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Mitigation of Cu with Copper tolerant plant growth promoting rhizobacteria in marigold (*Tagetes erecta*) plant growth

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ABSTRACT

Heavy metal poisoning is mostly caused by the emission of hazardous pollutants through various natural and anthropogenic activities. Copper (Cu) is an essential micronutrient for plants; it is required in relatively small amounts for proper growth and development. However, like many elements, copper also has negative effects on plants at its high concentration. Soil testing and proper agricultural practices can help prevent copper toxicity issues. In the present research, a PPL bioinoculant was prepared from three previously isolated and identified Cu-tolerant *Providentia, Paenibacillus*, and *Lysinibacillus* PGPR (plant growth promoting rhizobacteria) strains. *In vitro* testing of prepared PPL bioinoculant was done for its plant promotion activity and its effect on Cu accumulation by marigold (*Tagetus erecta*) plant using pot assay. Activities promoting plant growth, such as production of siderophores, indole acetic acid, hydrogen cyanide (HCN), and phosphate solubilization were measured *in vitro*. PPL bioinoculant influenced the growth of marigold plant both directly and indirectly. The introduction of a PPL bioinoculant under Cu stress led to a decrease in Cu phytotoxicity, while the root length, shootlength, fresh weight and dry weight of the marigold plant increased in comparison to the uninoculated control. In addition, compared to uninoculated control plants, the total Cu accumulation was also increased in marigold plant inoculated with PPL bioinoculant. According to our findings, metal tolerant PGPR are a very promising resource that could provide great bio-inoculants to increase the effectiveness of phytoremediation.

Key words: *Bioinoculant, Heavy metal, Metal tolerant PGPR, Pollutant*

Introduction

Industrial metal pollution poses a significant threat to human health, ecosystems, and the environment. Major sources of metal pollution include waste disposal, manufacturing, metal processing, and mining. Heavy metals like lead, mercury, arsenic, cadmium, chromium, copper, zinc, nickel, and aluminum are commonly discharged during industrial processes. Atmospheric deposition can result from industrial emissions emitting gases and metal particles into the atmosphere. Metals from industrial sites can contaminate surrounding water bodies by seeping into groundwater and surface water (Ritter *et al*., 2002). Soil contamination can harm ecosystem species and interfere with plant growth. Metal contamination

([1]Assistant Professor, [2]Research Guide) affects plant and animal life in both terrestrial and aquatic habitats, disrupting food networks and causing a decline in biodiversity. Government and environmental organizations establish standards and regulations to regulate and track metal emissions from industrial operations. Regulations pertaining to waste management, emission limitations, and effluent requirements aim to reduce the environmental impact of industrial operations (Ashrafi *et al.*, 2015). Adopting cleaner technologies, stringent regulatory measures, and sustainable waste management practices are essential components of a comprehensive strategy to address metal pollution from industrial sites. Phytoremediation, a technique that uses plants to eliminate, stabilize, or lower contaminants in soil, water, or the atmosphere, is an economical and environmentally friendly approach. Certain plants, known as hyperaccumulators, can absorb and accumulate high quantities of metals in their tissues from contaminated soil. Phytoremediation can be effective when combined with an all-encompassing site remediation plan (Devendrapandi *et al.*, 2024). Plant growth promoting rhizobacteria (PGPR) can enhance stress tolerance, facilitate nutrient uptake, and protect plants from diseases. PGPR strains can solubilize metal compounds insoluble in the soil, increasing their availability for uptake by plants. The rhizosphere's microbial community is influenced by PGPR, fostering plant growth and metal remediation. Research on using PGPR in metal phytoremediation is still in its preliminary stages, and the efficiency of specific bacteria may vary depending on the type of plant, metal pollutants, and the site's characteristics. Incorporating PGPR-assisted phytoremediation into an all-encompassing remediation plan could improve the efficiency and sustainability of metal-contaminated site restoration.

The present investigation reveals that metal tolerant rhizobacterial bioinoculants moderately remove Cu from the soil. Under the testing conditions, the marigold plants which were supplemented with Cu tolerant PGPR exhibited better Cu removal efficiency compared to the uninoculated plants from soil.

