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Morphometric and biochemical responses of rice seedlings to heavy metal stress mitigated by *Bacillus subtilis*

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ABSTRACT

This study evaluated the role of *Bacillus subtilis* in mitigating cadmium (Cd) and arsenic (As) stress (100 mM) in three rice cultivars (ADT 36, CO 51, and TPS 5). Twelve bacterial species were isolated from soil, with *Bacillus* spp. selected for their adaptability and plant growth-promoting traits. Heavy metal stress significantly reduced root and shoot growth, biomass, pigment levels, and protein and proline contents, while increasing phenol, soluble sugars, and Na^+ accumulation. Cd caused stronger growth inhibition, whereas As had a greater impact on pigment biosynthesis. Inoculation with *B. subtilis* enhanced seedling vigor, improved pigment retention, restored protein and proline levels, and reduced phenol and sugar accumulation across cultivars. Ionic balance was also improved, with lower Na^+ and higher Ca^{2+} and K^+ compared to stress plants. Among the cultivars, CO 51 and TPS 5 showed the strongest recovery, while ADT 36 exhibited moderate tolerance. Overall, *B. subtilis* effectively alleviates heavy metal–induced phytotoxicity in rice by improving morphometric, biochemical, and ionic traits, demonstrating its potential for sustainable phytoremediation.

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops worldwide, providing nutrition to more than half of the global population. In addition to its significance in human diet, rice serves as a model plant for understanding stress physiology and plant–microbe interactions due to its well-characterized genome and wide adaptability (Khush, 2005). However, rice productivity is increasingly threatened by environmental stresses, among which heavy metal contamination is a major concern. Rapid industrialization, mining, excessive use of agrochemicals, and wastewater irrigation have led to the accumulation of heavy metals such as cadmium (Cd) and arsenic (As) in agricultural soils (Ali *et al.,* 2013; Rahman *et al.,* 2007).

These toxic elements are readily absorbed by rice roots and translocated to aerial parts, posing serious risks not only to plant growth and yield but also to food safety and human health (Zhao *et al.,* 2010).

Heavy metals adversely affect plant morphometric traits such as shoot and root length, biomass accumulation, and leaf development. At the biochemical level, they impair photosynthetic pigments, reduce protein synthesis, disturb ion balance, and trigger the accumulation of phenols and soluble sugars as stress markers (Singh *et al.,* 2016; Nagajyoti *et al.,* 2010). Additionally, they induce oxidative stress by promoting the overproduction of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, and membrane damage (Gill and Tuteja, 2010).

Plants possess innate tolerance mechanisms such as proline accumulation, antioxidant enzyme activity, and selective ion uptake; however, these are often insufficient under prolonged or high levels of metal stress (Sharma *et al.,* 2012). Therefore, external interventions are required to enhance tolerance and maintain plant productivity in contaminated soils.

Plant growth-promoting rhizobacteria (PGPR) have emerged as effective biological tools to alleviate heavy metal toxicity and promote plant growth. Among them, *Bacillus subtilis* is of particular interest due to its spore-forming ability, resilience to environmental fluctuations, and multiple plant growth-promoting traits (Radhakrishnan *et al.,* 2017). It is known to enhance nutrient availability, solubilize phosphate, produce phytohormones such as indole-3-acetic acid (IAA), and secrete siderophores that reduce heavy metal bioavailability in soils (Idris *et al.,* 2007). Moreover, *B. subtilis* has been reported to mitigate the negative effects of heavy metals by regulating ion homeostasis, stimulating proline and protein metabolism, and maintaining pigment stability in plants (Vardharajula *et al.,* 2011; Chen *et al.,* 2009).

Understanding the combined effects of heavy metal stress and *B. subtilis* inoculation on rice is crucial for developing eco-friendly strategies to improve crop performance in contaminated soils.

