



Ameliorative Effect of *Bacillus subtilis* on Growth Attributes and Antioxidant Defense Capacity in Tomato (*Solanum lycopersicum* L.) Under Zinc Toxicity: An Outstanding PGPR to Achieve Heavy Metal Resistance

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Abstract

The growing problem of heavy metal contamination in agricultural soils poses a serious threat to the world. The accumulation of zinc (Zn) metal in the soil causes Zn stress in plants, inhibiting their growth and productivity. In order to mitigate heavy metal stress, the use of plant growth-promoting rhizobacteria (PGPR) such as *Bacillus subtilis* is an appealing option due to its favorable effects on plant physiology and biochemistry. This study evaluated the potential of PGPR strain *B. subtilis* (Bs) IMG22, which is used as a beneficial inoculant for enhancing growth and stress tolerance in tomato (*Solanum lycopersicum* L.) plants exposed to different levels of Zn toxicity (0.5 and 5 mM). The results revealed that Zn stress adversely influenced the physiological and biochemical parameters in tomato. The Zn-stressed plants showed a decline in growth attributes, leaf relative water content (RWC), and photosynthetic pigment contents. Under Zn stress, the enzymatic activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX); the contents of protein and proline; and the levels of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) increased. On the other hand, *B. subtilis* IMG22 inoculation attenuated the levels of oxidative stress indicators and considerably boosted the growth attributes, photosynthetic pigment contents, protein amount, proline accumulation, and antioxidant enzyme activities in Zn-stressed plants compared to uninoculated plants. Therefore, it was concluded that *B. subtilis* is an ideal candidate for mitigating Zn-induced heavy metal stress, as it promoted tomato growth and enhanced antioxidant enzyme activities and proline content against Zn-induced oxidative stress.

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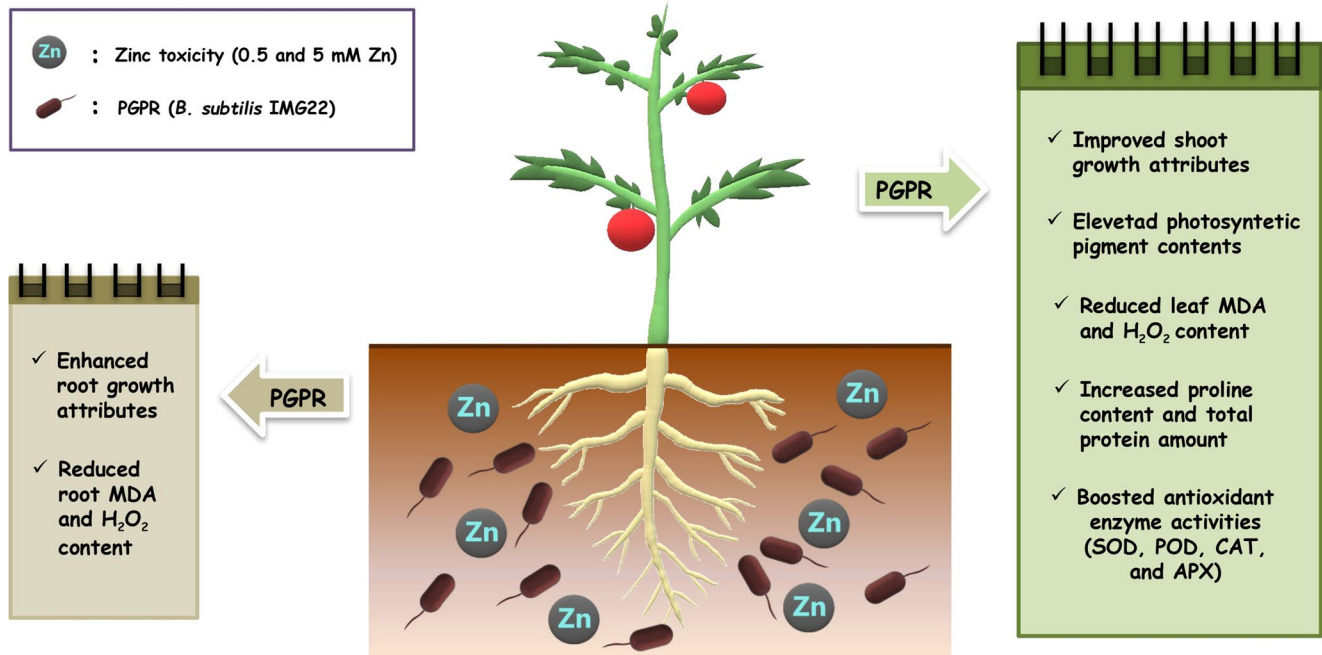
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Graphical Abstract



Highlights

- Excess zinc (Zn) disrupted the ecological and biochemical parameters in tomato.
- *B. subtilis* reduced the elevated MDA and H_2O_2 contents caused by the excess Zn.
- Inoculation with *B. subtilis* increased proline and photosynthetic pigment contents.
- *B. subtilis* inoculation boosted SOD, POD, CAT, and APX activities.
- PGPR strain *Bacillus subtilis* IMG22 inoculation ameliorated Zn toxicity in tomato.

Keywords *Bacillus subtilis* · Plant growth-promoting rhizobacteria · Zinc · Tomato · Heavy metal stress · Antioxidant enzyme

1 Introduction

Zinc (Zn), one of the most bioavailable and mobile metals, is an essential plant micronutrient required for normal physiological processes (Kaur et al. 2024). Zinc plays a vital role in plant metabolism by affecting the catalytic parts of various enzymes. These enzymes activated by Zn are involved in carbohydrate metabolism, protein synthesis, the maintenance of cellular membrane integrity, the regulation of auxin synthesis, and the formation of pollen. Moreover, Zn mediates the regulation and maintenance of gene expression necessary for resistance to abiotic stresses in plants (Hafeez et al. 2013; Barrameda-Medina et al. 2014). Zn, also known as a heavy metal ion, is a trace element in normal concentrations within the optimum range; however, in higher concentrations, it is highly toxic to the metabolic activities of plants (Kalaivanan and Ganeshamurthy 2016; Zajackowska et al. 2020). In addition to its toxic effect, it also limits plant growth at insufficient concentrations.

Hence, the homeostasis of the Zn metal ion, which is a cofactor of many enzymes in plant physiology, must be maintained by keeping its concentration at certain threshold levels (Hamzah Saleem et al. 2022). A zinc level of 100–400 $mg\ kg^{-1}$ dry weight (DW) is considered the toxicity threshold for plants (Natasha et al. 2022). The consequences of Zn metal toxicity in plant cells are leaf chlorosis, necrotic brown spots on the leaves, reduction of root growth, lipid peroxidation, nucleic acid damage, and eventually oxidative stress-induced cell death (DalCorso 2012; Angulo-Bejarano et al. 2021; Dobrikova et al. 2021).

