A Comparison of Tillage Techniques on Selected Soil Microbes Within the Rhizosphere of Wheat

A Comparison of Tillage Techniques on Selected Soil Microbes Within the Rhizosphere of Wheat

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Abbreviations

AM	Arbuscular mycorrhizal
PGPR	Plant growth promoting rhizobacteria
GRSP	Glomalin related soil protein
CT	Conventional tillage
ZT	Zero tillage
RT	Reduced tillage
SOM	Soil organic matter
SOC	Soil organic carbon
OC	Organic carbon
LOI	Loss on ignition
MHB	Mycorrhizal helper bacteria

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Abstract

This thesis reports on the implications of Conventional tillage regimes with a focus upon Arbuscular Mycorrhizal (AM) fungi for their contribution to soil chemical, physical and biological attributes, including aggregation, abundance and contribution to organic material.

Soil inversion of conventional tillage (CT) treatments was compared with soil management practices involving zero tillage (ZT) for the growing season between September 2018 and September 2019 within Hertfordshire, UK, focusing upon alterations to AM fungal relationships between glomalin and soil aggregation, host-fungal symbiosis, and interactions with rhizobacteria. Identified sites, matched on similarities of crop histories and fertiliser type and application, were sampled in grid formats to maximum soil depths of 40cm.

Sampled soils of both applied tillage practices indicated variances in soil glomalin and soil aggregation with sampling depth as well as between tillage regimes. Top soil (<10cm) glomalin and aggregation was seen to be homogenised throughout the zone of tillage in CT soils, whereas ZT soils varied with sampling depth. Correlations between glomalin and soil aggregates were seen to diminish in CT managed soils, accompanied by reductions to quantified fungal biomass, when compared with ZT treatments which did not produce such reductions.

Examined root sections of winter wheat, grown under each tillage treatment, indicated reduced AM fungal symbiosis in CT managed soils compared with ZT soils. Further investigations were able to indicate glyphosate application in ZT treatments may have produced profound reductions to AM fungal symbiosis. Furthermore, the type of fertiliser was seen to influence the degree to which AM fungi associated with wheat root tissues.

Application of Selected rhizobacterial inoculants (*Bacillus* spp.) to sampled soils from each tillage practice, with planted Zulu winter wheat, affected AM fungal symbiosis in stained root tissues as well as influencing above and below ground wheat biomass. Monitoring of selected rhizobacteria by qPCR gave further indications to the persistence of select *Bacillus* spp. rhizobacteria in wheat producing soils.

1.0 Introduction

Tilling soils is an ancient practice has been dated back to Ancient Egyptian texts (Mazoyer and Roudart, 2006). At this point in agricultural history, many ploughs were drawn my oxen, however, earlier examples of land preparation via tilling shows human drawn ploughs in labour intensive food production (Lal, 2003). In the 18th century, technological developments aided in the beginnings of mechanisation within agriculture, but it was not until 1837 when steel mouldboard ploughs were developed that land previously thought to be unsuitable for farming was used for crop production (Nicholson and Shaw, 2000). As agricultural machinery developed further, soils became more intensively managed to maintain food production for a growing global population (Hughes, 2011). In modern times, agricultural research is exploring alternate methods to soil management, with potential movements away from plough-based methods (Blanco-Canqui and Lal, 2008; Li et al. 2012).

The influences of tillage towards the microbial diversity of soil is an emerging area of investigation published in literature. Diversity alterations, resultant of tillage, have several consequences to developing crops within arable soils, such as reduced crop yield. DEFRA (2020) reported a lack and need for studies to compare tillage types and the influences each have on selected soil attributes, such as organic matter, the diversity of the microbiome, and overall soil quality (Wright et al. 1996; Wang et al. 2017). The soil microbiome has been reported to have a large range of crop development influences, many of which have yet to be fully understood. Some literature has also postulated that the application of selected rhizobacteria can be used as biofertilisers, the application of microorganisms to increase crop growth or reduce pathogen infectivity, however, the exact physiological mechanisms by which biofertilisers influence crop development is an emerging area of research. Throughout literature, the adoption of a conservational land management practice, having microbiome diversity protective properties, is preferred over a land management practice that is more soil invasive. Within the UK, 9.06 million hectares is used for agricultural production. Of this, 54% of land is used for arable crop production, with 41% of land used as permanent grassland. However, only an estimated 8% of UK arable land is managed by zero tillage (ZT) (DEFRA, 2019), the absence of soil inversion.

The primary purpose of tillage is to prepare soils for the cultivation of a crop. One of the practices available is conventional tillage (CT) which typically employs a mouldboard plough to a maximum depth of 30cm, however, a maximum depth of 20cm is usual in UK CT land management practices (AHDB, 2020). Soil disturbance from tillage practices, especially

CT, reduce the initial colonisation of AM fungi and other beneficial organisms to the root systems of developing plants, as well as rhizosphere community establishment and their contributions to soil quality and plant health (Douds et al., 2000; Curaqueo et al., 2011; Galvez et al., 2001)

Plant health and soil quality are highly dependent upon interactions between biological, chemical and physical attributes of soils, having influences on overall crop yields and productivity (Li et al., 2012; Larson et al., 1994). The rhizosphere, a narrow region surrounding root systems and directly under the influence of root exudates, has a complex myriad of interactions between the host plant and microbiological constituents of the rhizosphere, having either positive, negative or neutral effects towards the host plant (Barea et al., 2005; Azcón et al., 2013; Berendsen et al., 2012). Furthermore, the rhizosphere is an area high in metabolic activity and mineral transfer resultant of the microbiome (Berendsen et al., 2012). Root exudates, such as organic acids, are deposited into the rhizosphere to facilitate their improved survival via influencing positive changes in their surroundings, through the process of rhizodeposition, with specific influences on the rhizosphere microbiome (Hartmann et al., 2008). The application of Plant Growth Promoting Rhizobacteria (PGPR) as a biofertiliser has the potential to increase productivity (Berruti et al., 2016; Nadeem et al., 2019). Plant root exudates can encourage the growth of species such as *Bacillus subtilis*, reported to have fungal pathogen control properties (Rahman et al., 2010). A further example comes from leguminous crops, such as beans, that encourage *Rhizobacteriaceae* to increase in abundance via root exudates. *Rhizobacteriaceae* have been shown to aid in the acquisition of soil nitrogen through the fixation of atmospheric nitrogen (Hernandez et al., 2009). Sugiyama and Yazaki (2012) later produced estimates that *Rhizobacteriaceae* annually fix an estimated 40 – 60 million tonnes of atmospheric nitrogen into soil nitrates. Arbuscular Mycorrhizal (AM) fungi, amongst other organisms in the rhizosphere, aid in the transfer of nutrients, including nitrates, to the host plant via root associations (Azcon et al., 2011).

Glomeromycota, the family of mycorrhizal fungi, has a long standing association with terrestrial plants, and is thought to have assisted in the land colonisation of early plants (Brundrett, 2002), with modern estimates of symbiosis with 200,000 plant species (Lee et

al., 2013). *Glomeromycota* encompasses AM fungi and endocytobiotic *Geosiphon pyrifomis*, of which forms a plant symbiosis through the incorporation of cyanobacteria (Schubler et al., 2007). AM fungi are obligate symbionts, existing inter and intracellularly. AM fungi have been studied for several decades for their contribution to arable crop production and implications on soil quality (Akyol et al., 2011).

Within the rhizosphere microbiome, Arbuscular Mycorrhiza (AM) fungi form symbiotic relationships with host plants through root cortical structures, arbuscules. Plant hormones, such as strigolactone and branching factors, stimulate the germination of AM fungal spores and the rapid proliferation of AM fungal cells in mycelia. Besserer et al. (2006) reported on the influences of strigolactone stimulation of the growth of AM fungi and found, upon receipt of strigolactone signalling, AM fungi increased mitochondrial density within mycelial cells and increased respiration rates, which correlated strongly with the increase in mycelial biomass. Strigolactone, however, is additionally responsible for stimulating parasitic weeds and plants such as Striga and Orobanche (Screparti et al., 2016).

1.1 Plant microbiota

The rhizosphere houses a large diversity of microorganisms that form positive, negative or neutral associations with a host plant (Yuan et al., 2015; Adeleke et al., 2010) and forms the plant biota. From this, a holobiont is formed – the formation and established multi relationships with a host from a multitude of organisms (Bulgarelli et al., 2013). Within the rhizosphere, bacteria outnumber fungi, however, fungal biomass is much greater than that of bacteria (Lee et al., 2013). Rhizosphere bacteria exert growth control over other species of bacteria (Barea et al., 2005), through the production of antibiotics (Elshakh et al., 2016), as well as fungal control (Cruz et al., 2012). An example of the biocontrol properties, and positive influences of the bacterial rhizosphere constituents, comes from the work of Chen et al. (2018) in the control of *Fusarium graminearum*. Chen et al. (2018) were able to show the secretion of phenazine-1-carboxamide from *Pseudomonas* spp. inhibited growth, biotoxcin production and virulence of *F. graminearum* in wheat infections.

Mycorrhizas are of importance to plant nutrition, function, productivity and soil quality (Flores et al., 2007). Mycorrhizas are typically either endomycorrhizal (EDM) or ectomycorrhizal (EM) and are distinguished microscopically by their colonisation of the rhizoplane (Douds et al., 2000) or intracellularly of root cortical cells (Druille et al., 2013a;

Berdeni et al., 2018), for EM and EDM respectively, with arbuscular mycorrhizal (AM) fungi as an example of EDM. EM are typically found in symbiosis with an estimated 10% of woodland and orchard tree species (Lombardo et al., 2019), being associated to *Basidiomycota*, *Ascomycota*, and *Zygomycota* classes of fungi (Lombardo et al., 2019; Oehl et al., 2011), with some studies being able to quantify these fungi, via molecular methodologies, findings 15 EM species associated with a single Beech tree (Weintraub et al., 2007).

AM fungi, forming EDM associations with host plant roots, have been studied for their benefits to crop production and soil quality through established intracellular nutrient exchange with host plants and branching mycelial into bulk soils. AM fungi are obligate biotrophic symbionts that form mutualistic relationships with host plants via their root systems (Lerat et al., 2003; Pepe et al., 2018). In the absence of a host, fungal mycelia have been shown to reduce in abundance in bulk soils from biomass assessments, however, AM fungal spores can remain in soils for longer periods without becoming non-viable (Hildebrandt et al., 2006; Long et al., 2017), a change from previous understanding of the vulnerability of AM fungal spores. Plant roots are known to be influential in shaping their own microbiome and rhizosphere, as well as having profound influences on greater reaches in soils (Castro-Sowinski et al., 2007). To accomplish this, roots produced chemical signals, hormones and organic acid root exudates, that are released into the root space encouraging migration and development of soil microbes towards the host plant root.

AM fungal associations with host plant root systems, in arable crops for example, have been shown to increase crop yields (Calonego et al., 2017), nutrient uptake and acquisition (Ghimire et al., 2017; Galvez et al., 2001) and improve soil quality (Mulvaney et al., 2009). AM fungi are highly sensitive to the management of arable land, especially from applications of tillage as a land preparation practice (Apesteguía et al., 2017). The AM fungal life cycle can provide understanding of the implications arable land management has on AM fungal populations in soils.

1.1.1 AM fungal life cycle

Figure 1.1 depicts the life cycle of AM fungi from developing root systems producing plant hormones and exudates (Figure 1.1a) to spores from other symbiotic bodies receiving more signals for growth and development (Figure 1.1e).

Strigolactone is the primary plant hormone (Figure 1.1a) that is responsible for the growth encouragement of AM fungal migration towards a root system in the initial stages of root colonisation. This is through fungal recognition of a host plant as well as plant recognition of a symbiotic fungus (Genre et al., 2013). Studies have shown the plant-fungus recognition pathway, via strigolactone, is more ancient than the plant-bacteria recognition pathway (Yoneyama et al., 2008), and is thought that bacterial recognition pathways evolved to mimic that of AM fungi (Genre et al., 2013).

AM fungal spores have thick walls (Figure 1.1b) allowing them to remain in the environment for prolonged periods of time (Wright 2005). Whilst AM fungal spores germinate from root exudate signalling, they can however, begin to germinate without signalling molecules being detected, both in proximity to and away from a host plant. Wright (2005) studied spore germination and found lab grown samples were able to germinate in the absence of a host in soils and *in vitro*. Wright (2005) additionally was able to show increases in spore germination with the presence of a host, indicating exudation encourages early symbiosis.

