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RESEARCH

Glyphosate Residues in Soil and Phosphate Fertilizer Affect Foliar Endophytic Microbial Community Composition and Phytohormone Levels in Potato

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ABSTRACT

Glyphosate, the active ingredient of glyphosate-based herbicides (GBHs), controls the growth of weeds by inhibiting the shikimate pathway, thereby interrupting amino acid biosynthesis in plants. However, several microbes have the shikimate pathway, and the effect of glyphosate on these non-target organisms is ignored. The action of GBHs is further complicated when used with other agrochemicals, such as phosphate fertilizers, often varying their mode of action depending on soil type or plant species. To address the impact of GBHs and phosphate fertilizers, we simulated agricultural application of GBHs and phosphate fertilizers in a field study, investigating the composition of endophytic microbial communities and correlation of phytohormone concentrations with the microbial diversity of potato (*Solanum tuberosum*). In leaves, glyphosate residues in soil from GBH treatment alone and in combination with phosphate significantly shifted the bacterial community, whereas phosphate alone and in combination with glyphosate significantly altered the composition of the fungal community. There were no significant changes in microbial communities in roots and tubers. Plants treated with GBHs showed higher ratios of potentially glyphosate-resistant bacteria, with *Xanthomonadaceae* and *Moraxellaceae* being more abundant. Additionally, phytohormone concentrations showed various correlations with bacterial and fungal diversity in different treatments. The study highlights the impact of GBH residues in soil, particularly in combination with phosphate fertilizers, on the composition of plant-associated microbial communities. Together with changes in phytohormone concentrations, plant health may be affected. Future studies could provide insights into whether these agrochemicals influence the plant microbiome, leading to changes in phytohormones or vice versa.

Keywords: EPSPS, herbicide, microbiome, pesticide, plant defense, plant hormones, Roundup, *Solanum tuberosum*

The agriculture industry relies heavily on agrochemicals such as pesticides, fertilizers, and plant growth stimulators to ensure removal of weeds, improve nutrient composition of soil, and increase yield. Although agrochemicals are targeted to improve crop productivity, the dependence on these chemicals has dire consequences, impacting biodiversity decline and ecosystem functioning in agricultural as well as non-agricultural landscapes (Russo et al. 2020). Regular application of high concentrations of agrochemicals in agricultural fields for many years has adversely affected soil health (Hussain et al. 2009; Mandal et al. 2020), soil microbiota (Meena et al. 2020), plant diversity, and primary productivity of agricultural crops (Russo et al. 2020). The consequences extend beyond target species to non-agricultural landscapes via the accumulation of agrochemicals in the food web (Hayes and Hansen 2017). Cascading effects from plants to herbivores and predators and transportation to natural aquatic and terrestrial ecosystems can endanger species diversity (Mancini et al. 2019) and risk health and quality of human life for generations (Devi et al. 2022; Peillex and Pelletier 2020).

Every year, approximately 65,000 tons of pesticides and 2.16 million tons of fertilizers are used in fields (Davydov et al. 2018). Among pesticides, glyphosate-based herbicides (GBHs) are the most used globally. Glyphosate, the active ingredient of GBHs,

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controls weed growth by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the key enzyme in the shikimate pathway (Leino et al. 2021). Once the enzyme is blocked, glyphosate eventually interrupts the supply of the precursor (chorismate) for the biosynthesis of three aromatic amino acids: tryptophan, phenylalanine, and tyrosine (Maeda and Dudareva 2012). These amino acids are essential in plants for the synthesis of proteins, growth promoters (e.g., phytohormones such as the auxin indole-3-acetic acid), volatile organic compounds, and secondary metabolites (tannins, anthocyanins, flavonoids) essential for plant defense against herbivores and pathogens (Fuchs et al. 2021; Tzin and Galili 2010). Blocking the production of these essential amino acids eventually kills off the weeds.

Glyphosate is assumed to degrade quickly via soil microorganisms into AMPA or sucrose (Borggaard and Gimsing 2008), though this is not always the case. Accumulating evidence shows that the rate of glyphosate degradation depends on environmental conditions and is slower in northern latitudes, where residues of glyphosate have been detected even years after the last application (Helander et al. 2012). Glyphosate residues in soil have been shown to affect diverse soil organisms and subsequently plants and their interactions (Aristilde et al. 2017; Fuchs et al. 2022b; Helander et al. 2018, 2019; Muola et al. 2021; Niemeyer et al. 2018; Rainio et al. 2020). Recently, the presence of bioactive glyphosate residues and degradation products has been confirmed to persist in soil from diverse agricultural habitats, drawing attention to potential effects on the biology and ecology of encountered organisms (Maggi et al. 2020).

Contrary to the widespread claim that GBHs are safe, glyphosate residues and degradation products (e.g., AMPA) persist in soil even after the recommended 2-week "safety period" (Helander et al. 2012). This could lead to non-target agricultural plants in fields absorbing glyphosate residues, impacting plant performance and physiological processes (Fuchs et al. 2021). Phytohormones and secondary metabolites regulate plant growth and development and exhibit intensive crosstalk, interacting with each other in a synergistic and antagonistic manner to regulate plant performance and plant physiological responses to biotic and abiotic challenges (Berens et al. 2017; Fuchs et al. 2022a). Disturbing the phytohormone balance may increase plant vulnerability to various stressors, which may be exploited by specific herbivores to circumvent plant defenses (Zarate et al. 2007). Furthermore, phytohormones and secondary metabolites are essential signaling molecules for successful establishment of the mutualistic interactions between plants and microorganisms (Liu et al. 2021) that play key roles in host plant survival. Among non-target organisms, animals are considered safe from the toxic effects of GBHs due to the absence of the shikimate pathway in animal cells. However, the toxicological effects of GBH and glyphosate residues in soil, water, and food are causing genotoxicity, neurotoxicity, DNA damage, and physiological as well as hormone disruptions in both invertebrates and vertebrates (Gill et al. 2018; Helander et al. 2023b; Peillex and Pelletier 2020; Puigbò et al. 2022).

Risk and safety evaluations of agrochemicals often do not consider their effects on microbes. Recently, several studies have emphasized the harmful effects of GBHs on microbes, as several bacteria and fungi have the shikimate pathway and are especially vulnerable to glyphosate (Leino et al. 2021; Rainio et al. 2020). The negative effects of glyphosate have been detected in soil microbes as well as microorganisms associated with plants and animals (Druille et al. 2016; Helander et al. 2018, 2019, 2023a; Mathew et al. 2023; Ruuskanen et al. 2020, 2023; Székács and Darvas 2018; van Bruggen et al. 2021). This is concerning because microorganisms associated with plant and animal tissues contribute to several life-sustaining functions, predominantly nutrition, stress tolerance, and immunity to disease (Bacon and White 2016). Depending on the structural characterization of the EPSPS enzyme in the shikimate pathway, microorganisms can be either susceptible or resistant to glyphosate (Leino et al. 2021; Mathew et al. 2022, 2023). Furthermore, microorganisms may change the sensitivity status to the herbicide via a single mutation in the active site of EPSPS (Rainio et al. 2021).

The impact of GBHs on non-target organisms is further complicated when interacting with other agrochemicals, principally fertilizers, especially in colder climatic zones (Helander et al. 2012). More than 40 million tons of phosphate fertilizers are applied to agricultural land globally. The interaction and competition between phosphate fertilizers and glyphosate for binding sites in soil have been extensively studied (Munira et al. 2016). Applying a GBH to the soil after a phosphate fertilizer leads to increased leaching of glyphosate into groundwater. Depending on pH conditions, soils with added phosphate fertilizers have reduced binding capacity for glyphosate for several years (Munira et al. 2016; Padilla and Selim 2019; Wang et al. 2005). Consequently, it is of special interest whether the effects of glyphosate residues decrease faster in soil when GBHs are applied after phosphate supplementation. However, the interaction between phosphate and glyphosate in soil remains unclear, especially with regard to the effects on plants and associated microbial communities.

