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Tillage, Glyphosate and Beneficial Arbuscular Mycorrhizal Fungi: Optimising Crop Management for Plant–Fungal Symbiosis

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Abstract: Zero till cropping systems typically apply broad-spectrum herbicides such as glyphosate as an alternative weed control strategy to the physical inversion of the soil provided by cultivation. Glyphosate targets 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in plants. There is growing evidence that this may have a detrimental impact on non-target organisms such as those present in the soil microbiome. Species of commercial importance, such as arbuscular mycorrhizal (AM) fungi that form a symbiotic relationship with plant roots are an important example. This study investigates the impact of soil cultivation and glyphosate application associated with conventional tillage (CT) and zero tillage (ZT) respectively on AM fungi populations under field and glasshouse conditions. Topsoil (<10 cm) was extracted from CT and ZT fields cropped with winter wheat, plus non-cropped control plots within the same field boundary, throughout the cropping year. Glyphosate was applied in glasshouse experiments at rates between 0 and 350 g L^{-1}. Ergosterol, an indicator of fungal biomass, was measured using high performance liquid chromatography before and after glyphosate application. Fungal root arbuscules, an indicator of AM fungi–root symbiosis, were quantified from the roots of wheat plants. Under glasshouse conditions root arbuscules were consistently higher in wheat grown in ZT field extracted soils ($P = 0.01$) compared to CT. Glyphosate application however inhibited fungal biomass in both the ZT ($P < 0.00001$) and CT ($P < 0.001$) treatments. In the absence of glyphosate, the number of stained root arbuscules increased significantly. Ergosterol levels, used as a proxy for fungal biomass, remained lower in the soil post glyphosate application. The results suggest that CT has a greater negative impact on AM fungal growth than ZT and glyphosate, but that glyphosate is also detrimental to AM fungal growth and hinders subsequent population recovery.

Keywords: arbuscular mycorrhizal fungi; ergosterol; glyphosate; symbiosis; tillage; root arbuscule

1. Introduction

Agrochemicals such as herbicides, fungicides, and pesticides have been used within agricultural systems for several decades with the aim to improve crop yield and sustain an ever-growing global population. Many existing studies of agrochemicals focus on human health [1,2] and higher level taxa such as arthropods, mammals, birds, and fish [3]. While such impacts are important, consideration of the soil microbiome, a critical component of the agro-ecosystem trophic complex, has been largely overlooked.

The broad-spectrum herbicide glyphosate is favoured as a weed control measure in zero tillage (ZT) cropping systems. Such systems do not have the capacity to implement weed control via soil inversion as is the case in conventional tillage (CT). Glyphosate (phosphomethyl glycine) inhibits the enzyme 5-endolpyruvylshikimate-3-phosphate (EPSP) synthase within growing plants. The enzyme is a component of the process that synthesises the amino acids tryptophan, phenylalanine, and tyrosine via the Shikimate pathway. These amino acids have critical roles in plant auxin and flavonoid synthesis [4] while tyrosine derived metabolites are critical for plant survival [5]. Critically, both the Shikimate pathway and EPSP synthase are found in most fungi and bacteria in addition to plants [1,6]. The impact of glyphosate application on the soil microbiome requires evaluation.

A limited number of recent studies investigate the impact of glyphosate on non-target plants, the soil microbiome and other microorganisms associated with fauna and flora [1,7], but present contradictory findings to earlier studies. For example, Giesy et al. [8] conclude that glyphosate had a negligible impact on the soil microbial community due to rapid microbial metabolism coupled with immobilisation through absorption onto soil particles. This has been supported by studies since that evaluate the half-life of glyphosate but is contradicted somewhat by the analysis of glyphosate behaviour in soil [9–12]. The application of phosphate fertilisers, a widespread management technique in conventional agriculture, induce the mobilisation of glyphosate adsorbed onto soil particles due to competition for adsorption sites [13]. This increases the duration of glyphosate persistence in soil extending the period when the impact on non-target organisms (NTOs) may be realised. Aminomethylphosphonic acid (AMPA), a metabolite of glyphosate, persists within the environment for longer [14], shown in Figure 1, and has superior mobility within soils [7]. Importantly, Giesy et al. [8] in an extension of their study of glyphosate ecotoxicity and NTOs to AMPA found that AMPA is equally as toxic to NTOs as glyphosate. Evidently the greater persistence, mobility and comparable ecotoxicity of AMPA is of greater concern to bacteria and fungi that share the Shikimate pathway and EPSP synthase [15], both of which are targeted by glyphosate when applied as a mode of weed control.

