agriculture

MDPI

Article

Glyphosate Bioremediation through the Sarcosine Oxidase Pathway Mediated by *Lysinibacillus sphaericus* in Soils Cultivated with Potatoes

Mario Pérez Rodríguez [1], Carol Melo [2], Elizabeth Jiménez [2] and Jenny Dussán [1,*]

[1] Microbiological Research Center (CIMIC), Department of Biological Sciences, Universidad de Los Andes, Bogotá 111711, Colombia; me.perez10@uniandes.edu.co

[2] Applied Biochemistry Research Group (GIBA), Department of Chemistry, Universidad de Los Andes, Bogotá 111711, Colombia; cp.melo1464@uniandes.edu.co (C.M.); e.jimenezd@uniandes.edu.co (E.J.)

* Correspondence: jdussan@uniandes.edu.co; Tel.: +57-(1)339-4949

Received: 6 August 2019; Accepted: 26 September 2019; Published: 8 October 2019

Abstract: Glyphosate-based herbicides (GBH) use has increased drastically over the last decade. This is true especially for potato crops due to their fast harvest cycle and high market demand. In 2015, the World Health Organization (WHO) classified glyphosate and its breakdown product amidomethylphosphonic acid (AMPA) as probably carcinogenic to humans, and it has been reported that these compounds disrupt the ecological and nutritional equilibrium of soils. However, microorganisms with the sarcosine oxidase gene, such as *Lysinibacillus sphaericus*, can degrade glyphosate through the Carbon-Phosphorus (C-P) pathway without leading to AMPA production. The aim of this study was to evaluate the addition of the plant growth-promoting bacteria (PGPB) *L. sphaericus* as a bioremediation agent in a potato crop sprayed with a GBH, in conjunction with the nitrogen fixation activity mediated by the bacteria. To that end, a GBH solution was used to treat a potato field, and different treatments (glyphosate (G), bacteria (B), bacteria+glyphosate (BG), and negative control (C)) were evaluated by measuring the glyphosate, AMPA, nitrates, and ammonium concentrations. BG treatment showed a 79% reduction of glyphosate concentration in soil, leading to minimal AMPA production, compared to the 23% reduction observed after G treatment. Furthermore, the ammonium concentrations were significantly higher in samples treated with BG and in C samples ($p < 0.005$). Therefore, we propose the addition of *L. sphaericus* as a good bioremediation strategy for soils sprayed with GBH.

Keywords: glyphosate; biodegradation; *Lysinibacillus sphaericus*; ammonium; nitrate; sarcosine; AMPA

1. Introduction

Glyphosate usage has been on the increase since the 1990s, to the point of it becoming the main active ingredient in most commercial herbicides sold in America [1]. Its action mechanism is based on the inhibition of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), as a result of which it blocks the synthesis of essential aromatic amino acids such as tyrosine, tryptophan, and phenylalanine [2]. EPSPS sensitivity to glyphosate depends on its class. Class I is categorized as sensitive and Class II as insensitive [3]. Class I is found in all plants and some Gram-negative bacteria, while Class II is widely distributed among bacteria [4]. Given EPSPS class distribution and efficiency, glyphosate acts as a broad-spectrum herbicide.

In recent decades, the development of glyphosate-tolerant crops and a rise in weed resistance [5] has led to the heavy use of glyphosate-based herbicides (GBH), causing glyphosate accumulation in soil and water all around the globe; this has led to GHB traces accumulating in edible products [6]. In the

beginning, it was not a concern for public health, because of GHB physicochemical properties such as fast degradation, strong adsorption into the soil, and target specificity [7,8]. Nevertheless, recent reviews of the Environmental Protection Agency (EPA) regulations and new reports on risks assessment of glyphosate exposure have posed doubts about the real toxicological effects of GBH [9]. In fact, in 2015, the International Agency for Research on Cancer (IARC) reclassified glyphosate and its breakdown product amidomethylphosphonic acid (AMPA) to Category 2A as "probably carcinogenic to humans" [10,11].

One of the principal problems of glyphosate in the soil is the mineralization mediated by soil microorganisms, leading to the release of AMPA, a more persistent and dangerous compound than glyphosate [11]. AMPA can induce oxidative stress in animals, disable DNA damage repair, and impair mRNA synthesis [12,13]. Glyphosate degradation processes in soil are mediated by microorganisms producing AMPA, sarcosine, and glycine as intermediaries, depending on the degradation pathway [14] (Figure 1). The most common pathway involves C-N bond oxidation, where microorganisms degrade glyphosate releasing AMPA and glyoxylate, which are later used as a carbon source [15]. However, there is another degradation pathway, through sarcosine oxidase, which leads to sarcosine and phosphate initially. Sarcosine is further processed to glycine instead of AMPA, which makes this pathway more eco-friendly [13,16]. Moreover, glycine can be used as a metabolite by other organisms [17]. The sarcosine oxidase pathway has been reported to be used by organisms such as *Pseudomonas spp*. [18] and *Lysinibacillus sphaericus* [16].

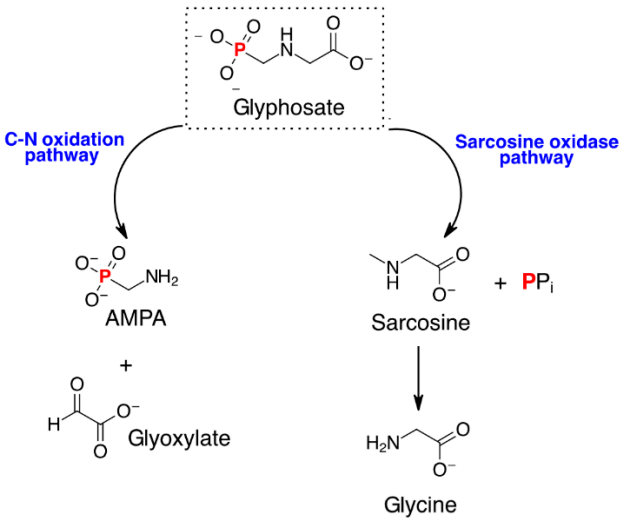

Figure 1. Biochemical pathways of glyphosate degradation by C-N oxidation and sarcosine oxidase.

The Gram-positive spore-forming bacteria *L. sphaericus* previously named *Bacillus sphaericus* [19] has been reported as a plant growth-promoting bacterium (PGPB) and a nutrient-enhancing bacterium because of its ability to solubilize phosphorus, its nitrogen fixation activity, and its production of phytohormones such as indole acetic acid (IAA) [20,21]. This bacterium has been used as a nutrient enhancer microorganism in fire-impacted soils [21], as a bioremediation landfarming agent of petroleum-derived hydrocarbons [22] and toxic metals, and has as a biocontrol for *Aedes aegypti* and *Culex quinquefasciatus* [23–28]. Furthermore, a previous study showed that *L. sphaericus* expresses the sarcosine oxidase gene [26] when exposed to glyphosate in a minimum salt medium (MMS) modified to lack phosphorous salts, thus limiting the phosphorous source to glyphosate. Results showed a release and rise of the concentration of phosphate groups, presumably through the degradation of glyphosate by the sarcosine oxidase pathway [16].