Morphometric and biochemical analyses provide key insights into plant physiological status and stress responses. While several studies have addressed the individual effects of heavy metals or PGPR on rice, comprehensive evaluations integrating growth, biochemical, and ionic parameters under heavy metal toxicity with *B. subtilis* inoculation remain limited. The present study, therefore, aims to investigate the morphometric and biochemical characteristics of rice seedlings exposed to cadmium and arsenic stresses, and to evaluate the role of *Bacillus subtilis* in mitigating their toxic effects. By integrating growth, pigment, biochemical, and ion analyses, this work seeks to highlight the potential of *B. subtilis* as a bioinoculant for enhancing rice tolerance to heavy metals and contributing to sustainable agriculture and phytoremediation.

MATERIALS AND METHODS

Sample collection

The soil samples were collected near form agricultural field. The samples were made at a depth within 5 cm from the surface of the soil. The collected soil samples were brought to the laboratory in sterilized polythene bags, handpicked, air dried and to pass through a

sieve (2mm). They was collected in a plastic container, sealed and stored at 4 °C until further use.

Isolation of microorganisms

Serial dilution technique was used for isolation of microorganism from soil sample. 1g of soil sample was suspended in 10 mL sterile distilled water and prepared stock. The 50 µL of the diluted sample (10^{-6}) was spread on nutrient agar plates.

Identification of the bacterial isolate

The microorganisms were identified based on colony characteristics. Gram staining methods was performed to check the morphology of the cells and spore chain were identified by spore staining methods and also biochemically characterized by IMVIC test, starch hydrolysis test, urea hydrolysis test, nitrate reduction test, hydrogen sulfide production test and cytochrome oxidase test (Aved *et al.,* 2008). On the basis of dominant group of bacteria, we identified the bacterial isolates as a *Bacillus subtilis*. The isolated bacterial strain was sub cultured on nutrient media and was incubated for 24h at 37°C in shaker to obtained culture density of 108 CFU mL^{-1} colony (Shah *et al.,* 2017).

Seed germination

Seeds of *Oryza sativa* cultivars cv. TPS 5, CO 51 and ADT 36 were collected from the Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India. The healthy and uniform seeds were surface sterilized using 3.5% sodium hypochlorite solution for 10 minutes.

The sterilized seeds were placed in a 9 cm-diameter Petri dish layered with Whatman No. 1 filter paper and 10 mL distilled water was added. The Petri dishes were sealed with Parafilm and placed inside a growth chamber for germination. After germination, seven-day-old seedlings were shifted to autoclaved sand in plastic pots (1kg sand per pot) and were grown for two weeks.

All pots were placed in a greenhouse with average 29 ± 1°Cand 24 ± 1°C temperatures for day and night, respectively, with average humidity 70% throughout the experiment. The experiment was laid out in complete randomized design (CRD) with 12thtreatments. According to the experimental design, 10mL bacterial suspension (108 CFU) was applied to each pot, whereas a $CdCl_2$ and As_2O_3 solution of 100mM concentration, with and without bacterial suspension, was applied to each pot of 21-day-old seedlings (Bal *et al.,* 2013).

Experimental design

The experiment consists of 12 pots using three rice varieties: ADT 36, CO 51, and TPS 5. Each variety is treated with two types of heavy metals—Cadmium ($CdCl_2$) and Arsenic (As_2O_3), both at a concentration of 100 mM. For each metal treatment, plants are either exposed to a bacterial suspension or not. Specifically, Pots 1 and 2 contain ADT 36 treated with $CdCl_2$, without and with bacterial suspension respectively; Pots 3 and 4 contain ADT 36 treated with As_2O_3, again without and with bacterial suspension. Similarly, Pots 5 and 6 include CO 51 treated with $CdCl_2$, and Pots 7 and 8 include CO 51 treated with As_2O_3, each pair differing by the presence or absence of bacterial suspension. Finally, TPS 5 is used in Pots 9 and 10 for $CdCl_2$ treatment and Pots 11 and 12 for As_2O_3 treatment, with and without bacterial suspension accordingly.

Morphometric analysis

After 15 days the grown plants were removed carefully and were separated into shoots roots. The lengths of the root, shoots were measured and fresh and dry weight was weighed (Navari-Izzo and Quartacci, 2001).

