

## RESEARCH ARTICLE

# Herbicide residues in soil decrease microbe-mediated plant protection

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Ecosystem services; fungal plant symbionts; herbivory; mutualism; trophic cascades; trophic interactions.

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**ABSTRACT**

- The residues of glyphosate are found to remain in soils longer than previously reported, affecting rhizosphere microbes. This may adversely affect crop and other non-target plants because the plant's resilience and resistance largely rely on plant-associated microbes. Ubiquitous glyphosate residues in soil and how they impact mutualistic microbes inhabiting the aboveground plant parts are largely unexplored.
- We studied the effects of herbicide residues in soil on *Epichloë* sp., which are common endophytic symbionts inhabiting aerial parts of cool-season grasses. In this symbiosis, the obligate symbiont subsists entirely on its host plant, and in exchange, it provides alkaloids conferring resistance to herbivores for the host grass that invests little in its own chemical defence.
- We first show decreased growth of *Epichloë* endophytes *in vitro* when directly exposed to two concentrations of glyphosate or glyphosate-based herbicides. Second, we provide evidence for a reduction of *Epichloë*-derived, insect-toxic loline alkaloids in endophyte-symbiotic meadow fescue (*F. pratensis*) plants growing in soil with a glyphosate history. Plants were grown for 2 years in an open field site, and natural herbivore infestation was correlated with the glyphosate-mediated reduction of loline alkaloid concentrations.
- Our findings indicate that herbicides residing in soil not only affect rhizosphere microbiota but also aerial plant endophyte functionality, which emphasizes the destructive effects of glyphosate on plant symbiotic microbes, here with cascading effects on plant–pest insect interactions.

**INTRODUCTION**

The increased use of agrochemicals has caused persistent pollution of soils with agrochemical residues at a global scale (Maggi *et al.* 2020; Riedo *et al.* 2021; Tang *et al.* 2021). Recent studies have highlighted the detrimental effects of herbicide residues in soil on the plant mutualistic microbes inhabiting the rhizosphere, showing decreased microbial viability and microbe-mediated benefits to their host (Helander *et al.* 2018; Mohamed *et al.* 2021; Riedo *et al.* 2021). The importance of microbes for plant productivity, protection, and performance, ranging from nutrient uptake to resilience against abiotic and biotic stressors, are among the most promising mechanisms to counteract the challenges of global change, including soil degradation and food security in sustainable agricultural practices (Kauppinen *et al.* 2016; Pozo *et al.* 2021).

Glyphosate (*N*-(phosphonomethyl)glycine) has become the dominant herbicide since its introduction in the 1970s (Duke & Powles 2008). Currently, glyphosate-based herbicides (GBH) are the best-selling herbicides because of their effectiveness and affordability, as well as their application together with glyphosate-resistant crop varieties (Duke & Powles 2008). Glyphosate kills plants by inhibiting 5-enolpyruvylshikimate-3-

phosphate synthase (EPSPS), an enzyme needed in the shikimate pathway for biosynthesis of the essential aromatic amino acids tryptophan, tyrosine, and phenylalanine, which form the basis of numerous primary and specialized plant metabolites (Hoagland & Duke 1982; Gomes *et al.* 2014; Fuchs *et al.* 2021). Biochemically, glyphosate contains a functional phosphate unit (P), which occupies similar binding sites in soil particles to phosphate fertilizer. Hence, the use of phosphate fertilizers, together with glyphosate, can promote glyphosate availability for plants but at the same time increases the risk to non-target plants by a promoted leaching into the environment (Hébert *et al.* 2019).

A recent study showed that a high proportion of microbes may be susceptible to glyphosate (Leino *et al.* 2021). The assessment, which is based on the taxonomic distribution and classification of the EPSPS enzymes, suggested that 57% of bacteria and 92% of fungi are potentially sensitive to glyphosate (Leino *et al.* 2021). As an example of a plant mutualistic microbe, the study of Leino *et al.* (2021) presented detailed results on the potential glyphosate sensitivity of 20 *Epichloë* fungal species. These fungi, which are commonly associated with Pooidae grasses, grow asymptotically in the intercellular spaces of aerial plant parts and benefit their host plants by