Potato (*Solanum spp.*), a member of the Solanaceae family, is the third most important crop for human consumption [29] and thus is important for agriculture and economics worldwide. *Solanum phureja*

is known as "papa criolla", lacks tuber dormancy, presents a short vegetative period and small tubers [30,31], and is currently cultivated only in central Peru, Venezuela, Ecuador, and Colombia [32], given that it only grows between 2300 and 2800 meters above sea level (m.a.s.l.). Because of the fast crop harvest cycles, the use of glyphosate-based herbicides is more intense for potato crops than in other plantations, leading to adverse effects on the environment by disrupting the ecological and nutritional equilibrium in the soil [14].

Considering that glyphosate can be degraded through the sarcosine pathway by *L.sphaericus*, this study aimed to evaluate the addition of the PGPB *L. sphaericus* as a bioremediation agent in a potato field treated with GBH, in relation to the activity of the nitrogen fixation metabolism of *L. sphaericus* in glyphosate-contaminated soil, in order to establish a bioremediation strategy for different soils irrigated with glyphosate.

2. Materials and Methods

2.1. Crop Experimental Design

The Bogota savanna—a montane savanna—is located in southwestern central Colombia (Cundinamarca) (Figure 2) and has an average altitude of 2550 m.a.s.l and a temperature between 0 °C and 24 °C. *S. phureja* seeds obtained from a local farmer were sown in a 6 × 12 m parcel in Gachancipá, Cundinamarca, Colombia (coordinates 4.981078, −73.883077).

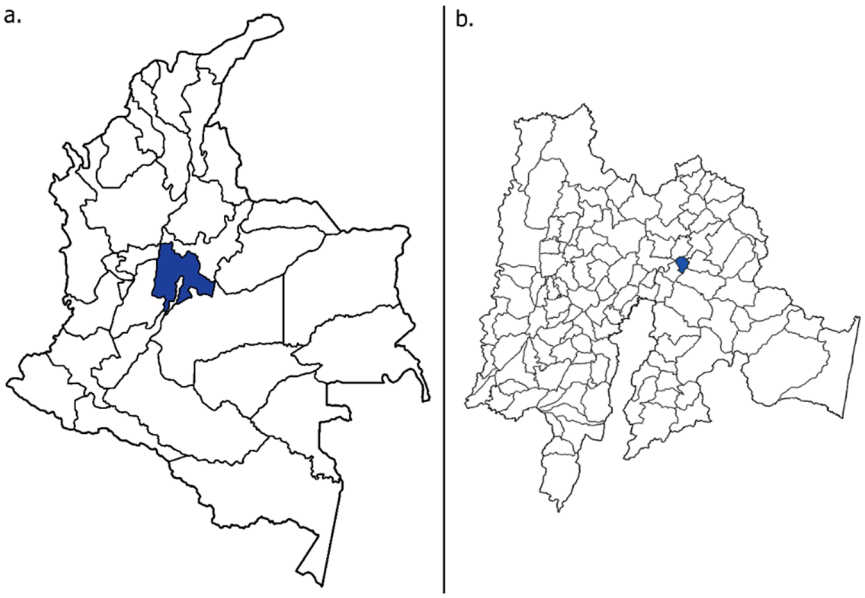

Figure 2. (**a**) Map of Colombia. Cundinamarca region highlighted in blue. (**b**) Map of Cundinamarca. Gachancipá highlighted in blue.

All seeds were sown on the same day in a 20 cm-deep by 20 cm-wide hole, 50 cm away from the other plants. Potato seedlings were obtained from a local farmer and were randomly assigned to the four treatments (30 plants per treatment = 120 experimental units). The treatments were identified as B for bacteria, BG for bacteria + glyphosate, G for glyphosate, and C for negative control (neither bacteria nor glyphosate) (Table 1 and Figure 3).

Table 1. Description of each treatment.

Treatment	B	G	BG	C
Description	Bacterial mixture containing five strains of *Lysinibacillus sphaericus*.	1.69 mg/mL glyphosate aqueous solution.	Bacterial mixture containing five strains of *L. sphaericus* and 1.69 mg/mL glyphosate solution.	Water from the same source used in the other treatments.

Figure 3. Potato crop treatment distribution in Gachancipá, Cundinamarca. A parcel was subdivided as to receive four different treatments. B: bacteria, G: glyphosate, BG: glyphosate + bacteria, C: negative control.

2.2. *L. sphaericus* Strains and Inoculum Preparation

For the treatments with *L. sphaericus*, an inoculum was made using a mixture of five different strains (Table 2). Each bacterial strain was cultivated in plate count agar (PCA) solid culture medium (2L) and incubated for 24 h at 30 °C. After incubation, the bacteria were scraped from the surface of the medium, collected in 100 ml of sterilized water, and stored at 4 °C until use. For the application to soil, all five strains were combined in a single volume with a final bacterial concentration of 1×10^{11} Colony-forming unit (CFU)/mL.

Table 2. *L. sphaericus* strains used for the mixture.

Strain	Source
CBAM5	Hydrocarbon-contaminated soil [23].
III (3) 7	Oak forest soil [26].
OT.4b.49	Native Colombian strain [19].
OT.4b.31	Native Colombian strain [23].
2362	WHO entomopathogenic reference strain [28].

2.3. Parcels Treatment Application

The parcel was subjected to four different treatments, as mentioned in (Table 1), i.e., B, BG, G, and C. After 30 and 60 days from sowing, each pertinent treatment was applied as shown in Figure 3. The glyphosate source used during this entire study was Roundup 747® (679 g/Kg of glyphosate corresponding to 747 g/Kg of N-(phosphonomethyl) glycine isopropylamine salt), made available on the market by Monsanto, São Paulo (Brazil).

2.4. Nitrate and Ammonium Determination

Ammonium and nitrates concentrations in soil samples were determined using commercial colorimetric kits (Ammonium Test, detection range 2.0 mg/L–150 mg/L, catalog number 100683, and Nitrate Test, detection range 0.9 mg/L–88.5 mg/L, catalog number 114773) and the spectrophotometer Spectroquant® NOVA60A both from Merck Millipore (Supelco), Darmstadt, (Germany).

At day 0 (before sowing the potatoes), four compound samples were randomly collected; then, 20 soil samples were collected on days 20, 40, and 60 around five different plants for each treatment (four compound samples each). Samples were collected with a small shovel every 20 days; each sample consisted of 100 g of soil collected at a depth of 15 cm.

2.5. Glyphosate and AMPA Determination by Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry (UHPLC–MS)

Glyphosate and AMPA were determined using UHPLC–MS/MS by a company (Primoris, Belgium CVBA) credited (BELAC EN-ISO 17025) in pesticide analysis. The method used was GGA-01. The standards used for chromatographic method calibration were isotope-labeled(^{13}C) glyphosate and AMPA, and the limit of quantification reported for the analysis was 0.01 mg/kg for both analytes. The chromatograms for each soil sample by treatment and day are available in the supplementary information section (Figures S1 and S2).

2.6. Glyphosate Determination by Ninhydrin Reaction

The quantification of glyphosate from soil involved three steps: (i) extraction from soil and standard addition method, (ii) ninhydrin derivatization, and (iii) quantification by standard addition. The extraction of glyphosate from soil was designed on the basis of the high cation exchange capacity (CEC, 24.405 meq/kg soil) determined for the soil where the potatoes were planted. The analysis was performed for three types of samples: Day 0 sample (glyphosate at time 0), Sample G (soil from G treatment at day 60), and Sample BG (soil from BG treatment at day 60). The analysis was a blinded experiment, so the identity of the samples was unknown during the experiment.