Chlorophyll determination

The chlorophyll content of plant leaves was estimated by the method of Arnon, (Arnon 1949). 100 mg of leaf samples were ground with 80% of acetone followed by centrifugation at 5000 rpm for 10 min. The supernatant was collected and its optical densities at 645 nm were measured using UV-visible spectrophotometer.

Estimation of total protein

500 mg of plant leaves were homogenized in 5 mL of phosphate buffer. The extract was centrifuged at 10000 rpm for 10 min and the supernatant was collected. The extract was precipitated by adding in equal volume of ice cold TCA. Then it was centrifuged

at 12000 rpm for 10 min. The pellet was collected and dissolved in 2 mL of NaOH (Bradford 1976). To 1 mL of the sample and 5 mL of the reagent were added and mixed thoroughly. The absorbance was read at 595 nm against the reagent blank. The amount of total protein in the sample was determined using a standard graph prepared from bovine serum albumin ranging from 10 to100 µg/mL.

Estimation of total lipids

The total lipid content extracted from the sample was determined by Folch *et al.,* 1957). Briefly, 500 mg of the plant leaves were taken and homogenized with 6 mL of chloroform: methanol. It was then transferred to a separating funnel and the organic phase was separated. To this, 2 mL of physiological saline was added and mixed well. The mixture was left undisturbed for overnight and about 0.5 mL of the lower chloroform phase containing lipid was collected in test tubes. The solvent was then allowed to evaporate at room temperature and the pellet was collected. 0.5 mL of concentrated sulphuric acid was added to the pellet and mixed well. The sample was closed and kept in a boiling water bath for 10 min and allowed to cool at room temperature. The resulting sample was taken and 5 mL of phosphovanillin reagent was added and mixed well than allowed to stand for 30 min and its measured at 520 nm. Standard graph was prepared using cholesterol ranging from 10 to 100 µg/mL.

Estimation of phenol

Phenol content was calculated following folin-ciocalteau method (Slinkard *et al.,* 1977). The blue colour developed in solution was read at 650 nm against a blank reagent. The concentrations of phenols were expressed as mg phenol g-1 tissue.

Total soluble sugar

Total soluble sugar contents were determined by the phenol-sulphuric acid method of Dey *et al.,* 1990) with slight modifications. Fresh plant material (50mg) was grounded in 3mL pre warmed 90% ethanol and incubated at 80°C for 60 min. The supernatant was transferred and the same procedure was repeated. Both supernatants were combined and added to 1:10.5% phenol, 5mL H_2SO_4, and 3mL distilled water under constant shaking conditions and incubated for 30 min. The absorbance was measured at 485nm using glucose as a standard.

Determination of proline

Free proline content was estimated by following the method of Bates *et al.* (1973). Fresh 0.5 gm root and shoot samples were homogenized in 5 ml of 3% sulpho salicylic acid using a mortar and pestle. About 2 ml of extract was taken in test tube and to it 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added. The reaction mixture was boiled in a water bath at 100°C for 30 min. After cooling the reaction mixture, 4 ml of toluene was added. After thorough mixing, the chromophore containing toluene was separated and absorbance of red color developed was read at 520 nm against toluene black on UV-visible spectrophotometer (Chemito, UV- 2600).

The proline concentration was determined using calibration curve and expressed as mg proline per g fresh weight of tissue.

Micronutrient estimation

Micronutrient analysis was carried out following the method described by Awan (2005) with slight modifications. Briefly, 50 mg of oven-dried plant material was digested with 2 mL of concentrated sulfuric acid (H_2SO_4) and 1 mL of hydrogen peroxide (H_2O_2). The mixture was heated until a clear solution with a single oily droplet remained. After cooling, the digest was diluted with 20 mL of distilled water and filtered through Whatman No. 1 filter paper. The concentrations of Na^+, Ca^{2+}, and K^+ ions in the filtrate were quantified using a flame photometer (Jenway PF7F).

RESULTS

Isolation and identification bacterial sp.

