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Glyphosate-based herbicide use affects individual microbial taxa in strawberry endosphere but not the microbial community composition

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Abstract

Aims: In a field study, the effects of treatments of glyphosate-based herbicides (GBHs) in soil, alone and in combination with phosphate fertilizer, were examined on the performance and endophytic microbiota of garden strawberry.

Methods and results: The root and leaf endophytic microbiota of garden strawberries grown in GBH-treated and untreated soil, with and without phosphate fertilizer, were analyzed. Next, bioinformatics analysis on the type of 5-enolpyruvylshikimate-3-phosphate synthase enzyme was conducted to assess the potential sensitivity of strawberry-associated bacteria and fungi to glyphosate, and to compare the results with field observations. GBH treatments altered the abundance and/or frequency of several operational taxonomic units (OTUs), especially those of root-associated fungi and bacteria. These changes were partly related to their sensitivity to glyphosate. Still, GBH treatments did not shape the overall community structure of strawberry microbiota or affect plant performance. Phosphate fertilizer increased the abundance of both glyphosate-resistant and glyphosate-sensitive bacterial OTUs, regardless of the GBH treatments.

Conclusions: These findings demonstrate that although the overall community structure of strawberry endophytic microbes is not affected by GBH use, some individual taxa are.

Significance and impact of study

Agrochemical residues in soil can shape the endophytic microbiota in crop plants.

Keywords: EPSPS, *Fragaria* × *ananassa*, herbicide, roundup, endophyte, microbiota, phosphate fertilizer, plant performance

Introduction

Glyphosate (*N*-phosphonomethyl glycine)-based herbicides (GBHs) are currently the most commonly used pesticides globally (Benbrook 2016). Glyphosate has been considered safe for nontarget organisms for two reasons. First, the effect of glyphosate is based on inactivation of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway inhibiting the production of aromatic amino acids phenylalanine, tyrosine, and tryptophan (Parthasarathy et al. 2018), which is an essential metabolic route in plants (Duke and Powles 2008) but is not present in animal cells (Williams et al. 2000, Helander et al. 2012). Second, glyphosate typically degrades in a few weeks or is adsorbed by soil solids; this limits its bioavailability and, thus, phytotoxicity (Sprankle et al. 1975). However, mounting evidence has revealed that there are ecological, environmental, and health risks of intensive GBH use that are largely related to the presence of glyphosate residues and degradation products in diverse agricultural habitats (Gill et al. 2018, Maggi et al. 2020, Leino et al. 2021). Still, it can be difficult to find a clear link between these risks and the use of GBHs because glyphosate and its degradation products can be retained and transported in ecosystems and their effects are often context dependent (Muola et al. 2021, Fuchs et al. 2022a).

Despite the relatively fast degradation of glyphosate under optimal conditions, glyphosate itself and its degradation products have been reported to persist in ecosystems even for years (Laitinen et al. 2009), especially in colder climates (Helander et al. 2012). In fact, an increasing number of studies have reported that, following the use of GBHs, residues of glyphosate and its degradation products are found in soil, water, crop plants, and even animal tissues (Bøhn et al. 2014, Bai and Ogbourne 2016, Helander et al. 2018, Silva et al. 2019, Ruuskanen et al. 2020). Further, even the use of field-realistic doses of GBH with a 2-week safety period has been shown to negatively affect the germination and growth of different crop plants (Helander et al. 2019). However, in other studies, low residues of glyphosate in soil have shown growth-promoting effects in some crop species (Cedergreen 2008, Brito et al. 2018, Helander et al. 2019, Fuchs et al. 2022b). The

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consequences of glyphosate residues for plants have shown to depend on plant species and weather conditions (i.e. via the degradation of GBHs) in addition to soil chemistry, especially in connection to soil phosphorus concentration (Helander et al. 2012). Glyphosate and inorganic phosphate are known to compete for sorption sites in a wide variety of soils (for instance, Sprankle et al. 1975, de Jonge and de Jonge 1999, de Jonge et al. 2001, Kanissery et al. 2015, Hébert et al. 2019). Depending on weather conditions and soil chemistry, inorganic phosphate can outcompete glyphosate for sorption sites in soil particles; this might affect the exposure of plants to glyphosate (Wang et al. 2005, Padilla and Selim 2019). Given the extensive use of both GBHs and inorganic phosphate fertilizers, it is essential to comprehensively understand how glyphosate residues—alone or in combination with phosphate fertilizers—affect factors associated with the health and fitness of crop plants.

In addition, recent studies have indicated that the risks of GBHs for nontarget organisms are not limited to the effects of active ingredient glyphosate alone. GBHs contain various co-formulants, in particular surfactants that enhance the uptake and translocation of active ingredients in weeds and possibly other organisms. Some of the co-formulants may be more toxic than glyphosate (Mesnage et al. 2019, Straw et al. 2021). The list of co-formulants added to commercial herbicide formulations is increasing, but the current safety regulations and laws specify that only the active ingredients are required to be tested for their toxicity to nontarget organisms. Information on the final composition of commercial products is defective because the information of co-formulants is treated as confidential trade secrets (Mesnage et al. 2019). Researchers can analyze the glyphosate residues in soil and nontarget organisms, but the residues and degradation time of the co-formulants remain unknown. This complicates the research on GBH effects on nontarget organisms.

Recent studies suggest that microorganisms may be one of the nontarget organisms at risk with GBH use, since the shikimate pathway is present in most fungi and bacteria (Bentley and Haslam 1990, Shehata et al. 2013, Leino et al. 2021, Rainio et al. 2021). This is corroborated by studies on the indirect negative effects of glyphosate residues on plants via soil microbiota, as well as microbes associated with plants and animals (Druille et al. 2016, Helander et al. 2018, 2019, Motta et al. 2018, Székács and Darvas 2018, van Bruggen et al. 2018, Ruuskanen et al. 2020, 2023). Plants and their associated microbiomes are increasingly being considered as co-evolving ecological entities, or holobionts, which can form a unit of selection in evolutionary processes (Saikkonen et al. 2020). The endophytic microbiota is tightly linked to the host plant, and changes in its composition and the abundance of individual microbial species can determine plant health (Nissinen et al. 2012). The intrinsic susceptibility of microbes to glyphosate can be determined based on the structural characterization of the EPSPS enzyme (Mathew et al. 2022). In fact, recent bioinformatics analyses have revealed that a large proportion of prokaryotes (including bacteria and archaea) are susceptible to glyphosate (Leino et al. 2021, Rainio et al. 2021), but no study has so far reported the proportion of glyphosate-sensitive and glyphosate-resistant microbes for any plant microbiome. Furthermore, in order to understand the potential risks of GBH use to the health and fitness of the plant holobiont, it is crucial to determine whether plant-associated glyphosate-sensitive and glyphosate-resistant endophytic microbes respond differently when exposed to glyphosate residues following the GBH applications.

