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Inoculation with the pH Lowering Plant Growth Promoting Bacterium *Bacillus* sp. ZV6 Enhances Ni Phytoextraction by *Salix alba* from a Ni-Polluted Soil Receiving Effluents from Ni Electroplating Industry

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Abstract: Soil contamination with Ni poses serious ecological risks to the environment. Several members of the *Salix* genus have the ability to accumulate high concentrations of Ni in their aerial parts, and thus can be used for the remediation of Ni-contaminated soils. Interestingly, the efficacy of Ni phytoextraction by *Salix* may be improved by the acidification of rhizosphere with rhizosphere acidifying bacterial strains. Therefore, the aim of this study was to assess the efficacy of bacterial strain *Bacillus* sp. ZV6 in the presence of animal manure (AM) and leaf manure (LM) for enhancing the bioavailability of Ni in the rhizosphere of *Salix alba* via reducing the pH of rhizosphere and resultantly, enhanced phytoextraction of Ni. Inoculation of Ni-contaminated soil with strain ZV6 significantly increased plant growth as well as Ni uptake by *alba*. It was found that the addition of AM and LM resulted into a significant increase in plant growth and Ni uptake by *alba* in Ni-contaminated soil inoculated with ZV6 stain. However, the highest improvements in diethylene triamine penta-acetic acid (DTPA) extractable Ni (10%), Ni removal from soil (54%), Ni bioconcentration factor (26%) and Ni translocation factor (13%) were detected in the soil inoculated with ZV6 along with the addition of LM, compared to control. Similarly, the enhancements in microbial biomass (92%), bacterial count (348%), organic carbon (organic C) (57%) and various enzymatic activities such as urease (56%), dehydrogenase (32%), β-glucosidase (53%), peroxidase (26%) and acid phosphatase (38%) were also significantly higher in the soil inoculated with ZV6 along with the addition of LM. The findings of this study suggest that the inoculation of Ni-contaminated soils with rhizosphere acidifying bacteria can effectively improve Ni phytoextraction and, in parallel, enhance soil health.

Keywords: acidification; rhizosphere; phytoextraction; bioavailability; bacteria; enzymes

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1. Introduction

Soil contamination with heavy metals (HMs) is caused by massive human activities such as smelting and mining of ores, electronic waste, industrial effluents, agrochemicals, military exercises and burning of fossil fuels [1]. Contamination of soil with Ni also results from lithogenic sources [2]. Regardless of the importance of Ni as a micronutrient at low concentrations, higher levels of Ni in soil pose severe environmental risks to plants, animals, humans, and water bodies. Considering these severe environmental risks, there is an urgent need to identify and optimize soil remediation technologies to manage Ni-polluted soils [2].

While addressing the limitations of traditional remediation approaches for Ni-polluted soils [3,4], one of the promising remediation approaches is phytoremediation by using

Ni hyperaccumulator plants [5,6]. Due to the production of high biomass, this approach is becoming popular among the researchers in recent years [7]. Moreover, these plants can accumulate a large quantity of Ni (>1000 μg g^{-1} dry weight) in their aerial parts without indicating toxicity symptoms [8,9]. Numerous researchers have confirmed the phenomenal potential of several species of willows (*Salix*) for the phytoextraction of Ni from contaminated soils [10–12].

The efficiency of Ni phytoextraction depends on several factors such as Ni bioavailability in the soil, soil physicochemical properties, environmental conditions, adaptation of the plants in terms of their survival and growth rate, and soil conditions such as drought, salinity, and waterlogging [12,13]. Apart from these factors, the pH of soil also plays a vital role in enhancing Ni bioavailability in the soil for its efficient removal by the phytoextraction [13]. Previously, several organic acids (OAs) and elemental sulfur have been used to increase the bioavailability of Ni in the soil and it's phytoextraction. However, several limitations such as high cost of these soil additives, leaching of Ni to groundwater, and its harmful effects on soil health may impede the success of the phytoextraction process [14,15].

Lowering the pH of rhizosphere is known to improve the bioavailability of HMs as well as their removal from the soil by different plants [1,15]. Interestingly, several species of plant growth promoting rhizobacteria(s) [PGPR(s)] have the ability to secrete chelators such as OAs and siderophores which reduce soil pH and enhance the plant available fraction of HMs in soil and their removal by the plants [16–18]. Phytoextraction assisted with PGPRs can improve growth and regulate the uptake of Ni in hyperaccumulator plants grown in Ni-contaminated soils [19–21]. Furthermore, the efficiency of the bacterial population for the removal of pollutants such as HMs from the contaminated soil can be increased by providing them with readily available sources of carbon and essential nutrients in the soil [22–24].

To date, insufficient research has been conducted about the role of rhizosphere acidifying PGPRs on reducing soil pH, exclusively in the *Salix alba* rhizosphere, and resultantly enhanced the phytoextration of Ni. In addition, the role of LM and AM, as sources of carbon and essential nutrients, for enhancing the activities of rhizosphere acidifying PGPRs and their effects on enhanced Ni phytoextraction by *Salix* is still unexplored. Therefore, a pot experiment was performed with the objectives: (1) to investigate the role of rhizosphere acidifying PGPRs for reducing soil pH, exclusively in the rhizosphere of *Salix alba*; (2) to evaluate the effect of reduced rhizosphere soil pH on the bioavailability and phytoextraction of Ni and; (3) to explore bio-stimulating role of LM and AM for enhancing the efficacy of Ni phytoextraction by *Salix alba* from a Ni-polluted soil formerly receiving Ni-rich effluents from an electroplating industry.

