MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Pseudomonas putida Endophytic Bacterium Isolated from *Typha domingensis* as Ecofriendly Tool to Remove Heavy Metal from Environment

***Mohammed S. Sultan*[1]*, El-Sayed F. El-Halawany*[1]*, Ashraf Elsayed*[1]*, Yasser A. El-Amier*[1]**

[1]*Botany Department, Faculty of Science, Mansoura University, Mansoura - 35516, Egypt*

*** Correspondence to:** *mohammedsoltan@gmail.com; Tel. +201118068857*

Received:16/2/2024
Accepted: 24/4/2024

Abstract: Heavy metal contamination has posed a serious threat to environmental stability because of its extreme toxicity and lack of biodegradability. Microbial remediation shown the most efficient and ecofriendly solution. The current study focuses on examination of *Pseudomonas putida* for its ability to tolerate heavy metals and its growth promoting activities. *Pseudomonas putida* was characterized according to morphology, biochemical, removal efficiency, and growth promoting. It was identified as *Pseudomonas putida* (PP077069). It showed a potential for the remediation of metal-containing wastewater with capacity to reduce the ecotoxicity of the effluent. It was Fe-, Cr-, and Cu-tolerant with MIC values (5, 7, and 3.5 mgl^{-1}). The highest removal efficiency was observed after five days (11.54, 18.62, and 10.04%), with metal uptake capacities (0.58, 1.3, and 0.35 mgl^{-1}), respectively. It is concluded that seed priming can be regarded as a promising approach for increasing the resistance of wheat seedlings. *Pseudomonas putida* had abilities to phosphate solubilization, siderophore, HCN production and IAA synthesis. Priming with *P. putida* had a significant stimulatory effect on the biochemical and physiological performance of wheat seedlings compared to unprimed seeds.

keywords: Bioremediation, Typha domingensis, HM-PGPB, Endophytes, bio-priming, Pseudomonas sp..

1.Introduction

The environment, ecosystems, and living beings are all at risk from heavy metal contamination. Water is an essential component of life and is becoming increasingly contaminated at an alarming rate by various human-caused activities because of rapid urbanization, industrialization, and population growth. Heavy metals have an adverse effect on land use, food quality, drinking water, and the food chain; putting human health and the ecosystem at risk. Heavy metals can disrupt lethal enzymatic activity, function as redox catalysts in the creation of reactive oxygen species, directly interfere with DNA and protein synthesis. Because heavy metal poisoning severely impairs plants' basic physiological functions, plants are among the creatures most affected. Traditional remediation techniques have numerous drawbacks, including high implementation costs, the production of mutagenic substances, and the use of harmful chemicals that are less environmentally friendly[2 ،1] .

Microbes are the basis of current bioremediation techniques because of their great potential, low cost, and environmental friendliness, especially at low metal concentrations. Bacteria develop resistance to heavy metals because of several interactions and the microbial communities usually exhibit changes in their morphology and physiology. Heavy metal tolerant plants have been shown to interact with endophytic bacteria that enhance their growth and adaptation conditions according to their abilities to produce indole acetic acid, gibberellins, siderophore, phosphate solubilization and other plant growth promoting enzymes [3].

Typha is a genus is one of the most important plants in phytoremediation. *Typha domingensis* has been used in phytoremediation due to their fast growth, large biomass, and ability to accumulate heavy metals mainly in their roots. The first study with Typha species was carried out by Aguilar and Cabriales [4], who isolated eight bacteria strains of the Proteobacteria including *Pseudomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., and *Ochrobactrum* sp. that colonize the roots. Endophytic bacteria belong to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes including *Bacillus* sp., *Microbacterium arborescens*, *Microbacterium* sp., *Rhizobium* sp., *Pantoea* sp., and *P. fluorescens* were isolated from the shoots and roots of *T. domingensis*.

Previous studies indicated that *Pseudomonas* genus can tolerate heavy metals according to their abilities to adapt to various environmental conditions. *Pseudomonas* genus comprises 66.7% of the total isolated endophytic bacteria. Previous studies showed that *Pseudomonas* species are part of endophytic communities in heavy metal hyper accumulator plants. The *Pseudomonas* genus, had capacity to adapt to multiple soil conditions, including heavy metals. Likewise, endophytic *P. azotoformans, P. fluorescens, P. gessardii, P. veronii* were isolated from the endosphere of *T. angustifolia*, *T. domingensis*, and *Typha* sp.. Shehzad and Fatima [5] isolated endophytic bacteria belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes from *T. domingensis* grown in wetlands to treat textile effluents contaminated with Ni, Fe, Cr, and Cd. They also had ability to promote the growth and adaptation of *T. domingensis* to the wetland conditions. Thus, the consortium has the biotechnological potential to be applied for plant-bacteria remediation purposes.