Bacterial species were isolated from soil samples and identified using various morphological and biochemical tests. The isolation was performed through serial dilution and plating techniques. A total of twelve bacterial species were identified, including

Pseudomonas putida, *Pseudomonas fluorescence*, *Pseudomonas alcaligenes*, *Pseudomonas aeruginosa*, *Azospirillum brasilense*, *Rhizobium leguminosarum*, *Xanthomonas maltophilia*, *Enterobacter* sp., *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus cereus*, and *Azotobacter* sp. Among these, species of *Pseudomonas* and *Bacillus* were predominant (Table 1). Soil microbes play a crucial role in nutrient cycling, maintaining soil structure, detoxifying harmful chemicals, controlling plant pathogens, and promoting plant growth. Therefore, these bacteria can enhance the phytoremediation capacity of plants and reduce the phytotoxicity of contaminated soils**.** *Bacillus* species were selected for further research because they were predominantly present in the soil samples, indicating high adaptability. They are well-known for their resilience, spore-forming ability, and plant growth-promoting properties, making them ideal candidates for studies on soil health, phytoremediation, and plant-microbe interactions.

Table 1. Bacterial species isolated from soil samples

#	Bacterial species identified	Group	Importance	Reference
1	*Pseudomonas putida*	*Pseudomonas*	Plant growth promotion, biodegradation, nutrient cycling	Stanier *et al.*, 1966; Timmis, 2002
2	*Pseudomonas fluorescence*	*Pseudomonas*	Produces fluorescent pigments, controls plant pathogens	Haas and Défago, 2005
3	*Pseudomonas alcaligenes*	*Pseudomonas*	Biodegradation of pollutants, soil health improvement	Watanabe *et al.*, 1998
4	*Pseudomonas aeruginosa*	*Pseudomonas*	Produces metabolites, potential biocontrol agent	Raaijmakers *et al.*, 2002
5	*Azospirillum brasilense*	*Azospirillum*	Nitrogen fixation, promotes root development	Okon and Labandera-Gonzalez, 1994
6	*Rhizobium leguminosarum*	*Rhizobium*	Symbiotic nitrogen fixation in legumes	Graham and Vance, 2000
7	*Xanthomonas maltophilia*	*Xanthomonas*	Plant-associated bacterium, some strains beneficial for bioremediation	Ryan *et al.*, 2009
8	*Enterobacter* sp.	*Enterobacter*	Nitrogen fixation, promotes plant growth	Shankar *et al.*, 2011
9	*Bacillus pumilus*	*Bacillus*	Produces antimicrobial compounds, biocontrol, soil resilience	Earl *et al.*, 2008
10	*Bacillus subtilis*	*Bacillus*	Spore-forming, plant growth promotion, pathogen suppression	Kloepper *et al.*, 2004
11	*Bacillus cereus*	*Bacillus*	Produces secondary metabolites, soil adaptation	Nicholson, 2002
12	*Azotobacter* sp.	*Azotobacter*	Free-living nitrogen fixer, improves soil fertility	Mrkovacki and Milic, 2001

Table 2. Comparative analysis of physiological parameters in *B. subtilis*-treated and untreated rice cultivars under cadmium stress

Cultivar	Treatment	Length		Fresh weight		Dry weight	
		Shoot	Root	Shoot	Root	Shoot	Root
ADT 36	Control	28.1±0.6	7.5±0.2	1.9±0.6	1.5±0.2	1.0±0.6	0.7±0.5
	Control+cd (100mM)	23.2±0.8	5.2±0.7	1.7±0.5	1.2±0.3	0.8±0.2	0.2±0.1
	B. subtilis+cd (100mM)	27.2±1.1	7.8±0.1	1.8±0.2	1.7±0.2	0.9±0.5	0.6±0.4
CO 51	Control	38.2±1.6	10.3±0.6	2.9±0.5	1.4±0.2	1.5±0.6	0.7±0.5
	Control+cd (100mM)	27.0±0.7	8.0±0.2	1.2±0.1	0.9±0.5	0.9±0.7	0.5±0.3
	B. subtilis+cd (100mM)	34.3±1.5	11.3±0.5	2.3±1.0	1.6±0.6	1.3±0.2	0.9±0.8
TPS 5	Control	32.5±1.2	9.2±0.5	1.9±0.2	0.8±0.2	1.0±0.5	0.4±0.2
	Control+cd (100mM)	29.7±1.4	6.2±0.7	0.9±0.4	0.3±0.5	0.5±0.4	0.1±0.1
	B. subtilis+cd(100mM)	36.5±1.9	9.4±0.8	1.3±0.5	0.9±0.7	0.9±0.2	0.5±0.2