2. Materials and Methods

2.1. Isolation of the Bacterial Strain ZV6

The strain ZV6 was isolated from a rhizospheric soil and tested for its potential to lower the pH in the aqueous and soil media. For estimating the potential of the strain ZV6 to lower pH in the aqueous media, it was allowed to grow in Nutrient Broth Medium [Meat extract (1.0 g L^{-1}), peptone (5.0 g L^{-1}), Sodium chloride (5.0 g L^{-1}), Yeast extract (2.0 g L^{-1})] and Lauria Bertani Medium [Sodium chloride (5.0 g L^{-1}), Tryptone (10.0 g L^{-1}), Yeast extract (5.0 g L^{-1})]. These sterilized media were inoculated with the strain ZV6 to develop an initial optical density (OD_{600}) of 0.05. In an incubator, the flasks were placed (28 °C, 150 rpm). The bacterial growth and the pH of the media were monitored over the incubation period (72 h). For the pot experiment, the optical density (OD_{600}) of the bacterial culture was maintained at 1.0.

2.2. Analysis of Ni-Polluted Soil

We collected soil samples (0–20 cm depth) from an arable land in Pakistan (31°40′18.5″ N 74°06′31.3″ E) polluted from the effluents of an electroplating industry. The homogenized air-dried soil sample was passed through a sieve with a diameter of 2 mm to remove debris

and stones. Physicochemical characteristics of this soil were determined after shade-drying. The soil properties are represented in Table 1.

Table 1. Physicochemical properties of the experimental soil [2].

Properties	Units	Values
Sand	%	16.0
Silt	%	41.0
Clay	%	43.0
pH (H_2O)	-	8.20
Organic matter (OM)	%	1.10
Calcium carbonate ($CaCO_3$)	%	3.10
Bicarbonate (HCO_3)	%	0.04
Electrical conductivity (EC)	dSm^{-1}	1.90
Cation exchange capacity (CEC)	$cmol_c\ kg^{-1}$	16.7
Nitrogen (N)	$mg\ kg^{-1}$	139.0
Phosphorus (P)	$mg\ kg^{-1}$	6.90
Potassium (K)	$mg\ kg^{-1}$	138.0
DTPA-extractable Ni	$mg\ kg^{-1}$	3.94
Total Ni	$mg\ kg^{-1}$	77.0

2.3. LM and AM Sources

Leaf manure and AM were purchased from Subhani Seeds (Office 506, Continental Trade Centre (CTC), Block 8, Clifton, Karachi, Pakistan) through https://www.daraz.pk/ (accessed on 1 June 2020). The properties of LM and AM are present in Supplementary Table S1. The composition of AM varies among animal type such as cattle, pigs, sheep, and poultry. Furthermore, other factors such as the age of animal, gender, health condition, feed stock used and rearing conditions also effect the composition of AM [25]. Similarly, the variations in the composition of LM are affected by leaf tenderness [26], nutrients in leaf, the involvement of earthworms and microorganisms during composting process [26,27], the ratio between carbon and nitrogen in the leaf, the source from where the leaves were obtained [28], composting time and the method used [29].

2.4. Pot-Scale Experiment

After the arrangement of LM, AM and the preparation of bacterial inoculum, the Ni-polluted soil was treated solely with LM, AM, and bacterial inoculum as well as a combination of LM and AM with bacterial inoculum (Table 2). The required quantities of LM, AM and bacterial inoculum were uniformly mixed with a small amount of soil by using spatula in a bucket. This homogenous mixture was amalgamated with the remaining soil using a mechanical shaker. After completing this process, 3 kg of soil was poured in the drained plastic container (9″ × 7″) with great care according to the treatment plan (Table 2). The experiment was run in triplicates. These pots were positioned in a randomized design. Afterward, the water was added manually in the pots and the soil was allowed to attain the ambient moisture status which was suitable for the plantation of *Salix* cuttings. Healthy *Salix* cuttings (length ≈ 12 cm, diameter ≈ 0.4 cm) were collected from Qadir Bukhsh nursery, Faisalabad, Pakistan. After that, each cutting was dipped in Indole-3-butyric acid gel (BOOST rooting gel, bought from sky seeds, Lahore, Pakistan) to promote rooting. Onward, one *Salix* cutting was cautiously placed into the soil in each pot. The cuttings of *Salix* sprouted after five days of their sowing in the soil. Depending on the climatic conditions, the experimental pots were regularly watered during the growth period. The plants were allowed to grow for 70 days. Before plants harvesting, the plant height (PtH) was measured by using a measuring tape.

Table 2. Treatment plan of the pot study.

Treatments	Abbreviations	Input Amounts of LM and AM (g kg^{-1} Soil)	Bacterial Inoculation
Control	**Control**	-	-
Bacteria	**B**	-	**10 mL bacterial suspension (OD_{600} = 1.0)**
Leaf Manure	**LM**	50	-
Bacteria + Leaf Manure	**B + LM**	50	**10 mL bacterial suspension (OD_{600} = 1.0)**
Animal Manure	**AM**	50	-
Bacteria + Animal Manure	**B + AM**	50	**10 mL bacterial suspension (OD_{600} = 1.0)**

2.5. Termination of Pot Experiment

The above ground portion of the plants were harvested by using a plant cutter. Later, the roots were cautiously retrieved from the soil and flushed thoroughly using tap water to remove soil particles adhered to the surface of roots. Root fresh weight (RFRW) and shoot fresh weight (SFRW) were determined instantly. To obtain dry weight (DRW), roots and shoots were oven-dried at 70 °C. This desiccated biomass was cleared through a 0.5 mm sieve after pulverizing in a milling machinery (IKA Werke, Staufen, Germany).