Plants have evolved mechanisms that effectively regulate metal homeostasis to cope with the adverse impacts of metal ion toxicity. Exclusion, sequestration, chelation, and upregulation of the antioxidant defense system are among the key mechanisms contributing to metal homeostasis (Angulo-Bejarano et al. 2021). To further support the survival of plants exposed to heavy metal stress, the application of plant growth-promoting rhizobacteria (PGPR) stands

out as a cost-effective and eco-friendly biological approach (Singh et al. 2018; Jain et al. 2020; Mushtaq et al. 2020; He et al. 2024). Several bacterial genera, such as *Azotobacter*, *Bacillus*, *Pseudomonas*, *Azospirillum*, *Rhizobium*, *Klebsiella*, *Enterobacter*, and *Serratia*, belong to the category of beneficial microorganisms known as PGPR (Saini et al. 2023). They promote beneficial impacts on plant growth and development through direct or indirect mechanisms (Parray et al. 2016; Iqbal et al. 2021). The direct mechanisms are nitrogen fixation, nutrient uptake, solubilization of complex nutrients, siderophore production, phytohormone biosynthesis, and improvement of the antioxidant system (de Lima et al. 2019; Chaffai et al. 2024). Induced systemic resistance against phytopathogens is one of the indirect mechanisms by which PGPR act as biocontrol agents (Nazir et al. 2018). *Bacillus* is one of the most prominent taxa among the well-known PGPR (Patel et al. 2023). *Bacillus subtilis*, a gram-positive, rod-shaped, obligate aerobe, and catalase-positive (+) bacterium, has the ability to produce a wide range of enzymes and metabolites that can support plant growth under stress conditions (Sagar et al. 2022).

Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated and consumed vegetable crops worldwide after the potato (Singh et al. 2018). It is well known that the yield and productivity of tomato are facing challenges caused by high levels of soil metals, such as Zn. However, despite the known negative effects of excessive zinc on tomato growth and development, there is limited research on the application of PGPR as a biological agent to mitigate these deleterious effects in tomato. The purpose of this study was to explore the potential of *Bacillus subtilis* (*Bs*) to support and stimulate the growth of tomato plants exposed to Zn metal stress. Based on studies on mitigating the harmful effects of heavy metal toxicity in plants using PGPR, we hypothesize the following: (1) *Bacillus subtilis* inoculation will improve growth characteristics and photosynthetic pigment content adversely affected by excessive Zn, (2) *Bacillus subtilis* will reduce excessive Zn-induced high MDA and H₂O₂ content, (3) the stress protection of *Bacillus subtilis* inoculation will be mediated through enhanced proline content and antioxidant enzyme activities (SOD, POD, CAT, and APX), (4) thus, inoculation with PGPR strain *Bacillus subtilis* IMG22 will ameliorate Zn toxicity in tomato. To test these hypotheses, we examined the morpho-physiological and biochemical aspects, including the development of tomato leaves and roots, oxidative damage, photosynthesis, and antioxidant defense capacity between uninoculated and inoculated tomato plants subjected to 0.5 and 5 mM Zn metal concentrations. Furthermore, the findings of this study offer a new perspective on the practical applications of the PGPR strain *B. subtilis* IMG22 in agricultural soils

as a sustainable and eco-friendly approach to improve the tolerance of plants cultivated under Zn-contaminated soil against Zn toxicity.

2 Materials and Methods

2.1 Preparation of the Bacterial Suspension and Soil Inoculation

In the present study, PGPR strain *Bacillus subtilis* IMG22 was selected for utilization. *Bacillus subtilis* bacteria were incubated in 50 mL nutrient broth medium at 37 °C in a 160 rpm shaking incubator for 18 h. For bacterial inoculation, an aqueous suspension containing the isolate at a concentration of 10⁵ cfu/mL was used (Mushtaq et al. 2020).

2.2 Plant Material and Experimental Design

Tomato (*Solanum lycopersicum* L.) variety “H-2274” was used as the experimental material in this study. Tomato seeds were immersed in a 10% sodium hypochlorite solution for surface sterilization and washed three times with distilled water. Five days after inoculation of the bacteria into the soil, tomato seeds were sown in each pot (24.5 cm x 16 cm). The type of soil used in the pots was peat soil. The physicochemical properties of the soil were: pH 5.9, moisture content 45%, electrical conductivity (EC) 335 µS/cm, N (3.07%), K (1163 mg kg⁻¹), P (7.396 mg kg⁻¹), and Zn (24.13 mg kg⁻¹). The experiment was carried out in a completely randomized design with three biological replicates. Experimental treatments were as follows: control (no bacterial inoculation, no zinc application), *Bs* (only bacterial inoculation; *Bacillus subtilis*), 0.5 mM Zn, 0.5 mM Zn + *Bs*, 5 mM Zn, and 5 mM Zn + *Bs*. The selected zinc concentrations for toxicity were based on reported levels in polluted agricultural soils (Kaur and Garg 2021). For the application of zinc (Zn), zinc sulfate (ZnSO₄·7H₂O) was used, and the application started one month after the plants were sown in the soil. Plants were irrigated with their respective treatments every two days in a growth chamber (DigiTech, PG34-3) under controlled conditions (16/8 h light/dark regime, 24/20°C day/night temperature, and 60% humidity). Following a one-month zinc treatment period, tomato plants were harvested. Root and shoot lengths (cm) and root and shoot fresh and dry weights (g) were measured on the day of harvest.

2.3 Determination of Relative Water Content

The relative water content (RWC) of tomato leaves was determined based on the method previously described by

Smart and Bingham (1974). After the fresh weights (FW) of the leaves were determined, turgid weights (TW) were measured by soaking in distilled water for 6 h. Following oven drying at 70 °C, dry weights (DW) were determined.

2.4 Measurement of Photosynthetic Pigment Content

Photosynthetic pigment (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid) contents were assessed according to the methods previously described by Arnon (1949) and Lichtenthaler and Buschmann (2001). Fresh leaf samples (0.05 g) were homogenized with 80% (v/v) acetone (15 mL), and the absorbances of the supernatants were read at wavelengths of 470, 645, and 663 nm using a spectrophotometer (AgileSpec™, Metertech Inc.).

2.5 Assessment of Oxidative Stress Indicators

As an indicator of lipid peroxidation caused by oxidative stress in plants, malondialdehyde (MDA) content in leaves and roots was analyzed by thiobarbituric acid assay following the technique of Cakmak and Horst (1991). In summary, 0.25 g of plant tissue was homogenized with 0.1% (w/v) trichloroacetic acid (TCA) (5 mL), and the homogenate was centrifuged at 10,000 g for 5 min. Subsequently, the supernatant (500 µL) was reacted with 0.5% (w/v) TBA dissolved in 20% (w/v) TCA (2 mL). The mixture was heated at 95 °C for 30 min and centrifuged at 10,000 g for 10 min after rapid cooling in an ice bath. The absorbance of the supernatant was recorded at wavelengths of 532 nm and 600 nm using a spectrophotometer.

