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Glyphosate decreases mycorrhizal colonization and affects plant-soil feedback

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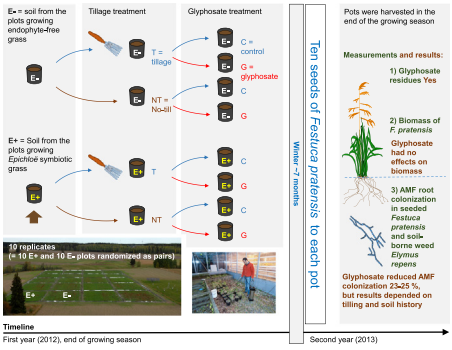
HIGHLIGHTS

- In northern ecosystems glyphosate residues are detected in crop plants the following growing season.
- Arbuscular mycorrhizal colonization is decreased in glyphosate treated plants.
- The magnitude of mycorrhizal reduction is dependent on tilling and soil history.

GRAPHICAL ABSTRACT



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ABSTRACT

Our aim was to study the effects of glyphosate, tilling practice and cultivation history on mycorrhizal colonization and growth of target (weeds) and non-target (crops) plants. Glyphosate, the world's most widely used pesticide, inhibits an enzyme found in plants but also in microbes. We examined the effects of glyphosate treatment applied in the preceding fall on growth of a perennial weed, *Elymus repens* (target plant) and a forage grass, *Festuca pratensis* (non-target plant) and their arbuscular mycorrhizal fungal (AMF) root colonization in a field pot experiment. Non-target plants were sown in the following spring. Furthermore, we tested if glyphosate effects depend on tillage or soil properties modulated by long cultivation history of endophyte symbiotic grass (E+ grass). AMF root colonization, plant establishment and growth, glyphosate residues in plants, and soil chemistry were measured. Glyphosate reduced the mycorrhizal colonization and growth of both target and non-target grasses. The magnitude of reduction depended on tillage and soil properties due to cultivation history of E+ grass. We detected glyphosate residues in weeds and crop plants in the growing season following the glyphosate treatment. Residues were higher in plants growing in no-till pots compared to conspecifics in tilled pots. These results demonstrate negative effects of glyphosate on non-target organisms in agricultural environments and grassland ecosystems.

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1. Introduction

Biocides, including pesticides, herbicides and fungicides, have been used in conventional farming practices for decades. While their use has increased crop production helping to feed globe's growing population, interest to study their risks has mainly focused on human health effects. The indirect effects of globally increasing use of biocides on non-target organisms are only rarely taken into account (Carvalho, 2016). This may translate into underestimation of the risks associated to globally increasing use of biocides to ecosystem functions and –services (Tilman et al., 2002).

Glyphosate, also known as N- (phosphonomethyl) glysine, is globally the leading herbicide of agriculture, horticulture, silviculture and urban environments in terms of both magnitude and broadness of usage (Helander et al., 2012; Myers et al., 2016). Glyphosate inactivates one part of the shikimate pathway, a metabolic route used by most plants, fungi, and bacteria for the biosynthesis of tryptophan, phenylalanine and tyrosine and molecules that require these essential proteins as precursors (Helander et al., 2012; Herman and Weaver, 1999). This pathway is not found in animal cells, and thus, glyphosate is considered to be safe for non-target organisms including vertebrates. In the connection to safe use of glyphosate against target plants, recent studies suggest possible indirect effects via soil on non-target plants, soil microbiota and microbes associated with plants and animals (Druille et al., 2013a, 2013b; Helander et al., 2012). Furthermore, the risks of the metabolites of glyphosate degradation such as AMPA [2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid], surfactants and other ingredients of commercial herbicide products, have been proposed to be even more toxic than the glyphosate alone (Folmar et al., 1979; Giesy et al., 2000; Gomes et al., 2016; Tsui and Chu, 2003).

Exposure of non-target organisms to glyphosate, its degradation products and other ingredients of herbicides has exponentially increased during the third millennium (Benbrook, 2016). The patent for herbicide use of glyphosate (Monsanto Company) launched under the trade mark Roundup in 1974. Since the Monsanto's patent expired outside the USA in 1991 and in the USA in 2000, several other major manufacturers have released numerous inexpensive glyphosate-based herbicides to the market. The expanding worldwide use of glyphosate from the 1990's is for the most part a consequence of development of genetically modified, glyphosate-tolerant strains of some of the most important crop species (Benbrook, 2016; Duke and Powles, 2008; Woodburn, 2000). Glyphosate tolerant crops allow weed control of the agricultural fields after germination of the crop plants. Furthermore, glyphosate-based herbicides have enabled the no-till cropping area to increase also outside the regions where genetically modified, glyphosate resistant cultivars are used. No-till farming enables farmers to sow crops without aggressive cultivation of soil. However, this requires that weeds are decimated by multiple and timely glyphosate applications. In Northern Europe, weed control before the farming season in early spring is commonly boosted by fall glyphosate applications to control cool season perennials such as *Elymus repens*. Noteworthy is that use of glyphosate is not limited to professional use in agriculture because herbicides are readily available for non-professional users.

Although the half- time of glyphosate is shown to be within a range of days to few weeks, its occurrence in the soil may be continuous due to frequent application (Primost et al., 2017) or degradation may be prolonged due to soil properties and other environmental factors (Bai and Ogbourne, 2016). For example, glyphosate competes with phosphate for adsorption sites in soil (Gimsing and dos Santos, 2005) and its degradation is stimulated by phosphorus (Laitinen et al., 2006, 2008). It may also form complexes with metal ions (Al, Mn, Zn, Fe) (Vereecken, 2005) and attach to soil particles. High microbial activity in the soil enhances the degradation rate of glyphosate. On the other hand, glyphosate may also affect microbiota and enzymatic activities in the soil (Carlisle and Trevors, 1988; Cherni et al., 2015; Imfeld and Vuilleumier, 2012; Krzysko-Lupicka and Sudol, 2008). Thus, for example soil management practices modulating soil biotic and abiotic characteristics may alter glyphosate degradation in soil (Alvarez and Steinbach, 2009; Doran, 1980). Increasing number of field studies lends support to the idea that glyphosate inactivation and degradation in soils can be much slower than generally believed. Glyphosate and its residuals have been found to stay in the soil varying lengths of time especially in ecosystems where winters are long and cold (Helander et al., 2012). For example, as much as 19% of glyphosate and 48% of AMPA have been detected as undecomposed 20 months after application in Finland (Laitinen et al., 2009). Slow decomposition rates could partially explain the high glyphosate-related contamination levels found in Scandinavian surface waters (Ludvigsen and Lode, 2001).