2.6. Analysis

2.6.1. Status of Ni in Plant Portions and Soil

Open flask digestion method was used to digest 1 g of ground roots and shoots in a mixture of HNO_3 and $HClO_4$ (2:1, *v*/*v*) [30]. Nickle concentrations in roots and shoots were estimated through the quantification of Ni in their digests on ICP−MS (PerkinElmer's NexION® 2000). The translocation factor (TF) and bioconcentration factor (BCF) values for Ni in *Salix* were calculated by following ratio Formulas (1) and (2) [31]:

$$\text{BCF} = [\text{Ni}]\ \text{shoot}/[\text{Ni}]\ \text{soil} \quad (1)$$

$$\text{TF} = [\text{Ni}]\ \text{shoot}/[\text{Ni}]\ \text{root} \quad (2)$$

Here, the Ni concentrations in roots and shoots were expressed in mg kg^{-1} DRW while the concentrations of Ni in the soil (total) as mg kg^{-1} DRW soil. In addition, the % removal of Ni from the soil was calculated via the Formula (3) [32].

$$\text{Ni removed from the soil (\%)} = \text{Ni contents in plants kg}^{-1}\ \text{soil/total Ni kg}^{-1}\ \text{soil} \times 100 \quad (3)$$

The bioavailable concentration of Ni in the soil was extracted with DTPA solution (0.005 M) [33]. Afterward, the ICP−MS was used to determine the concentration of Ni in DTPA-extract.

2.6.2. Assessment of Chlorophyll-a, Chlorophyll-b and Relative Water Content (RWTC)

Chlorophyll-a and chlorophyll-b in leaves were determined as described by Hiscox and Israelstam [34]. A 1 g sample of fresh leaf was homogenized in 20 mL of methanol chloroform water (12:5:3) and the Chlorophyll-a and Chlorophyll-b were measured at 664.5 nm and 647.4 nm, respectively, using a UV-Visible spectrophotometer (Analytik Jena SPECORD 200 PLUS).

We used equation 4 to compare turgid weight (TRW), RWTC, dry weight (DRW) and fresh weight (FRW) of leaves. Fresh leaves were initially weighed on a weighing balance and then were placed in dark for 24 h in deionized water. Later, the RWTC was calculated by the following formula [35].

$$\text{RWTC (\%)} = [(\text{FRW} - \text{DRW})/(\text{TRW} - \text{DRW})] \times 100 \quad (4)$$

2.6.3. Soil Enzymes

Dehydrogenase activity was measured by mixing 1 g of soil with 50 µL glucose solution (10 g L^{-1}), 1 mL TRIS buffer, and 0.2 mL 2,3,5-triphenyl tetrazolium chloride, followed

by incubation at 35 °C for 24 h. The soil was then extracted with 10 mL methanol and the concentration of Triphenylformazan in this soil extract was spectrophotometrically measured (λmax 485 nm). The results were expressed as μmol INTF (iodonitrophenylformazan) g^{-1} h^{-1}. The activity of phosphomonoesterase enzyme was determined according to the methods described by Paz-Ferreiro et al. [36] and Eivaz & Tabatabai [31]. A standard curve was drawn and it was observed that there was no substrate restriction in the reaction. The activities of alkaline phosphatase and β-glucosidase were measured by the methods of Eivazi and Tabatabai [37]. For β-glucosidase, we used ρ-nitrophenyl-β-D-glucopyranoside as a substrate while ρ-nitrophenyl phosphate for phosphatase enzymes. This reaction blend was kept at 37 °C for 1 h. Later, Tris (pH = 12, 0.02 mol L^{-1}) was added to pause the activity of β-glucosidase while a mixture of $CaCl_2$ and NaOH (0.5 mol L^{-1} each) was added to halt the alkaline phosphatase reaction. Then, the substrate cleavage was generated for ρ-nitrophenol glucoside (by β-glucosidase) and ρ-nitrophenyl phosphate (by phosphatase) that were recorded at 464 nm and 505 nm, respectively, using a UV-Visible spectrophotometer. The results were expressed as μmol PNF (p-nitrophenyl-β-D-glucopyranoside) g^{-1} h^{-1}. Kandeler and Gerber [38] methodology was used to measure the urease activity in the soil. For this purpose, 1 g moist soil was blended with 0.5 mL of urea solution and 4 mL of borate buffer (pH = 10) in reaction flasks. This mixture was incubated for 2 h. After 30 min, 1 M KCl (6 mL) was mixed, and later, sodium dichloroisocyanurate, Na salicylate/NaOH and deionized water were added in the filtrate. This mixture was placed at room temperature for 30 min to determine the ammonium content before estimating the optical density at 690 nm using a UV-Visible spectrophotometer. Acid phosphatase activity (mg PNP kg^{-1} h^{-1}) was estimated following the method of Tabatabai and Bremner [39]. The p-nitrophenol retrieved from 1.0 g of soil was calculated after 1 h of its incubation with a 0.025 M p-nitrophenyl phosphate substrate at 37 °C in 0.17 M universal buffer (4 mL) at pH 5. The results were expressed as mg PNP (p-nitrophenyl phosphate disodium) kg^{-1} h^{-1}.