Morphometric analysis

12 plants were used from four replicates to measure seedling vigour. Results showed that with the inoculation of *B. subtilis* the root/shoot length and fresh and dry weights increased in both cultivars under normal as well as under stress conditions. The Morphometric characteristics of *Oryza sativa* was analyzed in different experimental conditions. The root length, shoot length, fresh weight and dry weight were decreased in heavy metal exposed respected plants compared to control of experimental periods. The growth responses of three rice cultivars (ADT 36,

CO 51, and TPS 5) were evaluated under cadmium (Cd, 100 mM) and arsenic (As, 100 mM) stress, with or without *Bacillus subtilis* treatment. Both heavy metals significantly reduced shoot and root lengths, fresh weight, and dry weight compared to controls. In Cd-stressed plants, co-application of *B. subtilis* improved growth parameters across all cultivars, with CO 51 and TPS 5 showing the most notable recovery (Table 2). Similarly, under As stress, *B. subtilis* mitigated the inhibitory effects, restoring shoot and root growth, fresh weight, and dry weight closer to control levels (Table 3). Overall, the beneficial effect of *B. subtilis* was evident under both heavy metal stresses, enhancing plant tolerance and growth, though Cd caused slightly greater reductions than as in most cultivars.

Table 3. Comparative analysis of physiological parameters in *B. subtilis*-treated and untreated rice cultivars under arsenic stress

Cultivar	Treatment	Length		Fresh weight		Dry weight	
		Shoot	Root	Shoot	Root	Shoot	Root
ADT 36	Control	33.6±1.8	11.2±1.1	2.6±0.3	2.0±0.4	1.8±0.6	0.7±0.3
	Control+As (100 mM)	26.2±1.5	7.0±0.4	2.2±0.6	1.8±1.2	1.3±0.1	1.0±0.2
	B. subtilis+As(100 mM)	32.0±2.3	10.1±1.3	2.5±0.2	2.1±2.0	1.6±0.3	0.9±0.5
CO 51	Control	32.2±2.5	11.4±1.5	2.5±0.7	1.4±0.3	1.9±0.5	1.4±0.6
	Control+As (100 mM)	27.1±1.5	9.0±0.2	1.7±1.2	1.2±0.2	1.7±0.3	1.5±0.8
	B. subtilis+As (100mM)	31.5±1.8	12.7±1.2	2.2±1.6	1.6±0.5	1.8±0.1	1.4±0.2
TPS 5	Control	37.8±2.6	10.5±0.7	2.8±1.5	1.8±0.4	1.9±0.5	1.3±0.5
	Control+As (100 mM)	24.3±1.3	5.6±0.4	1.8±0.6	0.7±0.1	1.5±0.2	1.1±0.1
	B. subtilis+As (100mM)	35.8±2.2	11.6±0.8	2.6±1.2	1.9±0.5	1.8±0.6	1.5±0.4

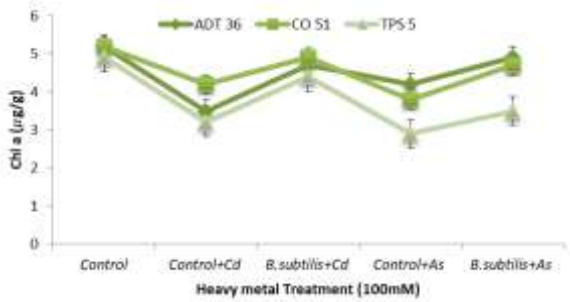

Fig. 1. Effect of *B. subtilis* on chlorophyll a of rice cultivars exposed to heavy metal stress