Here, the effects of GBH application and phosphate fertilizer individually and in combination were studied on plant performance and endophytic microbiomes in garden strawberry (*Fragaria* × *ananassa*). Endophytic bacteria and fungi of commercially important strawberry varieties have been shown to promote plant growth, as well as fruit quantity and quality. For instance, bacterial endophytes isolated from strawberry meristematic tissues (Dias et al. 2009), strawberry fruits (de Melo Pereira et al. 2012), and wild strawberry varieties (de Andrade et al. 2019) were found to produce growth hormones, solubilize phosphate, and fix nitrogen—all of which are important for improving plant performance. However, there is no information about the potential impacts GBH use might have for strawberry microbiota. Therefore, an experimental study was conducted to determine whether glyphosate residues in soil following the application of field-realistic doses of GBH alone, or in combination with phosphate fertilizer, alter strawberry performance and endophytic microbiota community composition of the roots and leaves. High-throughput sequencing of 16S rRNA gene and internal transcribed spacer (ITS) region was performed to characterize the taxonomic community composition of endophytic bacteria and fungi, respectively, in garden strawberries grown in GBH-treated and untreated soil, with and without phosphate fertilizer. In addition to analyzing the potential differences in structure and composition of endophytic bacterial and fungal communities in response to exposure to GBH alone and in combination with phosphate fertilizer, the indicator species analysis was used to identify "indicator taxa" characteristic to these treatments (Mouillot et al. 2002, Legendre 2013). Further, a novel bioinformatics tool, based on the type of EPSPS enzyme produced in the identified endophytic strawberry microbes, was utilized to determine the predisposition of the microbes to glyphosate. These results were compared with the alterations in microbiome detected in the field study to understand whether glyphosate-sensitive and glyphosate-resistant microbes respond differently when they are exposed to glyphosate residues. More specifically, the following hypotheses were tested: (1) GBH application affects the strawberry microbiome and decreases the abundance of glyphosate-sensitive microbes. (2) The effects of GBH application are systemic and affect the entire plant vascular system, but the root microbiome is more strongly affected than the leaf microbiome because roots are directly in contact with soil. In addition, we examined whether the application of inorganic phosphate fertilizer alters the effects of GBH application on plant performance and endophytic microbiota.

Materials and methods

Field experiment

The garden strawberry (*Fragaria* × *ananassa*) cultivar "Bounty" (Canada) was used to study the combined effects of GBH application and phosphate fertilizer on strawberry performance and the associated endophytic microbiota. To this end, a field experiment was conducted at Ruissalo Botanical Garden (University of Turku) in southwestern Finland (60°26′N, 22°10′E). In 2019, the mean annual temperature and precipitation in the area were 7.4°C and 741 mm, respectively (http://www.fmi.fi/en). The

experimental field (25 m × 50 m) was established in 2013 by mixing sand and peat with existing clay soil (pH 7.1) before tilling at a depth of 15 cm and fencing to exclude larger vertebrates from the area. The field was divided into alternating control and GBH treatment plots (10 plots each, measuring 23 m × 1.5 m), with 1.5-m buffer strips between the plots. Twice a year, the plots were tilled to a depth of 5 cm with a hand-held rotary tiller. Following this, the control plots were treated with tap water (5 L per plot), and the GBH plots were treated with Roundup Gold (glyphosate concentration: 450 g L^{-1}, CAS: 3864-194-0, application rate: 6.4 L ha^{-1} in 5 L of tap water per plot) using a hand-operated pressure tank with a plastic hood over the sprinkler tip to prevent the glyphosate from spreading outside the treatment plots. For the experiment, each of the 20 plots were divided into two: half of the plot was treated with phosphate fertilizer (Yara Ferticare; application rate: 80 g in 10 L of tap water), and the other half was treated with tap water. This resulted in four different treatments: control (C), phosphate fertilizer treatment (P), GBH treatment (G), and combined GBH and phosphate fertilizer treatment (G + P). All the plots were hand-weeded several times during the growing season to prevent plant competition. The plots were watered with a sprinkler located in the middle of the experimental field when needed throughout the growing season. The buffer strips between the plots were mowed several times during each field season to minimize weed invasion. A more detailed description about the establishment of the experimental field and its management, including GBH treatments, can be found in Helander et al. (2019).

The plant material used in this experiment was propagated from strawberry plants that were growing in the same experimental field in 2018. In September 2018, runners were collected and planted in small pots containing potting substrate (Kekkilä Taimimulta). After the runners had rooted, they were kept in the greenhouse until spring 2019 at a maximum temperature of +10°C under ambient light. Plantlets propagated from the runners originating from each treatment (C, P, G, and G + P) were randomly planted back under the same treatment conditions. In early June 2019, 3 weeks after GBH application and 1 month after phosphate application, 12 equal-sized plantlets were planted in each plot, i.e. 6 plantlets for each treatment. Out of 240 strawberry plantlets, 200 survived until the end of the growing season.

To study the combined effects of GBH application and phosphate fertilizer on strawberry growth and reproduction, the number of leaves was counted and considered as an indicator of plant growth. Since very few strawberries were flowering, but almost 90% were producing runners, vegetative reproduction was estimated by measuring the length of the produced runners in mid-August 2019. At the same time, soil was sampled from each subplot for analysis of glyphosate, and its degradation product aminomethylphosphonic acid (AMPA). Samples consisted of soil material that was ∼2.5 cm in diameter and 5 cm in depth and was air-dried before extraction and pooled according to treatment. Glyphosate and AMPA were extracted with aqueous acidified methanol followed by analysis via liquid chromatography coupled to mass spectrometry at GroenAgro (agrocontrol.nl).

Sampling for microbiota analysis

For microbiome analysis, one fully expanded, healthy leaf was collected and ∼100 mg of root material was dug up from one randomly selected strawberry individual in each treatment plot in early August 2019. Altogether, 10 replicates of each of the leaf and root per treatment was collected; thus, altogether 40 root and 40 leaf samples were collected. In the field, samples were placed in sterile plastic bags, stored on ice, and brought immediately to the laboratory for further processing.