increasing resilience under drought and salt stress, as well as enhancing resistance to herbivores (Saikkonen *et al.* 1998; Schardl *et al.* 2004; Fuchs *et al.* 2017; Bastias *et al.* 2021; Laihonon *et al.* 2022b). *Epichloë* endophytes are of agricultural importance, producing alkaloids that are known for their adverse effects on both invertebrate and vertebrate herbivores, thus preventing insect pest outbreaks and overgrazing (Jani *et al.* 2010; Kauppinen *et al.* 2016). Natural pest control measures, such as increasing plant resistance to herbivores by association with microbes, are discussed as key to reducing chemical pesticide use and increasing natural plant resilience in organic farming practices (Wille *et al.* 2019; Gruden *et al.* 2020; Pozo *et al.* 2021). Organic farming has been shown to successfully promote natural pest control when compared with conventional practices, which may be linked to higher microbial diversity and better functionality of plant symbiotic microbes (Muneret *et al.* 2018). Herbicides and other anthropogenic chemicals are slowly being recognized as possible reasons for the vanishing efficiency of ecosystem services and global insect biodiversity decline (Groh *et al.* 2022; Helander *et al.* 2023; Ruuskanen *et al.* 2023). In several plant species, glyphosate residues in soil have elicited substantial changes in phytohormone concentrations, which are key players in mediating plant chemical crosstalk with mutualistic symbiotic microbes and the defence response to herbivorous insects (Robert-Seilaniantz *et al.* 2011; Bastias *et al.* 2017; Fuchs *et al.* 2022a; Mathew *et al.* 2023).

In the current paper, we examine whether GBH residues in soil affect the protective function of the *Epichloë* endophyte in its host grass in defending against herbivores. We use the fungus *E. uncinata*, commonly inhabiting agronomically important meadow fescue (*Festuca pratensis*) (Kauppinen *et al.* 2016; Laihonon *et al.* 2022a), as a model symbiotum. In this symbiosis, the deterrence of herbivores is based primarily on strongly insecticidal loline alkaloids (*i.e.*, *N*-acetyllooline and *N*-formyllooline) (Easton *et al.* 2009). Concentrations of loline alkaloids are negatively correlated with aphid infestation, indicating their insecticidal function (Wilkinson *et al.* 2000).

Our study demonstrates the growth inhibiting effects of glyphosate-based herbicides on *Epichloë* fungal growth *in vitro*. Furthermore, our field study showed decreased production of loline alkaloids in host tissues of plants growing in soils with a history of GBH use. Lower loline alkaloid concentrations correlated with an increased plant colonization by phloem-feeding herbivores, exemplifying the cascading effects of herbicide residues in soil on trophic level interactions.

## MATERIAL AND METHODS

We analysed the effects of glyphosate on plant symbiotic fungal endophytes in two different ways. First, we tested pure glyphosate and glyphosate in the form of GBH on *E. uncinata* fungi by measuring the growth of fungal isolates during an *in vitro* assay. Second, we tested the effects of GBH on *E. uncinata* endophytes during an *in vivo* assay by growing *E. uncinata* symbiotic *F. pratensis* plants in soils that had experienced GBH treatment during the past 7 years, here through a common garden study. As responses, we measured endophyte-produced alkaloid concentrations, plant aboveground biomass, and aphid colonization of the host plants.

## Fungal growth assay

We conducted the fungal growth assay with *E. uncinata* isolated from *F. pratensis*. Plant individuals known to harbour *E. uncinata* were grown under greenhouse conditions for at least 1 year. To isolate fungal mycelium, we placed surface-sterilized (3% bleach for 3 min, followed by 70% ethanol for 1 min) plant leaf pieces of approximately 1 cm<sup>2</sup> onto potato dextrose agar (PDA). After 3–7 days, fungal mycelium started to grow out from the cut parts of the leaf onto the PDA. *E. uncinata* identification was confirmed microscopically. We placed 1 × 1 mm fungal mycelia in the center of PDA Petri dishes (diameter 9 cm) with low or high concentrations of either pure glyphosate (Sigma-Aldrich, *N*-(Phosphonomethyl) glycine 99% purity, and analytical standard quality), glyphosate in the form of GBH Roundup Gold® (Glyphosate acid: 36% w/v (potassium salt: 44.1% w/v; Monsanto), or glyphosate/GBH-free controls). Glyphosate/GBH was carefully mixed with 60 °C PDA before pouring into Petri dishes. The low and high glyphosate/GBH treatments contained 0.45 g l<sup>-1</sup> (G1/GBH1) and 0.90 g l<sup>-1</sup> (G2/GBH2) glyphosate, respectively, simulating realistic field concentrations of applied glyphosate. Each treatment was replicated ten times. The Petri dishes were stored in a dark growth chamber at a constant temperature of 21 °C. To estimate fungal growth, we measured the diameter of the colony from two directions in a 90° angle six times during the growth period of 26 days. The first measurement took place immediately after plating the fungal colonies to ensure the comparable size of each colony at the start of the experiment.