The procedure described by Velikova et al. (2000) was employed to determine hydrogen peroxide (H₂O₂) content. Plant material (0.5 g) was homogenized in 0.1% (w/v) TCA (5 mL) in an ice bath. Following a 15-min centrifugation of the homogenate at 12,000 g, the supernatant (0.5 mL) was reacted with 10 mM potassium phosphate buffer (0.5 mL, pH 7.0) and 1 M potassium iodide (KI) (1 mL). A spectrophotometer set at 390 nm was used to read the absorbance of the supernatant.

2.6 Analysis of Protein Amount and Proline Content

Protein amount in leaf tissues was quantified following the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The extraction buffer (2 mL), which contained 50 mM phosphate buffer, 1 mM EDTA, and 1% PVP, was used to homogenize 0.1 g of leaf tissue. The homogenate was centrifuged at 20,000 g for 20 min at +4 °C to obtain the supernatant, and the supernatant was used for performing antioxidant enzyme activity assays.

Proline content was measured based on the technique described by Bates et al. (1973). 0.5 g of leaf tissue was homogenized in 3% (w/v) sulfosalicylic acid (10 mL), and the homogenate was centrifuged at 10,000 g for 15 min. After incubating the supernatant (2 mL) with acid ninhydrin (2 mL) and glacial acetic acid (2 mL) at 100 °C in a water bath for 1 h, the reaction was stopped by rapidly cooling it in an ice bath. The reaction mixture was extracted with toluene (4 mL), and the absorbance values of the aspirated layer were recorded at 520 nm in a spectrophotometer.

2.7 Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity was determined by analyzing its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm according to the protocol of Giannopolitis and Ries (1977). Peroxidase (POD) activity was estimated by guaiacol oxidation at 470 nm, following the method of Hammerschmidt et al. (1982). Catalase (CAT) activity was assessed by observing the decomposition of H₂O₂ at 240 nm, adopting the procedure of Lima et al. (2002). Ascorbate peroxidase (APX) activity was measured by monitoring the decrease in absorbance at 290 nm using the method previously defined by Nakano and Asada (1981).

2.8 Statistical Analysis

The experiments were repeated thrice biologically, and data were subjected to analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in the GraphPad program (GraphPad Prism 9). Values are the mean ± standard deviation (SD), and differences between treatments were regarded as statistically significant at the $p < 0.05$ level. Statistically significant values are represented by different letters and standard deviations (SD) by the "±" sign.

3 Results

3.1 Impact of *B. subtilis* and Zn Stress on Tomato Growth Attributes and Relative Water Content

Exposure to Zn stress had a considerable toxic effect on the tomato plants; therefore, tomato growth was significantly restricted at 0.5 and 5 mM Zn toxicity. Relative to the control, the shoot length of plants under 0.5 and 5 mM Zn stress decreased by 6.1% and 11.9%, respectively. Under non-stressful conditions, there was a significant increase (13.4%) upon *B. subtilis* treatment. Furthermore, *B. subtilis* inoculation enhanced shoot length by 13.6% at 0.5 mM Zn stress and 12.2% at 5 mM Zn stress compared to uninoculated

Table 1 Effect of *B. subtilis* inoculation on shoot and root parameters of tomato plants under zinc toxicity

	Shoot Length (cm)	Root Length (cm)	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root DW (g)
Control	34.5±0.71c	38.8±1.06a	15.75±0.36b	3.472±0.08b	1.606±0.13a	0.275±0.02b
<i>Bs</i>	39.1±0.18a	39.3±0.35a	17.75±0.40a	3.871±0.02a	1.855±0.02a	0.405±0.05a
0.5 mM Zn	32.4±0.18d	27.5±0.71c	10.95±0.16cd	1.016±0.03d	1.221±0.02b	0.167±0.00c
0.5 mM Zn+ <i>Bs</i>	36.8±0.35b	36.1±0.18b	11.75±0.54c	1.644±0.08c	1.297±0.05b	0.238±0.03bc
5 mM Zn	30.4±0.53e	23.6±0.18d	9.55±0.37d	0.515±0.04e	1.074±0.10b	0.133±0.02c
5 mM Zn+ <i>Bs</i>	34.1±0.18c	35.3±0.35b	12.08±0.51c	1.038±0.03d	1.300±0.06b	0.180±0.01bc

FW Fresh Weight, DW Dry Weight. Values are mean ($n=3$)±SD. Different letters in the same column are significantly different at $p<0.05$ among the treatments according to Tukey's multiple comparisons test

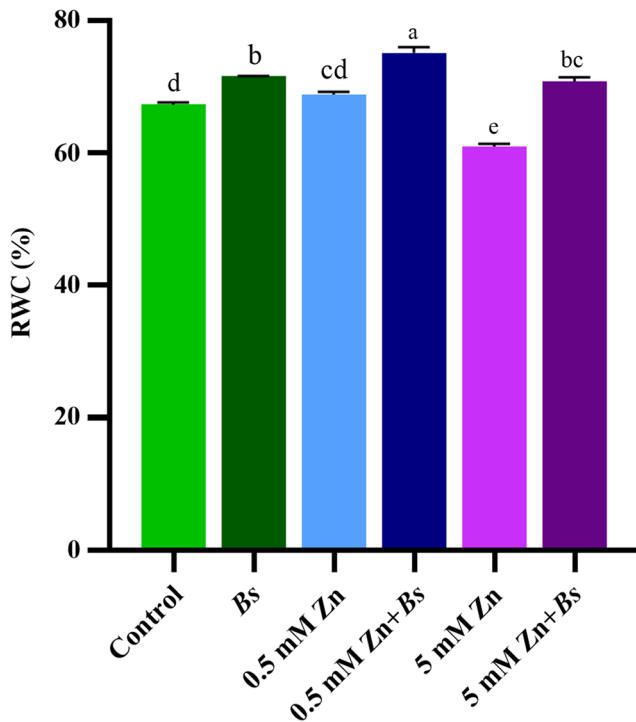


Fig. 1 Effect of *B. subtilis* inoculation on the relative water content (RWC) in the tomato leaves under zinc toxicity. Values are mean ($n=3$)±SD. Different letters on the bars represent significant differences ($p<0.05$) among treatment means according to Tukey's multiple comparisons test

plants at the same Zn level. Similarly, Zn stress led to a sharp decline (29.1% and 39.2%, respectively) in root length relative to the control at both concentrations. In comparison to uninoculated plants, *B. subtilis* inoculation improved root length (31.3% and 49.6%, respectively) at both Zn levels (Table 1).