The main aim of our study was to examine the effects of GBH application with and without phosphate fertilization on endophytic microbial communities and their association with changes in phytohormone levels in potato (*Solanum tuberosum*) (Fuchs et al. 2022a). We answered the following research questions: (i) Does GBH application affect endophytic bacterial and fungal communities differently in roots, tubers, and leaves of potato? (ii) Does GBH application affect the abundance of bacteria and fungi in roots, tubers, and leaves of potato based on their sensitivity to glyphosate? (iii) Does the interaction between glyphosate residues and phosphate fertilizer affect bacterial and fungal community compositions in potato roots, tubers, and leaves and their association with phytohormone concentrations? Hence, in this study we tested the following hypotheses: (i) GBH application in soil affects the endophytic microbial communities in roots, tubers, and leaves of potato and decreases the abundance of glyphosate-sensitive microbes; (ii) the effects of GBH application on potato endophytic microbial communities increase when phosphate fertilizer is added to GBH-treated soil.

MATERIALS AND METHODS

Experimental design. The experiment was conducted at the field site of the University of Turku Ruissalo Botanical Garden (60°26′N, 22°10′E) in southwestern Finland in 2019. The soil type in the field was medium clay with high organic matter content (>120 g kg^{-1}) and pH 5.9 (Hagner et al. 2019). During the experiment (June to August 2019), the average temperature was 17.0°C and the average precipitation was 53.9 mm. Our experimental setup had four treatment groups: control, GBH, phosphate, and GBH with phosphate. The field had been treated with the GBH Roundup Gold (Bayer, Leverkusen, Germany) twice a year since 2013, simulating standard agricultural practices. The study site consisted of 20 plots (23 × 1.5 m each), with 10 GBH-treated plots alternating with 10 control plots. The soil was treated in May 2019 with the standard dose of Roundup Gold (450 g $liter^{-1}$ glyphosate isopropylamine salt, CAS Registry Number 38641-94-0, application rate 6.4 liters/ha dissolved in 3 liters of tap water per plot). The control soil was sprayed with the same amount of tap water (3 liters per

plot) (Helander et al. 2019). Before GBH treatment, each plot was divided in half, and alternating subplots were treated with phosphate (Ferticare, Yara International, Norway) 80 g in 10 liters of water per subplot. The control subplots were treated with water only. Weeds were manually removed from all plots to minimize plant competition in the treatments.

We monitored the degradation and persistence of glyphosate by taking soil samples at a late stage (15 August 2019) of the experiment. Detected concentrations of glyphosate residues and AMPA have been published previously (Mathew et al. 2023). We planted six potato tubers (*S. tuberosum* var. *Ditta*, organically grown) in each subplot for the four treatment groups (total 240 plants) 24 days after the soil had been treated with GBH (6 June 2019). We monitored chewing insect herbivory (no aphids were recorded, and larger mammalian herbivores were excluded by a fence) using a scale of 0 to 3 and recorded plant height, number of stems, and (at the end of the experiment) fresh weight of tubers and dry weight of aboveground biomass (21 August 2019).

Sample collection. Root, tuber, and leaf tissues were collected 60 days after planting (6 August 2019) by destructively sampling one potato plant per subplot of each treatment (40 samples per tissue altogether). Approximately 1 g of leaf (one fully developed central leaflet and two leaflets on either side of the central leaflet from a centrally located leaf) and 250 to 300 mg of root tissues were cut from the plant using sterile scissors, and one tuber was separated. The samples were placed in plastic bags on ice and immediately brought to the lab. Each tissue sample was washed thoroughly with tap water and cut into smaller pieces. Approximately 100 mg of each sample was weighed, and care was taken to include tuber tissue samples with peels. Sterilization of the samples was done under a laminar flow hood by washing in 70% ethanol for 1 min followed by soaking in 3% chloramine T (Klorilli; Kiilto, Tampere, Finland) for 3 min and then rinsing three times in sterile Milli-Q water (EMD Millipore, Burlington, MA) for 1 min. After drying, the samples were transferred to 2-ml microcentrifuge tubes and stored immediately at –80°C to proceed with DNA extraction. Control plates were prepared to check the efficiency of sterilization by plating 100 µl of the water from the last rinse on Reasoner's 2A agar plates and incubating at room temperature for 2 weeks. None of the plates showed any microbial growth.

We sampled one randomly chosen potato plant from each subplot for phytohormone analysis (80 samples) 63 days after planting. Approximately 200 mg of leaf material was collected per plant. To minimize variation between plants, we performed a homogeneous cutting of a central leaflet from one fully developed leaf located centrally on each plant, which was then immediately transferred to a 2-ml microcentrifuge tube and flash frozen in liquid nitrogen. Samples were stored at –80°C until preparation for phytohormone analysis.

DNA extraction. The frozen tissue samples underwent two 30-s runs in a TissueLyser II (QIAGEN, Hilden, Germany) with two 3.2-mm chrome-steel beads and 0.1-mm glass beads for rupturing of the tissue followed by homogenization. DNA was extracted from homogenized samples using an InviSorb Spin Plant Mini Kit (STRATEC Biomedical Systems, Birkenfeld, Germany). The concentrations of DNA samples were analyzed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and diluted to 30-µl aliquots at a concentration of 30 ng/µl.

16S rRNA gene-targeted polymerase chain reaction for bacterial community analysis. Variable regions V6 to V8 of the bacterial 16S rRNA gene were amplified from potato DNA samples using a nested polymerase chain reaction (PCR) approach. The first round of PCR using the primers 799F (5′-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett 2001) and 1492R (5′-GGYTACCTTGTTACGACTT-3′)—modified from that used by Lane (1991)—included 30 ng of sample DNA, 1× PCR buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.3 µM of each primer, and 2,000 U/ml GoTaq DNA Polymerase (Promega, Madison, WI) in a 30-µl reaction volume. A second round of PCR was performed with M13-tagged 1062F (5′-ACGACGTTGTAAAAGTCAGCTCGTGYYGTGA-3′) (Ghyselinck et al. 2013) and M13-tagged 1390R (5′-CATTAAGTTCCCATTAACGGGCGGTGTGTRCAA-3′) (Zheng et al. 1996) primers with similar PCR conditions, with the exception of 1:10 dilution of the first PCR product as template DNA. This was followed by another round of PCR to tag the PCR products with barcodes and P1 adapter sequences for Ion Torrent sequencing (Thermo Fisher Scientific) (Mäki et al. 2016) as well as 1:1 dilutions of PCR products from the second PCR round as a template. The amplification profile for the first round of PCR was 3 min of initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 1 min. Final extension was carried out at 72°C for 5 min. The same protocol was followed for second and third rounds of PCR but with 25 and 8 cycles, respectively. All PCRs had negative controls. The amplicons were analyzed on 1.5% agarose gel after each round of PCR amplification to ensure the efficiency of PCR and the quality of amplicons.

Internal transcribed spacer gene-targeted sequencing for fungal community analysis. The internal transcribed spacer (ITS) regions from fungal endophytes were PCR-amplified using M13-tagged ITS7F (5′-ACGACGTTGTAAAAGTGARTCA TCGAATCTTTG-3′) and M13-tagged ITS4R (5′-CATTAAGT TCCCATTATCCTCCGCTTATTGATATGC-3′) (Ihrmark et al. 2012) primers. The 30-µl reaction mixture contained 30 ng of sample DNA, 1× PCR buffer, 0.2 mM dNTPs, 0.3 µM of each primer, and 1,250 U/ml GoTaq DNA Polymerase. The amplification procedure was 5 min of initial denaturation at 95°C followed by 35 cycles of denaturation, annealing, and extension at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, respectively. Final extension was carried out at 72°C for 7 min. The second round of PCR was performed using a 1:10 dilution of the first PCR product as a template and barcode primers and P1-tailed reverse primers. All other PCR components were the same as the first round of PCR, with the exception that amplification steps were repeated for only eight cycles. All PCR cycles had negative controls along with samples. The amplicons were analyzed on 1.5% agarose gel after each round of PCR amplification.

