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Isolation of Glyphosate-Resistant Bacterial Strains to Improve the Growth of Maize and Degrade Glyphosate under Axenic Condition

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Abstract: Glyphosate is a non-selective herbicide that is used to control perennial weeds in agriculture. However, its vast application may result in glyphosate residues in the food chain. Due to its toxicity to non-target organisms, glyphosate-contaminated soils needed to be remediated, and bioremediation is a conventional remedial method. The success of this depends on the isolation of bacteria with the ability to degrade glyphosate. The goal of this study was to isolate glyphosate-degrading bacteria from the rhizosphere of maize and wheat with a repeated application history of glyphosate for 5–10 years and test their roles in promoting the growth of maize (*Zea mays*) and glyphosate degradation in vitro. Eleven isolated bacteria were inoculated, and their role in plant growth was compared at different levels (100 and 200 mg/kg) of glyphosate. The results revealed that *E. ludwigii* improved the highest shoot length by 26% and the root length by 34% compared to the control at 100 mg/kg. The relative water contents in leaves significantly improved by 58% using *P. aeruginosa* at 100 mg/kg. The maximum electrolyte leakage from leaves significantly reduced by 73% using *E. ludwigii* at 100 mg/kg compared to the control (uninoculated). A high-pressure liquid chromatography instrument was used to assess the glyphosate concentrations. The highest degradation of glyphosate was observed in treatments inoculated with *E. ludwigii* (99 and 40%), *P. aeruginosa* (95 and 39%), *K. variicola*, (91 and 38%) *E. cloacae* (92 and 38%), and *S. liquefaciens* (87 and 36%), respectively, at 100 and 200 mg/kg within 28 days. These five strains demonstrated a great potential for degrading glyphosate and promoting the growth of maize in vitro, and they will be further exploited for the biodegradation of glyphosate and the growth promotion of broader crop species in situ in the near future.

Keywords: biodegradation; glyphosate; maize; organophosphates; pesticides toxicity; plant-growth-promoting rhizobacteria

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1. Introduction

Agriculture persists as the most important means of global livelihood, despite recent declining trends. It consisted of 28% of global employment and 37% of the world's land area in 2018 [1]. Pest pressures pose great challenges to crop production; for instance, yield losses from weeds alone accounted for more than 70% of losses in *Arachis hypogaea* and *Glycine max* [2]. Weeds which grow in maize (*Zea mays*) crop, as well as the presence of *Palmer amaranth*, can greatly reduce the growth of maize. It has been reported that the presence of *P. amaranth* from 0.5 to 8 plants m^{-1} of row can reduce maize yield by 11 to 91% [3]. A

glyphosate-containing chemical compound (N-phosphonomethyl glycine) is one of the most broadly used weedkillers in agriculture. Commercially available glyphosate products naturally contain iso-propylamine salt of glyphosate for the eradication of unwanted plants in sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), beans (*Phaseolus vulgaris*), maize (*Zea mays*), and tomatoes (*Solanum lycopersicum*) [4]. The worldwide usage of transgenic varieties (maize) that show resistant to glyphosate (RR: Roundup Ready ®) has contributed to the extensive usage of glyphosate-containing compound [5]. The application rates of glyphosate vary in different crops, for instance, 3.9 kg/ha on soybean, 6.6 kg/ha on corn, 0.4 kg/ha on cotton, and 0.7 kg/ha on wheat [6]. At these application rates, measured glyphosate residues have exceeded 0.5 mg/kg soil in American and European crop fields, with between 0.01 and 500 µg/L in drains, lakes, ditches, wetlands, and ponds in the USA, and between 0.02 and 5200 µg/L in underground water and large streams [7]. The application of herbicide for the eradication of weed is viable and widespread in current agriculture production [8]. In Pakistan, nearly 1100 tons of glyphosate was imported in 2015, while this figure increased to 1700 tons in 2016 (The Express Tribune, 2017). Between 1974 and 2014, the share of the total consumption of glyphosate among different herbicides was only 1.5% globally, but this rose to 71.6% during 2005–2015 [9]. Glyphosate can prevent stomatal conductance, nodular metabolism, and the accumulation of its residue in roots, tubers, and bulbs [10]. The majority of glyphosate residues (at least 90%) were observed in the upper 15 cm of the soil. Such residues are the main contaminants to soil microbial activity and root absorption by non-target plants [11]. Mertens et al. [12] proposed that non-resistant glyphosate soybeans, especially during primary growth, due to glyphosate drift, reduced the nodulation activity and shoot growth of plants even during the frequent application of nitrates, which ultimately reduced the yield. Glyphosate may also interfere in the micro-nutrition of plants. Field studies in the United States and Brazil have observed that the routine application of glyphosate can lead to manganese (Mn), iron (Fe), and zinc (Zn) deficiencies in various species of crops [13]. The frequent utilization of glyphosate to soybeans crops in low-Fe-content fields exacerbated Fe and chlorine (Cl) deficiency [14]. Glyphosate can cause oxidative stress and lipid peroxidation in the roots of willow trees [15]. Lipid peroxidation in biological membranes is the most obvious symptom of plant oxidative stress and is closely related to the composition and constancy of cell membranes [16,17]. The intensive use of herbicides in cropping systems is a general practice and thus raises environmental concerns [18]. Due to the huge scale and rigorous use of glyphosate and its accumulation in the natural environment and edible products, numerous major concerns have surfaced in recent years regarding the detrimental side effects of glyphosate in plants, animals, and humans [19]. Based on recent reports on the potential chronic side effects of glyphosate [20], the World Health Organization reclassified glyphosate as probably carcinogenic to humans in 2015. The inoculation of glyphosate-tolerant plant-growth-promoting rhizobacteria (PGPR) is an evolving approach to decrease the harmful effects of glyphosate. Plant growth and yield are improving considerably due to PGPR inoculation [21]. Several PGPR have been identified which may improve plant growth through the production of different enzymes or by improving the uptake of macro and micronutrients from rhizosphere by numerous direct mechanisms such as the production of auxin, nitrogen fixation, and the solubilization of phosphorus [22]. Secondary mechanisms are also influenced by PGPR via the production of catalase, oxidase, and siderophores, as well as by reducing pathogenic activities through the production of lytic enzymes [23]. Various microorganisms are known for their plant-growth-promoting characteristics, such as *Acinetobacter*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, and *Serratia* [24]. The excessive usage of glyphosate results in it accumulating in the environment (soil, surface, and groundwater) and ultimately causing contamination [25].

