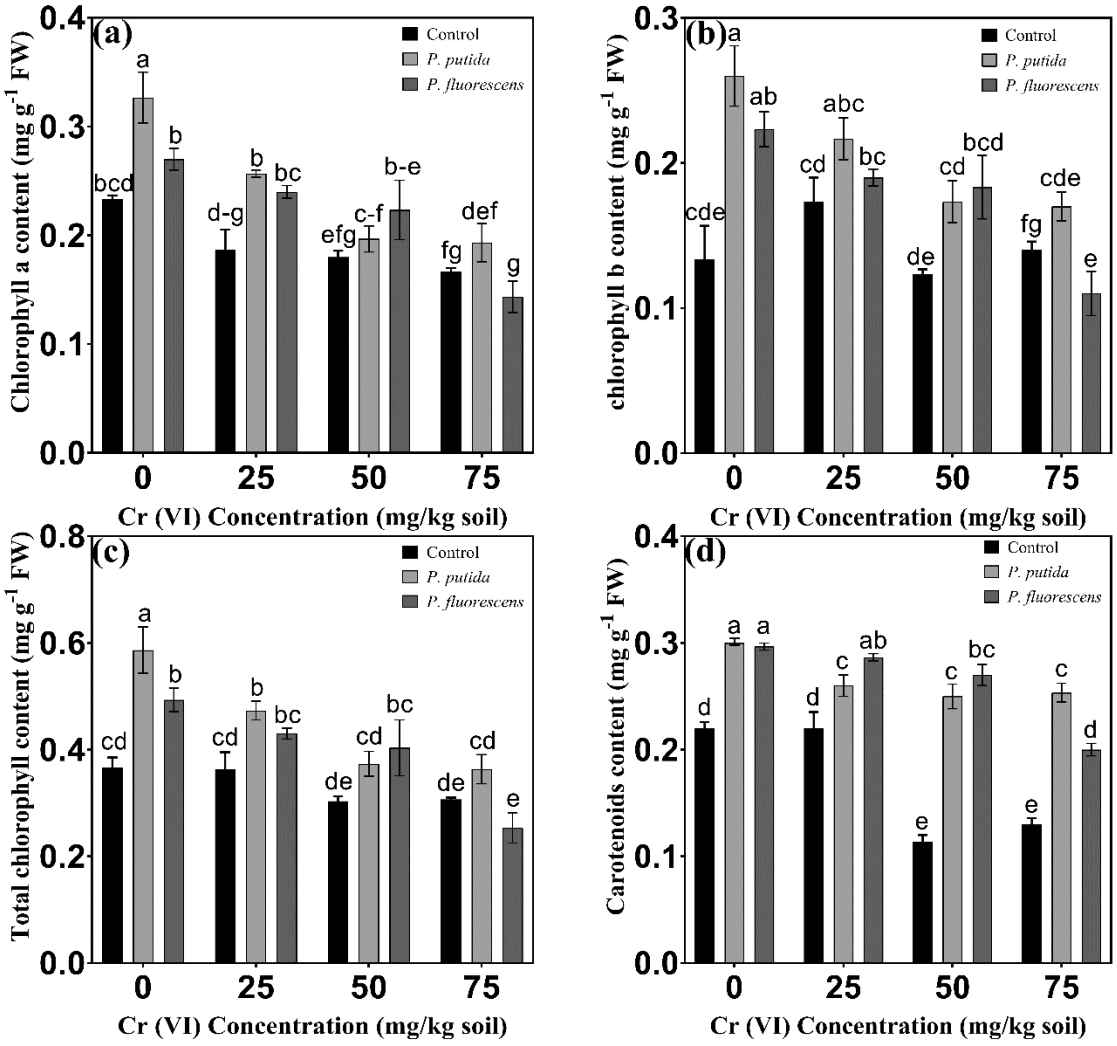


Fig. 3. Effect of Cr(VI) stress and PGPB inoculation on RWC and MSI in *S. marianum*. (a) relative water content (RWC, %) and (b) membrane stability index (MSI, %) across four Cr(VI) levels. Values are means ± SE (n=3). Different letters above bars indicate statistically significant differences among treatments based on Duncan's multiple range test (P≤0.05).