Library preparation and sequencing. The concentration of PCR products was checked on a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). Volumes constituting 30 ng of DNA from each sample were pooled to prepare an equimolar library. Library samples were size-fractionated on Pippin Prep (Sage Science, Beverly, MA) using a 2% agarose gel cassette (Marker B), selecting 350- to 500-bp amplicons. The purified libraries were sequenced on Ion 314 Chip v2 in an Ion Personal Genome Machine (Thermo Fisher Scientific).

Hormone extraction and quantification. Frozen samples (approximately 200 mg fresh weight) were homogenized in liquid nitrogen with a mortar and pestle and weighed. Phytohormones were extracted with cold (–20°C) methanol/water/formic acid (15:4:1 vol/vol/vol) as described by Dobrev and Vankova (2012) and Djilianov et al. (2013). The following phytohormones were analyzed using high-performance liquid chromatography (UltiMate 3000; Thermo Fisher Scientific) coupled with a 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Thermo Fisher Scientific): benzoic acid, salicylic acid, phenylacetic acid,

indole-3-acetamide, indole-3-acetic acid, 2-oxindole-3-acetic acid, abscisic acid, phaseic acid, jasmonic acid, 1-aminocyclopropane-1-carboxylic acid, cytokinin, dihydrozeatin, *trans*-zeatin, *cis*-zeatin, ribosides, O-glycosides, and N-glycosides. Phytohormone quantification was carried out using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data were processed with Analyst software version 1.5 (Thermo Fisher Scientific). Here we refer to phytohormone analyses and results published by Fuchs et al. (2022a), which correspond to the plant material used for the present study.

Bioinformatics and statistical analyses. Sequences from both 16S rRNA (Supplementary Dataset S1) and ITS (Supplementary Dataset S2) gene sequences were individually grouped into amplicon sequence variants (ASVs), and their phylogenetic affiliation was determined using DADA2's naive Bayes classifier (R 4.1.1, DADA2 version 1.22.0) (Callahan et al. 2016). The reference taxonomies used for the 16S rRNA and ITS genes were SILVA 138.1 prokaryotic SSU (Quast et al. 2013) and UNITE general FASTA release for eukaryotes (version 8.3) (Abarenkov et al. 2021), respectively. These analyses were performed using the 'nextflow' pipeline (version 22.10.1) and the 'ampliseq' pipeline (version 2.4.0) (Straub et al. 2020) on the Puhti supercomputing cluster (BullSequana X400; Atos, Bezons, France) made available by the Center for Scientific Computing (Espoo, Finland).

The R packages 'mia' (version 1.8.0) (Ernst et al. 2023) and 'vegan' (version 2.6-4) (Oksanen et al. 2022) were used to measure the alpha and beta diversities. The R package 'vegan' was also utilized for preliminary data checks using rarefaction curves and assessing the significance of community shifts using permutational multivariate analysis of variance (PERMANOVA). The ASVs were agglomerated to appropriate taxonomic levels using functions in the 'mia' package. Differentially abundant taxa were identified, after applying a 10% prevalence threshold, using a combination of five differential abundance estimators: 'ALDEx2' version 1.32.0 (Fernandes et al. 2013), 'ANCOM-BC' version 2.2 (Lin and Peddada 2020), 'MaAsLin2' version 1.13.0 (Mallick et al. 2021), 'LinDA' version 1.1 (Zhou et al. 2022), and 'DESeq2' version 1.40 (Love et al. 2014). Nearing et al. (2022) found that there was high variation in the output of differential abundance tools across numerous 16S rRNA data sets. Thus, following their advice, we used the consensus of multiple preselected tools for the analysis. The estimators queried at a significance level of $P < 0.05$ after Benjamini-Hochberg correction, and responses in which more than three of the five estimators indicated a significant response were considered significant.

The EPSPS enzyme can be classified as sensitive or resistant to glyphosate based on amino acid markers present on the active site of the protein. The ASVs from 16S and ITS sequencing in this study provided reliable taxonomic information but lacked information regarding the exact EPSPS type of the microbes. Rainio et al. (2020) demonstrated high taxonomic conservation of EPSPS and potential sensitivity of the taxa to glyphosate. Based on the method described by Mathew et al. (2022), bacterial species were mapped against EPSPS sequences from the Alignable Tight Genomic Clusters database (Kristensen et al. 2017) and fungal species were mapped against the Pfam database (El-Gebali et al. 2019). Sensitivity to glyphosate was then scored based on a range of 0 (resistant: no sequences in the taxonomic group were putatively sensitive to glyphosate) to 1 (sensitive: all known sequences in the taxonomic group were putatively sensitive to glyphosate). There were a range of in-between values (i.e., taxonomic groups that contained sequences that were estimated to be sensitive, resistant, or unknown). To account for this, cutoff values of <0.2 and >0.8 were used to indicate potential resistance and potential sensitivity, respectively.

Associations between microbial community compositions and measures of plant structure and performance (aboveground and belowground plant weights, plant height, number of stems, herbivory, and phytohormone levels) were analyzed using PERMANOVA and correlation analyses. Correlation of the Shannon diversity of the microbiota at the family taxonomic level and plant metrics between different treatment groups were compared using Fisher's z (Fisher 1925) and Zou's confidence interval (Zou 2007), as implemented in the R package 'cocor' (version 1.1-4) (Diedenhofen and Musch 2015). All statistical analyses were conducted using the open source software R 4.3.0.

RESULTS

Taxonomic distribution of microbial communities in potato. A total of 368,520 bacterial 16S rRNA genes were sequenced to saturation (Supplementary Fig. S1) and clustered into 2,686 ASVs belonging to 25 phyla, 126 orders, and 198 families. The main bacterial phyla found in the endophytic communities of potato leaf, root, and tuber were *Proteobacteria* and *Bacteroidota*. Typical bacterial orders included *Sphingomonadales* and *Cytophagales* in leaves and *Burkholderiales* and *Rhizobiales* in roots and tubers (Fig. 1A). At the family level, the prominent taxa included *Hymenobacteraceae* and *Sphingomonadaceae* in leaves and *Methylophilaceae* in roots as well as *Comamonadaceae*, *Rhizobiaceae*, *Pseudomonadaceae*, and unclassified groups in both roots and tubers (Supplementary Fig. S2A).

In addition, 306,991 fungal ITS regions were sequenced to saturation (Supplementary Fig. S3) and clustered into 1,210 ASVs belonging to 9 phyla, 83 orders, and 170 families. The main fungal phyla found in the endophytic communities of potato were Ascomycota, Basidiomycota, and Glomeromycota in the roots. Typical fungal orders included Pleosporales in all plant tissues, Capnodiales and Tremellales in leaves, and Glomerellales and Helotiales in roots and tubers (Fig. 1B). The family-level classification included Bulleribasidiaceae and Cladosporiaceae in leaves and Glomerellaceae and Helotiaceae in roots and tubers, with an increased abundance of Plectosphaerellaceae in tubers (Supplementary Fig. S2B). Both bacteria and fungi showed similar community compositions in the belowground plant tissues (roots and tubers), which differed from the aboveground leaves.

Glyphosate and phosphate affected bacterial but not fungal diversity. The bacterial diversity (Fig. 2A) was significantly higher in response to the GBH with phosphate treatment on leaves (Wilcoxon test and Benjamini-Hochberg procedure $P < 0.01$) and the phosphate treatment on tubers (Wilcoxon test and Benjamini-Hochberg procedure $P < 0.001$). The fungal community diversity was not affected by any of the treatments (Fig. 2B).