Glyphosate remaining in soils can have diverse and unpredictable consequences on ecosystem functions and services (Watrud et al., 2011) especially via changes in microbial communities and their interactions with other organisms. All plants are associated with numerous microbes inhabiting both below and aboveground plant tissues. In this paper we focus on arbuscular mycorrhizal fungi (AMF) inhabiting plant roots and *Epichloë* endophytic fungi (E+) living systematically and asymptomatically in aboveground plant tissues (Saikkonen et al., 1998). Both of them are common symbionts in grassland ecosystems dominated by Pooidae grasses but functionally they differ from each other. Mycorrhizal fungi are soil-borne in contrast to *Epichloë* fungi dispersed vertically from the plant to offspring via seeds (Saikkonen et al., 2004). Both are commonly thought to be plant mutualists in many environments but benefits to host plants are different. AMF improve growth and performance of plants by increased nutrient and water uptake (Sanders and Fitter, 1992; Smith and Read, 2008) whereas the role of *Epichloë* species is multifaceted, context dependent and partly unknown (Saikkonen et al., 1998). *Epichloë* species can enhance plant growth and reproduction, and modulate chemical ecology of the symbiotum e.g. by producing herbivore- and pathogen-deterring alkaloids (Bastías et al., 2018; Hamilton et al., 2012; Saikkonen et al., 2016). By altering the amount and quality of litter, and plant exudates into the soil, *Epichloë* species associated with aboveground tissues of the host grass can affect soil biology and chemistry affecting subsequent plant performance (García-Parisi et al., 2017; García-Parisi and Omacini, 2017). Plant-soil feedback is a process through which plants alter soil biotic and abiotic properties, which then affect plant performance (Klironomos, 2002; Bauer et al., 2015). Plant root-soil feedbacks have increasingly attracted attention, but potential above-ground microbial mediated plant-soil feedback shifts have received less attention (Bastías et al., 2018; Kulmatiski et al., 2008).

Here we study if (1) glyphosate usage in the fall, (2) tillage and (3) plant-soil feedback due to cultivation history of *Epichloë* colonized grass individually or jointly affect AMF colonization and performance of target perennial weed grass (*Elymus repens*) and annually sown non-target forage grass (*Festuca pratensis*) in the next growing season. We hypothesize that, in addition to tillage, glyphosate negatively affects the perennial weed grass as the target of the fall application. Based on the observation that glyphosate and its residues can remain in soils over the winter frosts and cool summers over the years, we assume that glyphosate residues can be traced from the plants, and these residues negatively affect plant performance and AMF in the growing season subsequent to the application. Taken into account that grass symbiotic *Epichloë* endophytes may suppress AMF (García-Parisi et al., 2017), we also hypothesized that soil properties modulated by long cultivation history of *Epichloë* endophyte symbiotic (E+) grasses are unfavorable for AMF colonization in weed survivors and forage grasses.

2. Materials and methods

2.1. Soil for the experiment

The soil used in this study was collected from a long-term field experiment in Jokioinen, Finland (60° 49′ N, 23° 30′ E) in October 2012.

The experiment comprising of ten blocks with two paired plots (25 m × 39 m) seeded with either endophyte symbiotic (E+) or endophyte-free (E−) meadow fescue, *Festuca pratensis* L., cultivar 'Kasper', in 2006. At the time of the soil sampling for the present study, the frequency of E+ plants was 80–90% and 0–3% in the E+ and E− plots, respectively. The aerial cover of meadow fescue had decreased from 100% to 75% in E− and 98% in E+ plots due to invasion of weeds such as the couch grass, *Elymus repens* (L.) Gould, which constituted more that 50% of the weed biomass at the time of the soil sampling (Saikkonen et al., 2013). According to Mikola et al. (2016) study, soil C and N contents were similar in E+ and E− plots in the experiment. The field is classified as Stagnosol and has sandy clay soil texture. For more detailed description for the long-term field experiment, see Huitu et al. (2014), Gundel et al. (2017), Mikola et al. (2016) and Saikkonen et al. (2013). Soil for the present study was dug from the 10 paired (E+/E−) plots cutting an 18.5 cm diameter, 17.0 cm deep block of soil, including the above-ground vegetation, and placing the soil clod into plastic pot. We sampled 8 soil blocks from each E+ and E− meadow fescue plots. The 160 pots with soil blocks were transferred to University of Turku Ruissalo Botanical Garden (60° 26′ N, 22° 10′ E).

2.2. Treatments and experimental design

To simulate tillage (T), topsoil (0–10 cm) in 80 pots was turned upside down and mixed using a small shovel. In the no-till (NT) treatment pots the soil structure was left untouched. Glyphosate in form of commercial formulation Roundup®Gold (450 g/l, active ingredient isopropylamine salt), 5 l/ha diluted in tap water (1:40) was then sprayed using a hand-operated pressure tank with a manual sprayer to 20 till (T) and 20 no-till (NT) pots of both endophyte origin soils (E + and E−). The recommended annual dose of glyphosate was sprayed at once in October 4th 2012 in contrast to customary glyphosate application in no-till fields (first in the end and then beginning of the growing seasons) to test the over-winter effects of glyphosate treatment in the soil. From the control pots we hand-weeded all green growth to avoid the disturbance of topsoil, and they received the same amount of tap water as the glyphosate treatment pots. The pots were randomized in a common garden with a fence around to prevent herbivore (e.g. rabbits) grazing. The mean monthly temperatures were below freezing from December 2012 to March 2013 and the ground had permanent snow cover.

