The potential of *Bacillus subtilis* and phosphorus in improving the growth of wheat under chromium stress

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**Abstract**

**Aim:** Hexavalent chromium (Cr⁺⁶) is one of the most toxic heavy metals that have deteriorating effects on the growth and quality of the end product of wheat. Consequently, this research was designed to evaluate the role of *Bacillus subtilis* and phosphorus fertilizer on wheat facing Cr⁺⁶ stress.

**Methods and Results:** The soil was incubated with *Bacillus subtilis* and phosphorus fertilizer before sowing. The statistical analysis of the data showed that the co-application of *B. subtilis* and phosphorus yielded considerably more significant (p<0.05) results compared with an individual application of the respective treatments. The co-treatment improved the morphological, physiological and biochemical parameters of plants compared with untreated controls. The increase in shoot length, root length, shoot fresh weight and root fresh weight was 38.17%, 29.31%, 47.89% and 45.85%, respectively, compared with untreated stress-facing plants. The application of *B. subtilis* and phosphorus enhanced osmolytes content (proline 39.98% and sugar 41.30%), relative water content and stability maintenance of proteins (86.65%) and cell membranes (66.66%). Furthermore, augmented production of antioxidants by 67.71% (superoxide dismutase), 95.39% (ascorbate peroxidase) and 60.88% (catalase), respectively, were observed in the Cr⁺⁶ – stressed plants after co-application of *B. subtilis* and phosphorus.

**Conclusion:** It was observed that the accumulation of Cr⁺⁶ was reduced by 54.24%, 59.19% and 90.26% in the shoot, root and wheat grains, respectively. Thus, the combined application of *B. subtilis* and phosphorus has the potential to reduce the heavy metal toxicity in crops.

**Significance and Impact of the Study:** This study explored the usefulness of *Bacillus subtilis* and phosphorus application on wheat in heavy metal stress. It is a step toward the combinatorial use of plant growth–promoting rhizobacteria with nutrients to improve the ecosystems’ health.

**Keywords**
*B. subtilis*, chromium, heavy metal, PGPR, phosphorus application wheat
INTRODUCTION

Heavy metals have been causing severe challenges to crops for many decades. They enter the environment from natural and anthropogenic sources, including industrial waste agrochemicals, mining and vehicle exhausts. Once heavy metals enter the environment, they become permanently accumulated in the food chain and, by bio-magnification, become more toxic and cause serious health issues in animals and humans (Wu et al., 2021). Chromium (Cr) has been considered the seventh most abundant element present on Earth. Fossil fuel burning, electroplating, pigment oxidants, tanneries, pulp, paper production, fertilizers, sewage sludge and steel production are the primary sources of Cr release into the environment. Chromium is severely toxic to plants, and it drastically reduces plant development and growth. Hexavalent Cr is among the most mutagenic and carcinogenic heavy metal pollutants (Gupta et al., 2022; Lei et al., 2021). Fei et al. (2022) reported that the amount of Cr is approximately 72 mg/kg in agricultural soils, but it varies in different regions. Cr disturbs normal growth processes of plants, including chlorophyll content, germination, shoot and root length, net photosynthesis and stomatal conductance. Its accumulation in plants causes chlorosis, inhibition of respiration and photosynthesis and delays the process of germination. Its toxicity in wheat is beyond the threshold level and wheat is highly susceptible to chromium stress (Diwan et al., 2012; Lei et al., 2021; Vishnupradeep et al., 2022). Furthermore, the uptake of Cr-contaminated food results in serious health problems in humans, including kidney and lung diseases, different types of cancers, skin and respiratory diseases, gastrointestinal problems and anaemia (Akcin, 2021).

Phosphorus (P) is one of the essential fertilizer components for improving crop yield. Phosphorus fertilizer has been added in the fields to improve crop yield. The phosphorus application reduces the uptake and accumulation of heavy metals in plants while improving the growth of plants, which helps ensure better soil fertility, maintains soil structure, reduces the chances of soil erosion and stabilizes contaminated sites (Ahmad et al., 2018). Finally, the phosphorus application improves the nutritional status of plants and makes them resistant to heavy metal stress. It was demonstrated that the application of phosphorus restored the diversity of plants in the heavy metal contaminated areas (Huang et al., 2020). The use of phosphorus increases fresh weight, leaf area, proline, glycine betaine, total soluble sugar, chlorophyll a and chlorophyll b in plants (De Andrade et al., 2021). It has been observed that the phytoase-producing bacteria from Himalayan soils possess the ability to solubilize inorganic phosphate, can produce phytoase, IAA (Indole-3-acetic acid, a form of auxin), and ammonia, as well as increase the availability of P and ammonia, leading to enhanced plant growth. Joint application of P and zinc (Zn) fertilizer increased essential oil content in Matricaria recutita by 50% field capacity, as well as improved other traits of plants and resulted in better growth and yield (Jeshni et al., 2017). Another study showed that the use of P improves the uptake of nutrients, increases proline, sugar, amino acids and chlorophyll content and enhances stress tolerance in Annona squamosal. It is also effective for reducing heavy metal stress in wheat (De Andrade et al., 2021).