Along with the increase in zinc concentration, shoot fresh weight decreased by 30.5% and 39.4%, respectively, compared to the control. On the other hand, *Bs*-inoculated plants without any stress treatment had higher shoot fresh weight (12.7%) than the control. *B. subtilis* inoculation at 5 mM Zn toxicity also noticeably increased shoot fresh weight by 26.4% relative to those exposed to 5 mM Zn toxicity alone. Although plants exposed to both Zn levels showed a

decrease in shoot dry weight (24% and 33.1%, respectively) compared to the control, no significant difference was noted between the *Bs*-inoculated and uninoculated groups (Table 1).

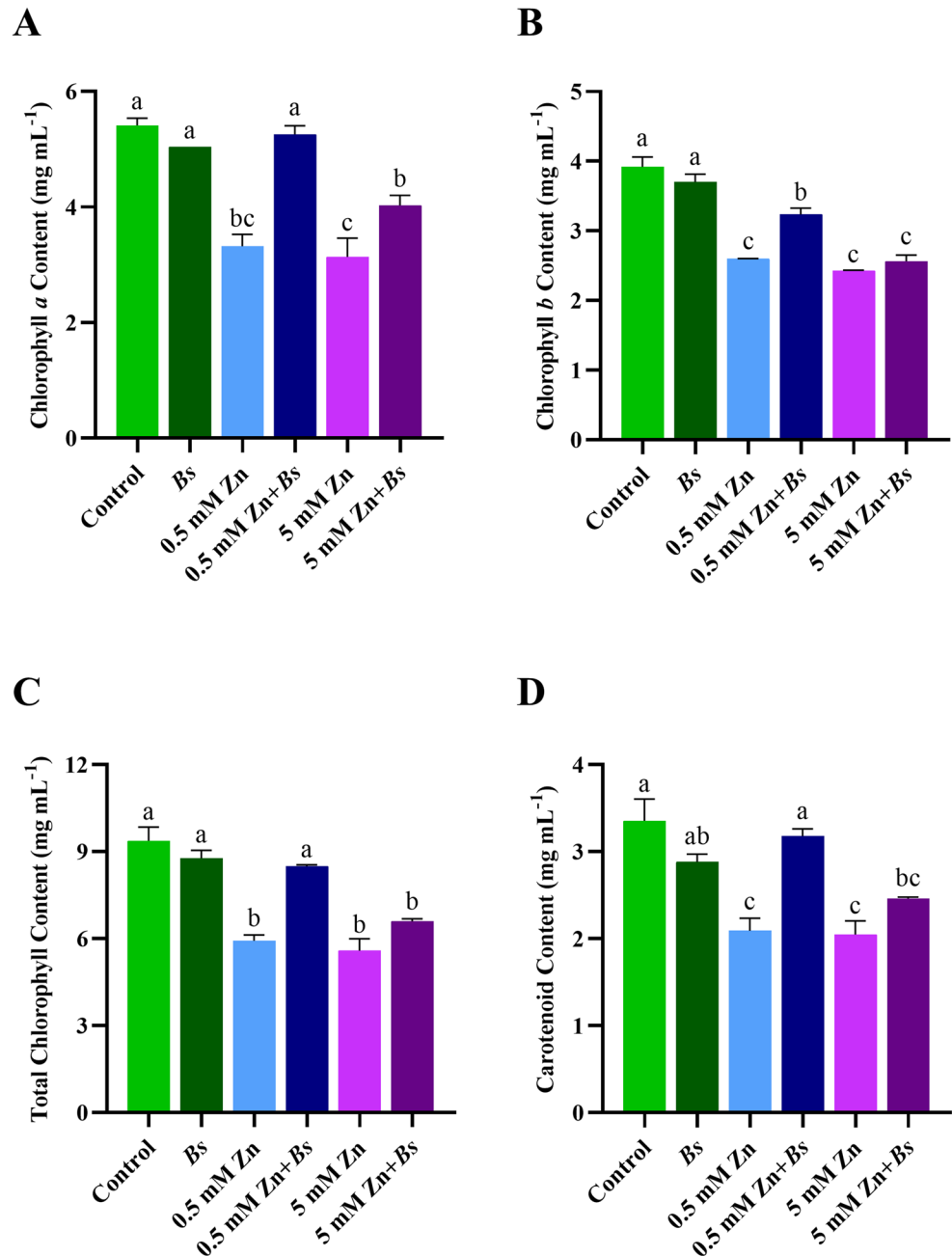
A severe reduction in root fresh weight (70.7% and 85.2%, respectively) was observed under both Zn concentrations relative to the control. However, plants inoculated with *B. subtilis* had higher root fresh weight than uninoculated plants at the same stress level. In addition, both Zn levels decreased root dry weight by 39.3% and 51.7%, respectively, compared to the control. Furthermore, unstressed plants inoculated with *B. subtilis* had higher root dry weight (47.1%) than the control. *Bs*-inoculated plants under Zn stress were not significantly different from uninoculated stressed plants in terms of root dry weight, but an increasing trend was recorded (Table 1).

While 0.5 mM Zn stress did not alter the relative water content in comparison to the control, 5 mM Zn stress significantly reduced it. However, *B. subtilis* inoculation resulted in an elevated relative water content as compared to uninoculated plants at the same stress level (Fig. 1).

3.2 Photosynthetic Pigment Content in Response to *B. subtilis* and Zn Toxicity

Both Zn levels reduced chlorophyll *a* (38.6% and 42%, respectively), chlorophyll *b* (33.7% and 38%, respectively), total chlorophyll (36.9% and 40.4%, respectively), and carotenoid (37.7% and 39%, respectively) contents, compared to the control. On the other hand, *B. subtilis* inoculation at 0.5 mM Zn concentration exhibited quite impressive results in terms of photosynthetic pigment content. Plants inoculated with *B. subtilis* at 0.5 mM Zn toxicity had higher photosynthetic pigment contents, including chlorophyll *a* (58.1%), chlorophyll *b* (24.6%), total chlorophyll (43.4%), and carotenoid (51.9%) contents, relative to the uninoculated plants at the same Zn level. Furthermore, *B. subtilis* inoculation at the 5 mM Zn level markedly increased chlorophyll *a* content (28.3%) as compared to the uninoculated plants at the same Zn level, while it did not induce any significant changes in

Fig. 2 Effect of *B. subtilis* inoculation on the photosynthetic pigments, including chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C), and carotenoids (D) under zinc toxicity. Values are mean ($n=3$) $\pm$ SD. Different letters on the bars represent significant differences ($p<0.05$) among treatment means according to Tukey's multiple comparisons test