The samples were washed with tap water, air-dried, weighed, and surface sterilized in a laminar air flow hood. Approximately 100 mg of root and leaf tissue was washed in 70% ethanol (1 min) and then 3% sodium hypochlorite solution (3 min), rinsed thrice with sterile distilled water (1 min per rinse), and air-dried. The samples were then transferred to 2-mL microcentrifuge tubes and stored at −80°C until further processing. A negative control was prepared by plating 100 μL of the water from the last rinse on Reasoner's 2 Agar (R2A) plate. Plates were kept at room temperature to monitor microbial growth.

DNA extraction

Frozen samples were homogenized twice on a bead mill homogenizer (Bead Ruptor 96 Well Plate Homogenizer, OMNI International US) for 30 s. DNA extraction was carried out using the Invisorb Spin Plant Mini Kit (STRATEC Biomedical AG, Germany) according to the manufacturer's instructions. The DNA concentration was adjusted to 30 ng μL^{-1}.

16S rRNA gene-targeted PCR for bacterial community analysis

The variable regions V6–V8 of the bacterial 16S rRNA gene were amplified by the nested PCR approach. In the first round of PCR, the primers 799F (AACMGGATTAGATACCCKG; Chelius and Triplett 2001) and 1492R [GGYTACCTTGTTACGACTT; modified from Lane (1991)] were used to eliminate chloroplast amplification. The PCR reaction solution contained 30 ng DNA, 1 × PCR buffer, 0.2 mM dNTPs, 0.3 μM of each primer, and 2000 U mL^{-1} of Go*Taq* DNA polymerase (Promega, WI, USA) in a 30-μL reaction volume. This was followed by PCR with the primers M13-1062F (TGTAAACGACGGCCAGTGTCAGCTCGTGYYGTGA) (Ghyselinck et al. 2013, Mäki et al. 2016) and 1390R (ACGGGCGGTGTGTRCAA) (Zheng et al. 1996) for sample barcoding. The PCR components were the same as in the first PCR, except for the template, which was 1:10 dilution of the first PCR product. This was followed by a third round of PCR with IonA-barcode-M13 primers (Mäki et al. 2016) 1390R-P1 for sample tagging. The PCR reactions were the same as those for the first two PCRs, except that a 1:1 dilution of the second PCR product was used as the template. The protocol for the first PCR were as follows: one cycle of 3 min of initial denaturation at 95°C; 35 cycles each of denaturation at 95°C for 45 s; annealing at 54°C for 45 s; extension at 72°C for 1 min; and final extension at 72°C for 5 min. The same protocol was followed for the second and third PCRs, except the number of cycles were reduced to 25 and 8 cycles, respectively. The amplicons were analyzed on 1.5% agarose gel.

ITS region-targeted PCR for fungal community analysis

The ITS regions of fungal endophytes were amplified using the primers M13-ITS7F (TGTAAAACGACGGCCAGTGTGARTCATCGAATCTTTG) and ITS4R (TCCTCCGCT-

TATTGATATGC) (Ihrmark et al. 2012). The 30-μL PCR reaction mixture contained 30 ng of sample DNA, 1 × PCR buffer, 0.2 mM dNTPs, 0.3 μM of each primer, and 1250 U mL^{-1} Go*Taq* DNA Polymerase (Promega, WI, USA). The amplification was initial denaturation at 95°C for 5 min, 35 cycles of denaturation, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and followed by a final extension at 72°C for 7 min. The second round of PCR was performed using a 1:10 dilution of the first PCR product as template with IonA-barcode-M13 as the forward primer and ITS4-P1 (CCTCTCTATGGGCAGTCGGTGATTCCTCGCTTATTGATATGC) as the reverse primer. All the other PCR components were the same as those used in the first round of PCR, but denaturation, annealing, and extension steps were repeated for only 8 cycles instead of 35. The amplicons were analyzed on 1.5% agarose gel.

Library preparation and sequencing

The PCR products were quantified on the Agilent 2100 Bioanalyzer system, and 30 ng of target amplicons (16S rRNA gene/ITS region) of each sample were pooled to prepare the library. To eliminate plant mitochondrial amplicons and small fragments, amplicons of size 350–550 bp were collected by size fractionation in Pippin Prep (Sage Science, MA, USA) using a 2% agarose gel cassette (Marker B). The purified libraries were subjected to emulsion PCR (Ion OneTouch™ 2 System, ThermoFisher Scientific Ltd) and sequenced on Ion 314™ Chip v2 in the Ion Personal Genome Machine™ (ThermoFisher Scientific Ltd).

Bioinformatics and statistical analyses

The sequence reads were processed using CLC Genomics Workbench 11.0 with a Microbial Genomics Module (Qiagen, Denmark). Low-quality sequences were filtered, and high-quality reads were trimmed to 250 bp and aligned. Reads with 97% sequence identity were clustered into operational taxonomic units (OTUs). Taxonomic classification of OTUs was done using the reference databases RDP 16S rRNA training set 16 for bacteria and UNITE Fungal ITS trainset 7.1 for fungi (https://rdp.cme.msu.edu) (Wang et al. 2007). To reduce noise from randomly occurring OTUs, low-abundant OTUs with combined abundance of <10 reads were eliminated. All statistical analyses were conducted separately for bacterial and fungal OTU datasets.

The effect of tissue type (root or leaf) and treatment (control, phosphate, GBH, or GBH with phosphate) on overall structure and composition of bacterial and fungal communities was analyzed using permutational multivariate analysis of variance (PERMANOVA) (Anderson 2017) based on Bray–Curtis dissimilarity matrix of square-root transformed data. The results were visualized using principal coordinate analysis (PCoA). To evaluate the diversity of bacterial and fungal communities, species richness and Shannon diversity index (H′) were calculated using Univariate Diversity Indices (DIVERSE). The above statistical analyses were conducted using the PRIMER 7 + PERMANOVA software (primer-e.com). To assess differences in microbial diversity based on treatment and tissue type, we performed ANOVA and glht post-hoc tests on the obtained Shannon values using the software R 3.6.1 and the package "multcomp" (Hothorn et al. 2020).

