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Effect of flax rhizobacteria on germination and seedling growth under copper toxicity

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Abstract

Plant growth promoting rhizobacteria (PGPR) supports plant growth by decreasing heavy metal levels in soil through methods that alter metal pathways and convert toxins into safe forms. Five rhizobacterial isolates (FLX-1, FLX-2, FLX-3, FLX-4, and FLX-5) isolated from flax rhizosphere were identified using MALDI-TOF-MS techniques based on morphology, biochemistry, and plant growth-promoting properties. Four isolates (FLX-1, FLX-2, FLX-3, FLX-5) capable of fixing nitrogen, four (FLX-1, FLX-3, FLX-4, FLX-5) dissolving inorganic phosphate, and three (FLX-2, FLX-4, FLX-5) producing IAA and HCN. Additionally, in this study, the effects of 5 different rhizobacteria and copper ($CuSO_4$) doses (0, 5, 10, 15, and 20 mM) on growth and development parameters of flax seeds were determined under fully controlled climate chambers. Moreover, Different rhizobacteria treatments significantly improved growth and development parameters in flax seed compared to the control group. It was observed that increasing copper doses resulted in a significant decrease in parameters such as germination rate, mean germination time, germination power index, root length, and shoot length in bacterial treatments. When evaluating the responses of different rhizobacteria treatments to copper heavy metal in flax seed germination, it was determined that the treatment of *Pseudomonas chlororaphis* FLX-5 yielded better results compared to other treatments and was effective in reducing the negative effects of copper in terms of the tested germination characteristics. In conclusion, the current study suggests that *Pseudomonas chlororaphis* FLX-5 may efficiently be used in tolerating copper toxicity, indicating that further research is needed in this area.

Keywords: Flax, Copper, PGPR, Germination, *Pseudomonas chlororaphis*

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INTRODUCTION

Flax (*Linum usitatissimum* L.) is a dicotyledonous plant belonging to the Linaceae family. It has been one of humanity's oldest industrial crops, having been used for thousands of years. This economically valuable plant is grown in many parts of the world, including Asia, the Eastern Mediterranean, the Middle East, and India, and is known for its high fiber and oilseed content. Out of around 100 species globally, about 38 can be found in Türkiye (Kumral & Şimşek, 2023). The term "flax" in Latin means "extremely useful," and it contains omega-3 fatty acids, phenols, phytoestrogenic lignans, flavonoids, proteins, sterols, antioxidants, and fiber. Natural fibers derived from flax fibers generally have significant potential in the material industry (Omer et al., 2022). About 40% of flaxseed is oil, 28-30% is protein, 6% is mineral matter and ash, and the remaining portion is hull. Due to its alpha-linolenic acids (ALA), short-chain polyunsaturated fatty acids (SC-PUFA), phytoestrogenic lignans (secoisolariciresinol diglucoside or SDG), and other bioactive compounds, flax seed has preventive and therapeutic effects against various diseases such as cardiovascular diseases, diabetes, hyperglycemia, obesity, breast-prostate cancer, osteoporosis, and reproductive problems (Kauser et al., 2024).

Copper (Cu) is recognized as a vital micronutrient that plays a crucial role in the morphological, physiological, and biochemical processes of all plants. However, excessive Cu has negative effects on plant growth and productivity. In many parts of the world, heavy metal accumulation is the main factor limiting the yield of many plants, especially flax. It has been reported that many oilseed crops under heavy metal stress have decreased grain yield and oil yield (Fatnassi et al., 2015; Abdel Latef et al., 2020). The widespread use of certain copper (Cu)-based agricultural chemicals, such as chemical fertilizers, fungicides, herbicides, insecticides, and nematicides, has resulted in the accumulation of copper in the soil at varying concentrations (Schoffer et al., 2020). The use of Cu-containing compounds to control plant diseases has increased in the amount of copper in the soil (Ballabio et al., 2018). In some cases, the treatment of phosphate fertilizers has also significantly elevated Cu concentrations in the soil (Ruyters et al., 2013). High concentrations of copper in the soil can be toxic to certain soil microorganisms and hinder the mineralization of macro-nutrients like phosphorus (P) and nitrogen (N). Since the accumulated Cu in the soil cannot be broken down biologically or chemically, it poses a threat to the environment, food safety, and human health. Thus, copper has become a critical issue in the management of horticulture and agricultural products. The increasing pollution of heavy metals like copper in the soil leads to a decrease in the number and diversity of soil microorganisms, severely disrupting ecological balance. In such cases, only organisms resistant to heavy metals can survive, enabling them to remediate the contaminated soil (Azeez et al., 2015).

Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that actively colonizes plant roots and enhances plant growth and development through various mechanisms such as nitrogen fixation, phosphate solubilization, IAA production, and ACC deaminase production. PGPR reduces heavy metal concentrations in the soil through immobilization, chelation, active removal, biosorption, and bioaccumulation. In recent years, bioremediation of heavy metal-contaminated soil through PGPR has garnered significant attention for developing eco-friendly solutions to remove heavy metals from the ecosystem. Identifying the relationships of these bacteria with plants is extremely important for sustainable agriculture, particularly in determining their potential contributions in environmental stress conditions (Gupta et al., 2024). Excessive levels of heavy metals in contaminated soils diminish microbial activity, soil health, and crop productivity. Consequently, bioremediation is an alternative approach to managing soil pollution. This technique employs specific microorganisms to extract heavy metals from polluted soil or convert highly toxic forms into less toxic ones. Plant growth-promoting rhizobacteria are capable of surviving in soils contaminated with heavy metals, thus their use aids in bioremediation. Although research on metal tolerance of PGPRs has increased recently, studies on copper tolerance are still limited. Many studies with PGPR suggest that heavy metal resistance of local isolates should be investigated in different ecosystems and plant species. This study aims to identify the bacteria in the rhizosphere of *L. usitatissimum* using MALDI TOF MS and to further investigate their plant growth-promoting properties, as well as the effects of different copper levels and five PGPR treatments (FLX-1, FLX-2, FLX-3, FLX-4 and FLX-5) on the seed germination, root and stem growth, and physiological characteristics of flax plants.

MATERIALS AND METHODS

The experiment was conducted in a fully controlled climate room of the Department of Field Crops, Faculty of Agriculture, Ankara University in 2023. The flax seeds (*L. usitatissimum*) used in this study were obtained from the Department of Field Crops of Ankara University Faculty of Agriculture. Copper sulfate ($CuSO_4.5H_2O$, Merck) was used at concentrations of 0, 5, 10, 15 and 20 mM.

Sample collection

Rhizospheric soil samples were collected in June 2023 from *L. usitatissimum* in the experimental field of Ankara University, Faculty of Agriculture, Department of Field Crops (39°57'44.2"N, 32°51'36.7"E). The sample taken from plant root rhizosphere at a depth of 10 cm. Each soil sample was labelled and brought to the bacteriological Laboratory and studied under aseptic conditions.

Isolation of rhizobacteria

Rhizospheric bacteria were isolated from 1 g of dried soil samples by serial dilution method. The soil samples were homogenized in 10 ml of sterile isotonic saline water. Each 1 g of soil sample was mixed in 9 ml of 0.85 % saline (NaCl) sterile water and then homogenized in a shaker for 30 min. Each rhizospheric soil sample was diluted from 10^{-1} to 10^{-6}. These dilutions were spread on nutrient agar (NA) solidified in Petri dishes and incubated at 28 °C. After incubation, different single colonies were screened in Petri dishes containing NA medium to obtain a pure colony and conserved in stock solution at -85 °C for later use.