Given that phosphorus is a macronutrient essential for plant growth, its widespread and often unregulated use in agricultural fields leads to harmful environmental consequences. The runoff into adjacent watersheds contributes to eutrophication, the consequent increase in algal biomass in lakes, rivers and coastal ocean regions that often lead to the formation of “dead zones” in which oxygen has been depleted by microbial decomposition of decaying blooms. Economic damages due to the eutrophication of freshwaters worldwide have been estimated to be 2.2 billion annually (Fitria & Dhokhikah, 2021; Sena et al., 2021).

Wheat growth demands a high amount of phosphorus, which is typically externally applied in organic or/inorganic form because of its importance for the continual crop system. The combined application of a conventional P fertilizer with phosphate-solubilizing bacteria (PSBs), a class of plant growth-promoting rhizobacteria (PGPRs), was shown to be more efficient compared with separate applications. PGPRs can control the release of phosphorus and other nutrients in the rhizosphere and make a conducive environment for plant growth even under unfavourable conditions (Fahsi et al., 2021; Hassan et al., 2015).

PGPRs live in a symbiotic relationship with plants, increase plants’ competitiveness for space and nutrients, improve plant growth and enhance plant resistance to withstand external stresses. These microbes get their nutritional needs from root exudates and, in return, impart many benefits to plants, including nitrogen fixation, plant growth promotion, growth hormone production, lytic enzyme production and antagonism against pathogenicity (Subiramanli et al., 2020). Bacillus subtilis is one of the most important bacterial strains that can detoxify heavy metals and is helpful in the management of contaminated soil (Asfa et al., 2019). The use of B. subtilis and Pseudomonas pseudoalcaligenes increases stress tolerance in Glycine max by activating different defence systems and improving its growth (Yasmin et al., 2020). The PGPR treatment also reduces oxidative stress and helps convert the more toxic form of heavy metal ions into a less toxic form by oxidation-reduction reactions (Ke et al., 2021). The mechanisms include the
formation of polyphosphate, thiophosphate, glutathione and biofilm, which collectively protect roots from the damaging effects of heavy metals (Roy, 2022). Li et al. (2022) reported that the use of B. subtilis reduces the accumulation, bioavailability, translocation factor and bioaccumulation coefficient of cadmium (Cd) in Lolium multiflorum by 27.9%, 39.1%, 24.2% and 36.5%, respectively. Ge et al. (2022) also reported that the use of B. subtilis application reduces the bioavailability of Cd to Brassica rapa. It makes biofilms and activates Cd immobilization genes such as cadA, epsA and CumaA, leading to the reduction in the Cd content in edible parts by 50%. Bacillus plays an active role in the mineralization of heavy metals from their active to inactive forms. PGPR are implicated in soil remediation by decreasing the exchangeable fraction of metals. Such bioremediation is the most effective and environmentally friendly strategy (Govarthanan et al., 2013, 2016, 2018).

The scale of toxicities of heavy metals and their repercussions on flora and fauna warrant quick steps to devise cost-effective and sustainable ways for remediating these noxious compounds. Various strategies have been used for the remediation of heavy metals-polluted soil but the current priorities lie in the development of more environmentally friendly approaches. Bioremediation techniques involving the plant-microbe ecosystem have attracted the attention of many groups of researchers because of their cost-effectiveness and sustainability and have yielded better and long-lasting results. The plant-microbe combinations help to improve the metal remediation techniques (Subiramanil et al., 2020).

This study was designed to evaluate the role of PGPR on plants and to analyse the effect of synergistic application of PGPR and phosphorus on wheat plants facing heavy metal stress.

**MATERIALS AND METHODS**

**Soil sampling**

Soil samples were collected from the discharge point of Kohinoor Textile Mills (33.21°N and 73.28°E) in Rawalpindi, Pakistan. The soil samples were properly cleaned and placed in sterile labelled zipper plastic bags and were then stored in the refrigerator at 4°C. Soil pH was measured by mixing an equal amount of water and soil in a beaker, shaking vigorously and letting it sit. The pH was measured by a pH meter. To measure soil electrical conductivity (EC), the sample was mixed with 200 ml of distilled water, and after shaking, the solution was filtered, and EC was measured using an EC meter (Peech, 1947). The texture, organic matter and chromium content were also analysed. The soil sample (2 g) was mixed with 5 ml of HNO$_3$ and 5 ml of distilled water and heated in a digestion block for 30 min. The solution was reheated after adding HNO$_3$ until the oxidation process was complete. Then the sample was heated again to reduce the digestion liquid to 5 ml, and further H$_2$O$_2$ and H$_2$O were added to the sample, heated and evaporated to 5 ml. The sample was kept at room temperature, and after cooling, HCl was added to the sample and placed at 95°C for 15 min. The solution was filtered and diluted with water to make the final volume of 100 ml, and chromium analysis was done by atomic absorption spectrophotometer (Model; GBC 932 Plus) (Banks et al., 2005).