Photosynthetic pigments

Increasing Cr concentration significantly diminished the content of all photosynthetic pigments. Inoculation with either strain alleviated this reduction and enhanced pigment content relative to the control ($P < 0.05$). Under non-stressed conditions, *P. putida* elicited the most pronounced increase in total chlorophyll content (59.7% above the control). The superiority of *P. putida* in this respect was also evident under severe Cr stress, where both chlorophyll a and total chlorophyll contents were significantly higher than those recorded for *P. fluorescens* (Fig. 4 a,b,c). The most salient finding of this section pertained to carotenoids. Under moderate stress (50 mg kg^{-1}), both strains elevated carotenoid content by approximately 110% relative to the control. At the highest stress level, *P. putida* increased carotenoid content by 96.2% compared with the control (Fig.4d), reflecting the comparative advantage of this strain in preserving the antioxidant capacity of carotenoids under severe Cr stress.



246

247 **Fig. 4.** Effect of Cr(VI) stress and PGPB inoculation on photosynthetic pigment content in
248 *S.marianum.* (a) Chlorophyll a, (b) Chlorophyll b, (c) total chlorophyll, and (d) total carotenoids
249 (mg g^{-1} FW). Values are means ± SE (n=3). Different letters above bars indicate statistically
250 significant differences among treatments based on Duncan's multiple range test ($P \leq 0.05$).

251 **Effect of Cr stress and bacterial inoculation on biochemical traits**

252 **Antioxidant enzymes**

253 In this experiment, H_2O_2 accumulation and antioxidant enzyme activity increased with rising Cr
254 concentration, although inoculation with either strain attenuated this response. Under severe Cr

stress, H_2O_2 content peaked in non-inoculated plants (a 68.1% increase relative to the control; Fig. 5c). GPX and APX activities both increased with increasing Cr concentration; however, their response patterns to bacterial inoculation differed. Under severe Cr stress, inoculation with *P. putida* and *P. fluorescens* increased APX activity by 4-fold and 3-fold, respectively, relative to the control (Fig. 5a,b). Under moderate Cr stress, in contrast to the increase in APX, GPX activity declined in inoculated plants, particularly in those treated with *P. putida*.

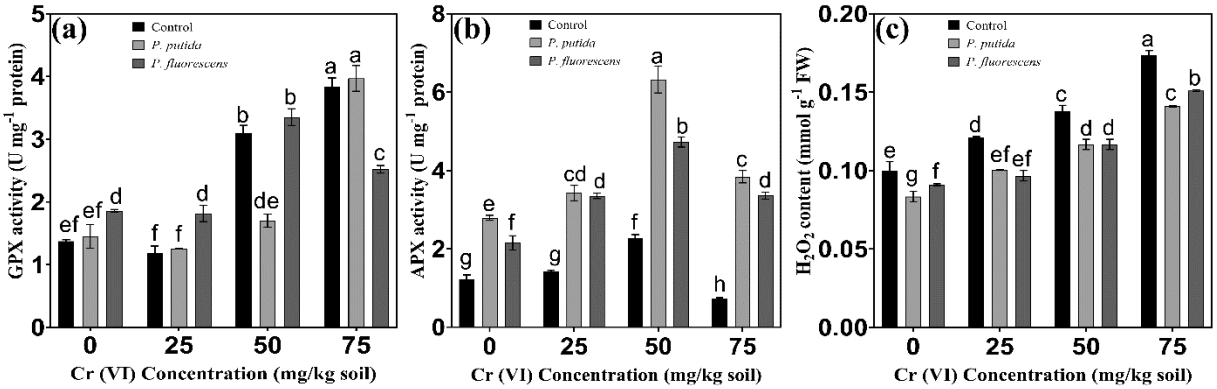


Fig. 5. Effect of Cr(VI) stress and PGPB inoculation on Hydrogen peroxide content and antioxidant enzyme activities in *S. marianum*. (a) guaiacol peroxidase (GPX) and (b) ascorbate peroxidase (APX) activity (U mg^{-1} protein), (c) Hydrogen peroxide content (H_2O_2, mmol g^{-1} FW). Values are means ± SE (n=3). Different letters above bars indicate statistically significant differences among treatments based on Duncan's multiple range test ($P \leq 0.05$).