2.6.1. Extraction of Glyphosate from the Soil and Standard Addition Method

Soil samples for each treatment were dried in an oven at 80 °C for 5 h because of differences in humidity; 10.0 g of soil was accurately weighted for each treatment and extracted with 25 mL of a solution containing sodium phosphate (0.4 M) and trisodium citrate (0.4 M) [33]. To perform the standard method, each sample was subjected to spiking with a glyphosate standard at the following concentrations: 0 mg/mL, 5.56×10^4 mg/mL, 8.33×10^4 mg/mL, and 1.11×10^5 mg/mL. Each concentration was prepared in triplicate, and the concentrations were chosen considering that glyphosate–ninhydrin adduct values must fit into a linear dynamic range after the dilution process that takes place during ninhydrin derivatization. The solutions were placed in a shaker at room temperature for 16 h and centrifuged at 4500 rpm for 80 minutes. If soil suspension persisted, the supernatant was filtered with a 0.22 μm filter. Then, 90 μL of each extract was diluted to 1 mL with Type I water for derivatization with ninhydrin.

2.6.2. Ninhydrin Derivatization

A ninhydrin working reagent 2.5% (*w*/*v*) was prepared by dissolving 1.25 g of ninhydrin in 50 mL of a solution containing water Type I + acetate buffer (0.4 M, pH = 5.5) (3:1 *v*/*v*). A 5% (*w*/*v*) sodium molybdate (Na_2MoO_4) solution was prepared by dissolving 2.50 g of the anhydrous salt Na_2MoO_4 in 50 mL of Type I water.

Derivatization was performed at a final volume of 3 mL with a diluted solution of extract + ninhydrin working solution + 5% Na_2MoO_4 (1:1:1 *v*/*v*/*v*). These solutions were heated for 30 minutes. The solution was cooled to room temperature and transferred to a cuvette, and the absorbance was read at 567 nm.

2.6.3. Quantification by Standard Addition

To determine the glyphosate concentration for each dataset, a linear regression was adjusted, and then, by extrapolation to zero, the concentration was cleared [34]. The basis of this extrapolation is that it is at this point that the minimal matrix effect will be found.

To construct the linear regression, the dilution carried out with the extraction solution was considered to calculate the final concentration of the standard added during the spiking step. The final concentrations used for the linear regression were: 0 mg/L, 5 mg/L, 7.5 mg/L, and 10.0 mg/L. Finally, the concentration of glyphosate in soil was calculated considering the concentration obtained by the extrapolation procedure, the volume of extraction solution, and the amount of soil used in the extraction.

2.7. DNA Extraction and Sarcosine Oxidase Gene Presence

DNA extraction was performed for each treated soil using the ZR Soil Microbe DNA MiniPrepTM kit (Catalog No D6001, ZYMO RESEARCH), following the manufacturer's instructions. In order to determine the presence of the sarcosine oxidase gene in the potato crop soil, several polymerase chain reactions (PCR) were performed using the primers SarcF 5′-CGTGTGAAACCTGGAAAACGTGGT-3′ and SarcR 5′-TAGCGGCTACATGAACACCTGCT-3′. The sequences, temperature, and cycles parameters from a previous investigation were followed [16]. Electrophoresis of the PCR products was performed in a 1.7% agarose gel for proper visualization, followed by Sanger sequencing and a BLASTn analysis to confirm the presence of the sarcosine oxidase gene.

2.8. Follicular Area Image Analysis

Follicular area analyses were performed with ImageJ software (National Institutes of Health, Maryland, United States of America). An aerial photograph of the plot at day 60 (Figure 9) was used to determine the relative follicular area of each plant, classified by treatment.

2.9. Statistical Analysis

Ammonium, nitrate, and glyphosate concentrations determined by the ninhydrin reaction were analyzed by analysis of variance (ANOVA), followed by the Tukey-Kramer test (HDS). Before these tests were carried out, homoscedasticity was tested by the Bartlett test, and normality was tested using the Shapiro test (Table S1). The grouping was carried out according to the significant difference between the treatment means and the control group.

The glyphosate concentration determined by the ninhydrin reaction was also analyzed by ANOVA and the Tukey Honestly-significant-difference (HSD)-test. Normality and homoscedasticity were proven with the Shapiro-Wilk test and the Bartlett test, respectively, before the application of ANOVA. The follicular area data did not accomplish the homoscedasticity and normality assumptions; thus, a nonparametric test was applied to this dataset. The follicular area was analyzed using the Kruskal-Wallis test and the Wilcoxon rank-sum test. All the statistical tests were performed used a significance value (α) of 0.05 to reject the null hypothesis. Statistical analyses were performed with R software. All results from the statistical tests performed can be found in the supplementary information.

3. Results and Discussion

According to our field experiments, the addition of the PGPB from *L. sphaericus* to a potato crop with GBH showed that glyphosate was degraded through the sarcosine pathway and that its activity in the nitrogen cycle, which was determined by the concentration of ammonium and nitrates in the soil, was evident during crop cultivation.

3.1. Nitrate and Ammonium Concentrations

The results of soil nitrate concentrations showed significant differences between the times of analysis (ANOVA: F_{D20} = 10.12, P_{D20} = 0.0042, F_{D40} = 72.75, P_{D40} = 0.00003, F_{D60} = 22.29, P_{D60} = 0.00030) (Table S1). Nevertheless, on day 20, significant differences were found only between C and B treatments and G and B treatments; at day 40 only the C and BG treatments showed no significant differences; and at day 60, no significant differences were found between C and BG treatments and C and G treatments (Figure 4a) (Table S2). The results of soil ammonium concentration were similar to those of nitrate concentration, showing significant differences at days 20, 40, and 60 (ANOVA: F_{D20} = 10.38, P_{D20} = 0.0039, F_{D40} = 37.71, P_{D40} = 0.00004, F_{D60} = 15.82, P_{D60} = 0.0010) (Table S3). However, on day 20, significant differences were found between C and G, C and B, and C and BG treatments; at day 40, no significant differences were found between BG and G treatments; at day 60, significant differences were found between the control and all treatments, but no significant differences were found between the treatments (Table S4). The mean concentration of nitrate and ammonium were as expected across the whole time of analysis; when nitrogen is fixed in the form of ammonium, it is promptly turned into nitrates that can be used by the plants, establishing a trend whereby, when nitrate concentration decreases, ammonium increases, and vice versa [21,35]. On day 20, there was a clear rise in ammonium concentration and a decrease in nitrate concentrations. At day 60, the opposite happened, and ammonium concentration started to drop as the nitrate concentration started to rise [21] (Figure 4). These results show that GBH did not inhibit the fixing of atmospheric nitrogen to ammonium or the oxidation of ammonium to nitrate. In other words, PGPB increased nutrients in the soil, which could contribute to the growth of potato crops treated with B and BG.