**Isolation of bacterial strains and their analysis**

Bacterial strains were isolated by serial dilution. 0.1 g of the soil sample was mixed with 9 ml of 1% saline distilled water and vortexed. Nutrient agar medium was used, and bacterial colonies were examined after 24 and 48 h. The purification of individual bacterial colonies was done by the quadrant streaking method. The agar dilution analysed all of the selected isolates for their tolerance to heavy metals. In this process, freshly growing cultures were streaked on agar containing Petri plates amended with K$_2$Cr$_2$O$_7$ at different concentrations (10, 15 and 25 mg/L). Heavy metal tolerance was determined by the appearance of bacterial growth after 3–4 days of incubation (Jett et al., 1997). To analyse siderophore production, bacterial strains were grown on Chrome Azurol S agar at 28°C for 48 h and observed for the appearance of an orange zone as an indication of siderophore activity (Bhusan et al., 2013). Tryptic soy broth medium was used to grow bacterial strains for 24 h at 200 rev min$^{-1}$. Bacterial strains were inoculated in Dworkin and Foster media supplemented with ACC and incubated at 30°C for 48 h (200 rev min$^{-1}$). The production of α-ketobutyrate was calculated using the standard curve of α-ketobutyrate (Penrose & Glick, 2003). The phosphate solubilization ability of various isolates was estimated by observing their activity on agar plates containing tricalcium phosphate. The agar medium was poured into Petri plates, and after solidification, the isolates were spot inoculated on the center of agar plates. Next, the plates were incubated for 5 days at 28°C. The phosphate solubilization index was measured as the ratio of total diameter (colony + halo zone) to the diameter of control (colony) (Pikovskay, 1948). The most tolerant bacterial strain A3 was sequenced and the sequence information was submitted to the National Center for Biotechnology Information. It was identified as B. subtilis accession number MW 316052.
Experimental layout

The soil was collected from an undisturbed field area near the glasshouse of the Department of Botany, PMAS Arid Agriculture University Rawalpindi, Pakistan. The soil sample was analysed for pH, texture, EC, organic matter, nutrients and Cr content by following the methods mentioned in the soil sampling section (Table 1). The soil sample (5 g) was mixed with 10 mM CaCl₂ (50 ml) containing various dilutions of KCl₂ (0.2, 0.4, 1, 2, 4 mmol/L). The sample was shaken thoroughly and allowed to sit for 24 h. After filtration, the analysis of potassium was done by a flame photometer (Abu, 2016). 5 g of soil were mixed with 100 ml of NaHCO₃ reagent and placed on a reciprocating shaker at 20°C for 30 minutes to measure phosphorus. The sample was filtered, and 5 ml soil extract solution was treated with 1.5 M H₂SO₄ (5 ml). Subsequently, ammonium molybdate was added to the sample, and absorbance was measured at 880 nm. An appropriate blank control was also prepared. The standard solution of phosphorus amount was used to make the curve. To measure nitrogen content, 0.5 g soil was treated with 5 ml H₂SO₄, a selenium catalyst (0.4%) and 0.1 g salicylic acid mixed with agitation. After 30 min, 0.1 g of zinc dust was added to the solution and heated at 310°C for 4.5 h. The sample was cooled, and H₂SO₄ was added to make the volume up to 5 ml. Next, 5 ml distilled water was added, and auto-analysis was done as described by Faithfull (2002). The bacterial strain was grown in Luria-Broth medium, and it was inoculated in the soil at the rate of 10⁻⁹ CFU per ml. Ammonium phosphate was used at the rate of 2%. The soil was incubated for 30 days, and subsequently, round plastic pots (diameter 7 cm, height 8 cm) were filled with that soil (8 kg/pot). Two wheat varieties were selected, i.e., NARC 2011 and NARC 2009. Wheat seeds were surface sterilized using 0.5% sodium hypochlorite (for 5 min) and washed with sterile distilled water. The sowing was performed in October when average temperatures were 21 ± 5°C. Treatments included; T₀ = control, T₁ = Cr⁴⁺⁶ stress, T₂ = Phosphate + Cr⁴⁺⁶ stress, T₃ = B. subtilis + Cr⁴⁺⁶ stress, T₄ = Phosphate + B. subtilis + Cr⁴⁺⁶ stress. When the plants reached the stem elongation stage, chromium stress was applied using 25 mg/L K₂Cr₂O₇ solutions (1000 ml solution per pot). Leaf samples were collected after 20 days of imposing stress and analysed for morphological, physiological and biochemical parameters.

Analysis of plant length and biomass

Plants were uprooted, and the length of shoot and roots was measured using a meter rod. The plants were washed with distilled water, and their fresh weights were taken after separating their roots and shoots. They were placed in an oven at 65°C for 1 week, and dry weight was measured (Karimzadeh et al., 2021).

Leaf chlorophyll contents

To measure leaf chlorophyll content, 0.2 g flag leaves were homogenized with 80% acetone, followed by absorbance determination with a spectrophotometer. The absorbance measurements were taken at 663 nm and 645 nm. Calculations were done using the formula below (Bruuinsma, 1963).

\[
\text{Chlorophyll a} = 12.7(A₆₃₃) - 2.7(A₆₄₅).
\]
\[
\text{Chlorophyll b} = 22.9(A₆₄₅) - 4.79(A₆₃₃).
\]

Determination of proline content

For the determination of proline content, the protocol of Bates et al. (1973) was used. Plant material (0.5 g) was homogenized in 4 ml sulfosalicylic acid, and after filtration, the supernatant was placed in separate test tubes, having ninhydrin (2 ml) and acetic acid (2 ml). The whole mixture was shaken. The samples were boiled in the water bath for 1 h, then 4 ml toluene was added to the solution, and after mixing, the upper layer was used to measure absorbance at 520 nm. The standard curve of proline was used for the estimation of proline content in the sample.