Identification of bacterial isolates

Physiological, biochemical tests, Gram staining, and spore formation tests of the bacterial isolates were examined using methods described by Bashan et al. (1993). MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) was used for bacterial identification. Microorganisms were identified by their unique molecular fingerprints by the MALDI Biotyper CA System. Protein profiles of microorganisms' biomolecules (such as protein, peptide, sugar, and polymer) were ionized and then passed through an electric and/or magnetic field in this method. These profile spectra were compared graphically to

reference microorganisms in the system's database to accurately identify them at genus and species level (Sivri & Öksüz, 2019).

Determination of Plant Growth-Promoting Characteristics

Evaluation of Phosphate Solubilizing Bacteria

The bacterial isolates were tested for their ability to solubilize phosphate according to the methods of Pikovskaya (1948) using Pikovskaya agar (PKA) medium (0.2 g L^{-1} NaCl, 10 g L^{-1} glucose, 0.2 g L^{-1} KCl, 5 g L^{-1} $Ca_3(PO_4)_2$, 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} $MgSO_4.7H_2O$, 0.002 g L^{-1} $FeSO_4.7H_2O$, 0.5 g L^{-1} yeast extract, 0.002 g L^{-1} $MnSO_4.H_2O$, and 1000 ml distilled water). The plates were inoculated with bacteria and incubated at 28±2 °C for 5 days. Colony, formed a clear halo zone around them, indicating phosphate solubilization. The experiments were performed in triplicate. The Phosphate solubilization index (PSI) was determined using measurements taken after seven days of growth from a point inoculation on a PKA medium at 28±2 °C (Meena et al., 2015).

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Halo zone diameter.}}{\text{Colony diameter}}$$

Identification of Nitrogen Fixing Isolates

Determination of the nitrogen fixation abilities of the isolates was used according to the protocol specified by Park et al. (2005). The isolates were streaked on NA medium and incubated at 28 ºC for 24 hours. Thereafter incubation, the fresh isolates were incubated for 4 days at 28±2 ºC by inoculating them on Petri dishes having solid Burk's N-free medium (0.05 g L^{-1} Na_2SO_4, 10 g L^{-1} glucose, 0.2 g L^{-1} $CaCl_2$, 0.41 g L^{-1} KH_2PO_4, 0.52 g L^{-1} K_2HPO_4, 0.0025 g L^{-1} Na_2MoO_4. $2H_2O$, 0.1 g L^{-1} $MgSO_4.7H_2O$, 0.005 g L^{-1} $FeSO_4.7H_2O$, and 1.8 g L^{-1} agar 1 L dsH_2O) by line culturing method. These growing isolates on Burk's N-free medium were evaluated as positive.

Evaluation of HCN Producing Isolates

The HCN production assay was carried out according to the method of Bakker and Schippers (1987). The bacteria were inoculated on NA medium and filter papers impregnated with picric acid (0.5% picric acid, 2% sodium carbonate) placed on the edge of the Petri plate. Petri dishes were tightly closed with paraffin and incubated at 28±2 °C for 4 days. At the end of the incubation, yellow colored picric acid-impregnated papers turned brown, which was considered a positive result (Deshwal & Kumar, 2013).

Determination of Isolates Producing Indole-3-Acetic Acid (IAA)

Sarwar and Kremer (1995) protocol was used to assess the isolates' capacity to produce Indole-3-Acetic Acid (IAA). Bacterial cultures were grown for 48 hours at 36±2 °C. These fresh cultures were centrifuged for 30 min at 3000 rpm. The supernatant (2 ml) was combined with 4 ml of the Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) and two drops of orthophosphoric acid. Pink appearances indicated the presence of IAA which was determined spectrophotometrically (Shimadzu UVmini-1240 Spectrophotometer) at 530 nm.

Seed treatment

Flax seeds were placed in 10 % sodium hypochlorite (NaOCl) solution, for 15 minutes. It was followed by five times rinsing with distilled tap water to ensure sterilization. The bacterial strains stored at -85 °C were inoculated on NA medium and incubated at 30±2 °C for 24 h. The pure cultures were grown on Nutrient Broth (NB) medium containing 4 % sugar. The concentration of the prepared bacterial solutions was adjusted to 10^8 cfu/ml with sterile distilled water. The seeds were then treated with bacterial suspensions for 3 h under sterile conditions. The sterile seeds were not treated with bacteria in the control group (Heinonsalo et al., 2004) (Figure 1A).

Germination tests

Petri dishes and whatman paper number 1 were used as germination material. The seeds were germinated by placing them between 2 pieces of sterile whatman paper in each sterile Petri dish. 20 seeds were placed in each Petri dish and treated with 15 ml of respective copper concentrations. Sterile distilled water (0mM) was used as the control treatment. Petri dishes were covered with parafilm to prevent moisture loss. The research was established according to the factorial experimental design in randomized plots with three replicates and 20 seeds in each replicate (Figure 1B). Thereafter, the flax seeds were germinated in dark condition (25 °C and 65% relative humidity) for 14 days. Four days after the seeds were transferred to Petri dishes, an average of 10 ml sterile distilled water was added to each Petri dish for 14 days. The germinated seeds were counted on each day and the seed with a rootlet length of 2 mm was considered as germinated following "International Seed Testing Association (ISTA 1993)"(Ertekin et al., 2020). Germination percentage (%), mean germination time, germination power index, root length and stem length (cm), root and stem fresh weight and root and stem dry weight (mg) were analysed. After the flax seeds were placed in the germination medium, the counts were made every day for 14 days and the germination characters stated below were calculated.

Germination Percentage (GP) (%): Counted every day and calculated by dividing the total number of germinated seeds by the number of seeds taken for germination after 14 days (Pordel et al., 2019).

Mean Germination Time (MGT): Calculated by dividing the number of germinated seeds on the counting day by the number of counting days (Dezfuli et al., 2008).

Germination Power Index (GPI): Calculated by multiplying the sum of the average stem length and average root length by the total germination percentage (Pordel et al., 2019).

The seedlings were taken out of climate cabinets to measure their root and shoot lengths.

Root and Stem Fresh Weight (mg plant^{-1}): The root and stem fresh weight of the plants was determined by weighing them on a precision scale at the end of the germination test.

Root and Stem Dry Weight (mg plant^{-1}): They were dried in an oven at 70 °C for 48 hours and weighed on a precision scale to determine the root and stem dry weight of the plants.