Estimation of potential sensitivity to glyphosate

To determine the predisposition of the identified endophytic strawberry microbes to glyphosate, we utilized a novel bioinformatics tool that is based on the type of EPSPS enzyme produced in microbes (Mathew et al. 2022). EPSPS can be classified as potentially sensitive or resistant to glyphosate based on amino acid markers present at its active site (Leino et al. 2021). However, in this study, the microbiome was studied based on an analysis of the 16S rRNA gene (bacteria) and ITS region (fungi). Thus, there was no information about the type of EPSPS sequence or the exact bacterial or fungal strain or species. Therefore, a probabilistic approach, based on the method of Mathew et al. (2022), was used to estimate the potential sensitivity of the strawberry endophytic microbe to glyphosate. First, bacterial species were mapped onto two precomputed datasets of EPSPS sequences available at EPSPSClass webserver at this link: https://ppuigbo.me/programs/EPSPSClass (Leino et al. 2021). Previous studies have demonstrated high taxonomic conservation of the EPSPS sequence and potential sensitivity to glyphosate (Rainio et al. 2021). Accordingly, bacterial and fungal species were mapped onto EPSPS sequences from the ATGC (Kristensen et al. 2017) and PFAM (El-Gebali et al. 2019) databases, respectively. A probabilistic score of sensitivity to glyphosate ranging between 0 (resistant: no sensitive sequences to glyphosate were found) and 1 (sensitive: all known sequences in the taxonomic group were sensitive to glyphosate) was calculated. Evidently, there is a range of in-between values, i.e. taxonomic groups that contain sequences that are sensitive, resistant, and unknown. In order to account for this, a cut-off of <0.2 and >0.8 was used to indicate resistance and sensitivity, respectively.

The distribution of potentially glyphosate-sensitive and glyphosate-resistant bacterial and fungal OTUs under different treatment conditions compared to the control conditions was estimated using the *z*-test and kernel density distribution with Excel and the R package "density," respectively.

Indicator species analysis

Indicator species analysis was used to test the differences in abundance and frequency of each OTU in the samples collected from different treatments. While PERMANOVA focuses on the entire microbial community structure, indicator species analysis provides a tool to analyze the potential effects of glyphosate residues alone and in combination with phosphate fertilizer on individual OTUs in order to identify "indicator taxa" for different treatments (e.g. Fortunato et al. 2013, Tedjo et al. 2016). The filtered OTU dataset was analyzed with indicator species analysis with the R package "labdsv" and the "ìndval" test (Dufrêne and Legendre 1997) to identify OTUs specific to each of the treatments (control, phosphate, GBH, and GBH with phosphate). The indicator values range from 0 to 1, with better indicators having higher values for the respective treatment and P-value < 0.05. Both abundance and frequency are used to determine whether an OTU is characteristic for strawberry root or leaf under a given treatment condition.

Plant growth and vegetative reproduction

The combined effects of GBH application and phosphate fertilizer on strawberry growth and vegetative reproduction were analyzed by using generalized and general linear mixed models, respectively (PROC GLIMMIX, SAS 9.4). Poisson

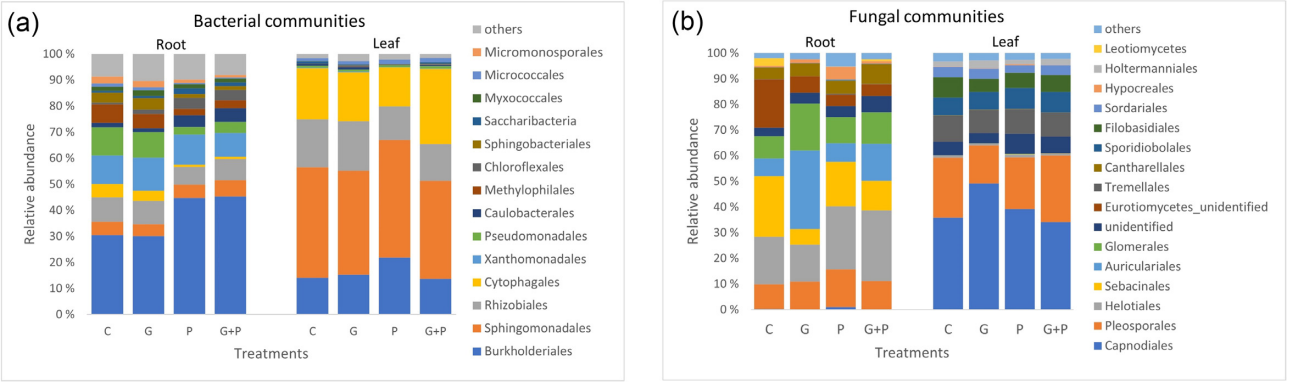

Figure 1. Order-level taxonomic distribution of endophytic microbial communities in the roots and leaves of garden strawberry (*Fragaria* × *ananassa*). Bacterial communities (a) and fungal communities (b) in the control (C), GBH (G), phosphate fertilizer (P), and GBH with phosphate fertilizer (G + P) treatment groups. Highly abundant orders are presented in the figure, and minor taxa are grouped together as "Others."

distribution and log link function were used in the strawberry growth model. Strawberry growth was measured in terms of the number of leaves at the end of the growing season, and vegetative reproduction was measured as the length of the runners produced. In the models, treatment was used as a fixed factor and plot as a random factor to eliminate the potential effects of environmental variation in the experimental field. In addition, to control for the effect of plant size on vegetative reproduction, the number of leaves was included as a covariate in the model for analyzing the combined effects of glyphosate residues and phosphate fertilizer on vegetative reproduction. Finally, the normality and equality of variances of the residuals were assessed by visual examination and Levene's test, respectively.

Results

Glyphosate residues in soil

The levels of glyphosate and its degradation product AMPA were markedly higher in soil samples from the GBH treatment than in the control treatment samples, irrespective of whether phosphate was added. In soil samples from the control and phosphate treatments, glyphosate and AMPA concentrations were below the quantification limit (0.01 mg kg^{-1} for glyphosate and 0.05 mg kg^{-1} for AMPA). The glyphosate concentration was 0.06 mg kg^{-1} in the GBH treatment samples and 0.07 mg kg^{-1} in the GBH with phosphate treatment samples, and the AMPA concentration was 1.9 mg kg^{-1} in the GBH treatment samples and 1.8 mg kg^{-1} in the GBH with phosphate treatment samples.

Taxonomic distribution of microbial communities

Regarding taxonomic distribution of the strawberry bacterial community, 140 912 bacterial sequence reads were clustered into 911 OTUs belonging to 17 phyla, 65 orders, and 106 families. The main bacterial phyla found in the strawberry endophytic community were Proteobacteria and Bacteroidetes. The major orders in the strawberry root samples were Burkholderiales, Xanthomonadales, Rhizobiales, and Pseudomonadales, with Burkholderiales showing higher relative abundance in root samples from the phosphate and GBH with phosphate treatments (Fig. 1a). In the leaf bacterial community, 95% was distributed among the orders Burkholderiales, Sphingomonadales, Rhizobiales, and Cytophagales (Fig. 1a).