Determination of soluble sugar and protein

Soluble sugar and protein amounts were determined by the method described by Dubois et al. (1951). The plant material (0.5 g) was mixed in 80% of 10 ml ethanol, and the mixture was heated at 80°C for 60 min. The extract (0.5 ml) was mixed with 18% of 1 ml phenol and incubated

| TABLE 1 Analysis of different parameters of soil used in pot experiment |
|-----------------------------------------------|
| **Parameters** | **Data** |
| pH | 7.1 |
| Texture | Clay sandy loam |
| Ec | 1.92 ds/m |
| Organic matter | 0.56% |
| P (extractable) | 3.6 ppm |
| N (extractable) | 23 ppm |
| K (extractable) | 145 ppm |
| Cr (extractable) | 0.00 mg/kg |
BACILLUS AND PHOSPHOROUS IMPROVE WHEAT IN CHROMIUM

for 60 min. After that 2.5 ml of sulphuric acid was added to the mixture, and the absorbance was taken at 490 nm. The standard curve of glucose was used for calculation. To find soluble protein, 0.2 g plant material was sampled and ground in 4 ml sodium phosphate buffer; the extract was collected after centrifugation. In 0.5 ml extract, 3 ml of Coomassie Blue dye (Bio-Rad) and 0.5 ml of distilled water were added. The absorbance was measured at 595 nm (Bradford, 1976).

Membrane stability index and relative water content

The membrane stability index (MSI) was calculated using the Bhusan et al. (2013) method. The leaf discs (100 mg) were rinsed with tap water and chopped into pieces. Next, these pieces were placed in a water bath at 40°C for 30 min. The EC (C1) was recorded by an EC meter. After 10 min the samples were placed in a water bath at 100°C, and EC was calculated (C2). The formula used for calculation is given below.

\[
MSI = \left[ 1 - \left( \frac{C1}{C2} \right) \right] \times 100
\]

Relative water content was measured by following the protocol of Dhanda and Sethi (1998). The leaves were dipped in water for 24 h, and their turgid weight was measured. Leaves were dried for 48 h at 70°C, and their dry weight was measured. The following formula was used for calculation:

\[
RWC = \left[ \frac{(\text{Fresh weight} - \text{dry weight})}{(\text{turgid weight} - \text{dry weight})} \right]
\]

Analysis of antioxidant enzymes

The Giannopolitis and Ries (1977) method was used for superoxide dismutase (SOD) estimation. The fresh plant material (0.5 g) was homogenized in 10 ml phosphate buffer (pH = 7). The samples were centrifuged at 15,000 rev min⁻¹ for 15 min, and the supernatant (0.1 ml) was added to two sets of test tubes (A and B). Next, 0.1 ml riboflavin stock, and 3 ml buffer (EDTA + NTB + methionine) were added and mixed. Set A test tubes were kept in fluorescent light (40 W), and set B was kept in the dark for 8 min until the conversion of yellow colour to dark. The absorbance was measured at 560 nm for both sets. Enzyme extract was prepared to measure ascorbate, peroxidase and catalase activity. The sample (1 g) was treated with 10 ml extraction buffer (1% polyvinyl polypyrrolidone in 50 mM potassium phosphate buffer). The homogenized mixture was centrifuged at 15,000 g for 30 minutes, and the supernatant was collected. The ascorbate peroxidase reaction mixture was prepared using 100 mM phosphate buffer, 100 μl enzyme extract, 0.1 mM EDTA, 0.062 mM H₂O₂ and 0.3 mM ascorbic acid. The activity of ascorbate peroxidase was calculated by measuring the oxidation rate of ascorbate at 290 nm (Nakano & Asada, 1981). The reaction mixture for catalase was prepared by using 200 μl of 50 mM K-phosphate buffer, 250 μl enzyme extract, 100 μl of H₂O₂ (60 nM) and 450 μl DH₂O. To initiate the reaction, H₂O₂ was added to the mixture. The decrease in absorbance was taken at 470 nm (Ravi Kiran & Aruna, 2010).

Analysis of heavy metals in wheat

The plant samples were collected at the milking stage to find the content of heavy metals in roots, shoots and wheat seeds. The uptake of heavy metals was measured by using the method of Ouzounidou et al. (1997). The dried sample (0.6 g) was digested with HNO₃ (5 ml), and after 12 h, H₂O₂ (0.5 ml) was added to the sample and placed in a muffle furnace at 155°C for 4.5 h. The remaining product was dissolved in distilled water to the final volume of 30 ml. Atomic absorption spectrophotometry (Model GBC 932 Plus) was used to measure heavy metals in every sample individually.

Statistical analysis

To analyse data, Statistix 8.1 software was used for statistical analysis. ANOVA was applied, the design was completely randomized, Tukey honest significant test was used, and post-hoc was also done to measure the difference among treatments. Additional statistical analyses were employed to determine differences between the NARC-2009 and NARC-2011 varieties.

RESULTS

Heavy metal tolerance and plant growth–promoting characteristics of bacterial strains

In this research, heavy metal–contaminated soil samples were collected from the effluent point of Kohinoor Textile Mills in Rawalpindi, Pakistan. Bacterial strains were isolated and tested for Cr tolerance in the medium (Table 2). It was observed that all strains showed tolerance to 10 mg/L Cr stress while only three bacterial strains grew in the presence of 15 mg/L Cr concentration. Two strains named A2 and A3 tolerated 25 mg/L Cr stress. The plant growth–promoting characteristics like siderophore, ACC deaminase production and phosphate solubilization...
were also tested for all bacterial strains. The A3 strain performed better in all assessments. Its morphological appearance was rough, opaque, cloudy and white in colour. The A3 strain was selected for inoculation in wheat after its sequencing by 16S rRNA. It was subsequently identified as *B. subtilis* accession No. MZ 461936.