Materials and Methods

Copper tolerance level of PGPR

Three Cu tolerant PGPR strains viz. *Providentia*, *Paenibacillus*, and *Lysinibacillus* spp. were tested for their highest Cu tolerance limit by grid plate method. PGPR strains were spot inoculated on Lauria Bertani (LB) agar plates supplemented with Cu in the range of 200 ppm to 1400 ppm with difference of 200 ppm. Cu was used in the form of copper sulphate ($CuSO_4$, $5H_2O$: AR grade) under this study.

Plant growth promotion activity

Plant growth promoting activities of three Cu tolerant PGPR spp. were tested for IAA production by Salkowsky's reagent (Gorden and Weber, 1951), phosphate solubilization using Pikovskya's agar plate (Gaur, 1990), siderophore production by CAS method (Schwan and Neilands, 1987), production of ammonia by Nesseler reagent (Cappuccino and Sherman, 1992) and production of HCN by adapting method of Lorck (Lorck, 1948).

Preparation of PPL bioinoculant (carried based consortium)

The carrier-based consortium was prepared using a consortium of selected three Cu metal-tolerant PGPR strains. The 5ml culture suspension of each PGPR ($8*10^8$ cells /ml) was mixed in sterile 50 g of carrier i.e., compost, charcoal and soil (1:1:2) aseptically. The prepared carrier based consortium was placed in a polythene bag and kept at room temperature (RT) till its further use in pot experiment. It was labeled as PPL bioinoculant.

Copper treatment on marigold plant

On the basis of metal tolerance, two different concentrations of copper (200 mg/kg of soil and 400 mg/kg of soil) were selected for further pot assay experiment. The soil samples were treated with two different concentrations of heavy metal (Cu) and PPL bioinoculant. The negative control (NC), i.e. soil without metal and PPL bioinoculant and metal control (MC), i.e. soil with metal but without PPL bioinoculant were also maintained. The test and controls used were as given below (Table 1).

Five seeds of Marigold (*Tagetes erecta*) were sown in plastic pots (pot diameter 17.5 cm, bottom diameter 11.5 cm, height 13.5 cm) filled with approximately 1 kg of respective soil mixture. The pots were irrigated with 100 ml distilled water every second day. At the end of three months plants were harvested for further study.

Table 1. Details of test and control in marigold plant

Treatment code	Details	Details of treatment
NC	Negative control	Soil
MC 1	Metal control 1	Soil + Cu: 200mg/kg^{-1}
T 1	Test 1	Soil + Cu: 200mg/kg^{-1} + PPL bioinoculant
MC 2	Metal control 2	Soil + Cu: 400mg/kg^{-1}
T 2	Test 2	Soil + Cu: 400mg/kg^{-1} + PPL bioinoculant

Plant growth parameters

At the conclusion of the experiment, marigold plants were meticulously removed from the pots. Deionized water was used to clean the plant roots in order to eliminate any debris from the root surface. Different parameters like plant shoot length, root length; fresh weight and dry weight were measured. To determine the dry biomass, all plant parts were dried at 70 °C until a consistent weight was reached.

Analysisof Cu

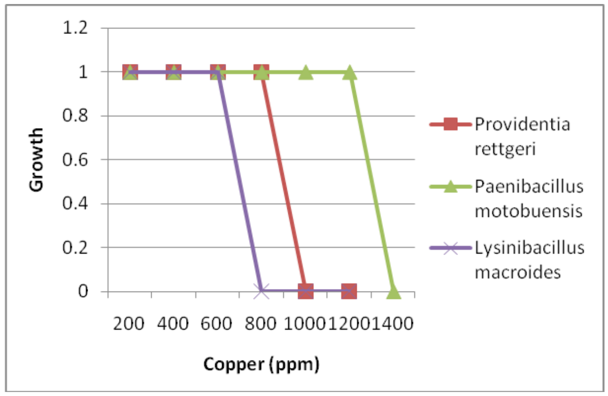
Soil and dried plant shoot sample were homogenized with mortars and pestles separately. A final sample of 0.5 g was digested by adding 10 ml HNO3 -HCl mixture (3:1, v/v) in a microwave digestion unit at 200 °C and 90-bar pressure for 30 min. After the digestion, the sample was diluted to 50 ml with 1 % HCl and directly analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES).The bioaccumulation factor (BF) was also calculated as follows to evaluate the metal accumulation in plants.