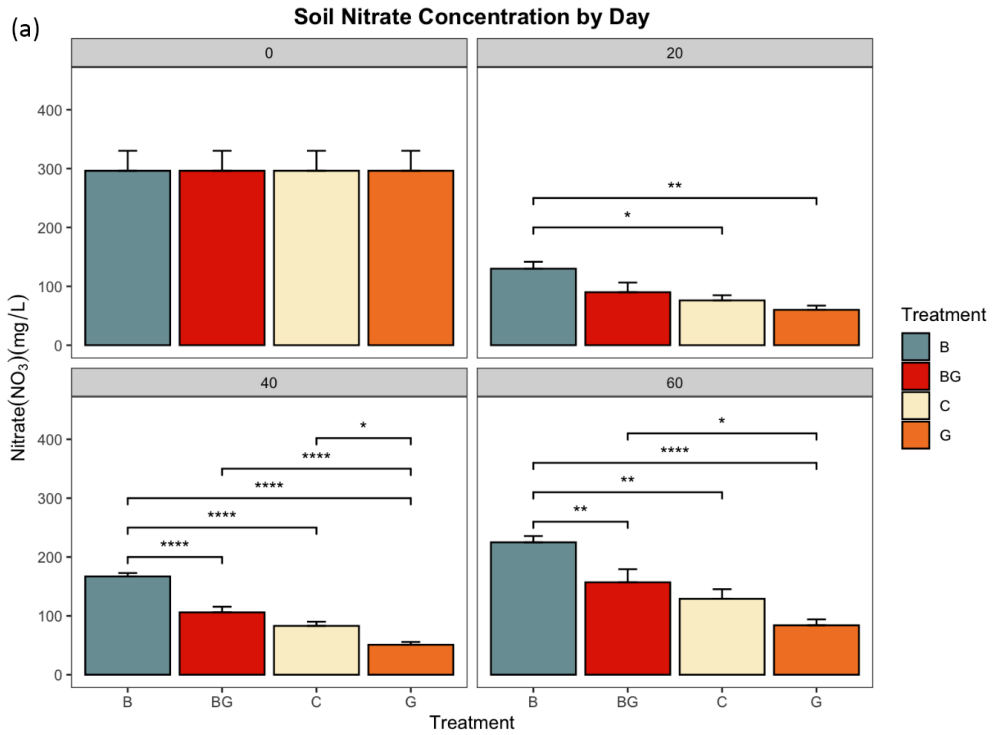

Figure 4. *Cont.*

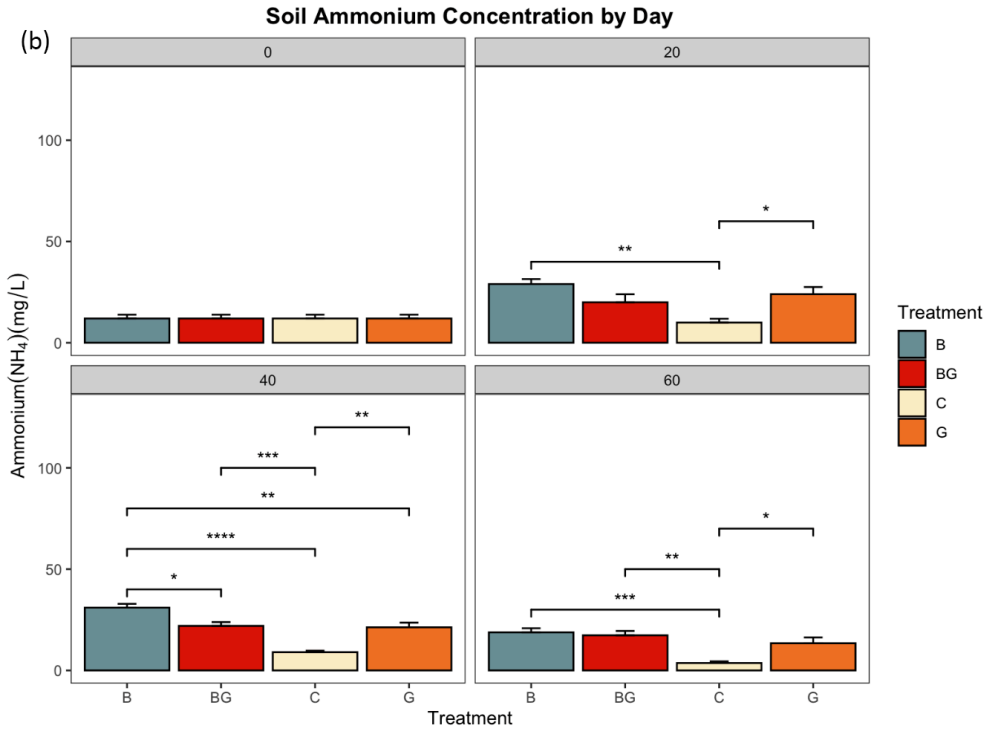

Figure 4. Nitrate and ammonium concentrations across time. Nitrate concentration (**a**) and ammonium concentration (**b**) in soil were determined by a colorimetric assay at days 0, 20, 40, and 60. Statistical analysis was performed using ANOVA (one way). Results are shown as the mean ± SEM, Significance level: 0.05 (*), 0.01 (**), 0.001 (***), <0.001 (****).

3.2. *Glyphosate Degradation Quantified by UHPLC-MS/M*

The chromatograms of UHPLC-MS/M analyses (Figures S1 and S2) showed high reproducibility in the retention times in comparison to the standards and acceptable peak resolution for glyphosate and AMPA.

In Figure 5, there is an apparent decrease in the concentration of glyphosate as determined chromatographically. The BG treatment showed a 79% reduction in glyphosate concentration in soil, compared to the 23% reduction observed for the G treatment. The reduction with the G treatment may be related to the leaching process and material degradation through the C-N oxidation pathway and possible further breakdown of glyphosate that was not detected.

AMPA concentration for BG and G treatments showed a similar pattern to that of glyphosate concentration. AMPA concentration was low in BG treatments (78% of the initial concentration) on day 30.

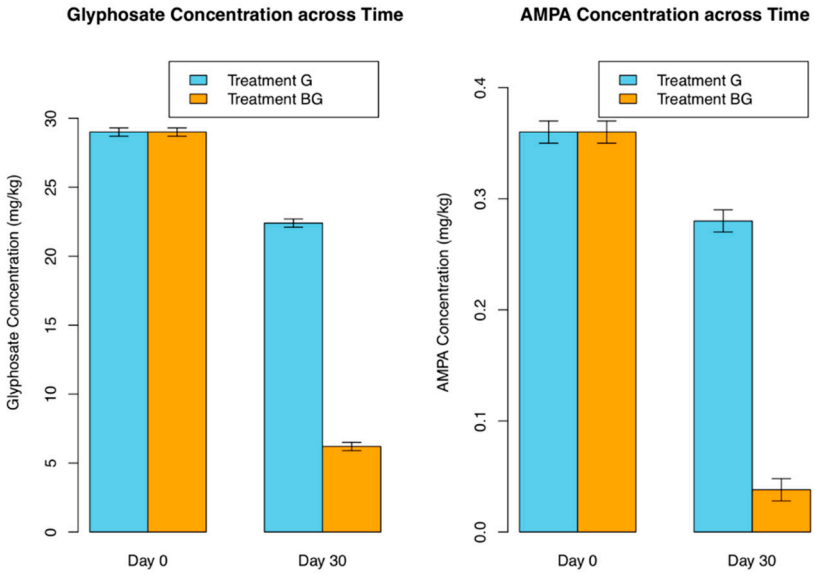

Figure 5. Determination of glyphosate and amidomethylphosphonic acid (AMPA) concentrations by UHPLC–MS/MS. Glyphosate and AMPA concentrations were determined at days 0 and 30 when the G and BG treatments were applied to the parcel. BG: bacteria-glyphosate, G: glyphosate (roundup 747). Results are shown as the mean ± SEM.