Through continued strigolactone exudation from the host plant, AM fungal mycelia continue to grow towards root structures (Figure 1.1c) via increased cellular density of mitochondria and cell proliferation (Tamasloukht et al., 2003). Soil phosphate concentrations have been measured as having an influence on mycelial growth. Lower soil phosphates increase hyphal growth and branching, whilst increasing plant exudation (Douds and Nagahashi, 2000; Akiyama et al., 2005) once fungal mycelia reach the root surface of the host plant, and forms an appressorium at the site for intercellular fungal growth and the establishment of plant-fungal symbiosis (Douds and Nagahashi, 2000). AM fungi have been shown to produce an appressorium with root cells that have become detached from the main root structure and are termed ‘phantom’ roots in the case of AM fungal appressorium formation (Aarle et al., 2002). This shows that root exudation plays little to no part in the initial infection pathway of AM fungi in the formation of intracellular root complements (Tamasloukht et al., 2003), rather strigolactone is an initial trigger for the pre-symbiosis. Furthermore, different crop species and further differences in crop cultivars produce variances in rhizodeposition of root exudates (Huang et al., 2014). Geel et al. (2016) explore

the differences in thirteen crop families and nine genera of AM fungi, with final conclusions presenting a possible twenty nine combinations of AM fungal-crop interactions with optimal AM fungal species for each crop family. These results can then be taken further to suggest field inoculation of AM fungal genera can be optimised for a selected crop.

The growth of hyphae through root structures, from the appressorium, marks the beginning of the symbiotic relationship between host plant and fungus. To establish the relationship, intracellular arbuscules must be formed (Figure 1.1d), producing a large surface area for nutrient exchange from fungal acquired soil nutrients provided to the host. Organelles of the plant cell must be modified in order to accommodate the arbuscule structure. Hyphae begin to penetrate root cortical cells and the plant plasma membrane invaginates (Taylor et al., 2015). The plant cell penetrating hyphae, the 'trunk', continues to branch whilst still enveloped by the plant cell membrane. After continued hyphal branching, the plant plasma membrane becomes the peri arbuscular membrane (Figure 1.3) and is the site of nutrient exchange (Ivano et al. 2012). The peri arbuscular membrane is continuous with the plasma membrane of the root cell; however, regulation of plant DNA expression has led to the modification of membrane proteins specialised for nutrient exchange (Bonfante and Anca, 2009). Alteration of plant DNA regulation began once fungal hyphae penetrate the cortical cell (Yamoto 2005) causing non transcribed regions of the plant chromosomes to decondense.

The mycelia of the main fungal body, branching into bulk soils will produce more spores (Figure 1.1e) for later germination having received exuded signal molecules. Mycelia itself is able to continue growth towards plant hormone and exudate signals creating further symbiosis with several other plants. When multi plant symbiosis is created, it may involve several plant species rather than conforming to a single plant species. An example of this comes about from symbiosis with wheat crops and emerging weed plants. In cases of woodland soils and EM fungi, multi plant symbiosis has been described as a 'Wood Wide Web' (Giovannetti et al., 2006).

Figure 1.2 demonstrates the interactions between AM fungi, the host plant and surrounding soils. Whilst host plant root systems can exude strigolactones, a plant hormone, to attract the growth of AM fungi (Akiyama et al., 2005), benzoxazinoids are produced to encourage chemotaxis of PGPR. Benzoxazinoids have a particularly strong chemotaxis

towards *Pseudomonas putida* (Neal et al., 2012). Table 1.1 provides some chemical groups exudated from host plant root systems in rhizodeposition responsible for the formation and communication with a rhizosphere microbiome.

Table 1.1 – Chemical groups of root exudates (Garcia et al. 2002; Canarini et al. 2019; Badri and Vivanco, 2009; Narasimhan et al. 2003; Zhao et al. 2005; Koo et al. 2005)

Chemical group	**Compounds**
Organic acid	Acetic, citric, malonic, oxalic, pyruvic, succinic, tartaric
Amino acid	Arginine, α/β-alanine, glutamine, glycine, lysine, methionine, serine
Enzyme	Amylase, invertase, phosphatase, protease
Growth factors	Amino benzoic acid, auxins, choline, inositol, nicotinic acid, thiamine
Phenolic acids	Caffeic acid, cinnamic acid, ferulic acid, salycillic acid, vanillic acid
Carbohydrates	Arabinose, fructose, galactose, glucose, maltose, raffinose, ribose, sucrose
Fatty acids	Linoleic acid, stearic acid, palmitic acid,
Nucleotides	RNA, mRNA
Glucosinolates	Cyclobrassinone, desulphoprogitrin, desulphoglucoalyssin
Sterols	Campesterol, sitosterol, stigmasterol
Flavonols	Quercitin, naringin, myricetin, kaempferol, rutin, genistein
Lignins	Catechol, benzoic acid, vanillin, coumaric acid

Primary metabolites, such as amino acids and sugars, are released through rhizodeposition into the rhizosphere (Badri et al., 2009). Primary metabolites are typically released from root tips due to their rapid growth and high metabolism (Sugiyama and Yazaki, 2012). This is further increased in soils which are nutrient deficient (Zhao et al., 2005) in an attempt to recruit a microbiome that can aid in the acquisition of soil bound nutrients that are typically inaccessible to the plant without microbiological help. Secondary

metabolites tend to be smaller molecules, with lower molecular weights, and in much greater abundance within the rhizosphere, as well as being able to target genes within the microbial population of the rhizosphere (Huang et al., 2014). In specific reference to benzoxazinoids, the production of the group of compounds aid in plant defence from pathogens and also alters the relationship between fungi and bacteria with host roots as well as inhibiting plant growth (Koo et al., 2002). Benzoxazinoids also deter herbivore attacks through increasing defensive responses involving salicyclic acid and jasmonic acid. Furthermore, benzoxazinoids decrease leaf size and total sugar content (Neal et al., 2012) and increase leaf toughness (Wouters et al., 2016). Such a change in leaf sugars and leaf toughness is produced as a defence mechanism against foraging insects (Maag et al., 2016) reducing the loss of photosynthetically produced carbohydrates. Furthermore, the reduction in leaf sugars is off set by the increase of exuded sugar into the rhizosphere in aid to increase pathogen inhibiting constituents of the rhizosphere microbiome (Badri et al., 2009), as well as through microbiome mediated whole plant defence and immune responses (Wang et al., 2017). This is an example of a positive plant-microbe exudate interaction.

Interactions of root exudates and AM fungi receive similar molecular communications from rhizobacteria on host plant roots (Huang et al., 2014). Root exudates further allow the AM fungus to recognise the species of host plant. Several studies (Wang et al. 2017; Van Geel et al. 2016; Nadeem et al. 2014; Berendsen et al. 2012; Adriana et al. 2007; Gralves et al. 2001) have indicated specific AM fungal species form symbiotic relationships with specific species of plant. This reinforces the notion that AM fungi can identify plant species based on molecules exuded from roots and thereby further adding to the specificity of their interactions. However, studies into this specificity of interactions are still yet to provide indications of the exact mechanism (Badri et al., 2009; Garcia et al., 2001). Through the interactions of exudates and AM fungi in rhizospheres and bulk soils, mycelia can receive molecular signals from other plants and symbiotic fungi leading to multi plant-fungal interactions from the same mycelial network (Yoneyama et al., 2008). Flavonoids have been shown to stimulate the branching of mycelia (Koo et al., 2005), however, literature forms a consensus on strigolactone inducing initial AM fungal symbiosis (Kretzschmar et al., 2012).

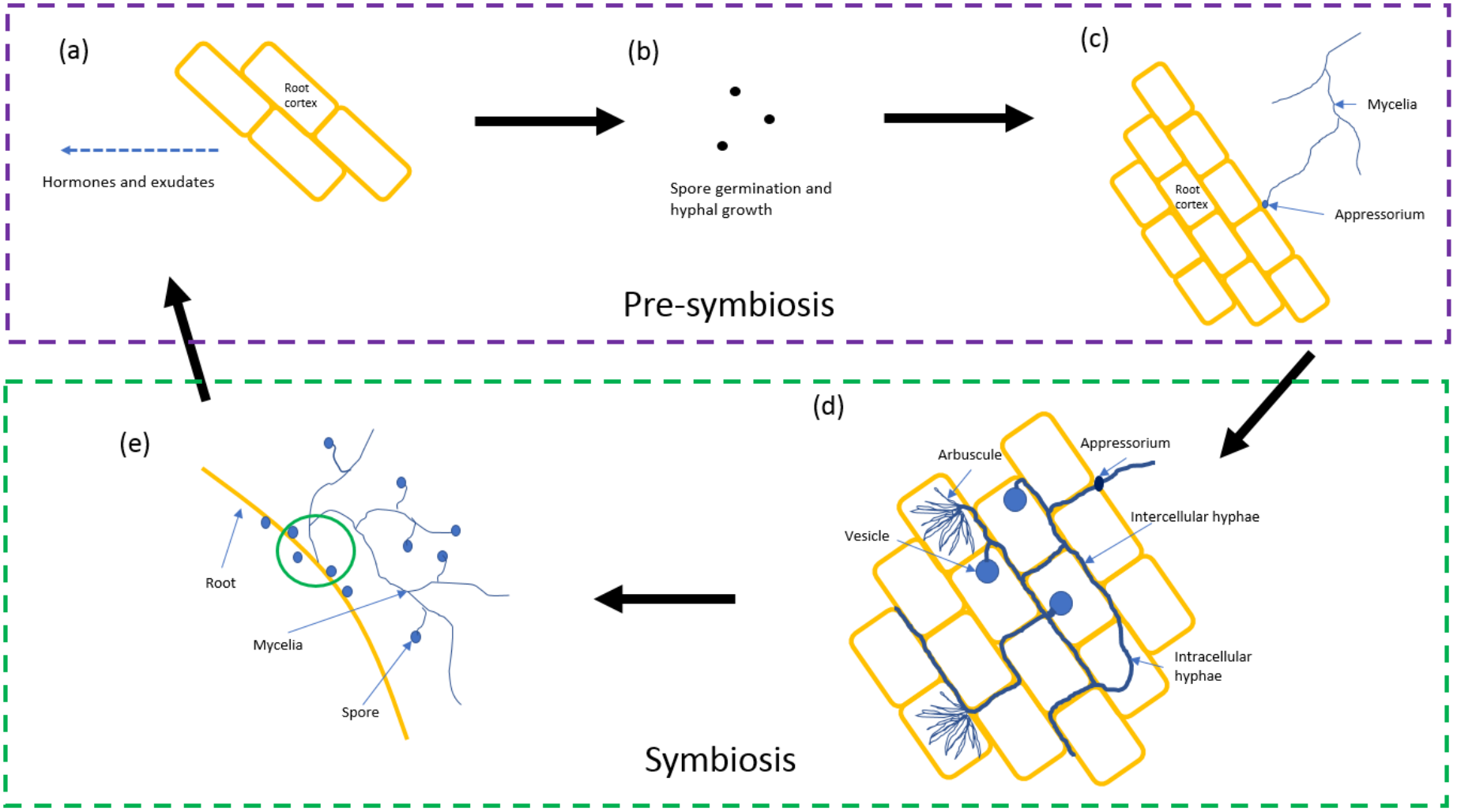

Figure 1.1 - Diagrammatic representation of the AM fungal life cycle through initial plant signalling (a), spore germination (b) and initial plant infection (c) in pre-symbiosis. Following infection, establishment of intracellular root cortical fungal structures (d) establishes plant-fungus symbiosis. Extra radiating mycelia are also available for growth and development from further plant exudated compounds to establish multi plant symbiosis