## Common garden study design

We conducted the common garden experiment at Ruissalo Botanical Garden (60°26' N, 22°10' E) in southwestern Finland during the field seasons in 2019 and 2020. The field has been used for glyphosate experiments following common agricultural practices since 2014. The soil is characterized as medium clay with high organic matter and a high phosphate affinity. The topsoil layer of the plot area consists of approximately 88% clay, 6% sand and 6% peat. Physicochemical properties of the soil were assessed as follows: pH between 6.0 and 6.5, calcium (between 2100 and 2600 mg l<sup>-1</sup>), phosphorus (between 4.0 and 6.0 mg l<sup>-1</sup>), potassium (between 200 and 250 mg l<sup>-1</sup>), magnesium (between 450 and 550 mg l<sup>-1</sup>) and sulfur (between 16 and 22 mg l<sup>-1</sup>) (eurofins.fi). The field consisted of alternating 12 GBH-treated and 12 non-treated control plots (23 × 1.5 m) (Helander *et al.* 2019). Half of each GBH plot and control plot was further assigned a phosphorus treatment. Thus, we had four soil treatments: control (C), phosphorus treatment (P), GBH treatment (G), and combined phosphorus and GBH treatment (PG). Before grasses were planted in the field on 4–5 June 2019, we treated the respective plots either with GBH, phosphate, or both; as a control, we sprayed tap water. We sprayed the plot area with Roundup Gold as a GBH treatment (glyphosate concentration 450 g l<sup>-1</sup>, CAS: 3864-194-0, application rate: 6.4 l ha<sup>-1</sup>, in 3 l tap water per plot, applied on 13 May), and for the control treatment, we sprayed only water (3 l tap water per plot). Before GBH treatment, we treated phosphate-assigned subplots with phosphate (P) (Yara Ferticare, 80 g in 10 l water per subplot, applied on replicates 1–5 on 29 April, on replicates 6–10 on 3 May, control subplots

treated with water only). In May 2020, when the plants were already growing in the soil, we sprayed the soil immediately surrounding the plants with concentrations of GBH, corresponding to the doses applied in May 2019, and phosphate was added as described above. Green plant parts did not have direct contact with GBH at any time.

We planted 12 endophyte-symbiotic meadow fescue plants (*F. pratensis* symbiotic with *E. uncinata*) in each soil treatment plot. The plants were grown from seedlings in the greenhouse and transferred to the experimental field in on 4–5 June 2019, 3 weeks after soil treatment with glyphosate. The plants were regularly watered throughout spring/summer to avoid drought stress. Weeds were regularly manually removed from the plot area to minimize potential plant competition in any of the treatments. Eight plants were regularly clipped for another study, while four plants per plot were only cut once for plant biomass harvest.

### Plant biomass

We harvested plants in the third week of September 2020 by cutting the entire aboveground plant tissue, including inflorescences with seeds. We determined the dry biomass (48 h at 65 °C) of the plants by pooling the biomass of the four plants (or less in case of mortality: two plants died in the early stage, one in the C and one in the G treatment) per plot (C, P, G, and PG) and then divided the total biomass per plot by the number of live plants in the plot.