$BF = M_{shoot} / M_{soil}$

Where M_{shoot} is the metal accumulation in plant shoots (mg kg^{-1}) and M_{soil} is the metal remained in soil (mg kg^{-1}). The copper analysis was carried out in Hi tech laboratory, Sangali, Maharashtra.

Results and Discussion

Copper tolerance limit of selected PGPR spp. were determined and Cu tolerance ability of selected PGPR spp. under study was as illustrated in Figure 1. The highest tolerance to Cu was exhibited by *Paenibacillus motobuensis* (1200 ppm) strain which was followed by *Providentiarettgeri* (800 ppm) and *Lysinibacillus macrolides* (600 ppm). The multiple metal tolerance including Cu and high efficiency of degradation by *Providentiarettgeri AVRB20* was reported by Anjanpriya *et al.*, 2022 and it was concluded that *Providentia* spp. are suitable for bioremediation because of its Cu tolerance property.



(1 is depicted as growth and 0 as no growth)
Fig. 1. Copper tolerance level of PGPR spp. under study

Govarthanan *et al.*, 2016 also reported that *Paenibacillus* spp. can tolerate 750 mg/l of Cu because of significant PGP traits. Copper tolerant *Lysinibacillus* spp. isolated from metal contaminated site was also reported by Jinal *et al.* in 2021.

Plant growth promotion activity

Three Cu tolerant PGPR strains were tested for their plant growth promotion activites. IAA and siderophore production ability of each strain was quantitatively measured. The results were as depicted in Table 2 and Figure 2 (results are presented in the form of mean ± Standard Error Mean (n = 3)).

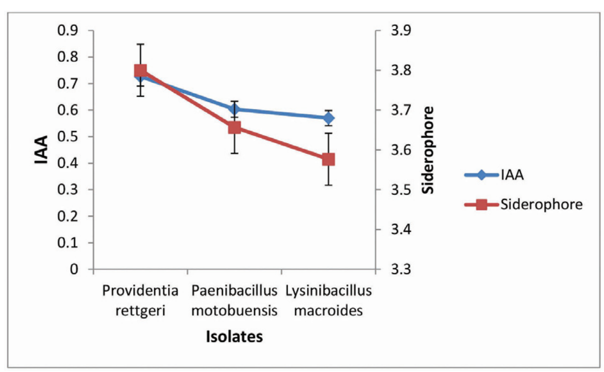


Fig. 2. IAA and siderophore production by Cu tolerant PGPR strains.

All three PGPR strains exhibited IAA, Siderophore ammonia production and phosphate solubilization ability while HCN production was exhibited by only *Providentia* and *Lysinibacillus* spp. Cavalcante da Silva *et al.* in 2020 also reported higher concentration of IAA production by *Providentia spp.* According to Manoj *et al*. (2020), plant growth promoting bacteria can enhance root elongation and cell proliferation through the production of IAA, which increases the root surface area available for uptaking of water and nutrients.

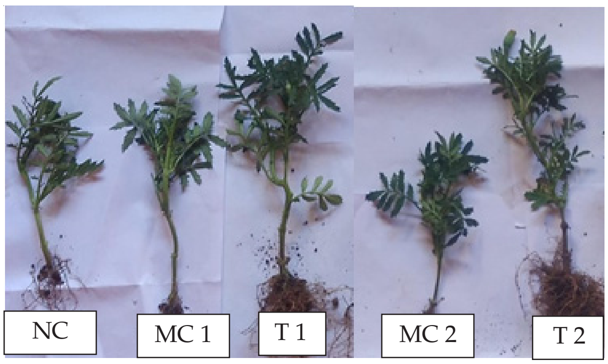


Fig. 3. Growth of marigold plant after treatment with Cu and PPL bioinoculants

Effect of PPL bioinoculant on growth and metal accumulation in marigold plant

Effect of Cu on overall growth of marigold plant was evaluated by pot assay. Plant growth parameters and Cu accumulation level in plant was assessed. Figure 4 (A) and (B) illustrate plant shoot and root length and weight after treatment while Figure 4 (C) displays the Cu accumulation by plants compared to control. Plants shoot length was same after exposure to 200 mg/kg^{-1} of Cu while it decreased by 25% on exposure to Cu at 400 mg/kg^{-1}. Similarly plant root length decreased by 20% and 15% under Cu stress at 200 mg/kg^{-1} and 400 mg/kg^{-1} respectively. Whereas after inoculation of plants with PPL bioinoculant in presence of Cu there was significant increase in plant shoot and root length. Total increase in shoot length was more in T 2 pot (41%) while 33% in T 1 pot. Similarly as depicted in Fig 4A root length was increased more in T 2 pot (59%) than T 1 pot (23%).