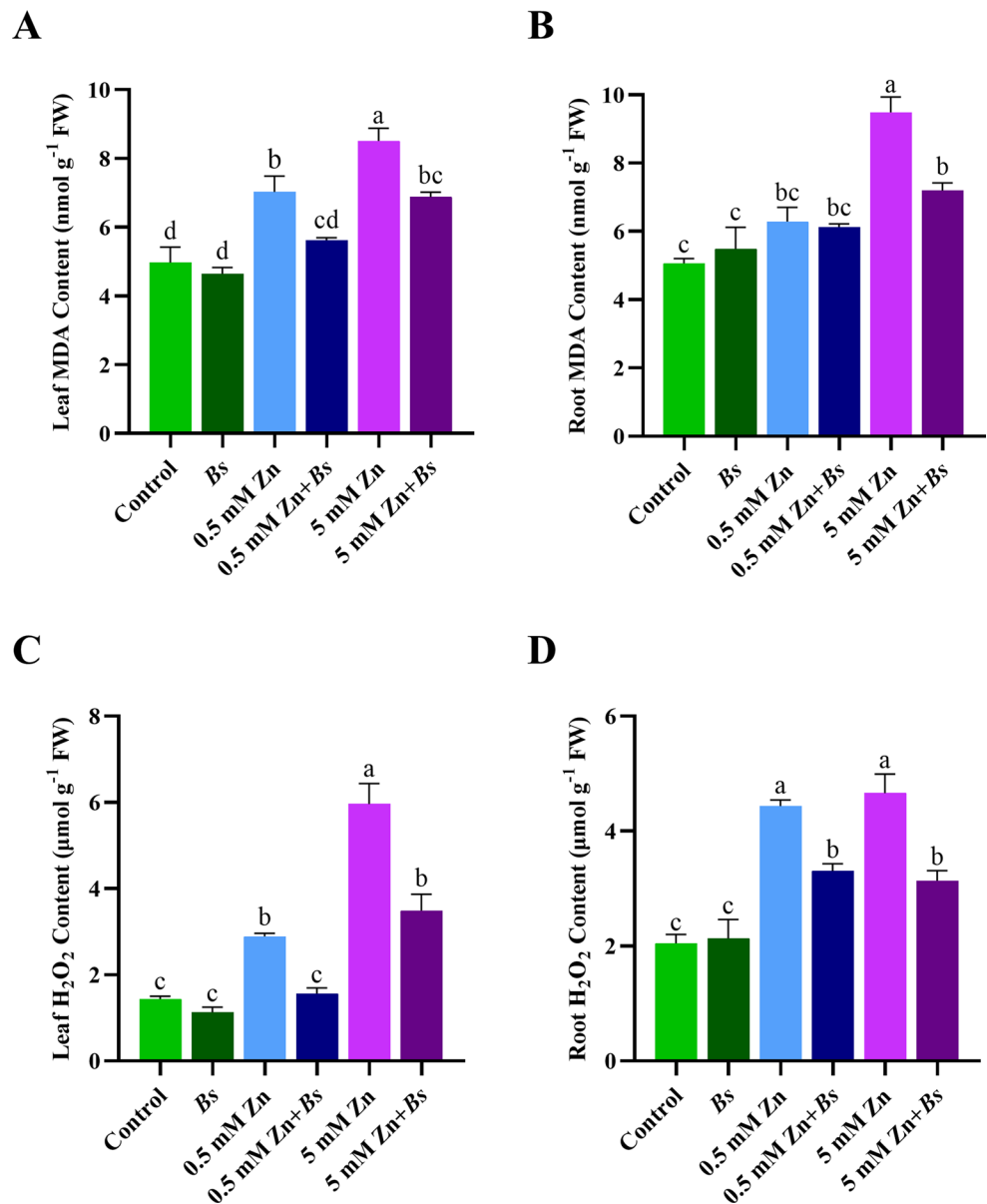
chlorophyll *b*, total chlorophyll, and carotenoid contents (Fig. 2A-D).

3.3 Effect of *B. subtilis* and Zn Stress on Oxidative Stress Indicators

Oxidative damage caused by zinc stress in tomato plants was detected in terms of alterations in endogenous hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) content. MDA content represents the degree of lipid peroxidation in plant membranes due to the ROS accumulation. Plants

treated with 0.5 and 5 mM Zn displayed a noticeable increase (41.4% and 71.4%, respectively) in leaf MDA content relative to the control. On the other side, *B. subtilis* inoculation reduced leaf MDA content by 20% at 0.5 mM Zn concentration and 19.2% at 5 mM Zn concentration compared to uninoculated plants at the same Zn level (Fig. 3A). In roots, MDA content remained unchanged between control and 0.5 mM Zn stress, but increased by 87.3% under 5 mM Zn stress. However, *B. subtilis* inoculation under 5 mM Zn stress reduced root MDA content by 24.1% relative to uninoculated plants (Fig. 3B).

Fig. 3 Effect of *B. subtilis* inoculation and zinc stress on the oxidative stress indicators, including leaf MDA (A), root MDA (B), leaf H_2O_2 (C), and root H_2O_2 (D). Values are mean ($n=3$) $\pm$ SD. Different letters on the bars represent significant differences ($p<0.05$) among treatment means according to Tukey's multiple comparisons test