To date, no other *T. domingensis* heavy metal tolerant bacterial strains have been identified to investigate their heavy metal tolerance. Regarding the ability of *P. putida* to tolerate heavy metals, many references is available, which facilitates comparisons and the investigation of novel microbial strains. The current research aims to characterize *P. putida* according to its tolerance to heavy metals (Fe, Al, and Cu) as well as its PGPB activities.

2. Materials and Methods

2.1. Isolation of Endophytic Bacteria

Pseudomonas putida was isolated from roots of *T. domingensis* plant collected from Faraskour drain, Damietta, Egypt using Luria Bertani (LB) agar medium (1.25 g yeast extract, 2.5 g peptone, 2.5 g sodium chloride, 3.75 g agar, and 250 ml distilled water) according to the procedure by Liu and Xu [6].

2.2. Determination of minimum inhibitory concentration

To assess MIC, purified bacterial strains were grown on LB media incorporated with different heavy metal concentrations (mgl-1) of Iron (Fe) in the form of iron sulfate ($FeSO_4.7H_2O$), Aluminum (Al) in the form of Aluminum sulfate ($Al_2(SO_4)_3.16H_2O$), and copper (Cu) in the form of copper sulfate ($CuSO_4.5H_2O$). The petri plates were incubated at 37 °C for 24 to 72 hrs [7].

2.3. Morphological and biochemical characterization

Pseudomonas putida was characterized by colony morphology, cell microscopic, and biochemical tests as catalase, oxidase, nitrate reduction, urease, hydrogen peroxide, phosphate solubilization activity, siderophore production, hydrogen sulphate, indole, gibberellins production, and hydrogen cyanide according to[8] .

2.4. Phylogenetic analysis

Pseudomonas putida was molecularly identified using the MicroSeq® 500 16SrRNA Bacterial Identification Kits methodology [9]. The sequence obtained in this investigation were submitted to the National Center for Biotechnology Information Gene Bank database (https://blast.ncbi.nlm.nih.gov/) and compared with the standard sequences available in the database. Finch TV (version1.4.0) and MEGA-X (version10.2.5) software were used to analyze the sequences, and Seaview software was used to create phylogenetic trees using the closest published type of strain sequences .

2.5. Germination Assay

Wheat seeds that were uniform in appearance sorted, surface sterilized for five minutes with one percent sodium hypochlorite, and then rinsed three times with deionized water. The seeds for the seed priming experiment treated with *P. putida* for two hours with shaking at 180 rpm, whereas the seeds for the control experiment repeatedly rinsed in distilled water under standard laboratory conditions. For the germination investigation, treated seeds put in a petri dish with three layers of cotton and incubated at 28 ºC for 14 days. Each petri plate included 20 treated seeds. Three repetitions of a completely randomized block design used for the experiment. Finally, the seedlings were recovered to determine the morphological characteristics, shoot, and root length after the 14 days of germination. The following equations used to calculate the main daily germination (MDG), germination value, seed vigor index (SVI), allometric coefficient (AC), and percentage of germination (GP) [10].

$$GP\,\% = (no.of\ germinated\ seeds)/(\text{no. of used seeds}) \times 100$$

$$SVI\,\% = (Seedling\ lenght)/2 \times GP\,\%$$

$$AC = (Root\ lenght)/(\text{Shoot lenght})$$

$$MDG = \frac{GP\,\%}{\text{no. of days to final germination}}$$

$$GV = \text{GP}\% \times \text{MDG}$$

2.6. Statistics

To evaluate the parameters affecting seed germination and wheat growth, the experiments were conducted in three biological duplicates with 20 seeds or seedlings each. The information shown as the mean and standard error (SEM). Statistically significant differences between the mean values assessed using CoStat's one-way ANOVA and an LSD test with a 0.05 probability level (version 6.311, CoHort Software, USA, www.cohort.com).