Both bacterial and fungal communities were affected by GBH and phosphate treatments. Redundancy analysis was performed on the bacterial and fungal communities using ASVs as the response variables and plant tissue, plot, and treatment as the explanatory variables testing the effect of GBH and phosphate treatment exposure on microbial communities. The bacterial community response was significant for the plant tissue, plot, and treatment (PERMANOVA $P < 0.0001$, < 0.05, and < 0.01, respectively), explaining 39.28% of the observed community variation in the first two axes of the constrained axis plot. The fungal community response was significant for the plant tissue (PERMANOVA $P < 0.0001$), explaining 20.96% of the observed community variation in the first two constrained axes.

The bacterial community was significantly affected by the GBH with phosphate treatment on the leaves and tubers (PERMANOVA $P < 0.01$). Additionally, the bacterial community was affected by

GBH treatment on the leaves (PERMANOVA $P < 0.001$) and phosphate treatment on the tubers (PERMANOVA $P < 0.01$). The fungal community response to the treatments was somewhat significant on the leaves and tubers (PERMANOVA $P < 0.05$ and < 0.1, respectively), explaining a respective 23.95 and 10.84% of the observed community variation. The fungal community was significantly affected by the phosphate and GBH with phosphate treatments on the leaves (PERMANOVA $P < 0.05$) (Fig. 3; Table 1; Supplementary Material S5.1 and S5.2).

GBH treatment affected relative abundance of certain bacterial taxa in potato leaves. The differential abundance analysis indicated that GBH treatment alone or in combination with phosphate significantly affected certain bacterial families in potato leaves but not in roots or tubers (Table 2). The relative abundance was lower for *Hymenobacteraceae* in response to GBH treatment and *Enterobacteriaceae* in response to phosphate treatment. The relative abundance was higher for *Flavobacteriaceae*, *Moraxellaceae*, *Xanthomonadaceae*, *Weeksellaceae*, *Sphingobacteriaceae*, and *Erwiniaceae* in response to GBH and *Comamonadaceae*, *Flavobacteriaceae*, *Moraxellaceae*, *Caulobacteraceae*, *Xanthomonadaceae*, *Sphingobacteriaceae*, and *Micrococcaceae* in response to GBH with phosphate treatment. Finally, *Chitinophagaceae* was relatively more abundant upon phosphate treatment in tubers. With regard to the fungal community, only two taxa, *Helotiaceae* and *Bulleraceae*,

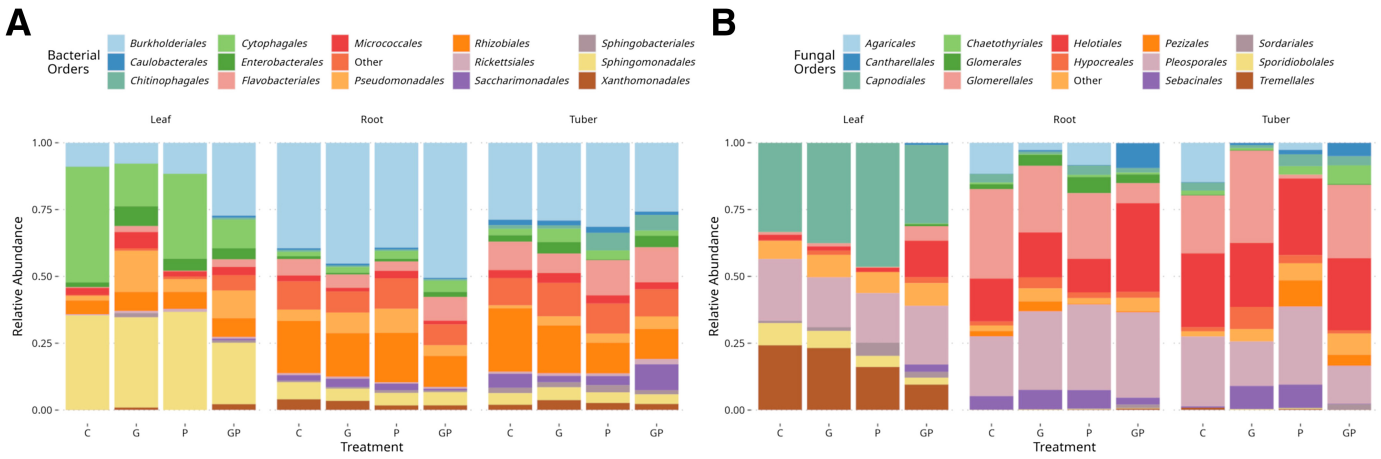

Fig. 1. Order-level taxonomic distribution of endophytic microbial communities in the roots, leaves, and tubers of potato plants averaged over 10 plants each. **A,** Bacterial communities and **B,** fungal communities in control (C), glyphosate-based herbicide (GBH) (G), phosphate (P), and GBH with P (GP) treatment groups. Amplicon sequence variants (ASVs) outside the 14 most abundant orders and those that could not be assigned taxonomy at the order level are included as "Other."

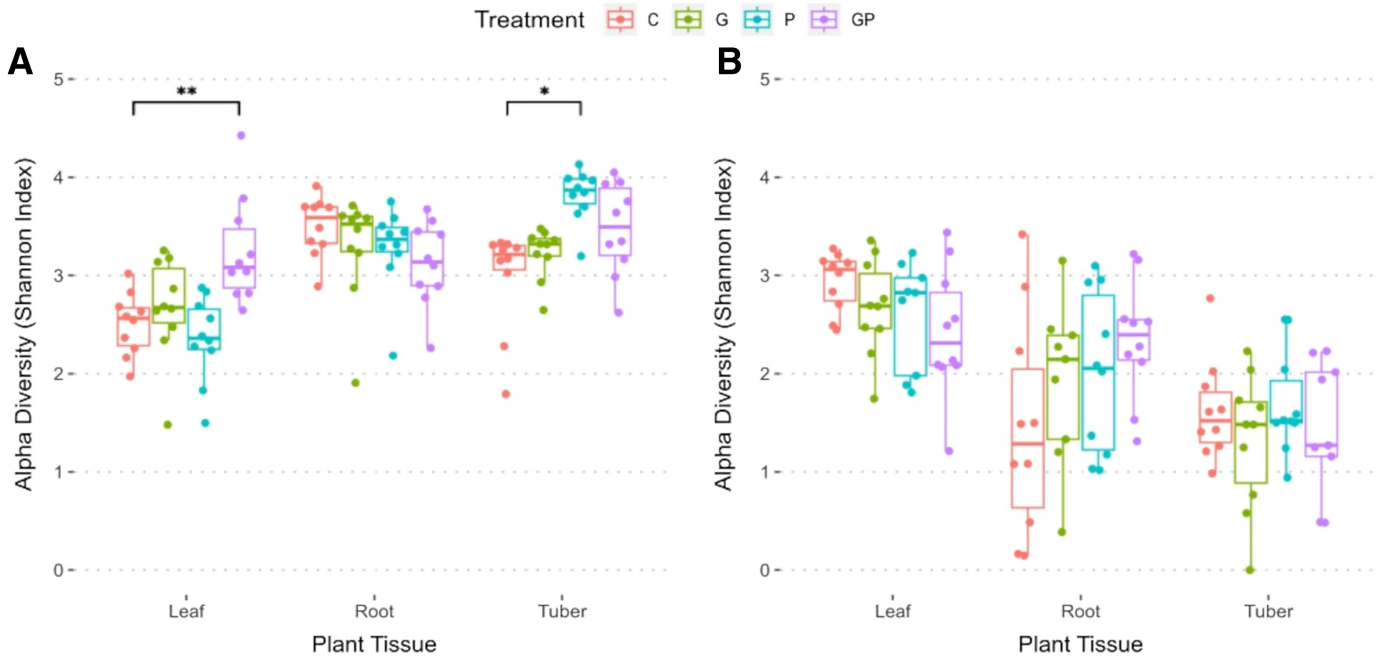

Fig. 2. Alpha diversity of microbial communities in different plant tissues comparing control (C) and glyphosate-based herbicide (GBH) (G), phosphate (P), and GBH with P (GP) treatment groups. **A,** Box plots of Shannon diversity indexes for bacterial communities. **B,** Box plots of Shannon diversity indexes for fungal communities. Comparisons were tested using Wilcoxon rank sum test, and results were corrected using the Benjamini-Hochberg procedure. $^{*}P < 0.01$, $^{**}P < 0.001$.

showed differential abundance, being relatively less abundant in leaves exposed to GBH treatment.