### Morphological parameters

Chromium treatment considerably decreased shoot length, root length and biomass of wheat (Figure 1). The application of phosphorus and *B. subtilis* had positive interaction and supported plants' development in unfavourable conditions. The plants treated with phosphorus showed an increase of 12.64% (NARC-2011), 7.55% (NARC-2011), 22.62% (NARC-2009) and 12.60% (NARC-2009) in shoot and root length, respectively, compared with untreated stress-exposed plants. The increase in these parameters by the inoculation of *B. subtilis* was 25.50%, 17.73% in NARC-2011 and 33.73% and 22.46% in NARC-2009, respectively. The co-application of phosphorus and *B. subtilis* further augmented the growth of plants. It increased shoot length and root length by 30.02% and 28.19% in NARC-2011 and 38.71% and 29.31% in NARC-2009, respectively. These treatments also enhanced the biomass of plants (*p* < 0.05). The application of phosphorus increased shoot fresh weight, shoot dry weight, root fresh weight and root dry weight by 9.48%, 11.06%, 29.41 and 58.49 in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. *Bacillus subtilis* also increased wheat biomass to 24.13%, 16.60%, 38.12% and 69.81% in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. *Bacillus subtilis* also increased wheat biomass to 24.13%, 16.60%, 38.12% and 69.81% in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. *Bacillus subtilis* also increased wheat biomass to 24.13%, 16.60%, 38.12% and 69.81% in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. *Bacillus subtilis* also increased wheat biomass to 24.13%, 16.60%, 38.12% and 69.81% in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. *Bacillus subtilis* also increased wheat biomass to 24.13%, 16.60%, 38.12% and 69.81% in NARC-2011 and 15.71%, 14.00%, 31.21% and 62.29% in NARC-2009, respectively, compared with untreated stress-facing plants. **TABLE 2** Heavy metal tolerance and plant growth-promoting characteristics of bacterial strains

| Bacterial strains | Cr-10 (mg/L) | Cr-15 (mg/L) | Cr-25 (mg/L) | Siderophore production | ACC deaminase | P solubilization |
|-------------------|--------------|--------------|--------------|------------------------|--------------|------------------|
| A1                | +            | −            | −            | +                      | +            | +                |
| A2                | +            | +            | +            | −                      | −            | +                |
| A3                | +            | +            | +            | +                      | +            | +                |
| A4                | +            | −            | −            | +                      | +            | −                |
| A5                | +            | +            | −            | +                      | +            | +                |

### Physiological parameters

The increase in plant biomass is linked with the rate of photosynthesis and chlorophyll content. In this study, the amount of chlorophyll a and b content was decreased by 51.60% and 43.15% in NARC-2011 and 50.99% and 41.81% in NARC-2009 compared with the control (Figure 2a,b). The supplementation of phosphorus increased the amount of chlorophyll a and b content by 16.89% and 12.96% in NARC-2011 and 43.70% and 18.96% in NARC-2009 compared with their stress-exposed controls. The inoculation of *B. subtilis* had a stronger positive effect compared with phosphorus alone. It enhanced the rate of chlorophyll synthesis by 31.75% (chlorophyll a, NARC-2011), 25.92% (chlorophyll b, NARC-2011), 62.91% (chlorophyll a, NARC-2009) and 31.01% (chlorophyll b, NARC-2009). The co-application of phosphorus and *B. subtilis* gave significantly more positive results compared with their separate application. When co-treated, they enhanced the amount of chlorophyll content by 52.70% (chlorophyll a, NARC-2011), 70.19% (chlorophyll a, NARC-2011), 31.01% (chlorophyll b, NARC-2009) and 43.10% (chlorophyll b, NARC-2009) compared with untreated stress-facing plants. The trend was phosphate < *B. subtilis* < phosphate + *B. subtilis* in both tested varieties of wheat.

The production of osmolytes was also increased in plants under stress conditions (Figure 2c,d). The separate application of phosphorus increased the proline and sugar content by 8.92% and 19.56% in NARC-2011 and 13.24% and 22.15% in NARC-2009 compared with the untreated one. The presence of *B. subtilis* in the plant's rhizosphere enhanced the synthesis of proline and sugar to 15.02% (NARC-2011), 31.52% (NARC-2011), 18.26% (NARC-2009) and 33.86% (NARC-2009), respectively. The maximum amount of proline and sugar was reported in plants receiving the co-application of phosphorus and *B. subtilis*, i.e., the increase was 17.83%, 39.98%, 19.63% and 41.30% compared with their respective control.