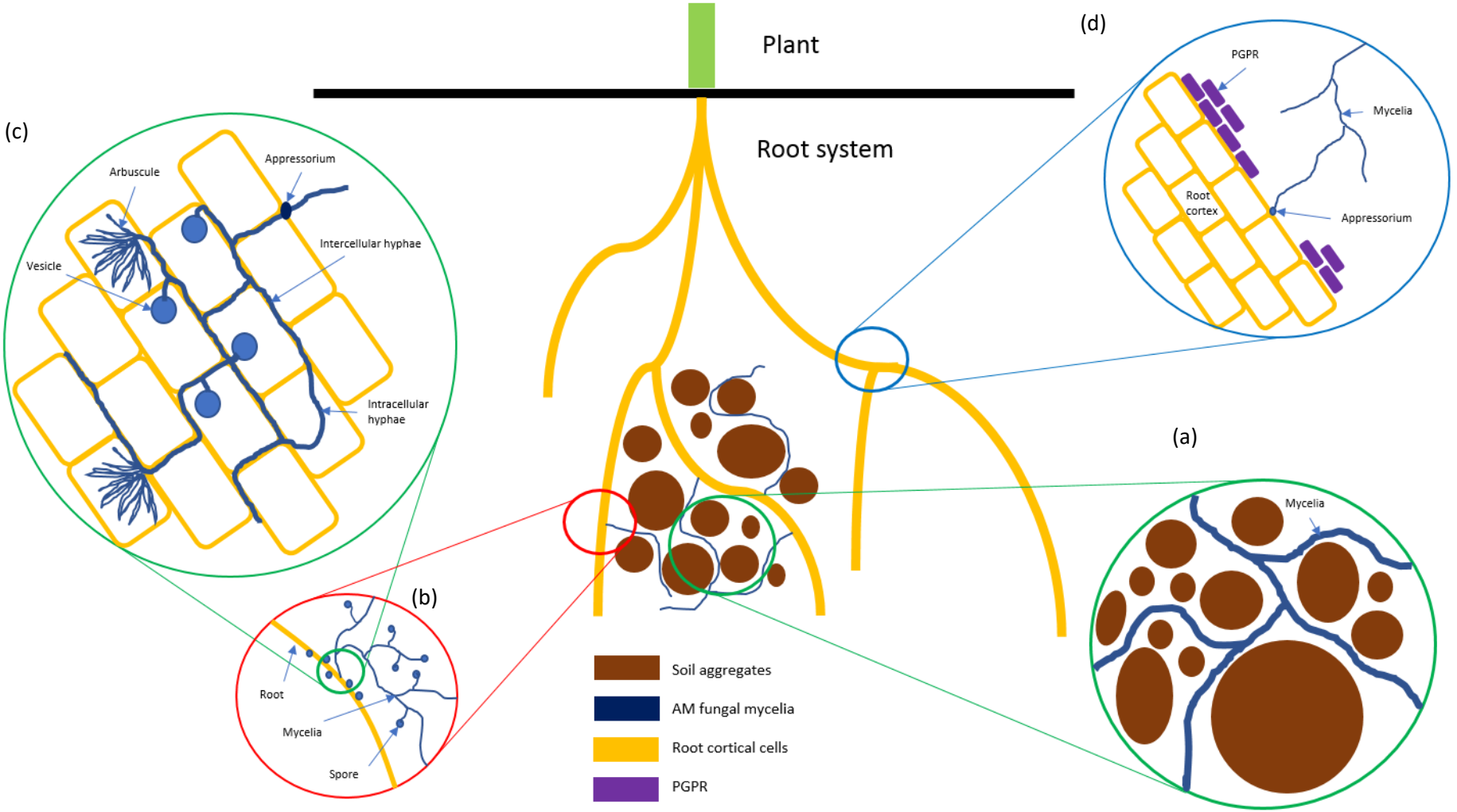

Figure 1.2 – *Diagrammatic representation of the associations between host plant and AM fungi both intracellularly and extracellularly. (a) Extraradiating AM fungal mycelia into the surrounding soils of the rhizosphere producing structural support of soil aggregates with glomalin deposited into the local space. (b) AM fungal growth in association with the host plant root and fungal spores distributed around the site of fungal penetration. (c) Intracellular components of the AM fungal symbiosis with the host plant producing arbuscules, the site of nutrient exchange, and vesicles. (d) Interactions between PGPR and AM fungi for the initial colonisation of a host root with AM fungi.*

1.2 Plant-AM fungus symbiosis

Through the establishment of a host-AM fungus relationship (Figure 1.1d), soil nutrients can be provided to the host via intracellular AM fungal arbuscules in exchange for photosynthetic carbohydrates. Current estimates suggest 20% of photosynthetically acquired hexose carbohydrates are provided to AM fungi (Lerat et al., 2003).

Soil nutrient availability can alter the establishment of symbiosis. Soils deficient in nitrates produce plants with increased root exudation and encourage further AM fungal root colonisation and symbiosis (Blanke et al., 2011). The reverse is additionally true, soils high in nitrates reduce the overall AM fungal level of symbiosis. Further to this, studies have been able to show that nutrient deficient soils, producing higher levels of host-fungus interactions, result in more carbon exchange via arbuscular structures giving strong indications that a mutual relationship between plant and fungus exists through symbiosis (Blanke et al., 2011; Olsson et al., 2010; Bucking and Shacher-Hill, 2005).

1.2.1 Nutrient exchange

1.2.1.1 Carbon

AM fungi require carbon compounds, in the form of carbohydrates, as part of their life cycle. They are, however, not able to produce the required carbohydrates alone. This brings about the need to establish close relationships with a host to provide the needed carbohydrates. Smith and Read (2008) showed this can be achieved through indirect means from increases in extraradical mycelia after a root has been colonised and forming symbiosis with several plants at any one time. Wang et al. (2017) produces an in-depth review of direct carbohydrate exchange from host to fungus. The movement of carbohydrates is regulated by the plant and, in part, by the fungus. Sucrose, the photosynthetically produced carbohydrate, is transported to the root tissue via the phloem. Within these tissues, sucrose is cleaved into glucose and fructose (Bago et al., 2000) AM fungi do not prosses the ability to cleave sucrose to glucose and fructose, so must be performed via the host (Wang et al., 2017; Schubert et al., 2004). From carbon sink studies of Doidy et al. (2012), it was shown that increases in root mycorrhizal colonisation causes the host plant to increase transported carbohydrates to root tissue. Whilst only 20% of photosynthetically produced carbohydrates have been shown to be provided to AM fungi via arbuscular structures in cortical root cells

(Lovelock et al. 2004; Vierheilig et al. 2005), the increase in transported carbohydrates reported by Doidy et al. (2012), however, still did not exceed 20% of total produced photosynthetic carbon of the host as previously reported (Schubert et al., 2004). From this, it can be drawn that host biomass and carbon dioxide intake must increase to maintain the demands of both the plant and the symbiotic AM fungi.

Bonfante and Anca (2009) showed changes in expressed membrane transporter proteins from transcribed regions of DNA stimulated from fungal colonisation and arbuscular formation. Sucrose transporter membrane proteins (SUT) and monosaccharide transporters (MST) have been shown to increase in fungal colonised root cells. However, these transport structures have also been seen to increase in the phloem, a region of root tissue that is not directly colonised by AM fungi (Wang et al., 2017). This, therefore, reinforces the notion of plant responses to AM fungal interactions being whole plant rather than a localised phenomenon. From carbohydrate exchange in root tissues, carbohydrates must be transported from intraradical mycelia to extraradical mycelia for the continued growth and development of branching hyphae and spores in soils. To achieve this, plant provided sugars are enzymatically converted into lipids, the main carbon storage molecule in AM fungi (Becard et al., 1991)., stored within vesicles additionally found within root tissues (Vierheilig et al., 2005). Bago et al. (2003) found the conversion of carbohydrates to lipids, back to carbohydrates, was only 50% efficient.

1.2.1.2 Nitrogen

The supply of nitrogen in soils, without fertiliser application, has been known to be a plant growth limiting factor (Crawford and Glass, 1998). Many studies involving the provision of nitrogen favour methodologies of labelled N^{15}. Johansen et al. (1996) were one of many that followed labelled nitrogen from soil, absorbed into extra radiating mycelia and incorporation into amino acids via the glutamine synthetase, glutamine oxoglyutarate aminotransferase (GS/GOGAT) cycle in the formation of arginine (Wang et al., 2017)

AM fungi are able to transfer organic nitrogen, NO_3^- and NH_4^+, to host plants and also increase soil organic nitrogen through organic matter decomposition (Hodge et al., 2001). From N^{15} labelled soil nitrate sources, it was shown that 21% of extra radiating mycelial sourced nitrogen was exchanged within root structures (Toussaint et al., 2004). It was then

later shown that between 30 – 50% of fungal acquired nitrogen was exchanged at root sites, with an estimated 75% reaching leaf tissue (Tian et al. 2010, Govindarajulu et al., 2005). Govindarajulu et al. (2005) was able to show the movement of arginine to intra radiating mycelia and its breakdown into urea and ornithine. The utilisation of urea during breakdown produces ammonia gas, and contributes to nitrogen loss from soils. However, most ammonia is released into the plant across arbuscular exchange sites, where ammonium transporters in AM fungi (GintAMT1, GintAMT2, and GintAMT3 in *R.irregularis*) are expressed in established symbiosis (Calabrese et al., 2016). Branches of the arbuscular structure have been found to express GmAMTH.1 and ATM2;3 on the peri arbuscular membrane rather than the 'trunk' of the mycelia (Breuillin-Sessoms et al., 2015). This indicates arbuscule branches are the site of nitrogen transfer. These transport structures are further expressed under low nitrogen conditions to facilitate further nitrogen transport, as well as AM fungal mediated acceleration of organic matter decomposition (Leigh et al., 2009). This strongly suggests reductions in AMF symbiosis at higher soil nitrogen levels.

1.2.1.3 Phosphorus

Phosphorus is a major plant nutrient and is provided to a host by AM fungal branching mycelia, increasing soil uptake surface area of phosphorus, in the form of inorganic phosphate, in exchange for photosynthetic carbon across the peri arbuscular membrane (Figure 1.3). Phosphorus transport has been shown to be induced from the establishment of symbiosis from manipulation of the *Pht1* family of genes responsible phosphate transport (Walder et al., 2016; Javot et al., 2007; Bucher, 2007). High levels of soil phosphate have been shown to reduce gene transcription for strigolactone production (Kobae et al., 2016) indicating reductions in symbiosis from excess soil nutrients. This has also been identified from calcium spiking inhabiting pre symbiosis pathways (Balzergue et al., 2013)

1.2.1.4 Potassium

Potassium is the most abundant element within the soil's composition (Garcia and Zimmerermann, 2014), however, potassium has low availability towards plants and is a growth limiting factor. Potassium also plays a crucial role in several plant functions, including membrane polarisation, plant growth, stomatal aperture functionality, as well as

environmental adaptation (Anschütz et al., 2014). Via particle induced X-ray emission studies (Olsson et al. 2008; Olsson et al. 2011), accumulation of potassium in AM fungi were seen in mycelia, vesicles and spores. Potassium transporter channels from AM fungi to host plant are still under investigation with only four known transports; three SKC-type transport and one KT/KUP/HAK transporter (Garcia and Zimmerermann, 2014; Casieri et al., 2013). Potassium, originating from AM fungi, has been shown to increase plant stress tolerances, especially in response to salinity (Rabie and Almadini, 2005). However, little is known regarding the mechanism of increased stress tolerance or the physiological pathway (Estrada et al. 2013; Garcia and Zimmerermann, 2014; Zhang et al. 2017).

Figure 1.3 – x1,000 magnification, of Vicker's® microscope, from lab controlled Zulu winter wheat growth showing AM fungal arbuscules (red), vesicle (green), peri arbuscular membrane (white arrow) and intracellular hyphae (yellow arrow). Imaged with a Bresser® HD microscope camera and stained with Sheaffer® blue (Wilkes et al., 2019).

1.3 AM fungal mycelia and soil structure

AM fungal mycelia extend into bulk soils, primarily in the search of nutrient sources to acquire for plant growth in exchange for photosynthetic carbohydrates (Bago et al., 2003). However, soil structure benefits from the extensions of mycelia from the formation of more stabilised soil macroaggregates from microaggregates formed from the presence of glomalin along the length of the fungal mycelia as an addition to structural support (Driver et al., 2005). Glomalin, a glycoprotein containing 20 – 30% carbon and remaining in soils in the range of 7 – 42 year (Lovelock et al., 2004) produced by AM fungi (Wright et al., 1996;

Rillig et al., 2002, Lovelock et al., 2004, Driver et al., 2005; Bendini et al., 2009; Adeleke, 2010; Singh et al, 2012; Walley et al., 2014; Wang et al., 2017; Prassad et al., 2018), was first discovered in 1996 by Sara F. Wright in a novel citrate extraction method (Gillespie et al., 2011). Glomalin was initially more difficult to extract and study due to its strong adhesive properties between soil aggregates, hindering its discovery and understanding. The identification and quantification of glomalin was performed via the Bradford assay and from monoclonal antibodies (Mab32B11) from crushed spores of AM fungi (Rillig et al., 2002).