Secondary metabolites, silymarin, and antioxidant capacity

Total phenolic, flavonoid, and phenolic acid contents

Cr concentration, bacterial inoculation, and their interaction significantly affected the contents of total phenols, flavonoids, and phenolic acids, as well as DPPH radical scavenging activity ($P < 0.05$). In contrast to photosynthetic pigments and plant biomass, which declined uniformly with increasing Cr stress, moderate Cr stress combined with bacterial inoculation maximized the production of these compounds. The highest total phenol content was recorded in the *P. fluorescens* treatment at the moderate stress level (50 mg kg^{-1}), reaching 9.97 mg GAE g^{-1} DW, which was 70% higher than that of the non-stressed control (Fig. 6a). For flavonoids, *P.*

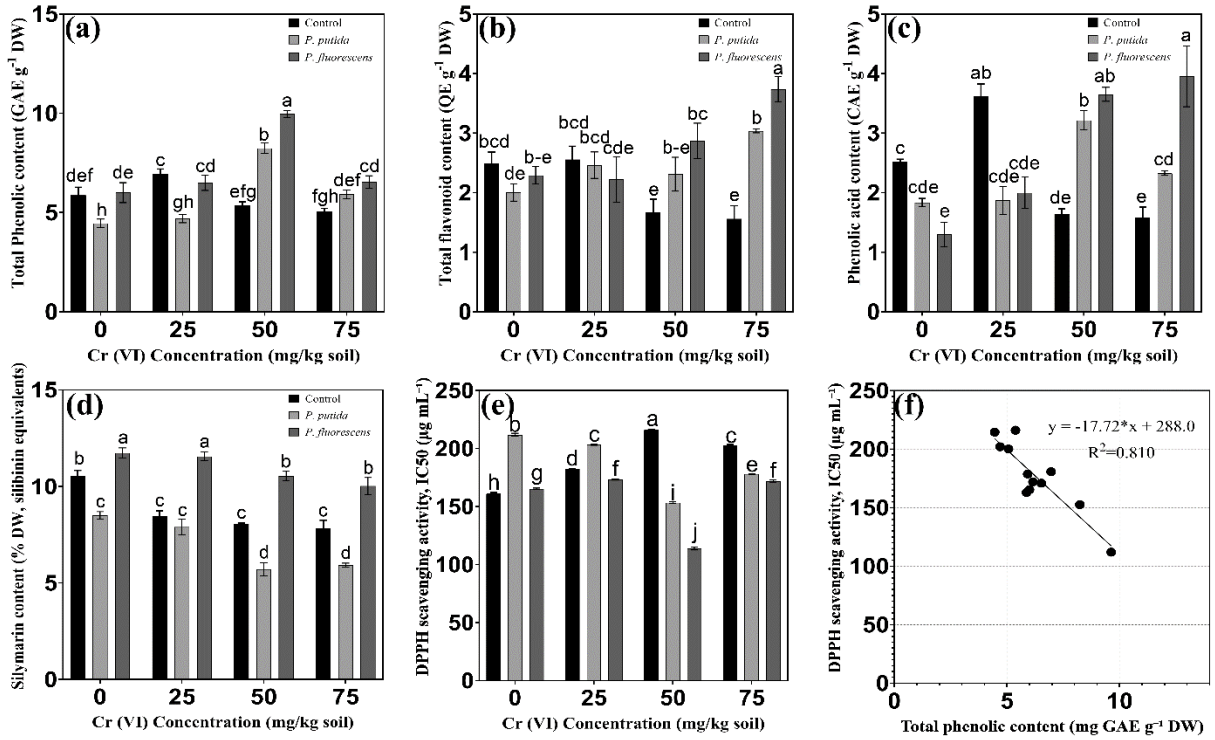
fluorescens also attained the highest value under severe stress (Fig. 6b). Phenolic acids followed a similar trend, with *P. fluorescens* exhibiting the highest content under severe stress (3.95 mg CAE g^{-1} DW) (Fig. 6c). overalls, *P. fluorescens* and *P. putida* demonstrated a comparative advantage in preserving and enhancing phenolic metabolites under moderate and severe stress.

Silymarin content

The findings regarding silymarin are of particular significance. The highest silymarin content was recorded in the *P. fluorescens* treatment under non-stressed or mildly stressed conditions (11.73%), whereas increasing Cr stress led to its gradual decline. Nevertheless, bacterial inoculation significantly enhanced silymarin content relative to the control at each stress level, a finding that indicates this system retains the capacity to produce the valuable medicinal compound silymarin even under moderate contamination (Fig. 6d).