2.3. Plant analyzes

In the beginning of the growing season in June 9th 2013, all vegetation was removed from the pots. Then 10 seeds of endophyte-free (E−) *F. pratensis* cultivar 'Kasper' were sown into each pot and marked with a toothpick to enable recording of their establishment success. The pots were left to grow until August 16th 2013, when all vegetation was cut and divided into *F. pratensis* plants sown in the beginning of the growing season and weeds (all other plants), and weighed. The above-ground *F. pratensis* and weed samples from each pot were then frozen and freeze-dried before homogenization. Then pots were taken into laboratory where the grass roots were carefully separated from the soil.

2.4. Extraction and glyphosate quantitation

50 mg of the fine plant powder was weighed into 2 mL eppendorf tubes. 1.3 mL of milli-Q water was added and the sample extracted for 7 h in a shaker in a cold room. Extracts were centrifuged, 500 μl sample was taken and 145 μl of internal standard (31.2 μg/ml $^{13}C_2/^{15}N$ isotope-labelled glyphosate) was added, vortexed for 5 min and filtered through 0.2 μm PTFE filters into UPLC vials. Extracts were analyzed by ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) by the Waters Acquity Xevo UPLC triple quadrupole mass spectrometer. For the analyses, separate multiple reaction monitoring (MRM) methods were developed for the detection of unlabelled (168 > 150, 20 V, 10 eV; 168 > 81, 20 V, 16 eV) and labelled glyphosate (171 > 153, 24 V, 12 eV; 171 > 81, 24 V, 16 eV). UPLC was operated with a Dionex Acclaim® Polar Advantage II RP-HPLC column using acetonitrile (A) and 0.1% aq. formic acid (B) as eluents: 0.0–0.1 mins, 0.1% A in B (isocratic); 0.1–1.0 mins, 0.1–20% A in B (linear gradient); 1.0–4.0 mins, 20% A in B (isocratic); column wash and stabilization. The mass spectrometer was operated in the negative mode with 0.7 kV capillary voltage, 650 °C desolvation temperature, and 1100 l/h desolvation gas flow. Glyphosate was quantified from 4 control and 20 glyphosate treatment *F. pratensis* samples, and from 4 control and 16 glyphosate treatment weed samples, with a separately prepared quantitation curve of glyphosate.

2.5. AMF root colonization

From each pot two 5–10 cm *F. pratensis* and *E. repens* root segments (when available) were randomly picked and washed under tap water. The root segments were cleared with 10% KOH for 15 min at 90 °C, placed in 1% HCl for 10 min and then stained with 0.05% lactic–glycerol–Trypan Blue for 5 min at 100 °C (Phillips and Hayman, 1970). Total of 10–20 root fragments (ca. 1-cm long) from each plant were mounted on slides in a polyvinyl alcohol–lactic acid–glycerol solution and examined under microscope at 200 × magnification. Root colonization was observed by counting the total colonized roots and the root length containing arbuscules and vesicles (McGonigle et al., 1990).

2.6. Soil analyses

Soil samples were taken from the depth of 5–10 cm from 36 glyphosate (G) and 38 control (C) pots. The soil was air dried, sieved and sent to Natural Resources Institute Finland (Jokioinen) laboratory for pH, Ca, K, Mg and P analyses. Due to unfortunate event in the storage facilities, the soil samples for glyphosate detection were destroyed and thus not available for analyzes.

2.7. Statistical analyses

Linear models were applied to study the factors affecting final biomasses of *F. pratensis* and weeds. The models included first the glyphosate treatment, endophyte status and tilling of soil and their interactions. However, the residuals of the model followed the normal distribution only after soil phosphorus level was included in the model. Furthermore the model for weeds (biomass excluding *F. pratensis*) required a square root transformation and exclusion of a single pot with the highest biomass to follow the assumptions of the model. Proportion of established *F. pratensis* seedlings exposed to each of the treatments (soil properties modulated by long cultivation history of E+ grass, glyphosate treatment and tilling) were studied separately using χ^2 – tests.

The effects of soil properties modulated by long cultivation history of E+ grass, the glyphosate treatment and tilling of soil on mycorrhizal fungal structures of *F. pratensis* and *E. repens* were analyzed using generalized linear models (R 3.3.2 package glm). The number of detected arbuscules, vesicles and hyphae were regarded as Poisson distributed variables and the total number of examined root segments was used as an offset-variable; thus the estimated proportion was the estimated number of colonizations out of the examined root segments. In case the Poisson-model showed statistically significant overdispersion, a Quasi-Poisson distribution was used instead. All interactions between the predictors were first included in the model, but all interactions that were not statistically significant were dropped out. Model assumptions were studied and interactions interpreted using the R packages lsmeans and visreg. For more illustrative presentation, percentage of mycorrhizal colonization is shown in bar graphs with arcsine – transformed *t*-test comparisons.

Linear models were also used to study the effects of the glyphosate treatment, endophyte status and tilling on soil nutrients and pH. Model assumptions were studied and all non-significant interactions and predictors were dropped out.

3. Results

3.1. Weed responses

Glyphosate treatment significantly reduced weed biomass (consisting mainly of *Elymus repens*) in pots that had received glyphosate treatments 10 months earlier compared to control pots (Fig. 1; $t = 2.96$, df = 154.2, p = 0.004). Overall effects of tillage on weed biomass remained insignificant, but tillage affected weed biomass interactively with glyphosate treatment. In no-till treatments, weed biomass was 23% lower in glyphosate treated soils compared to control but the difference was statistically insignificant ($t = 1.15$, df = 77.4, p = 0.255), while in tilled soils the difference was markedly higher becoming statistically significant ($t = 3.27$, df = 72.9, p = 0.002)(Fig. 1). The weeds grown in glyphosate treated soils had 46% lower biomass than the weeds in control soils (Fig. 1). Weed biomass was lower in pots with soil cultivation history of E+ grasses compared to soil cultivation history with E− grasses ($t = 2.49$, df = 146.6, p = 0.014)(Fig. 1), which is in concordance with the earlier study showing that weed species coverage was higher in E− plots compared to E+ plots in the experimental field from where the soil was originally collected (Saikkonen et al., 2013).