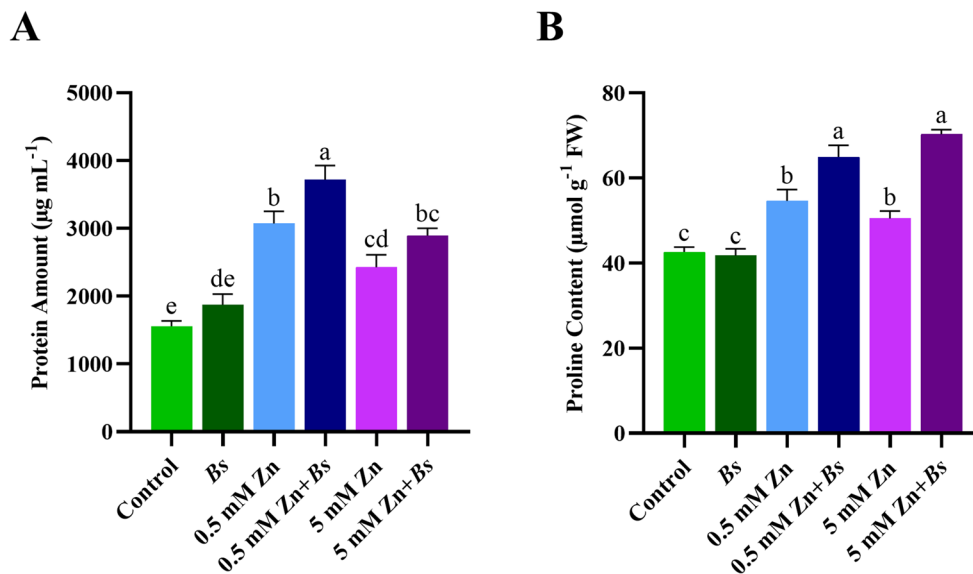


The changes in leaf H_2O_2 content induced by Zn stress and *B. subtilis* inoculation were quite similar to the changes observed in leaf MDA content. In comparison to the control, both Zn concentrations increased leaf H_2O_2 content by 100.9% and 314.3%, respectively, with the increase becoming more evident as the zinc concentration rose. *B. subtilis* inoculation at both Zn levels reduced leaf H_2O_2 content by 45.9% and 41.4%, respectively, as compared to uninoculated plants (Fig. 3C). In a similar manner, exposure to 0.5 and 5 mM Zn led to a drastic increase in root H_2O_2 content (117.1% and 127.9%, respectively) relative to the control. *Bs*-inoculated plants had 25.4% lower root H_2O_2 content at 0.5 mM Zn concentration and 32.7% lower root H_2O_2 content at 5 mM Zn concentration than uninoculated plants (Fig. 3D).

3.4 Influence of *B. subtilis* and Zn Stress on Protein Amount and Proline Content

Plants exposed to 0.5 and 5 mM Zn concentrations had higher total protein amounts than the control. The increase in total protein amount at the 0.5 mM Zn concentration (97.2%) was more pronounced than the increase at 5 mM Zn concentration (55.9%). Inoculation with *B. subtilis* at 0.5 mM Zn level further increased the total protein amount (21.1%) as compared to uninoculated plants at the same Zn level. Additionally, although there was no significant alteration in total protein amount between *Bs*-inoculated and uninoculated plants at 5 mM Zn toxicity, an increasing trend was noted in *Bs*-inoculated plants (Fig. 4A).

Fig. 4 Effect of *B. subtilis* inoculation on the protein amount (A) and proline content (B) in the tomato leaves under zinc toxicity. Values are mean ($n=3$) $\pm$ SD. Different letters on the bars represent significant differences ($p<0.05$) among treatment means according to Tukey's multiple comparisons test



Relative to the control, both Zn levels substantially increased proline content by 28.2% and 18.5%, respectively. Besides that, *B. subtilis* inoculation at both Zn levels further augmented the elevated proline content induced by 0.5 and 5 mM Zn levels (Fig. 4B).

3.5 Antioxidant Enzyme Activities in Response to *B. subtilis* and Zn Toxicity

The efficiency of *B. subtilis* on SOD, POD, CAT, and APX activities, which constitute the major enzymatic components of the antioxidant defense system of plants, was examined in tomato under zinc stress. In comparison to the control, both Zn levels significantly increased SOD activity by 33.9% and 47.7%, respectively. Additionally, *B. subtilis* inoculation enhanced SOD activity by 28.5% at the 0.5 mM Zn level and 40.3% at the 5 mM Zn level relative to uninoculated plants (Fig. 5A).

Similar to SOD activity, 0.5 and 5 mM Zn concentrations elevated POD activity by 98.7% and 76.9%, respectively, compared to the control. Furthermore, groups inoculated with *B. subtilis* at both Zn levels had higher POD activity (35.4% and 24.3%, respectively) than uninoculated groups (Fig. 5B).

In parallel with the increase in zinc concentration, CAT activity dramatically increased by 100% and 326.7%, respectively, compared to the control. *B. subtilis* inoculation under non-stressful conditions also resulted in increased CAT activity (73.4%) relative to the control. Likewise, *B. subtilis* inoculation at both Zn levels further elevated CAT activity by 46.7% and 26.6%, respectively, as compared to uninoculated plants at the same Zn level (Fig. 5C).

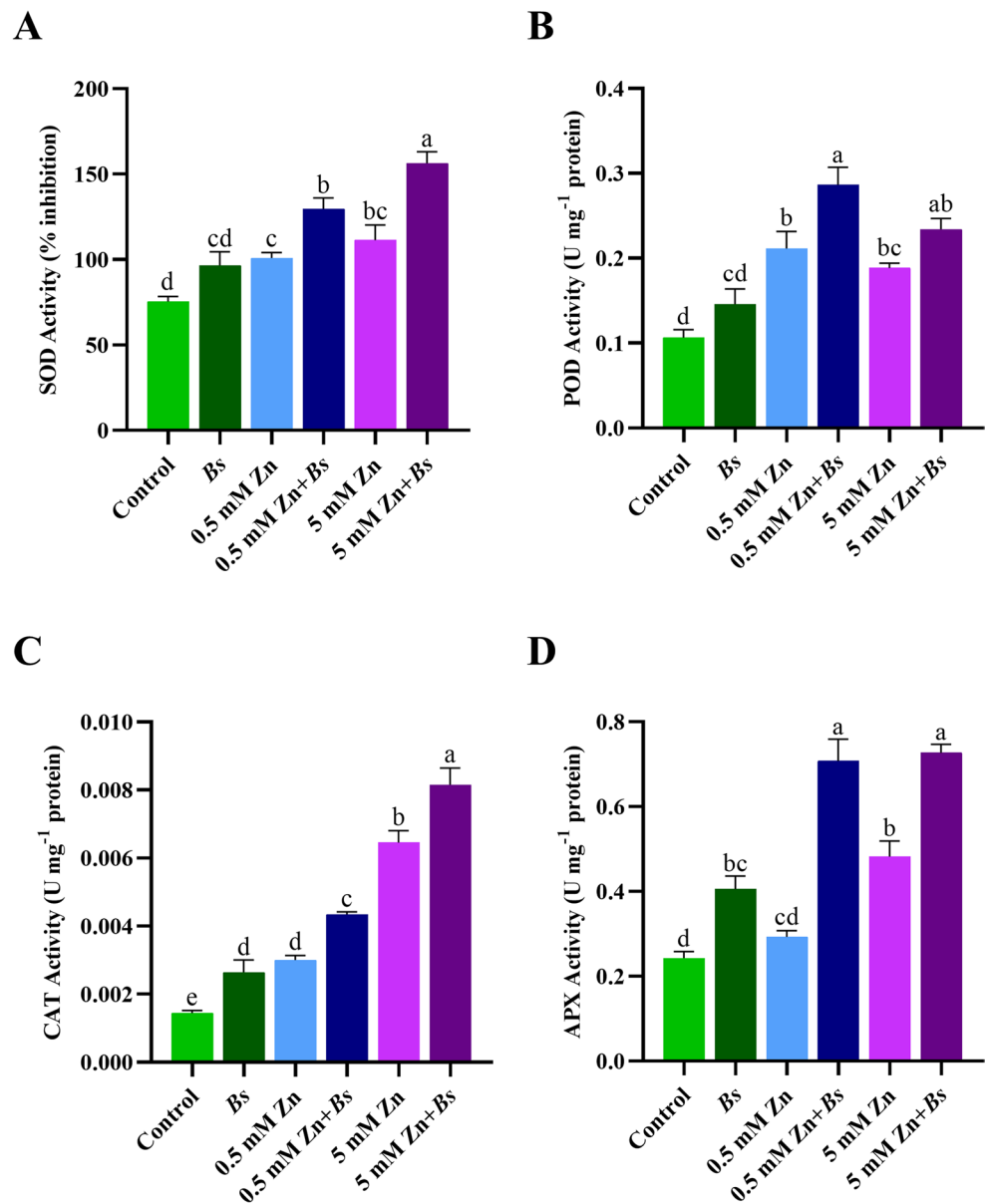
No significant change in APX activity was recorded between 0.5 mM Zn and the control, whereas APX activity