Regarding taxonomic distribution of the fungal community, 196 410 sequence reads were clustered into 363 OTUs representing 5 phyla, 52 orders, and 90 families. The root fungal communities belonged to the phyla Ascomycota, Basidiomycota, and Glomeromycota, while the leaf communities were dominated by the phyla Ascomycota and Basidiomycota. Further, root fungal communities mainly belonged to the Pleosporales, Helotiales, Sebacinales, Auriculariales, and Glomerales orders, while the orders Capnodiales, Pleosporales, Tremellales, Sporidiobolales, and Filobasidiales were dominant in the leaf communities (Fig. 1b). The relative abundance of Sebacinales were lower and that of Auriculariales were higher in the root communities as a consequence of GBH treatments in soil (Fig. 1b).

Impact of glyphosate and phosphate on the diversity and composition of microbial communities

The Shannon diversity index for measuring the richness and uniformity of bacterial and fungal communities showed that bacterial communities present in the roots were more diverse than those in the leaves. Phosphate treatment resulted in an increase in the diversity of bacterial communities in the roots (Table 1, Supplementary Fig. S1), while neither tissue type nor treatment had an effect on the diversity of fungal communities (Table 1). The structures of both bacterial and fungal communities were significantly different between the roots and leaves ($P = 0.001$ according to PERMANOVA, Table 2). Comparison of root bacterial communities between the treatment (Table 3) with PCoA showed that phosphate fertilizer treatment significantly shaped bacterial communities in the roots (Table 3, Fig. 2a) but not in the leaves (Table 3, Fig 2b). In contrast, GBH treatment did not have a significant impact on bacterial community structure in either root or leaf tissues (Table 2). None of the treatments impacted the structure of the fungal communities (Table 2, Fig. 2c and d).

In silico analysis of the glyphosate sensitivity of microbial community members

The dataset contains 389 bacterial OTUs that are potentially sensitive to glyphosate, 376 OTUs that are potentially resistant, and 147 OTUs with unknown sensitivity status. The ratio of potentially glyphosate-sensitive bacteria to potentially glyphosate-resistant bacteria indicate higher abundance of

Table 1. Results of a linear model of the effects of tissue type (root or leaf) and treatment (control, GBH, phosphate fertilizer, or GBH with phosphate fertilizer) on the Shannon diversity of endophytic bacterial and fungal communities of garden strawberry (*Fragaria* × *ananassa*).

Response variable	Explanatory variable	F	*Df*	*P*
Bacteria	GBH	0.267	1/68	0.607
	Phosphate	1.260	1/68	0.266
	Tissue (root/leaf)	344.93	1/68	<0.001
	GBH × phosphate	0.023	1/68	0.558
	GBH × tissue	0.044	1/68	0.477
	Phosphate × tissue	8.265	1/68	0.005
	GBH × phosphate × tissue	0.017	1/68	0.656
Fungi	GBH	0.092	1/68	0.408
	Phosphate	0.039	1/68	0.473
	Tissue (root/leaf)	0.051	1/68	0.342
	GBH × phosphate	0.027	1/68	0.488
	GBH × tissue	0.002	1/68	0.863
	Phosphate × tissue	0.014	1/68	0.617
	GBH × phosphate × tissue	0.056	1/68	0.320

Table 2. Results of PERMANOVA analysis of the effect of plant tissue (root or leaf) and treatment (control, GBH, phosphate fertilizer, or GBH with phosphate fertilizer) on the composition of endophytic bacterial and fungal communities in garden strawberry (*Fragaria* × *ananassa*).

Response variable	Source	df	Pseudo-F	*P*	Unique perms
Bacteria	Tissue	1	42.92	0.001	998
	Treatment	3	1.54	0.001	996
	Tissue × treatment	3	1.49	0.001	995
Fungi	Tissue	1	65.46	0.001	999
	Treatment	3	0.92	0.707	998
	Tissue × treatment	3	1.03	0.370	997

Table 3. Results of pairwise *t*-test for analysis of the difference in the composition of endophytic bacterial communities between different treatments in the roots and leaves of garden strawberry (*Fragaria* × *ananassa*).

Tissue	Treatment	*t*	*P*	Unique perms
Root	C vs. G	1.020	0.357	993
	C vs. P	1.484	0.001	989
	C vs. G + P	1.629	0.001	998
	P vs. G	1.484	0.001	988
	P vs. G + P	1.064	0.166	988
	G + P vs. G	1.607	0.001	994
Leaf	C vs. G	1.008	0.358	986
	C vs. P	1.076	0.181	990
	C vs. G + P	0.999	0.39	990
	P vs. G	1.078	0.187	991
	P vs. G + P	0.936	0.667	991
	G + P vs. G	1.011	0.365	992

C = control, G = GBH, P = phosphate fertilizer, and GBH + P = GBH with phosphate fertilizer.

potentially glyphosate-sensitive bacteria in the roots than in the leaves (Supplementary Table S2). The Kernel density plot indicated that the addition of phosphate fertilizer tended to affect the abundance of bacterial OTUs belonging to both potentially glyphosate-sensitive and glyphosate-resistant communities in the roots (Fig. 3a). A *z*-test confirmed a significant increase in the abundance of potentially glyphosate-resistant and glyphosate-sensitive bacterial OTUs in root samples from both phosphate fertilizer treatments compared to the control treatment. In the leaves, no significant difference in the glyphosate sensitivity of OTUs was observed between the treatments (Fig. 3b, Supplementary Table S1).

Analysis of the EPSPS enzyme in fungal communities showed that 212 OTUs were potentially sensitive to glyphosate, while the remaining 156 OTUs were unclassified. Thus, the findings do not indicate any impact of GBH or phosphate fertilizer on glyphosate sensitivity of the fungal community.

Impact of phosphate and glyphosate on bacterial and fungal community members

To identify OTUs associated with the effects of glyphosate residues in soil, indicator species analysis was used to test for tissue-specific differences in the abundance and/or frequency of bacterial and fungal OTUs between the control and GBH treatment samples, and between the phosphate and GBH with phosphate treatment samples (Table 4). In the roots, eight bacterial OTUs were enriched in the control samples, where five of these OTUs were classified as potentially glyphosate resistant. Six bacterial OTUs were enriched in the GBH treatment samples, out of which five were classified as potentially glyphosate resistant (Table 4). In the leaves, seven bacterial OTUs were enriched in the control treatment samples, where three were classified as potentially glyphosate sensitive represent the bacterial genus *Hymenobacter*. None of the leaf bacterial OTUs were enriched in the GBH treatment samples (Table 4).