From AM fungal mycelia and soil glomalin, soil stability is increased with corresponding reductions in soil wind and water erosion, resulting in an overall increase to soil quality (Guo et al., 2016). A plot experiment by Li et al. (2012) further explored this from soil inoculants of AM fungi and found increased aggregate stability and a maintenance of a neutral soil pH. This is an example of how soil inoculants of AM fungi improve soil physical attributes, aggregation, through biological means, a key co-operation that defines soil quality (Larson and Pierce 1994; Li et al. 2012). The presence of glomalin is additionally a key component of AM fungal contribution to the improvement of soil quality.

1.3.1 Function of glomalin in soils

Of the 250 species of known AM fungi, an estimated 90% of all species are capable of producing glomalin (Lee et al., 2013). Glomalin produces improvements to soil microaggregates and leads to reductions in soil erosion (Lambardo et al., 2019). Improvements to micro soil aggregates can also be seen from the presence of fungal mycelia that physical bind soil aggregates together, seen in Figure 1.2a. Glomalin has adhesive properties between soil aggregates and particulates, whilst being comprised of an estimated 30 – 40% (USDA, 2002) carbon, 3 – 5% nitrogen, 4 – 6% hydrogen, 30 – 50% oxygen, 0.03 – 0.1% phosphorus and 0.8 – 8.8% iron (Sharifi et al., 2018). Following from this, Glomalin Related Soil Protein (GRSP) comprises a large proportion of SOC (Lovelock et al., 2004), of which is very stable (Riling et al., 2001). Some studies have estimated that glomalin contributes 5% of the total SOC measured within a soil sample (Lovelock et al., 2004), with a range of longevity in soils between 7 and 42 years (Wright and Upadhyaya, 1996). Glomalin's ability to remain in soils for several decades comes about from the water insoluble characteristics and resistance to heat degradation from structural similarities to heat shock protein (Wright et al., 1996). Further studies have additionally been able to show

that increases in atmospheric carbon dioxide can produce increases in soil glomalin concentrations (Zhang et al., 2015; Wang et al., 2017). This is thought to occur from the photosynthetically fixed carbon, in the form of carbohydrates, being transported to root systems and provided to the present symbiotic fungi (Asmelash et al., 2016). From the provided plant acquired carbohydrates, AM fungi utilise this as a source of carbon and produce glomalin (Asmelash et al., 2016; Adeleke, 2010). Glomalin has the added function of structural support for fungal mycelia (Driver et al., 2005), but can be damaged and broken by the application of land management practices such as CT. This had negative connotations for soil quality from the reductions of soil aggregation leading to increased soil erosion from water and wind.

1.5 Plant Growth Promoting Rhizobacteria and AM fungal interactions

Interactions between PGPR and AM fungi can have benefits to overall plant health and productivity. Perez-de-Luque et al. (2017) investigated the interactions of PGPR and AM fungi on host plant immune defences, whilst noting that PGPR-AM fungal interactions were little understood.

Vafadar et al. (2014) investigated PGPR applications (*B. polymyxa, P. putida, A. chroococcum*) with an AM fungus (*G. intraradices*) and found that PGPR applications alone, and in combination with AM fungi, increased root and shoot biomass when compared with an uninoculated control. Furthermore, PGPR applications were noted to increase urease, dehydrogenase and sucrase activity in soils improving overall soil quality (Karthikeyan et al., 2016) in the presence of AM fungi.

Mycorrhizas have been studied to have growth and development assistance from Mycorrhizal Helper Bacteria (MHB). Mycorrhizal Helper Bacteria are associated with root interaction sites as well as radiating mycelia (Klett et al., 2007). Their main functions include, stimulating mycorrhizal formation as well as interact with AM fungi already in symbiosis to further increase fungal biomass and host-fungus interactions (Karthikeyan et al., 2016). Through the interactions of MHBs, root cortical cells are subject to endoglucanase, cellobiose, hydrolase, pectase, hyase and exylanase to assist fungal penetration (Karthikeyan et al. 2016; Labbe et al. 2014; Frey-Klett et al. 2007; Duponnois 1992).

Rahman et al. (2011) studied the effects of *B. subtilis*, in combination with AM fungi, on plant development and nutrient transfer to the fungal host plant. Results showed that plant height and tillers where increased along with improvements to nitrogen, phosphorus and potassium availability, however, reduced sodium was noticed and contributed to mitigation of salt stress.

1.5.1 PGPR influence on glomalin production

Associations between PGPR and AM fungi have been the topic of several studies in relationship to increasing soil carbon, via increased production of glomalin, to further improve soil quality (Apesteguía et al., 2017). Soil inoculants of PGPR, for the improvement of AM fungal produced glomalin, can be accomplished via complimentary applications of PGPR. The inoculation of *Acinetobacter junii* to tomato and bell pepper crops, Padmavathi et al. (2015) was able to demonstrate the increase to crop biomass, AM fungal root colonisation and glomalin in controlled environmental growth conditions. Adeleke (2010) inoculated wheat (*Triticum aestivum*) with several PGPR isolates: *Pseudomonas cepacian, P. aeruginosa, P. putida and P. fluorescence* and found increases to crop biomass with some individual inoculants, but othera were antagonistic against AM fungi leading to reduced quantities of glomalin. Further, Adeleke (2010), under aseptic conditions co-inoculated plants withall PGPR *Pseudomonas* spp. together with AM fungi and showed an increase in glomalin; however, co-inoculation in non-sterilised soils this increase was not observed

1.6 Tillage

Through the application of invasive conventional land management practices, such as conventional tillage (CT), there is building body of evidence to suggest the inability of AM fungi to survive the homogenisation and aeration of a soil profile (Sosa-Hermandez et al., 2019). Kabir (2005) identified that the employment of intensive tillage practices is a major reducing factor of AM fungal abundance and diversity, with later suggestions by Sosa-Hermandez et al. (2018) to move away from such intensive practices as CT. Sale et al. (2015) investigated the diversity and abundance of AM fungi via soil spore analysis to a depth of 40cm from a reduced tillage (RT) and CT system. Their findings supported the work of Kabir (2005) showing a reduction in abundance and diversity from the application of an invasive

soil management regime. However, Sale et al. (2015) also was able to show that the diversity of abundance of AM fungi was greater in soils deeper than 40cm. Within the UK, CT typically inverts soil to a maximum depth of 20cm (AHDB, 2020) but can invert soils to a maximum depth of 30cm with a plough pan found between 30 – 40cm deep. Findings of Kabir (2005) and Sale et al. (2015), support the view that CT has a negative effectby damaging and fragmenting the soil AM fungal community and thereby reducing the symbiotic nature of AM fungi. A potential solution for this is to manage soils through a zero tillage (ZT) practice, a practice that removed the mycelial damaging soil inversion. Compared with soil inversions of CT, ZT has very little to no soil disturbance. A possible drawback of a ZT system is that it increases in soil bulk density making the, use of agrochemicals for weed control and the reduced mobility of nutrients and fertilisers through a soil profile more problematic (Sosa-Hermandez et al., 2019).

Table 1.2 provides a comparative description between tillage types and their impacts on soil. From the management of soils, resultant of CT, soil aggregation is reduced and leads to increases in soil erosion by means of wind and water (Zheng et al., 2018). Whereas in ZT soils, reduction of erosion and aggregation is seen. From improved aggregation, the decomposition of crop residue is protected (Zhao et al., 2018), whilst having an additional benefit of improving soil aggregation (Sun et al., 2018; Lu et al., 2018). Cittenden et al. (2015) investigated the importance of tillage management on the stability of soil aggregates and found soil organic carbon (SOC) serves to increase aggregation, whilst Sheehy et al. (2015) were able to show SOC has greater aggregate stabilising properties for macroaggregates and should be used as an indicator for carbon loss resultant of tillage management practices. Through a series of longer term experiments, improvements of ZT have been seen to increase micro-aggregates and improvements to total SOC levels (Dai et al., 2015; Lu et al., 2018). This leads to reductions in soil erosion from wind and water, with reductions from carbon dioxide emissions additionally seen.

Table 1.2 – Summary of select tillage types and their associated mechanical impact characteristics (Ita et al. 2014; Moussa-Machraoui et al. 2010; Moroke et al. 2009; Grange et al. 2005; Stanila et al. 2013; Saglam et al. 2020)

Tillage type	Equipment employed	Tillage characteristics	Impact
Conventional	Mouldboard plough	• Maximum 30cm soil inversion • Loosens and aerates soil • Buries crop residue from previous crop	• Reduces AM fungal abundance and diversity • Increase in soil erosion • Reduce soil carbon and increase carbon dioxide emissions • Increases in soil aeration • Reduction of soil bulk density in zone of tillage • Reduces weeds • Reduces select fungal pathogens
Reduced	Rotary disc	• Maximum 15cm soil disturbance	• Reduced soil erosion compared to conventional tillage • Not always effective against weed reduction
Strip	Specialist equipment • Disk opener • Coulter	• Maximum 15cm soil disturbance • Only soils to be drilled are disturbed • Soil drying effect of conventional tillage	• Weed reduction can be difficult • More passes of heavy equipment may be required • Removal of a cover crop may be difficult • Aerates top soil

			• Soil carbon, nitrogen and phosphorus are lower than zero tillage
Zero	Direct seed drill	• Direct seed drilling into top soil • Little to no soil disturbance • No suitable for soils with poor water drainage • Crop residue remains on surface of soil	• Increases AM fungal abundance and diversity • Decreases in soil erosion • Increase soil carbon • Increase in soil bulk density • Increase in select fungal pathogens • Agrochemicals required to reduce weeds

1.6.1 Tillage and soil quality

The application of CT, through the preparation of a seed bed via a mouldboard plough, reduces AM fungal abundance and, therefore, reduces the amount of mycelia available to receive stimuli from plant strigolactone production (Brito et al., 2012), as well as modifying soil chemical, physical and biological properties (Apesteguía et al., 2017), the attributes of which are included in the calculation of soil quality. Many investigations into soil study the effects of tillage by comparing CT practices with ZT practices. Physical soil attributes, such as bulk density, aggregation and aeration, are directly influenced by tillage (Hajabbasi et al., 2000) and have been reported to be largely detrimental to overall soil quality through the loss of aggregation and reduction in bulk density (Osunbitan et al., 2005). To counter this, ZT land management aids in the maintenance of soil aggregation (Teodor et al, 2011). However, bulk density is increased with reductions to soil aeration to similar depths as inverted soils in CT (AHDB, 2020).

The influences of tillage towards AM fungi have been discussed previously, however, the alterations to AM fungi resultant of tillage is best explored through the detrimental impacts to soil's aggregate stability and the establishment of AM fungal host symbiosis.

1.6.1.1 Tillage and Water Stable Aggregates

An increasing body of research exists for the effects of tillage on Water Stable Aggregates (WSA) via tillage implications to glomalin. Wilson et al. (2009) noted high correlations between soil aggregation and carbon sequestration with increases in AM fungal abundance. Lovelock et al. (2004) produced estimates of between 3% and 5% of soil carbon and nitrogen, respectively, is attributed to glomalin. Hontoria et al. (2009) studied differences between CT and ZT systems in respect to WSA and extractable, Bradford reactive, glomalin. Hontoria et al. (2009) were able to show ZT had nearly a third higher stabilised soil aggregates and 3 to 5 times more extractable glomalin.

Sharifi et al. (2018) produce experimental rationale that glomalin had a greater influence on improved soil aggregation than organic matter contents. Additionally, Shafiri et al. (2018) were able to show a positive correlation between glomalin and WSA. This was also shown earlier by Curaqueo et al. (2011). Whilst tillage does influence the amount of glomalin within soils, the plant species to which hosts AM fungi alters the quantity of

glomalin deposited in soils. Therefore, crop type can improve or reduce initial glomalin levels, and relate to changes in WSA. However, the application of tillage can reduce glomalin through microbial metabolism. This not only reduces soil carbon, but reduces aggregate adhesion leading to increases in soil erosion.