Phytoremediation is the most important and emerging practice; within this practice, a microbial-assisted approach is used to degrade and decompose organic pollutants, a process known as rhizoremediation [26]. The bacterial strains which possess a plant-growth-promoting capability in glyphosate-polluted soil play a primary role in phytoremediation even under harsh environmental conditions [27]. Glyphosate-tolerant bacterial strains, also known as PGPR, which exist in soil possess combined effects, such as the capability to degrade glyphosate residue and secrete growth-promoting hormones [28]. Due to these combined effects, PGPR have been used as an alternative to degrade xenobiotics in the pursuit of sustainable agriculture. *Pseudomonas* and *Enterobacter* are the most studied PGPR, having the capability to improve growth and development in agricultural interest [29]. The biodegradation of glyphosate is primarily accomplished by soil microorganisms. The breaking of the C-N bond through the enzyme glyphosate oxidoreductase results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate in the first route [30]. Glyphosate oxidoreductase is actually a flavoprotein that uses FAD as a cofactor, which is decreased at its effective site by glyphosate. Under aerobic circumstances, oxygen is employed as a cofactor, whereas anaerobic environments use ubiquinone and phenazine methosulfate as electron acceptors [31]. Glyphosate oxidoreductase and C–P lyase are the key enzymes used for the biodegradation of glyphosate and its intermediate metabolite AMPA in microbes [32]. Through C-P lyase activity, AMPA is transformed to methylamine, which is then turned into formaldehyde by the methylamine dehydrogenase enzyme. Methanol is formed when formaldehyde combines with water or hydroxyl radicals. As a result, glyphosate biodegradation produces carbon dioxide, phosphate, ammonia, and methanol at the end [33]. Several glyphosate-degrading bacterial strains have been identified, but the most prevalent bacterial strains tested in liquid culture medium are *Pseudomonas fluorescens*, *Arthrobacter atrocyaneus*, and *Flavobacterium* sp. [34]. These bacteria have been used for degrading xenobiotics in the pursuit of agriculture. Previous studies mainly focused on the degradation of glyphosate through few bacterial strains in liquid culture medium, for instance, *Enterobacter cloacae* K7 degraded 5 mM of glyphosate in liquid culture within 24 h of application [35], *Ochrobactrum* sp. GDOS degraded 3 mM of glyphosate within 60 h after application [36], and *Burkholderia vietnamiensis* AO5-12 degraded 50 mg/L of glyphosate in liquid culture within 24 h [37]. Additionally, very little knowledge has been reported in the literature regarding the beneficial impact of glyphosate-degrading bacterial strains on plant growth promotion in a highly contaminated environment. It is hypothesized that the application of glyphosate-degrading PGPR can improve plant growth and degrade glyphosate residue from contaminated soil. The current study has ascertained the role of glyphosate-degrading rhizobacteria as inoculants, shown that they are proficient in degrading glyphosate, reducing glyphosate phytotoxicity, and improving the morphological and physiological characteristics of maize plants in glyphosate-spiked sand under an axenic condition.

2. Materials and Methods

2.1. Collection of Soil Samples

Rhizosphere (maize and wheat) samples were collected (December 2019–March 2020) from four different cities, Bahawalpur, Rahim Yar Khan, Faisalabad, and Multan (Pakistan), with a repeated glyphosate application history of 5–10 years. These collected soil samples were transferred into bags (Ziplock) and stored at 4 °C until further usage. Glyphosate-tolerant rhizobacterial strains were isolated and purified using an enrichment culture technique in which glyphosate was used as the only source of carbon. For this purpose, the rhizosphere soil samples (5 g) were added to a mineral salt media (MSM) [38] which consisted of KH_2SO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; Na_2SO_4, 5.8 g; and $MgSO_4.7H_2O$, 0.25 g in deionized water supplemented with Focht-trace-element-containing glyphosate (100 mg/L) and placed into a shaking incubator at 130 rpm and 28 °C for 14 days. After 14 days, we transferred 1 mL of inoculum to the freshly prepared MSM with glyphosate (150 mg/L) and again placed them into a shaking incubator under the same conditions

described above for 14 days. After that, the abovementioned process was repeated but the glyphosate concentration was enhanced to (200 mg/L). After 14 days, 300 µL of inoculum was poured on the MSM agar plate using a serial dilution technique and that was incubated for 48 h at 28 ± 2 °C under an aerobic environment to obtain glyphosate-tolerant bacterial strains.

2.2. Plant-Growth-Promoting Characteristics of Glyphosate-Degrading Bacterial Strains

The qualitative plant growth promotion characteristics of isolated bacterial strains were determined using the following protocols. General purpose media was used to determine the indole-3-acetic acid (IAA) production [39]. Siderophores production was checked according to the method described by Smith et al. [40]. The production of catalase was revealed as per the method explained by Janda [41]. Phosphorus solubilization was analyzed according to the method illustrated by Mehta and Nautiyal [42]. Exopolysaccharide determination was analyzed via the method explained by Ashraf et al. [43]. The chitinase activity was checked according to the method explained by Akeed et al. [44]. 1-aminocyclopropane-1-carboxylic acid (ACC) was determined by the method explained by Mehboob et al. [45]. The root colonization test was performed according to the procedure outlined by Simons et al. [46].

2.3. Analytical Procedure for Glyphosate Determination

Then, 10 g of sand spiked with glyphosate was taken from a plastic jar and was added into 50 mL centrifuge tubes; we then added 20 mL of 0.01 M KH_2PO_4 and this was shaken for 2 h on a rotary shaker. This process was followed by centrifugation at 8000 g for 10 min, after which the supernatants were filtered through a 0.22 µm syringe filter and transferred for the derivatization process. For the derivatization process, 1 mL of filtrate was added in centrifuge tubes (25 mL), then there was the addition of 1 mL of 0.02 M FMOC-Cl and 2 mL of 0.05 M borate buffer. The mixture was shaken at 4 g for 1 h on an end-to-end shaker, after which 2 mL of diethyl ether was added to each tube and this was vortexed for 2 min to remove unreacted FMOC-Cl. The organic layer was discarded, and the aqueous solution was transferred to GC vials for a further determination of glyphosate using high-pressure liquid chromatography (HPLC) [25]. All the chemicals were of analytical grade. Acetonitrile (HPLC grade), diethyl ether, glyphosate (99.7%), and FMOC-Cl (97%) were obtained from Sigma Aldrich® (Germany).

Glyphosate residue was determined using the Sykam HPLC system (Gewerbering 15 86922 Eresing, Germany) equipped with UV/Vis (Model S 3345) DAD (diode array detector); pump system (Model S1125G), a reverse-phase analytical column C18 (Sykam), and a column oven (Model S 4120). Acetonitrile (HPLC grade) and 0.05 M of KH_2PO_4 mixture (30:70 *v/v*) were used in isocratic mode for the mobile phase. The running time was 15 min, with a flow rate of 0.7 mL/min and a column temperature of 40 °C, while the injection volume was 20 µL. For the determination of glyphosate residue using the HPLC-DAD detector, 2 wavelengths, 210 and 315 nm, were used. The retention time of glyphosate was 3 min. Data were obtained and investigated using clarity chromatography computer software. For quantification, a calibration curve was constructed using the known quantities of glyphosate standards [47]. Glyphosate biodegradation was checked after 7-, 14-, and 28-day intervals, and the degraded concentration was calculated using Equation (1).

$$\text{Glyphosate degraded } (\text{mg kg}^{-1}) = T_0 - T_1 \quad (1)$$

where T_0 represents the glyphosate concentration at 0 h and T_1 represents the glyphosate concentration present in the sample.

2.4. Evaluation of Plant Growth Promotion under Axenic Conditions

Jar experiments were conducted to evaluate the biotic and abiotic degradation of glyphosate and the growth promotion characteristics of maize at 100 (level 1) and 200 (level 2) mg/kg concentrations of glyphosate in spiked sand. The location of the experiment

was a growth room within the Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan, where the location's latitude was 31.434321 and longitude was 73.071174, and the height from sea level was 186 m. The source of sand was Chenab River, with the location's latitude being 31.763844 and its longitude being 72.984714. A surface sterilization method was used by bathing the hybrid maize seed (P1429) in ethanol (95%) and $HgCl_2$ (0.2%) for at least 3 min in order to eliminate any bacterial entity [48]. The sand to be utilized was weighed and 500 g of sand was filled in plastic jars (400 cm^3) and double autoclaved; the respective isolated bacterial inoculum was prepared with a cell density of 10^{-7} CFU/mL measured on a spectrophotometer (OD = 600) and we added (5 mL) during the sowing of the maize (*Zea mays*) seed, with a total of 8 seeds per pot. After complete germination, the plants were thinned down to one plant per pot. Glyphosate 100 and 200 mg/kg was filter-sterilized (0.22 µm), mixed with distilled autoclaved water, and spiked on the sand in the respective treatments. The jars were incubated in a growth room (16 h light at 28 °C, 8 h darkness at 20 °C, 80% relative humidity (RH). Hoagland nutrient solution (half Strength) was applied within three-day intervals to maintain nutrients in the sand, while normal irrigation was maintained by autoclaved distilled water [49]. The jar trial was conducted in two experimental sets with the following layout: (1) control (non-inoculated) spiked with 100 mg/kg and 200 mg/kg of glyphosate concentration, and (2) the inoculation of isolated bacterial strains individually in respective jar spiked with 100 mg/kg and 200 mg/kg of glyphosate. A summary of the experimental design is displayed in Table 1. All treatments were arranged in a complete randomized design (CRD) with three replicates. A zero-time sample was collected after the application of glyphosate to analyze and maintain the 100 and 200 mg/kg concentration of glyphosate in spiked sand. Sand samples were taken within 7-, 14-, and 28-day intervals for the determination of the biotic and abiotic degradation of glyphosate according to the analytical procedure abovementioned in Section 2.3. Thirty days post-sowing, the plant samples were collected to calculate the morphological and biological parameters at the time of harvesting.