### Loline extraction and quantification

We collected plant material for loline extraction simultaneously with the August count of aphid numbers in 2020. We analysed loline alkaloids from 10 plots per treatment consisting of a pooled sample of plant material of all four plants (or less in case of mortality) per plot, which resulted in 40 samples. We harvested five pseudostem parts between the third and fourth internode, at a length of approximately 10–15 cm, along with two branching leaves, here cut to a length of approximately 6–8 cm (for alkaloid tissue distribution in endophyte-symbiotic meadow fescue, see Justus *et al.* 1997). Plant samples were immediately flash frozen in liquid nitrogen and stored at –80 °C until lyophilization. We determined loline concentrations via a method modified from Pan *et al.* (2014) using gas chromatography coupled with mass spectrometry (GC–MS). In brief, alkaloids were extracted with NaOH (100 µl, 1 M) and 1000 µl chloroform, here containing 50 µg ml<sup>-1</sup> internal standard compound quinoline, from approximately 100 mg lyophilized and finely ground leaf material, by shaking for 12 h (VWR incubating mini shaker; 300 min<sup>-1</sup>). Thereafter, the extracts were centrifuged (14,000 g, 15 min; Eppendorf centrifuge 5415D) and the liquid phase was filtered through a PTFE syringe filter (0.45 µm) into 1.5-ml glass vials (Thermo Fischer). Then 1 µl was injected using an AS 1310 autosampler (Thermo Fischer) into a gas chromatograph (Trace 1310; Thermo Fischer) coupled to a mass spectrometer (TSQ 8000 EVO; Thermo Fischer) equipped with a HP5MS column (30 m × 0.25 mm × 0.25 µm; J&W Scientific). GC operating conditions were as follows: helium flow 1.0 ml min<sup>-1</sup>, initial temperature 75 °C, ramp one 10 °C min<sup>-1</sup> to 160 °C, ramp two 4 °C min<sup>-1</sup> to 200 °C, ramp three 10 °C min<sup>-1</sup> to 300 °C,

hold at 300 °C for 5 min. MS detection was performed in the range of 50–250 *m/z* in EI mode, with an ionization energy of 70 eV. We processed the data with Xcalibur® software (Thermo Fischer Scientific). Peaks were assigned to *N*-Formylloline and *N*-Acetylloline by retention time and MS spectra comparison with authentic standard compounds. Quantification was achieved by peak area of quantifier ions: Quinoline (129 *m/z*), *N*-Formylloline, and *N*-Acetylloline (82 *m/z*). Concentrations were calculated in µg g<sup>-1</sup> dry weight.

### Aphid herbivory

We recorded the natural aphid colonization twice per month on the plants throughout spring/summer 2020. The majority of aphids were from the genus *Aphis*, where numbers stayed below ten individuals per plot during June and July until the numbers peaked at the beginning of August. We recorded the presence of aphids on each plot and counted the number of aphids per plot (reported are values from the August count corresponding to the time point of sampling for alkaloids). On all aphid-infested plots, we recorded 10 or more aphids per plant. We encountered only negligible counts of parasitized aphids or aphid-feeding predatory insects or other invertebrate herbivores.

### Glyphosate and aminomethylphosphonic acid (AMPA) analyses

We recorded the degradation and persistence of glyphosate by taking soil samples on 15 August 2019, approximately 3 months after GBH application in spring. The soil samples were taken from each subplot, and the samples were pooled. Each soil sample was approximately 2.5 cm in diameter and 5 cm in depth. The sampled soil material was air-dried before analysis for glyphosate and its degradation product, AMPA. Extraction was performed with aqueous, acidified methanol, which was followed by analysis via liquid chromatography coupled with mass spectrometry (LC–MS/MS) at GroenAgro (agrocontrol.nl) (Ruuskanen *et al.* 2020).

### Statistics

Statistical analyses for biomass, aphid numbers, and alkaloid concentrations were performed using linear models and subsequent one-way ANOVA and *post-hoc* tests with the software R version 4.0.2. Treatments were compared with the control with the ‘glht’ function from the package ‘multcomp’ in the software R, which provides a *post-hoc* test (Dunnnett), comparing each treatment to the control (Hothorn *et al.* 2020). For loline alkaloid analyses, we included plant biomass as a covariate in a linear model, which was not significant and was removed from the model. All analyses included plot as a random factor in a linear model, which did not significantly affect the response variables. Correlation was performed based on Pearson’s product–moment correlation and visualized with the package ‘ggplot2’ (Wickham 2016). We calculated the growth rates for each individual sample (fungal growth assay) by fitting a linear model and estimating the slopes: here, the increase in growth per week. Subsequently, we used a one-way ANOVA followed by a Tukey *post-hoc* test to compare growth rates between the treatments.

## RESULTS

### Fungal growth inhibited by glyphosate and glyphosate-based herbicides

Our results show the growth-inhibiting effects of glyphosate and GBH on *E. uncinata* fungal growth *in vitro* (Fig. 1a and b). The growth of *E. uncinata* mycelia on Petri dishes with high GBH concentrations was completely inhibited, and in low GBH concentrations, it was strongly inhibited, indicating the indisputable antimicrobial effect of GBH (Fig. 1). Glyphosate alone decreased growth by approximately 75% in high glyphosate concentration and approximately 50% in a low glyphosate concentration, indicating that glyphosate itself strongly suppresses the growth of *E. uncinata* (Fig. 1b).