After harvest fresh weight of plant was more in T 1 pot (4.8 g) than T 2 pot (0.8 g).The dry weight of plant was also more in T 1 pot (1.8 g) followed by T 2 pot (1.5 g). There was significant increase in fresh and dry weight of plant inoculated with PPL bioinoculant (T 1 pot) (Fig.4 B).

BF is a measure of how well a plant accumulates metal in its shoots. In general, the sequence for enhanced peaks for bioaccumulation pattern was as illustrated in Figure 4C. Cu was accumulated more in T 1(0.81) followed by T 2 (0.66). Whereas BF in absence of PPL bioinoculant least in both metal control. Ju W *et al.* in 2020 also demonstrated that by improving soil quality and plant tolerance to Cu, rhizobacterial inoculation promoted Cu phytoextraction during EDDS-enhanced phytoremediation. Mani *et al* in 2016 reported that co-inoculation of *P. putida* and *T. thiooxidans* had a significant effect on plant biomass and concluded that integrated vermicompost, elemental sulphur and microbial co-inoculation were responsible for enhanced clean-up of Cd and Pb-contaminated soils.

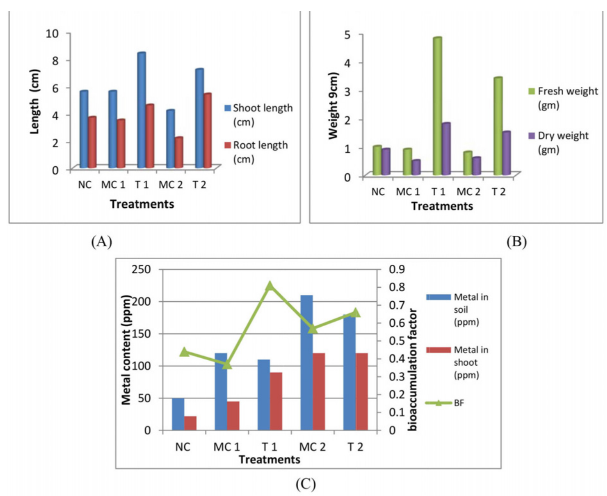


Fig. 4. Plant growth parameters and metal accumulation (A) Shoot and Root length (B) Fresh and dry weight (C) Bioaccumulation factor

Table 2. Plant growth promoting activities by copper tolerant PGPR strains.

	IAA (mcg/ml)	Siderophore	Phosphate solubilization	Ammonia production	HCN production
Providentia rettgeri	0.72±0.04	3.8±0.26	+	+	+
Paenibacillus motobuensis	0.60±0.08	3.65±0.11	+	+	-
Lysinibacillus macroides	0.57±0.04	3.57±0.21	+	+	+

Three strains (EhS7, RA1, and RA2) isolated from soil contaminated with heavy metals discovered to display several PGP activities and multimetal resistance. Additionally, these strains were encouraging the phyto stabilization of Cu and Cd-polluted soils as reposted in Ke *et al.*, 2021.

Conclusion

The growth and biomass of marigold plant reduced in artificially metal contaminated soil. Nevertheless, the introduction of Cu metal tolerant PGPR bioinoculant (*Providentia, Paenibacillus,* and *Lysinibacillus spp.*) with ability to produce IAA and siderophore, reduced the negative impacts of Cu on marigold plant and improved plant growth as well as biomass. There was a correlation between the rise in plant growth, biomass and reduction in metal stress along with higher accumulation of heavy metals by marigold plants in presence of PGPR bioinoculant. Thus heavy metal tolerant PGPR strains can be a great choice for bacterially assisted phytoremediation, which is an enhanced restoration method for contaminated soils.

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Conflict of Interest- None

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