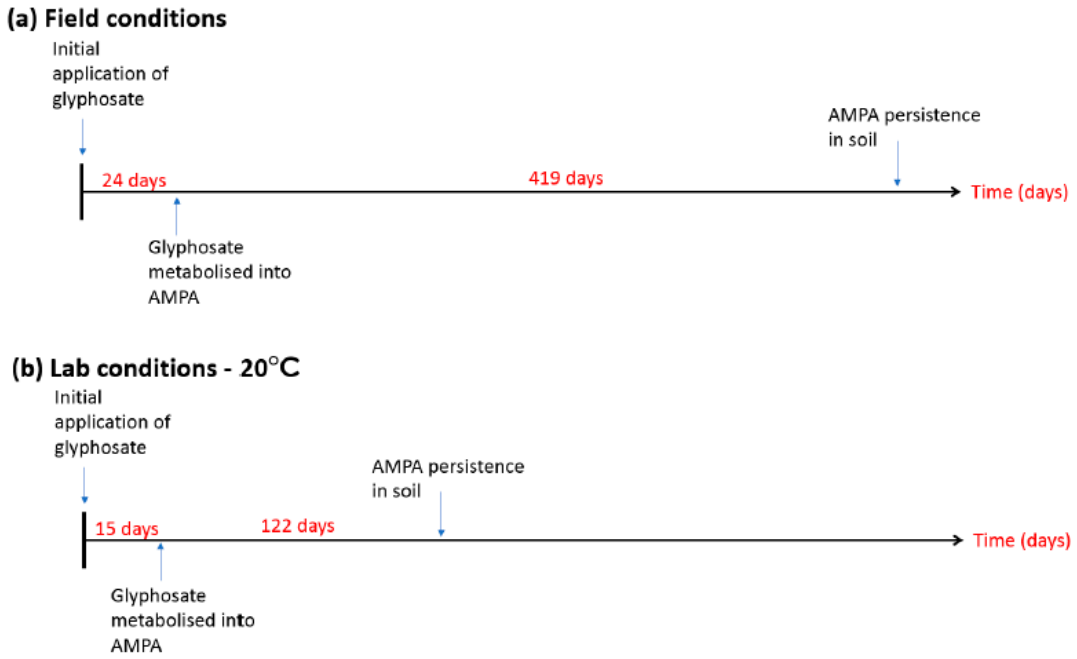

Figure 1. Timeline of glyphosate metabolism and Aminomethylphosphonic acid (AMPA) persistence in soils from both field soils (**a**) and laboratory controlled soils (**b**), constructed from data provided by the Pesticide Properties DataBase (PPDB) [3].

Arbuscular mycorrhizal (AM) fungi are obligate biotrophic symbionts that form mutualistic relationships with a host plant via root systems [16]. While obligate biotrophs tend to be plant

pathogens (fungi that extract nutrients from living plant cells), the symbiotic component of AM fungi renders the relationship beneficial to both plant and fungi. Soil nutrients are assimilated by AM fungi and transferred to the host plant. Photosynthesis derived carbohydrates are provided by the host plant to the AM fungi in exchange. Furthermore, AM fungal established symbiosis benefits the soil structure through the production of glomalin, an adhesive glycoprotein stabilising microaggregates [17], and increased biomass of mycelia that provide further soil stability to macroaggregates. In the absence of a host, AM fungal mycelia have been shown to reduce in abundance [16]. The spores of AM fungi, however, can remain in soils for long periods, up to 90 days [18], without losing viability. Where soils are managed appropriately, this reservoir of AM fungal spores may be harnessed to benefit crop growth and improve the resilience of crop production systems to factors such as increased drought risk and salt stress. Plant roots are known to be influential in shaping their own microbiome and rhizosphere, as well as having a substantial influence on the chemical and physical attributes of soils at greater depths within the soil profile [19]. Plant microbiome and rhizosphere development is facilitated by root produced chemical signals (hormones and organic acid root exudates) released by the plant into the root space that encourage the migration and development of soil microbes within the vicinity of the host plant root system. When glyphosate is applied for the control of weeds, molecules of active substance may potentially be deposited within this same rhizosphere space via translocation through the plant [12,20].

The effect of glyphosate on AM fungi has significant implications toward the maintenance of a functional soil microbiome within the plant rhizosphere but literature on the topic remains sparse. The present analysis aims to evaluate, under field and controlled glasshouse conditions, the effect of tillage and glyphosate on AM fungal symbiosis through the quantification of intracellular fungal root structures and ergosterol as a measure of fungal biomass. A comparison of CT and ZT soil management techniques will be made.

2. Materials and Methods

2.1. Sample Sites

The study site consisted of two commercial farms in Hertfordshire, UK: Farm A (near Hitchin, nearest climate station Rothamsted) mean annual rainfall 712.3 mm, mean minimum and maximum annual temperature 6.0–13.7 °C; Farm B (Hatfield, nearest climate station Rothamsted) mean annual rainfall 712.3 mm, mean minimum and maximum annual temperature 6.0–13.7 °C)[21]. Both crops were managed for commercial purposes and a realistic representation of current arable crop and soil management protocols. Further, this management had been undertaken in the longer-term, a period of over eight years at the time of sampling. Farm A implemented CT, soil inversion with a mouldboard plough to a depth of 20 cm. Farm B practiced ZT by direct seed sowing (John Deere® (Langar, Nottinghamshire, UK) 750A direct drill). Glyphosate (360 g L^{-1} active ingredient, 3.0 L ha^{-1}, CleanCrop Hoedown® (Agrovista UK Ltd., Nottingham, UK) was applied as a weed control measure to the ZT crop during seed-bed preparation in September. Soil cores (10 cm deep) were extracted in a 50 m^2 grid formation across each field, with locations determined by handheld GPS (Garmin® eTrex10, Garmin (Europe) Ltd., Southampton, UK). The soil texture, determined by the methodology described by Brown and Wherrett (2015) [22], was sandy loam in all sample locations on both farms. Samples were taken at four identical times to represent an entire cropping year at each farm: in the autumn (September) immediately one week before seedbed preparation, post seedbed preparation in late winter (February) when the crop was tillering (GS22 [23]), late spring (May) at the beginning of ear formation (GS39 [23], with the fourth and final set of samples taken immediately post-harvest during the summer in August. Fields were selected based on farm records provided by participating land managers. Fields were identified where the recent crop history, rate, and type of supplementary crop nutrients were identical for each, with both farms following RB209 [24] recommendations for winter wheat on a sandy loam soil. Further soil samples were taken from a species poor grass margin immediately adjacent to the crop

perimeter at each farm to provide a non-cropped control. The control eliminated all crop production operations. The elimination of individual crop management methods, e.g., glyphosate application were conducted in a set of glasshouse experiments described in Section 2.3.

2.2. Experimental Design

Twenty winter wheat plants (Zulu variety) were sampled from each farm field site during February 2019 (GS22 [23]), the root systems maintained and subject to subsequent staining (Section 2.6) to identify AM fungal wheat root arbuscules. A further 20 wheat plants (GS39 [23]) were sampled in May 2019. For a second experiment (Section 2.3) a total of 20 kg (10 kg per farm) of topsoil (<10 cm from the soil surface) was extracted from the centre of each field study site on the two farms during the post-harvest sampling phase in August 2019. This sampling period equated to a timeframe of 12 months after initial seedbed preparation via either mouldboard plough in the CT soils or the application of glyphosate to the ZT soils. For each sample site the sampled field soil was subdivided into 100 g portions and added to 10 circular plant pots (6 cm radius × 9 cm depth), 20 pots in total, 10 replicates per treatment. A single winter wheat seed of Zulu variety was planted 2 cm below the soil surface in each pot, watered twice a week with 200 mL and kept in controlled glasshouse conditions at 25 °C and 37% relative humidity for a total of 30 weeks. To compensate for variables associated with potential soil nutrient depletion, urea (CH_4N_2O), potassium chloride (KCl), and sodium hydrogen phosphate (Na_2HPO_4) were applied to the soil surface at 560 μg cm^{-2} five weeks after initial seed planting. This quantity of fertiliser equates to the recommended field fertiliser rates used at both study sites.