The amount of protein was decreased in plants facing chromium stress (Figure 2e). The decrease was 64.01% and 62.26% in NARC-2011 and NARC-2009, respectively. It was observed that phosphorus and *B. subtilis* enhanced...
protein synthesis by 32.47%, 71.67% in NARC-2011 and 40.93% and 75.45% in NARC-2009, respectively. In contrast, the maximum amount of protein was found in plants receiving the co-application of phosphorus and \textit{B. subtilis}, at 76.84% and 86.65% compared with the un-inoculated ones. Chromium stress also decreased the membrane stability (56.25%) and relative water content (34.24%) in plants (Figure 2f,g). Phosphorus treatment improved the membrane stability by 40.00% (NARC-2011) and 42.85% (NARC-2009), as well as water content in stress-exposed plants by 12.5% (NARC-2011) and 15.68% (NARC-2009), respectively. \textit{Bacillus subtilis} inoculation also remarkably increased the membrane stability (59.52%) and water content (23.52%). However, co-inoculation of phosphorus and \textit{B. subtilis} further enhanced the membrane stability to 62.85% (NARC-2011) and 66.66% (NARC-2009), and relative water content to 29.16% (NARC-2011) and 31.37% (NARC-2009).

**Biochemical parameters**

The rate of antioxidants production, including SOD, ascorbate peroxidase (APX) and catalase (CAT), was also
FIGURE 2 Role of phosphorus and *Bacillus subtilis* on physiological attributes of wheat. (a) chlorophyll a, (b) chlorophyll b content, (c) proline content, (d) sugar content, (e) protein content, (f) membrane stability index, and (g) relative water content of wheat varieties.
measured in both wheat varieties (Figure 3). The data analysis showed that the individual and combined application of phosphorus and \textit{B. subtilis} significantly ($p < 0.05$) improved the activity of antioxidants in plants facing chromium stress. Phosphorus application enhanced the amount of SOD by 25.75% and 27.68% in NARC-2011 and NARC-2009 compared with untreated stress ($\text{Cr}^{+6}$) facing plants. \textit{Bacillus subtilis} also increased the rate of SOD synthesis by 41.71% (NARC-2011) and 54.58% (NARC-2009). The combined inoculation of phosphorus and \textit{B. subtilis} considerably improved the synthesis of SOD in plants, i.e., 60.41% and 67.71% increase was noted in NARC-2011 and NARC-2009, respectively, compared with untreated stress ($\text{Cr}^{+6}$) facing plants.

The activity of APX and CAT was also increased by the treatment of phosphorus to 61.04%, 21.47%, 64.28% and 24.91% in NARC-2011 and NARC-2009, respectively, compared with untreated stress ($\text{Cr}^{+6}$) facing plants. The improvement in APX and CAT levels following the inoculation of \textit{B. subtilis} was 73.19%, 34.03%, 79.15% and 50.19% in NARC-2011 and NARC-2009, respectively. The co-application of phosphorus and \textit{B. subtilis} once again yielded more pronounced results, that is, the increase was 91.05%, 55.21%, 95.39% and 60.88% in NARC-2011 and NARC-2009, respectively, compared with untreated stress ($\text{Cr}^{+6}$) facing plants.

### Heavy metal content

The amount of \text{Cr}^{+6} was analysed in the shoot, root and grains of wheat to evaluate the effect of phosphorus and \textit{B. subtilis} in reducing the toxicity and uptake of heavy metals in plants (Figure 4). Phosphorus amendments reduce the amount of \text{Cr}^{+6} in shoots and roots by 31.99%, 17.28%, 33.70% and 19.07% compared with untreated stress ($\text{Cr}^{+6}$) facing plants. While the use of \textit{B. subtilis} further reduced the amount of \text{Cr}^{+6} in shoot and roots of wheat, that is, 40.40%, 24.32%, 42.61% and 25.67% decrease was observed in both wheat varieties. However, the co-application of phosphorus and \textit{B. subtilis} strongly reduced the uptake and accumulation of \text{Cr}^{+6} in plants. They decreased the amount of \text{Cr}^{+6} by 54.24%, 28.71%, 59.19% and 30.53%. The content of \text{Cr}^{+6} was also significantly reduced in the grains of wheat. The decrease was 73.71% and 74.16% by using phosphorus, while \textit{B. subtilis} decreased the amount of \text{Cr}^{+6} by 80.93% and 83.57% compared with untreated stress ($\text{Cr}^{+6}$) facing plants. The co-application of phosphorus and \textit{B. subtilis} considerably reduced the amount of \text{Cr}^{+6} in wheat grains (86.45% and 90.26% decrease was noted, respectively), and it produced the best results in both wheat varieties.

**FIGURE 3** Role of phosphorus and \textit{Bacillus subtilis} on (a) superoxide dismutase, (b) ascorbate peroxidase, and (c) catalase content in wheat varieties.
DISCUSSION

Heavy metals pollution is a severe threat to the environment, including agricultural soils. Heavy metals are toxic to soil structure and pose a severe issue to vegetation, including food crops and other plants. Heavy metal toxicity affects several cellular and biochemical processes in plants. Crops grown in heavy metal contaminated soil are hazardous for living organisms when consumed because they contain an excessive amount of toxic metals (Sanaei et al., 2021). Plants uptake heavy metals and store them in their vegetative parts, which becomes hazardous when consumed by humans. Due to rapid industrialization, the soil gets contaminated by heavy metals (Ghasemzadeh et al., 2022). Heavy metals stress disturbs stomatal regulation, thus reducing CO₂ availability to plants. It also adversely affects the electron transport chain and inhibits the rate of photosynthesis (Vishnupradeep et al., 2022). Chromium is considered one of the most toxic heavy metals for plants and animals. It affects the soil’s biogeochemical activities. It has been reported that chromium was the most abundant among toxic heavy metals, and its concentration frequently exceeded the threshold limits (Naz et al., 2021; Shreya et al., 2020). Most often, the discharged chromium is gathered at the thin topsoil layer. Its contamination in the agricultural lands often results in low fertility, lack of proper structure, decreased aeration and low yield because of disturbed plant growth. Chromium disrupts normal processes of plants, including germination, shoot and root length, net photosynthesis and stomatal conductance. Its accumulation in the plant causes chlorosis, decreases respiration and photosynthesis and delays germination (Habiba et al., 2019).