3.2. Festuca pratensis establishment and growth

Overall establishment success was 51% in the *Festuca pratensis* seeds sown in the experimental pots eight months after the glyphosate treatment. *F. pratensis* established equally well on glyphosate (G) treatment and control (C) pots regardless of endophyte status of the previous grass cover (E+/E−). However glyphosate affected *F. pratensis* establishment interactively with tillage; plants established better in no-till (NT) (60%) compared to tilled (T) (43%) glyphosate treated soils ($\chi^2 = 11.6$, df = 1, p < 0.001), while in control soils there was no difference (NT: 53%, T: 47%, $\chi^2 = 1.2$, df = 1, p = 0.199).

At the end of the study the glyphosate treatment had different consequences for *F. pratensis* biomass depending on cultivation history of soils (tillage and cultivation history of E+/E− grass). Overall, tilling did not significantly affect the biomass of the *F. pratensis* plants (glyphosate: 3.46 g, control: 3.03 g; $t = 1.26$, df = 137, p = 0.210) although tilling decreased biomass both in control (no-till: 3.30 g, tilled: 2.73 g) and glyphosate (no-till: 4.30 g, tilled: 2.54 g) treatments (Fig. 2). The decrease was, however, statistically significant in glyphosate treated soils (glyphosate treatment: $t = 3.43$, df = 65.4, p = 0.001; control: $t = 1.61$, df = 64.0, p = 0.113) (Fig. 2). In tilled (T) soils the grasses grew equally well in glyphosate treated and control soils while in no-till (NT) and glyphosate treated soils *F. pratensis* biomass was 32% higher compared to control soils (Fig. 2). Similarly to tillage, the cultivation history of soils with either E+ or E− grass affected *F. pratensis* growth interactively with glyphosate treatment (Fig. 2). In glyphosate treated soils *F. pratensis* biomass was not statistically significant (endophyte-free E−: 3.49 g, endophyte symbiotic E+: 3.42 g). In contrast, *F. pratensis* plants produced 41% less biomass in the control soils with cultivation history of E+ grass (E−: 3.62 g, E+: 2.44 g; $t = 3.31$, df = 75.8, p = 0.001)(Fig. 2). Taking into account the cultivation history of soils only, biomass of *F. pratensis* plants was highest in no-till (NT) pots with soil that had cultivation history of E− grass (Fig. 2).

3.3. AMF colonization of grasses

Mycorrhizae were found in 37% and 35% (total mycorrhizal colonization), and arbuscules in 28% and 19% of *F. pratensis* and *E. repens* root segments, respectively. Vesicles were detected in 5% of the root segments in both species examined.

Glyphosate treatment decreased the total mycorrhizal colonization by 23% in *F. pratensis* ($t = 3.03$, df = 34.1, p < 0.0047) and 25% in *E. repens* ($t = 3.33$, df = 66.5, p < 0.0014) (Fig. 3) and reduced the percentage of arbuscules (*F. pratensis* 44%; $t = 6.48$, df = 43.5, p < 0.001 and *E. repens* 27%; $t = 2.81$, df = 69.0, p = 0.006) (Fig. 4) in both species. The effects of glyphosate treatment on vesicles depended on the plant species. Glyphosate did not affect the vesicles in *E. repens*, but increased the proportion of root segments containing vesicles 48% compared to controls in *F. pratensis*.

In the case of *F. pratensis*, the effect of glyphosate on arbuscular colonization varied interactively with tillage and endophyte status (Table 1). To elucidate these interactive effects, we calculated the model-based estimated proportions of root segments with arbuscules

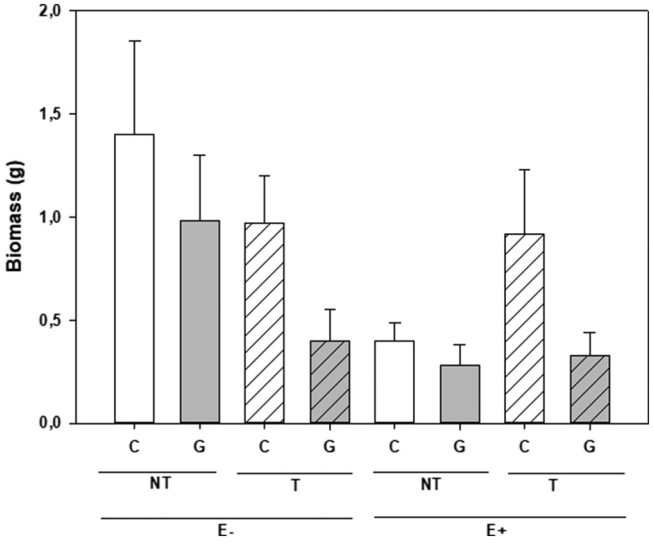

Fig. 1. Weed biomass (mean ± S.E.) in soil that had been exposed to long term grass cover without the endophyte (E−) or with the symbiotic endophyte (E+), in no-tilled (NT) or tilled (T) pots with glyphosate (G) or control (C) treatment. In pairwise comparisons, only NT treated C plants had significantly more biomass compared to G plants (E−: $t = 2.12$, df = 23.0, p = 0.044; E+: $t = 2.79$, df = 9.1, p = 0.021).

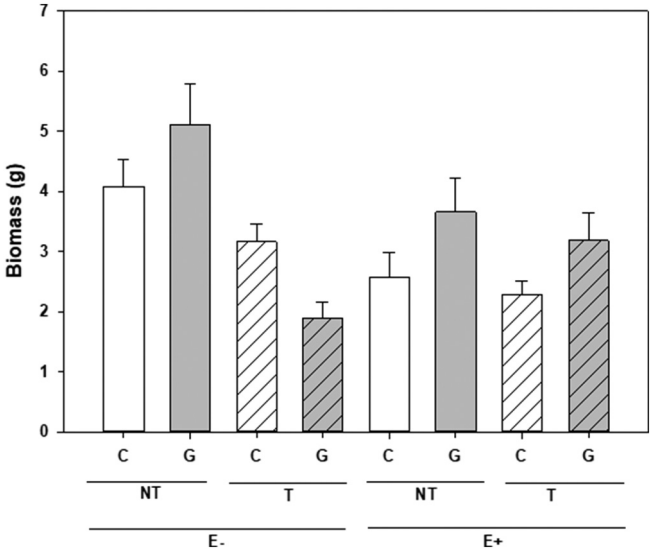

Fig. 2. *Festuca pratensis* biomass (mean ± S.E.) in soil that had been exposed to long term grass cover without the endophyte (E−) or with the symbiotic endophyte (E+), in no-tilled (NT) or tilled (T) pots with glyphosate (G) or control (C) treatment. In pairwise comparisons, only plants in E− & T treatment had significantly more biomass ($t = 3.16$, df = 0.003, p = 37.8) compared to glyphosate-treated plants.