Table 1. Summary of experimental design.

Bacterial Strains	Glyphosate-Spiked Sand [a]	Glyphosate-Spiked Sand [a]
Control (non-inoculated)	100 mg/kg	200 mg/kg
WAG1	100 mg/kg	200 mg/kg
WAG2	100 mg/kg	200 mg/kg
WAG3	100 mg/kg	200 mg/kg
WAG4	100 mg/kg	200 mg/kg
WAG5	100 mg/kg	200 mg/kg
WAG6	100 mg/kg	200 mg/kg
WAG7	100 mg/kg	200 mg/kg
WAG8	100 mg/kg	200 mg/kg
WAG9	100 mg/kg	200 mg/kg
WAG10	100 mg/kg	200 mg/kg
WAG11	100 mg/kg	200 mg/kg

[a] All experimental units were carried out in triplicate.

2.5. Determination of Morphological and Physiological Parameters

Morphological parameters, i.e., the shoot and root length, shoot, and root fresh biomass, were determined after harvesting, while the above and below ground dry biomasses were determined after they were sun-dried and then placed in oven at 65 °C for 24 h. Physiological parameters, i.e., the gas exchange, were recorded 25 days after the germination of maize. These include the photosynthetic rate (A), stomatal conductance (gs), substomatal conductance (Ci), and transpiration rate (E) using CIRAS-3, a portable photosynthesis system (PLC3, USA). Chlorophyll pigments were determined using a chlorophyll meter (SPAD) 502 Plus, Spectrum technologies Inc., Paxinos, PA, USA [50].

2.6. Relative Water Content (RWC)

González and González-Vilar [51] developed the following formula for the determination of the relative water content as shown in Equation (2):

$$\text{Relative water content (RWC)} = (\text{FW} - \text{DW}) \div (\text{FTW} - \text{DW}) \quad (2)$$

where FW = fresh weight, DW = dry weight, and FTW = fully turgid weight. The leaf weight after it achieved 100% humidity for 48 h at 4 °C was designated to be the fully turgid weight.

2.7. Electrolyte Leakage

The electrolyte leakage was analyzed using the method outlined by Garraway et al. [52]. A uniform disk of fresh leaf was cut and dipped into a culture tube with 5 mL of distilled water (D.I.). Culture tubes were placed in an orbital shaker for 4 h at 28 ± 2 °C. The solution's conductivity caused by the leakage of ions from leaf was quantified, representing the 1st reading. After that, the solution in culture tubes was sterilized at 121 °C for 20 min in an autoclave and we again measured the solution's conductivity, representing the 2nd reading of the total ions present in the leaf disk. The % of total ions released through the leaf disk was computed from the subsequent formula as shown in Equation (3):

$$\text{Electrolyte leakage} = (\text{1st Reading} \div \text{2nd Reading}) \times 100 \quad (3)$$

The seed germination rate was checked in Petri plates and was determined by each of the plates containing 8 maize seeds (*Zea mays*) with 3 replications. Then, 10 ml of inoculum with a different concentration of glyphosate (100 and 200 mg/L) was poured in each Petri plate and incubated at 29 ± 2 °C for 72 h. Seed germination (%) was assessed according to the International Seed Testing Association Plant Evaluation Manual. The germination rate of the seeds was determined as the ratio of the number of germinated seeds to the total number of the tested seeds multiplied by 100 [53].

2.8. Statistical Analysis

All the experimental set-up was carried out in triplicate under a completely randomized design in factorial design. The treatment means were compared using Tukey's HSD test at $p < 0.05$ using XLSTAT software [54]. Pearson's correlation was applied to analyze the correlation among different parameters on RStudio 2022.07.1 Build 554. Principal component analysis (PCA) was used to check the positive and negative correlation between the isolated bacterial strains and parameters using OriginPro 2022b software [55].

2.9. Bacterial Identification

Freshly prepared colonies (48 h) were sent to Macrogen, Korea, for the identification of isolated bacterial strains. Genomic DNA was isolated according to the Macrogen protocol and was used as a template for polymerase chain reaction (PCR). The amplification of 16S ribosomal rRNA gene was achieved using precise primers through polymerase chain reaction (PCR). The forward primer consisted of the following base pairs: 27F 5′ (AGA GTT TGA TCM TGG CTC AG) 3′, and the reverse primer consisted of the following base pairs: sequence 1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3′ [56]. The PCR conditions were 94 °C for 5 min, 94 °C for 1 min, and 50 °C for 1 min, followed by 30 cycles of 72 °C for 90 s, with a final step at 72 °C for 10 min. The result of the sequencing was submitted to GenBank for BLAST analysis to generate the accession number.

3. Results

3.1. Isolation and Selection of Glyphosate-Degrading Bacteria

Upon multiple enrichments of glyphosate in various soil samples, 11 bacterial strains were isolated from the 10 different enrichments. Typically, a few colonies were obtained from each culture plate. These 11 glyphosate-degrading bacteria represent diverse rhizobacteria in different locations and crop types. They were further screened for plant growth promotion characteristics including indole acetic acid, siderophore production, catalase, phosphorus solubilization, exopolysaccharides, chitinase, 1-aminocyclopropane-1-carboxylic acid (ACC), and root colonization activity, which are summarized in Table 2. These 11 rhizobacterial strains, designated as WAG1, WAG2, WAG3, WAG4, WAG5, WAG6, WAG7, WAG8, WAG9, WAG10, and WAG11, were used in this study.

Table 2. Plants growth regulator traits of 11 glyphosate-degrading bacteria.

Strain Name	Gram Stain	Indole Acetic Acid	Siderophore	Catalase	P Solubilization	Exopolysaccharides	Chitinase	1-Aminocyclopropane-1-Carboxylic Acid	Root Colonization
WAG1	+ve	+	+	+	+	-	-	+	+
WAG2	-ve	+	+	+	+	+	+	+	+
WAG3	+ve	+	+	-	+	+	-	+	-
WAG4	-ve	+	+	+	+	+	-	+	+
WAG45	-ve	+	+	+	+	+	-	+	+
WAG6	-ve	+	+	+	+	-	-	+	+
WAG7	+ve	+	+	+	+	+	+	+	+
WAG8	+ve	-	+	+	+	+	-	+	+
WAG9	-ve	+	+	+	+	+	-	+	+
WAG10	-ve	+	+	+	+	+	-	+	-
WAG11	-ve	+	+	+	+	+	-	+	+

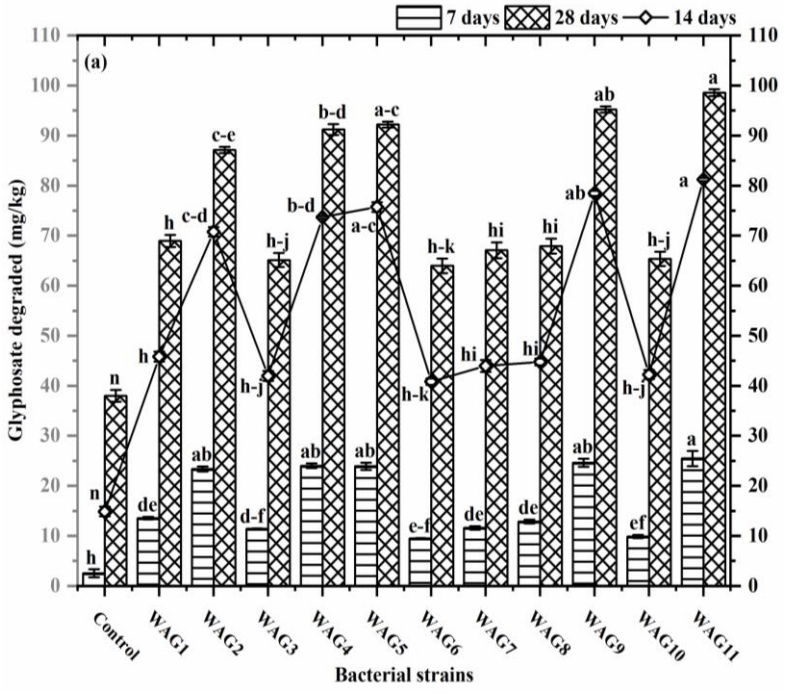
+ = character present, - = character absent.