3.3. Glyphosate Determination by the Ninhydrin Reaction

The determination of glyphosate by the ninhydrin reaction with the standard addition method was performed in three samples: Day 0 sample (glyphosate irrigation at time 0), sample G (soil from G treatment at day 60) and sample BG (soil from BG treatment at day 60). Glyphosate concentration was determined by using a linear regression model (Figure S3) and method parameters (Table S5). The difference in glyphosate content between samples Day 0 and G (day 60) was 12.7% (Figure 6). This difference may arise from glyphosate biotransformation or a leaching process [14,36]. The difference in glyphosate content between samples Day 0 and BG (day 60) was 21.6% (Figure 6). The difference between treatments (8.9%) suggests an active role of *L. sphaericus* in decreasing the amount of glyphosate in soil.

Results from ANOVA (Table S5) and the Tukey-Kramer test signaled significant differences only between Day 0 and BG treatments, confirming the role of *L. sphaericus* in glyphosate degradation in soil. Although the differences between the percentages of remaining glyphosate determined with the ninhydrin method and the chromatographic method by an authorized glyphosate determination company in Colombia are not comparable, both methodologies suggest an active role of *L. sphaericus* in the decrease of glyphosate in soil. These results can be attributed to the different sensitivity and sensibility of the methods used. The ninhydrin reaction, because of the ability of this chromophore to react with primary and secondary amines in the reaction conditions used [37,38], has a lesser degree of selectivity than the chromatographic method. In order to accurately distinguish glyphosate degradation with the ninhydrin method, a sample of B treatment that has never been in contact with glyphosate must be used as a blank to further discern the amount of absorbance that can be attributed to the presence of *L. sphaericus* vs. the presence of glyphosate.

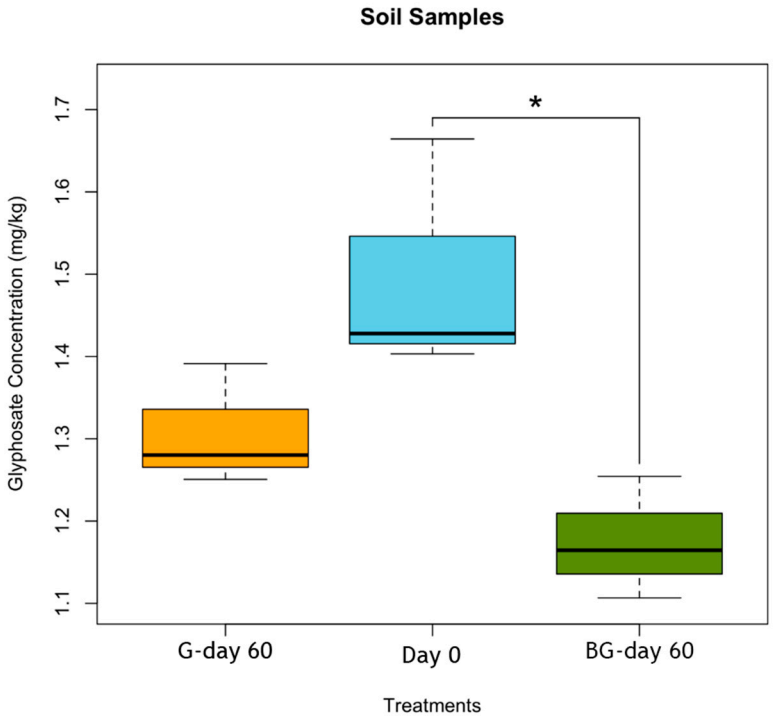

Figure 6. Boxplot of glyphosate concentration for different treatments. Glyphosate was determined by the standard addition method at days 0 and 60 for BG and G treatments. BG: bacteria-glyphosate, G: Glyphosate (roundup 747). Statistical analysis was performed using ANOVA (one-way) Results are shown as the mean ± SEM, and datasets were analyzed by the Tukey-Kramer test, with significance level: 0.05 (*).

3.4. Sarcosine Oxidase Gene Presence

After DNA extraction from potato cropland, PCR assays confirmed the amplification of sarcosine alpha subunit [26] in the bacteria-amended samples. The amplification of the sarcosine gene showed an amplicon corresponding to a fragment between approximately 70 bp for the B and BG treatments (Figure 7). Additionally, the BLASTn showed an identity in the treatments containing *L. sphaericus* between 95% and 98% (Table 3) as well as an E-value of 3e-19 for B and 3e-18 for BG.

Table 3. Blastn results for the PCR products.

BLAST Result	Accession	Genome Region	B		BG	
			Identity (%)	Query Cover	Identity (%)	Query Cover
L. sphaericus strain 2362, complete genome.	CP015224.1	3365804–3365863	98.33	83%	95.38	89%
L. sphaericus III(3)7, complete genome.	CP014856.1	264109–264168	98.33	83%	95.38	89%
L. sphaericus strain OT4b.25, complete genome.	CP014643.1	1131747–1131688	98.33	83%	92.96	98%
L. sphaericus C3-41, complete genome.	CP000817.1	117009–116950	98.33	83%	92.96	98%

These results are consistent with those of AMPA determination, where the BG treatment showed a minimal concentration of this metabolite in comparison to the concentration observed in the G treatment. In fact, the presence of AMPA after G treatment suggests the presence of other bacteria with the ability to metabolically process glyphosate by the C-N oxidation pathway (Figure 1). The presence of these bacteria could explain the presence of AMPA in the samples subjected to BG treatment; more interestingly, the minute amounts of AMPA in BG-treated samples compared to G-treated samples suggest that *L. sphaericus* outperforms other bacteria in glyphosate processing.

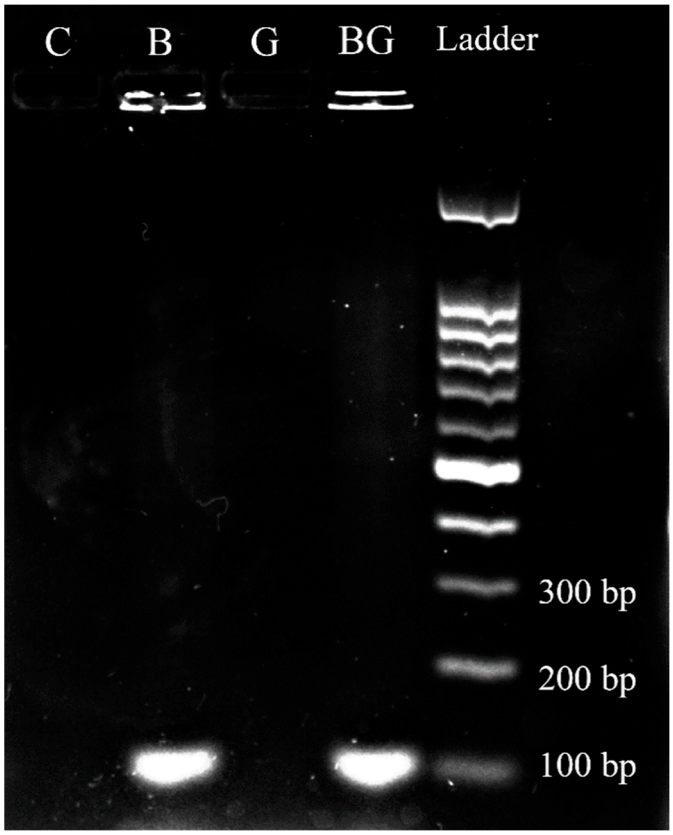

Figure 7. Gel electrophoresis of sarcosine oxidase. PCR amplification of the sarcosine oxidase gene product visualized in a 1.7% agarose gel.