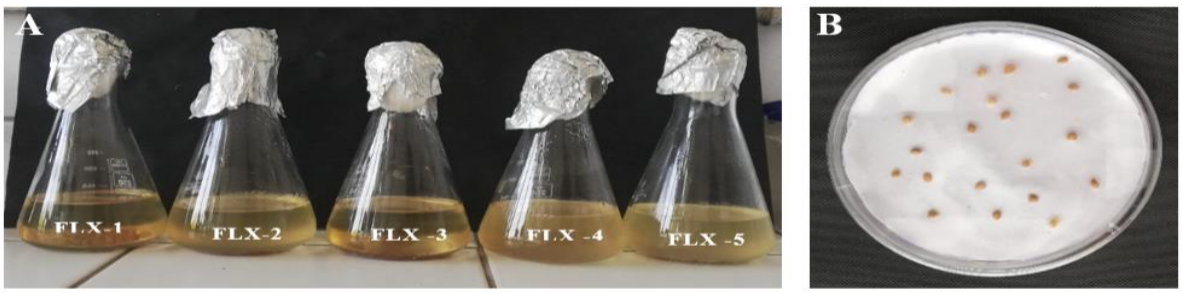

Figure 1. Preparation of bacterial culture in NB medium(A) and application of PGPR-Cu to flax seeds (B)

Statistical Analysis

Statistical analysis of the data was performed using JMP Pro 17.0 statistical software. Normally distributed dependent variables were presented as mean ± Standard Error (X±Sx). The differences in the mean amounts of variables were determined using the Tukey test (Genç & Soysal, 2018).

RESULTS AND DISCUSSION

Identification of isolates

Five bacterial isolates were identified based on biochemical, morphological, and MALDI-TOF MS analysis in the present study. The isolates were characterized as species within the genera *Bacillus*, and *Pseudomonas*. Among all isolates, 2 showed Gram (+) reaction. The catalase test was positive for all isolates, whereas the oxidase test was positive for 4 isolates except for *Bacillus thuringiensis* FLX-1. The sporulation ability of the isolates was examined and the results showed that two isolates (*Bacillus thuringiensis* FLX-1 and *Bacillus mojavensis* FLX-4) produced spores and the other isolates (*Pseudomonas libanensis* FLX-2, *Pseudomonas chlororaphis* FLX-5 and *Pseudomonas boreopolis* FLX-3) did not. Additionally, Motility tests for all other isolates were positive. The morphological and biochemical results of the isolates are presented in Table 1.

Table 1. Morphological and biochemical traits of isolates

Bacterial isolate code	Isolates	Morphological Characteristic				Biochemical Characteristic		
		Gram reaction	KOH 3%	Colony color	Motility	Catalase	Oxidase	Spore forming
FLX-1	*Bacillus thuringiensis*	+*	-	cream	+	+	-	+
FLX-2	*Pseudomonas libanensis*	-	+	white	+	+	+	-
FLX-3	*Pseudomonas boreopolis*	-	+	white	+	+	+	-
FLX-4	*Bacillus mojavensis*	+	-	Milky white	+	+	+	+
FLX-5	*Pseudomonas chlororaphis*	-	+	white	+	+	+	-

Note: * +, positive; –, negative

The MALDI-TOF MS identification method, is based on the detection of intracellular molecules, especially ribosomal proteins at the genus, species, and strain level (Solntceva et al., 2021). It is one of the most reliable tools used to identify microorganism species in fields such as medicine, food, and agriculture due to its high accuracy and rapid results (Stîngu et al., 2008; Vega-Castano et al., 2012). Çelikten and Bozkurt (2018) used the MALDI-TOF method to identify 120 bacteria they isolated from the wheat rhizosphere to investigate plant growth-promoting bacteria. Öksel et al. (2022) used the MALDI-TOF MS method to identify bacteria in wheat rhizospheres. Similarly, Ünlü et al. (2023) used MALDI-TOF MS to identify bacterial strains isolated from the

alfalfa rhizosphere as belonging to the genera *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Lysinibacillus*, *Acinetobacter*, and *Enterobacter*. In a recent study, Güler, (2024) used the MALDI TOF MS method to identify phosphate solubilizing bacteria isolated from different medicinal aromatic plants.

Plant growth-promoting properties of isolates

The isolates exhibited various plant growth-promoting traits. Four isolates (FLX-1, FLX-2, FLX-3, and FLX-5) demonstrated nitrogen-fixing ability, four (FLX-1, FLX-3, FLX-4, and FLX-5) solubilized inorganic phosphate, three (FLX-2, FLX-4, and FLX-5) produced IAA, and HCN (Figures 2 and 3). Phosphorus is a key element that restricts plant growth as well as nitrogen. PGPR assists plants absorb more phosphate by transforming it from insoluble to soluble forms. The Bacillus and Pseudomonas genera are the most influential bacteria that typically solubilize phosphate by producing organic acids (Qingwei et al., 2023). According to Rawat et al. (2021), the most prevalent phosphate-solubilizing bacteria in the rhizosphere are *Bacillus*, *Enterobacter*, and *Pseudomonas*. *Bacillus thuringiensis* FLX-1, *Pseudomonas boreopolis* FLX-3, *Bacillus mojavensis* FLX-4 and *Pseudomonas chlororaphis* FLX-5 dissolved inorganic phosphate in the current study. P solubilization index of bacterial isolates is defined to be between 3.56 to 5.47 on PKA agar medium. According to the Phosphate solubilization index of bacterial isolates in PKA agar medium, the maximum value was obtained from *Pseudomonas chlororaphis* FLX-5 with 5.47. This was followed by *Pseudomonas boreopolis* FLX-3 with 4.19 and *Bacillus mojavensis* FLX-4 with 3.85, respectively (Table 2).

Mukhtar et al. (2017) determined that *Bacillus safensis* PSB5 and *B. megaterium* PSB12 from wheat rhizosphere solubilized high phosphate levels. Prajakta et al. (2019) reported that *Bacillus mojavensis* PB-35 had a phosphate solubilization ability among 95 rhizobacterial strains isolated from soybean rhizosphere. Similarly, it was determined that *Bacillus mojavensis* FLX-4 had a higher phosphate solubilization ability (3.85) in the current study. Aliyat et al. (2022) reported that bacteria collected from the phosphate mining area were capable of dissolving various forms of phosphate (tricalcium phosphate, aluminum phosphate, and iron phosphate), with the maximum phosphate solubilization index (PSI) being 4.12 from *Pseudomonas brassicacearum* BT125. Similarly, it was determined that *Pseudomonas boreopolis* FLX-3 had a high (4.19) phosphate solubilization index in the current study. Amri et al. (2023) determined 28 phosphate-solubilizing bacteria from soil samples collected from different regions of Tunisia. They found that the solubilization index percentages of these bacteria ranged 2.14 to 3.51%, with the maximum PSI percentage belonging to *Pseudomonas fluorescens*. In the present study, it was determined that the phosphate solubilization index in the Pseudomonas genus was between 4.1 and 5.4. These results align with the findings of Roychowdhury et al. (2019), and Blanco-Vargas et al. (2020), which demonstrated that the solubilization index among various bacterial isolates, including *Pseudomonas* spp., ranged 2.56 to 4.50.

IAA controls and regulate plant growth, development, senescence, cell division, elongation, and fruiting. Approximately 80 % of PGPR are capable of producing IAA (Zahroya et al., 2020). In the present study, *Pseudomonas libanensis* FLX-2, *Bacillus mojavensis* FLX-4, and *Pseudomonas chlororaphis* FLX-5 produced varying amounts of IAA, as determined spectrophotometrically at 530 nm. The ability of Pseudomonas and Bacillus strains to produce IAA has been demonstrated in previous studies (Myresiotis et al., 2014; Rizvi & Khan 2017). Zainab et al. (2020) determined that *Bacillus gibsonii* (PM11) and *Bacillus xiamenensis* (PM14) strains inoculated on flax seeds produced high levels (79 µM ml^{-1} ve 91µM ml^{-1}) of IAA. Devanathan et al. (2021) reported that *Bacillus* sp. in the rhizosphere of *Capsicum annum* L. produced high levels of IAA. Khatami et al. (2023) reported that rhizospheric *Bacillus* sp. synthesized high amounts of IAA. Likewise, *Bacillus mojavensis* FLX-4 produced IAA in the present study (Table 2).