Wheat is susceptible to chromium stress (Ali et al., 2018). The present study was designed to find the amelioration ability of phosphate fertilizer and PGPRs for wheat crops under chromium stress. PGPRs have a strong ability to tolerate heavy metal stress both in culture medium and contaminated soil. Due to their beneficial PGP characteristics, they enable the plants to tolerate metal stress and promote the growth of crops, including wheat. Heavy metal contaminated soil was used to isolate the PGPR, and B. subtilis was selected based on its high heavy metal tolerance ability. It was further evaluated for the analysis of plant growth-promoting characteristics (Roy, 2022). This study elaborates on the positive impact of the co-application of phosphorus and B. subtilis on wheat.

Chromium is severely noxious to plants, and it drastically reduces plant development and growth. Hexavalent Cr is more mutagenic and highly carcinogenic for many crops. Perveen et al. (2012) reported that the vegetables
irrigated with wastewater accumulated more concentrations of Cr ranging from 3.74 to 3.95 mg/kg compared with control that contained only 0.004 mg/kg of Cr. All these noxious metals have been proposed to produce serious health problems, including kidney and lung diseases, different types of cancers, skin and respiratory diseases, gastrointestinal issues and anaemia (Zeng et al., 2020). In this study, it was observed that the use of B. subtilis and phosphorus enhanced biomass production in plants, and the co-application of phosphorus and B. subtilis acts in synergism to reduce the toxic effect of stress. Phosphorus is an essential macronutrient, and the inoculation of phosphorus-solubilizing bacterial strains enhanced P availability and uptake in plants. The use of phosphorus fertilizer increased the biomass of plants under unfavourable conditions (Gullap et al., 2014) and the growth of Corchorus capsularis by improving the rate of photosynthesis and reducing oxidative stress under unfavourable conditions (Saleem et al., 2020). It was also observed that PGPR possessing ACC deaminase activity increased the growth of Cajanus cajan and Triticum aestivum. It also reduced the toxic effects of Cd stress on plants (Singh & Kumar, 2014).

Moustaine et al. (2016) reported the ability of different strains of Bacillus sp. to boost both roots and shoot growth of T. aestivum compared with plants without the PGPR inoculation. It was noted that Pantoeaagglomerans bacterial strains produced promising results in terms of fresh weight and chlorophyll content of wheat. Swarnalakshmi and Prasanna (2013) reported combined inoculation of mixtures and bio inoculants (i.e., Azotobacter chroococcum + Anabaena torulosa + Pseudomonas striata and/or Anabaena torulosa) were best over single inoculation and chemical fertilizer in terms of nutrient uptake and plant growth. Baris et al. (2014) revealed that the plants receiving mixed inoculation of PGPR (Bacillus megaterium, B. subtilis, Azospirillum brasilense) had a high uptake of plant nutrients compared with the untreated ones. The application of phosphorus-solubilizing bacteria and 50% recommended phosphate fertilizer considerably increased shoot length (21%), shoot dry weight (97%), phosphorus uptake (122%) and yield (50%) of O. sativa (Rawat et al., 2021). The co-application and selenium (Se) and Bradyrhizobium japonicum improved plant biomass, osmolytes content and antioxidant activity. Collectively, these treatments enhance the availability and uptake of nutrients in plants, which enhances the growth of plants in unfavourable conditions (Nemat et al., 2020).

It was observed that there was a negative correlation between plant growth and Cr stress. Cr downregulates numerous physiological attributes, causing a reduced rate of photosynthesis, transpiration and decreased water using efficiency. Heavy metal toxicity leads to a reduction in chlorophyll pigment production and protein contents in plants. The decrease in chlorophyll content was 25.83% and 22.77% in Nicotiana tabacum facing heavy metal stress (Zhang et al., 2020). Saleem et al. (2020) reported that phosphorus improved biomass, photosynthetic pigments, gaseous exchange attributes and yield and quality of end product in crops. Phosphorus fertilizer enhanced the content of chlorophyll pigments and decreased the bioavailability of heavy metals in the rhizosphere, making them less available to the roots directly, which improved plant growth. Furthermore, chromium toxicity disturbs the metabolic processes in plants. Mushtaq et al. (2021) reported that the cumulative use of press mud and PGPR increases photosynthetic rate, root length and shoot length by 31%, 37% and 19% in Abelmoschus esculents. They converted the Cr (VI) to Cr (III), which is less toxic, and the negative effect of Cr toxicity was reduced. The application of ACC deaminase producing PGPR and Fe-fortification enhances the amount of chlorophyll a and b content by 51.1% and 55.5%, respectively, in the presence of Cr stress. This increase was due to the improved uptake of nutrients from the soil. The amount of potassium, phosphorus and nitrogen in leaves and roots was increased by 3.40-fold, 183.3%, 64.7%, 97.3%, 122.2% and 25.6% compared with control (Danish et al., 2019). Hamid et al. (2021) reported that salinity stress decreases the amount of chlorophyll content and the rate of photosynthesis of Helianthus annuus by 43–53% and 39–53%, respectively. While the combined application of B. subtilis and biogas slurry improved the chlorophyll content, photosynthetic rate and transpiration rate by 78%, 84% and 59%, respectively, compared with untreated control. This increase was due to reduced ROS, improved phosphorus uptake and the production of growth hormones. The co-application of B. subtilis and biogas slurry maintains water content in plants, and this study also corroborated our findings.