3. Results

3.1. Characterization of Rhizospheric Acidifying Bacterial Strain Bacillus sp. ZV6

The plant growth-promoting acidifying bacterial isolate ZV6 was isolated from maize rhizosphere. The BLASTn analysis of the 16S rDNA gene of this bacterial strain indicated that this strain had more than 98% homology with the genus *Bacillus*. Moreover, in a phylogenetic tree constructed by neighbor-joining method, this strain was grouped with the *Bacilli* strains (Supplementary Figure S1). Based on the BLASTn and the phylogenetic analyses, this bacterial strain was designated as *Bacillus* sp. ZV6 (GenBank Accession No. OM920551). The strain ZV6 had an excellent potential to reduce the pH of the medium. While studying the growth and the potential to reduce the pH in nutrient broth and LB broth media, the strain ZV6 was found to grow efficiently in both media and significantly reduce their pH (Supplementary Figure S2). As a result of the growth of the strain ZV6, the pH of the nutrient broth medium was reduced from 7.4 to 5.4 over an incubation period of 72 h. Over the same incubation period, the pH of the LB medium was reduced from 5.5 to 7.0.

3.2. Changes in pH of Rhizosphere and Bulk Soils

The pH of the rhizospheric soil ranged from 7.02 to 8.02, while from 7.51 to 8.20 for bulk soil (Figure 1). Except for LM and AM, remaining treatments remarkably decreased the pH of bulk and rhizospheric soils as compared with the control. Surprisingly, the decrease in pH values of the bulk and rhizospheric soils was recorded by 0.69 and 0.98 units in the B + LM treatment.

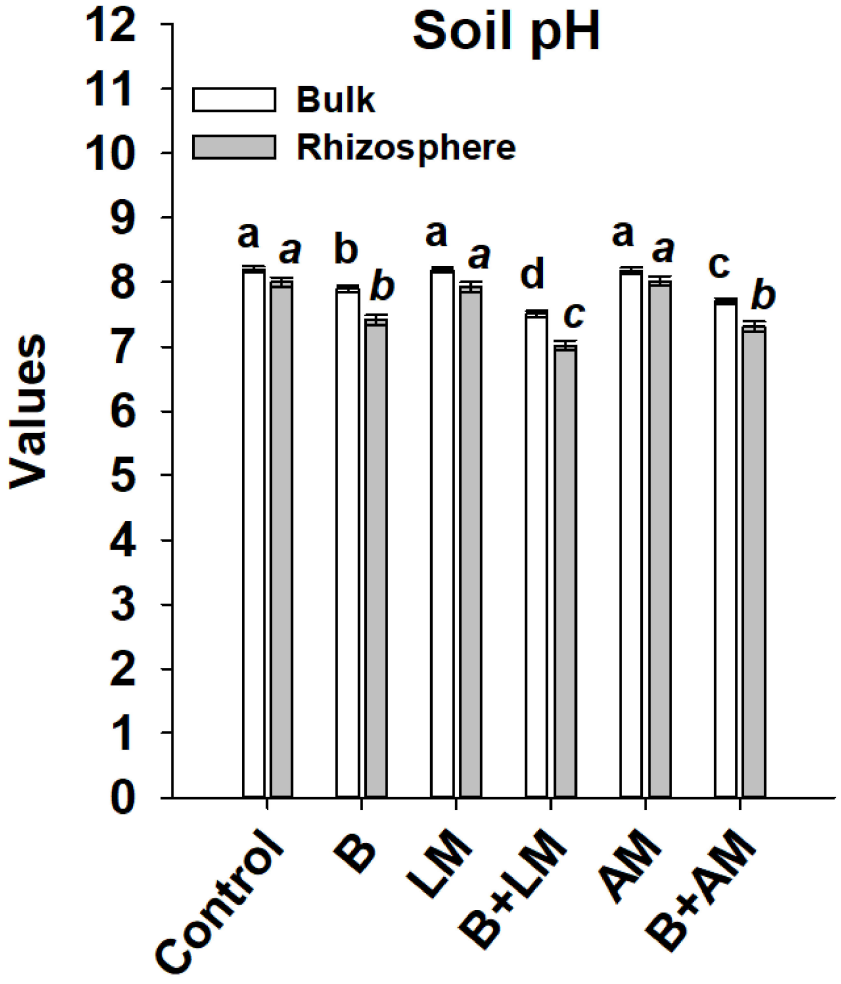

Figure 1. Variations in pH values of bulk and rhizospheric soils as affected by adding LM, AM, and *Bacillus* sp. ZV6 inoculum. Values denoted by the same letter do not differ significantly ($p < 0.05$). The error bars show the standard error of the mean (n = 3).

3.3. Growth, Biomass, Chlorophyll and Relative Water Contents

The growth of the plants in terms of PtH, shoot DRW and root DRW were found to range from 81.1 to 98.3 cm, from 5.36 to 6.76 g pot^{-1}, and from 1.32 to 1.56 g pot^{-1}, respectively (Table 3). The B, B + LM and B + AM treatments significantly enhanced PtH, shoot DRW, and root DRW of plants as compared to control plants. The highest increments in PtH by 21% and 15% as well as root DRW by 18% and 14% were found in B + LM and B + AM treatments, respectively. The B + LM treatment also resulted in the maximum significant increase in shoot DRW by 26%, compared to control.

The contents of chlorophyll-a, chlorophyll-b and RWTC in leaves ranged from 1.25 to 1.60, from 0.90 to 1.26 mg g^{-1} FRW, and from 72.1% to 85.0%, respectively (Table 3). With exception of AM, all other treatments significantly ($p < 0.05$) amplified the contents of chlorophyll-a and chlorophyll-b, relative to control. Interestingly, the maximal augmentation in chlorophyll-a by 28% and 22%, while in chlorophyll-b by 41% and 31% were observed in B + LM and B + AM treatments, respectively. Except LM and AM, the remaining treatments significantly elevated RWTC in leaves as compared with control. The maximal augmentation in RWTC contents in the leaves by 18%, 13%, and 10% was observed in B + LM, B + AM, and B treatments, relative to untreated control.