Table 2. IAA production and phosphate solubilization index (PSI) values of isolates

Bacterial isolate code	Isolates	IAA Production[1] (OD at 530nm) (X±Sx)	Phosphate Solubilization Index[2] (PSI) (X±Sx)
FLX-1	*Bacillus thuringiensis*	ND*	3.56±0.08[a]
FLX-2	*Pseudomonas libanensis*	12.45±0.60[a]	ND
FLX-3	*Pseudomonas boreopolis*	ND	4.19±0.22[b]
FLX-4	*Bacillus mojavensis*	10.39±0.65[a]	3.85±0.07[a]
FLX-5	*Pseudomonas chlororaphis*	16.61±0.18[b]	5.47±0.08[c]

*ND: Not Detected, Mean differences were statistically classified as significant ($p< 0.05$).

Nitrogen-fixing bacterial genera commonly found in the rhizosphere include *Rhizobium*, *Sinorhizobium*, *Bacillus*, *Pseudomonas*, *Azoarcus*, and *Burkholderia* (Shah et al., 2021). The current study demonstrated that *Bacillus thuringiensis* FLX-1, *Pseudomonas libanensis* FLX-2, *Pseudomonas boreopolis* FLX-3, and *Pseudomonas chlororaphis* FLX-5 have the ability to fix nitrogen. Reetha et al. (2014) determined that the *Pseudomonas fluorescens* strain isolated from the onion rhizosphere carried out nitrogen fixation ability. Afa et

al. (2020) reported that among 20 *Pseudomonas* isolates isolated from the rhizosphere of *Allium ascalonicum*, 6 of them (Twt04, Twb11, Tt01, Lw07, Lt01, and Lp03) fixed a high amount of nitrogen**.** Alnefai et al. (2020) determined that 8 isolates identified using MALDI-TOF MS showed strong nitrogen-fixing ability. Similarly, Singh et al. (2023) determined that *Pseudomonas koreensis* CY4 and *P. entomophila* CN11 isolated from sugarcane rhizosphere fixed nitrogen.

Some soil bacteria produce a volatile secondary metabolite called HCN. It inhibits electron transport pathways and energy sources in cells, resulting in pathogen death (Manasa et al., 2017). In the present study, *Pseudomonas libanensis* FLX-2, *Bacillus mojavensis* FLX-4 and *Pseudomonas chlororaphis* FLX-5 produced HCN. Ahmad et al. (2008) reported that 50% of Bacillus and 88% of Pseudomonas in the plant rhizosphere could produce HCN. Quesssaoui et al. (2017) determined that *P. fluorescens* Q110B and *P. fluorescens* Q036B, taken from the tomato rhizosphere, produced HCN. Similarly, Halimursyadah et al. (2023) determined that *P. fluorescens* produced HCN among 37 isolates from the patchouli rhizosphere. Singh et al. (2019) determined that *B. thuringiensis* SF 23, *P. aeruginosa* SF 44, *B. subtilis* SF 48, and *B. subtilis* SF 90 isolates produced HCN. The plant growth-promoting properties of the isolates are presented in Figures 2 and 3.

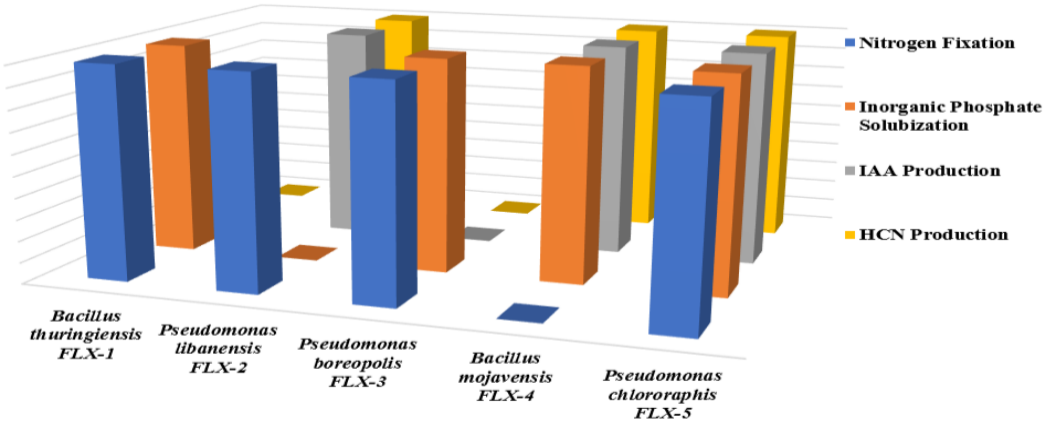

Figure 2. Distribution of plant growth promoting properties of isolates

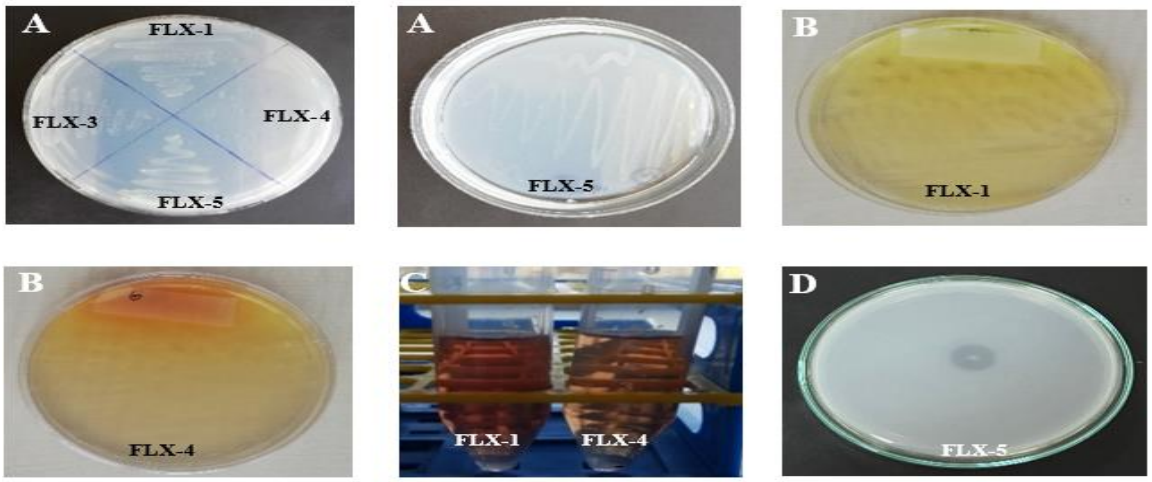

Figure 3. Plant growth promoting traits of the isolates. A: Nitrogen fixation B: HCN production C: IAA production D: Inorganic phosphate solubilization.