Phytohormone levels, plant biomass, and herbivory. Phytohormone and aboveground biomass results were retrieved from the study by Fuchs et al. (2022a) and compared with control values. Phosphate treatment alone increased salicylic acid concentrations (Supplementary Tables S1 and S2). GBH with phosphate increased the auxin phenylacetic acid and decreased the abscisic acid precursor phaseic acid and cytokinin ribosides (CK_R). GBH alone increased concentrations of jasmonic acid and abscisic acid and decreased cytokinin oxides (CK_O) and ribosides. Plant performance measured as dry biomass at the end of the season showed an increase in the biomass of plants growing in phosphate-treated soil with GBH (Supplementary Fig. S4). Herbivore damage remained negligible throughout the season.

Treatments affected certain microbiota–phytohormone associations. GBH, GBH with phosphate, and phosphate treatments significantly reduced the correlations between tuber bacteria and jasmonic acid, leaf bacteria and phenylacetic acid, and tuber fungi and dihydrozeatin, respectively (Fig. 4A to C; Supplementary Material 5.3), whereas the GBH with phosphate treatment significantly increased the correlation between leaf fungi and jasmonic acid levels (Fig. 4D; Supplementary Material 5.3). Neither PERMANOVA nor differential abundance analysis of hormone levels binned into two categories (high/low) yielded any significantly differentially abundant taxa.

Potentially glyphosate-resistant bacterial families were relatively more abundant than glyphosate-sensitive bacterial families in potato leaves in all treatments. Based on the type of EPSPS enzyme, the in silico analysis estimated the potential sensitivity of prominent bacterial taxa in potato to glyphosate. The bacterial communities in leaves showed a higher relative abundance of ASVs of taxonomic groups with members known to have glyphosate resistance compared with ASVs assigned to a taxonomic group with members known to have glyphosate sensitivity in all three treatments compared with controls. In the roots and tubers, there were no major shifts in the ratios of potential glyphosate-resistant to potential glyphosate-sensitive taxa (Fig. 5A). The most abundant glyphosate-resistant bacterial taxa were *Sphingomonadaceae*, *Rhizobiaceae*, and *Moraxellaceae* in leaves and *Xanthomonadaceae* in underground tissues. The most abundant potentially glyphosate-sensitive bacterial families were *Microbacteriaceae* and *Comamonadaceae* in leaves and *Methylophilaceae*, *Comamonadaceae*, and *Oxalobacteraceae* in roots and tubers.

The fungal community analysis showed ASVs assigned to fungal taxa known to have glyphosate-sensitive members, members with undetermined sensitivity to glyphosate, and members with negligible putative glyphosate resistance (Fig. 5B). Therefore, it is difficult

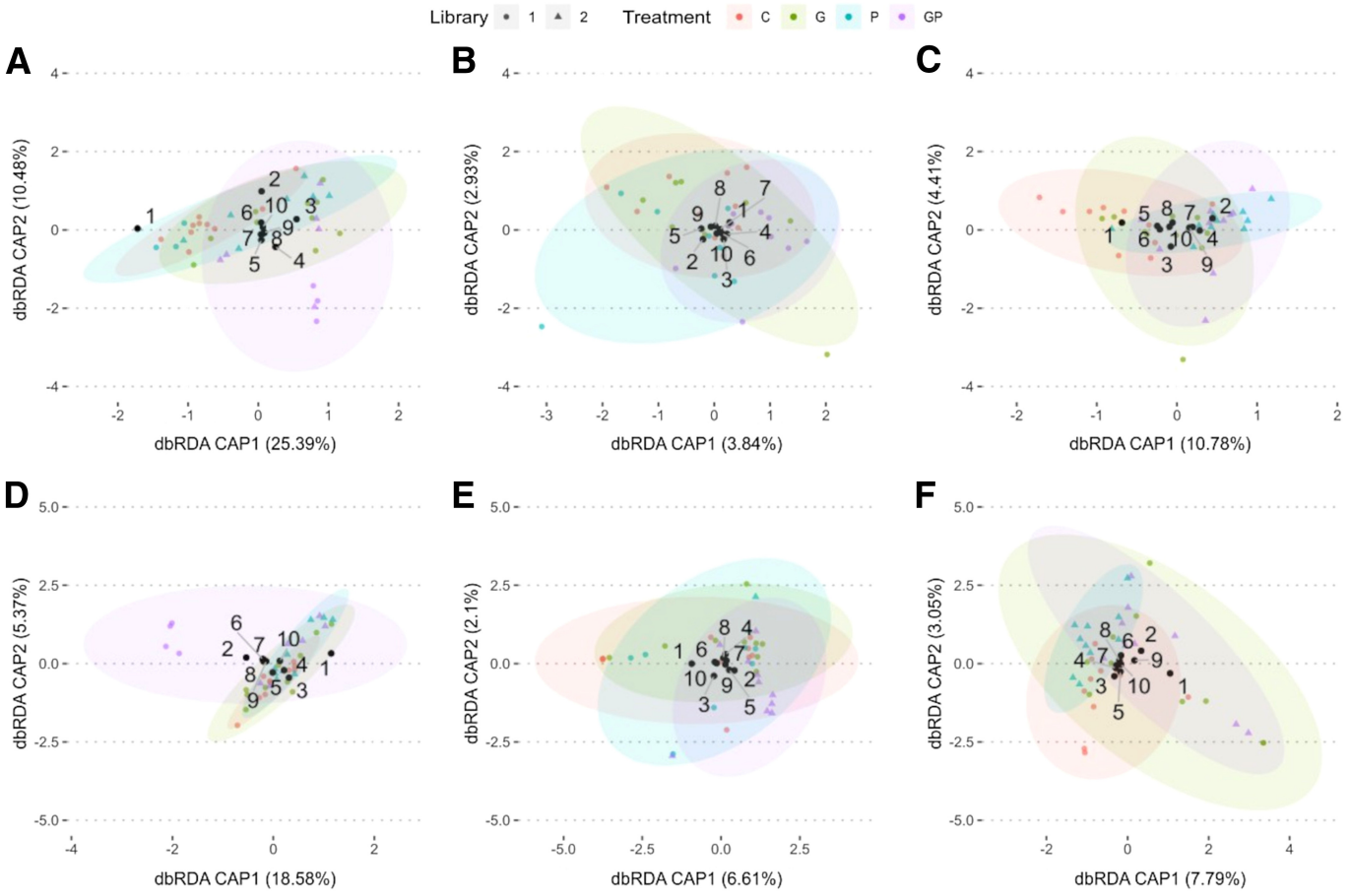

Fig. 3. Samples and 10 most variable taxa plotted on the first two main axes from redundancy analysis of the bacterial community response to glyphosate-based herbicide (GBH) (G), phosphate (P), and GBH with P (GP) treatments in **A,** leaves, **B,** roots, and **C,** tubers as well as fungal community response to the same treatments in **D,** leaves, **E,** roots, and **F,** tubers. Treatment type is indicated by the color of the symbol and 95% confidence interval ellipse. CAP = constrained analysis of principal coordinates; dbRDA = distance-based redundancy analysis. The 10 most variable taxa in each subfigure are shown in Table 1.

to determine whether glyphosate residues in soil or phosphate treatments affected the functionality of the fungal community.