for the three-way interaction (G × T × E+). Glyphosate decreased the proportions of root segments with arbuscules from 41% (C) to 21% (G) in plants grown in no-tilled soils (NT) with the cultivation history of E− grass, from 36% to 16% in plants grown in tilled soils (T) with history of endophyte symbiotic grasses (E+) and from 33% to 21% in plants grown in no-till (NT) soils with history of endophyte symbiotic grasses (E+). In contrast, glyphosate decreased arbuscules only from 23% to 20% in tilled soils (T) with history of endophyte free (E−) grass.

In *E. repens*, soil tillage or long-term grass cover history with endophyte symbiotic grass was not affecting total mycorrhizal colonization or fraction of root length containing arbuscules and vesicles.

3.4. Glyphosate residues in plants

At the end of the study, we detected glyphosate residues of 0.24 ± 0.45 mg/kg DW (dry weight)(mean ± S.D.) in *F. pratensis* plants, which were sown to pots (n = 20) eight months after the glyphosate treatment. The *F. pratensis* plants growing in the control pots (n = 4) did not contain detectable amounts of glyphosate. Grasses growing in glyphosate treated no-till (NT) soils had higher residues (0.32 ± 0.63 mg/kg DW; n = 10) than grasses in glyphosate treated tilled (T) soils (0.24 ± 0.45 mg/kg DW; n = 10).

The weeds growing in glyphosate treated soils (n = 16) had 2.31 ± 3.24 mg/kg DW (mean ± S.D.) of glyphosate in contrast to 0.03 ± 0.03 mg/kg DW (mean ± S.D.) in weeds grown in control soils. Similarly to *F. pratensis*, weeds growing in no-till (NT) soils had higher glyphosate residues (2.73 ± 3.92 mg/kg DW; n = 8) than weeds in tilled (T) soils (1.89 ± 2.60 mg/kg DW; n = 10).

3.5. Soil chemistry

Measured soil chemicals were not statistically significantly affected by glyphosate (G/C) or tilling (T/NT) treatments. The soil pH was 6.2 ± 0.2 (mean ± S.D.), and Ca, K, Mg and P contents were 3112 ± 381 mg/l, 547 ± 105 mg/l, 538 ± 116 mg/l, and 17 ± 5.5 mg/l, respectively. The soil pH correlated with contents of several nutrients. Soil pH was positively correlated with Mg and Ca contents (Mg: $t = 2.2$, df = 54.4, p = 0.032, Ca: $t = 2.6$, df = 63.9, p = 0.010; pH: $t = 1.9$, df = 69.6, p = 0.068) and negatively with K contents in soils that had a long-term cultivation history of E+ grass.

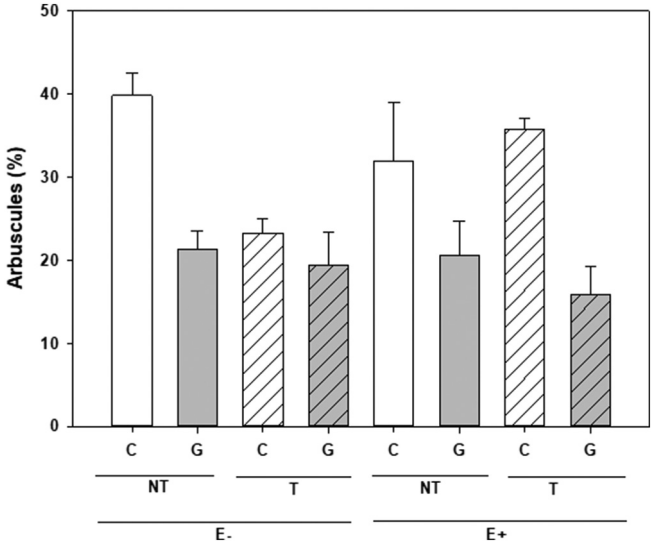

Fig. 3. *Festuca pratensis* mycorrhizal arbuscules (mean ± S.E.) in plants growing in soil that had been exposed to long term grass cover without the endophyte (E−) or with the symbiotic endophyte (E+), in no-tilled (NT) or tilled (T) pots with glyphosate (G) or control (C) treatment. In pairwise comparisons, C plants had significantly more arbuscules compared to G plants in combinations E− & NT ($t = 5.22$, df = 10.4, p = 0.0003) and E+ & T ($t = 5.64$, df = 5.4, p = 0.002). See text and Table 1 for more detailed models.

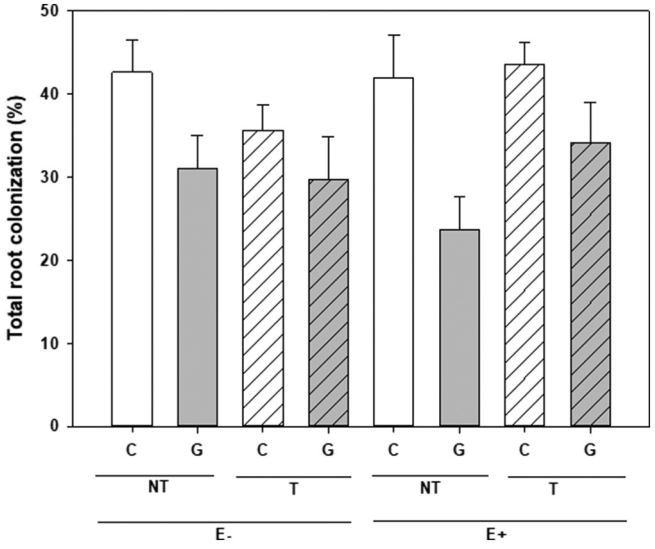

Fig. 4. *Elymus repens* total mycorrhizal colonization (mean ± S.E.) in plants growing in soil that had been exposed to long term grass cover without the endophyte (E−) or with the symbiotic endophyte (E+), in no-tilled (NT) or tilled (T) pots with glyphosate (G) or control (C) treatment. In pairwise comparisons, only weeds in NT control treatments had significantly more colonization in C plants compared to G plants (E−: $t = 2.12$, df = 23.0, p = 0.044; E+: $t = 2.79$, df = 9.1, p = 0.021) in NT treatment.