notably increased by 98.2% in 5 mM Zn toxicity. Unstressed plants inoculated with *B. subtilis* also had higher APX activity (66.8%) than the control. Moreover, *B. subtilis* inoculation boosted APX activity by 141.4% at 0.5 mM Zn toxicity and 50.7% at 5 mM Zn toxicity, as compared to uninoculated plants (Fig. 5D).

4 Discussion

Plant growth-promoting rhizobacteria (PGPR) consist of rhizosphere bacteria that can enhance plant growth in various challenging environmental conditions (Kumar et al. 2019). Due to the detrimental effects of zinc-contaminated soils on plants, inoculating such soils with PGPR can promote plant growth and mitigate the toxic effects of zinc (Islam et al. 2014; Jain et al. 2020). It is well known that Zn toxicity hinders plant growth and development (Ramakrishna and Rao 2015; Zajaczkowska et al. 2020). Likewise, in our study, 0.5 and 5 mM Zn metal stress showed toxicity symptoms that inhibited shoot and root growth and had negative effects on tomato growth. Zn metal toxicity is mainly associated with modifications in root physiology. During Zn toxicity, root thickening, root blunting, and the restriction of root cell division and elongation cause the inhibition of root growth (Rout and Das 2009). This is supported by our results, which show that increased zinc concentrations inhibit root elongation. Additionally, excess Zn stress resulted in the formation of a purplish-red color on tomato leaves, which is associated with phosphorus deficiency induced by Zn stress (Emamverdian et al. 2015). However, as mentioned earlier, PGPR can stimulate plant growth and development in challenging environmental circumstances by inducing root elongation, regulating endogenous plant hormone levels, and enhancing

Fig. 5 Effect of *B. subtilis* inoculation and zinc stress on the antioxidant enzyme activities of tomato leaves, including SOD (A), POD (B), CAT (C), and APX (D). Values are mean ($n=3$) $\pm$ SD. Different letters on the bars represent significant differences ($p<0.05$) among treatment means according to Tukey's multiple comparisons test



mineral and water uptake (He et al. 2024). In the current study, PGPR strain *B. subtilis* IMG22 inoculation enhanced the growth attributes of tomato under Zn toxicity. Previous studies have also achieved similar findings, documenting that PGPR improve the hindered growth characteristics of various plants under abiotic stress conditions, such as heavy metals (El-Meihy et al. 2019), drought (Begum et al. 2022), and salinity (Sapre et al. 2022).

Excessive Zn concentrations also affect the water relations of plants, lowering the relative water content (RWC) of plant tissues and leading to a slowdown in transpiration rates. Furthermore, they induce severe dehydration, also known as physiological drought, due to overaccumulation of Zn in the above-ground shoots (Kaur and Garg 2021). In agreement with previous studies (Sood et al. 2020; Li et al.

2023), our study demonstrated that 5 mM Zn stress reduced the RWC in tomato. This decline in the RWC of leaves could probably be due to the decrease in absorbent hair surfaces of the roots or could be attributed to the disturbance of the water transport process mediated by a blockage in the xylem vasculature. The loss of turgor that accompanies the decrease in water content also results in lower cell expansion, consequently stunting the growth of the plant (Kaur and Garg 2021). On the other hand, as stated by previous PGPR studies in maize (Ali et al. 2022) and radish (Ahmad et al. 2025), our study indicated that *B. subtilis* inoculation elevated the RWC of tomato at both Zn concentrations, even exceeding the control level. Hence, it can be deduced that *B. subtilis* IMG22 may elevate water absorption of tomato by stimulating the formation of elongated roots.

For capturing light, a critical step in photosynthesis where solar energy is converted into chemical energy, plants have two types of photosynthetic pigment classes: chlorophylls (Chl) and carotenoids (Car) (Rai et al. 2016). Therefore, if these light-absorbing pigments are damaged, photosynthesis is directly impacted. The Chl content in plants declines due to the substitution of Mg^{2+} in the chlorophyll structure by divalent (2+) metals such as Zn. Because of the lower electron release capacity of Zn-substituted chlorophylls, the photosynthetic electron transport chain and eventually the whole photosynthesis machinery may be disrupted (Souri et al. 2019). Additionally, excess Zn concentrations diminish the efficiency of Photosystem II (PSII) and hinder CO_2 fixation by lowering the carboxylase activity of the RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzyme (Mousavi et al. 2013; Ghori et al. 2019). Aligned with previous studies in cotton (Anwaar et al. 2015), rice and maize (Janeeshma et al. 2021), and white mustard (Repkina et al. 2023), the current study indicated that chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents decreased in tomato as a result of Zn stress. However, *B. subtilis* IMG22 inoculation improved photosynthetic pigment contents disrupted by Zn stress, with this effect being more prominent at the 0.5 mM Zn level. In a similar manner to our findings, Yasmin et al. (2020) reported that *B. subtilis* SRM-3 and *Pseudomonas pseudocaligenes* SRM-16 inoculations elevated chlorophyll *a*, chlorophyll *b*, and carotenoid contents in soybean under salinity stress. Desoky et al. (2020) also indicated that *B. subtilis* inoculation increased chlorophyll *a*, chlorophyll *b*, and carotenoid contents, net photosynthetic rate, transpiration rate, and stomatal conductance in spinach under lead and cadmium stress.