Antioxidant capacity

PGPB inoculation improved the antioxidant capacity (DPPH IC_{50}) under Cr stress. The lowest IC_{50} value (i.e., the highest antioxidant activity) was observed following application of the *P. fluorescens* strain under moderate stress (113.89 µg mL^{-1})(Fig. 6e). Linear regression analysis revealed a significant negative correlation between total phenol content and IC_{50} (Fig. 6f). For each unit increase in total phenol content, IC_{50} decreased by 17.73 µg mL^{-1}, underscoring the central role of phenolic compounds in enhancing the antioxidant capacity of the plant, although the contributions of flavonoids, phenolic acids, and silymarin should not be discounted.



295

296 **Fig. 6.** Effect of Cr(VI) stress and PGPB inoculation on secondary metabolite accumulation and antioxidant
297 capacity in *S. marianum*. (a) total phenolic content (mg GAE g^{-1} DW), (b) total flavonoid content (mg QE
298 g^{-1} DW), (c) phenolic acid content (mg CAE g^{-1} DW), (d) silymarin content (% DW, silibinin equivalent),
299 (e) DPPH radical scavenging activity (IC_{50}, µg mL^{-1}), and (f) linear regression between total phenolic
300 content and DPPH IC_{50} values. Values in panels a–e are means ± SE (n=3). Different letters above bars
301 indicate statistically significant differences among treatments based on Duncan's multiple range test
302 ($P \leq 0.05$).

303 **Effect of PGPB on Cr accumulation in plant tissues and Cr depletion from soil**

304 PGPB inoculation significantly affected Cr content in shoot, roots, and soil. The overall pattern
305 indicates that PGPB enhanced Cr accumulation in roots while reducing it in shoot, implying that
306 both bacterial strains restricted the translocation of Cr from roots to shoots and promoted its
307 retention in belowground tissues. Across all stress levels, the highest Cr accumulation in roots
308 occurred in plants inoculated with *P. putida*. Under moderate stress, root Cr concentration in *P.*
309 *putida*-inoculated plants reached 37.87 mg kg^{-1} DW, which was 64.2% higher than that of non-

inoculated controls. Under severe stress, this value exceeded that of the control by 68.6%. In contrast, PGPB significantly reduced Cr accumulation in shoot (Fig. 7a).

Effect of PGPB on quantitative phytoremediation indices (BCF, BF, and TF)

PGPB inoculation exerted highly significant effects on key phytoremediation indices ($P < 0.001$). Integrated analysis of these indices indicates that *Silybum marianum* predominantly behaved as a Phytostabilizer (TF < 1, BCF > 1), and that inoculation with either strain, particularly *P. putida*, reinforced this strategy by increasing BCF and decreasing TF (Fig. 7c, d,e). The most striking finding of this section was the BCF value obtained with *P. putida* under mild stress (6.72), which was 138.3% higher than that of the non-inoculated control and considerably above the phytostabilization threshold (BCF > 1). Under moderate and severe stress, BCF in *P. putida*-inoculated plants exceeded the control by 59.3% and 67.9%, respectively, indicating the sustained capacity of this strain to stabilize Cr in the roots (Fig. 7d). In contrast, BF declined following PGPB inoculation under severe Cr stress. BF values for plants inoculated with *P. putida* and *P. fluorescens* were 1 and 1.35, respectively, both lower than that of the control plants (BF = 1.61). These findings reflect a shift in the distribution of Cr within the plant. The translocation factor (TF) corroborated these results. *P. putida* significantly reduced TF under moderate and severe stress, whereas non-inoculated plants and those inoculated with *P. fluorescens* exhibited similar TF values under severe stress (Fig. 7c,d,e).