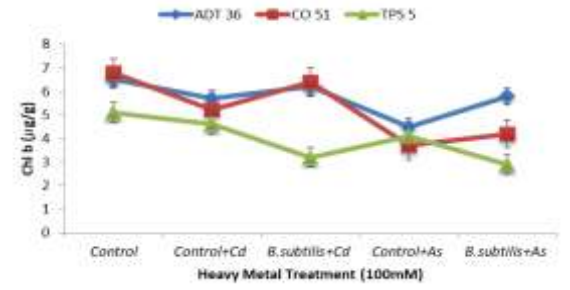

Fig. 2. Effect of *B. subtilis* on chlorophyll b of rice cultivars exposed to heavy metal stress

Pigment analysis

Cadmium and arsenic stresses markedly influenced the pigment content of the three rice cultivars studied, with a general trend of reduction in chlorophyll a, chlorophyll b, and carotenoids compared to control plants. In ADT 36, control plants recorded 5.2 µg/g chlorophyll a, 6.5 µg/g chlorophyll b, and 410 µg/g carotenoids. Exposure to cadmium (100 mM) resulted in a decline of these pigments (3.5, 5.7, and 350 µg/g, respectively). Inoculation with *Bacillus subtilis* under cadmium stress partially alleviated this reduction, restoring pigment levels to 4.7 µg/g (Chl a), 6.2 µg/g (Chl b), and 380 µg/g (carotenoids). Similarly, arsenic stress induced a sharper decline (4.2, 4.5, and 280 µg/g, respectively), which was also mitigated by *B. subtilis* treatment (4.9, 5.8, and 320 µg/g, respectively).

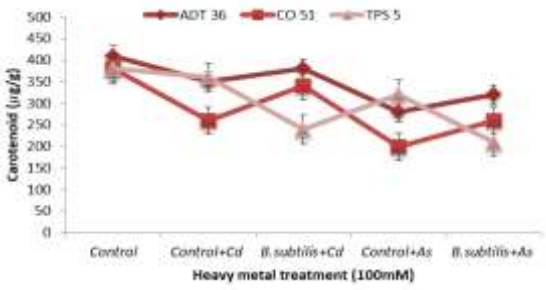

Fig. 3. Effect of *B. subtilis* on carotenoid of rice cultivars exposed to heavy metal stress

In CO 51, control plants showed 5.2 µg/g chlorophyll a, 6.8 µg/g chlorophyll b, and 380 µg/g carotenoids.

Cadmium stress reduced these values to 4.2, 5.2, and 260 µg/g, whereas *B. subtilis* inoculation enhanced them to 4.9, 6.4, and 340 µg/g. Arsenic stress caused a more pronounced reduction (3.8, 3.7, and 200 µg/g), but again, *B. subtilis* application improved the pigment content to 4.7 µg/g, 4.2 µg/g, and 260 µg/g, respectively. In TPS 5, pigment levels under control conditions were 4.9 µg/g (Chl a), 5.1 µg/g (Chl b), and 380 µg/g (carotenoids). Cadmium exposure reduced these pigments to 3.2, 4.6, and 360 µg/g. Interestingly, *B. subtilis* inoculation improved Chl a to 4.4 µg/g but led to comparatively lower Chl b (3.2 µg/g) and carotenoids (240 µg/g). Arsenic stress also decreased pigments (2.9, 4.1, and 320 µg/g), and although *B. subtilis* treatment offered slight recovery, pigment levels remained low (3.5, 2.9, and 210 µg/g, respectively). Taken together, the results demonstrate that both cadmium and arsenic stresses substantially reduced pigment biosynthesis in all three rice cultivars, with arsenic exerting a stronger inhibitory effect than cadmium. Inoculation with *B. subtilis* alleviated the adverse effects of heavy metals and enhanced pigment retention, particularly in ADT 36 and CO 51, while TPS 5 exhibited comparatively weaker recovery. This suggests cultivar-specific differences in stress tolerance and responsiveness to microbial inoculation (Fig. 1 to 3).