3. Results

3.1. Isolation and characterization

Pseudomonas putida (PP077069) a heavy metal resistant endophytic bacteria that obtained from the roots of *T. domingensis* plant which collected from contaminated environment with higher amounts of heavy metals than the permissible limits set by the WHO using standard methods. It was characterized according to morphology, microscopic, biochemical, and 16S rRNA gene sequence as shown in Figure (1). *Pseudomonas putida* had a circular shape, entire margin, convex elevation, smooth creamy texture, whitish pigmentation, and gram-negative bacteria (Figure 1c).

Likewise, it showed a positive ability to produce oxidase, catalase, lipase, cellulase, amylase, indole acetic acid, gibberellic acid, nitrate reductase, and phosphate solubilization, siderophore and hydrogen cyanide. Likewise, it showed a negative result to produce urease and hydrogen sulphate. According to the qualitative analysis the selected endophytic bacteria could improve growth due to its ability to produce IAA and other plant-growth promoting hormones (Table 1).

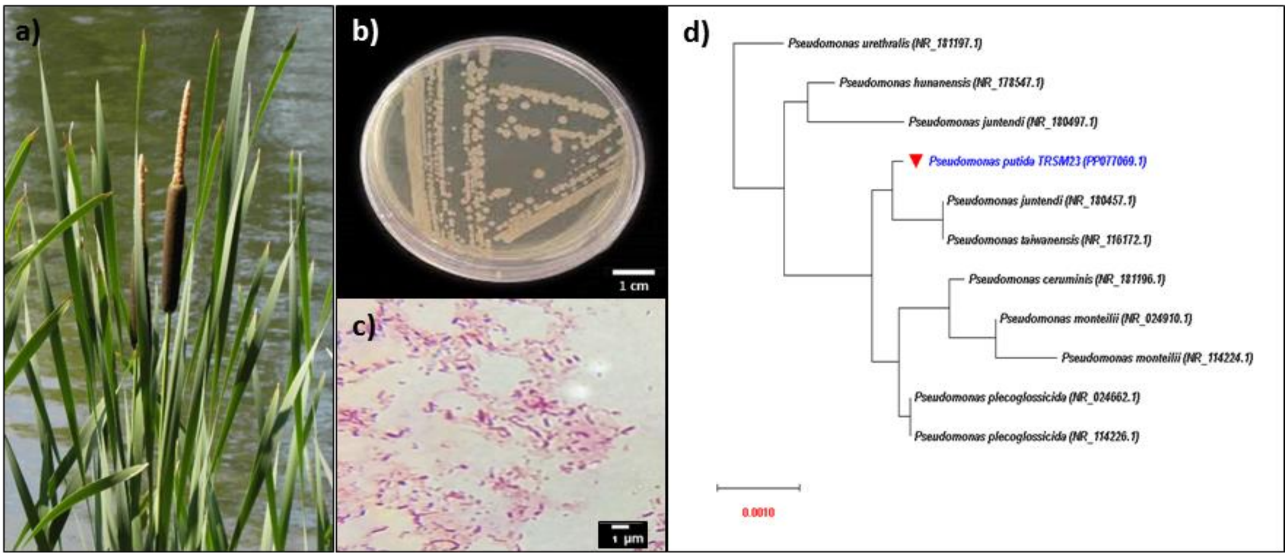

Figure 1. (b) Morphological, (c) microscopically, and (d) phylogenetic tree characterization of *P. putida* isolated from (a) *T. domingensis* plants collected from wastewater drains.

Table 1. Biochemical characteristics of colonies and cells of *P. putida* isolated from *T. domingensis* plants growing in wastewater drains.

Biochemical characterization					
Oxidase	**Catalase**	**Lipase**	**Cellulase**	**Amylase**	**Urease**
+	+	+	+	+	-
Plant growth promoting enzymes					
IAA	**Gbs**	**PS**	**NR**	**SP**	**HCN**
+	+	+	+	+	+

IAA: Indole acetic acid, Gbs: Gibberellic acid, PS: Phosphate solubilization, SP: Siderophore production, and HCN: Hydrogen cyanide production.