In the comparison between phosphate and GBH with phosphate treatments, 11 bacterial OTUs were enriched in root samples from phosphate treatment. A total of 18 bacterial OTUs were enriched in root samples from the GBH with phosphate treatment: 10 were classified as glyphosate resistant, 5 were classified as glyphosate sensitive, and 3 were unclassified (Table 4). OTUs belonging to Burkholderiales were enriched in both GBH and GBH with phosphate treatment

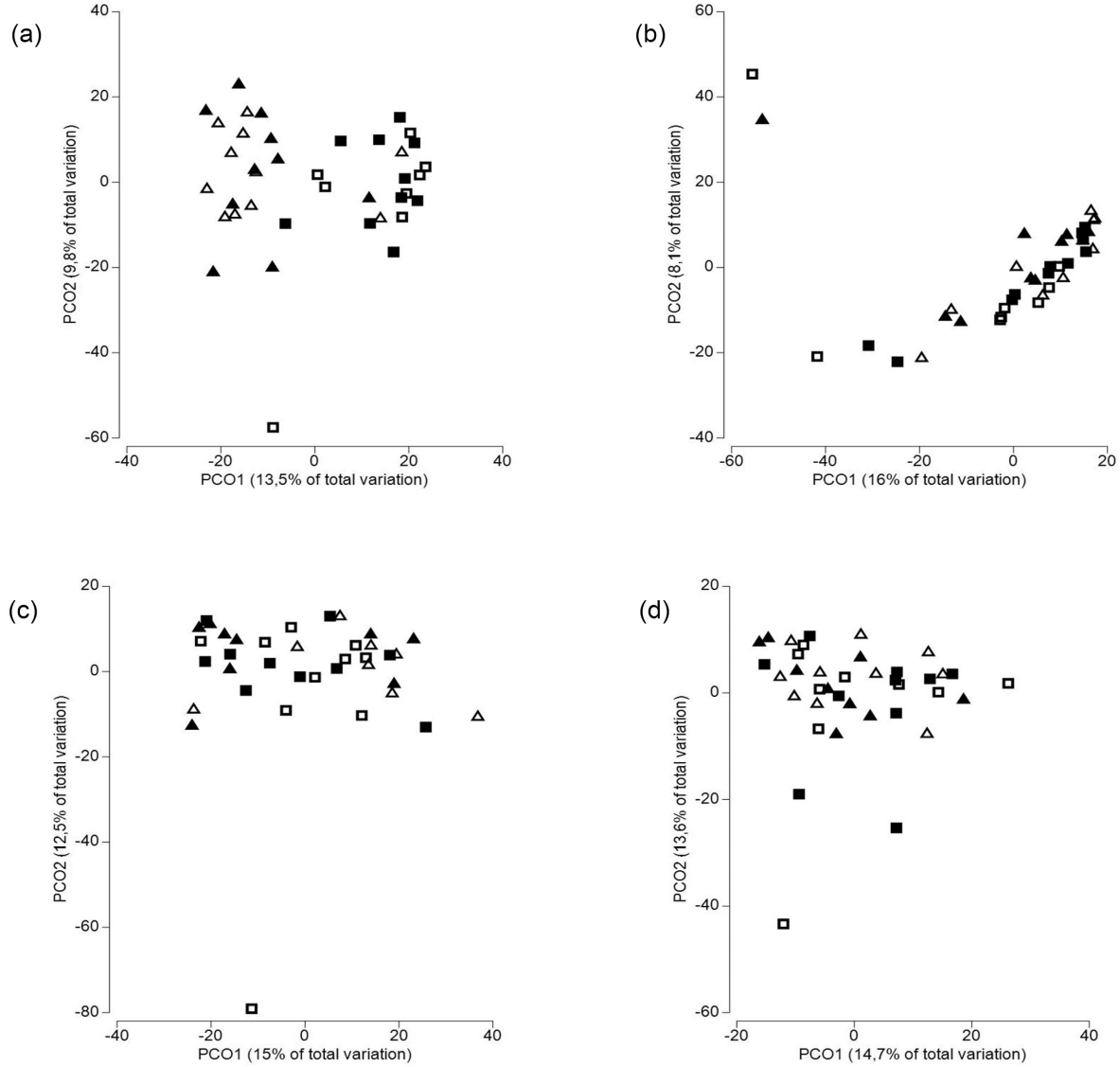

Figure 2. Results of PCoA showing the impact of different treatments on the structure of microbial communities in the endosphere of garden strawberry (*Fragaria* × *ananassa*). The GBH (G) (▲), phosphate fertilizer (P) (□), and GBH with phosphate fertilizer (G + P) (■) treatments are compared with control treatment (Δ) in terms of their effects on bacterial communities in the roots (a), bacterial communities in the leaves (b), fungal communities in the roots (c), and fungal communities in the leaves (d).

(Supplementary Table S3). In the leaves, one glyphosate-resistant and one glyphosate-sensitive bacterial OTU were enriched in the phosphate treatment samples, while two glyphosate-sensitive bacterial OTUs were enriched in the GBH with phosphate treatment samples (Table 4). Further details on taxonomic classification and potential glyphosate sensitivity of bacterial indicator OTUs for each treatment is provided in Supplementary Table S3.

In the comparison of root fungal OTUs between control and GBH treatment, 11 fungal OTUs were enriched in the control treatment samples. A total of 6 out of the 11 OTUs enriched in the control treatment samples belonged to the order Sebacinales, five of them were classified as potentially glyphosate sensitive. Two potentially glyphosate-sensitive fungal OTUs representing *Tetracladium* (Pleosporales) and *Paraphoma* (Agaricales) genera were enriched in the GBH treatment samples (Table 4). In the leaves, two of the three fungal OTUs enriched in the control samples belonged to genus *Leucosporidium*. Regarding the comparison between phosphate treatment and GBH with phosphate treatments in roots, three fungal OTUs were enriched in the phosphate treatment samples. Two of the OTUs were classified as potentially glyphosate sensitive and one had unknown sensitivity status. Two fungal OTUs of unknown sensitivity status were enriched in the GBH with phosphate treatment samples (Table 4). No indicator species were identified for leaf fungal OTUs in any of these treatments (Table 4). Details on fungal indicator taxa for each treatment are provided in Supplementary Table S4.