3.5. Addition, Mitigation, and Nitrogen Fixation Activity of L. sphaericus in the Potato Crop

The addition of *L. sphaericus* mitigated the herbicide effect of glyphosate (Figure 8). Only 18.75% of the plants treated with glyphosate (G) managed to survive this 60-day experiment, in contrast with what observed for the plants treated with glyphosate and *L. sphaericus* (BG), whose survival rate significantly increased to 50%. Furthermore, the bacterial treatment (B) was associated with the highest survival rate at 90.6%, which was even higher than that observed for the control, though only by 9.35%.

Figure 9 presents a clear visual difference in the follicular area between the plants in each treatment. Only five plants resisted the addition of the herbicide (G treatment) and showed a reduced foliar area in contrast to plants that received the other treatments. The C and the BG treatments showed similar follicular areas, but the bacteria (B)-treated plants visually presented a greater follicular area than plants receiving any of the other treatments.

This is shown by the follicular area distribution in Figure 10, where G treatment presented the lowest mean follicular area among the treatments and B treatment had the greater mean follicular area. The results of the Kruskal-Wallis sum-rank test and post-hoc Dunn test showed significant differences among relative follicular areas by treatment; B treatment presented significant differences with respect to all other treatments (Table S6).

These results show both the mitigation of the herbicide effect mediated by the bacteria and the growth-promoting role played by *L. sphaericus* in the crop. Due to the nitrogen fixation role of *L. sphaericus*, differences in nitrogen and ammonium concentrations between the control and the treatments with bacteria (B, BG) were observed across the whole study (Figure 4), consisting in higher nitrate and ammonium concentrations in the samples treated with bacteria than in the control. Furthermore, nitrate concentrations at day 60 in the bacteria-treated samples were close to the respective concentrations before the treatment (day 0), and the ammonium concentrations at day 60 in the samples

treated with bacteria (B, BG) were significantly higher than on day 0. These findings reveal that *L. sphaericus* was able to enhance the nitrate and ammonium concentrations in the soil to even higher values than the ones before the study started and that the presence of glyphosate did not have a negative or inhibitory effect on the nitrogen fixation ability of *L. sphaericus*. Therefore, we propose *L. sphaericus* as a bioremediation agent for soils irrigated with glyphosate.

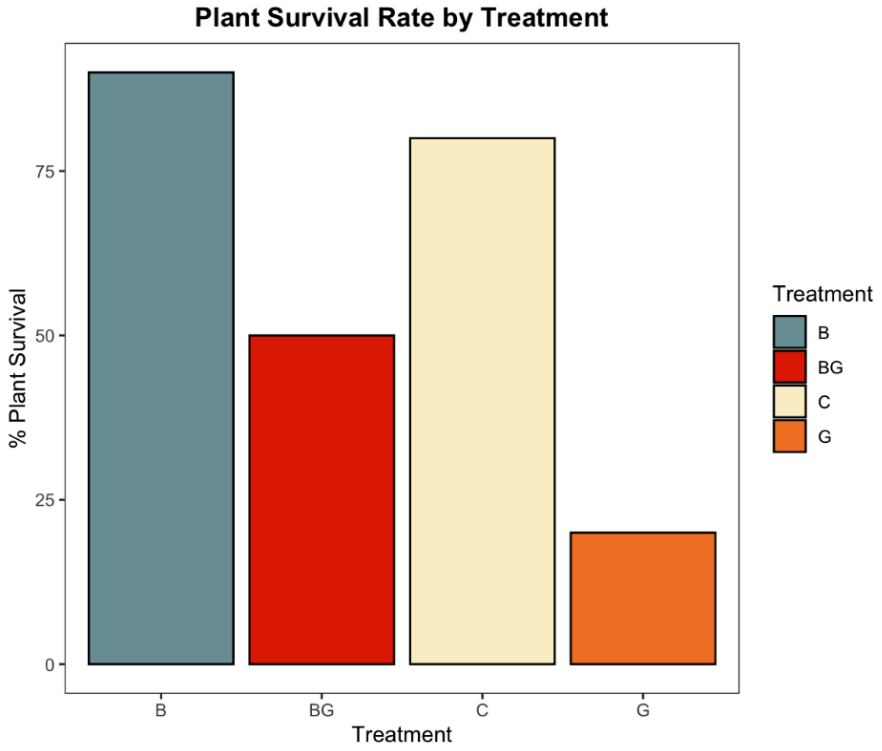

Figure 8. Final plant survival rate. Plants were analyzed for survival with respect to the treatment received.

Figure 9. Aerial photograph of the crop distribution at day 60.

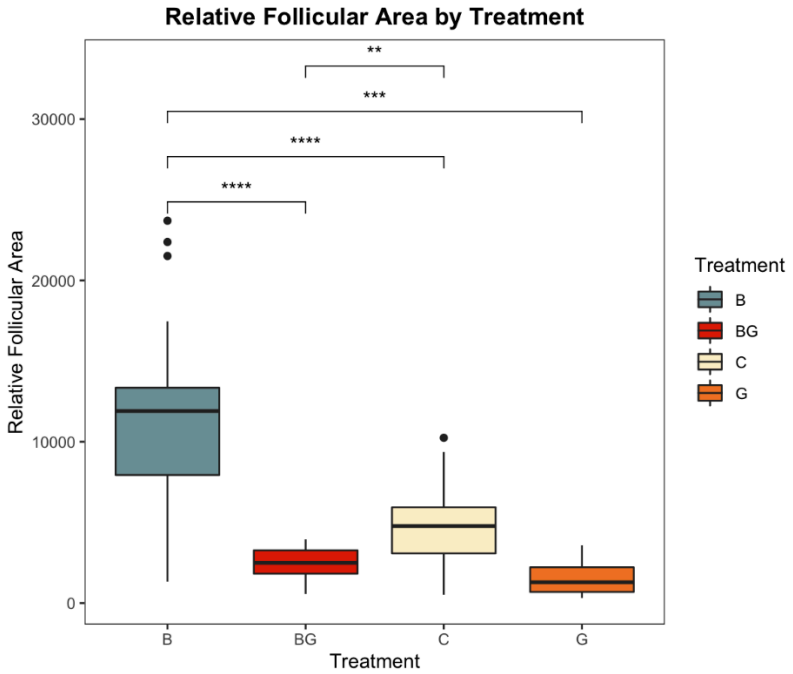

Figure 10. Boxplot of relative follicular area by treatment. The aerial photograph was analyzed by Image J and used to determine the relative follicular area by each treatment. Datasets were analyzed by the Tukey-Kramer test, with significance level: 0.01 (**), 0.001 (***), <0.001 (****)".