Proline and sugar are essential osmoprotectants accumulated in plant cells in response to different stresses. In this study, proline and sugar increased significantly by applying phosphorus and B. subtilis in Cr exposed plants. These treatments also maintained membrane stability and relative water content of both wheat varieties. These results were supported by the study of Khanna et al. (2019). They reported that PGPR supplementation increased the content of proline, reducing sugars, carbohydrates and free amino acids 54.8%, 64.5%, 94% and 63%, respectively. The increase in phenol, flavonoids, polyphenols and anthocyanin was also significant compared with the control. The cumulative application of nutrients with biofertilizer improved chlorophyll content, osmolyte production and antioxidant activities in plants. This improvement was linked with the activation of genes responsible for the synthesis of these metabolites. These treatments reduced the toxicity of heavy
metals by bio-sorption and cation exchange capacity. The production of osmolytes like proline and sugar decreased the toxic effects of free radicals and helped maintain membrane stability. These osmolytes also do osmotic adjustment and conserve water in plants. When the amount of osmolytes increases in plants, they absorb and retain enough amount of water that is required for cell turgidity and various other physiological functions. These osmolytes also combat the negative effects of reactive oxygen species (Aroug et al., 2016; Morcillo & Manzanera, 2021).

Plants produce different antioxidants to cope with stress conditions, but when exposed to the high concentration of heavy metals, they cannot adequately maintain cellular homeostasis. The decrease in antioxidant activities may be due to the denaturation of the proteins and distortion of the structure of enzymes, which causes the reduction in their activity. In this study, the application of phosphorus and B. subtilis increased the production of SOD, APX and CAT in wheat plants. The literature also reported that the inoculation of PGPR (Pseudomonas aeruginosa) releases plant growth regulators like HCN, indole acetic acid, siderophore and various other metabolites that improve plant growth in heavy metal (Ni and Cr) stress. They also increased the synthesis of SOD, GR, CAT and APX in plants and decreased the heavy metal uptake by roots and their subsequent accumulation in plant tissues (Saif & Khan, 2018). The inoculation of Rhizobium also increased SOD, POD and CAT in leaves and roots of Vicia faba facing copper (Cu) stress (Fatnassi et al., 2015). When the plants experience stress, they upregulate their immune responses, and the free radicals are detected by the rhizosphere-associated microbes, activating molecular patterns for reducing the damaging effects of stress. It was observed that the use of phosphate-solubilizing bacterial strains and phosphate fertilizer increased catalase and peroxidase activity in Mentha piperita by 16% and 52%, respectively (Seif Sahandi et al., 2019).

The application of phosphorus increases the fertility of the soil, maintains soil pH and increases the diversity of vegetation. It also reduces the toxicity of heavy metals in plants, increases plant resistance toiotic and abiotic factors and helps the heavily metal-polluted ecosystems recover (Huang et al., 2020). It was noted that many studies showed that microorganisms resist, absorb and endure heavy metals by mechanisms such as re-wiring their metabolic pathways, producing secondary metabolites, and their valance state (Devi & Kumar, 2020). In this study, the co-application of phosphorus and B. subtilis considerably decreased the accumulation of Cr$^{6+}$ in shoot, root and wheat grains. Such findings were also documented by Wani and Khan (2010), who stated that the inoculation of Bacillus sp. decreases chromium uptake in the root, shoot and grains of Cicer aretianum. Moreover, Pseudomonas putida reduced the accumulation of Cr, Pb, Ni and Cd in leaves and grains of Zea mays. These PGPR control the movement of heavy metals and bind them to make them unavailable. They also prevent the leaching of heavy metals and modulate the movements of ions through the membrane transporters; all these factors, in turn, contribute to the low uptake and accumulation of heavy metals in plants (Bano & Javed, 2021). Bacillus subtilis has a powerful potential to decrease heavy metal stress in contaminated soils and improve plant growth under heavy metal stress through different mechanisms. Here, we observed that the individual and combined applications of phosphorus and B. subtilis increased the morphological, physiological and biochemical attributes of plants. The co-application showed considerable improvement in plants facing chromium stress. The uptake and accumulation of Cr$^{6+}$ were also significantly ($p<0.05$) decreased under these treatments. The direct and indirect mechanisms used by phosphorus and B. subtilis were responsible for the better growth of plants in stress conditions. The mechanisms of B. subtilis’ action include the formation of polyphosphates, thiophosphates, glutathione and biofilm, which protects roots from the damaging effects of heavy metals. The use of P increases nutrient uptake, proline, sugar, amino acids, chlorophyll content and stress tolerance in plants. They showed strong potential to ameliorate heavy metal stress in food plants. We conclude that the combined use of the nutrients in combination with PGPR holds promise to enhance their positive effects on the crop yield and soil health.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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