3.2. Minimum inhibitory concentration, removal efficiency and metal uptake of *P. putida*

As shown in Figure (2) *P. putida* was exposed to several concentrations of different metal ions Fe (2.5 and 5 mgl^{-1}), Al (5 and 7 mgl^{-1}), and Cu (2 and 3.5 mgl^{-1}), respectively. The results showed the highest dose of the lethal concentration, where *P. putida* showed the maximum resistance for Fe, Al, and Cu with MIC values 5, 7, and 3.5 mgl^{-1}. This indicated the inhibition of growth of the bacterial isolates at higher concentration of heavy metals.

The removal efficiency increased slowly up in the first day then speeded up, while the uptake rate of heavy metal was analyzed by considering the remaining concentration in the supernatants of the microbial culture. After five days of incubation as shown in Figure (3) (b), *Pseudomonas putida* showed the maximum removal of Fe at 2.5 and 5 mgl^{-1} metal ions with values (27.71 and 11.54 %), and it achieved an uptake capacity with values (0.69 and 0.58 mgl^{-1}). On the other hand, it showed the maximum removal of Al at 5 and 7 mgl^{-1} metal ions with values (31.74 and 18.62 %), and it achieved an uptake capacity with values (1.6 and 1.3 mgl^{-1}). While the maximum removal of Cu at 2 and 3.5 mgl^{-1} metal ions showed values (22.41 and 10.04 %), and it achieved an uptake capacity with values (0.45 and 0.35 mgl^{-1}).

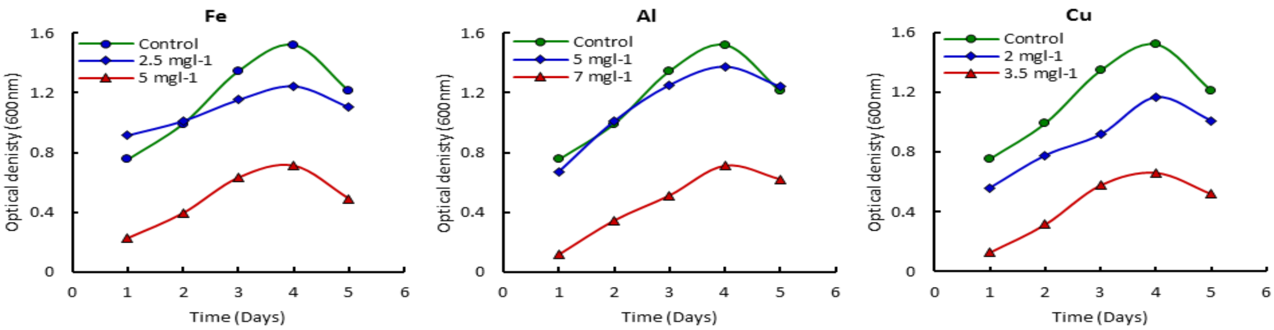

Figure 2. Minimum inhibitory concentration (MIC) of *P. putida* isolated from *T. domingensis* against heavy metals ions.

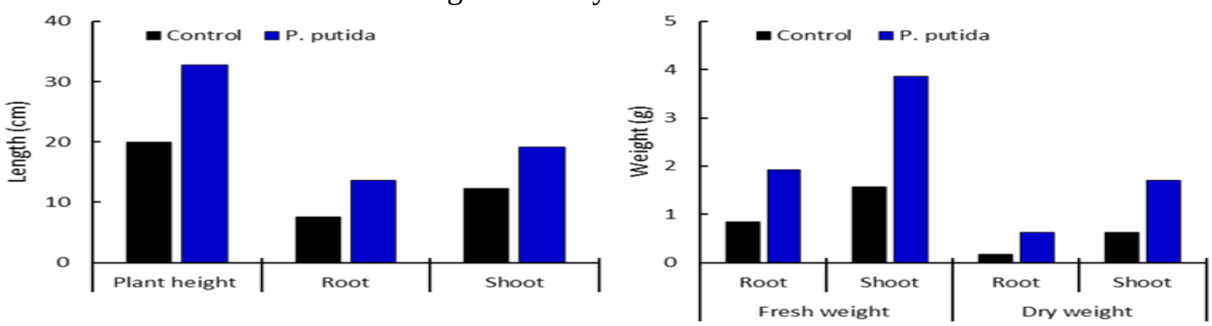

Figure 4. Effect of *P. putida* on the growth parameters (length, fresh, and dry weight of the roots and shoots) of 14-day-old seedlings under typical circumstances.