Table 3. Plant height (A), shoot DRW (B), root DRW (C), chlorophyll-a (D), chlorophyll-b (E) contents, and RWTC (F) of *Salix* after applying bacteria and organic amendments (LM and AM) in a Ni-contaminated soil. Values denoted by the same letter do not differ significantly ($p < 0.05$). The error bars show the standard error of the mean (n = 3).

Treatments	Plant Height (A)	Shoot DRW (B)	Root DRW (C)	Chlorophyll-a (D)	Chlorophyll-b (E)	RWTC (F)
	(cm)	(g pot^{-1})		(mg g^{-1} FRW)		(%)
Control	81.1 ± 2.05 **d**	5.36 ± 0.13 **d**	1.32 ± 0.02 **d**	1.25 ± 0.04 **d**	0.90 ± 0.02 **d**	72.1 ± 1.82 **d**
B	91.0 ± 2.28 **bc**	5.82 ± 0.14 **bc**	1.43 ± 0.03 **bc**	1.42 ± 0.04 **bc**	1.12 ± 0.05 **bc**	79.0 ± 1.99 **abc**
LM	87.6 ± 2.22 **bcd**	5.69 ± 0.14 **cd**	1.40 ± 0.03 **bcd**	1.39 ± 0.04 **c**	1.05 ± 0.04 **c**	77.3 ± 1.94 **bcd**
B + LM	98.3 ± 2.48 **a**	6.76 ± 0.17 **a**	1.56 ± 0.03 **a**	1.60 ± 0.03 **a**	1.25 ± 0.04 **a**	85.0 ± 2.14 **a**
AM	84.4 ± 2.14 **cd**	5.51 ± 0.14 **cd**	1.37 ± 0.03 **cd**	1.37 ± 0.05 **cd**	0.94 ± 0.03 **d**	75.0 ± 1.88 **cd**
B + AM	93.0 ± 2.34 **ab**	6.16 ± 0.15 **b**	1.50 ± 0.04 **ab**	1.52 ± 0.04 **ab**	1.17 ± 0.03 **ab**	81.5 ± 2.05 **ab**

3.4. Ni Distribution in Salix, BCF and TF Values, and Ni Removed from the Soil

As shown in Figure 2, the Ni concentrations in shoots and roots ranged from 81.4 to 102.3 mg kg^{-1} DRW and from 70.6 to 78.3 mg kg^{-1} DRW, respectively. However, the contents of Ni in shoots and roots were ranging from 0.42 to 0.69 and from 0.09 to 0.12 mg pot^{-1}, respectively. Besides LM and AM, the other treatments significantly augmented Ni concentrations in plant shoots and roots, whereas, except AM treatment, the contents of Ni in roots and shoots were increased compared to control. The maximum increase in the concentrations of Ni in shoots and roots by 26% and 11%, respectively, were found in B + LM treatment compared to the control.