4. Discussion

Our results demonstrate that glyphosate use can have indirect long term consequences on non-target plants and microbes in the environments where the degradation of the glyphosate is retarded due to seasonally cold climatic conditions. As predicted, glyphosate applied on plant canopy and tillage efficiently eradicated weeds mostly comprising of perennial species such as *Elymus repens*. Against manufacturer's directions claiming that glyphosate degrades within few weeks after application (Borggaard and Gimsing, 2008; Giesy et al., 2000), we detected glyphosate residues in plants in the subsequent growing season as well as reduced AMF associated with grasses. In our model forage grass *Festuca pratensis* these negative effects depended, however, on mechanical and biological cultivation history of soils suggesting that abiotic and biotic environmental factors may alone or interactively have impact on how glyphosate affects the non-target plants and their associated microbes.

Glyphosate and mechanical tilling are both effective methods for weed control, and together they reduced the weed biomass more than either of them alone (Fig. 1). Establishment success of the non-target *F. pratensis* seeds was not affected six months after the glyphosate

Table 1
Effect of glyphosate (G) or control (C) treatment on arbuscular colonization of *Festuca pratensis* in tilled (T) or no-tilled (NT) soils with a history of endophyte symbiotic (E+) or endophyte free (E−) grasses.

	Estimate	p
Intercept	−0.894	
Glyphosate (G)	−1.600	0.001***
Endophyte (E+)	−1.482	0.233
Tilled (T)	−1.099	0.001***
G × T	−1.830	0.011*
G × E+	−1.027	0.410
T × E+	−1.582	0.003**
G × T × E+	−1.578	0.009**
AIC: 295.75		
Residual deviance 56.7, df = 41		

treatment while tilling reduced the grass seed establishment by 10%. Presumably lower establishment rate also led to lower biomass production of *F. pratensis* in tilled pots. The examined biotic environmental factors, i.e. long-term cultivation history of either endophyte symbiotic or endophyte-free grass were not affecting plant establishment.

However, biotic environmental factors clearly had consequences on plant biomass. The long-term cultivation history of endophyte symbiotic grass (E+) of the field from where the experimental soil was collected, reduced the biomass of weeds and the seeded forage grass via plant-soil feedback when the soil was mechanically or chemically untreated. The lower biomass of weeds could partly be explained by lower number of weeds due to competitive superiority of E+ grass hindering weed invasions (Saikkonen et al., 2013). However, the lower biomass of our experimental forage grass, endophyte-free *F. pratensis*, suggests that long-term cultivation history of E+ grass has modulated soil properties unfavorable for its conspecific either via plant-soil feedbacks or allelopathic effects modulated by grass-symbiotic *Epichloë* species (García-Parisi et al., 2017; Saikkonen et al., 2015) and/or cultivation practices.

In this study, we were primarily interested in possible long-term consequences of glyphosate use on mycorrhizal colonization in plants. We detected that glyphosate application significantly reduced total mycorrhizal colonization and specifically arbuscules in roots of both target and non-target grasses (Figs. 3 and 4). The reduction of arbuscules results in a loss of functionality in the symbiosis because nutrients between the plant and the fungus are exchanged in the arbuscules (Smith and Gianinazzi-Pearson, 1988). Glyphosate was applied on targeted weed plants in fall. Therefore glyphosate could have directly affected mycorrhizal inoculum (spores and external hyphae) in the soil, and thus lowered mycorrhizal colonization of the roots (Druille et al., 2013a, 2013b). In addition, the availability of carbohydrates to support the mycorrhizal partner within the roots can be limited in the glyphosate weakened grass (Druille et al., 2013b). In contrast to weeds, the non-target *F. pratensis* grasses were sown to the glyphosate treated and control soils six months after the glyphosate applications in the beginning of the following growing season. Thus, the detected low AMF colonization in *F. pratensis* plants grown in glyphosate treated soil indicates decreased availability of inocula, altered microbial interactions in soils or weakened grass performance due to glyphosate residues in soils. The plants grew equally well on glyphosate treated and control soils, however, suggesting that glyphosate residues in soils alter microbial community or alter microbial activity rather than constrain resource allocation to mycorrhizal partner in grasses.

We detected three-way interaction among glyphosate, tillage and cultivation history of either E− or E+ plants on AMF. Contrary to previous studies suggesting that tillage (Kabir et al., 1997; Schalamuk and Cabello, 2010) and cultivation history of E+ grass (García-Parisi et al., 2017) increase mycorrhizal susceptibility to glyphosate, in this study we detected comparable proportions of root segments with arbuscules in all glyphosate treated plants. Instead, mycorrhizal colonization was equally low in plants grown in tilled control soil with long-term cultivation history of E− grass compared to glyphosate treated soils. Because of the relatively modest number of replicates the importance of these abiotic and biotic environmental interactions to AMF remains to be solved in future studies.

Our study supports the previous findings that the risks of glyphosate use on non-target organisms can be higher than presumed because glyphosate and its degradation products can retain in soils and later accumulate in non-target plants (Helander et al., 2012; Laitinen et al., 2006). Recent studies have also suggested that glyphosate sorption and degradation in soils is highly variable depending on soil properties, climate and weather conditions (Aparicio et al., 2013; Helander et al., 2012; Laitinen et al., 2006). Our present study clearly demonstrates that in environments with seasonally cold climate with snow cover during the winter, glyphosate residues can be detected in perennial target plants and non-target plants in the growing season following the spraying. In the case of examined perennial weeds, glyphosate residues in above-ground plant parts can be explained by transportation of remains from the underground parts of the perennial weeds survived and overwintered in the soil to the re-growing shoots in the spring. However, glyphosate residues in non-target *F. pratensis* plants seeded in the spring demonstrate that glyphosate can be transported from soils to new emerging seed-borne plants. The ten-fold higher glyphosate remains detected in the perennial weeds compared to non-target *F. pratensis* plants is a likely consequence of direct exposure of them to glyphosate treatment and six months' longer time window to absorb glyphosate residues from the soils.