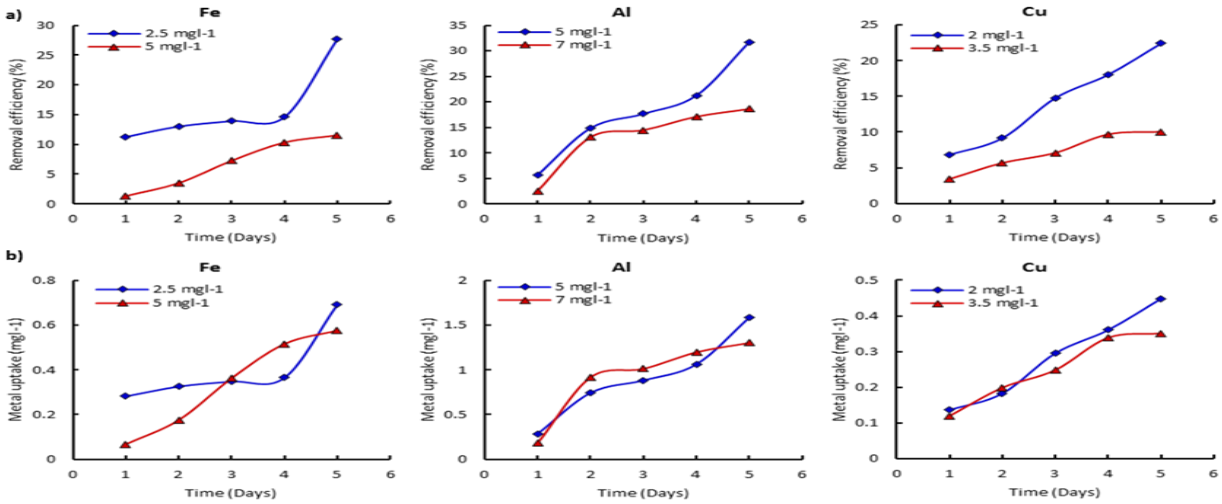

Figure 3. a) Removal efficiency (%) and **b)** metal uptake (mgl^{-1}) of *P. putida* for heavy metals from liquid medium containing Fe, Al, and Cu by absorption spectroscopy.

Table 2. Effect of *P. putida* on the plant height, allometric coefficient, seed vigor index, germination percentage, mean germination time, and germination value of 14-day-old seedlings under typical circumstances. The bars represent the SEM means (n = 20) at probability level of p < 0.05.

Seed morphological characteristics					
Treatments	**SVI**	**AC**	**GP%**	**MDG**	**GV**
Control	746.25±30.60^{b}	0.62±0.15^{a}	75.00±7.10^{b}	5.36±3.14^{b}	401.79±68.10^{b}
P. putida	1640.00±61.1^{b}	0.72±0.09^{b}	100.00±1.20^{c}	7.14±0.89^{b}	714.29±112.30^{c}
LSD$_{0.05}$	83.208***	0.202**	9.674***	3.947**	165.334***
Plant biochemical					
Treatments	**Photosynthetic pigments (µgl^{-1})**			**Oxidative enzyme (µgl^{-1})**	
	Chl. a	**Chl. b**	**CART**	**PO**	**PPO**
Control	1.56±0.15^{b}	1.16±0.21^{b}	1.30±0.17^{a}	1.12±0.08^{c}	0.96±0.02^{b}
P. putida	2.13±0.02^{a}	2.01±0.12^{a}	1.72±0.36^{a}	3.87±0.22^{a}	1.54±0.38^{a}
LSD$_{0.05}$	0.299**	0.298***	0.548ns	0.321***	0.457*

SVI: Seed vigor index, AC: Allometric coefficient, GP: Germination percentage, MDG: Main daily germination, GV: Growth value, Chl. a: Chlorophyll (a), Chl. b: Chlorophyll (b), CART: Carotenoids, PO: Oxidative phosphorylation, PPO: polyphenol oxidase.

3.4. Growth parameters

The application of *P. putida* on wheat seeds caused an increase in growth parameters, but higher than the un-inoculated ones, due to the production of plant growth regulators (IAA, GA3, PS, NR, SP, and HCN) (Table 1). In vitro germination experiment revealed that wheat seeds have better germination percentages under normal conditions. In contrast the application of *P. putida* enhanced up to 25 % or more of the length of wheat seedlings relative to the control during the early stages of ontogenesis. Compared with the control of un-inoculated plants that was 19.9 cm in height, 0.85 and 0.18 g root fresh/dry weight, and 1.57 and 0.62 g shoot fresh/dry weight. Results showed an increase with seedling height (32.8 cm), root fresh/dry weight (1.93 and 0.63 g), shoot fresh/dry weight (3.86 and 1.71 g) of seeds treated with *P. putida* Table (2).