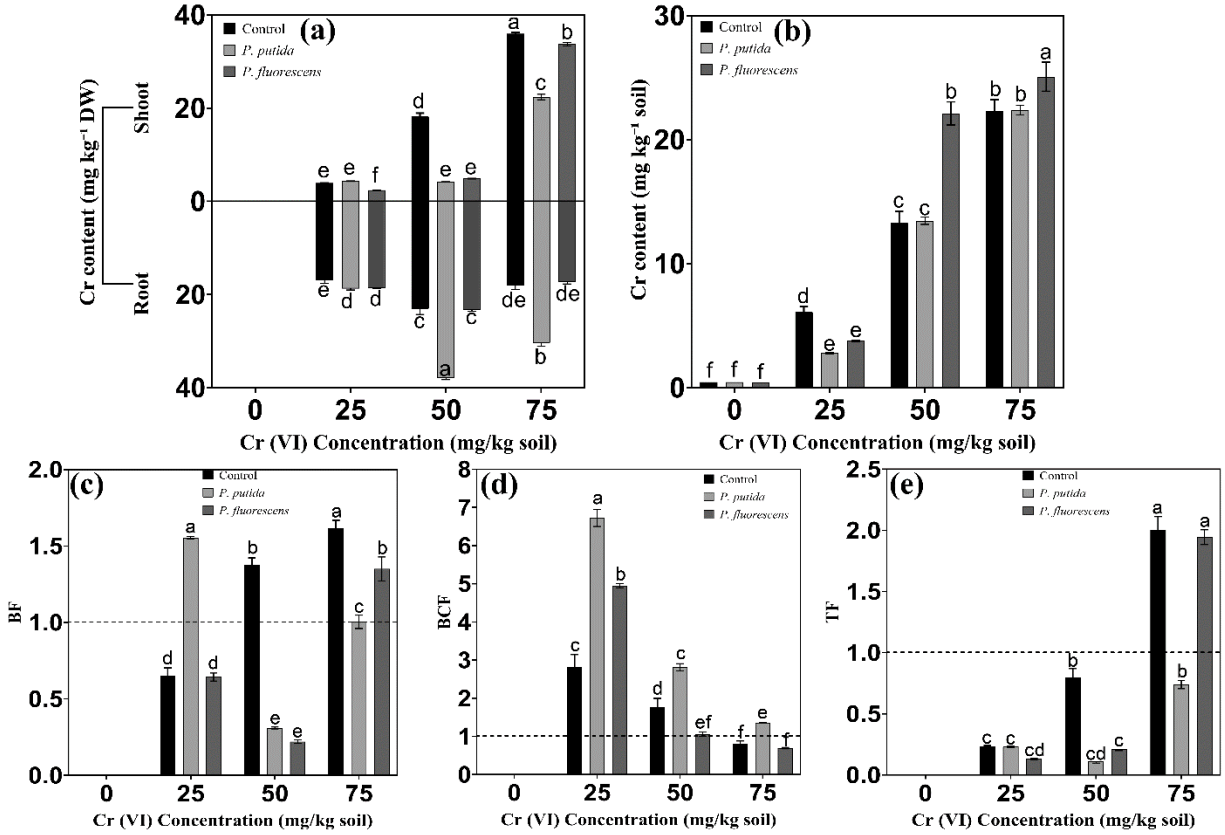


Fig. 7. Effect of PGPB inoculation on chromium accumulation, soil Cr remediation, and phytoremediation indices in *S. marianum*. (a) Cr concentration in root and shoot tissues (mg kg^{-1} DW), (b) residual Cr in soil after the growth period (mg kg^{-1} soil), (c) bioaccumulation factor (BF), (d) bioconcentration factor (BCF), and (e) translocation factor (TF. Values are means ± SE (n=3). Different letter(s) indicate statistically significant differences among treatments based on Duncan's multiple range test ($P \leq 0.05$).

Discussion

Stability of bacterial colonization under chromium stress: strain-specific differences and their implications

The higher rhizosphere colonization by *P. fluorescens* under moderate and severe chromium stress, compared with *P. putida*, which performed better under non-stress conditions, indicates

fundamental differences between the two strains in their chromium tolerance capacity. This pattern is consistent with the report of Ramírez-Díaz et al. (2008), who demonstrated that *P. fluorescens* exhibits higher expression of chromium resistance genes, including *chrA* (chromate efflux pump) and *chrR* (chromate reductase). Reduction of Cr(VI) to Cr(III) by this bacterium may alleviate intracellular oxidative burden, thereby facilitating bacterial survival in contaminated environments (DeLeo & Ehrlich, 1994), although *P. putida* is not devoid of these genes either (Cánovas et al., 2003; Tasleem et al., 2023). The present results suggest that, under the experimental conditions employed here, the expression level or functional efficiency of these systems was greater in *P. fluorescens*.