4. Conclusions

This study showed the mitigation of the effects of glyphosate in the presence of *L. sphaericus*, which might have positively influenced the degradation of glyphosate via the sarcosine pathway, leading to minimal AMPA production and increasing the survival rate of the plants. Also, a growth-promoting role was evidenced, as the plots treated with bacteria showed a greater follicular area than the ones treated without bacteria. These results indicate that the presence of glyphosate does not seem to intercede with the nitrifying role of *L. sphaericus*, as the nitrate and ammonium concentrations were as expected across the whole study.

Furthermore, we demonstrated that a decrease in the amount of glyphosate in the soil with bacterial amendment correlated with a reduction of the concentration of the AMPA metabolite, which confirmed that the degradation occurred through the sarcosine oxidase pathway. The ability of *L. sphaericus* to degrade glyphosate thus limiting the production of AMPA, in addition to its growth-promoting effects on soil, offers a new alternative for bioremediation of GBH-treated croplands. A closer insight into the effect of microbial nitrogen enhancers on crops exposed to glyphosate or similar herbicides is needed to better respond to potential adverse implications the use of these herbicides may have for the environment, food sustainability, and public health. As such, we suggest that the treatment of potato crops with *L. sphaericus* could be beneficial in reducing the side effects of glyphosate breakdown products on soil and health and in enhancing nitrate and ammonium concentrations in the soil. We also propose the addition of *L. sphaericus* as a good bioremediation strategy for soils sprayed with GBH.

It should be noted that, although the most common degradation pathway involves the C-N bond oxidation [14], *L. sphaericus* manages to degrade glyphosate via the sarcosine oxidation pathway and, in comparison with other microorganisms that also degrade glyphosate via the sarcosine pathway [39–41], *L. sphaericus* does not represent a risk to the health of nearby animals and plants, as it is not considered a pathogenic microorganism [42]. In addition to this, the fact that it is an endemic microorganism already used with success in other biodegradation processes in Colombia [22], its application does not seem to represent a threat to the ecology of soils.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/9/10/217/s1, Figure S1: Glyphosate UHPLC-MS/M chromatograph; Figure S2: AMPA UHPLC-MS/M chromatograph; Figure S3: Glyphosate concentration linear regression model. Table S1: Statistical analysis of soil nitrate concentration by day; Table S2: *Post-hoc* analysis of soil nitrate concentration; Table S3: Statistical analysis of soil ammonium concentration by day; Table S4: *Post-hoc* analysis of soil ammonium concentration; Table S5: Glyphosate determination by the ninhydrin reaction; Table S6: Statistical analysis of relative follicular area by treatment.

Author Contributions: Conceptualization, M.P.R., and J.D.; formal analysis, M.P.R.; funding acquisition, J.D.; investigation, M.P.R., and J.D.; methodology, M.P.R., E.J., C.M., and J.D.; project administration, J.D.; resources, J.D. and. E.J.; supervision, J.D.; validation, M.P.R., and C.M.; visualization, M.P.R., E.J., and C.M.; writing—original draft, M.P.R., and C.M.; writing—review and editing, M.P.R., C.M., E.J., and J.D.

Funding: This research was funded by the Research Fund at the School of Science at Universidad de Los Andes for the Financing of Projects: IPINV-2017-51-1433, "FAPA -P17160322012" and the Microbiological Research Center (CIMIC). APC was funded by the School of Science at Universidad de Los Andes and the Microbiological Research Center (CIMIC).

Acknowledgments: We would like to acknowledge the CIMIC group for their support and help during the development of this investigation. We also thank Tiziana Laudato for the English grammar corrections provided, Tito Peña for the critical revision, and Ricardo Villegas for the aerial photographs used in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Ouided, B.; Abderrahmane, B. Isolation and characterization of glyphosate-degrading bacteria from different soils of Algeria. *Afr. J. Microbiol. Res.* **2016**, *7*, 5587–5595. [CrossRef]
2. Glycine, W.; Stizolobium, E.; De Glyphosate, B.; Glycine, R. De Biodegradation of Glyphosate in Rhizospheric Soil Cultivated with Glycine max, Canavalia ensiformis and Stizolobium aterrimum. *Planta Daninha* **2009**, 781–787.
3. Funke, T.; Healy-Fried, M.L.; Han, H.; Alberg, D.G.; Bartlett, P.A.; Schönbrunn, E. Differential inhibition of class I and class II 5-enolpyruvylshikimate-3- phosphate synthases by tetrahedral reaction intermediate analogues. *Biochemistry* **2007**, *46*, 13344–13351. [CrossRef] [PubMed]
4. Sutton, K.A.; Breen, J.; Russo, T.A.; Schultz, L.W.; Umland, T.C. Crystal structure of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase from the ESKAPE pathogen Acinetobacter baumannii. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2016**, *72*, 179–187. [CrossRef] [PubMed]
5. Wang, W.; Xia, H.; Yang, X.; Xu, T.; Si, H.J.; Cai, X.X.; Wang, F.; Su, J.; Snow, A.A.; Lu, B.R. A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (Oryza sativa) without herbicide. *New Phytol.* **2014**, *202*, 679–688. [CrossRef] [PubMed]
6. Battaglin, W.A.; Meyer, M.T.; Kuivila, K.M.; Dietze, J.E. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. *J. Am. Water Resour. Assoc.* **2014**, *50*, 275–290. [CrossRef]
7. Cuhra, M.; Bøhn, T.; Cuhra, P. Glyphosate: Too Much of a Good Thing? *Front. Environ. Sci.* **2016**, *4*, 1–14. [CrossRef]
8. Gaupp-Berghausen, M.; Hofer, M.; Rewald, B.; Zaller, J.G. Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Sci. Rep.* **2015**, *5*, 1–9. [CrossRef]
9. Vandenberg, L.N.; Blumberg, B.; Antoniou, M.N.; Benbrook, C.M.; Carroll, L.; Colborn, T.; Everett, L.G.; Hansen, M.; Landrigan, P.J.; Lanphear, B.P.; et al. Is it time to reassess current safety standards for glyphosate-based herbicides? *J. Epidemiol. Community Health* **2017**, *71*, 613–618. [CrossRef]
10. International Agency for Research on Cancer Monograph Working Group, IARC, Lyon, France. Evaluation of five organophosphate insecticides and herbicides. In *Some Organophosphate Insecticides and Herbicides*; IARC: Lyon, France, 2015; ISBN 978-92-832-0178-6.
11. Van Bruggen, A.H.C.; He, M.M.; Shin, K.; Mai, V.; Jeong, K.C.; Finckh, M.R.; Morris, J.G. Environmental and health effects of the herbicide glyphosate. *Sci. Total Environ.* **2018**, *616–617*, 255–268. [CrossRef]
12. Cressey, D. Widely used herbicide linked to cancer. *Nature* **2015**. [CrossRef]