Furthermore, our results emphasize the importance of cultivation practices to glyphosate accumulation in soils and transportation into plants. We detected higher glyphosate residues in the weeds and non-target *F. pratensis* grasses growing in no-till soils compared to conspecifics in tilled soils. In the field scale this might translate into higher risks of accumulating glyphosate residues in no-till agricultural practices, in which the soils are regularly sprayed with glyphosate for weed control instead of or complementary to mechanical tillage. On the other hand, tillage increases the risk of glyphosate leaching from agricultural field to surrounding non-target ecosystems. Other consequences of tillage which should be taken into account in future studies include modulated chemical composition and microbial communities of the soils. For example, amount of phosphorus in soils and thus the free binding sites for glyphosate (Borggaard and Gimsing, 2008) may differ in till and no-till top-soil, and changes in microbial soil community due to glyphosate application may affect the degrading of glyphosate (Schafer et al., 2014).

5. Conclusion

Our results demonstrate that the effects of chemical and mechanical agricultural practices on ecosystem services can exceed the year of application. Here we showed that glyphosate negatively affects plant beneficial AMF in target weeds and non-target forage grasses. These results call attention to more comprehensive understanding of the effects of glyphosate on other plant associated microbes and microbial communities in soils. For example, soil microbes are known to play an important role in glyphosate degradation (Ayansina and Oso, 2006), and thus changes in their frequencies and community composition may affect the amount of available glyphosate for crop plants and weeds in the soil. Beside biotic factors also the abiotic environment is important in determining actions of the glyphosate in the soil. Depending on the initial soil microbiota, glyphosate may differently modify the outcome of microbial composition for glyphosate degradation and for the weeds and crop plants. Specifically in non-target *F. pratensis* AMF colonization was decreased interactively with biotic factors including the symbiotic fungus of the previous field cover plant, and abiotic factors including cultivation techniques. The core of a matter in maintaining fertile soils for sustainable crop production is to provide favorable environment for the soil and plant beneficial microbes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.377.

References

Alvarez, R., Steinbach, H.S., 2009. A review of the effects of tillage systems on some soil physical properties, water content, nitrate availability and crops yield in the Argentine Pampas. Soil Tillage Res. 104, 1–15.

Aparicio, V.C., De Gerónimo, E., Marino, D., Primost, J., Carriquiriborde, P., Costa, J.L., 2013. Environmental fate of glyphosate and amminomethylphosphonic acid in surface waters and soil of agricultural basins. Chemosphere 93, 1866–1873.

Ayansina, A.D.V., Oso, B.A., 2006. Effect of two commonly used herbicides on soil microflora at two different concentrations. Afr. J. Biotechnol. 5, 129–132.

Bai, S.H., Ogbourne, S.M., 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ. Sci. Pollut. Res. 23, 18988–19001.

Bastías, D.A., Martínez-Ghersa, M.A., Newman, J.A., Card, S.D., Mace, W.J., Gundel, P.E., 2018. The plant hormone salicylic acid interacts with the mechanism of anti-herbivory conferred by fungal endophytes in grasses. Plant Cell Environ. 41, 395–405.

Bauer, J.T., Mack, K.M.L., Bever, J.D., 2015. Plant-soil feedbacks as drivers of succession: evidence from remnant and restored tallgrass prairies. Ecosphere 6, 1–12.

Benbrook, C., 2016. Trends in glyphosate herbicide use in the Unites States and globally. Environ. Sci. Eur. 28 (3). https://doi.org/10.1186/s12302-016-0070-0.

Borggaard, O.K., Gimsing, A.L., 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64, 441–456.

Carlisle, S.M., Trevors, J.T., 1988. Glyphosate in the environment. Water Air Soil Pollut. 39, 409–420.

Carvalho, F.P., 2016. Agriculture, pesticides, food security and food safety. Environ. Sci. Pol. 9, 685–692.

Cherni, A.E., Trabelsi, D., Chebil, S., Barhoumi, F., Rodriques-Llorente, I.D., Zribi, K., 2015. Effect of glyphosate on enzymatic activities, Rhizobiaceae and total bacterial communities in an agricultural Tunisian soil. Water Air Soil Pollut. 226, 2263.

Doran, J.W., 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am. J. 44, 765–771.

Druille, M., Cabello, M., Omacini, M., Golluscio, R.A., 2013a. Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. Appl. Soil Ecol. 64, 99–103.

Druille, M., Omacini, M., Golluscio, R.A., Cabello, M.N., 2013b. Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. Appl. Soil Ecol. 72, 143–149.

Duke, S.O., Powles, S.B., 2008. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64, 319–325.

Folmar, L.C., Sanders, J.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol. 8, 269–278.

García-Parisi, P.A., Omacini, M., 2017. Arbuscular mycorrhizal fungi can shift plant-soil feedback of grass-endophyte symbiosis from negative to positive. Plant Soil 419, 13–23.

García-Parisi, P.A., Lattanzi, F.A., Grimoldi, A.A., Druille, M., Omacini, M., 2017. Three symbionts involved in interspecific plant-soil feedback: epichloid endophytes and mycorrhizal fungi affect the performance of rhizobia-legume symbiosis. Plant Soil 412, 151–162.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup® herbicide. Rev. Environ. Contam. Toxicol. 167, 35–120.

Gimsing, A.L., dos Santos, A.M., 2005. Glyphosate. In: Nowack, B., Van Briesen, J.M. (Eds.), Biogeochemistry of Chelating Agents. ACS Symposium Series. vol. 910. American Chemical Society, Washington, DC, USA, pp. 263–277 ISBN 978-0841238978.

Gomes, M.P., Le, Manac'h S.G., Maccario, S., Labrecque, M., Lucotte, M., Juneau, P., 2016. Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants. Pestic. Biochem. Physiol. 130, 65.

Gundel, P.E., Helander, M., Garibaldi, L.A., Vazquez De Aldana, B.R., Zabalgogeazcoa, I., Saikkonen, K., 2017. Direct and indirect effects of the fungal endophyte *Epichloë uncinatum* on litter decomposition of the host grass, *Schedonorus pratensis*. Plant Ecol. 218, 1107–1115.