Plant growth and vegetative reproduction

Overall, experimental plants had 10 ± 0.3 leaves and 68 ± 4 cm runners. None of the treatments affected strawberry size, as indicated by the number of leaves, or vegetative reproduction (plant size: $F_{3,\,178} = 0.22$, $P = 0.8814$; vegetative reproduction: $F_{3,\,173} = 1.76$, $P = 0.1561$). The effect

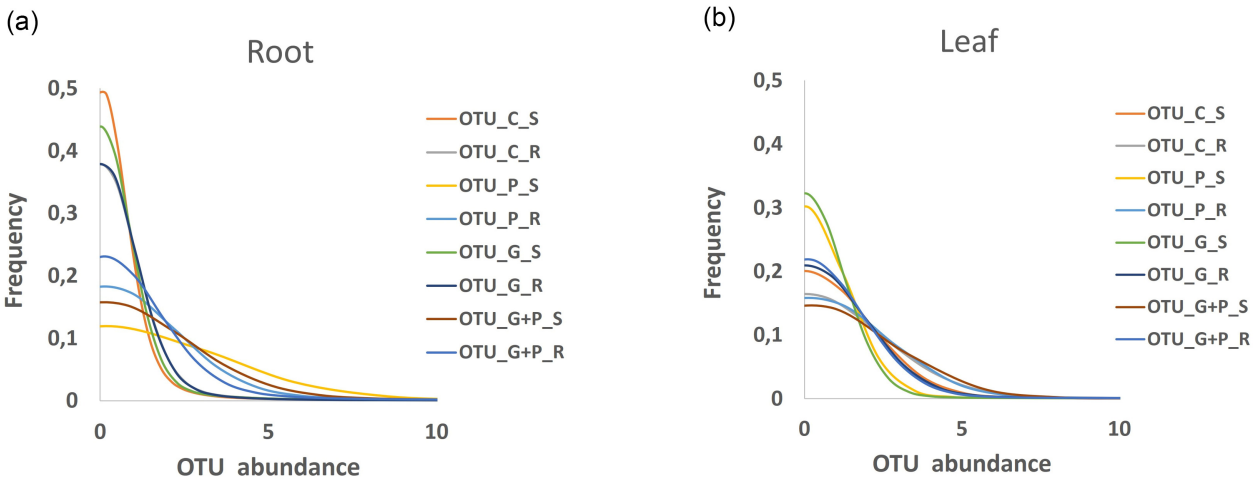

Figure 3. Kernel density distribution of the abundances of glyphosate-sensitive and glyphosate-resistant endophytic bacterial OTUs according to treatment. The abundances of glyphosate-sensitive (S) and glyphosate-resistant (R) endophytic bacterial OTUs in the roots (a) and leaves (b) of garden strawberry (*Fragaria* × *ananassa*) are shown for the control (C), GBH (G), phosphate fertilizer (P), and GBH with phosphate fertilizer (G + P) treatments.

Table 4. Summary of indicator species analyses indicating the number of OTUs with potential glyphosate sensitivity (S), potential glyphosate resistance (R), and unknown glyphosate sensitivity status (U) for different treatments and tissues.

			Number of indicator species			
			C vs. G		P vs. G + P	
Bacteria	Roots	S	2	1	7	5
		U	1	0	1	3
		R	5	5	3	10
	Leaves	S	4	0	1	2
		U	0	0	0	0
		R	3	0	1	0
Fungi	Roots	S	7	2	2	0
		U	4	0	1	2
		R	0	0	0	0
	Leaves	S	1	0	0	0
		U	2	0	0	0
		R	0	0	0	0

The detailed results of indicator species analysis are provided in Supplementary Tables S3 and S4. Comparisons were performed between control (C) and GBH (G), and phosphate fertilizer (P) and GBH with phosphate fertilizer (G + P) treatments.

of plant size on runner production differed across treatments, as indicated by the significant treatment–plant size interaction ($F_{3,\,173} = 3.07$, $P = 0.0291$). Plant size and runner production were not correlated with each other in the GBH treatment, but for all the other treatments, they were positively correlated (control: $r = 0.49$, $P = 0.0003$, $n = 50$; GBH treatment: $r = 0.01$, $P = 0.9235$, $n = 49$; phosphate treatment: $r = 0.55$, $P < 0.0001$, $n = 50$; GBH with phosphate treatment: $r = 0.43$, $P = 0.0017$, $n = 50$). These findings indicate that larger plants produced more runners under all treatment conditions, except for GBH treatment.

Discussion

GBH application in soil did not cause shifts in the diversity or the overall community structure of strawberry endophytic bacteria or fungi and did not affect plant performance or vegetative reproduction. Similar to these findings, GBH application had no effect on the richness of endophytic microbial communities or the composition of the endophytic fungal community of a perennial weed (Ramula et al. 2022) or root-associated soil microbial communities of corn and soybean (Kepler et al. 2020), although other studies have reported shifts in soil- and plant-associated microbes in response to glyphosate residues and/or GBH applications [reviewed by van Bruggen et al. (2018)]. Despite the finding that GBH application did not have effect on the overall community structure of strawberry microbiota, GBH application shifted the relative abundances and/or frequencies of several fungal and bacterial OTUs in garden strawberry, especially in strawberry roots. This supports the hypothesis that the root microbiome is more affected than the leaf microbiome because roots are directly in contact with soil and, thus, glyphosate residues. A large proportion of the observed shifts in the abundance of bacterial OTUs were related to the potential resistance/sensitivity of their taxon to glyphosate. Interestingly, the ratio of sensitive-to-resistant bacteria in different plant tissues indicates that there are more bacterial taxa potentially sensitive to glyphosate in strawberry roots than in strawberry leaves. Although this might further explain the observed shifts in abundance and/or frequency of endophytic bacteria in the root, it is contrary to the prediction of Rainio et al. (2021) that bacteria that are more exposed to glyphosate are intrinsically more resistant to it. However, the lifestyle of plant-associated endophytic bacteria might protect them from direct exposure (Rainio et al. 2021). Lastly, results of this study show that the use of phosphate fertilizer significantly shaped the root but not leaf endophytic bacterial communities and increased the abundance of both glyphosate-resistant and glyphosate-sensitive OTUs, regardless of the presence of glyphosate residues in soil. This agrees with previous findings that showed soil phosphorus levels affected rhizo- and endospheric microbiota in the roots but did not affect the community structure of shoot bacteria (Finkel et al. 2019).