At the bars represent the SEM means (n = 20) P 0.05 levels, statistical analysis revealed significant treatments differences. Results indicated an increased in seed germination percentage of treated seeds and showed a maximum seed germination percentage of 100% comparing with the control that was 75% (Table 2). On the seed vigor index, there were noticeable differences between the various seed priming treatment and the control. Treated seeds with *P. putida* showed the maximum seed vigor index of (1640) compared with control (746.25). On the other hand, the germination value for treated seeds had higher germination values (714.29) than the control (401.79). Likewise, higher AC value (0.72) recorded comparing with the control that showed AC value (0.62) as showed in Table (2).

Data presented in Table 2 indicated that photosynthetic pigments increased in treated seeds with *P. putida* because of its ability to promote growth parameters. The values of photosynthetic pigments of treated seeds chlorophyll (a) (2.13 μgl^{-1}), chlorophyll (b) (2.01 μgl^{-1}), and for carotenoids (1.72 μgl^{-1}) were increased, comparing with the un-inoculated control that was 1.97 μgl^{-1} for chlorophyll (a), 1.26 μgl^{-1} for chlorophyll (b), and 1.32 μgl^{-1} for carotenoids (Table 2). The application of endophytic bacteria *P. putida* on wheat causes significant increase in peroxidase and polyphenol oxidase enzymes comparing with the un-inoculated ones. Treated plants with *P. putida* showed the highest PO values (3.87 μgl^{-1}) and PPO (1.54 μgl^{-1}), comparing with the control that gave values 1.12 μgl^{-1} for PO, and 0.96 μgl^{-1} for PPO (Table 2).

4. Discussion

A few studies explored bacterial communities associated with *Typha* sp. growing in natural environments. Bacterial heavy metal removal property has great application in environmental point of view. It is possible that these bacterial species could have coevolved with the plant to be adapted to a specific arid habitat that is nutrient poor. In response to environmental condition such as pH, temperature, and salinity which may provide a survival advantage [11]. Earlier studies reported the predominance of Gram-negative bacteria in the tissues of various plants [12].

Endophytic bacterial diversity has been reported for several agricultural field and wastewater chromium, lead, and arsenic resistant *E. coli*, *Bacillus* sp., *Pseudomonas* sp., and *Micrococcous* sp. was reported. Endophytic *P. azotoformans*, *P. fluorescens*, *P. gessardii*, *P. veronii* were isolated from *T. angustifolia*, *T. domingensis*, and *Typha* sp. Likewise, the MIC of few bacterial isolates against lead and cadmium was reported. *Pseudomonas putid*a showed high resistance to metal ions. The high heavy metal tolerance of bacterial isolates may be due to their adaptation to high concentrations in the *T. domingensis*. It has previously been shown that high heavy metal concentrations in soils favor the survival of bacteria with multiple resistant mechanisms [3]. Heavy metal endophytic bacteria improving the efficiency of phytoremediation of polluted sites. The removal efficiency of Pb by *B. vesicularis* decreased when concentration increased. The highest uptake and removal efficiency of *T. viride* and *P. fluorescens* was reported [13].

Endophytic bacteria reported to enhance plant health, growth, development and tolerance to organic pollutants and heavy metals presence in soil or water through several mechanisms and production of plant growth-promoting hormones. Priming seeds with beneficial bacterial inoculums are an enticing ecological tactic to boost germination rates under adverse environmental conditions. Priming generally affects a variety of physiological systems in seeds and it is characterized by primed plants' quicker and more effective responses to stressors. Heavy metal tolerant bacteria promote the growth of host plants in contaminated sites and improve phytoremediation processes [14]. The increase in seed vigor index brought on by seed priming closely complies with findings from other researchers [10]. An increase in photosynthetic activity followed by increase in plant growth and the relationship between total chlorophyll content in bacterial treated plants [10].