In this context, the production of extracellular polymeric substances (EPS) appears to be another factor that may account for the greater persistence of *P. fluorescens*. By forming a physicochemical matrix in the rhizosphere, bacterial EPS protects cells from direct contact with Cr ions (Balíková et al., 2022). It should be noted, however, that most available evidence on EPS-metal binding has been obtained under liquid culture conditions (Wu et al., 2021), and the contribution of this mechanism in chromium-contaminated soil rhizosphere warrants further direct investigation.

Importantly, the smaller decline in strain populations at the root surface relative to the bulk rhizosphere points to a protective effect exerted by the root. Root exudates, by lowering the pH at the root surface and supplying dissolved organic carbon, create a more favorable microenvironment for bacterial survival (Compant et al., 2010; Xiao et al., 2023). These findings are consistent with the report of Dell'Amico et al. (2005) that metal-resistant bacterial densities at the root surface were twice those in the rhizosphere.

Under severe stress, *P. fluorescens* maintained a higher population than *P. putida* at the root surface; nevertheless, despite its lower population, *P. putida* exerted stronger beneficial effects on plant growth traits and phytoremediation. This discrepancy is presumably related to differences in single-cell metabolic activity a hypothesis that merits evaluation in future studies.

Chromium stress and bacterial inoculation: dose-dependent effects on plant growth and biomass

The pronounced, dose-dependent reduction in shoot and root biomass under Cr stress observed in this study is consistent with the multiple mechanisms underlying chromium toxicity. Chromium inhibits plant growth by disrupting mineral nutrition, impairing photosynthetic efficiency, and inducing oxidative stress (Shahid et al., 2017; Shanker et al., 2005). The decrease in shoot dry weight at the highest stress level (75 mg kg^{-1}) indicates that this concentration approached or exceeded the tolerance threshold of *S. marianum*, a condition under which endogenous defense systems can no longer counteract the multifaceted toxicity of chromium (Zulfiqar et al., 2023).

The key finding of this section was the shift in the superiority of the two strains in response to stress intensity. *P. fluorescens* exerted a greater beneficial effect under non-stress and mild-stress conditions, whereas *P. putida* performed better under moderate and severe stress, particularly with respect to root biomass. Both strains possess the capacity to reduce Cr(VI) to Cr(III), produce siderophores, facilitate mineral uptake, synthesize IAA, and exhibit ACC deaminase activity (AL-Huqail et al., 2025; Sayel et al., 2014; Tirry et al., 2021). These differential responses likely reflect differences in the mechanisms and stimulatory effects of the strains with respect to plant growth promotion under varying stress intensities.

Role of PGPB in Membrane Integrity, Water Status, and Photosynthetic Pigments under Chromium Stress

The concurrent declines in MSI, RWC, and photosynthetic pigments under chromium (Cr) stress reflect a continuous physiological cascade. Chromium disrupts membrane integrity through lipid peroxidation and ROS generation (Pandey et al., 2009; Shahid et al., 2017). This disruption, in turn, impairs osmotic regulation and reduces cellular water content, leading to stomatal closure, restricted CO_2 availability, and ultimately oxidative damage to chloroplasts, as well as competition between Cr and essential elements required for chlorophyll synthesis particularly Mg, Fe, and N thereby diminishing photosynthetic capacity (Sharma et al., 2020; Vernay et al., 2007). The 63.8% reduction in MSI at the highest stress level represented the most severe Cr-induced effect in this study, co-occurring with a marked decline in carotenoids. Beyond their role in light harvesting, carotenoids also contribute to the antioxidant protection of the photosynthetic apparatus (Jahns & Holzwarth, 2012).

In this study, the effects of the bacterial strains on RWC and MSI were nearly identical across different stress intensities. It appears that PGPB-mediated protection of membrane integrity and plant water status is primarily exerted through pathways that are less sensitive to stress severity. The most plausible mechanism in this regard is the reduction of Cr(VI) bioavailability via its bio reduction to Cr(III), as well as complexation by bacterial extracellular polymeric substances (EPS) (Ahemad, 2015).

The most notable finding in this section was the 110% increase in carotenoid content in both bacterial treatments under moderate stress, compared with a 48.2% decrease in control plants. The disproportionate response of carotenoids relative to chlorophylls appears consistent with their dual role as photosynthetic pigments and antioxidant defense components (Latowski et al., 2011). In this context, PGPB may have facilitated this response by improving Fe availability through siderophore production, since iron is essential for the activity of enzymes in the MEP pathway of carotenoid biosynthesis (Rajkumar et al., 2010).