Indicator species analysis revealed that there was a shift in the abundance and/or frequency of certain bacterial and fungal OTUs in response to GBH treatments. Bacterial OTUs in leaves that were enriched in the control and phosphate (i.e. glyphosate residue free) treatment samples were mainly from

the orders Rhizobiales (Alphaproteobacteria) and Cytophagales (Bacteroidetes). Most of the Cytophagales OTUs were classified under the genus *Hymenobacter*, which is known to be a consistent member of the leaf core microbiome (Grady et al. 2019, Ares et al. 2021). A clear taxonomical pattern in the root bacterial OTUs in response to the GBH treatment was not found. For instance, OTUs enriched in both the GBH treatment and control treatment samples belonged to the order Burkholderiales. The explanation behind this might be that the sensitivity or resistance of EPSPS to glyphosate can vary within the same bacterial taxon, even at the species level, as reported previously (Leino et al. 2021). Remarkably, 83% and 56% of the root bacterial OTUs enriched in the GBH treatment and GBH with phosphate fertilizer treatment samples, respectively, were characterized as being potentially glyphosate resistant, and only 17% and 28%, respectively, were potentially glyphosate sensitive. This is in line with the hypothesis that the abundance of glyphosate-sensitive microbes is decreasing as a response to glyphosate residues in soil. The observed loss in abundance of these potentially glyphosate-sensitive bacterial OTUs might be caused by the mechanism of action of glyphosate residues targeting the EPSPS enzyme in the shikimate pathway and, thereby, inhibiting the growth and/or reproduction of these bacterial OTUs. On the other hand, bacteria may easily become resistant to glyphosate as a result of a single mutation in the EPSPS active site, and this would result in a higher proportion of glyphosate-resistant bacteria in glyphosate-exposed environments (Rainio et al. 2021).

Contrary to the low sensitivity of bacterial communities to glyphosate, most fungi (92%) are known to be sensitive to glyphosate (Leino et al. 2021). In line with this, 212 out of 369 fungal OTUs in this study were found to be potentially glyphosate sensitive, while the remaining could not be classified. However, the response of the EPSPS protein to glyphosate has been determined mainly in plants and bacteria, as both have a unidomain EPSPS protein; in contrast, fungal EPSPS contains multiple domains that can lead to variable responses to glyphosate (Mathew et al. 2022). Accordingly, we found potentially sensitive fungal OTUs that were enriched in the GBH treatment samples, while other potentially sensitive fungal OTUs were also enriched in other treatments.

Indicator species analysis of strawberry fungal OTUs showed a consistent taxonomic trend at the order and family levels in response to GBH treatments. Most root fungal OTUs enriched in the control treatment samples represented the fungal families Sebacinaceae and the arbuscular mycorrhizal fungal (AMF) family Glomeraceae. This is in line with previous studies that have reported that GBH application can decrease AMF abundance as well as root colonization in different systems (Zaller et al. 2015, Druille et al. 2016, Helander et al. 2018). In leaves, two out of three fungal OTUs enriched in the control treatment samples were from the order *Leucosporidium*, which has been reported to be part of the phyllosphere microflora of several plant species (Bálint et al. 2013, Wang et al. 2016, Suryanarayanan and Shaanker 2021). Reduction in the abundance of important plant-associated fungal taxa following GBH treatment may affect plant resilience to a multitude of stressors (van Bruggen et al. 2021), but more directed studies are needed to test this. However, since the exact EPSPS sequences and, thus, potential sensitivity of several responding OTUs from our samples are not known, further empirical and theoretical studies are needed to fine-tune the classification of the EPSPS enzyme in fungi in order to determine more precisely their susceptibility to glyphosate.

Phosphate and glyphosate are known to compete for the same adsorption sites in soil. As a result, phosphate fertilizer applications have been demonstrated to increase the mobility of glyphosate in soil and lead to increased uptake of glyphosate by plant roots (Bott et al. 2011, Gomes et al. 2015). This has the potential to increase the harmful effects of glyphosate on nontarget organisms. However, in the current study, phosphate was not found to mediate the effects of GBH residues on plant microbiota or performance. Phosphate fertilizer treatment did not have effect on strawberry performance, but it affected the diversity, community composition, and abundance of bacterial OTUs in strawberry roots. In accordance with these findings, phosphate addition has been shown to increase root bacterial diversity in two model plant species (Bodenhausen et al. 2019). Furthermore, recent studies on *Arabidopsis* indicate that phosphate availability has an effect on the colonization of roots by soil bacteria (Zuccaro 2020).

Given the extensive use of GBH in agriculture and horticulture, as well as glyphosate persistence in ecosystems (Helander et al. 2012, Maggi et al. 2020), both wild and cultivated plants are likely to be exposed to glyphosate residues. In addition, the effects of various co-formulants on plants and other nontarget organisms after GBH use cannot be ruled out (Mesnage et al. 2019). An increasing number of studies are showing that the effects of commercial herbicide formulations on nontarget organisms can be stronger than the effects of the active ingredient alone (Helander et al. 2019, Straw et al. 2021). Although our results fail to differentiate whether the results are the outcome of the effect of the active ingredient or co-formulants, the use of GBHs is justified in experiments because it corresponds to the actual weed control. Changes in endophytic microbiota may function as a reliable indicator of persistent, environmental stressor-mediated changes in plants. However, the results of this study did not indicate any major changes in the overall endophytic microbiome mediated by soil GBH treatment. Still, certain bacterial and fungal taxa, especially in strawberry roots, responded to GBH treatments. Although these changes were not reflected in the measured plant performance traits in this short-term experiment, it remains to be answered whether these GBH-driven alterations in strawberry microbiota may affect other plant functions and, eventually, plant resilience. This study is the first one to report the proportion of potentially glyphosate-resistant and glyphosate-sensitive bacteria in any plant species. Further studies are needed to understand the glyphosate-resistance mechanisms for fungi and how differences in sensitivity/resistance to glyphosate, together with long-term exposure to GBH application, affect the composition and diversity of endophytic microbial communities. Strong selection pressures due to the intensive GBH use might cause rapid evolution of glyphosate-sensitive bacteria possibly having consequences on plant–microbe interactions and thus on plant health.

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Supplementary data

Supplementary data is available at *JAMBIO* online.

Conflict of interest

No conflict of interest declared.

Author contributions

A.M. and M.H. conceived and designed the study. S.A.M. conducted microbiome experiments and A.M. conducted plant performance experiments. R.N., S.A.M., B.F., P.P., and A.M. conducted data analysis. S.A.M., A.M., and B.F. drafted the manuscript. All authors contributed to editing and proofreading the manuscript.

Data availability

The datasets generated during the study are available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.cvdncjt7k).

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