DISCUSSION

Our study indicates that GBH residues in soil alone or in combination with phosphate fertilizer significantly altered endophytic bacterial community composition in potato leaves but not in roots and tubers. In addition, the ratio of the abundance of potentially glyphosate-resistant bacteria to potentially glyphosate-sensitive bacteria was estimated to be relatively higher in leaves of potato compared with roots and tubers grown in soil treated with GBH and GBH with phosphate.

The levels of glyphosate and AMPA were markedly higher in soil treated with GBH compared with controls (Mathew et al. 2023) when sampled at the end of the growing season (mid-August 2019). In addition to slow degradation of GBH applied to soil at the beginning of the study, the experimental field has been treated with GBH since 2013, simulating agricultural practices of adding agrochemicals every year, and could have accumulated glyphosate residues, elevating levels in the soil or affecting the soil microbiome (Bueno de Mesquita et al. 2023; Caggìa et al. 2023; Lorch et al. 2021). The interaction between GBHs and phosphate fertilizers is complicated because phosphate availability influences glyphosate's presence in soils due to shared adsorption mechanisms. European soils have been fertilized with phosphorus for decades at levels greater than what is utilized by crops (Valkama et al. 2009). Excess accumulation of phosphate fertilizers reduces the capacity of soils to adsorb glyphosate or phosphate, leading to remobilization of glyphosate residues in soil (Laitinen et al. 2009). Thus, understanding the dynamics of interaction between GBHs and fertilizers is crucial, especially in northern latitudes, where seasonal variations impact soil chemistry, microbial activity, and glyphosate's ecological consequences (Helander et al. 2012).

The bacterial and fungal community structure was significantly different in the aboveground leaves compared with the belowground plant tissues, roots, and tubers. Plant tissue is a major determining factor compartmentalizing microbial communities, providing physical and chemical niches for certain bacterial taxa (Given et al. 2020; Tkacz et al. 2020; Trivedi et al. 2020), thereby engaging in symbiotic or mutualistic interactions benefiting plant growth and development (Hassani et al. 2020). Potato leaves had a higher relative abundance of *Sphingomonadales* and *Cytophagales*, which are typical orders found in the phyllosphere and endosphere of most plants. The plant growth-promoting *Burkholderiales* and *Rhizobiales* were the most dominant endophytic taxa in the roots, as has also been observed in several other plants (Ares et al. 2021; Given et al. 2020; Santoyo et al. 2016; Tian et al. 2017). With regard to the fungal community, Pleosporales, Capnodiales, Tremellales, and Helotiales were the most abundant orders and are commonly found in tissues of several plants in the Arctic (Zhang and Yao 2015). It is noteworthy that the fungal family Plectosphaerellaceae, commonly found in the roots of potato and soils from potato fields (Giraldo and Crous 2019), was highly abundant in the treatments with GBH compared with the controls. Plectosphaerellaceae includes numerous plant pathogenic genera, such as *Plectosphaerella cucumerina*, one of the main genera causing wilt, particularly in potato (Alam et al. 2021) and several other crops in tropical and temperate regions (Carrieri et al. 2014; Gilardi et al. 2012; Mirtalebi et al. 2022). Shifts in bacterial community composition due to GBH may imbalance plant beneficial bacteria that otherwise control pathogens, possibly risking plant health (Johal and Huber 2009; van Bruggen et al. 2021).

The bacterial diversity in potato was higher in leaves after GBH with phosphate treatment and in tubers treated with phosphate. In our previous study on strawberries (*Fragaria* × *ananassa*), bacterial diversity was higher in roots exposed to combined GBH and phosphate treatment (Mathew et al. 2023). The bacterial community composition also shifted in strawberry roots in response to phosphate treatment. Comparison of these two studies shows that GBH and phosphate treatments alter microbial diversity in different plant tissues depending on the host plant. The increase in bacterial diversity in potato tubers could be attributed to their direct contact with GBH or phosphate, although the diversity in potato roots remained unaffected. Remarkably, the bacterial diversity in potato leaves increased in the GBH with phosphate treatment even though the leaves were not exposed to any of the treatments directly. The fungal diversity did not change significantly in any treatment. Fungal communities are generally considered more stable than bacterial

TABLE 1
Ten most variable bacterial and fungal taxa in leaves, roots, and tubers of potato in response to glyphosate-based herbicide (GBH), phosphate, and GBH with phosphate treatments as depicted in Figure 3[a]

Taxon number	Leaf 16S	Root 16S	Tuber 16S	Leaf ITS	Root ITS	Tuber ITS
1	*Hymenobacteraceae*	*Comamonadaceae*	*Rhizobiaceae*	Cladosporiaceae	Glomerellaceae	Plectosphaerellaceae
2	*Sphingomonadaceae*	*Moraxellaceae*	*Flavobacteriaceae*	Helotiales_fam_ incertae_sedis	Helotiales_fam_ incertae_sedis	Helotiaceae
3	*Pseudomonadaceae*	*Methylophilaceae*	*Enterobacteriaceae*	Bulleribasidiaceae	Pleosporales	Psathyrellaceae
4	*Comamonadaceae*	*Oxalobacteraceae*	*Chitinophagaceae*	Didymellaceae	Plectosphaerellaceae	Phaeosphaeriaceae
5	*Methylophilaceae*	*Rhizobiaceae*	*Blattabacteriaceae*	Sporidiobolaceae	Ceratobasidiaceae	Glomerellaceae
6	*Morganellaceae*	*Weeksellaceae*	*Oxalobacteraceae*	Pleosporales	Tricholomataceae	Pezizaceae
7	*Yersiniaceae*	*Flavobacteriaceae*	*Comamonadaceae*	Glomerellaceae	Phaeosphaeriaceae	Helotiales_fam_ incertae_sedis
8	*Moraxellaceae*	*Pseudomonadaceae*	*Saccharimonadales*	Helotiaceae	Sebacinales	Massarinaceae
9	*Rhizobiaceae*	*Xanthobacteraceae*	*Saccharimonadaceae*	Serendipitaceae	Helotiaceae	Exidiaceae
10	*Microbacteriaceae*	*Microscillaceae*	*Pseudonocardiaceae*	Sordariaceae	Psathyrellaceae	Hyaloscyphaceae

[a] ITS = internal transcribed spacer.

communities, and fungi may resist glyphosate effects via mechanisms more complicated than bacterial responses (Tall and Puigbò 2022). The treatments also triggered significant changes in bacterial community composition, with GBH treatments affecting composition more in leaves and phosphate treatments affecting composition more in tubers. Likewise, phosphate treatments significantly impacted fungal community composition. Changes in bacterial diversity and microbial community composition could be due to translocation of glyphosate residues, AMPA, and phosphate via phloem and accumulation at active meristematic tissues, such as roots, tubers, and young leaves, depending on herbicide amounts, environmental factors, and plant species (Helander et al. 2019; Pantigoso et al. 2020; Singh et al. 2020). The composition of fungal communities was also shifted in leaves in the GBH with phosphate treatment. In our study, the GBH treatment with or without phosphate clearly shaped the endophytic microbial community in leaves and tubers, depicting the direct effects of glyphosate residues translocated from the soil and accumulated in these plant tissues. The lack of significant change in root microbial diversity may also be attributed to this translocation of glyphosate residues away from the roots, as

TABLE 2
Microbial families responding to glyphosate-based herbicide (GBH), phosphate (P), and GBH and P (GP) treatments in potato leaves and tubers[a]