Effect of Chromium Stress and PGPB on Redox Homeostasis and the Antioxidant Defense System

In this experiment, the progressive accumulation of H_2O_2 in control plants with increasing Cr concentration indicates the growing inability of endogenous antioxidant systems to maintain redox homeostasis. Chromium exacerbates ROS production through Fenton-like reactions and disruption of the chloroplastic electron transport chain (Panda & Choudhury, 2005; Shanker et al., 2005). Although GPX activity was induced under moderate to severe stress, this response appeared insufficient to suppress oxidative stress. Conversely, the sharp decline in APX activity in control plants under severe stress was likely attributable to enzyme denaturation or depletion of ascorbate pools (Sharma & Dubey, 2005) a phenomenon indicative of impaired peroxidase functionality in the absence of bacterial strains.

Under moderate Cr stress, a divergent response in peroxidase activity was observed in plants inoculated with *P. putida*, such that GPX activity decreased by 44.9% while APX activity increased by 179.7%, with a concomitant suppression of H_2O_2 accumulation. It appears that *P. putida*, by modulating the oxidative burden, employed the APX ascorbate pathway as the preferred

mechanism for H_2O_2 scavenging. Under severe stress, both bacterial strains markedly enhanced APX activity, which was directly associated with reduced H_2O_2 accumulation, a finding consistent with previous reports (Haghmadad Milani et al., 2023; Mellidou et al., 2021). The underlying mechanisms governing this antioxidant enhancement likely involve the induction of antioxidant gene expression via plant–bacteria signaling cascades, coupled with improved bioavailability of critical cofactor elements, such as Fe (for the heme moiety in peroxidases) and P (for the generation of ATP and reduced glutathione within the ascorbate–glutathione cycle) (Kayoumu et al., 2023; Ramírez et al., 2013).

Secondary Metabolites, Silymarin, and Antioxidant Capacity: The Complex Interplay between Chromium Stress and Bacterial Inoculation

Phenolic compounds exhibited a biphasic pattern in response to Cr stress, whereby total phenol, flavonoid, and phenolic acid contents peaked under moderate stress and declined under severe stress. This trend is likely associated with the dual mechanism of heavy metal action, since under mild to moderate stress, reactive oxygen species act as signaling molecules that stimulate the phenylpropanoid pathway through activation of phenylalanine ammonia-lyase (PAL) (Jakubowska et al., 2025; Michalak, 2006), whereas under severe stress, structural cellular damage and severe metabolic disturbances preclude the continuation of biosynthetic activities (Zhang et al., 2022).

Unlike other phenolic compounds that were induced under stress, silymarin declined with increasing Cr stress, a pattern consistent with the study by Amjadi et al. (2025), who reported reduced leaf silymarin in *S. marianum* inoculated with *P. putida*. Silymarin biosynthesis requires the coordinated activity of PAL, CHS, and specific peroxidases (AbouZid & Osama, 2013; Dong & Lin, 2021), and this pathway competes with other phenylpropanoid routes, including lignin and suberin synthesis, which are prioritized under severe stress. It appears that phenylpropanoid precursors are redirected toward structural defense pathways rather than toward the production of specialized flavonolignans and silymarin (Sharma et al., 2019; Vogt, 2010).

In this experiment, the distinction between the two bacterial strains with respect to silymarin has important practical implications. *P. fluorescens* maintained or increased silymarin content relative

to the corresponding control at all stress levels, recording the highest value under non-stressed or mildly stressed conditions, whereas *P. putida* exerted a reducing effect on silymarin under moderate and severe stress. This finding, together with the superior phytostabliztation efficiency of *P. putida* (higher BCF, greater biomass), reveals an important practical trade-off: for soil remediation purposes, *P. putida* is the superior choice; however, for systems aimed at integrating phytostablization with the preservation of the medicinal value of *S. marianum*, *P. fluorescens* or a combination of both strains represents a more favorable strategy. This conclusion provides a novel framework for optimizing bacterial strain selection based on management objectives.