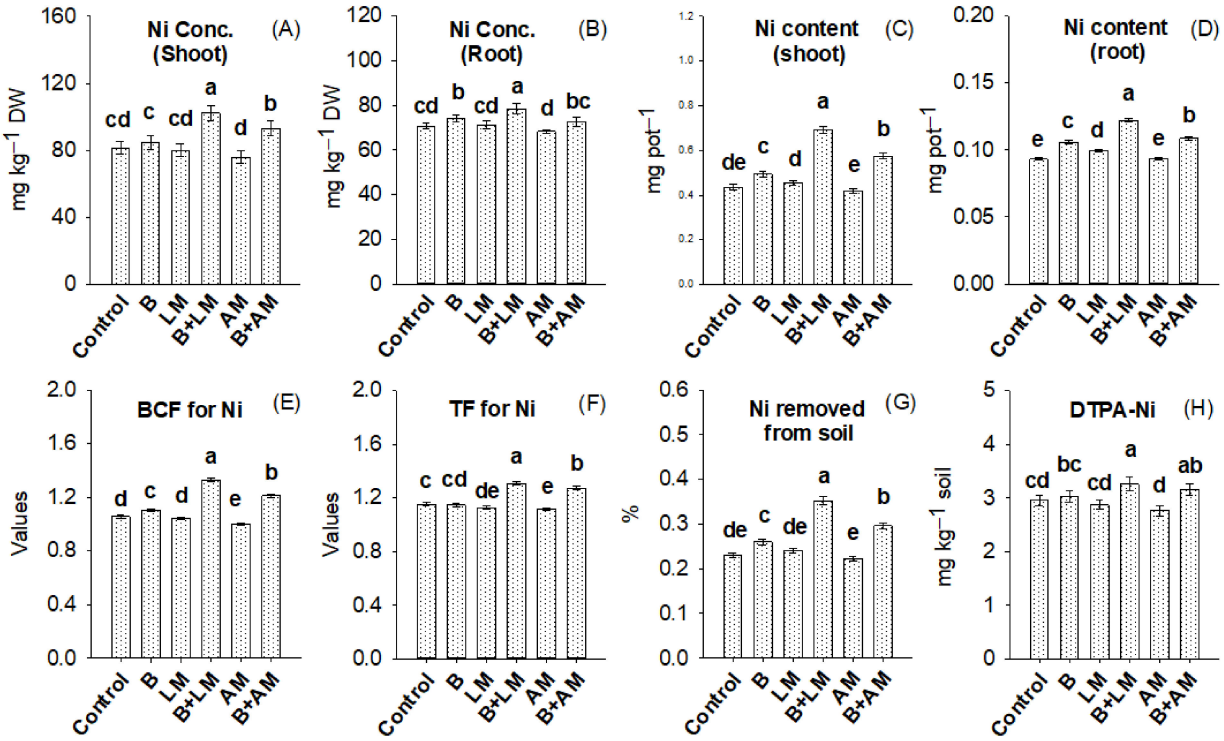

Figure 2. The concentrations of Ni in shoot (**A**) and root (**B**), contents of Ni in shoot (**C**) and root (**D**), BCF (**E**) and TF (**F**) values of Ni, DTPA–Ni (**H**) and Ni removal from the soil (**G**) as affected by adding LM, AM and *Bacillus* sp. ZV6 inoculum in Ni-contaminated soil. Values denoted by the same letter do not differ significantly ($p < 0.05$). The error bars show the standard error of the mean (n = 3).

The BCF and TF values for Ni ranged from 1.00 to 1.33 and from 1.12 to 1.31, while Ni removed from soil and DTPA−Ni from 0.22 to 0.35% and from 2.77 to 3.26 mg kg^{-1} soil,

respectively, among all of the treatments (Figure 2). Compared to control, all treatments increased Ni removed from the soil and DTPA–Ni, except for LM and AM. The highest increments by 54% and 10% in the values of Ni removed from soil and DTPA–Ni, respectively, were observed in B + LM treatment, compared to control.

3.5. Soil Enzymatic Activities, Bacterial Count, Microbial Biomass, and Organic C

The data associated with the activities of dehydrogenase, β-glucosidase, acid phosphatase, urease, and peroxidase were in the ranges from 1.31 to 1.73 µmol INTF g^{-1} h^{-1}, from 0.19 to 0.29 µmol PNF g^{-1} h^{-1}, from 23.3 to 32.2 mg PNP kg^{-1} h^{-1}, from 2.20 to 3.43 µmol N–NH_4^+ g^{-1} h^{-1}, and from 7.30 to 9.21 mol g^{-1} h^{-1}, respectively, in post-harvest soil (Figure 3). Each treatment significantly enhanced the activities of all soil enzymes, except for AM treatment, in case of β-glucosidase, acid phosphatase, and peroxidase activities, compared to control. Interestingly, the highest increments up to 32%, 56%, 53%, 38%, and 26%, in the activities of dehydrogenase, urease, β-glucosidase acid phosphatase, and peroxidase were noted in B + LM treatment relative to the control.

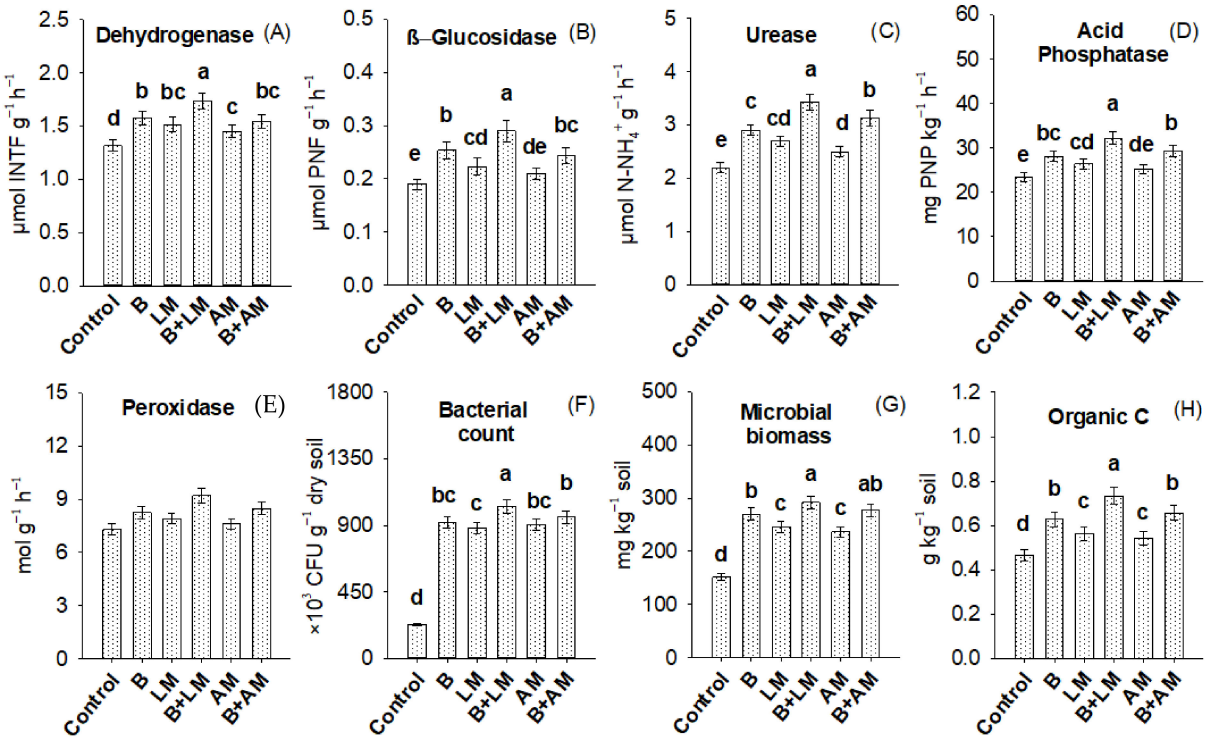

Figure 3. Activities of dehydrogenase (**A**), β-glucosidase (**B**), urease (**C**), acid phosphatase (**D**), peroxidase (**E**), bacterial count (**F**), microbial biomass (**G**) and organic C (**H**) as affected by adding LM, AM, and *Bacillus* sp. ZV6 inoculum. Values denoted by the same letter do not differ significantly (at $p < 0.05$). The error bars show the standard error of the mean (n = 3).