Table 4. Effect of *B. subtilis* on biochemical content of rice cultivars exposed to heavy metal stress

Cultivar	Treatment	Heavy metal	Bio chemicals			
			Protein (mg/g)	Proline (µmol/g)	Phenol (%)	Soluble sugar (mg/g)
ADT 36	Control	-	29.2±3.1	16.1±1.5	2.34±0.05	9.2±0.8
	Control+Cd	100 mM	24.2±2.6	12.0±1.9	3.36±0.09	17.1±1.3
	B. subtilis+Cd	100 mM	27.1±2.5	15.1±1.6	2.87±0.07	12.1±0.8
	Control+As	100 mM	19.0±1.8	11.2±1.1	3.55±0.05	16.0±1.4
	B. subtilis+As	100 mM	25.7±2.5	14.6±1.5	2.32±0.01	9.5±1.1
CO 51	Control	-	28.5±2.8	19.4±1.7	2.53±0.01	6.2±0.1
	Control+Cd	100 mM	19.0±1.5	9.0±1.2	2.97±0.02	15.7±1.4
	B. subtilis+Cd	100 mM	24.4±2.9	13.6±1.8	2.21±0.02	7.0±1.0
	Control+As	100 mM	21.0±1.2	11.2±1.3	3.67±0.06	16.5±1.2
	B. subtilis+As	100 mM	26.6±1.8	16.5±1.5	2.73±0.07	13.2±1.8
TPS 5	Control	-	34.6±2.5	21.6±1.8	2.15±0.01	5.7±0.8
	Control+Cd	100 mM	26.7±1.3	10.6±1.1	3.32±0.02	17.3±1.5
	B. subtilis+Cd	100 mM	32.6±2.8	15.8±1.9	2.97±0.04	12.6±1.2
	Control+As	100 mM	24.0±2.2	12.0±1.1	3.15±0.09	15.8±1.4
	B. subtilis+As	100 mM	29.2±2.7	17.2±1.5	2.28±0.05	6.3±1.1

Biochemical content

Heavy metal exposure significantly altered the biochemical profile of all three rice cultivars. In ADT 36, cadmium and arsenic stresses reduced protein content (from 29.2 to 24.2 and 19.0 mg/g, respectively) and proline levels, while increasing phenol and soluble sugar accumulation.

Inoculation with *Bacillus subtilis* improved protein and proline contents while reducing phenols and sugars compared to stressed controls, indicating stress alleviation. In CO 51, protein levels dropped sharply under Cd (19.0 mg/g) and As (21.0 mg/g) compared to control (28.5 mg/g), while phenol and soluble sugar levels increased. *B. subtilis* inoculation enhanced protein (up to 26.6 mg/g under As), restored proline, and reduced soluble sugar accumulation, suggesting a protective role. In TPS 5, the control plants recorded the highest protein (34.6 mg/g) and proline (21.6 µmol/g). Both Cd and As stresses caused reductions in protein and proline with a concomitant rise in phenol and soluble sugars. *B. subtilis* treatment notably restored protein (32.6 and 29.2 mg/g under Cd and As, respectively) and proline levels while lowering phenol and sugar contents. Overall, cadmium and arsenic stresses decreased protein and proline while enhancing phenol and soluble sugars across cultivars. *B. subtilis* inoculation mitigated these effects, with TPS 5 showing the strongest recovery in protein and proline, while ADT 36 and CO 51 also displayed significant improvements (Table 4).

Ionic content

Heavy metal stress caused notable alterations in ion homeostasis across the three rice cultivars. In ADT 36, cadmium and arsenic stresses increased Na^+ levels (16.2 and 25.7 ppm vs. 10.8 ppm in control) with a simultaneous decline in Ca^{2+} and K^+ contents. Inoculation with *Bacillus subtilis* reduced Na^+ accumulation and improved Ca^{2+} and K^+ uptake, partially restoring ionic balance. In CO 51, Cd and As stresses elevated Na^+ while reducing Ca^{2+} and K^+. Application of *B. subtilis* effectively lowered Na^+ levels (9.0 and 11.1 ppm under Cd and As, respectively) and enhanced Ca^{2+} and K^+ compared to stressed plants, indicating improved ion regulation. Similarly, in TPS 5, Cd and As treatments led to higher Na^+ and reduced Ca^{2+} and K^+ contents. *B. subtilis* inoculation markedly lowered Na^+ and improved Ca^{2+} and K^+ concentrations, with values approaching control levels. Overall, heavy metals disrupted ionic balance by increasing Na^+ and reducing essential Ca^{2+} and K^+. *B. subtilis* treatment alleviated these effects, maintaining better ion homeostasis in all cultivars, with CO 51 and TPS 5 showing stronger recovery than ADT 36 (Fig. 4).