Germination tests

Germination Percentage (GP)

The study found that with each increase in copper concentration, the germination percentage decreased, and the flax seeds treated with bacteria germinated faster compared to the control group. This outcome can be attributed to the gradually increasing copper (Cu) stress, which exerts a toxic influence on seed germination and leads to a reduction in germination rate; however, bacterial treatment appears to be effective in alleviating this negative impact. In the evaluation made according to the rhizobacteria-Cu treatments, it was determined that the germination percentage ranged from 49.8% (control treatment) to 77.1% (FLX-5 rhizobacterium treatment) according to the average doses of Cu (Figure 4A, Table 3). Additionally, the maximum germination percentage for copper doses was 76.8% in the control group (0 mM), while the minimum germination percentage was 60 % after 20 mM treatment.

Mean Germination Time (MGT)

It was determined that the average germination time increased as the copper concentration increased in other bacterial treatments except for the FLX-2 rhizobacterium treatment. The evaluation based on the rhizobacteria-Cu treatments, it was determined that the average germination time ranged from 3.59 days (FLX-1 rhizobacterium treatment) to 2.89 days (FLX-2 rhizobacterium treatment) (Figure 4B). When considering the average copper doses, the maximum average germination time was 3.63 days in the control (0 mM) group, while the minimum germination time was detected on the 20 mM treatment after 2.8 days.

Germination Power Index (GPI)

The evaluation made according to the rhizobacteria-Cu treatments showed that the germination power index varied between 826.2 (FLX-5 rhizobacterium treatment) and 229.2 (control treatment) (Figure 4C). Additionally, considering the averages of copper doses, the maximum germination power index was 1116.5 in the control (0 mM) treatment, while the minimum germination power index was determined as 30.8 on 20 mM treatment.

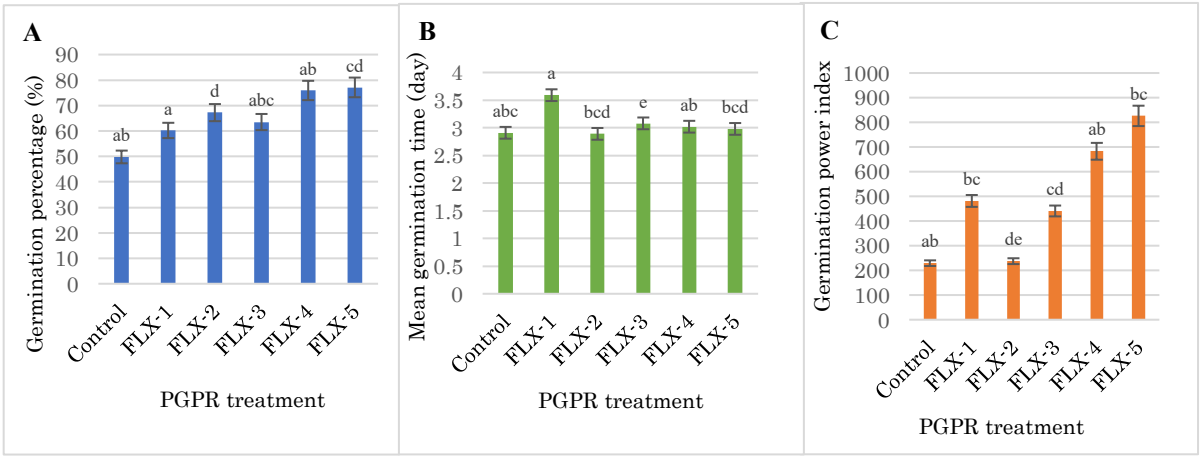

Figure 4. Effect of PGPR treatments on germination percentage (A), mean germination time (B) and germination power index (C) of flax seed

Many studies indicate that excessive copper accumulation in plants like corn, beans, cucumbers, and tomatoes negatively impacts plant growth (Mediouni et al., 2006; Feil et al., 2020). Copper stress in plants create biomass mobilization through glucose-fructose release, which prevents the breakdown of starch or sucrose in tissues and reduce seed germination. It has also been reported that copper toxicity inhibits seed germination by down-regulating the activity of α-amylase or enolase (Bajpai et al., 2021). Widawati and Suliasih (2018) reported applying PGPR (*Azospirillum* sp., *Azotobacter* sp., *Bacillus* sp.) to sorghum seeds promoted seed germination parameters as well as root and shoot growth in vitro. Similarly, Ghorbel et al. (2023) determined that inoculating barley seeds with the *Glutamicibacter* sp. MD36 strain, which has plant growth-promoting properties, increased barley germination under Cu heavy metal stress by 77 % at 50 mM, 63 % at 100 mM, and 38 % at 150 mM.

Germination percentage coefficient, germination percentage, germination power index, stem length and root length decreased due to the increase in Cu concentration; mean germination time was prolonged except for *Pseudomonas libanensis* FLX-2 treatment. It was determined that *Pseudomonas chlororaphis* FLX-5 bacteria treatment significantly increased the germination percentage, mean germination time, and germination power index compared to the control treatment. According to Zainab et al. (2020), IAA-producing rhizobacteria accelerate phytoremediation efficiency and increase the root and stem development of the plant. This improvement in germination parameters can probably be attributed to the ability of *Pseudomonas chlororaphis* FLX-5 to solubilize phosphate, produce IAA, and fix nitrogen. On the other hand, it was determined that *Pseudomonas*

boreopolis FLX-3 bacteria treatment increased the germination percentage, average germination time, and germination power index.

Evaluation of root and stem lengths

The maximum root length was determined as 5.14 cm after treatment with the FLX-5 bacteria, and the minimum root length was determined as 1.75 cm in the control treatment in this study. Moreover, considering the average copper doses, the maximum root length was determined as 7.33 cm in the control (0 mM) treatment; the minimum root length was determined as 0.35 cm in the 20 mM treatment (Table 3).

It was determined that *Pseudomonas chlororaphis* FLX-5 and *Bacillus mojavensis* FLX-4 strains provided longer roots compared to the control group in this study. In particular, it was determined that *Pseudomonas chlororaphis* FLX-5 strain sharp improvement in root length (5.14 cm) compared to control treatments. Whereas, the minimum root length (3.09 cm) was determined on plants treated with *Bacillus thuringiensis* FLX-1 (Figure 5A). In the evaluation made according to the bacteria-Cu treatments, the longest stem length of 4.91 cm was noted after treatment with *Pseudomonas chlororaphis* FLX-5 strain, and the shortest stem length of 2.10 cm was noted on the control treatment. Moreover, According to the average copper doses, the longest stems of 6.91 cm were noted on the control (0 mM) treatment. The minimum stem length of 0.27 cm was noted on the 20 mM treatment in the current study (Figure 5B).