### Glyphosate residues in the soil

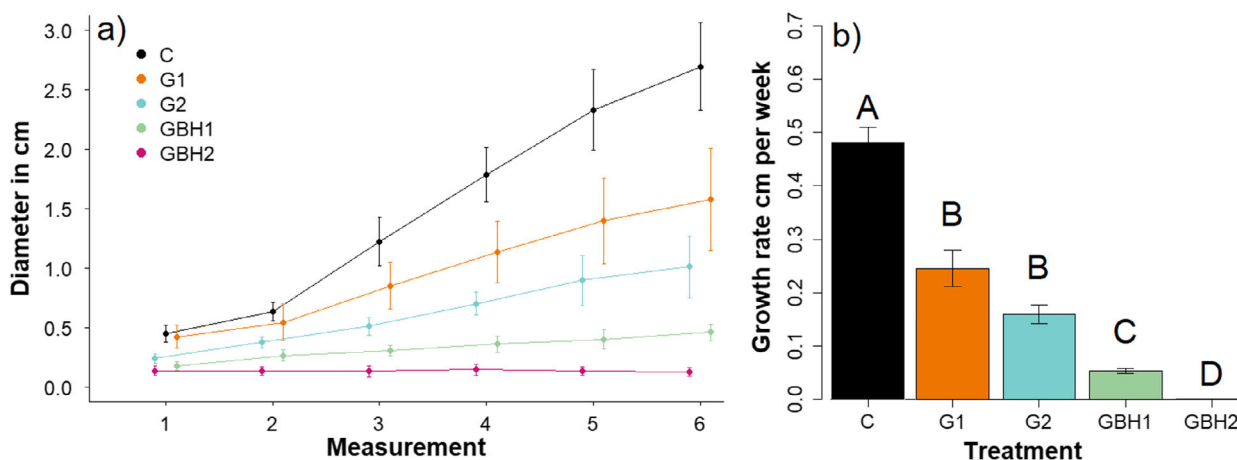
We demonstrated that glyphosate residues remaining in soils with a preceding history of GBH use can predispose plants to herbivory in the real world. With a long-term field experiment, we showed that, contrary to the alleged rapid degradation of GBH in soils, both glyphosate and its degradation product aminomethylphosphonic acid (AMPA) concentrations remained higher in GBH-treated soils compared with control (C) soils, irrespective of phosphate (P) treatment, approximately 3 months after the last application of GBH. On average, we detected 1.5 and 1.7 mg kg<sup>-1</sup> glyphosate from GBH (G)-treated and phosphate- and GBH (PG)-treated soils, respectively. AMPA concentrations in soils from the G and PG treatments were 1.3 and 1.5 mg kg<sup>-1</sup>, respectively. In contrast, both glyphosate residues and AMPA remained below the quantification limit (concentrations <0.01 mg kg<sup>-1</sup> for glyphosate and <0.05 mg kg<sup>-1</sup> for AMPA) in soil samples collected from C and P treatments at all sampling time points.