Nautiyal et al. (2019) studied the influence of glomalin in regards to its contribution to soil carbon stores and was able to show a significant positive relationship with organic carbon contents of soils ($P < 0.001$) and aggregate stability. This conforms to the "glue" description of glomalin, by Wright and Upadhyaya (1996), acting on soil aggregates aiding in the formation of macroaggregates from microaggregates. Whilst the reported findings of Nautiyal et al. (2019) are important, Nautiyal et al. (2019) is unable to consider the effects of tillage and land management practices on the quantities of glomalin in soils and alterations to glomalin's adhesive properties. However, Sheehy et al. (2015) produces direct comparisons between soil aggregates of three tillage practices (conventional, reduced and zero till) and is able to give clear indications that zero till practices maintain a greater proportion of macroaggregates, contribution to more stable soils and erosion resistant, than soil aggregates in conventional till. Sheehy et al. (2015) additionally commented on the percentages of soil organic matter between the tillage practices and noted zero till to have greater soil carbon when compared to conventional and reduced till, with conventional till having the lowest quantifiable soil carbon. Ample literature is able to comment and explore the effects of tillage on soil aggregation or soil carbon stores between differing types of applied tillage, however, very few studies have been able to comment and produce rationale regarding the direct influence of tillage against glomalin and how such an influence relates to soil aggregation with potential suggestions for which tillage practice produces the greatest benefits to overall soil quality. Shariffi et al. (2018) explored the relationship between glomalin and tillage or fire land management in the Zagros forests of Iran. The tillage depth if Shariffi et al. (2018) is equivalent to CT in European countries. However, Sariffi et al. (2818) was unable to comment on the effects of such agricultural practices on soil aggregates but were able to conclude that applying tillage to soils did reduce the amount of quantifiable glomalin with it the system. Wright et al. (2007) produced one of the few studies that attempts to compare tillage on glomalin and aggregate stability of soils. The results of Wright et al. (2007) indicate ZT constantly increased quantities of glomalin and

soul aggregates compared with CT treated soils. However, the presented data does not give a clear indication whether or not there is a significant difference between ZT and CT treatments through the relationship between glomalin and soil carbon. Therefore there is highly contradictory evidence throughout much of the literature as to the nature of the effects between ZT and CT tillage practices on soil aggregates and glomalin. For example, Hontoria et al. (2009) in a study of olive groves in central Spain, shows it is difficult to directly compare their study with a perennial plant against the majority of literature that has focused upon arable cereal cultivation. Whilst Hontoria et al. (2009) concluded that glomalin had a positive influence on soil aggregation, they was not able to produce data with sufficient statistical support, and had to leave the connection between glomalin, tillage and soil aggregates open to question that required further investigation.

1.6.1.2 Tillage and AM fungal symbiosis

AM fungal mycelia act as the primary inoculum of host plant roots (Bernola et al., 2018). Applying tillage to soils breaks the delicate mycelial networks (Brito et al., 2012). Applying CT levels of soil inversion homogenises a maximum depth of 30cm, 20cm typical in the UK, dramatically fragmenting mycelial networks across depths resulting in a detriment to AM fungal survival and a tendency to reduced oxygen content whereby soils become increasingly anaerobic (Kapoor, 2017). The dilution of mycelia causes delays in root colonisation (Douds and Nagahashi, 2000) as the network of hyphae needs to redevelop. Additionally, reduced levels of resources will be available to the fragments of mycelia and can result in the death of many mycelial fragments further reducing the amount of AM fungi in CT soils (Castillo et al., 2006). Furthermore, this has negative detrimental implications towards soil aggregation.

Anaerobic and facultatively anaerobic soils do not provide adequate environmental conditions to support a population of AM fungi, however, a population of AM fungi can be found in such non-optimal conditions from the establishment of associations with a host plant root network that acts as a life support system at soil depths greater than 50cm. Such a life supporting role of the host plant comes about from the provision of plant derived carbohydrates and oxygen supplied via the root system (Bernola et al., 2018).

ZT practices do not damage the mycelial networks in top soils (<10cm) allowing them to remain intact and able to begin root colonisation for host-fungal symbiosis (Wang et al.,

2017). Studies by Galvez et al. (2001) and Sharma-Poudyal et al. (2017) are able to show agricultural land management practices of a more conservation orientation, away from CT, have positive growth implications for soil fungi in both abundance and diversity. Sharma-Poudyal et al. (2017), via molecular identification methods, specifically names *Humicola, Cryptococcus, Cadophora* and *Hydondontaceae* as being more abundant in ZT practices compared to CT sampled soils. Whilst these are part of a wider soil microbiome, they are not, however, classified as AM fungi.

Through the work of many others, the reduction of a diverse soil fungal community has been reported (Akiyama et al., 2008; Driver et al., 2005; Bonfante and Acna, 2009). This focuses primarily on fungal biomass within soils. An example of a fungal biomass marker, used for biomass estimations, comes about from ergosterol. Ergosterol acts as a cell membrane stabilising and support molecule in a similar way to the functions of cholesterol in mammalian cell membranes (Weete et al., 2010). Biosynthesis of ergosterol has high demands on AM fungal metabolism but is off set by ergosterol's ability to enable the fungus to withstand climatic conditions and variances (Dupont et al., 2012).

1.7 Rationale and objectives

Employment of a tillage regime based on soil inversion shows it to be effective against emergent weeds and the control there of, with the application of glyphosate in zero till providing an alternative to soil invasion weed management. The implications of reduced tillage on AM fungi is still an emerging area of understanding with a sparse number of studies reporting on its effects towards AM fungal mediated relationships, as well as key components such as the relationship between glomalin and soil aggregation. Understanding these relationships is fundamental to maintaining a sustainable soil agroecosystem.

The alterations of AM fungal-plant host symbiosis, resultant of tillage, has implications to reduce nutrient acquisition and soil physical attributes with further implications towards crop biomass and development. Comparisons between CT and ZT managements practices have yet to be explored in relation to AM fungal-host relations and has potential to have critical impacts AM fungal diversity and abundance. A small number of published works are able to link arbuscules, and other intracellular fungal components of symbiosis, with fertiliser applications, suggesting fertiliser type can hinder or improve fungal

symbiosis to some degree (Balzergue et al., 2013; Battaglin et al., 2005; Battini et al., 2017), however, the implications of tillage are not discussed.

Furthermore, tillage strongly influences the soil microbiome via alterations of aeration through a soil profile. Such a change in soil microbiota further impacts the microbial associations with AM fungi and the measured effects of AM fungi as mediated by constituent soil microbes. Published literature has considered rhizobacteria and PGPR effects on crop yields (Sharma et al., 2019), however, implications of tillage towards the abundance and diversity of rhizobacteria and PGPR are not reported to the same degree, nor are their associations and interactions with AM fungi in an arable setting.

The present project aims at addressing the resultant influences of tillage against AM fungal populations and associations with a host crop in arable soils, along with their chemical and physical contributions to soil properties. This will, in part, be determined from the assessment of the relationship between AM fungi biomass, glomalin and water stable soil aggregates in a soil inversion tillage practice, such as CT, and a conservative practice such as ZT. A further determination of the influences of tillage towards AM fungi will be demonstrated through the quantification of intracellular root cortical AM fungal arbuscular structures, the impact of present AM fungal host root associations on overall root length, as well as through the evaluation of compounds exuded by the roots in the recruitment of a rhizosphere microbiome community. Within this root rhizosphere community, it is hypothesised that the influences of rhizobacteria to promote and/or inhibit fungal growth and its host associated properties is important. The testing of this hypothesis however has not as yet been addressed in the published literature. Therefore, the present project will also aim to address the interactions between selected rhizobacteria and AM fungi in relation to plant growth, as measured through above and below ground biomass, and the degree of intracellular AM fungal root structures as mediated by rhizobacteria along with the persistence and presence of select rhizobacteria correlated to quantifiable AM fungal attributes.

To further assess the aims of the project, selected hypotheses will be tested for the determination of the effect's tillage has upon AM fungal populations within arable soils. These include the evaluation of glomalin as an indicator of AM fungi and, in conjunctions

with quantified ergosterol, to be used for the purpose of AM fungal biomass quantification. Further hypotheses to be tested are as follows:

1. That conventional tillage will have a detrimental effect on AM fungi, which will reduce wheat productivity via biomass differences, compared to zero tillage.
2. Glyphosate used in zero till management is detrimental to AM fungi irrespective of tillage application.
3. The application of select rhizobacteria will affect AM fungal colonisation of the root differently in soils of different tillage managed soils.

2.0 Methods

The present study incorporates the investigative approaches of three experimental chapters (Chapters 3 to 5). Each chapter aims to ascertain the influences of tillage and their respective land management practices upon soil AM fungi, with implications of fungal abundance and diversity on soil chemical and physical properties between tillage treatments. Effects exerted by AM fungi towards crop growth from each respective tillage practice will also be assessed.

Two farms in Hertfordshire, with similar climatic conditions and crop rotations were selected with their main variable being the tillage in which they practice (CT or ZT). Soils were sampled in 2 fields of winter wheat plus grass headlands within the same field boundary, acting as control samples (CTC (CT control) and ZTC (ZT control)), at four depths (0-40cm) in a regular grid over a period of 12 months to determine if there are differences in AM fungal populations between tillage treatments, and if seasonal variation exists. Soil samples were analysed in the laboratory to determine soil physical and chemical properties with potential to influence AM fungi populations: bulk density, soil texture, soil moisture, pH, and nutrients (nitrogen, phosphate and potassium). Glomalin was used as a proxy indicator of AM fungal growth, and ergosterol was assessed as an indicator of fungal biomass. Experimental approaches employed for each chapter investigation are given in Table 2.1.

Table 2.1 – Summary of the respective analytical method from field sampled soils, laboratory analysis and manipulative experimental protocols in the determination of the influences upon AM fungi and their respective contributions to soil chemistry, physics and biology result of applied tillage and land management practices between conventional (CT) and zero tillage (ZT) treatments.

Chapter	Experimental approach	Description of experimental approach	Experiment location	Section
3	Field sampling	Gather arable soils from CT and ZT treatments for comparatives between the land management practices	Field	2.1
	Soil texture	Confirm the soil texture given in farmer's records via soil texture triangle	Laboratory	2.2.1
	Moisture quantification	Oven dry soils	Laboratory	2.2.3
	Organic matter by loss on ignition	Heat soils in muffle furnace to 400°C for 18 hours	Laboratory	2.2.4
	Nitrogen, phosphate and potassium (NPK) testing	Measure nitrogen (pre made HACK 339 testing kit), potassium and phosphate in arable soils	Laboratory	2.2.6
	Water Stable Aggregates	Separate micro and macroaggregates from sampled soils of each tillage treatment	Laboratory	2.2.7
	pH	To determine the relationship between pH and correlate soil pH with ergosterol and glomalin quantities	Laboratory	2.2.8
	Bulk density	Mass of sampled soils of known volume	Laboratory	2.2.9
	Ergosterol HPLC to determine ergosterol	Measure the total amount of live fungi within soils at each sample depth and location	Laboratory	2.3.3
	Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein as an indicator of soil AM fungi population	Laboratory	2.3.5 and 2.3.6

	Seed washing	Remove seed surface fungal and bacterial contaminants	Laboratory	2.2.5
4	Fungal identification	Identify soil isolated fungi	Laboratory	2.3.2
	Ergosterol HPLC	Measure the total amount of live fungi within soils	Laboratory	2.3.3
	Root staining with Sheaffer blue	Quantify intracellular mycorrhizal fungal structures in root cortical cells	Laboratory	2.3.4
	Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein	Laboratory	2.3.5 and 2.3.6
	In vitro glomalin production to soil pH	Quantity soil glomalin and fungal biomass over a soil pH range	Laboratory and growth cabinet	2.3.3, 2.3.5 2.3.6 and 2.3.7
	Fungal influence of root length	AM fungal influence on root length from established AM fungal-host relationship between different nutrient sources	Growth cabinet	2.3.8
	Root exudate HPLC	Rhizodeposited compounds from winter wheat into rhizosphere soils	Laboratory	2.3.9
	Glyphosate	Growth inhibition on non target organisms	Glasshouse	2.4.4
	Controlled growth conditions	Maintain a constant environment for the sustained growth of winter wheat	Glasshouse and growth cabinet	2.4.1
5	Fungal identification	Identify soil isolated fungi	Laboratory	2.3.2
	Ergosterol HPLC	Measure the total amount of live fungi within soils	Laboratory	2.3.3
	Root staining with Sheaffer blue	Quantify intracellular mycorrhizal fungal structures in root cortical cells	Laboratory	2.3.4
	Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein	Laboratory	2.3.5 and 2.3.6

	Rhizobacterial organisms	Known rhizobacteria from a commercially available mixture	Laboratory	2.3.10
	Identification of *Bacilli* spp. rhizobacteria	API identification of *Bacilli* spp. from Section 2.3.10	Laboratory	2.3.11
	qPCR of rhizobacteria and AM fungi	Presence and persistence of selected rhizobacteria and AM fungi in sampled soils	Laboratory	2.3.12
	Glomalin and root mass	Glomalin as a proxy indicator of AM fungal biomass associated with wheat roots	Laboratory	2.4.2
	Nutrient applications and root arbuscules	Influence of nutrient type, as fertiliser applications, with AM fungal root cortical cells	Glasshouse	2.4.3 and 2.3.4
	Rhizobacteria and glomalin	Rhizobacterial influences on AM fungal biomass determined via glomalin quantification	Glasshouse	2.4.5
	Rhizobacterial and root arbuscules	Rhizobacterial influences on AM fungal-host interactions	Glasshouse	2.4.6
	Rhizobacteria and ergosterol	Rhizobacterial influences on AM fungal biomass determined via ergosterol quantification	Glasshouse	2.4.7
	Rhizobacterial and AM fungal inoculant to Zulu variety winter wheat	Inoculant induced AM fungal-host association effects on wheat biomass between CT and ZT	Glasshouse	2.4.8

2.1 Sample sites

2.1.1 Study 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—same weather parameters as Farm A) (UK Meteorological office, 2018). Both were commercially managed farms, with current management undertaken over a minimum period of 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® 750A direct drill, Illinois, USA). Farm A had practiced conventional tillage exclusively for over 10 years, whilst zero tillage was practiced exclusively for 8 years on Farm B. It is acknowledged that differences between management regime were not exclusively limited to tillage, such as the use of glyphosate (360g L^{-1} active ingredient, 3.0L ha^{-1}, CleanCrop Hoedown® (Agrovista UK Ltd., Nottingham, UK)) in ZT land management and not in CT practices.