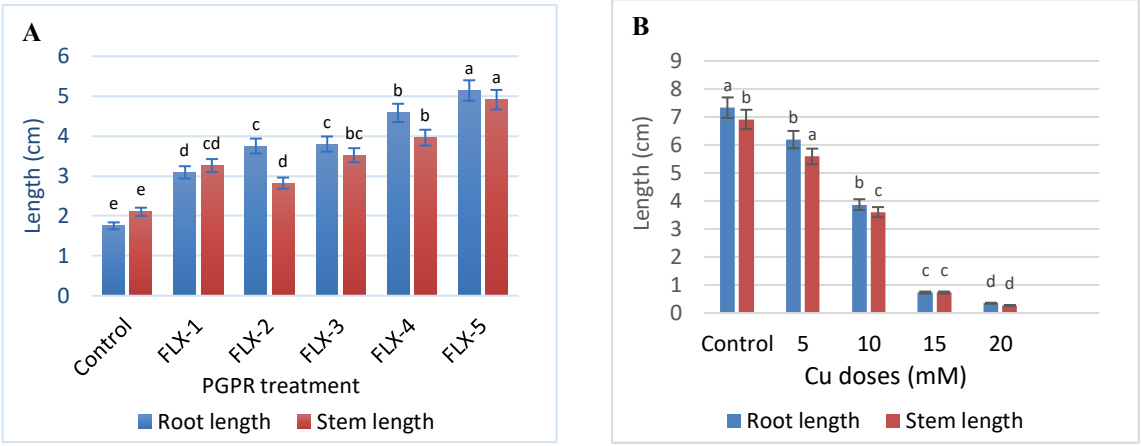

Figure 5. Effect of PGPR (A) and Cu (B) treatments on root and shoot development from flax seed

PGPR strains are beneficial microorganisms that influence the bioavailability of heavy metals (Gupta et al., 2024). Rizvi et al. (2020) investigated the Cr biosorption capacity of *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Azotobacter chroococcum*. They determined that *B. subtilis* biosorbed Cr at a record rate of 96% at 25 g mL^{-1}. El-Saadony et al. (2024) determined that the treatment of *Pseudomonas azotoformans*, *Serratia rubidaea*, *Paenibacillus pabuli* and *Bacillus velezensis*, which were isolated from heavy metal contaminated soils and determined to have plant growth promoting properties, to pepper plants grown under heavy metal stress (Cd, Cu, Pb and Ni) increased photosynthetic pigment contents, relative water content and membrane stability index and germination parameters compared to plants without bacteria treatment.

Many researchers have reported that PGPR can tolerate heavy metals, break down or clean up soil pollutants, and convert them into less toxic forms (Nazli et al., 2020; Zhu et al., 2023). It has been noted that PGPR bacteria reduce the phytotoxicity of metal ions by sharing the metal load resulting from bioaccumulation in the presence of heavy metals (Zaidi et al., 2006). Pandey et al. (2013) reported that *Bacillus* species with plant growth promoting properties stimulated rice seed germination under copper stress and increased root and shoot lengths in rice plants compared to the control. According to Patel et al. (2016), the use of PGPR capable of adsorbing heavy metal ions is an eco-friendly alternative to chemical metal chelators. Hansda et al. (2017) determined that the plant growth-promoting *Kocuria* sp. CRB15 bacterial strain was resistant to various heavy metals like Cu, Ni, Cd, Zn, and Pb. Elsakhawy et al. (2019) reported that the halophilic bacteria *Chromohalobacter salexigens* KT989776 significantly improved all germination parameters of flax seeds under different heavy metal and salt concentrations. A study conducted by Abbaszadeh-Dahaji et al. (2021) reported that PGPR strains (*Pseudomonas cedrina* K4 and *Stenotrophomonas* sp. A22) were effective in reducing Cu toxicity in corn and sunflower grown in Cu-contaminated soil. It has been reported that the presence of toxic levels of copper in the soil prevents the growth and development of newly developing rootlets, resulting in decreases in root and shoot lengths (Sfaxi Bousbih et al., 2010; Pena et al., 2011). Soudek et al. (2010) determined that high levels of copper slowed down the root development of the flax plant when they studied the effects of different doses of various heavy metals (cadmium, cobalt, copper, zinc, nickel, lead, chromium, and arsenic) on its germination.

Minuț et al. (2022) reported that *Pseudomonas* sp. treatment increased the root and shoot lengths of flax by 178.38 % and 15.08 %, respectively, compared to the control treatment. In the current study, it was determined that *Pseudomonas libanensis* FLX-2, *Pseudomonas boreopolis* FLX-3 and *Pseudomonas chlororaphis* FLX-5 treatments increased root lengths by 114 %, 117 % and 193 %, respectively, compared to the control treatment. In addition, in the current study, it was determined that the maximum stem length was 4.91 cm after treatment with in the FLX-5 treatment, while the minimum stem length was 2.10 cm in the control group. This can be attributed to the ability of *Pseudomonas chlororaphis* FLX-5 to produce IAA and solubilize phosphorus. This result is consistent with Rizvi and Khan (2017), who investigated the effect of Cu stress on wheat germination by PGPR *Pseudomonas aeruginosa* strain CPSB1, which has the ability to produce IAA and solubilize phosphorus.

Evaluation of root and stem weights

In the evaluation of root fresh weight according to the bacteria-Cu treatments, the maximum root fresh weight was obtained from the FLX-5 bacteria treatment with 18.2 mg, while the minimum root fresh weight was obtained from the control treatment with 12.8 mg in the present study. According to the average values for copper doses, the maximum root dry weight was 18.9 mg in the control group (0 mM), followed in descending order by 5 mM (16.65 mg), 10 mM (15.05 mg), 15 mM (12.78 mg), and 20 mM (11.36 mg) concentrations (Table 3).

Many researchers have shown that PGPR treatments enhance the number of leaves, root and shoot length, fresh and dry weight, proline, and chlorophyll content. For example, in the study conducted by Yıldırım et al. (2015), it was reported that PGPR treatment in cucumber significantly increased stem diameter, seedling height, shoot and root weight compared to the control. Idder et al. (2019) stated that inoculating *Vicia faba* plant with *Pseudomonas* P1, P7 and P15 strains under copper stress significantly increased the root-stem fresh weight of the plants compared to uninoculated plants. Similarly, Madline et al. (2021) determined that the inoculation of growth-promoting rhizobacteria (*Mesorhizobium tamadayense*, *Enterobacter xiangfangensis*, *Pseudomonas azotifigens*, and *Streptomyces caelestis*) isolated from copper mining soils increases the root and shoot development of *Peganum harmala* and *Lactuca sativa* seeds.

There are many studies indicating that *Pseudomonas* sp. and *Bacillus* sp. bacteria, which are abundant in the soil, increase product yield by promoting plant growth. For example, In a similar study, Abbaszadeh-Dahaji et al. (2021) reported that inoculating corn seeds with *Pseudomonas fluorescens* P22 and *Pseudomonas* sp. Z6, isolated from copper-contaminated soils, increased shoot dry weight by 12 % and 16 %, respectively, and shoot length by 20 % and 22 %, compared to the control. According to Omer et al. (2022), they reported that the treatment of *Pseudomonas geniculata*, which supports plant growth, to flax seeds increased root and shoot lengths and fresh and dry weights in flax compared to the control group. In the current study, it was determined that *Pseudomonas libanensis* FLX-2 strain treatment provided an 11 % increase in root fresh weight compared to the control treatment, but had no effect on stem fresh weight (Figure 6A). On the other hand, it was determined that *Pseudomonas chlororaphis* FLX-5 strain treatment increased the root and shoot fresh weights by 42 % and 23 %, respectively, compared to the control. Figure 6 shows the fresh weight of the roots and stems of flaxseed under different PGPR and copper treatments.