3.2. Comparison of Glyphosate Degradation Efficiency of Selected Strains

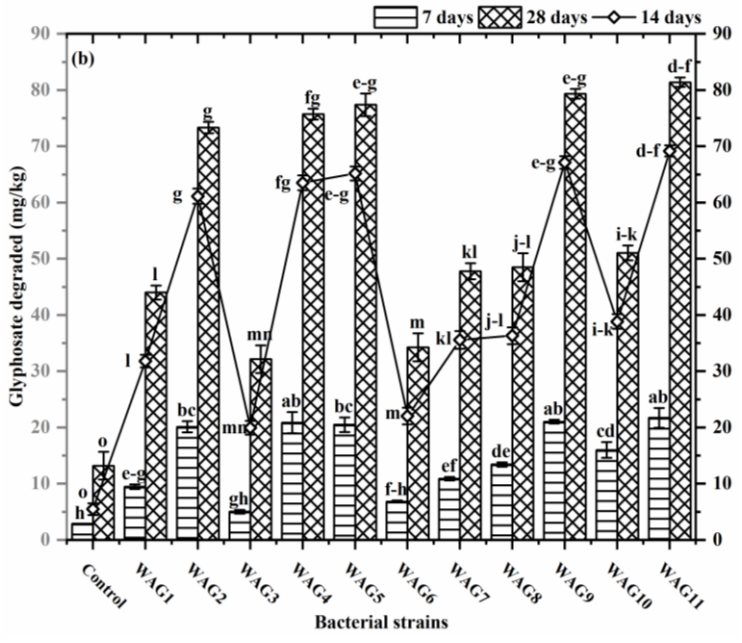
To compare the glyphosate degradation efficiency, the 11 strains were exposed to 2 different levels of glyphosate (100 and 200 mg/kg), as displayed in Figure 1a,b. Among these 11 strains, WAG2, WAG4, WAG5, WAG9, and WAG11 strains significantly outperformed other strains in terms of glyphosate degradation at all 3 time points (day 7, 14, and 28) and both glyphosate levels, as showed in Figure 1a,b. WAG11 had the highest degradation efficiency and degraded 98.6% and 40.7% glyphosate after 28 days at level 1 (100 mg/kg) and level 2 (200 mg/kg) of glyphosate, respectively. WAG9 degraded 95.2% and 39.7% after 28 days at level 1 and 2. WAG4 degraded 91.2% and 37.9% glyphosate at level 1 and 2 of glyphosate. WAG5 degraded 92.2% and 38.7% at level 1 and 2. WAG2 degraded 87.1% and 36.7% at level 1 and 2 of glyphosate. The glyphosate degradation was 38% at level 1 and 6.6% at level 2 in the control (without bacterial inoculation) after 28 days.

3.3. Growth Promotion of Maize Plant by the Addition of Selected Bacteria

Interestingly, these five strains (WAG2, WAG4, WAG5, WAG9, and WAG11) also showed the stronger promotion of shoot length and weight compared to the other six strains at both glyphosate levels, as presented in Table 3. All 11 strains yielded a longer shoot length and higher shoot weight than the control after 28 days. For example, the shoot length of maize with WAG11 is 18 cm longer than that of the control at level 1, as summarized in Table 3. The measurement of root length and weight also showed that these five bacterial strains (WAG2, WAG4, WAG5, WAG9, and WAG11) resulted in better root development than the other strains, as shown in Table 3. For example, maize with WAG11 increased the root length by 141% compared to the control (uninoculated) at level 1 of glyphosate. At level 2 of glyphosate, the root length of maize with WAG11 was also longer than the control, but the growth improvement at level 2 was not as profound as at level 1.



(**a**)



(**b**)

Figure 1. Removal of glyphosate by eleven isolated bacteria in autoclaved sand containing 100 mg/kg glyphosate (**a**) and 200 mg kg glyphosate (**b**) at 7, 14, and 28 days, respectively. The control contained the same culture medium without bacterial inoculation. Results represent the mean value of three replicates and error bars indicate the standard deviations. Treatment means sharing the same letter(s) do not differ significantly at $p < 0.05$.

Table 3. Effect of isolated bacterial strains on morphological characteristics of maize plants in glyphosate-spiked sand.

Bacterial Strains	Shoot Length (cm)		Root Length (cm)		Shoot Fresh Weight (g)		Root Fresh Weight (g)	
	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg
Control	30.3 ± 0.88 f-i	17.3 ± 0.33 j	3.2 ± 0.15 hi	2.27 ± 0.12 i	1.7 ± 0.1 c-e	1.03 ± 0.03 f	1.2 ± 0.06 ef	0.95 ± 0.1 f
WAG1	32 ± 1.15 e-h	27.3 ± 0.88 hi	5.57 ± 0.12 b-d	3.53 ± 0.29 g-i	2.3 ± 0.06 ab	1.67 ± 0.14 de	1.87 ± 0.14 cd	1.68 ± 0.08 c-e
WAG2	42 ± 0.57 bc	35 ± 1.15 d-f	6.6 ± 0.1 ab	5.33 ± 0.09 b-f	2.52 ± 0.06 a	2.17 ± 0.09 a-d	2.47 ± 0.09 ab	2.02 ± 0.06 b-d
WAG3	34.3 ± 0.88 d-g	26 ± 1 i	4.6 ± 0.71 c-g	4.27 ± 0.42 e-h	2.23 ± 0.09 a-d	1.33 ± 0.07 ef	1.83 ± 0.07 cd	1.65 ± 0.1 c-e
WAG4	44.7 ± 0.66 ab	36 ± 0.57 d-f	7.33 ± 0.09 a	5.57 ± 0.12 b-d	2.53 ± 0.09 a	2.18 ± 0.02 a-d	2.53 ± 0.03 ab	2.07 ± 0.09 b-d
WAG5	44.3 ± 0.88 ab	36.7 ± 1.45 c-e	7.3 ± 0.1 a	5.53 ± 0.2 b-e	2.53 ± 0.11 a	2.17 ± 0.05 a-d	2.52 ± 0.13 ab	2.06 ± 0.03 b-d
WAG6	34 ± 1.52 d-g	28 ± 0.57 hi	5.93 ± 0.12 b	3.83 ± 0.18 gh	2.2 ± 0.15 a-d	1.65 ± 0.16 de	1.9 ± 0.11 cd	1.57 ± 0.17 de
WAG7	37.3 ± 0.33 c-e	27.3 ± 0.88 hi	5.43 ± 0.2 b-e	4.13 ± 0.18 f-h	2.17 ± 0.12 a-d	1.67 ± 0.14 de	1.83 ± 0.07 cd	1.63 ± 0.07 de
WAG8	37.3 ± 1.85 c-e	29 ± 1.15 g-i	5.5 ± 0.38 b-e	4.37 ± 0.35 d-h	2.27 ± 0.12 a-c	1.83 ± 0.13 b-e	1.88 ± 0.04 cd	1.7 ± 0.06 c-e
WAG9	45.7 ± 0.88 ab	36.7 ± 0.33 c-e	7.47 ± 0.13 a	5.6 ± 0.17 b-d	2.57 ± 0.09 a	2.19 ± 0.06 a-d	2.57 ± 0.09 ab	2.1 ± 0.15 b-d
WAG10	37.3 ± 0.88 c-e	29 ± 1 g-i	5.77 ± 0.09 bc	4.27 ± 0.09 e-h	2.26 ± 0.09 a-c	1.77 ± 0.12 b-e	1.77 ± 0.07 cd	1.6 ± 0.15 de
WAG11	48.3 ± 2.39 a	38.3 ± 0.88 cd	7.73 ± 0.34 a	5.73 ± 0.09 bc	2.7 ± 0.06 a	2.23 ± 0.08 a-d	2.73 ± 0.03 ab	2.2 ± 0.15 a-c
HSD value ($p \leq 0.05$)	5.88		1.2954		0.5731		0.5445	

Results represent the mean value ± standard deviation of three replications. Treatment means sharing same letter(s) within the column are statistically non-significant according to Tukey's HSD test at $p \leq 0.05$.