After iron (Fe), Zn is the second most prevalent transition metal in living organisms and participates in the function of most enzymes (Broadley et al. 2007). By interacting with phospholipids and sulfhydryl groups of membrane proteins, Zn preserves the integrity of cellular membranes and maintains the structural orientation of macromolecules (Tsonev and Cebola Lidon 2012). Despite its beneficial features, toxic concentrations of Zn increase the production of reactive oxygen species (ROS) in plants. MDA is produced during the lipid peroxidation process initiated by ROS attack, and it is a marker indicating the magnitude of oxidative injury within the cell (Anjum et al. 2015). In this study, Zn toxicity caused oxidative damage by increasing MDA and H_2O_2 contents, and these findings were supported by the studies of Goodarzi et al. (2020) and Dobrikova et al. (2021). On the other side, PGPR can lessen abiotic stress-induced oxidative damage via enhancing the activities of ROS-detoxifying enzymes (Nivetha et al. 2021). Our study demonstrated that *B. subtilis* IMG22 inoculation reduced MDA and H_2O_2 contents under 0.5 and 5 mM Zn

toxicity. Similarly, Patel et al. (2023) stated that *B. subtilis* ER-08 inoculation lowered the MDA and H_2O_2 contents in fenugreek under salt and drought stress. Maslennikova et al. (2023) also documented that *B. subtilis* 10–4 reduced electrolyte leakage, MDA, and H_2O_2 contents in wheat under cadmium stress.

Heavy metal-induced stress, such as zinc, mostly decreases the content of proteins in plants (Ramakrishna and Rao 2012), but also triggers catabolic reactions by elevating the activity of hydrolytic enzymes such as protease and RNase. Exposure to toxic levels of Zn can disrupt the mechanisms of protein formation in plants and even further decrease the incorporation of amino acids necessary for protein synthesis, eventually leading to a decline in protein levels (Sidhu 2016). However, in the present study, 0.5 and 5 mM Zn toxicity increased total protein amount. To regulate the uptake and intracellular concentrations of metal ions in response to heavy metal stress, the most common strategy used by plants is the synthesis of membrane transporters and thiol-containing chelating compounds (Hossain and Komatsu 2013). This involves increasing the abundance of molecular chaperones, phytochelators, and metallothioneins, as well as synthesizing defense proteins for effective ROS scavenging (Hasan et al. 2017; Sevgi and Leblebici 2025). Hence, to withstand heavy metal stress, plants increase endogenous protein levels by synthesizing these stress-responsive proteins (Anjum et al. 2015). Moreover, in our study, PGPR strain *B. subtilis* IMG22 inoculation generally further augmented total protein amount under Zn stress. Likewise, Ilyas et al. (2022) noted that *B. subtilis* inoculation enhanced protein content in wheat under chromium stress. Sahile et al. (2021) also reported that *Bacillus cereus* inoculation increased total protein content in soybean under cadmium stress. These findings suggest that PGPR may help plants cope with the detrimental effects of heavy metal stress by augmenting stress-responsive proteins.

Proline is a water-soluble amino acid accumulated in the tissues of plants subjected to abiotic stresses such as drought, heat, cold, salinity, and heavy metals (Siddique et al. 2018). Being one of the most prevalent compatible compounds in plants, proline is regarded as both an excellent osmolyte and an antioxidant defense molecule as a ROS scavenger (Kaur and Asthir 2015). Besides that, it acts as a molecular chaperone that stabilizes the structure of proteins and maintains their integrity, and also serves as a metal chelator that chelates heavy metals in plants by forming a non-toxic metal-proline complex (Ghosh et al. 2022). Consistent with the findings of Khan et al. (2019) and Badiaa et al. (2020), in the present study, 0.5 and 5 mM Zn levels increased proline content. Additionally, PGPR strain *B. subtilis* IMG22 inoculation further increased proline content

under Zn stress. PGPR inoculation was also documented to elevate the proline content in plants under cadmium (Shah et al. 2020) and chromium stress (Akhtar et al. 2021). Therefore, it is proposed that PGPR can assist the thriving of plants in heavy metal-contaminated soils by accumulating osmotic substances such as proline. It is also noteworthy to mention that PGPR can alleviate heavy metal stress by secreting siderophores. Siderophores, which are low-molecular-weight chelating agents, form complexes with various heavy metals, including Cd, Fe, Pb, and Zn (He et al. 2024).

In plants, heavy metals damage cellular biomolecules through oxidative stress by triggering excessive ROS production. By utilizing various antioxidant enzymes such as SOD, POD, CAT, and APX, which function at the cellular and subcellular levels, plants combat these stressors that impair cell metabolism, performance, and vitality (Maleki et al. 2017). The adaptation of plants to heavy metal stress is linked to the enhanced activity of antioxidant enzymes such as SOD, POD, CAT, and APX, as well as higher proline levels. In line with the results of dos Santos et al. (2020) and Du et al. (2020), our study showed that excess Zn enhanced SOD, POD, CAT, and APX activities. Furthermore, our study revealed that *B. subtilis* IMG22 inoculation further boosted the enhanced SOD, POD, CAT, and APX activities caused by excess Zn. Consistently, Mush-taq et al. (2020) indicated that *B. subtilis* S4 increased SOD, CAT, and APX activities in pumpkin subjected to arsenic toxicity. Fatima et al. (2025) also recorded that *B. subtilis* IAGS174 inoculation increased SOD, POD, and CAT activities in sunflower exposed to salt and lead stress. Accordingly, it can be inferred that *B. subtilis* activates the antioxidant defense system of plants, including SOD, POD, CAT, and APX, in order to counteract heavy metal stress.

Collectively, these investigated mechanisms — higher content of photosynthetic pigments, less damage to membrane lipids, and elevated antioxidant enzyme activities and proline content — explain the improved plant growth performance and stress tolerance in tomato plants inoculated with *B. subtilis* IMG22 under zinc stress. These findings highlight the effectiveness of *B. subtilis* IMG22, used as a bioinoculant to mitigate zinc toxicity.

5 Conclusion

This study revealed that zinc (Zn) stress has toxic effects on the growth and biochemical properties of tomato plants. However, plant-bacteria interaction led to impressive results in plant growth and overcoming challenging environmental conditions. *Bacillus subtilis* IMG22 inoculation regulated the fundamental ecophysiological and

biochemical processes of tomato plants through elevated photosynthetic pigment content, proline content, and antioxidant enzyme activities, as well as lowered hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels. In conclusion, the findings of the present study demonstrated that *Bacillus subtilis* IMG22 has great potential as a cost-effective and eco-friendly approach to stimulate tomato growth and mitigate the negative effects of zinc toxicity on tomato plants. Plant growth-promoting rhizobacteria (PGPR), a good alternative to chemical fertilizers, may be the pioneers of the next green revolution, but the long-term effect of these bacteria on the structure of the soil microbial community is an issue that needs to be carefully evaluated. Our study provides valuable knowledge about how *Bacillus subtilis* inoculation mitigates zinc toxicity in tomato at physiological and biochemical levels. However, further research should focus on investigating its effects on molecular pathways and soil microbial communities to elucidate its potential benefits in promoting plant growth and reducing zinc toxicity.

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Data Availability All data generated or analyzed during this study are included in this article.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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