13. Sviridov, A.V.; Shushkova, T.V.; Ermakova, I.T.; Ivanova, E.V.; Epiktetov, D.O.; Leontievsky, A.A. Microbial degradation of glyphosate herbicides (Review). *Appl. Biochem. Microbiol.* **2015**, *51*, 188–195. [CrossRef]
14. Bai, S.H.; Ogbourne, S.M. Glyphosate: Environmental contamination, toxicity and potential risks to human health via food contamination. *Environ. Sci. Pollut. Res.* **2016**, *23*, 18988–19001. [CrossRef] [PubMed]
15. la Cecilia, D.; Maggi, F. Analysis of glyphosate degradation in a soil microcosm. *Environ. Pollut.* **2018**, *233*, 201–207. [CrossRef] [PubMed]
16. González-Valenzuela, L.E.; Dussán, J. Molecular assessment of glyphosate-degradation pathway via sarcosine intermediate in Lysinibacillus sphaericus. *Environ. Sci. Pollut. Res.* **2018**, *25*, 22790–22796. [CrossRef] [PubMed]
17. Hove-Jensen, B.; Zechel, D.L.; Jochimsen, B. Utilization of glyphosate as phosphate source: Biochemistry and genetics of bacterial carbon-phosphorus lyase. *Microbiol. Mol. Biol. Rev.* **2014**, *78*, 176–197. [CrossRef] [PubMed]
18. Guyton, K.Z.; Loomis, D.; Grosse, Y.; El Ghissassi, F.; Benbrahim-Tallaa, L.; Guha, N.; Scoccianti, C.; Mattock, H.; Straif, K.; International Agency for Research on Cancer Monograph Working Group. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *Lancet Oncol.* **2015**, *16*, 490–491. [CrossRef]
19. Gómez-Garzón, C.; Hernández-Santana, A.; Dussán, J. Comparative genomics reveals Lysinibacillus sphaericus group comprises a novel species. *BMC Genom.* **2016**, *17*, 709. [CrossRef]
20. Martínez, S.A.; Dussán, J. *Lysinibacillus sphaericus* plant growth promoter bacteria and lead phytoremediation enhancer with *Canavalia ensiformis*. *Environ. Prog. Sustain. Energy* **2018**, *37*, 276–282. [CrossRef]
21. Aguirre-Monroy, A.M.; Santana-Martínez, J.C.; Dussán, J. Lysinibacillus sphaericus as a Nutrient Enhancer during Fire-Impacted Soil Replantation. *Appl. Environ. Soil Sci.* **2019**, *2019*, 1–8. [CrossRef]
22. Hernández-Santana, A.; Dussán, J. Lysinibacillus sphaericus proved to have potential for the remediation of petroleum hydrocarbons. *Soil Sediment Contam.* **2018**, *27*, 538–549. [CrossRef]
23. Peña-Montenegro, T.D.; Dussán, J. Genome sequence and description of the heavy metal tolerant bacterium Lysinibacillus sphaericus strain OT4b.31. *Stand. Genomic Sci.* **2013**, *9*, 42–56. [CrossRef] [PubMed]
24. Lozano, L.C.; Dussán, J. Synergistic Activity Between S-Layer Protein and Spore–Crystal Preparations from Lysinibacillus sphaericus Against Culex quinquefasciatus Larvae. *Curr. Microbiol.* **2017**, *74*, 371–376. [CrossRef] [PubMed]
25. Gómez-Garzón, C.; Hernández-Santana, A.; Dussán, J. A genome-scale metabolic reconstruction of Lysinibacillus sphaericus unveils unexploited biotechnological potentials. *PLoS ONE* **2017**, *12*, e0179666. [CrossRef] [PubMed]
26. Rey, A.; Silva-Quintero, L.; Dussán, J. Complete genome sequencing and comparative genomic analysis of functionally diverse Lysinibacillus sphaericus III(3)7. *Genom. Data* **2016**, *9*, 78–86. [CrossRef] [PubMed]
27. Rojas-Pinzón, P.A.; Dussán, J. Efficacy of the vegetative cells of Lysinibacillus sphaericus for biological control of insecticide-resistant Aedes aegypti. *Parasit. Vectors* **2017**, *10*, 231. [CrossRef]
28. Hernández-Santana, A.; Gómez-Garzón, C.; Dussán, J. Complete Genome Sequence of Lysinibacillus sphaericus WHO Reference Strain 2362. *Genome Announc.* **2016**, *4*, e00545-16. [CrossRef] [PubMed]
29. International Potato Center. *Why Are Potatoes Important?* International Potato Center: Lima, Perú, 2017.
30. Spooner, D.M.; Núñez, J.; Trujillo, G.; Herrera, M.d.R.; Guzmán, F.; Ghislain, M. Extensive simple sequence repeat genotyping of potato landraces supports a major reevaluation of their gene pool structure and classification. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 19398–19403. [CrossRef] [PubMed]
31. Ghislain, M.; Andrade, D.; Rodríguez, F.; Hijmans, R.J.; Spooner, D.M. Genetic analysis of the cultivated potato Solanum tuberosum L. Phureja Group using RAPDs and nuclear SSRs. *Theor. Appl. Genet.* **2006**, *113*, 1515–1527. [CrossRef] [PubMed]
32. Spooner, D.M. The potato: Evolution, biodiversity and genetic resources. J.G. Hawkes. *Am. Potato J.* **1990**, *67*, 733–735. [CrossRef]
33. Sun, L.; Kong, D.; Gu, W.; Guo, X.; Tao, W.; Shan, Z.; Wang, Y.; Wang, N. Determination of glyphosate in soil/sludge by high performance liquid chromatography. *J. Chromatogr. A* **2017**, *1502*, 8–13. [CrossRef] [PubMed]
34. Saxberg, B.E.H.; Kowalski, B.R. Generalized Standard Addition Method. *Anal. Chem.* **1979**, *51*, 1031–1038. [CrossRef]

35. Bhattacharjee, R.B.; Singh, A.; Mukhopadhyay, S.N. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: Prospects and challenges. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 199–209. [CrossRef] [PubMed]
36. Borggaard, O.K.; Duke, S.O.; Powles, S.B. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: Areview. *Pest Manag. Sci.* **2008**, *63*, 1100–1106. [CrossRef] [PubMed]
37. Nagaraja, P.; Bhaskara, B.L. Direct Sensitive spectrophotometric assessment of carbofuran using dapsone as a new chromogenic reagent in formulations and environmental samples. *Eclet. Quim.* **2006**, *31*, 43–48. [CrossRef]
38. Friedman, M. Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences. *J. Agric. Food Chem.* **2004**, *52*, 385–406. [CrossRef]
39. Moneke, A.; Okpala, G.; Anyanwu, C. Biodegradation of glyphosate herbicide in vitro using bacterial isolates from four rice fields. *Afr. J. Biotechnol.* **2010**, *9*, 4067–4074.
40. Arfarita, N.; Imai, T.; Kanno, A.; Yarimizu, T.; Xiaofeng, S.; Jie, W.; Higuchi, T.; Akada, R. The potential use of trichoderma viride strain FRP3 in biodegradation of the herbicide glyphosate. *Biotechnol. Biotechnol. Equip.* **2013**, *27*, 3518–3521. [CrossRef]
41. Cheloufi, R.; Messaadia, H.; Alayat, H. Biodegradation of herbicides by Pseudomonas aeruginosa in two soils types of the Bou Namoussa irrigable perimeter (Algerian Extreme Northeast): Effects on mineral nutrition (P_2O_5 and NO_3^-). *J. Mater. Environ. Sci.* **2017**, *8*, 2513–2521.
42. Rivera, S.; Dussán, J. Trial evaluation of toxicity and pathology in murine tissues using Bacillus sphaericus Colombian strain. *Actual Biol.* **2008**, *30*, 125–134.

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).