Each field in Farm A and B was selected based on the presence of a comparable crop history, and type and application rate of supplementary crop nutrients, with both farms following RB209 (AHDB, 2018) recommendations for winter wheat growth and development.

2.1.2 Sampling approach

A regular 50m grid was constructed for sampling fields of both CT (Figure 2.1 – 65 field and 5 control sampling points) and ZT (Figure 2.2 – 54 field and 5 control sampling points), with 260 and 216 samples for each tillage practice respectively across 10, 20, 30 and 40cm sampling depths per sampling period. Soils were sampled at regular intervals throughout the growing season between September 2018 to August 2019 (n = 864 (ZT); n = 1,040 (CT) over the growing year). All soils were sampled with a Dutch auger, with each soil sample accounting for a volume of 450cm^3.

2.2 Determination of environmental parameters

2.2.1 Soil texture

Soil texture was determined by the methodology described by Brown and Wherrett (2015). In brief, 50g of soil previously passed through a 2mm sieve mesh, were suspended in a 1:2 ratio of soils and water (w/v) in a pre-weighed beaker. The suspension was agitated for 30 seconds to ensure all particles were homogenised throughout. The suspension was allowed to rest at room temperature for 30 seconds before liquid being decanted into a second pre-weighed beaker. The first sample was dried at 60°C and is representative of the sand fraction of soils. The second beaker was allowed to rest for a maximum of 30 minutes, and indicative of the silt fraction. Remaining liquid was decanted into a third beaker and discarded (clay fraction), whilst the second beaker was dried at 60°C until dry. Once samples had dried and been re-weighed, percentage representation of total sample was calculated. Clay fractions were calculated from the remaining percentage after determination of sand and silt fractions. Percentages were compared against the soil texture triangle Brown and Wherrett (2019).

2.2.2 Meteorological conditions

Meteorological data was requested from Rothamsted Research e-RA archive for periods between August 2018 and September 2019, with focus on soil temperature under grassland and bare soils corresponding to the sampled permanent grass margin control and within crop areas respectively, at depths of 10, 20, 30 and 50cm.

2.2.3 Moisture quantification

A total mass of 100g sampled soils were placed into a pre-weighed aluminium trays and allowed to dry to completion within a drying oven set at 60°C for 18 hours. Drying to completions was tested using a single sample and weighing at 2 hour intervals until a consistent weight was recorded a total of 3 times.

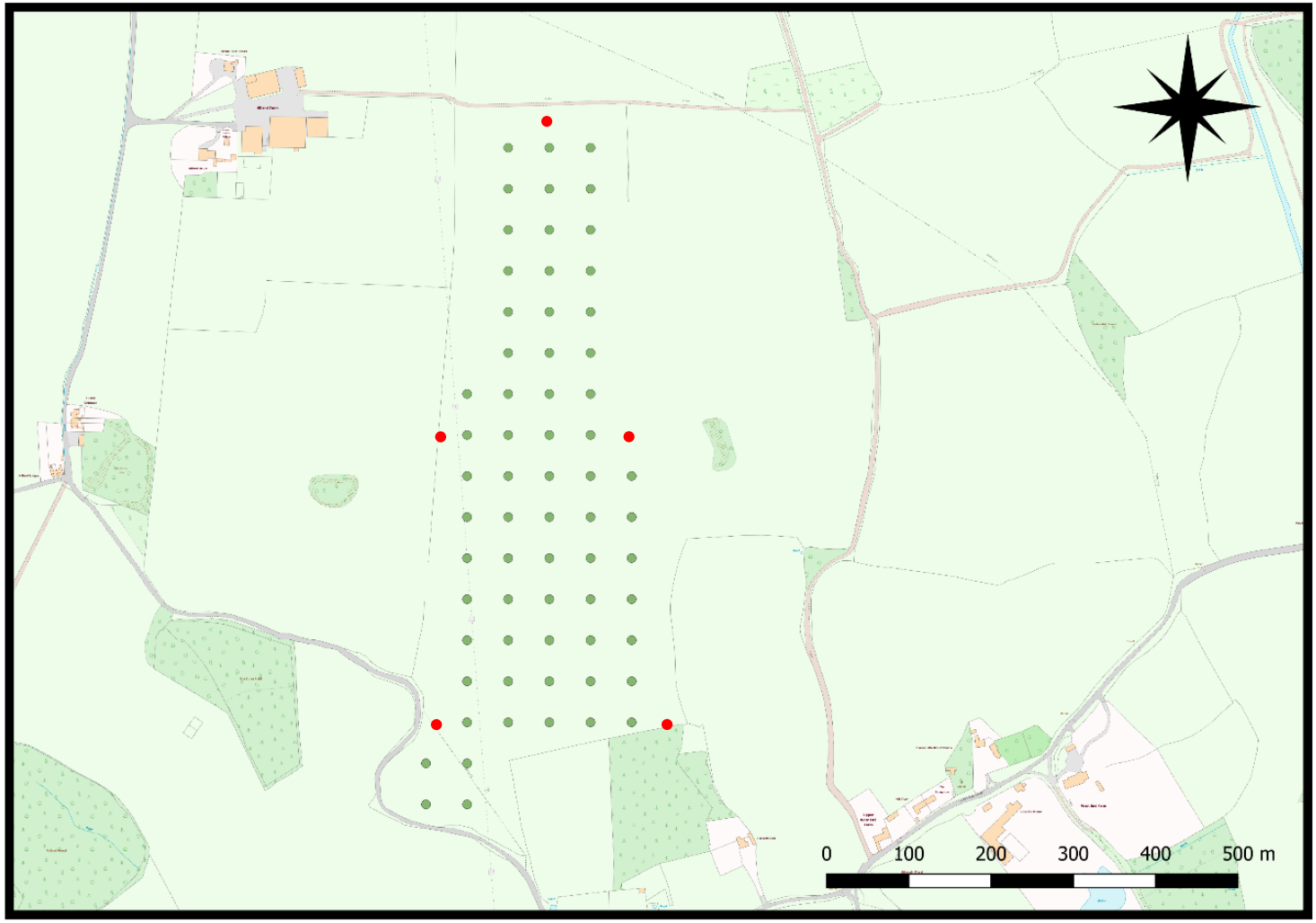

Figure 2.1 – Sampling map of Conventional Tillage site (Farm A – located in central Hertfordshire) with 64 field sample points (green), constructed from a 50 metre grid, and 5 control sample point (red). Control samples were selected by their proximity to field sample points, whilst remaining exterior to the zones of tillage treatment.

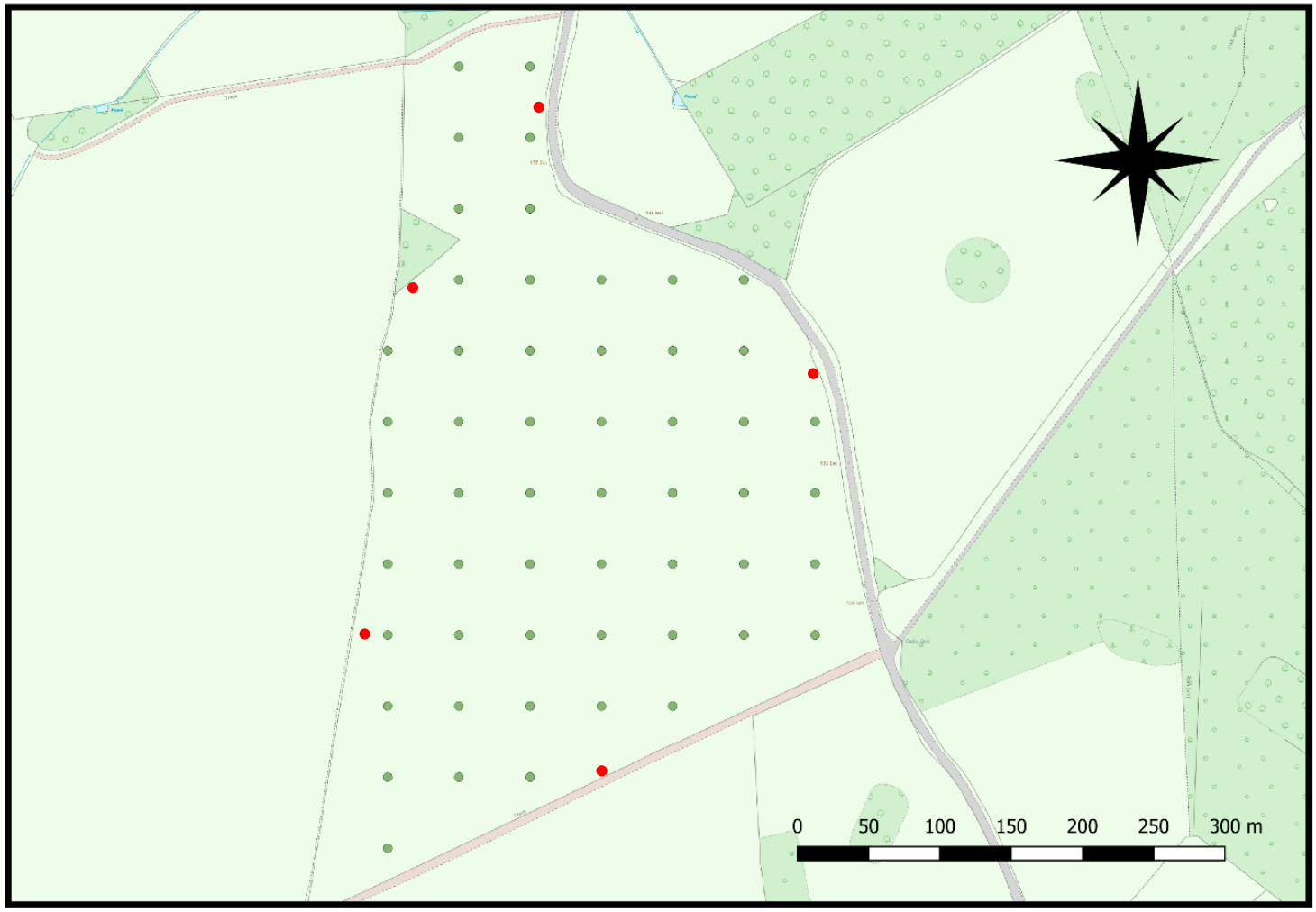

Figure 2.2 - Sampling map of Zero Tillage site (Farm B – located in central Hertfordshire) with 54 field sample points (green), constructed from a 50 metre grid, and 5 control sample point (red). Control samples were selected by their proximity to field sample points, whilst remaining exterior to the zones of tillage treatment.

2.2.4 Organic matter analysis via Loss on Ignition

A modified Loss on Ignition (LOI) method from Myrbo et al (2001) was adopted as follows; 5g of dried field sampled soils were weighted out into crucibles and left in a muffle furnace at 400°C for 18 hours. After sufficient time had elapsed, allowing the furnace to cool, samples were re-weighed and percentage difference was calculated.