Hamilton, C., Gundel, P.E., Helander, M., Saikkonen, K., 2012. Endophytic mediation of reactive oxygen species and antioxidant activity in plants: a review. Fungal Divers. 54, 1–10.

Helander, M., Saloniemi, I., Saikkonen, K., 2012. Glyphosate in northern ecosystems. Trends Plant Sci. 17, 569–574.

Herman, K.M., Weaver, L.M., 1999. The shikimate pathway. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 473–503.

Huitu, O., Forbes, K.M., Helander, M., Julkunen-Tiitto, R., Lambin, X., Saikkonen, K., Stuart, P., Sulkama, S., Hartley, S., 2014. Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores. Front. Plant Sci. 5 (478). https://doi.org/10.3389/fpls.2014.00478.

Imfeld, G., Vuilleumier, S., 2012. Measuring the effects of pesticides on bacterial communities in soil: a critical review. Eur. J. Soil Biol. 49, 22–30.

Kabir, Z., O'Halloran, I.P., Fyles, J.W., Hamel, C., 1997. Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization: hyphal density and mycorrhizal root colonization. Plant Soil 192, 285–293.

Klironomos, J.N., 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature 417, 67–70.

Krzysko-Lupicka, T., Sudol, T., 2008. Interactions between glyphosate and autochthonous soil fungi surviving in aqueous solution of glyphosate. Chemosphere 71, 1386–1391.

Kulmatiski, A., Beard, K.H., Stevens, J.R., Cobbold, S.M., 2008. Plant–soil feedbacks: a meta-analytical review. Ecol. Lett. 11, 980–992.

Laitinen, P., Siimes, K., Eronen, L., Rämö, S., Welling, L., Oinonen, S., Mattsoff, L., Ruohonen-Lehto, M., 2006. Fate of the herbicide glyphosate, glufosinate-ammonium, phenmedipham, ethofumesate and metamitron in two Finnish arable soils. Pest Manag. Sci. 62, 473–491.

Laitinen, P., Siimes, K., Rämö, S., Jauhiainen, L., Eronen, L., Oinonen, S., Hartikainen, H., 2008. Effects of soil phosphorous status on environmental risk assessment of glyphosate and glufosinate-ammonium. J. Environ. Qual. 37, 830–838.

Laitinen, P., Rämö, S., Nikunen, U., Jauhiainen, L., Siimes, K., Turtola, E., 2009. Glyphosate and phosphorous leaching and residues in boreal sandy soil. Plant Soil 323, 267–283.

Ludvigsen, G.H., Lode, O., 2001. Results from the agricultural and environmental monitoring program of pesticides in Norway 1995–1999. Fresenius Environ. Bull. 10, 470–474.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J.A., 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115, 495–501.

Mikola, J., Helander, M., Saikkonen, K., 2016. No effects of *Epichloë* endophyte infection on nitrogen cycling in meadow fescue (*Scherodonus pratensis*) grassland. Plant Soil 405: 257–264. https://doi.org/10.1007/s11103-015-2711-2.

Myers, J.P., Antoniou, M.N., Blumberg, B., Caroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vanderberg, L.N., Vom Saal, F.S., Wv, Welshons, Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ. Health 15 (19). https://doi.org/10.1186/s12940-016-0117-0.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 158–161.

Primost, J.E., Marino, D.J.G., Aparicio, V.C., Costa, J.L., Carriquiriborde, P., 2017. Glyphosate and AMPA, "pseudo-persistent" pollutants under real-world agricultural management practices in the Mesopotamic Pampas agroecosystem, Argentina. Environ. Pollut. 229, 771–779.

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: a continuum of interactions with host plants. Annu. Rev. Ecol. Syst. 29, 319–343.

Saikkonen, K., Wäli, P., Helander, M., Faeth, S.H., 2004. Evolution of endophyte-plant symbioses. Trends Plant Sci. 9, 275–280.

Saikkonen, K., Ruokolainen, K., Huitu, O., Gundel, P.E., Piltti, T., Hamilton, C.E., Helander, M., 2013. Fungal endophytes help prevent weed invasions. Agric. Ecosyst. Environ. 165, 1–5.

Saikkonen, K., Mikola, J., Helander, M., 2015. Endophytic phyllospere fungi and nutrient cycling in terrestrial ecosystems. Curr. Sci. 109, 121–126.

Saikkonen, K., Young, C.A., Helander, M., Schardl, C.L., 2016. Endophytic *Epichloë* species and their grass hosts: from evolution to applications. Plant Mol. Biol. 90:665–675. https://doi.org/10.1007/s11103-015-0399-6.

Sanders, I.R., Fitter, A.H., 1992. Evidence for differential responses between host-fungus combinations of vesicular–arbuscular mycorrhizas from a grassland. Mycol. Res. 96, 415–419.

Schafer, J.R., Hallett, S.G., Johnson, W.G., 2014. Rhizosphere microbial community dynamics in glyphosate-treated susceptible and resistant biotypes of giant ragweed (*Ambrosia trifida*). Weed Sci. 62, 370–381.

Schalamuk, S., Cabello, M., 2010. Effect of tillage systems on the arbuscular mycorrhizal fungi propagule bank in soils. In: Arya, A., Perelló, A.E. (Eds.), Management of Fungal Plant Pathogens. CAB International, pp. 162–170.

Smith, S.E., Gianinazzi-Pearson, V., 1988. Physiological interactions between symbionts in vesicular–arbuscular mycorrhizal plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 221–244.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis. 3rd ed. Academic Press, London.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677.

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.

Vereecken, H., 2005. Mobility and leaching of the glyphosate: a review. Pest Manag. Sci. 61, 1139–1151.

Watrud, L.S., King, G., Londo, J.P., Colasanti, R., Smith, B.M., Waschmann, R.S., Lee, E.H., 2011. Changes in constructed *Brassica* communities treated with glyphosate drift. Ecol. Appl. 21, 525–538.

Woodburn, A.T., 2000. Glyphosate: production, pricing and use. Pest Manag. Sci. 56, 309–312.