### Endophyte-mediated plant protection reduced by glyphosate-based herbicides in soil

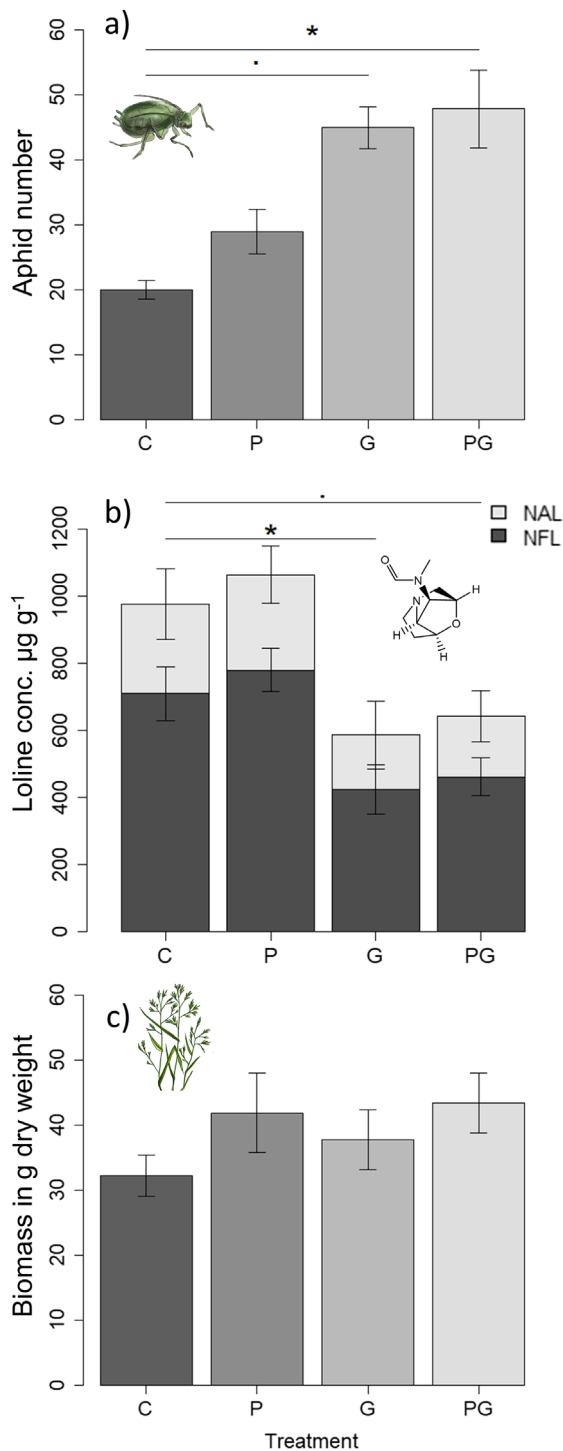
Soils with a GBH history had markedly increased aphid numbers on plants (Fig. 2a), while GBH reduced loline concentrations in *Epichloë* endophyte-symbiotic plants (Fig. 2b, Table 1) compared with plants growing in phosphate-treated and control soils (Fig. 2b). Half of the plants on GBH plots were aphid-infested compared with only 33% of the control plots, and GBH treatment approximately doubled the number of aphids on the plants (Fig. 2a, Table 1). *Post-hoc* tests showed a significantly higher number of aphids on plants growing in soil treated with phosphate and GBH (PG) compared with plants growing in control soil (C), when taking into account only plots with aphid occurrence (Fig. 2a, Table 1). Loline concentrations negatively correlated with aphid number across all aphid-infested plots (Fig. 3), demonstrating their insecticidal impact. Plant size did not explain the significantly higher proportion of aphid incidences. GBH and phosphorus treatment solely and jointly slightly increased plant biomass (Fig. 2c, Table 1).

## DISCUSSION

Glyphosate residues are detected in diverse habitats globally, and their destructive effect on the rhizosphere is increasingly being revealed (Nivelle *et al.* 2018; Maggi *et al.* 2020; Tang *et al.* 2021; van Bruggen *et al.* 2021). Despite slightly increasing plant biomass (hormesis: see Brito *et al.* 2018), our results highlight the potential susceptibility of *Epichloë* endophytes to glyphosate (Leino *et al.* 2021). Further, our results reveal that commercial GBH may also contain other microbicidal ingredients, as demonstrated by the significant fungal growth reduction caused by GBHs compared to pure glyphosate. Our field results demonstrate that the effects of GBH residue



**Fig. 1.** Fungal growth rate of *E. uncinata* decreases on glyphosate-containing PDA medium. (a) Weekly measured fungal colony diameter growing *in vitro* on potato dextrose agar (PDA) containing two concentrations of pure glyphosate (G1 = low, G2 = high) or glyphosate-based herbicide (GBH1 = low, GBH2 = high) compared with a glyphosate-free control (C). (Visual representation of data points from the same time point were shifted for better visibility). (b) Mean ( $\pm$ SE) growth rate of fungi compared with a one-way ANOVA and Tukey *post-hoc* test. Letters in (b) indicate statistically significant differences.  $P < 0.05$ .