2.2.5 Seed varieties

Winter wheat (variety: Siskin) 98% germination rate and no chemical pre-treatments was supplied by KWS, UK Ltd. A second winter wheat variety (Zulu) was sourced from Farm A as farm saved seed. These were used throughout all experimental approaches.

2.2.6 Soil nutrient testing

2.2.6.1 Nitrogen

Nitrogen quantification was performed using 5g of dried soil and 0.2g calcium sulphate in a total volume of 20ml deionised water, whilst being agitated at regular intervals for 30 minutes. After filtration, 1ml of filtrate was used with HACH LCK 339 nitrate testing kits. Samples were prepared and analysed via manufacturer's instructions.

2.2.6.2 Phosphate

To 20ml phosphate extraction solution (Appendix 8.2), 1g of dried soils was added and shaken at regular intervals over a 30 minute period. After filtering the soil suspension, 10ml of filtrate was added to 20ml deionised water and 2ml molybdate solution in a new clean beaker, with an addition of a single spatula of ascorbic acids in excess, before heating to the boil over a Bunsen flame. A blue/green colour change was observed in samples containing phosphate.

After samples had cooled to room temperature, photospectrometry (model: Cecil 1021) at a wavelength of 650nm absorbance was conducted, using deionised water to reference the photospectrometer. A standard curve was constructed using known phosphate concentrations.

2.2.6.3 Potassium

Ensuring soils have been sieved through a 2mm mesh, 1g of dried soil was placed in a 100ml conical flask with the addition of 20ml extraction solution (Appendix 8.3). Agitate the soil and extraction solution for 60 seconds to produce a soil suspension. After allowing the suspension to settle for 20 minutes, agitation and rest stages were repeated twice more. A final agitation of the suspension was carried out before filtering through Whatman No.1 filter paper. If filtrate was cloudy, or contained soil debris, a single spatula of activated carbon was added prior to agitation and re-filtering. Filtrate was adjusted to a pH between 5 and 7 with sparing additions of 32% sodium hydroxide solution. A total of 30ml of 2:1 EDTA disodium salt and 20% formaldehyde respectively was added for 3ml of pH adjusted filtrate in test tubes and vortexed briefly at maximum speed. Sodium tetraphenylborate (1ml 5% w/v) was added and vortexed for 30 seconds at maximum speed. Extraction solution was used as a reference point during photospectrometry (model: Cecil 1021) at a wavelength of 690nm absorbance. Spectrograph readings were compared to a constructed standard curve of known concentrations.

2.2.7 Percentage Water Stable Aggregates

Dry soil (100g) was passed through a 0.2mm sieve with sparing amounts of deionised water sprayed over the surface. The following equation was used in order to determine the total percentage of stable aggregates:

$$\text{Equ 1: Water Stable Aggregate } (\%) = \frac{Mass\ of\ soil\ remaining\ on\ sieve\ (g)}{Total\ mass\ of\ soils\ origianlly\ placed\ on\ sieve\ (g)} \times\ 100$$

2.2.8 pH

A 1:1 ratio of deionised water to dried soils was produced and allowed to stand for 30 minutes with regular agitation. A calibrated Hannah pH probe was submerged 1cm below the meniscus and allowed to acclimatise for 20 seconds before a reading was taken.

2.2.9 Bulk density

A container with known volume (450cm^3) and mass, was inverted and inserted into the soil and carefully extracted without damaging the container and preserving the total

volume. The sample was weighed, and container mass subtracted. Bulk density was calculated through the following equation:

$$\text{Equ 2: } Bulk\ density\ (g\ cm^{-3}) = \frac{Mass\ (g)}{Volume\ (cm^3)}$$

2.3 Laboratory protocol

2.3.1 Seed washing

Wheat seeds were washed in 100ml 10% sodium hypochlorite solution for 30 minutes, followed by rinsing in 100ml sterilised deionised water for 5 minutes in triplicate.

2.3.2 Identification of soil fungi

Sparing amounts of soil were spread over the surface of an antibiotic (1000 U penicillin, 1000mg/L streptomycin) nutrient agar (Breckland Scientific, UK – Appendix 8.5) and antibiotic (1000 U penicillin, 1000mg/L streptomycin) Czapek dox agar (Fisher Scientific) and incubated at 25°C for a total of 2 weeks with daily viewing of agar plates. Unique morphologies were removed from each plate, as an agar plug, and transferred to new antibiotic nutrient agar (Breckland Scientific, UK – Appendix 8.5) with the same incubation conditions. Once developed sufficiently, a piece of transparent adhesive tape was placed over the top of the fungal culture and place over a microscope slide containing a single drop of India ink, viewed microscopically (Vickers® compound microscope) and identified via a fungal key (Navi et al., 1999)

Samples that were identified to have AM fungal properties were later identified via molecular means via DNA extraction from a culture grown in nutrient broth (Breckland Scientific – Appendix 8.5). The soil DNA purification kit GeneAll® (CamBio) was employed for the total extraction of fungal DNA from broth grown culture. Extracted fungal DNA was sent to Eurofins® for ITS 1 (forward: GGAAGTAAAAGTCGTAACAAGG & reverse: GCTGCGTTCTTCATCGATGC) and ITS 2 (forward: GCATCGATGAAGAACGCAGC & reverse: TCCTCCGCTTATTGATATGC) region sequencing to provide genera or species identification. Genera and species identification was performed through the report generating services of Eurofins®.

2.3.3 Ergosterol HPLC

A modified methodology from Millie-Lindblom and Tranvij (2003) was used to determine soil ergosterol levels. In brief, 1g sub-sample of each field sampled soil was freeze dried using a ChechaTech system LSB40 freeze drier chamber, Edwards RV5 vacuum pump and MicroModulyo freeze drier (Thermo Scientific) for a 21 hour cycle as of Appendix 8.6. Of the freezer dried soil, 150mg was weighed into 50ml centrifuge tubes. Potassium hydroxide was added to HPLC grade methanol (Fisher Scientific) until 10% w/v was achieved. To each centrifuge tube, 4ml of KOH in methanol and 1ml cyclohexane was added and sonicated in an ultrasonic water bath for 15 minutes before subject to reflux at 70°C for no longer than 2 hours with tube lids slightly opened. Once samples had cooled to room temperature, 1ml of Milli-Q water was added with a further 4ml of cyclohexane. Samples were vortexed at maximum speed for 60 seconds and centrifuged at 1000 x g for 60 seconds. The cyclohexane fraction was transferred to a cleaned test tube and incubated in a water bath at 70°C overnight in a fume cupboard. Once cyclohexane had evaporated to completion, 1ml HPLC grade methanol was added to each tube and incubated at 40°C for 15 minutes. Each sample was then filtered through 0.2µm nylon membrane syringe filters (Chromatography direct, Runcorn, Cheshire, UK) directly into HPLC vials and ran through the chromatographic system. HPLC was ran using a H5C18-25QS (4.6 x 250mm Interchrim, Montluçon Cedex, France) column with guard column (Phenomenex KJ0-4282 SecurityGuard analytical guard cartridge system, fitted with a AJ0-7510 cartridge). Eluent used comprised of 100% HPLC grade methanol (Fisher Scientific) at a flowrate of 1ml min^{-1} for the total run time of 15 minutes, with an injection volume of 10µl. Ultraviolet (UV) detection was set at a wavelength of 282nm. Ergosterol produced a peak at a retention time of 8.1 minutes.

Ergosterol (Sigma) standards were ran at known concentrations to construct a standard curve for soil ergosterol quantification.

2.3.4 AM fungal root component staining

Intracellular root arbuscules and vesicles were examined after roots were left fully submerged in a formaldehyde, acetic acid, alcohol (FAA) and deionised water solution, 10:5:50:35 respectively, for 24 hours. Roots were removed and rinsed with deionised water prior to autoclaving. Root systems, containing small quantities of soils, were subject to sonication at 42KHz for 10 minutes and further rinsed in deionised water. If small amounts

of soils still adhered to root systems, a soft fine paint brush was used to remove debris. Root systems were submerged in 5% hydrochloric acid for 30 minutes and incubated at 60°C in a water bath. After cooling to room temperature, root material was sectioned into 1cm sections. Five 1cm root sections were each allowed to stain in 0.4% trypan blue in Phosphate Buffered Saline (PBS) (Fisher Scientific®) and another 5 x 1cm root sections were stained in 10% Sheaffer® blue ink in 25% glacial acetic acid (Vierheilig et al, 1998) for 3 minutes.

Samples were viewed initially at a total magnification of x40 using a Vickers® compound microscope. Counting of stained root vesicles and arbuscules was performed at a total magnification of x100. Images of the samples were taken with a Bresser® HD microscope camera.

2.3.5 Glomalin extraction and analysis

Glomalin was extracted via a modified methodology from Wright and Upadhyaya (1996) to measure total soil glomalin (TSG). Briefly, 1g of soil was suspended in 8ml 50mM trisodium citrate dihydrate (Sigma) and kept at autoclave conditions (121°C 15psi) for 60 minutes. Soils were then centrifuged at 1000 x g for 2 minutes to remove suspended soil particles. Supernatant was further centrifuged at 6800 x g for 10 minutes, a total of three times to remove impurities within the sample. Of the centrifuged sample, 1 ml was used for the Bradford protein assay according to manufacturer's instructions, as well as manufacturer led standard curve construction, at a photospectrometer (Cecil 1021) absorbance of 595nm. In brief, 1 ml of Bradford reagent was added to 1 ml of extraction solution and quantified via photospectrometry and standard curve, constructed from egg white albumen at the stated concentrations. Bradford assay allows for more concentrated glomalin extractions to be quantified via 30 µl of extractant and 1.5 ml of Bradford reagent, being quantified in an identical manner.

2.3.6 Total glomalin estimation

Firstly, bulk density was calculated by recording the mass of soil sampled from a known volume. Volume of sample area was calculated for 0.1 m^3. Mass of glomalin was converted from result of the Bradford assay in section 2.5 and multiplied by the known

volume of extraction solution added to each gram of tested soil. The following equation was constructed to find the total mass of glomalin with the field sampled soils of the cropping area, an area typically under trafficked by heavy agricultural machinery, and calculated for the desired volume:

Equ 3: $$Estimated\ mass\ of\ glomalin\ (g) = [Soil\ volume\ (cm^3) \times Bulk\ density\ (g\ cm^{-3})] \times [Mass\ of\ Bradford\ reactive\ glomalin\ (g\ ml^{-1}) \times Volume\ of\ extraction\ solution\ (ml)]$$

2.3.7 In vitro glomalin production over a pH range

Top soils (<10cm) were removed from Bayfordbury Field Station, Hertfordshire, sampled as an undisturbed soils absent of arable practices acting as a control, and double sterilised before drying overnight at 60°C, ensuring minimal moisture remained. Soil glomalin was extracted until the Bradford reagent was unable to detect glomalin and re-dried overnight at 60°C. 50g of dried soils were placed in 6 x 250ml beakers pH adjusted (calibrated glass membrane HANNAH pH probe), using 75% v/v glacial acetic acid and sodium carbonate powder. Soils were adjusted to cover the pH range between 4 – 9 by drop wise additions of neat glacial acetic acid and 0.1g sodium hydrogen carbonate. Soils were again dried overnight at 60°C prior to the addition of 20ml deionised water, pH checked and adjusted if needed, then double autoclaved (125°C, 15 p.s.i for 15 minutes) for sterility. Previously isolated and cultured *Rhizophagus* spp., from nutrient agar (Breckland Scientific, UK – Appendix 8.5) at 25°C for 1 week, was transferred to each 50g pH adjusted soil sample, via a 1cm^2 agar plug, whilst the control pH samples of the same range were left sterile. All soils were incubated at 25°C with 1g samples tested in triplicate for glomalin and fungal biomass (ergosterol HPLC, Section 2.3.3.) every week for a total of 6 weeks.

2.3.8 Fungal influence of root length

Water agar was produced to 4g L^{-1} with either 200ppm urea, potassium chloride or sodium hydrogen phosphate, or produced without the addition of a nutrient source to a total volume of 100ml in a 250ml conical flack with a cotton wool plug in the neck of the flask. Zulu wheat was subject to seed washing as of Section 2.3.1. A single seed was placed aseptically in the centre of the agar and grown in a controlled environment as described in

Section 2.4.1. Each nutrient set up comprised of 12 individual samples. A further 12 samples of each nutrient and seed combination were produced, but this time they were grown in the presence of a 1cm^2 agar plug of a previously isolated fungus (Section 2.3.2). All samples were grown for a total of 12 weeks.