Maize treatments inoculated with the respective bacterial strains showed a higher shoot and root fresh weight than the control. While the maize amended with WAG11, WAG9, WAG5, WAG4, and WAG2 showed a 58.82, 50.98, 49, 49.02, and 48.43% higher shoot fresh weight, respectively, compared to the control, the root fresh weight of maize amended with WAG11, WAG9, WAG5, WAG4, and WAG2 was improved by 127, 113, 110, 111, and 101%, respectively, compared to the control (uninoculated) at level 1 of glyphosate, as presented in Table 3. The growth of maize with bacterial amendment was significantly improved compared to that of the control at both levels of glyphosate. A better improvement of the shoot and root length and root and shoot fresh weight was observed at level 1 of glyphosate compared to level 2 of glyphosate. The plant growths were significantly improved in all the treatments (with each of the 11 bacteria) compared to the control (without bacterial inoculation) at both levels of glyphosate (100 mg/kg and 200 mg/kg).

3.4. Effect of Bacterial Amendment on Maize Physiology

The application of 11 isolated bacterial strains significantly reduced the electrolyte leakage caused by glyphosate stress (100 mg/kg) on maize plants compared to the control. Moreover, the 11 bacterial strains' inoculation significantly improved the chlorophyll SPAD value and relative water content compared to the control at level 1 (100 mg/kg) and level 2 (200 mg/kg), as summarized in Table 4. The maximum electrolyte leakage was reduced by 59.18% on average with the amendment of WAG2, WAG4, WAG5, WAG9, and WAG11. At level 2 of glyphosate, the maximum electrolyte leakage reduction for the same five bacterial strains was averaged at 43.05% compared to the control. The inoculation of 11 isolated bacterial strains significantly improved the gas exchange parameters, such as the photosynthetic rate, transpiration rate, stomatal conductance, and sub-stomatal conductance compared to the control at level 1 and level 2, as summarized in Table 5.

Table 4. Effect of isolated bacterial strains on physiological characteristics of maize plants in glyphosate-spiked sand.

Bacterial Strains	Electrolyte Leakage (%)		Relative Water Contents (%)		Chlorophyll SPAD (%)	
	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg
Control	61 ± 1.15 c	78.7 ± 1.33 a	51 ± 1.15 g-i	38 ± 0.57 j	23.93 ± 0.78 hi	16.83 ± 0.93 k
WAG1	42.7 ± 0.66 de	70.3 ± 1.2 b	57 ± 1.15 fg	49 ± 0 hi	29.2 ± 0.5 fg	20.93 ± 0.8 i-k
WAG2	27.3 ± 1.2 f	47.7 ± 1.76 d	66.67 ± 1.2 cd	58.67 ± 2.72 ef	33.33 ± 0.43 b-f	28.77 ± 0.52 g
WAG3	45.3 ± 1.2 de	68 ± 3.2 bc	59 ± 2.07 ef	48.33 ± 1.33 hi	27.57 ± 0.6 gh	20.97 ± 0.96 i-k
WAG4	24.7 ± 0.88 f	45.7 ± 1.45 de	74.33 ± 0.88 ab	65 ± 0.57 c-e	36 ± 0.26 a-c	30.73 ± 0.94 d-g
WAG5	26 ± 1.52 f	46.3 ± 1.2 d	74 ± 1.52 ab	64.67 ± 1.45 c-e	34.5 ± 0.49 a-d	29.33 ± 0.33 fg
WAG6	42.7 ± 0.66 de	68.3 ± 2.32 bc	59.67 ± 0.66 ef	50.33 ± 0.66 g-i	29.43 ± 0.44 fg	22.37 ± 1.46 ij
WAG7	38.7 ± 1.2 e	61 ± 1.15 c	55 ± 1.15 f-h	45.33 ± 0.88 i	29 ± 0.55 g	23.2 ± 1.39 ij
WAG8	43.3 ± 1.2 de	67.7 ± 0.66 bc	54 ± 1.15 f-h	46.67 ± 1.2 i	28.27 ± 0.86 g	19.53 ± 0.52 jk
WAG9	22 ± 1.52 fg	43.3 ± 1.2 de	80.67 ± 1.33 a	69 ± 1.15 bc	37.3 ± 1 ab	31.7 ± 0.4 c-g
WAG10	38.3 ± 1.2 e	73.3 ± 0.88 ab	60 ± 1.52 d-f	45 ± 0.57 i	29.53 ± 0.49 e-g	21.33 ± 1.34 ij
WAG11	16.3 ± 0.33 g	41 ± 1.15 de	76.33 ± 0.88 a	66.67 ± 1.45 cd	40.4 ± 0.95 a	33.77 ± 0.82 b-e
HSD value ($p \leq 0.05$)	7.585		6.9887		4.3161	

Results represent the mean value ± standard deviation of three replications. Treatment means sharing same letter(s) within the column are statistically non-significant according to Tukey's HSD test at $p \leq 0.05$.

Table 5. Effect of isolated bacterial strains on gas exchange parameters of maize plants in glyphosate-spiked sand.

Bacterial Strains	Photosynthetic Rate (umole CO_2 m^{-2} S^{-1})		Transpiration Rate (mmol H_2O m^{-2} S^{-1})		Stomatal Conductance (mmol H_2O m^{-2} S^{-1})		Sub-Stomatal CO_2 Concentration (μmol^{-1})	
	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg
Control	28 ± 1.15 c–f	18 ± 1.15 h	7.2 ± 0.51 c–e	4.5 ± 0.36 i	451 ± 7.2 e–i	344 ± 6.63 j	149 ± 11.32 jk	102 ± 6 l
WAG1	29 ± 2.07 c–e	20 ± 1.33 gh	7.5 ± 0.41 c–e	4.7 ± 0.41 g–i	452 ± 10.06 e–i	401 ± 11.51 h–j	167 ± 11.81 h–k	133 ± 10.63 kl
WAG2	32 ± 0.57 b–d	24 ± 1 e–h	8.1 ± 0.15 a–d	6.2 ± 0.06 e–h	503 ± 9.8 b–e	434 ± 6.9 f–i	280 ± 11.92 bc	250 ± 15.1 cd
WAG3	29 ± 1.72 c–e	20 ± 0.33 gh	7.4 ± 0.11 c–e	4.9 ± 0.14 f–i	475 ± 23.81 d–f	405 ± 15.61 h–j	152 ± 13.51 i–k	126 ± 1.15 kl
WAG4	35 ± 0.88 a–c	28 ± 0.66 c–f	8.6 ± 0.27 a–c	6.5 ± 0.09 d–f	543 ± 16.99 a–c	466 ± 6.46 e–h	290 ± 12.65 a–c	250 ± 14.38 c–e
WAG5	34 ± 1.45 b–d	27 ± 0.66 d–g	8.4 ± 0.4 a–c	6.4 ± 0.09 e–g	538 ± 19.84 a–d	460 ± 4.68 e–h	290 ± 11.88 a–c	256 ± 16.88 b–d
WAG6	29 ± 0.88 c–e	20 ± 0.88 gh	7.8 ± 0.21 b–e	4.7 ± 0.53 hi	468 ± 19.88 e–h	406 ± 6.46 g–j	225 ± 12.43 d–f	184 ± 14.67 f–j
WAG7	29 ± 1.72 c–e	21 ± 1.76 f–h	7.5 ± 0.14 c–e	5 ± 0.34 f–i	472 ± 18.26 d–g	422 ± 5.75 f–i	205 ± 13.17 e–h	180 ± 14.37 g–j
WAG8	29 ± 1 c–e	20 ± 1.2 gh	7.7 ± 0.33 b–e	5 ± 0.12 f–i	455 ± 13.83 e–h	385 ± 13.32 ij	217 ± 14.1 d–g	181 ± 17.05 f–j
WAG9	37 ± 1 ab	30 ± 1.52 b–e	9.3 ± 0.35 ab	7 ± 0.41 c–e	558 ± 10.53 ab	477 ± 5.15 c–f	299 ± 12.37 ab	256 ± 15.43 b–d
WAG10	29 ± 1.45 c–e	21 ± 1.33 gh	7.4 ± 0.29 c–e	5.2 ± 0.24 f–i	455 ± 15.7 e–h	403 ± 12.29 h–j	195 ± 11.51 f–i	160 ± 17.24 h–k
WAG11	41 ± 1.33 a	32 ± 2.07 b–d	9.7 ± 0.32 a	7.2 ± 0.38 c–e	598 ± 5.27 a	509 ± 4.89 b–e	325 ± 4.46 a	275 ± 12.45 bc
HSD value ($p \leq 0.05$)	7.2316		1.6838		67.436		44.86	