2.3. Glyphosate Applications on Fungal Biomass

Extracted field soil in the absence of a wheat plant was sub divided into 10 units of 50 g and replicated in triplicate for each tillage treatment (n = 60 total). For each 50 g replicate 1 g of soil was removed and analysed for the quantity of ergosterol measured via high performance liquid chromatography (HPLC) analysis (Section 2.4) and a standard curve generated. Glyphosate was applied in a 1 mL dose to the surface of the remaining soil at rates equivalent of between 0 and 350 g L^{-1}. The soil was sampled at three day intervals and the HPLC procedure repeated.

2.4. Ergosterol HPLC

Soil ergosterol levels (Tillage experiment: n = 100 per treatment ZT and ZTC, n = 200 total; Glyphosate application experiment: 10 glyphosate concentrations of three replicates each, five sample days, two tillage treatments n = 300 total) were determined using a modified methodology originally developed by Millie-Lindblom and Tranvij [25]. A 1 g sub-sample of each field sampled soil was freeze dried using a ChechaTech System® (MechaTech®, Bristol, UK) LSB40 freeze drier chamber, Edwards® RV5 vacuum pump (Thermo Fisher Scientific®, Loughborough, Leicestershire, UK) and MicroModulyo® (Thermo Fisher Scientific®, Loughborough, Leicestershire, UK) freeze drier. The duration of each cycle was 21 h. Of the freezer dried soil, 150 mg was weighed into 50 mL centrifuge tubes. Potassium hydroxide was added to HPLC grade methanol (Thermo Fisher Scientific®, Loughborough, Leicestershire, UK) until 10% w/v was achieved. To each centrifuge tube, 4 mL of KOH in methanol and 1 mL cyclohexane was added and sonicated in an ultrasonic water bath for 15 min before incubation at 70 °C for a maximum of 2 h. Samples were cooled to room temperature and 1 mL of Milli-Q water was added with a further 4 mL of cyclohexane, vortexed at maximum speed for 60 s then centrifuged at 1000× g for 60 s. The cyclohexane fraction was transferred to a clean test tube and all cyclohexane evaporated, before 1 mL of HPLC grade methanol added and each tube incubated at 40 °C for 15 min then filtered through 0.2 μm nylon membrane syringe filters (Chromatography Direct®, Runcorn, Cheshire, UK) into HPLC vials and running through the chromatographic system. The HPLC ran using a H5C18-25QS (4.6 × 250 mm Interchrim®, Montluçon Cedex, France) column with guard column (Phenomenex® (Macclesfield, Cheshire, UK) KJ0-4282 SecurityGuard analytical guard cartridge system, fitted with an AJ0-7510 cartridge). The effluent analysed comprised of 100%

HPLC grade methanol (Thermo Fisher Scientific®, Loughborough, Leicestershire, UK) at a flowrate of 1 mL min^{-1} for 15 min, with an injection volume of 10 µL. Ultraviolet (UV) detection was set at a wavelength of 282 nm. Ergosterol produced a peak at a retention time of 8.1 min and standards were run at known concentrations (10 µg mL^{-1} to 1000 µg mL^{-1}) to allow the construction of a standard curve for soil ergosterol quantification.

2.5. Determination of Fungal Biomass

Fungal biomass was determined from measured ergosterol concentration using Equation (1) [26]:

$$\begin{gathered}\text{Fungal biomass (FB) (µg/g)} = \text{Ergosterol (µg/g)} \times \text{f} \times \text{Rf} \\ \text{Where } f = 250, \text{ and Rf is the recovery factor (1.61).}\end{gathered} \quad (1)$$

2.6. Root Staining

Root staining was performed following the protocol developed by Wilkes et al. [27] to identify wheat root AM fungal arbuscules. Root samples (n = 20 for CT and ZT, two sampling periods, n = 80 total) were fixed in a FAA (10% Formaldehyde, 50% Alcohol, 5% Acetic acid *v/v*) solution for 24 h, autoclaved in deionised water and incubated at 60°C in 5% *v/v* hydrochloric acid for 1 h. Roots were sectioned into 5 × 1 cm sections and stained with 10% *v/v* Sheaffer® Blue ink (Providence, RI, USA) in 5% glacial acetic acid for 3 min before subject to root squash and viewed under a light microscope at ×100 magnification.

2.7. Statistical Analysis

Differences in counts of stained intracellular AM fungal arbuscules between tillage treatments and sample dates in the field and glasshouse, and annual measured ergosterol derived from ZT and ZTC managed soils were analysed using a multi-way analysis of variance (ANOVA) with post-hoc t-testing. All statistical tests were carried out using R commander® (Hamilton, ON, Canada).

3. Results

The number of AM fungal wheat root arbuscules was higher in ZT compared to CT managed soils (Figure 2) although not significantly (multi-way ANOVA: F = 2.60,3 y = 19; P = 0.11). Post-hoc t-testing indicated a significant difference between tillage (F = 2.77,1 y = 19; P = 0.01) for the post-seedbed preparation sample period (September 2019) only. Quantified root arbuscules were noted to have increased in both tillage treatments during pre-harvest (May 2019) sampling but did not indicate a significant increase in either treatment (F = 2.60,3 y = 19; P = 0.16). Post-hoc t-test comparisons between sampling periods (post seedbed preparation versus pre-harvest) of the same tillage treatment (CT versus CT or ZT versus ZT) were not significantly different (F = 2.60,1, y = 19; P = 0.26).