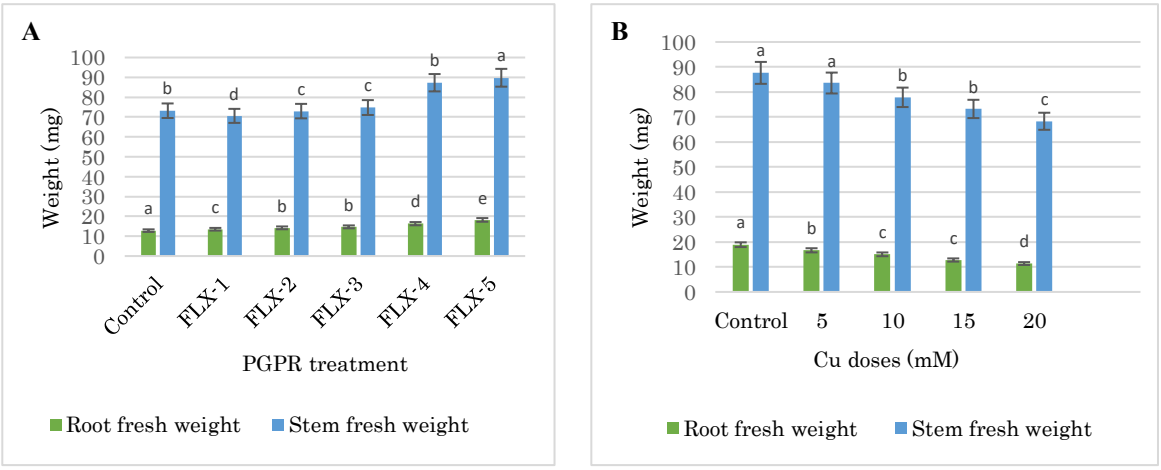

Figure 6. Effect of PGPR (A) and Cu (B) treatments on root and shoot fresh weight of flax seedlings

In the evaluation of root dry weight according to the bacteria-Cu treatments, the maximum root dry weight was determined in FLX-5 bacteria with 1.24 mg, and the minimum root dry weight was determined in FLX-1 bacteria with 1.05 mg in the current study. According to the average copper doses, the maximum root dry weight was determined as 1.50 mg in the control (0 mM) treatment, followed by 5 mM (1.42 mg), 10 mM (1.26 mg), 15 mM (1.0 mg) and 20 mM (0.77 mg) concentrations in decreasing order. In the evaluation of stem dry weight according to bacterial treatments, the maximum stem dry weight was determined as 10.4 mg in FLX-5 bacteria, and the minimum body dry weight was determined as 10.08 mg in FLX-2 bacteria. In addition, according to copper concentrations, the maximum stem dry weight was determined as 12.19 mg in the control (0 mM) treatment, followed by 5 mM (11.72 mg), 10 mM (10.93 mg), 15 mM (9.12 mg) and 20 mM (6.90 mg) concentrations in decreasing order (Table 3). According to our results, the isolated *Pseudomonas chlororaphis* FLX-5 not only promotes plant growth but also exhibits resistance to different copper concentrations, thereby exerting a positive effect on flax seed germination. Furthermore, *Pseudomonas chlororaphis* FLX-5 may possess significant potential for the remediation of areas contaminated with copper heavy metal.

Numerous studies have shown that inoculating plants with PGPR causes significant changes in various growth parameters, such as plant height, root and stem dry weight (Mazhar et al., 2020; Liu et al., 2022). Moreover, It has been reported that strains belonging to the Bacillus genus, which are abundant in the plant root, increase root development by producing nitrogen fixation and IAA, and thus play a role in plant development (Myresiotis et al., 2014). In a study conducted by Zainab et al. (2020), it was reported that the treatment of *Bacillus gibsonii* (PM11) and *Bacillus xiamenensis* (PM14), which have the ability to produce IAA and ACC-deaminase, to flaxseed significantly increased the root and shoot length, fresh and dry weight, proline and chlorophyll content of the plant. Similarly, Ke et al. (2021) reported that inoculating the grass plant with *Bacillus thuringiensis* and *Bacillus cereus* isolates obtained from Cu and Cd contaminated mines increased the stem and root biomass by 11.49 % and 44.50 %, respectively. Our current study revealed that treatment of *Bacillus thuringiensis* FLX-1 and *Bacillus mojavensis* FLX-4 strains increased stem length by 55 % and 88 %, respectively, compared to the control group. When evaluating fresh root and fresh stem weights, it was determined that the *Bacillus thuringiensis* FLX-1 strain treatment resulted in a 15 % increase in fresh root weight compared to the control group, while it had no positive effect on fresh stem weight (Figure 7A). On the other hand, the *Bacillus mojavensis* FLX-4 strain treatment was observed to provide increases of 27 % and 19 % in fresh root and fresh stem weights, respectively, compared to the control group. Figure 7 presents data on root and stem dry weight of flaxseed in different PGPR and Cu treatments. Table 3 shows the effects of PGPR and Cu treatments on parameters such as root-shoot length, root fresh-dry weight and shoot fresh-dry weight.

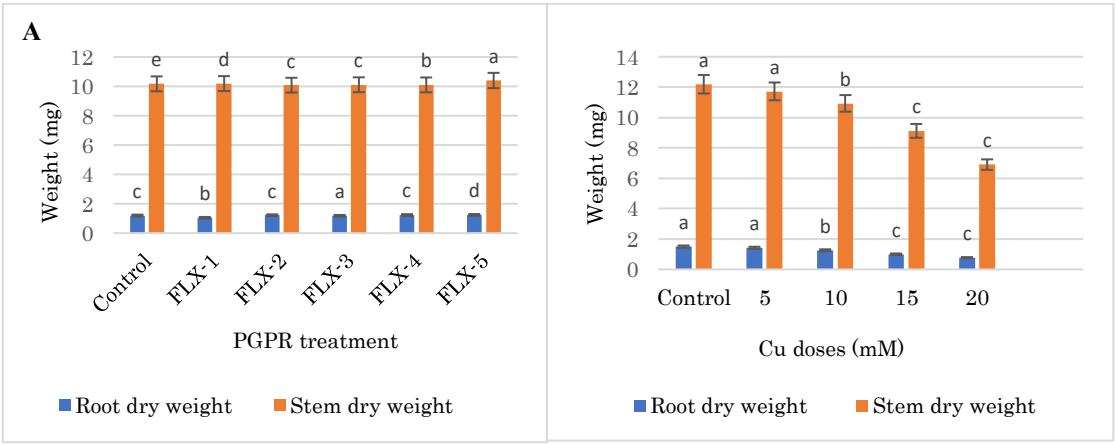

Figure 7. Effect of PGPR (A) and Cu (B) treatments on root and stem dry weight of flax seedlings

Table 3. Effects of PGPR and Cu treatments on root-shoot length, root fresh-dry weight and shoot fresh-dry weight of flax seedlings.