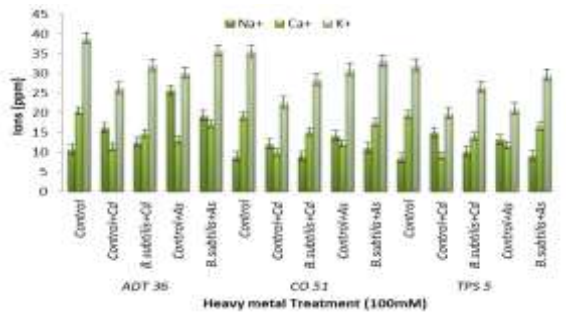

Fig. 4. Effect of *B. subtilis* on Ionic content of rice cultivars exposed to heavy metal stress

DISCUSSION

In the present study, a total of twelve bacterial species were isolated from soil samples, including *Pseudomonas putida*, *P. fluorescens*, *P. alcaligenes*, *P. aeruginosa*, *Azospirillum brasilense*, *Rhizobium leguminosarum*, *Xanthomonas maltophilia*, *Enterobacter* sp., *Bacillus pumilus*, *B. subtilis*, *B. cereus*, and *Azotobacter* sp. Among these, species of *Pseudomonas* and *Bacillus* were predominant, suggesting their wide adaptability and competitive advantage in soil environments.

Similar findings have been reported by earlier studies, where *Pseudomonas* spp. were frequently isolated from agricultural soils due to their ability to utilize diverse carbon sources and produce secondary metabolites (Compant *et al.*, 2010).

Soil microbes are well recognized for their critical role in nutrient cycling, maintaining soil structure, detoxifying harmful chemicals, suppressing plant pathogens, and enhancing plant growth (Van *et al.*, 2008). Their association with plants is also known to improve phytoremediation potential by reducing the phytotoxicity of contaminated soils through metal immobilization, production of siderophores, and secretion of organic acids (Glick, 2012).

In this study, *Bacillus* species were chosen for further investigation because of their predominance in the collected soil samples, indicating high adaptability under varying soil conditions. *Bacillus* spp. are spore-forming, resilient to environmental stresses, and have strong plant growth–promoting properties such as phosphate solubilization, production of phytohormones, and secretion of antimicrobial compounds (Chen *et al.*, 2009). Their well-documented role in biocontrol and phytoremediation makes them promising candidates for studies aimed at improving soil health and sustainable crop production.

CONCLUSION

The present study demonstrated that cadmium and arsenic stresses adversely affected rice cultivars by reducing pigment levels, protein and proline contents, and essential ions (Ca^{2+}, K^+), while increasing phenols, soluble sugars, and Na^+ accumulation. These alterations reflect oxidative stress and ionic imbalance caused by heavy metal toxicity. Inoculation with *Bacillus subtilis* effectively mitigated these negative effects by enhancing pigment retention, restoring protein and proline levels, reducing phenol and sugar accumulation, and maintaining ion homeostasis. Among the cultivars tested, TPS 5 exhibited stronger intrinsic tolerance and better recovery, while ADT 36 and CO 51 also showed significant improvements under *B. subtilis* treatment.

Overall, the findings highlight the potential of *Bacillus subtilis* as a promising bioinoculant for alleviating heavy metal stress and improving plant growth and resilience. Its spore-forming ability, adaptability, and plant growth-promoting properties make it a valuable tool for sustainable agriculture and phytoremediation in contaminated soils.

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