Images of the stained intracellular AM fungal arbuscules from winter wheat roots grown in the field in CT and ZT treatment soils are given in Figure 3. Limited fungal root structures are present in the CT treatment root tissues from previously sampled field wheat roots (Figure 3b) relative to the ZT treatment (Figure 3a). There are however, extraradiating mycelia from the rhizoplane.

The number of arbuscules present on stained winter wheat root sections grown in controlled glasshouse conditions in soil extracted from CT and ZT fields at weeks 15 (GS21 [23]) and 30 (GS39 [23]) were greatest in ZT managed soils (multi-way ANOVA: (F = 51.123,3 y = 19; $P = 1.17 \times 10^{-6}$) (Figure 4), also showing that CT treatments reduce the number of root arbuscular structures. The number of AM fungal structures was greater in glasshouse grown wheat (Figure 4) compared to field grown wheat (Figure 2) for both tillage treatments (multi-way ANOVA: F = 306.42,5 y = 19; $P = 2.20 \times 10^{-16}$). The soils used to generate the data in Figure 4 were sampled before another annual application of glyphosate and tillage, resulting in these soils having been void of the mycorrhizal damaging active ingredient for over 12 months. Additionally, in the absence of glyphosate application, greater numbers of quantifiable AM fungal root structures were produced in the glasshouse experiments when compared

to the field sampled wheat roots, with a 41.6-fold increase, equating to 4057.14% over field sampled wheat roots, at week 30. Conventional tillage soils however, increased 21.3 fold in the glasshouse at week 30 (Figure 4) compared to field sampled wheat tissues (Figure 2), equating to a 2053.85% increase to quantifiable AM fungal root structures in the absence of an additional cultivation treatment. An example of a stained root section from glasshouse grown winter wheat 20 weeks post germination (GS30 [23]) is given in Figure 5.

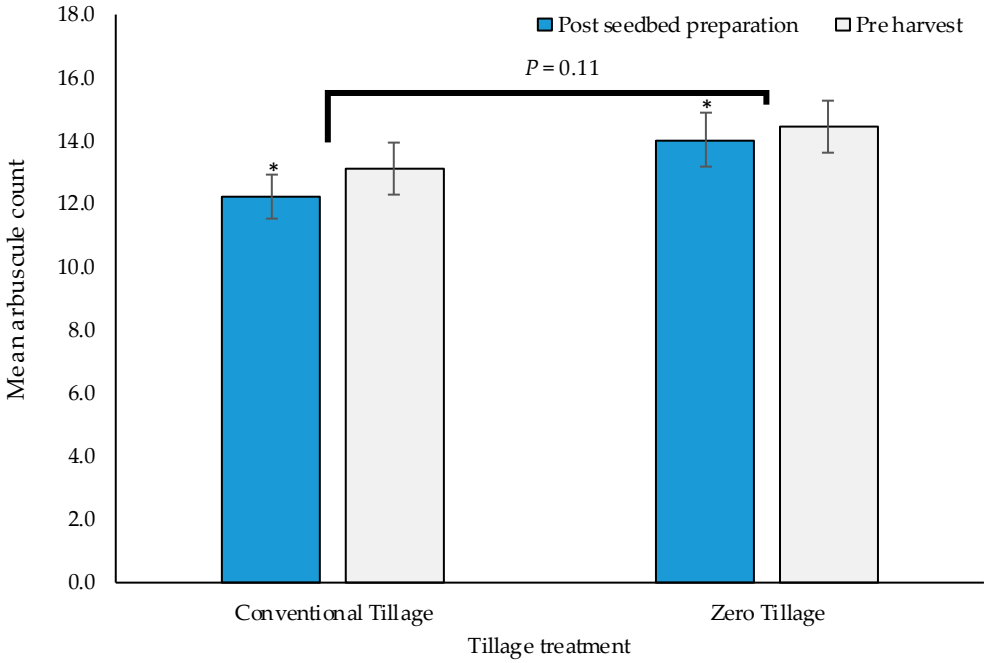

Figure 2. Average (n = 20 per sampling period per tillage treatment) stained intracellular arbuscular mycorrhizal (AM) fungal arbuscules post seedbed preparation and pre-harvest between conventional and zero tillage managed soils growing winter wheat. Multi-way ANOVA: F = 2.60,3 y = 19; P = 0.11); (*) post-hoc t-test (F = 83.89,1 y = 19; P = 0.01). Error bars = Standard Error of Mean (SEM).

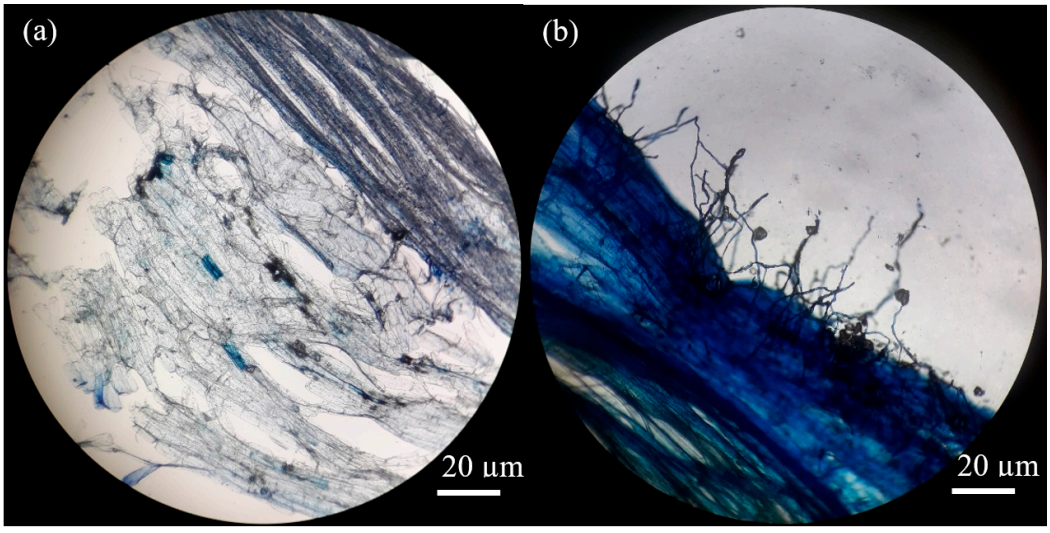

Figure 3. Winter wheat root sections (1 cm length) stained with Sheaffer® blue, sampled from (**a**) zero tillage (ZT) and (**b**) conventional tillage (CT) soils in February. Imaged with a Samsung Galaxy® (Seoul, South Korea) Android 3J camera smartphone at a magnification of ×40 of a Vickers® (York, UK) compound microscope.