Results represent the mean value ± standard deviation of three replications. Treatment means sharing same letter(s) within the column are statistically non-significant according to Tukey's HSD test at $p \leq 0.05$.

3.5. Effect of Bacterial Supplement on Seed Germination of Maize

The inoculation of 11 bacterial strains in the existence of glyphosate considerably enhanced germination rates and root emergence compared to the control at level 1 and 2. The germination of maize seed was clearly observed in WAG2, WAG4, WAG5, WAG9, and WAG11 treatments, which showed a 50 to 75% improved germination rate in relevance to the control at level 1, but at level 2, WAG2, WAG4, WAG5, WAG9, and WAG11 showed a 150 to 200% improved germination compared to the respective control (uninoculated). For the control, the germination rate at level 2 was only half of level 1. Additional seed germination data are available in Table 6.

Table 6. Effect of isolated bacterial strains on seed germination rate in glyphosate-spiked sand.

Bacterial Strains	100 mg/kg [a]	200 mg/kg [a]
Control	4 ± 0.57 cd	2 ± 0.57 e
WAG1	5 ± 0 bc	3 ± 0 de
WAG2	7 ± 0 a	6 ± 0 ab
WAG3	5 ± 0.57 bc	4 ± 0 cd
WAG4	7 ± 0 a	6 ± 0 ab
WAG5	6 ± 0 ab	5 ± 0 bc
WAG6	5 ± 0 bc	4 ± 0 cd
WAG7	5 ± 0 bc	3 ± 0.57 de
WAG8	3 ± 0 de	3 ± 0 de
WAG9	7 ± 0 a	5 ± 0 bc
WAG10	4 ± 0 cd	3 ± 0.57 de
WAG11	7 ± 0 a	5 ± 0 bc
HSD value ($p \leq 0.05$)	1.4132	

[a] Results represent the mean value ± standard deviation of three replications. Treatment means sharing same letter(s) within the column are statistically non-significant according to Tukey's HSD test at $p \leq 0.05$.

3.6. Correlation Analysis

The Pearson correlation matrix heatmap revealed a strong correlation among glyphosate degradation and plant morphological and physiological characteristics as displayed in Figure 2a (100 mg/kg) and Figure 2b (200 mg/kg). The shoot fresh weight was highly positively correlated to glyphosate degradation after 28 days at 100 mg/kg (r = 0.98), and the root fresh weight showed a strong correlation to glyphosate degradation after 28 days at 100 mg/kg (r = 0.99). Electrolyte leakage was strongly negatively correlated with glyphosate degradation after 14 days at 100 mg/kg (r = −0.98). Furthermore, the photosynthetic rate correlated with the chlorophyll SPAD value at 100 mg/kg (r = 0.96).

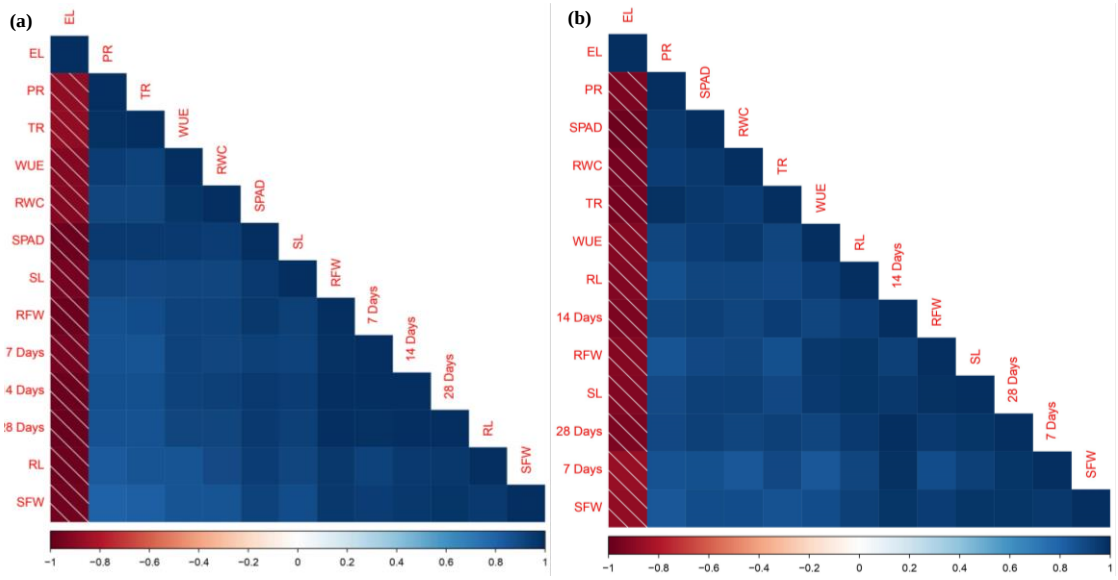


Figure 2. Pearson's correlation matrix heat map shows correlations among different parameters in axenic experiments with (**a**) 100 and (**b**) 200 mg/kg glyphosate. 7 days = degradation of glyphosate at 7 days, 14 days = degradation of glyphosate at 14 days, 28 days = degradation of glyphosate at 28 days, SL = shoot length, RL = root length, SFW = shoot fresh weight, RFW = root fresh weight, PR = photosynthetic rate, EVT = transpiration rate, WUE = water use efficiency, RWC = relative water content, EL = electrolyte leakage, SPAD = chlorophyll SPAD value.

3.7. Glyphosate-Degrading Bacteria with Plant Growth Promotion Capability

The PCA analysis showed that strains WAG2, WAG4, WAG5, WAG9, and WAG11 are positively correlated with most of the parameters used in this study (Figure 3). The important parameters include the degradation of glyphosate, shoot length, root length, shoot and root fresh weight, photosynthetic rate, water use efficiency, transpiration rate, relative water content, electrolyte leakage, and chlorophyll SPAD value at 100 and 200 mg/kg. When the preceding parameters were considered, strains WAG2, WAG4, WAG5, WAG9, and WAG11 clearly outperformed the remaining bacterial strains. These five bacterial strains were further characterized based on their 16S rRNA gene sequences. WAG2 was identified as *Serratia liquefaciens* with a GenBank accession number of MW375469, WAG4 as *Klebsiella variicola* with a GenBank accession number of MW375470, WAG5 *as Enterobacter cloacae* with a GenBank accession number of MW375471, WAG9 as *Pseudomonas aeruginosa* with a GenBank accession number of MW375472, and WAG11 as *Enterobacter ludwigii* with a GenBank accession number of MW375473.