Taxon number	Microbiota	Part	Comparison	Family	Treatment	Score	Median	IQR
1	Bacteria	Leaf	GBH:C	*Erwiniaceae*	C	3	0	0
2	Bacteria	Leaf	GBH:C	*Erwiniaceae*	GBH	3	27.5	33.25
3	Bacteria	Leaf	GBH:C	*Flavobacteriaceae*	C	5	0	0
4	Bacteria	Leaf	GBH:C	*Flavobacteriaceae*	GBH	5	8	11.25
5	Bacteria	Leaf	GBH:C	*Hymenobacteraceae*	C	3	1,361.5	711.75
6	Bacteria	Leaf	GBH:C	*Hymenobacteraceae*	GBH	3	107.5	498.25
7	Bacteria	Leaf	GBH:C	*Moraxellaceae*	C	3	0	0
8	Bacteria	Leaf	GBH:C	*Moraxellaceae*	GBH	3	19	26
9	Bacteria	Leaf	GBH:C	*Sphingobacteriaceae*	C	5	0	0
10	Bacteria	Leaf	GBH:C	*Sphingobacteriaceae*	GBH	5	23	26.75
11	Bacteria	Leaf	GBH:C	*Weeksellaceae*	C	3	0	9.25
12	Bacteria	Leaf	GBH:C	*Weeksellaceae*	GBH	3	29	47.25
13	Bacteria	Leaf	GBH:C	*Xanthomonadaceae*	C	3	0	0
14	Bacteria	Leaf	GBH:C	*Xanthomonadaceae*	GBH	3	8	29.5
15	Bacteria	Leaf	GP:C	*Caulobacteraceae*	C	4	0	0
16	Bacteria	Leaf	GP:C	*Caulobacteraceae*	GP	4	25	35
17	Bacteria	Leaf	GP:C	*Comamonadaceae*	C	5	52.5	31.25
18	Bacteria	Leaf	GP:C	*Comamonadaceae*	GP	5	474	491
19	Bacteria	Leaf	GP:C	*Flavobacteriaceae*	C	5	0	0
20	Bacteria	Leaf	GP:C	*Flavobacteriaceae*	GP	5	48.5	48.75
21	Bacteria	Leaf	GP:C	*Micrococcaceae*	C	4	0	0
22	Bacteria	Leaf	GP:C	*Micrococcaceae*	GP	4	29.5	23.75
23	Bacteria	Leaf	GP:C	*Moraxellaceae*	C	3	0	0
24	Bacteria	Leaf	GP:C	*Moraxellaceae*	GP	3	53	80.25
25	Bacteria	Leaf	GP:C	*Sphingobacteriaceae*	C	4	0	0
26	Bacteria	Leaf	GP:C	*Sphingobacteriaceae*	GP	4	37	47.5
27	Bacteria	Leaf	GP:C	*Xanthomonadaceae*	C	4	0	0
28	Bacteria	Leaf	GP:C	*Xanthomonadaceae*	GP	4	80.5	74
29	Bacteria	Leaf	P:C	*Enterobacteriaceae*	C	5	20	109
30	Bacteria	Leaf	P:C	*Enterobacteriaceae*	P	5	0	0
31	Bacteria	Tuber	P:C	*Chitinophagaceae*	C	3	15.5	33.5
32	Bacteria	Tuber	P:C	*Chitinophagaceae*	P	3	201	308
33	Fungi	Leaf	GP:C	Bulleraceae	C	3	35	24.5
34	Fungi	Leaf	GP:C	Bulleraceae	GP	3	0	10.5

[a] Differential abundance tests are based on five estimators, 'ALDEx2', 'ANCOM-BC', 'MaAsLin2', 'LinDA', and 'DESeq2', with $P < 0.05$ after adjusting for multiple testing using the Benjamini-Hochberg correction. Score column indicates the number of estimators finding significant differential abundance. Median and interquartile range (IQR) of counts are also included. C = control.

these residues do not accumulate in roots sufficiently to induce a significant impact on microbial diversity.

The increased ratio of relative abundance of glyphosate-resistant bacteria to glyphosate-sensitive bacteria in leaves but not in roots and tubers for all three treatments compared with controls is also noteworthy. The shift in the bacterial community toward glyphosate resistance could be one of the tolerance mechanisms toward glyphosate, thereby maintaining its functional contribution to the host plant (Hertel et al. 2021). However, the similar ratio of glyphosate-resistant bacteria to glyphosate-sensitive bacteria in roots in glyphosate treatments compared with controls is similar to our previous study in strawberries (Mathew et al. 2023). This could possibly be due to their lifestyle within plant tissues in comparison with free-living bacteria that are more exposed to glyphosate application (Rainio et al. 2021). The differential abundance of certain prominent bacterial families between the treatment groups has been highlighted in leaves and tubers, which might also be attributed to their mechanism of glyphosate resistance. For instance, *Moraxellaceae* and *Xanthomonadaceae* were identified as potentially glyphosate-resistant, contributing to their relatively higher abundance in GBH and GBH with phosphate treatments. However, the higher relative abundance of other bacterial taxa in the treatments compared with controls cannot be clearly justified based solely on their potential response to glyphosate.

Microbial plant associates are also often affected by phytohormone concentrations in plants. The sites for accumulation of glyphosate residues (i.e., meristematic tissues) are also sites for the production of phytohormones, and the buildup of glyphosate residues has been shown to cause phytohormone fluctuations in plants (Gomes et al. 2014). Jasmonic acid concentrations negatively correlated with bacterial abundance in tubers in the GBH treatment and positively correlated with fungal abundance in leaves