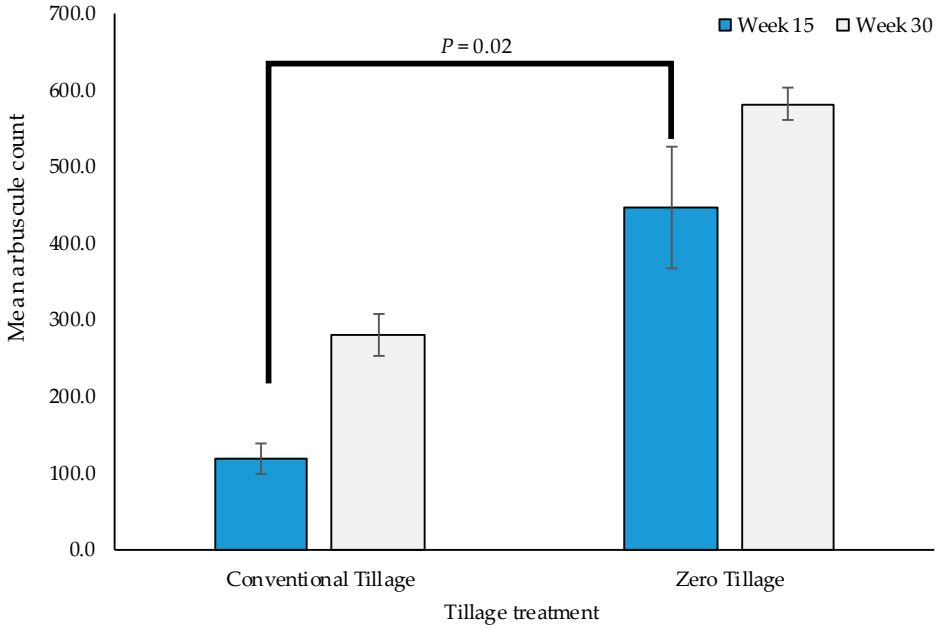

Figure 4. Average (n = 20 per sampling period per tillage treatment) stained intracellular AM fungal arbuscules from glasshouse grown winter wheat after 15 and 30 weeks. Multi-way ANOVA (F = 51.123,3 y = 19; $P = 1.17 \times 10^{-6}$). Error bars = one SEM.

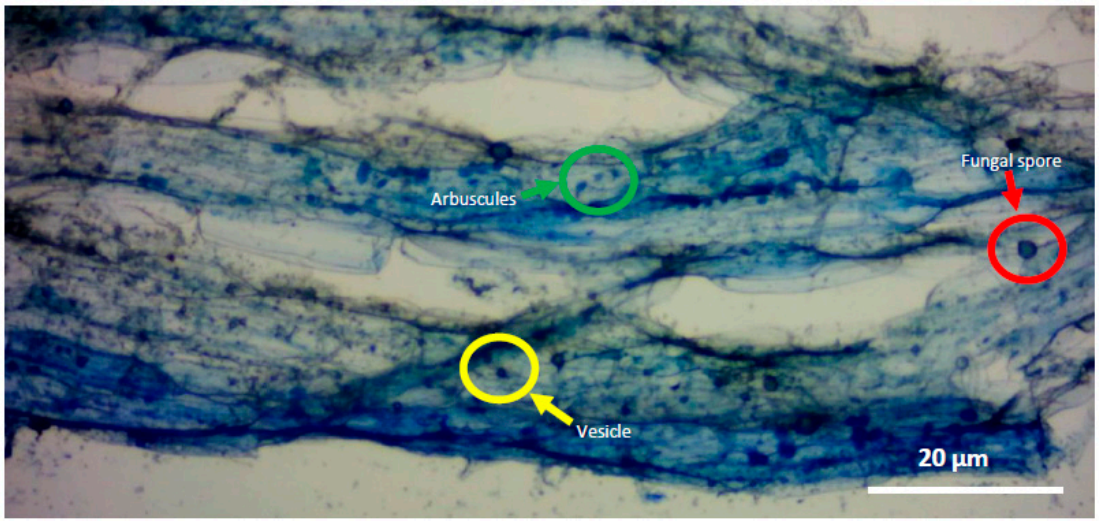

Figure 5. Winter wheat root sections (1 cm length) grown in ZT soil under controlled glasshouse conditions stained with Sheaffer® blue at 20 weeks post germination. Stained structures are indicated in coloured circles. Images were recorded using a Bresser® (Rhede, Germany) HD microscope camera under a total magnification of ×100 of an Apex® (Chippenham, Wiltshire, UK) compound microscope.

Mean ergosterol in the ZT soils was consistently greater in the ZTC sampling area (control soils sampled within the same field boundary, but outside the cropping area and area of glyphosate application), summarised in Figure 6 (multi-way ANOVA: F = 19.68,7 y = 24; $P = 9.46 \times 10^{-8}$). Greatest differences in quantified ergosterol between ZT and ZTC soils were noted in the post seedbed preparation and pre-harvest stages (post-hoc t-test, Figure 6). The decline observed in ergosterol throughout the sampling year in both the ZT and ZTC was greater in the ZT soils in receipt of glyphosate.