Chromium Accumulation, Distribution Pattern, and Phytostabilization Efficiency

The preferential accumulation of chromium in roots relative to shoots, characterized by BCF > 1 and TF < 1 across most treatments, indicates that *S. marianum* predominantly employs a phytostabilization strategy. This mechanism is linked to the capacity of root cells to reduce Cr(VI) to Cr(III) via endogenous reducing agents such as ascorbate and glutathione (Lytle et al., 1998), a process that lowers Cr mobility and facilitates its sequestration by carboxyl and hydroxyl functional groups in the root cell wall (Usman et al., 2020). This compartmentalization pattern offers an important ecophysiological benefit: plants that compartmentalize Cr in subterranean tissues maintain sufficient biomass without exhibiting aerial toxicity, thereby sustaining a higher long-term phytoremediation capacity (Sajad et al., 2020; Shahandeh & Hossner, 2000). A concordant root-retention pattern under heavy metal stress was observed by Papadimou et al. (2024) in *S. marianum* exposed to cadmium.

Inoculation with PGPB significantly reinforced this root stabilization pattern, with *P. putida* demonstrating a pronounced superiority over the co-evaluated strain. Under mild Cr stress, *P. putida*-treated plants achieved a BCF of 6.72 (a 138.3% increase over the non-inoculated control), well exceeding the threshold for effective bioaccumulation (BCF > 1). This highlights the remarkable capability of this strain to immobilize Cr in belowground tissues. *P. putida* is recognized for its ability to reduce Cr(VI) to Cr(III) directly within the rhizosphere (Ackerley et al., 2004), thereby curbing metal bioavailability and mobility prior to root uptake. Furthermore,

the augmented root surface area and biomass documented in inoculated plants expand the total binding capacity for Cr immobilization (Glick, 2012). In addition, the putative stimulation of chelating peptide biosynthesis (e.g., phytochelatins) in root tissues may enhance vacuolar sequestration capacity (Ma et al., 2016), a hypothesis that warrants dedicated mechanistic validation in future studies. Our findings align with previous reports, including Tirry et al. (2021), who documented a 49% increase in root Cr accumulation in alfalfa inoculated with *Pseudomonas* sp., and Akhtar et al. (2021), who recorded a 2.71-fold increase in root Cr retention in *Brassica nigra* assisted by *Bacillus cereus*.

Conclusion

This study demonstrates that inoculation of *Silybum marianum* with *Pseudomonas putida* and *Pseudomonas fluorescens* significantly alleviates a broad spectrum of Cr (VI) induced toxicities at morphological, physiological, and biochemical levels. Under the highest Cr stress, both bacterial strains improved membrane stability by 40%. A reduction in GPX activity, concurrent with up to a fourfold increase in APX activity and a simultaneous decline in H2O2 accumulation, points to a PGPB-mediated reprogramming of the preferred antioxidant detoxification pathways. Furthermore, bacterial inoculation enhanced carotenoid content by 110% under moderate stress, while bolstering total phenolic and non-enzymatic antioxidant capacities. In this plant- microbe system, *S. marianum* primarily adopted a phytostabilization strategy (BCF>, TF<1), which was markedly reinforced by PGPB application, driving the BCF up to 6.72 in the *P. putida* treatment.

A pivotal finding of this work is the identification of a practical functional trade-off between the two evaluated strains, enabling a targeted, application-driven bioinoculant selection: *P. putida* emerges as the superior candidate for maximizing phytoremediation efficiency, particularly in moderately to severely contaminated soils, whereas *P. fluorescens*, owing to its superior preservation of silymarin content across all stress regimes, is ideally suited for integrated systems aimed at coupling soil decontamination with the sustainable production of high-value medicinal biomass. As these findings were derived from controlled greenhouse trials using individual strains, field-scale validation and evaluations across broader microbial consortia, including dual co-inoculation of both strains, remain essential next steps.

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Declarations

Conflict of interest

The authors of this study declare that they have no conflict of interest

Author Contributions

AP: Data collection and analysis, performed all experiments, measured and recorded data, performed the statistical analysis, wrote the paper; AG: conceived and designed the experiments and analysis, performed the statistical analysis, wrote the paper, performed text mining analysis, discussed the results and contributed to the final manuscript; MM: conceived and designed the experiments, established and refined the microbial culture and inoculation techniques **,** wrote the paper.

Data Availability

Data will be made available on request.

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