Data of bacterial count, microbial biomass, and organic C content in the post-harvest soil ranged from 229.1 to 1026.3 × 10^3 CFU g^{-1} dry soil, from 151.6 to 291.7 mg kg^{-1} soil, and from 0.46 to 0.73 g kg^{-1} soil in whole treatments (Figure 3). Each treatment significantly augmented the bacterial count, microbial biomass, and organic C content compared to control. The highest increments by 348% and 57% in the bacterial count and organic C were found in B + LM treatment, while microbial biomass was augmented up to 92% and 83% in B + LM and B + AM treatments, respectively, compared to the control.

4. Discussion

4.1. Changes in pH of Broth Media and the Salix Bulk and Rhizospheric Soil Portions

In this study, a plant growth-promoting bacterial strain *Bacillus* sp. ZV6 having the potential to reduce the pH of the aqueous media was isolated from the rhizosphere of maize plant. The isolation of the strain ZV6 will be a new addition in the potential plant-growth-promoting and pH reducing bacterial strains belonging to genus *Bacillus,* which is one of the most studied and ubiquitous genera for multiple beneficial functions in the environment [40–42]. The strain ZV6 showed an excellent potential to reduce the pH in the aqueous media and the soil. A similar decrease in pH in response to bacterial inoculation has also been reported for a few other *Bacilli* bacterial strains, including *Bacillus aryabhattai, Bacillus pumilus* and *Bacillus cereus* [41,43]. Another study reported that the Ni-resistant bacteria significantly reduced the pH of soil [44]. One of the possible reasons for the reduction of pH of the media as well as the soil in response to the bacterial growth might be the production and release of OAs by the bacterial strains, which results in acidification of the soil as well as aqueous media [45]. Such acidification of the soil results into an improvement in the availability of nutrients to the plants.

After the experiment, the lowest pH values of both bulk and rhizosphere portions of soil were found in B + LM treatment. Furthermore, the pH value of rhizospheric soil was 0.4 unit lower than the bulk soil in B + LM treatment (Figure 1). Previous studies have reported lower pH values of rhizosphere soil in rice [46] and mung bean [1], compared to the bulk soil portion. Likewise, bacterial inoculum resulted in significant reductions in the values of rhizosphere soil pH than bulk soil of *Brassica juncea* [1]. Furthermore, the inoculation of PGPRs contributed to the acidification of rhizosphere soil than the bulk soil of different plants [46,47]. The lower values of rhizospheric soil pH in comparison with bulk soil pH are as a result of the balance among the release of H^+ and HCO_3^- (OH^-) in plant roots which is dependent on the ratio of uptake of cations and anions. Higher uptake of anions by the roots results in the excretion of higher concentrations of HCO_3^- than H^+ which results in the elevation of rhizosphere soil pH. However, the acidification of rhizosphere soil occurs when higher concentrations of cations are taken up by the roots than anions [48]. Since the LM was rich in cationic nutrients (Supplementary Table S1), the higher uptake of cations by *Salix* roots resulted in the secretion of H^+ which resulted in the acidification of rhizosphere. Furthermore, lower value of rhizosphere soil pH when compared to bulk soil pH in *Salix alba* is also attributed to the secretion of several low-molecular weight organic acids (LMWOAs) such as oxalic acid, formic acid, malonic acid, lactic acid, malic acid, acetic acid, maleic acid, citric acid, fumaric acid, and succinic acid in the vicinity of rhizosphere [49].

4.2. Growth, Biomass, Chl-a, Chl-b, and RWTC

In our experiment, the highest significant improvements in the PtH, shoot DRW, and root DRW, chlorophyll-a, chlorophyll-b and RWTC in *Salix* were observed in B + LM treatment, compared to control (Table 3). A previous study has reported that addition of LM (5 ton ha^{-1}) significantly improved plant growth and yield parameters of *Capcicum annuum* L. [50]. Similarly, Ni resistant PGPR (*Kluyvera ascorbate*), under the toxicity of Ni, Pb and Zn, was shown to enhance the development of tomato, Indian mustard and canola [51]. In another field study, okra growth and yield as well as the concentrations of K, Ca, Fe, Zn, Cu and vitamin C were improved in the pods of okra after amending the soil with green manure [52]. Previously, inoculation of Ni-contaminated soil with two PGPRs (*Bacillus* sp. CIK-516 and *Stenotrophomonas* sp. CIK-517Y) improved radish (*Raphanus sativus*) biomass and chlorophyll contents [19]. Furthermore, improvements in fresh and dry biomass of *Eruca sativa* grown in Ni-contaminated soil due to the inoculation of *Pseudomonas putida* was also reported [51,53]. Likewise, in a pot study, the addition of LM in the soil showed significant positive influences on the growth and yield of okra including PtH, fruit length, fruits number $plant^{-1}$ and individual fruit weight [54].