5. Conclusion

Pseudomonas putida associated with *T. domingensis* roots growing in heavy metal-contaminated environments could be a biotechnological tool to improve plant growth. Therefore, in this study, *P. putida* was screened based on Fe, Al, and Cu tolerance and to their activities related to plant growth promotion. In this study, we thoroughly examined the early seedling strategy in wheat in typical conditions from the perspective of morphological performances. Priming with *P. putida* may be the greatest remedy for the reduced and delayed germination of fresh or preserved wheat seeds caused by the hardness of the seed. This bacterial strain can be effectively employed to remove harmful heavy metals from the ecosystem and maintain ecological equilibrium.

6. References

1 Sun, Q., Y. Li, L. Shi, R. Hussain, K. Mehmood, Z. Tang, and H. Zhang. (2022).Heavy metals induced mitochondrial dysfunction in animals: Molecular mechanism of toxicity. Toxicology, 469: p. 153136.

2 Pal, A., S. Bhattacharjee, J. Saha, M. Sarkar, and P. Mandal. (2022) Bacterial survival strategies and responses under heavy metal stress: A comprehensive overview. Critical Reviews in Microbiology,. **48(3)**: p. 327-355.

3 Shuaib, M., N. Azam, S. Bahadur, M. Romman, Q. Yu, and C. Xuexiu. (2021) Variation and succession of microbial communities under the conditions of persistent heavy metal and their survival mechanism. Microbial Pathogenesis,. 150: p. 104713.

4 Aguilar, J.R.P., J.J.P. Cabriales, and M.M. Vega. (2008.) Identification and characterization of sulfur-oxidizing bacteria in an artificial wetland that treats wastewater from a tannery. International *Journal of Phytoremediation*, **10(5)**: p. 359-370.

5 Shehzadi, M., K. Fatima, A. Imran, M. Mirza, Q. Khan, and M. Afzal. (2016) Ecology of bacterial endophytes associated with wetland plants growing in textile effluent for pollutant-degradation and plant growth-promotion potentials. Plant Biosystems-An *International Journal Dealing with all Aspects of Plant Biology*, **150(6)**: p. 1261-1270.

6 Liu, K.,-J., and Xu, X.,-D. (2013)First report of gray leaf spot of maize caused by Cercospora zeina in China. Plant Disease, **97(12)**: p. 1656-1656.

7 Resmi, G., S. Thampi, and S. (2010).Chandrasekaran. Brevundimonas *vesicularis*: A novel bio-sorbent for removal of lead from wastewater.

8 Zhong, Q., C. Cruz-Paredes, S. Zhang, and J. Rousk. (2021).Can heavy metal pollution induce bacterial resistance to heavy metals and antibiotics in soils from an ancient land-mine? *Journal of hazardous materials,* **411**: p. 124962.

9 Sanchez-Porro, C., H. Tokunaga, M. Tokunaga, and A. (2007.)Ventosa. Chromohalobacter japonicus sp. nov., a moderately halophilic bacterium isolated from a Japanese salty food. *Int J Syst Evol Microbiol*, **57**(Pt 10): p. 2262-2266.

10 Tania, S.S., M.S. Rhaman, and M.M. Hossain. (2020) .Hydro-priming and halo-priming improve seed germination, yield, and yield contributing characters of okra (Abelmoschus esculentus L.). Trop. Plant Res, **7**: p. 86-93.

11 Sabry Sultan, M., A. Elsayed, and Y. Ahmed El-Amier. (2023) .First Report of Endophytic Bacteria Isolated from Senecio glaucus L., Egypt. LA GRANJA. Revista de Ciencias de la Vida, **38(2)**: p. 82-95.

12 Maqsood, Q., N. Hussain, M. Mumtaz, M. Bilal, and H.M. Iqbal, (2022) Novel strategies and advancement in reducing heavy metals from the contaminated environment. Archives of Microbiology, **204(8)**: p. 478.

13 Bhattacharya, A. and A. Gupta. (2013) Evaluation of Acinetobacter sp. B9 for Cr (VI) resistance and detoxification with potential application in bioremediation of heavy-metals-rich industrial wastewater. Environmental Science and Pollution Research, **20**: p. 6628-6637.

14 Jiang, D., M. Tan, Q. Guo, and S. Yan. (2021)Transfer of heavy metal along food chain: a mini-review on insect susceptibility to entomopathogenic microorganisms under heavy metal stress. Pest Management Science,. **77(3)**: p. 1115-1120.