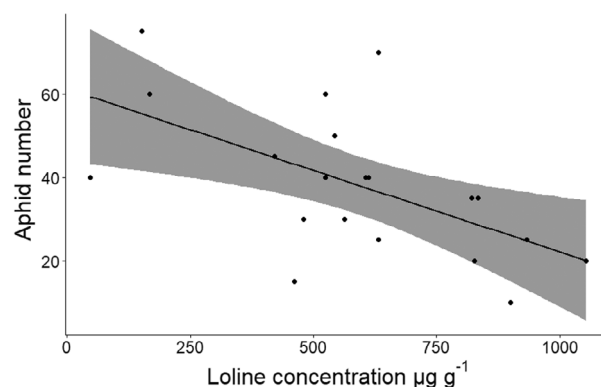


**Fig. 2.** Responses of plant biomass, loline alkaloid production, and aphid colonization to phosphate and glyphosate-based herbicides. (a) Aphid number (mean  $\pm$  SE) counted on plants of each treatment, here including only plants with aphids at sampling time (percentage of colonized plots per treatment: C: 25%, P: 42%, G: 42%, PG: 58%). (b) Sum of loline alkaloids (mean  $\pm$  SE) per sample compared with one-way ANOVA and *post-hoc* (glht comparison between all treatments to the control); *N*-Formylloline (NFL, dark grey), *N*-Acetyllooline (NAL, light grey). (c) Aboveground biomass (in g dry weight) per plant (mean  $\pm$  SE); For statistics see, Table 1. \* $P \leq 0.05$ ,  $P \leq 0.1$ . Treatments: C, Control; P, Phosphate; G, Glyphosate; PG, Phosphate and Glyphosate.

**Table 1.** Results of ANOVA and Tukey *post-hoc* tests on plant biomass, aphid number, and loline concentration to the treatments: phosphate (P) and glyphosate (G) and their combination (PG) (See Fig. 2). *N*-formylloline (NFL), *N*-acetyllooline (NAL), sum of both loline alkaloids (Loline sum).

response variable	explanatory variable	ANOVA			glht ( <i>P</i> -value)		
		F	df	P	P-C	G-C	PG-C
Shoot biomass	G	0.508	42	0.480	/	/	/
	P	2.501	42	0.121			
	P:G	0.177	42	0.676			
Aphid number	G	9.334	16	<b>0.007</b>	0.722	0.087	<b>0.040</b>
	P	0.396	16	0.538			
	P:G	0.186	16	0.672			
NFL conc.	G	15.705	36	<b>&lt;0.001</b>	0.855	<b>0.031</b>	0.070
	P	0.498	36	0.485			
	P:G	0.043	36	0.836			
NAL conc.	G	14.714	36	<b>&lt;0.001</b>	0.928	<b>0.029</b>	0.080
	P	0.045	36	0.508			
	P:G	0.001	36	0.980			
Loline sum	G	15.812	36	<b>&lt;0.001</b>	0.873	<b>0.028</b>	0.068
	P	0.496	36	0.486			
	P:G	0.026	36	0.872			

Significant *P* values are highlighted in bold.



**Fig. 3.** Negative correlation between loline alkaloid concentration (sum of both lolines) and aphid number per plot across all treatments, based on a Pearson correlation test ( $t = -3.0347$ ,  $df = 18$ ,  $P = 0.00713$ ,  $r = -0.58$ ).

concentrations, which are similarly found across all European soils (Silva *et al.* 2018), are not limited to reducing below-ground microbe viability but may also negatively affect productivity of a plant's beneficial microbial symbiont growing in the aerial plant endosphere, leading to a decrease in plant protection against aphids. Glyphosate residues in soil have previously been found to modify plant resistance to aboveground herbivory *via* changes in leaf quality (Ramula *et al.* 2022).

Our findings indicate compromised plant–endophyte symbiosis, which may be the result of either a direct effect of GBH residues in the soil on the endophytic fungus *in planta*, or an indirect effect caused by modulation of rhizosphere and/or plant physiological and biochemical processes (Newman *et al.* 2016; Helander *et al.* 2018; Fuchs *et al.* 2021; Fuchs *et al.* 2022a; Keronen *et al.* 2023), with cascading consequences on the *Epichloë*–grass symbiosis. In previous research, equivalent glyphosate residues in soil have been shown to modify soil

and rhizosphere microbiota (including mycorrhizal fungi) (Busse *et al.* 2001; Helander *et al.* 2018) and negatively affect plant performance (Helander *et al.* 2019; Muola *et al.* 2021; Fuchs *et al.* 2022b). A previous study with potted plants found glyphosate residues in the green leaf material of meadow fescue (*F. pratensis*) and the weedy grass (*Elymus repens*) during the growing season following the glyphosate treatment (Helander *et al.* 2018). This suggests that glyphosate can be taken up from the soil into plant roots and then translocated *via* the phloem throughout the plant, which may cause direct contact between glyphosate and the endophyte *in planta* and explain the observed negative effect on *Epichloë* endophytes and alkaloid biosynthesis in the grass endosphere. However, this is rather unlikely, since we would expect to see an additional herbicidal effect decreasing plant biomass.