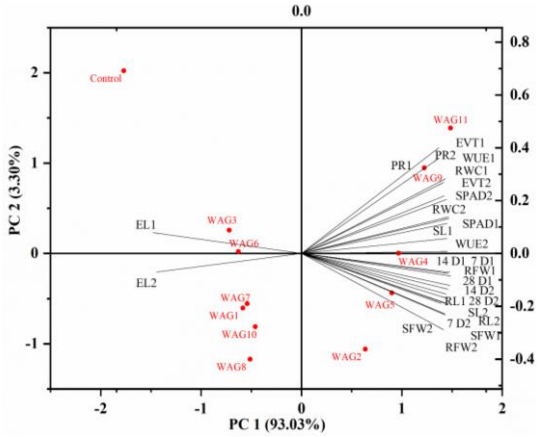


Figure 3. Principal components analysis showing positive and negative correlations between eleven different bacteria and parameters in the experiments with 100 and 200 mg/kg glyphosate. 7 D1 = degradation of glyphosate after 7 days at 100 mg/kg, 14 D1 = degradation of glyphosate after 14 days at 100 mg/kg, 28 D1 = degradation of glyphosate after 28 days at 100 mg/kg, 7 D2 = degradation of glyphosate after 7 days at 200 mg/kg, 14 D2 = degradation of glyphosate after 14 days at 200 mg/kg, 28 D2 = degradation of glyphosate after 28 days at 200 mg/kg, SL1 = shoot length at 100 mg/kg, SL2 = shoot length at 200 mg/kg, RL1 = root length at 100 mg/kg, RL2 = root length at 200 mg/kg, SFW1 = shoot fresh weight at 100 mg/kg, SFW2 = shoot fresh weight at 200 mg/kg, RFW1 = root fresh weight at 100 mg/kg, RFW2 = root fresh weight at 200 mg/kg, PR1 = photosynthetic rate at 100 mg/kg, PR2 = photosynthetic rate at 200 mg/kg, EVT1 = transpiration rate at 100 mg/kg, EVT2 = transpiration rate at 200 mg/kg, WUE1 = water use efficiency at 100 mg/kg, WUE2 = water use efficiency at 200 mg/kg, RWC1 = relative water content at 100 mg/kg, RWC2 = relative water content at 200 mg/kg, EL1 = electrolyte leakage at 100 mg/kg, EL2 = electrolyte leakage at 200 mg/kg, SPAD1 = chlorophyll SPAD value at 100 mg/kg, SPAD2 = chlorophyll SPAD value at 200 mg/kg.

4. Discussion

The growth of plants can be affected by environmental pollutants such as the presence of excessive salts, heavy metals, pesticides, and herbicides residue in soil, which can alter plant chemistry, growth, and biomass production [57]. Eleven bacterial strains efficient for degrading glyphosate were isolated from glyphosate-contaminated soil using an enrichment technique. Among them, five strains, WAG2, WAG4, WAG5, WAG9, and WAG11, showed a superior performance on the chlorophyll content (SPAD), electrolyte leakage, relative water contents, photosynthetic rate, stomatal and sub-stomatal conductance, and shoot and root length and weight in sand under axenic conditions. They were identified as *Serratia liquefaciens*, *Klebsiella variicola*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and

Enterobacter ludwigii. It was reported previously that *Enterobacter* sp. and *Pseudomonas* sp. could degrade 2-aminoethylphosphonate, pentachlorophenol [58], and chlorpyrifos. *Serratia marcescens* to degrade organophosphorus compound pesticides, i.e., fenitrothion, chlorpyrifos, and parathion was studied in MSM and in three different characteristics of soils. *Serratia marcescens* could use all pesticides at a concentration of 50 mg/L as the individual carbon source when grown in MSM 70.5%, 58.9%, and 82.5% of the initial quantity of fenitrothion, chlorpyrifos, and parathion, respectively, which was degraded within 14 days [59]. Zhang et al. [60] found that when 50 mg/L of atrazine was added in culture media, 81.5% was degraded through *Klebsiella variicola* in 11 days.

The current study indicates that isolated bacterial strains are able to degrade glyphosate at two different concentrations of glyphosate (100 mg/kg and 200 mg/kg) in most cases; 87.1% to 98.6% of glyphosate at level 1 and 36.7% to 40.7% at level 2 was removed after 28 days of application, while only five strains were very efficient against glyphosate degradation in sand under axenic conditions. However, it is important to point out that at 200 mg/kg of glyphosate, degradation was very low (40.7%) after 28 days due to greater stress on the bacterial culture which resulted from their lack of adaptation to molecule metabolism, mainly considering the toxicity generated by the glyphosate. Hertel et al. [61], with *Bacillus subtilis*, showed that 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase does not facilitate any changes that enhance the resistance of the enzyme to glyphosate. On the other hand, any variation in the amino acid sequence of the *B. subtilis* EPSP synthase might decrease the enzyme activity significantly to permit the survival of the bacteria. There is a chance that the excessive toxicity of glyphosate may alter the structural change in the amino acids of the isolated bacterial strains and reduce the microbial biomass in spiked sand. Nguyen et al. [62] reported that soil microbial biomass was significantly lower at glyphosate concentrations of 10–100 mg/kg. In greenhouse experiments, the application of glyphosate at the recommended dosage (<10 mg/kg) has recently been found to affect enzymatic activity and microbial composition in plant rhizospheres as well as in bulk soil [63]. Disturbing consequences of glyphosate on earthworms and their interactions with symbiotic mycorrhizal fungi have also been reported [64]. The consortium of *Pseudomonas aeruginosa* and *Bacillus cereus* degraded 85% glyphosate at 7.2 mg mL^{-1} after 45 days [65]. Few bacterial strains secrete an oxidoreductase enzyme, which degrades glyphosate into APMA and glyoxylate such as *Flavobacterium* sp. strain GD1, *Arobacterium radiobacter*, and *Arthrobacter atrocyaneus* ATCC 13,752 [66]. *Stenotrophomonas acidaminiphila* Y4B completely degraded 50 mg/L of glyphosate with a degradation efficiency of over 98% within 72 h [67].

The breakdown of carbon phosphorus converts glyphosate into sarcosine, which eventually forms formaldehyde and glycine in a reaction initiated by sarcosine oxidase. Certain bacterial strains were found to have carbon phosphorus lyases activity, such as *Pseudomonas* sp. and *Arthrobacter* sp. strain GLP-1 [68]. It is possible that isolated bacterial isolates *Serratia liquefaciens, Klebsiella variicola, Enterobacter cloacae, Pseudomonas aeruginosa*, and *Enterobacter ludwigii* also breakdown glyphosate through both AMPA and sarcosine pathways. It has been reported that glyphosate can be degraded by *Pseudomonas* sp. and *O. anthropic* using two different mechanisms. These two bacterial strains utilized glyphosate as a source of phosphorus and carbon [69]. However, WAG2 (*Serratia liquefaciens*), WAG4 (*Klebsiella variicola*), WAG45 (*Enterobacter cloacae*), WAG9 (*Pseudomonas aeruginosa*), and WAG11 (*Enterobacter ludwigii*) might utilize glyphosate as a source of carbon and phosphorus within a phosphorus starving environment. This is the first report on the capacity of *Serratia liquefaciens*, *Klebsiella variicola*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Enterobacter ludwigii* to degrade glyphosate and also promote the growth of plants in sand under an axenic condition within 28 days.