The application of glyphosate to both CT and ZT soils under glasshouse conditions significantly inhibited the growth of fungi (multi-way ANOVA: F = 8.38,99 y = 2; $P = 1.07 \times 10^{-8}$), as measured by ergosterol, at all concentrations applied (Tables 1 and 2). Baseline ergosterol levels were 300 µg L^{-1} and

750 µg L^{-1} in CT and ZT soils, respectively. Lower glyphosate concentrations, 10 g L^{-1}, were noted to allow fungal biomass to increase after six days in the ZT soil and nine days in the CT soil. Fungal growth was, however, reduced in the CT extracted soils (soil managed in the field through practices that did not use glyphosate) at glyphosate applications in the glasshouse of 50 g L^{-1} active ingredient and above (post-hoc *t*-test, $F = 1.71{,}19$ $y = 2$; $P = 0.03$). Glyphosate concentrations of 10 g L^{-1} in CT soil did not show reductions in fungal biomass to the same degree ($F = 2.04{,}19$ $y = 2$; $P = 0.12$). Fungal biomass in ZT extracted soil, soils typically managed with the application of glyphosate, showed significant reductions with all glyphosate applications of 10 g L^{-1} and above (post-hoc *t*-test: $F = 1.71{,}19$ $y = 2$; $P = 0.02$). The quantified fungal biomass was consistently reduced by 2.2–2.5-fold in CT soils compared to ZT treated soils (Table 2).

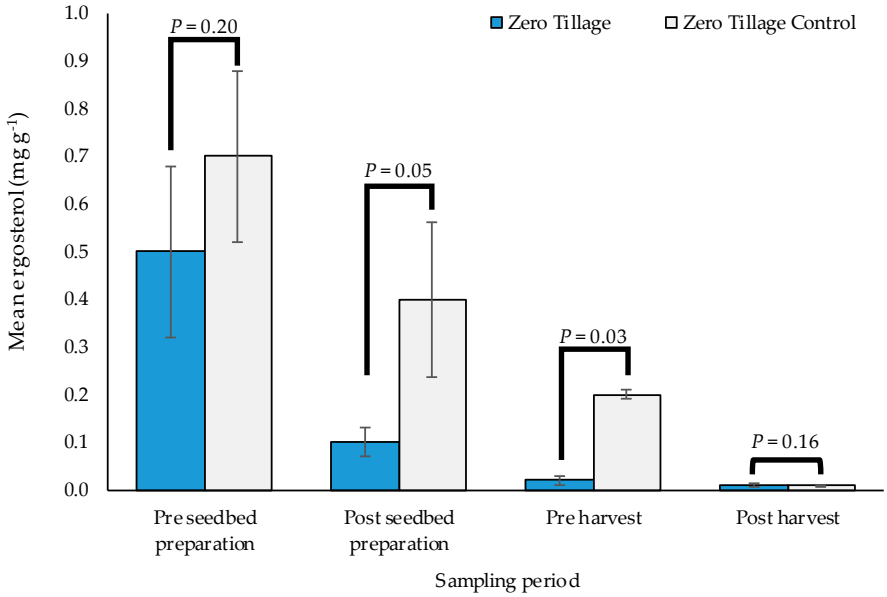

Figure 6. Mean ($n = 200$) ergosterol (mg g^{-1}) extracted from zero tillage and zero tillage control soils over one cropping year (September 2018–August 2019). Multi-way ANOVA: significant difference ($F = 19.68{,}7$ $y = 24$; $P = 9.46 \times 10^{-8}$). Post-hoc *t*-test: significant difference between the ZT and ZTC treatments post seedbed preparation ($F = 1.83$, 1 $y = 24$; $P = 0.05$) and pre-harvest ($F = 1.71$, 1, $y = 24$; $P = 0.03$). Error bars = one SEM.

Table 1. Mean ($n = 300$) change in ergosterol concentration (µg mL^{-1}) measured in CT and ZT soils at day 3, 6, 9, and 12 compared to the original ergosterol concentration (µg mL^{-1}) measured at day 0 before glyphosate application. for 10 different glyphosate application rates. A positive value indicates an increase in ergosterol concentration compared to day 0. Ergosterol concentration was significantly reduced in both tillage treatments with increased glyphosate application (multi-way ANOVA: $F = 8.38{,}99$ $y = 2$; $P = 1.07 \times 10^{-8}$).

	Conventional Tillage					**Zero Tillage**				
	Day									
Glyphosate (g L^{-1})	**0**	**3**	**6**	**9**	**12**	**0**	**3**	**6**	**9**	**12**
0	0.0	10.0	30.0	60.0	75.0	0.0	20.0	25.0	40.0	60.0
10	0.0	−10.0	0.0	15.0	52.0	0.0	0.0	10.0	25.0	30.0
50	0.0	0.0	0.0	5.0	15.0	0.0	0.0	0.0	10.0	20.0
75	0.0	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	5.0
100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
150	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
200	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
250	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
300	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
350	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. Calculated fungal biomass (from Equation (1) [26]) for glyphosate applied soils of CT and ZT treated soils under controlled glasshouse conditions, indicating fungal growth inhibition from glyphosate application above 75g L^{-1} over 12 days.

Glyphosate Concentration (g L^{-1})	Final (Day 12) Fungal Biomass (mg) per Tillage Treatment	
	CT	ZT
0	150.9	326.0
10	141.7	314.0
50	126.8	310.0
75	122.7	308.0
100	120.7	301.9

4. Discussion

The impact of tillage and glyphosate application on AM fungi using root arbuscule count and soil ergosterol as indicators has been evaluated for field grown crops and with glasshouse experiments. The field crops were commercially grown and in receipt of standard crop management protocols including annual tillage or glyphosate application. The glasshouse grown crops used soil extracted from the field sites but allowed for the manipulation of management including the elimination of the subsequent tillage or glyphosate applications, or the application of glyphosate at variable dosage rates. The impact of such management on AM fungal population recovery could then be assessed.