An increasing number of studies have shown how sublethal glyphosate doses can affect metabolic pathways in plants and microorganisms (Lydon & Duke 1989; Duke 2018; Fuchs *et al.* 2021), in particular the biosynthesis of phytohormones, phenolic compounds, or alkaloids (Ossipov *et al.* 2003; Gomes *et al.* 2014; Rainio *et al.* 2020; Fuchs *et al.* 2022a; Fuchs *et al.* 2022b). Many of these compounds are specialized metabolites and essential for directing plant communication to interacting species and are predominantly important for fungal symbionts to successfully establish a symbiosis with the host plant, as shown for *Epichloë* endophytes and cool-season grasses (Bastías *et al.* 2018; Scott *et al.* 2018). Here, in particular salicylic acid and jasmonic acid are key drivers, which were both affected by glyphosate (Fuchs *et al.* 2022a). Consequently, a glyphosate-mediated disruption in the biosynthesis of plant signalling molecules may inhibit fungal growth *in planta* and the production of alkaloids. A previous study demonstrated that disrupted signalling between the grass and its fungal endophyte can turn mutualism into antagonism, emphasizing the fine balance of the symbiotum (Eaton *et al.* 2010).

Another possibility is that GBH residues in the soil inhibit the biosynthesis of plant-derived metabolites, which serve as precursors in the biosynthesis of loline alkaloids. However, the biosynthesis of proline, an essential amino acid for loline biosynthesis, was found to be rather stimulated by glyphosate treatment (Gomes *et al.* 2017). Further studies are needed to understand the complexity of the mechanism through which GBH residues in soil can directly or indirectly affect biological interactions (Brühl & Zaller 2021).

The complexity and long-term effects of intensified herbicide use on ecosystems and on global biodiversity during the past few decades have only recently been recognized (Helander *et al.* 2012; Brühl & Zaller 2019; Fuchs *et al.* 2021; Muola *et al.* 2021; Riedo *et al.* 2021; Groh *et al.* 2022). However, increasing levels of GBH and their degradation products are found in a wide range of environments across the globe (Laitinen *et al.* 2009; Maggi *et al.* 2020; Tang *et al.* 2021). The consequences for ecosystem functions and services are unforeseeable and can turn against agricultural productivity (Riedo *et al.* 2021; Wittwer *et al.* 2021). The direct insecticidal effects of sublethal doses of herbicides on insects and the indirect effects on insect communities in response to altered plant coverage or community have been observed (Hill *et al.* 2012; Egan *et al.* 2014; Sharma *et al.* 2018; Kraus & Stout 2019). In contrast, here, we show that by affecting a plant's mutualistic

fungus, glyphosate residues in soil increase the susceptibility of plants to insect pests. This suggests that herbicide usage can lead to a vicious circle of an increased need for chemical pest control by counteracting microbe-based integrated pest management practices (van der Werf *et al.* 2020).

## CONCLUSIONS

Plant beneficial microbes, such as *Epichloë* endophytes, provide prospective tools to mitigate pest problems and circumvent the heavy use of pesticides, as well as playing a key role in shaping sustainable agriculture of the future (Kauppinen *et al.* 2016; Blundell *et al.* 2020; Pozo *et al.* 2021; Riedo *et al.* 2021). Our results indicate the vulnerability of plant-microbe symbioses to herbicide residues in soil, highlighting that the use and mechanisms of herbicides need urgent review through more holistic approaches covering: (i) the long-term effects of herbicides, including their persistent residues, (ii) the effects on multi-trophic and multi-species ecological interactions, and (iii) how the accumulating agrochemical pollution affects ecosystem services and insect biodiversity in agricultural ecosystems and beyond. Further studies are required to unravel the mechanistic links behind the inhibition potential of herbicide residues in soils on loline biosynthesis in *Epichloë* symbiotic plants.

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## AUTHOR CONTRIBUTIONS

BF, KS and MH performed the field study, MH performed the *in vitro* assay, BF, AD and BY developed the method for alkaloid quantification, BF performed the alkaloid analyses, BF analysed the data, BF wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## DATA AVAILABILITY STATEMENT

Data are available from the authors upon reasonable request.

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