The current study found that the isolated bacteria WAG2 (*Serratia liquefaciens*), WAG4 (*Klebsiella variicola*), WAG45 (*Enterobacter cloacae*), WAG9 (*Pseudomonas aeruginosa*), and WAG11 (*Enterobacter ludwigii*) were able to increase the maize shoot length by 39–59% and the maize root length by 106–141%, while the shoot and root fresh weight were improved by 48–59% and 105–127%, respectively, in the inoculated sand. This has not been reported

in earlier axenic studies. Biodegradation will not only retrieve the glyphosate-polluted soil but will also improve the fertility of the soil. These microorganisms improve plant growth through plant hormones and the solubilization of insoluble nutrients in the soil [70]. The free-living rhizobacterium *P. rhizophila* S211 has a great potential to increase plant development by enhancing nitrogen fixation, phosphate solubilization, ACC deaminase, and phytohormone synthesis according to in vitro bio-chemical studies and genomic research [71]. *Pseudomonads* have been shown in many studies to effectively colonize roots and reduce soil-borne fungal infections by releasing iron-chelating pyoverdines [72]. Bacterial inoculated plants have revealed that an increased biomass, foliar area, photosynthetic pigment content, and cell membrane integrity might all be linked to a greater adaptability and less oxidative damage [73]. In the presence of herbicide, the inoculation of bacteria, *Azospiril*-lum, or *Pseudomonas* boosted the plant phytohormone concentration [74]. The increase in the endogen content of jasmonic acid (JA) in inoculated plants in comparison with the non-inoculated ones indicates that there could be a better response signal before the herbicide presence because this phytohormone modulates the responses to stress and development. The levels of IAA and abscisic acid (ABA) were greater in *Azospirillum* inoculation, suggesting that the presence of this bacteria elevates the levels of these phytohormones [75]. The synthesis of growth regulators is one of the reasons for *Azospirillum's* stimulatory effects on plant development [76]; such regulators have been discovered in the supernatants of these bacterial cultures. IAA produced by bacteria can modify the phytohormone content of plants, leading to their growth stimulation [77]. Glyphosate-degrading bacteria can also produce IAA hormone that can help to increase plant growth [78]. Plants secrete specialized secondary metabolites, for instance, coumarins for *Arabidopsis thaliana*, which can improve Fe mobilization and generate reactive oxygen species to subdue root bacterial communities that contend with plants for Fe [79]. It has been reported that reactive oxygen species (ROS) control the plant cell redox system and stimulate the roots' elongation [80]. Correspondingly, the stress generated in the presence of reactive nitrogen and reactive oxygen species was detected to change the root structure and promote its branching. It was expressed that *Pseudomonas* and *Enterobacter* inoculation expanded the lateral roots of maize plant [81]. The development of lateral roots was also revealed by the *Bacillus* spp. inoculated *Cucumis sativus* [82]. Tomato plant with a high resistance to *Ralstonia solanacearum* enriches *Flavobacterium spp*. in the soil to subdue pathogens [83]. Plant-growth-promoting rhizobacteria improve the biodegradation of glyphosate as well reestablish soil abiotic and biotic components [84].

The current study reported that the inoculation of these five bacteria improved the chlorophyll content, relative water content, gas exchange parameters, such as the photosynthetic rate, stomatal conductance, transpiration rate, and sub-stomatal conductance. However, electrolyte leakage was reduced in the inoculated treatments. Plant-growth-promoting rhizobacteria have been effectively used to decrease stress in plants in contaminated soils. The inoculation of PGPR may ascertain the soil nutrient status, leading to enhanced plant growth [85]. It has been reported that the improved growth and development of plants is directly linked to different growth-promoting mechanisms [86]. The colonization of PGPR in rhizosphere increases the production of root exudates such as vitamins (Vi), organic acids (OA), carbohydrates (CBH), high-molecular-weight polymers (HMWP), and amino acids (AA), which eventually stimulate growth and microbial activities. The expression of numerous nitrogen-cycling genes was identified in rice roots, which revealed the role endophytes in the processes of nitrogen fixation, nitrification and denitrification [87]. Maize plants inoculated with pesticide-degrading bacterial strains substantially promote the sub-stomatal conductance and photosynthetic rate. The photosynthetic rate (A) performs a vital role in the control of carbon dioxide through stomatal opening by increasing the intracellular carbon dioxide concentration in leaves [75]. The accumulation of ROS in leaves is mostly reliant on the balance among ROS production and detoxification through numerous enzymes scavenging ROS. The inoculation of isolated bacterial strains initially reduced the stress of glyphosate in plant roots, which could lead to the improvement of enzymes

scavenging ROS that ultimately lowered photoinhibition [88]. The results stated that the inoculation of isolated bacterial strains reduces the effect of ROS on plants' physiological characteristics. Therefore, we assume that the surge in photosynthesis is associated with bacterial inoculation at a specific phase of growth and development.

The seed germination rate indicated that isolated bacterial strains WAG2 (*Serratia liquefaciens*), WAG4 (*Klebsiella variicola*), WAG45 (*Enterobacter cloacae*), WAG9 (*Pseudomonas aeruginosa*), and WAG11 (*Enterobacter ludwigii*) increased the seed germination rate by 25–75% in 100 mg/kg, while the increasing concentrations of glyphosate by 100–200 mg/kg reduced the germination rate. The bacterial strains could consume glyphosate as the only source of carbon, and its carbon phosphorus lyase enzyme could degrade glyphosate in phosphorus-enriched environments. The results of the seed germination rate are consistent with those of Noumavo et al. [89] who found that the inoculation of bacterial strains significantly enhanced seed germination. Glyphosate and roundup significantly declined seed germination by reducing respiration rates in *D. wilsonii* seed. The production of antioxidants, such as catalase and ascorbate peroxidase (AP), ensures no accumulation of hydrogen peroxide (H_2O_2) in glyphosate-contaminated seeds [90]. It is usual practice to include a surfactant, such as polyethoxylated tallow amine (POEA), in glyphosate-based herbicide formulations to increase the bioavailability of the active herbicide component [91]. Although POEA is often found in soils, its possible impacts on soil bacteria have not been studied, even though such an exposure is extensively established for aquatic species [92]. Succinate, a root exudate, was used with POEA to test the *P. putida* KT2440, *P. putida* S12, and *P. protegens* Pf-5 growth rate. The application of POEA reduced the bacterial growth biomass by as much as 60% compared to that consisting only of succinate compound [93].

This is the first axenic report in sand of isolated bacteria which degrade glyphosate as a carbon source and produce growth-promoting hormones for the improvement of plant growth at a 100 to 200 mg/kg concentration of glyphosate. These strains have a remarkable capability for application in bioremediation and promoting the growth of crop plants. Based on the above research, we have set up a collective project to test the application of these bacterial strains in the crop field to check their capability of improving plant yield and the degradation of glyphosate.

5. Conclusions

Our in vitro study showed that the five bacterial strains WAG2 (*Serratia liquefaciens*), WAG4 (*Klebsiella variicola*), WAG45 (*Enterobacter cloacae*), WAG9 (*Pseudomonas aeruginosa*), and WAG11 (*Enterobacter ludwigii*) significantly improved plant growth and declined the glyphosate concentration in spiked sand, therefore reducing the herbicide content and suppressing its harmful effect on maize plants. These bacterial strains will be deposited in culture collection centers and will be available to other researchers to conduct further tests. The inoculation of bacterial strains with the ability to survive in herbicide-spiked sand by degrading glyphosate and improving plant growth in contaminated soils is a promising technology for agriculture applications. We plan to test these bacterial candidate strains in situ (field soils) to evaluate their actual impact on different crop plants in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13040886/s1, Table S1: Effect of isolated bacterial strains on seed germination rate in glyphosate spiked sand.

Author Contributions: All the authors contributed equally W.M.-U.-D. conducted research and wrote the initial draft, M.J.A. conceptualized and generated resources to conduct this research, S.B. conducted analysis and software analysis, H.N.A. performed statistical analysis and improved the first draft in writeup, M.F.N. wrote discussion and analyzed the data, and F.C. conducted the data analysis and improved the first draft. All authors have read and agreed to the published version of the manuscript.

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