Bacterial isolate code	**Cu (mM)**	**Root length** ** **(cm)** (X±Sx)	**Stem length (cm)** (X±Sx)	**Fresh Weight (mg)** (X±Sx)		**Dry Weight (mg)** (X±Sx)	
				Root	**Stem**	**Root**	**Stem**
Control	**0**	3.20±0.11^{a}	2.90±0.11^{b}	18.5±0.45^{a}	86.3±2.55^{a}	1.74±0.05^{a}	12.64±0.02^{a}
	5	2.44±0.10^{b}	3.70±0.12^{a}	16.0±0.35^{b}	84.8±0.95^{a}	1.59±0.03^{a}	12.21±0.01^{a}
	10	2.08±0.10^{b}	1.78±0.07^{c}	13.5±0.80^{c}	69.2±0.75^{b}	1.32±0.02^{b}	9.8±0.32^{b}
	15	0.76±0.09^{c}	1.96±0.09^{c}	8.5±0.25^{c}	68.5±1.40^{b}	0.76±0.04^{c}	8.06±0.03^{c}
	20	0.29±0.07^{d}	0.2±0.12^{d}	7.5±0.20^{d}	57.2±0.92^{c}	0.67±0.02^{c}	8.14±0.01^{c}
	Mean	**1.75^{e}**	**2.10^{e}**	**12.8^{a}**	**73.2^{b}**	**1.20^{c}**	**10.17^{e}**
FLX-1*	**0**	5.43±0.24^{a}	6.02±0.08^{a}	19.5±0.56^{a}	88.6±0.70^{a}	1.36±0.02^{a}	12.67±0.04^{a}
	5	4.66±0.20^{a}	4.23±0.07^{b}	16.4±0.15^{b}	80.1±0.62^{b}	1.28±0.03^{ab}	11.29±0.03^{b}
	10	3.78±0.17^{b}	5.13±0.12^{c}	13.7±0.30^{c}	74.7±0.72^{c}	1.16±0.02^{b}	10.55±0.02^{c}
	15	1.12±0.16^{c}	0.61±0.12^{d}	9.5±0.35^{d}	57.3±1.48^{d}	0.77±0.02^{c}	9.81±0.04^{d}
	20	0.46±0.09^{c}	0.32±0.02^{d}	8.4±0.15^{d}	52.3±1.26^{e}	0.72±0.07^{c}	6.67±0.03^{e}
	Mean	**3.09^{d}**	**3.26^{cd}**	**13.5^{c}**	**70.6^{d}**	**1.05^{b}**	**10.19^{d}**
FLX-2	**0**	8.21±0.12^{a}	5.54 ±0.14^{a}	17.3±0.15^{a}	85.5±1.65^{a}	1.60±0.04^{a}	11.98±0.06^{a}
	5	6.34±0.09^{b}	4.87±0.38^{a}	15.2±0.30^{b}	79.3±1.47^{ab}	1.45±0.01^{b}	12.23±0.02^{b}
	10	3.0±0.41^{c}	3.14±0.14^{b}	14.6±0.20^{b}	71.5±2.63^{bc}	1.39±0.03^{b}	10.58±0.05^{c}
	15	0.75±0.09^{d}	0.32±0.10^{c}	14.3±0.20^{b}	68.6±0.72^{c}	1.14±0.03^{c}	8.73±0.01^{d}
	20	0.47±0.06^{d}	0.27±0.04^{c}	9.6±0.20^{c}	60.1±0.56^{d}	0.56±0.02^{d}	6.88±0.01^{e}
	Mean	**3.75^{c}**	**2.82^{d}**	**14.2^{b}**	**73.0^{c}**	**1.22^{c}**	**10.08^{c}**
FLX-3	**0**	6.31±0.08^{a}	7.45±0.67^{a}	18.7±0.36^{a}	82.4±0.4^{a}	1.47±0.02^{a}	12.06±0.29^{a}
	5	7.54±1.58^{a}	5.88±0.12^{b}	16.4±0.25^{b}	76.5±0.68^{b}	1.31±0.04^{ab}	11.67±0.09^{a}
	10	4.40±0.28^{a}	3.43±0.06^{c}	13.7 ±0.30^{c}	74.4±0.41^{bc}	1.19±0.03^{bc}	11.45±0.02^{a}
	15	0.61±0.07^{b}	0.56±0.02^{d}	12.8±0.15^{cd}	71.5±1.08^{cd}	1.10±0.04^{c}	9.48±0.03^{b}
	20	0.37±0.07^{b}	0.30±0.11^{d}	11.9±0.26^{d}	69.2± 0.46^{d}	0.83±0.02^{d}	5.85±0.04^{c}
	Mean	**3.80^{c}**	**3.52^{bc}**	**14.7^{b}**	**74.8^{c}**	**1.18^{a}**	**10.11^{c}**
FLX-4	**0**	9.43±0.19^{a}	8.9±0.55^{a}	19.6±0.45^{a}	89.5±0.92^{a}	1.33±0.02^{a}	11.39±0.02^{a}
	5	7.56±0.07^{b}	5.8±0.34^{b}	17.3±0.55^{b}	88.7±0.72^{ab}	1.54±0.02^{b}	11.27±0.03^{b}
	10	4.67±0.08^{c}	4.19±0.11^{c}	16.9±0.26^{b}	87.0±0.25^{abc}	1.28±0.03^{b}	12.41±0.03^{b}
	15	0.9±0.30^{d}	0.62±0.18^{d}	14.1±0.35^{c}	86.1±0.58^{bc}	1.03±0.02^{c}	9.02±0.03^{c}
	20	0.38±0.03^{d}	0.33±0.10^{d}	13.6±0.26^{c}	85.2±0.41^{c}	0.97±0.06^{c}	6.45±0.04^{d}
	Mean	**4.58^{b}**	**3.96^{b}**	**16.3^{d}**	**87.3^{b}**	**1.23^{c}**	**10.10^{b}**
FLX-5	**0**	11.4±0.28^{a}	10.7±0.25^{a}	19.8±0.55^{a}	93.5± 0.37^{a}	1.55±0.01^{a}	12.45±0.01^{a}
	5	8.6±0.92^{b}	9.1±1.05^{a}	18.6±0.30^{ab}	92.1± 0.25^{a}	1.36±0.02^{b}	11.68±0.05^{b}
	10	5.3±0.26^{c}	4.2±0.20^{b}	17.9±0.35^{b}	90.4± 0.52^{b}	1.22±0.02^{c}	10.82±0.04^{c}
	15	0.28±0.08^{d}	0.35±0.06^{c}	17.5±0.35^{b}	87.3± 0.20^{c}	1.20±0.01^{c}	9.65±0.01^{d}
	20	0.14±0.02^{d}	0.21±0.03^{c}	17.2±0.20^{b}	85.7± 0.32^{c}	0.88±0.03^{d}	7.43±0.01^{e}
	Mean	**5.14^{a}**	**4.91^{a}**	**18.2^{e}**	**89.8^{a}**	**1.24^{d}**	**10.4^{a}**

*FLX-1: *Bacillus thuringiensis*, FLX-2: *Pseudomonas libanensis*, FLX-3: *Pseudomonas boreopolis*, FLX-4: *Bacillus mojavensis*, FLX-5: *Pseudomonas chlororaphis*. Each value represents the mean of at three replications **Values sharing the same letter within a column are not statistically different at the 0.05 level of confidence.

CONCLUSION

Heavy metal accumulation in soil due to industrial and agricultural waste is increasing, affecting soil fertility and food safety. Excess copper in soil harms plant growth. Plant growth-promoting rhizobacteria (PGPR) are used to reduce heavy metal toxicity by altering metal pathways and converting them into harmless forms. This study found that PGPR strains positively affect flax seed germination and physiological traits, and *Pseudomonas chlororaphis* FLX-5 treatment effectively reduced copper's negative effects.

Compliance with Ethical Standards
Peer-review
The authors declare that there is no ethical issue
Declaration of Interests
The authors declare that there is no conflict of interest
Author contribution
The authors declare that they have contributed equally to the article

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