Leaf manure acts as an alternate to chemical fertilizers for increasing the fertility status as well as chemical, biological and physical properties of the soil by the virtue of providing OM, essential nutrients and beneficial microorganisms to the soil [50]. Moreover, LM also supports the release of nutrients from the soil via several processes [55]. Organic manure amendments contain both organic and mineral ingredients that are vital for plant growth and the activation of biochemical phenomenon in the plants, such as metabolism, photosynthesis and chlorophyll production, thereby boosting the plant quality parameters and productivity [56,57]. All of these mechanisms improve the availability of essential nutrients and water to the plants [57]. Furthermore, PGPRs have been proven to enhance the plant yield, growth and root morphology [58]. The release of siderophores and phytohormones such as auxins by PGPRs positively influence the growth of plants [58]. Several previous studies also indicate that the growth and activities of the PGPRs are also enhanced as a result of organic amendments which ultimately result into further improvements in plant growth and physiological parameters [35,59].

4.3. Ni Distribution in Salix Plant, Values of BCF and TF, and Ni Removed from the Soil

The highest significant values of Ni concentrations and its contents in the roots and shoots, BCF, TF, Ni removed from the soil and DTPA-Ni were found in B + LM treatment as compared to control (Figure 2). The plants could be identified as accumulators ($1 < BCF < 10$) or hyperaccumulators ($BCF > 10$ and $TF > 1$) and excluders ($BCF < 1$ and $TF < 1$) of Ni based on their BCF and TF values [60]. The TF value higher than 1 shows that the metal has been translocated from the roots to the aerial portions of the plants [61]. In our experiment, the maximum BCF and TF values were observed in the B + LM treatment since, as noted, this treatment resulted in the highest DTPA-extractable Ni from the soil. Willow plants have been extensively examined for their ability to translocate metals from roots to aerial portions [62] and have already been reported for significantly higher mean TF values for Cd (1.709–11.37), confirming the capacity of willows to transport a large quantity of Cd from roots to aerial portions. Similarly, Yang et al. [63] found that leaf TF values for Cu and Zn exceeded one in four willow clones, and also that accumulation and translocation in woody species were specific metal-dependent. A study depicted that use of hyperaccumulator with PGPR can enhance the accumulation of Ni in shoot and root of plant [64]. Likewise, Yang et al. [63] found that hyperaccumulators have the ability to uptake higher concentrations of HMs to the aerial parts of the plants compared to roots. While enhanced phytoextraction can help the plants to accumulate HMs, plants also have an intrinsic system for insoluble HMs absorption via root exudates. Root exudates act as HMs reductases around the rhizosphere, with the inherent ability to decrease metal-organic complexes generated in the soil to transform these to free ions or metal complexes, allowing the roots to assimilate them more easily. Metal-hyperaccumulating plants possess unique inherent regulatory mechanisms that enable the plants to retain or hyperaccumulate surplus levels of HMs in various above-ground tissues instead of accumulating them in the root system. The capacity of hyperaccumulators to accumulate excessive metals is partly due to the constitutive amplification of metal carriers and the tendency to translocate HMs swiftly from the roots to aerial parts. Metal ligands perform essential functions in metal hyperaccumulating plants as well. Such metal hyperaccumulating plants could be used to remediate metal-contaminated soils [65].

4.4. Soil Enzymatic Activities, Bacterial Count, Microbial Biomass, and Organic C

Significantly, the highest activities of soil enzymes, microbial count, microbial biomass, and organic C were found in the B + LM treatment (Figure 3). Soil enzymes such as β-glucosidase, dehydrogenase, urease, acid phosphatase and peroxides are primarily secreted by soil microorganisms. Previous study has reported that the inoculation of microbes into HMs polluted soils enhances the activities of soil enzymes in them [66]. Likewise, it was observed that arbuscular mycorrhizal fungi and *Bacillus subtilis* BS1 inocula enhance the soil enzymatic activities [67]. Interestingly, another study reported that bacteria, algae,

fungi and protozoa secrete soil enzymes such as urease, cellulose dehydrogenase and invertase [68]. Moreover, the inoculation of *Serratia* spp. increases the bacterial count and microbial population in a Ni-polluted soil [69]. Likewise, the total number of bacteria were increased in a soil after receiving a bacterial inoculum [70]. Enzymes support plant growth and development via several processes such as mineralization of soil OM and mobilization of soil nutrients from fixed pools to make them available to the plants [71]. Furthermore, it has been reported that higher magnitude of bacterial root colonization leads to higher secretions of the enzymes [72]. Similarly, microbial biomass C depicts the overall activities of soil microorganisms [73]. The LM was rich in mineral nutrients and had higher contents of organic C (Supplementary Table S1). Supplementation of Ni-polluted soil with LM significantly increased the bacterial colonies via providing them essential nutrients and carbon source (Figure 3). It has been reported that the presence of bacteria communities and their root colonization have significant effects on the soil enzymatic activities [74]. Consequently, bacterial inoculation in HMs-stressed soil can result in higher enzymatic activities mainly due to the ability of bacteria to secrete more enzymes in the soil [72].

5. Conclusions

Results of this study reveals that the pH lowering capability of the PGPR *Bacillus* sp. ZV6 might be exploited not only to improve the growth of the *Salix alba* in the Ni contaminated soils but also its Ni phytoextraction capability. The use of LM, as an organic amendment, results into a significant improvement in the potentials of the bacterial strain as well as *Salix alba* for the enhancement of Ni phytoextraction from a Ni-contaminated soil and biological health of soil. Hence, the novel findings of this study could be helpful in designing green strategies for the remediation of soils contaminated with Ni and other HMs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14126975/s1, Figure S1: Neighbour joining phylogenetic tree of Bacillus sp. ZV16.; Figure S2: Growth of *Bacillus* sp. ZV16 in Nutrient Broth and LB broth media, and its impact on pH.; Table S1: Properties of leaf manure (LM) and animal manure (AM).

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