4.1. Glyphosate and Symbiosis

Root arbuscule counts were lower in field grown wheat plant roots under CT (Figure 2), albeit not significantly. Counts were significantly lower on the roots of wheat grown in CT soils in the glasshouse (Figure 4). This is most likely due to two key factors. Firstly, the process of soil inversion causing physical damage to the AM fungi hyphal structures [28]. Secondly, in ZT soils, the residual effect of glyphosate in the ZT wheat crop exerting an inhibitory effect on AM fungal growth (Tables 1 and 2). These two factors are removed in the glasshouse grown wheat. The residual effect of soil inversion however appears greater than that of glyphosate. Although higher than in CT, the arbuscule counts of wheat grown in ZT field soils did not increase significantly (there was no increase in fungal–root symbiosis) in the period after glyphosate application and before harvest. Winter wheat root samples grown under ZT field conditions grew in soil that had received a glyphosate application during initial land preparation, two weeks after samples were taken in September. Both CT and ZT soil extracted for use in the glasshouse experiment was collected post-harvest, 11 months after the previous glyphosate application and one month before a further glyphosate application and sowing of the following crop were due to commence. The lack of increase in fungal–root symbiosis in the field sampled wheat but not in the glasshouse samples implies that there is a negative residual effect associated with glyphosate on AM fungi. Glyphosate decomposition rates are reported as being 2–147 days in soils [3,29]. Residual glyphosate in the extracted soils used in the glasshouse experiments will have declined during this period, corroborated in part by the weed emergence that was noted at the time of soil collection. Unlike the soils in the field which will then receive a further glyphosate application, the ZT soils used for the control in the glasshouse experiments had no further application of glyphosate. The average arbuscule count on the roots of winter wheat grown in the glasshouse was significantly greater in the ZT relative to the CT treatment. This is due to the absence of firstly, soil inversion before soil was extracted from the field and secondly, the absence of the subsequent glyphosate application in the glasshouse normally associated with seed-bed preparation for the following crop. The removal of the glyphosate application under glasshouse conditions in the present study increased AM fungal symbiosis by a factor of 41.6 (5057.14%). This supports in part the conclusions of Zaller et al. [30] who also found that the application of glyphosate reduced AM fungal colonization and symbiosis but by a far smaller amount, 40%. It is acknowledged that environmental conditions present in the glasshouse experiments, such as temperature and soil moisture, are likely to favour AM fungal development

relative to the field environment. However, quantified ergosterol in the CT soils did not increase to the same degree as in the ZT soils. If the glasshouse environmental conditions were having a significant impact on the total amount of AM fungi present within the field sampled soils, the CT soil quantified ergosterol would have increased similar to that observed in the ZT soil. Although this study agrees broadly with the findings of [30] and acknowledges the potential impact of glasshouse conditions on growth rate, other factors such as the root staining protocol require consideration.

Both studies use comparable staining methodologies, Sheaffer® ink as opposed to trypan blue, except [30] use Sheaffer® black as opposed to Sheaffer® blue ink in the current study [23]. Veierheilig et al. [31] favoured Sheaffer® black but this has since been shown to be less effective for the complete assessment of fungal root structures [27]. The clarity of samples may be inhibited by soil debris, intra-radiating mycelia and inadequate homogenization of root cells on the microscope slide, all of which may obscure the quantification of fungal components. Sheaffer® blue allows a density gradient of stain to be viewed, allowing for a more reliable and accurate identification of stained structures. Additionally, a staining time of one minute [30] is not sufficient to allow stain penetration of root tissues. A staining time of three minutes [27] allows for a more thorough stain penetration. Furthermore, the use of a fixative solution permits the stain to be more selective against the target fungal structures and does not cause root cells to be additionally stained. This further allows the differentiation of desired structures to be viewed and quantified [27]. The lower percent reduction of arbuscules observed previously [30] may be due to a less effective staining protocol. The negative impact of glyphosate on AM fungi may have been underestimated, a 40% reduction in AM fungal arbuscules [30] compared with 5057.14% under glasshouse conditions in the present study.

4.2. Tillage and Symbiosis

Soils managed via CT had lower fungal arbuscules per centimetre of root tissue compared to ZT managed soils, significantly in the glasshouse grown wheat plants. As a soil management practice, CT would appear to be detrimental to overall AM fungal root colonisation. It concurs with the previous findings [31–36] that tillage is detrimental to fungal communities. Indeed Brito et al. [37], using operational taxonomic units (OTU), showed a 40% reduction in AM fungi due to soil inversion in CT compared with ZT. A difference of this magnitude was not evident in the field grown wheat in the current study due to a second potential factor, the negative impact of glyphosate on AM fungi in the ZT managed crops, as identified by the glasshouse experiments (Tables 1 and 2). The reduction in fungal abundance, measured via ergosterol as a biomass indicator, means that fewer fungi are present within soils and able to respond to the phyto-hormone strigolactone, the presence of which acts as a signal for initial root colonisation. This then reduces nutrient availability for the host plant as well as reducing the diversity and abundance of symbiotic fungi due to interference with their life cycle. Although lower in number, the fungal structures present in the root tissues of wheat sampled from CT managed crops had additional radiating mycelia from the rhizoplane compared to those in the ZT treatment. It suggests that the application of tillage has not only reduced the abundance of AM fungi, but produced a change in fungal species diversity [32,33]. Lu et al. [32] conclude that CT managed soils significantly reduce both fungal diversity and the abundance of *Glomus* spp., a well reported genera of AM fungi. A decline in *Glomus* spp. was correlated with reductions in soil aggregate stability due to the physical damage of fungal mycelia during the soil inversion process.

On a positive note, the CT arbuscule counts from Figure 4 of the present study indicate AM fungal recovery is possible when soils do not receive continued cultivation on an annual basis. The quantified arbuscules from field sampled wheat roots increased by 17% in the period after cultivation to pre-harvest showing the potential for gradual population recovery in the absence of further cultivation. This was further corroborated in the glasshouse experiments. Soil samples extracted after harvest from the CT managed fields with minimal disturbance were used to simulate the impact of continued crop growth in the absence of a further tillage application. Under these conditions the number of quantifiable fungal structures associated with AM fungi–root symbiosis per centimetre of root tissue increased.