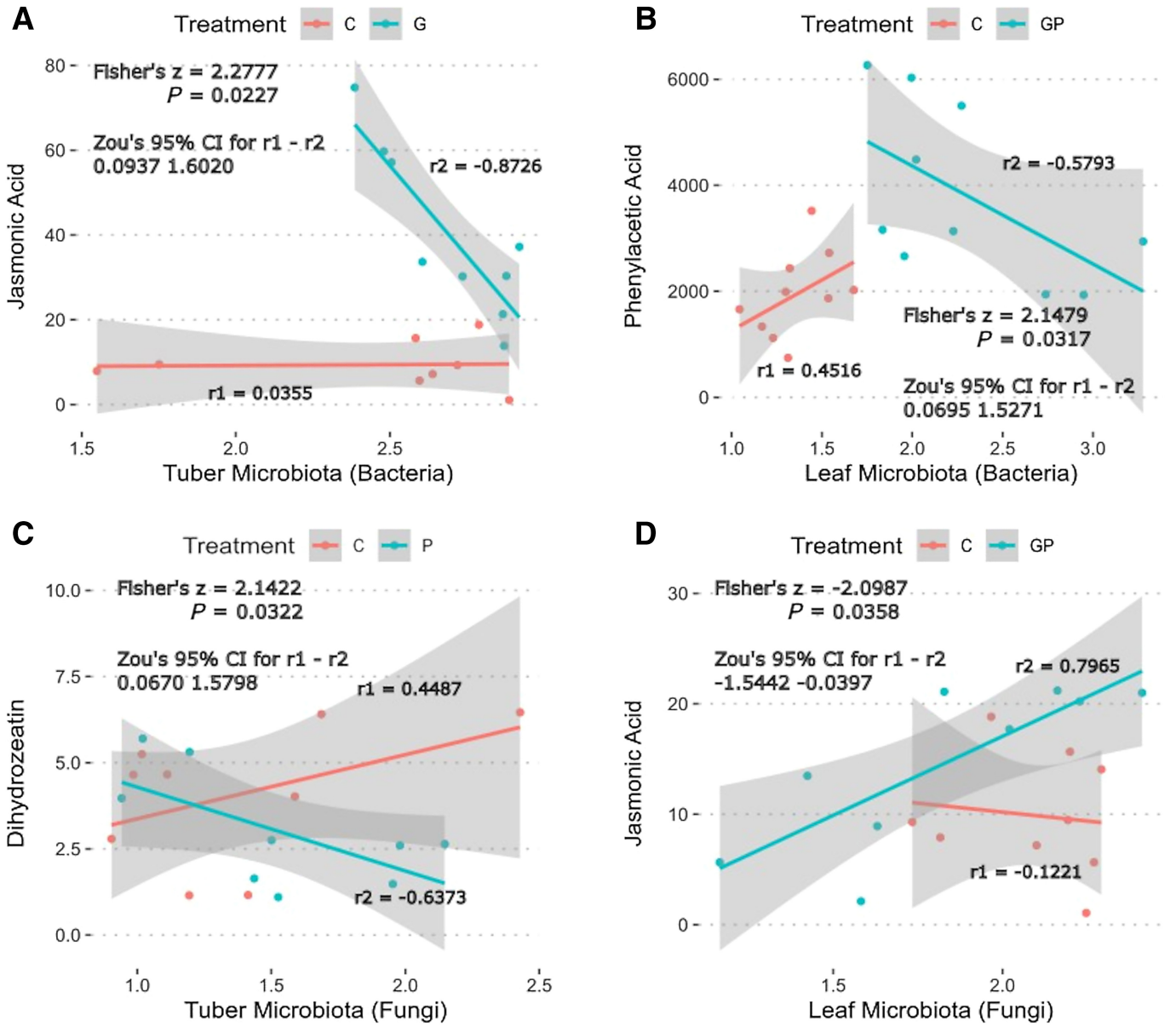

Fig. 4. Significant change in linear correlation among **A,** tuber bacteria and jasmonic acid, **B,** leaf bacteria and phenylacetic acid, **C,** tuber fungi and dihydrozeatin, and **D,** leaf fungi and jasmonic acid levels as a result of glyphosate-based herbicide (GBH) (G), phosphate (P), and GBH with P (GP) treatments (indicated on color scale). The significance of the change in correlation (Pearson) was measured using Fisher's *z* and Zou's confidence interval (CI) (null hypothesis of identical correlation is rejected when CI does not include 0).

in the GBH with phosphate treatment, indicating that the induction of jasmonic acid by GBH in soil alters the microbial abundance differentially in tubers and leaves (Fuchs et al. 2022a). This is in line with the results from a study on wheat, in which the microbial response to jasmonic acid was different between roots and leaves (Liu et al. 2017). Jasmonic acid is known to regulate the defense response against necrotrophic pathogens (Thaler et al. 2012) and has a crucial role in shaping the entire plant microbiome (Carvalhais et al. 2017). The auxin phenylacetic acid was negatively correlated with leaf bacterial abundance in the GBH with phosphate treatment, indicating its interactive function with bacterial plant associates (Kunkel and Harper 2018). The cytokinin dihydrozeatin was negatively correlated with endophytic fungi in tubers in the phosphate treatment and positively correlated in tubers in the control treatment, indicating that root-associated fungi may promote the production of plant growth hormones (Liu et al. 2023), but in the presence of phosphate fertilizer, this relationship is reversed. The accumulation of glyphosate residues in plant tissues, causing hormone disturbances, may shift the endophytic microbial diversity, as the chemical niche for the microbes may change. Alternatively, glyphosate residues and AMPA may be eliciting a shift in the diversity of glyphosate-sensitive microbes, which in turn affects phytohormone biosynthesis in the plant.

Conclusions. GBH alone and in combination with phosphate fertilizers significantly altered the structure of microbial communities in potato leaves compared with roots and tubers. Bacterial communities were mainly impacted by glyphosate residues, whereas fungal communities were impacted by phosphate fertilizers. The agrochemical treatments caused changes in phytohormone concentrations, which correlated with microbial diversity. Even though these phytohormone changes cannot be directly connected to abundances or functional changes to specific microbial taxa, the study clearly shows how pesticides and fertilizers impact microbial communities within plant tissues and interfere with phytohormone signaling. However, it remains to be elucidated whether the agrochemicals alter phytohormone signaling to shift microbial diversity or vice versa. Further studies looking into the impairment of host plant health and survival due to shifts in phytohormone concentrations as well as microbial community structure and function are essential.

Data availability. The sequence data are available at the National Center for Biotechnology Information Sequence Read Archive database under accession PRJNA1071005 (https://www.ncbi.nlm.nih.gov/bioproject/1071005).

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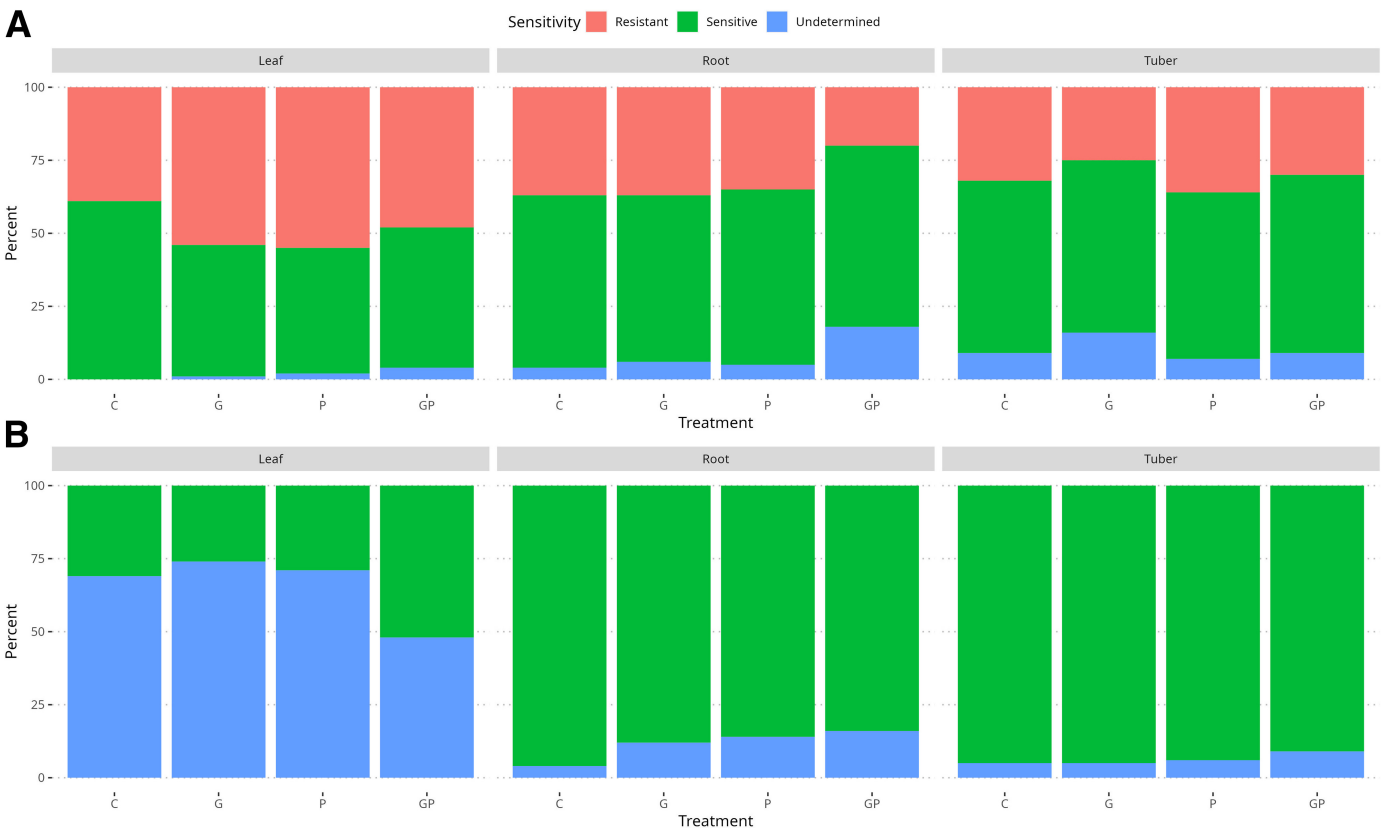

Fig. 5. Ratio of glyphosate-sensitive to glyphosate-resistant amplicon sequence variants (ASVs) for **A,** bacterial community and **B,** fungal community in potato leaves, roots, and tubers under glyphosate-based herbicide (GBH) (G), phosphate (P), and GBH with P (GP) treatment compared with control (C).

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