Soil inversion is implemented annually in the CT treatment, the time taken for the potential regeneration of AM fungal biomass post seedbed preparation and the extent of this recovery within any given cropping year is therefore of particular interest. The glasshouse experiment removed the equivalent of one cultivation associated with the following crop, i.e., increased the time elapsed since tillage to above 12 months in the CT treatment soil. It allowed for insights into the potential regeneration of fungal biomass in CT soils if tillage was not applied on an annual basis. The number of AM fungal arbuscules increased nearly 10-fold due to the elimination of this one equivalent tillage operation. An increase in plant–fungal symbiosis is evidently possible and within a relatively short timeframe in the absence of annual tillage.

4.3. Glyphosate and Ergosterol

Glyphosate inhibits the Shikimate pathway and EPSP synthase production in plants, but these pathways are also critical functions of bacteria and fungi, including AM fungi [1,4–6]. Between both tillage types sampled, CT and ZT, glyphosate was the only agro-chemical applied that differed between the sites. Bulk soil ergosterol levels, used as an indicator of fungal biomass, quantified pre-drilling and before glyphosate application were higher than in the samples taken following glyphosate application in the remainder of the sampling year. The quantity of ergosterol decreased by a factor of five, from 0.5 mg to 0.1 mg per 0.1 m^3. The grass margins represent soils void of inversion, aeration, disturbance, or chemical treatments. Ergosterol was higher relative to the ZT soil treatment but was reduced by a factor of 1.75 between sampling periods. This is most likely to be the result of a seasonal change in environmental and meteorological conditions, such as soil and air temperature [38], producing sub-optimal growth conditions [39]. A further factor is that although both the ZT and ZTC soils are within the same field boundary the ZTC plot area was mown after the third sampling period, pre-harvest, to allow easier access for agricultural machinery. Areas of soil became devoid of vegetation cover as a result. A loss of vegetation results in loss of potential AM fungal host root material, disrupting the AM fungal biotrophic life cycle [16] and causing the reduction in ergosterol and fungal biomass [25] observed in Table 1. In the ZT treatment however, not only is living plant tissue removed during crop harvest but by the application of glyphosate also. Further, glyphosate application removes both the living host and is detrimental directly to the Shikimate and EPSP synthase pathways important in AM fungi [8,15], as shown in the glasshouse experiments. It is acknowledged that glyphosate application is not the only variable between the ZT and ZTC treatment, this was addressed by the glasshouse experiments. Lower ergosterol levels were observed in all glasshouse treatments that received glyphosate relative to the control. Ergosterol increases over each three day period where glyphosate is not applied however the rate of increase is reduced at concentrations as low as 10 g L^{-1}, with no increase evident until after six days as opposed to three. A field application rate is equivalent to 75 g L^{-1} per 50 g soil, where a negligible increase was noted after day 12. No increase was observed at application rates of 100 g L^{-1} or above. The provision of a continued supply of living root biomass is critical in maintaining AM fungi populations, irrespective of management and land use.

4.4. Glyphosate Growth Inhibition

Although ergosterol was higher in soil extracted from farm B under ZT management compared to soils managed by CT, the rate of fungal growth over 12 days using these soils in glasshouse experiments was comparable (60 μg mL^{-1} ergosterol) for both tillage treatments. It would imply that recovery of fungi proceeds at a similar rate but due to the lower ergosterol initially within the CT treatment, an approximate additional 90 days is required under optimal soil conditions to reach levels comparable to those in the ZT treatment. Field conditions are rarely optimal for AM fungal growth [40] and the potential recovery time would be expected to be longer. In a commercial crop rotation a further tillage application associated with seed-bed preparation for the following crop will have occurred by this time, reducing fungal abundance to post harvest levels once again. Glyphosate also appears to reduce fungi populations compared to an untreated control albeit not to the same magnitude

but unlike tillage, has an associated residual inhibitory effect. The application of glyphosate in the glasshouse to CT extracted soils differs to the situation in field grown crops in that a CT crop does not typically receive glyphosate [41]. The associated soil fungi will not been pre-exposed to this herbicide and its mode of action, lending further support to the inference that glyphosate inhibits fungal growth. The rate of recovery will be inhibited where residual glyphosate is present in the soil, or potentially within 2–147 days post application [3,29]. Recovery post seedbed preparation may therefore be more rapid than recovery immediately post glyphosate application. From the perspective of the AM fungal life cycle, an increase in fungal biomass should have been measured across the sampling year due to an increase in host plant root mass combined with an increase in soil and air temperature improving the suitability of soil conditions to population growth [39]. The arbuscule count in wheat root samples extracted pre-harvest from the ZT field crop although more numerous were not significantly different to the CT treatment. Further, it did not have the magnitude of difference exhibited in the glasshouse comparison between tillage treatments where glyphosate was not applied to the ZT soil. The inhibition of fungal EPSP synthase by glyphosate evidently has the potential to significantly reduce fungal biomass, growth, and symbiosis establishment [1,15,30]. It has important implications for the management of ZT crops where a benefit may be realised by eliminating tillage but this benefit is then curtailed by the application of glyphosate. A further key variable is the presence of living plant root biomass, removed in both the CT and ZT treatments either physically or chemically. Permitting the persistence of non-crop plant species that do not impact on crop yield, for example common groundsel (*Senecio vulgaris* L.) [42] would provide an opportunity for AM fungi to maintain higher populations within the soil post-harvest but before development of more extensive root systems in the following crop. The impact on AM fungi populations of other reduced tillage approaches, for example strip-tillage [43,44], in which only the row of soil where the crop is sown is disturbed but alternative methods of weed control may be available, is worthy of evaluation in future work.

5. Conclusions

The present study provides evidence that tillage and glyphosate application for seedbed preparation or weed control are potentially detrimental to soil fungal abundance. AM fungi are in all probability sensitive to both tillage and glyphosate having shown reductions in symbiosis attributed to both treatments. Consequently, this compromises the benefits conferred by AM fungi to host plants including enhanced nutrient availability, and salt and drought tolerance, coupled with the greater plant resource expenditure to increase root biomass that would otherwise be off-set by the AM fungi–host relationship. Despite this, ZT is a preferable crop management strategy to CT despite the reduction in soil fungi populations due to glyphosate application. If alternative weed control measures could be identified, the full benefit of the crop–AM fungi relationship will be realised in zero tillage systems.

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