2.3.9 Root exudate HPLC

A modified method from Yuan et al (2015) was implemented to analyse low molecular weight organic acids via HPLC, using a H5C18-25QS (4.6 x 250mm Interchrim, Montluçon Cedex, France) column with guard column (Phenomenex KJ0-4282 SecurityGuard analytical guard cartridge system, fitted with a AJ0-7510 cartridge). Eluent used comprised of 5mM sulphuric acid (A) and 100% HPLC grade methanol (B) (Fisher Scientific) at a flowrate of 0.4ml min^{-1}. From 0 – 10 minutes into the run, eluent was run at 95% A and 5% B. For the next 50 minutes, eluent A was run at 90% with eluent B at 10%, giving a total run time of 60 minutes. Standard organic acid samples (citric, malonic, malic, succinic, oxalic, fumaric – Appendix 8.14) were produced at a concentration of 0.1M and 10µl of sample injected into the HPLC chromatographic system to ascertain retention times for each of the organic acids. Injected samples were subject to UV detection at a wavelength of 210nm.

Wet soils were weighed out to 1g in a 15ml Falcon centrifuge tube with 10ml deionised water added. Tubes were vortexed at maximum speed for 30 seconds, allowed to settle for 5 minutes and vortexed as previous. Samples were then centrifuged at 1000xg for 60 seconds before 1ml of supernatant was removed and filtered (0.2µm nylon membrane – Chromatography direct, Runcorn, Cheshire, UK) direct into HPLC vials. Filtered samples were analysed via HPLC as directed for standard organic acid samples.

2.3.10 Rhizobacteria organisms

A commercially available mixture (Tribus®) of known rhizobacterial *Bacilli* spp., Sold for their ability to promote plant growth, was selected. Known species include *B. subtilis, B. pumilis, B. amyloliquefaciens* at a combined cfu count of 10 billion per millilitre.

2.3.11 Identification of *Bacilli* species

A serial dilution of the Tribus® *Bacilli* spp. mixture was performed to reach a total cfu count of 200 per millilitre. The dilution was then subject to streak plating for purification on nutrient agar (Breckland Scientific, UK – Appendix 8.5) and incubated at 37°C for 24 hours. Each unique colony was subsequently grown on new nutrient agar, and incubated as previous, before growth in nutrient broth (Breckland Scientific, UK – Appendix 8.5) at 37°C for 24 hours. Confirmation of *Bacilli* spp. was achieved from the employment of API 20E (bioMerieux), following manufacturer instructions.

2.3.12 Molecular identification and quantification of AM fungi and rhizobacteria

Following manufacturer's instructions, Soil DNA purification kits (ThermoFisher, UK) were used to extract 0.2g rhizosphere soil (sampled as soil adhered to root systems upon sampling) for DNA extraction. Extracted DNA was combined with Environmental Master Mix 2.0 (ThermoFisher) as manufacturer directed. Custom TaqMan assays (ThermoFisher) for *B. subtilis* (Assay ID: APGZJG4)*, B. pumilus* (Assay ID: APT2DHH)*, B. amyloliquefaciens* (Assay ID: APWCZNA) and *R. intraradicies* (Assay ID: APYMNUJ) were produced. At the request the of the manufacture, the identification of TaqMan probes are unable to be provided. Each glasshouse sample was extracted in multiples of 4, one for each species to be identified. A 96 well plate was produced for each custom TaqMan assay with samples ran in triplicate for each sample of each species to be identified.

2.3.12.1 qPCR conditions

qPCR was performed using an Antigen thermocycler. qPCR thermo cycles were set to begin at 95°C for 10 minutes before beginning a 60 cycle sequence of 15 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C (Appendix 8.18). Fluorescence was recorded for SYBR analysis, with CT values compared to a constructed standard curve logarithmic of copy numbers (Appendix 8.19).

2.4 Controlled growth environments

2.4.1 Growth room conditions

Controlled growth conditions were maintained at a temperature of conditions at 25°C ±2°C and humidity of 35% ±3%, with light intensity of 1770lm. Growth conditions were chosen to support the growth of the developing crop, as well as speed up crop development due to COVID restrictions.

2.4.2 Glomalin and root mass

Zulu wheat was grown in J Arthur Bowers top soil® (Lincoln, UK) with 25% w/w compost of the same brand for a total of 22 weeks in controlled conditions set out in Section 2.7. Samples were taken weekly and roots, with rhizosphere soil (2mm), were subject to glomalin extraction (Section 2.17). Further samples were produced under the same growth conditions and with an identical ratio of top soil and compost from the same manufacturer. However, rhizosphere soil was carefully removed via passing root systems between finger and thumb, a maximum of 3 times, using a nitrile glove. Soils were then taken for glomalin extraction (Section 2.17) whilst roots were weighed before and after drying for dry mass quantification and homogenisation of data. Field sampled root systems were additionally processed in this manner.

2.4.3 Nutrient applications and arbuscule count

Purchased top soil and compost (J Arthur Bowers) was combined to produce 25% w/w compost and sub divided into 300g in autoclavable 500ml plastic beakers. Combination of top soil and compost was produced to provide mycorrhizal inoculants to purchased top soils. A single Zulu wheat seed was planted into each nutrient set up (urea, sodium hydrogen phosphate, potassium chloride). Nutrients were applied weekly in 0.2g increments up to 1g to the associated samples. Control samples were run alongside and received no nutrient addition. Root cortical fungal structures were stained and quantified as of Section 2.15.

2.4.4 AM fungal growth inhibition from glyphosate

Extracted field soil in the absence of a wheat plant was sub divided into 10 units of 50g and replicated in triplicate for each tillage treatment (n = 60 total). For each 50g replicate 1g of soil was removed and analysed for the quantity of ergosterol measured via high performance liquid chromatography (HPLC) analysis (Section 2.3.3) and a standard curve generated. Glyphosate was applied in a 1mL dose to the surface of the remaining soil at rates equivalent of between 0 and 350g L^{-1}. The soil was sampled at three day intervals and the HPLC procedure repeated.

2.4.5 Rhizobacteria effects on total glomalin

Field sampled soils were collected from their respective sites and set up as 100g into sterilised plastic beakers. Samples were taken prior to the introduction of 1 ml of bacterial isolate to their respective soils. Continuous sampling of once per week was carried out over a total period of 6 weeks. Glomalin was quantified via the procedure outlined in section 2.17.

2.4.6 Rhizobacteria effects on AM fungal root components

Purchased top soils (J Arthur Bowers) was weighted (300g) into sterile plastic beakers with a single seed of Zulu variety wheat, previously washed as of section 2.7. A total of 40 plants were produced and divided into control and rhizobacteria (Section 2.4.8) samples. Roots were destructively sampled once per week for a total of 10 weeks. Quantification of fungal root components was performed through staining as of section 2.15.

2.4.7 Rhizobacteria growth alterations towards AM fungi

Field sampled soils were collected from their respective sites and set up as 100g into sterilised plastic beakers. Samples were taken prior to the introduction of 1 ml of bacterial isolate to their respective soils. Continuous sampling of once per week was carried out over a total period of 10 weeks. Ergosterol was quantified through the methodology of section 2.11.

2.4.8 Rhizobacteria and AM fungal inoculant to Zulu variety wheat

Collected top soils from farms A and B were subdivide into 200g samples and potted in to cleaned plastic plant pots (70mm diameter x 90mm depth). A total of 80 samples (8 microbe inoculum in 5 replicates) were set up for each tillage type of the respective farm (conventional and zero tillage) to produce samples for collection at week 15 post germination and week 30. Potted soils were inoculated every 2 weeks with either AM fungus grown in nutrient broth (Breckland Scientific, UK – Appendix 8.5), at 0.5g ml^{-1} fungal mycelia and spores from mechanical blending, or rhizobacteria, identified from Section 2.3.11 (*B. subtilis, B. pumilis* and *B. amyloliquefaciens*), at a total 1 million cfu ml^{-1} determined by dilution plating on colony count media (Sigma, UK) from nutrient broth (Breckland Scientific, UK – Appendix 8.5) cultures, both applied at 2ml per pot. Additionally, combination treatments were produced between AM fungi and a rhizobacteria species at the same rate of inoculation. At 15 weeks, 20 samples from each tillage type were taken and subject to molecular identification and quantification, Section 2.3.4 and 2.3.12. Remaining samples were taken at 30 weeks and subject to the same molecular procedures.

2.5 Statistical analysis

Quantified attributes, of the described protocols and analysis above, were compiled into a spreadsheet dataset against tillage type, i.e. CT, ZT, CTC (CT control) and ZTC (ZT control), and sampling depth. T test statistics were employed for direct comparisons between quantified attributes of the same type between tillage types and their respective controls sampled from within the same field boundary, e.g. between ZT and CT, and between CT and CTC. ANOVA analysis was applied to provide indications of sampling depth significance for each individual tillage type with comparatives with their respective control samples for each measured attribute from Sections 2.2 and 2.3, environmental sample analysis and laboratory protocols respectively. Pearson's bivariate correlations were applied between quantified glomalin and all other measured soil attributes, e.g. ergosterol, WSA and nitrates, in order to determine the effects of tillage on potential relationships to increased soil glomalin concentrations.

Post hoc statistical analysis was applied to ANOVA significance from paired equal variance T tests compared to control samples. The Bonferroni factor was further applied to

the post hoc T test significance to indicate the degree of influence each set of samples had to the ANOVA significance. This provided a greater detail of which samples were producing the significant effects. Pearson's chi squared analysis were additionally applied to datasets that were noted to have a Pearson's correlation

All graphical representations of data were subject to Standard Error of Mean (SEM) for representative error bars.

3.0 Effect of tillage on the relationship between AM Fungi, Glomalin and water stable soil aggregates

3.1 Introduction

The present experimental chapter aims to test the hypothesis that CT, compared to ZT, will have a detrimental effect on AM fungi in the soil which will affect the soil's glomalin concentration and WSA. The investigation was designed as a comparative study between two commercially grown crops of wheat in fields of similar soil structure and aspects, through the following:

1. To investigate the relationship between glomalin, WSA, ergosterol and AM fungi throughout the growing season and at different soil depths in the two fields
2. To be able to measure changes of glomalin and WSA, resultant of tillage, over a whole growing season
3. To quantify the amount of AM fungi associated with wheat roots in the two fields
4. To investigate the impact of temperature, pH and fertilizer applications on glomalin, WSA and AM fungi in the different tillage systems
5. To measure changes on the proportion and contribution of glomalin in soil organic matter

Organic matter and mineral aggregates, along with pore spaces, construct the 3 dimensional structure of soils (Wu et al., 2014). Soil structure and stable aggregates have important roles in relation to carbon sequestration, nutrient storage and availability, as well as gas flow through a soil profile (Borie et al., 2008). This is important to the sustained development of crops in regards to the development of root systems (Brundrett, 2002). Soils that have fewer pore spaces and are more compact, have been shown to produce root systems that fail to reach greater depths to the detriment of plant growth. Furthermore, plant root systems have a strong influence on the formation of soil aggregates in both a physical supporting role as well as through the establishment of AM fungal associations which contribute through mycelial connectivity and the production of glomalin.

Soil's aggregate stability refers to its microaggregates (<0.25mm) which are under direct influence of root structures and the microbiome. Of the microbiome, the influence of fungi towards improving aggregate stability, measured as water stable aggregates (WSA), is higher than that of the influence of bacteria (Wu et al., 2014). AM fungal mycelia, able to grow through soil pore spaces, aids in the increase of fungal surface area acting to physically

bind soil particulates thereby improving soil aggregate stability. Furthermore, glomalin, produced by AM fungi (Wright and Upadhyaya 1996; Adeleke 2010; Bendini et al. 2009; Wang et al. 2017; Walley at el., 2013), acts as an aggregate adhesive (Wright et al., 1996). Glomalin has been quantified to improve overall soil aggregation of microaggregates (<0.25mm), whilst AM fungal mycelia bind microaggregates into macroaggregates (>2mm) (Bendini et al. 2009). Upon the death of AM fungal mycelia, glomalin deposited in bulk soils further increases the adhesion of microaggregates into macroaggregates (Driver et al., 2005). Glomalin further adds to soil by weight/volume carbon, specifically within rhizosphere soils (Lerat et al., 2003), whilst also providing structural support to AM fungal mycelia spanning pore spaces (Driver et al., 2005). Structural support properties of glomalin allow mycelia to withstand slight adjustments to soil structure from actions such as compaction from heavy agricultural machinery and builds resilience into the soil ecosystem.

Schindler et al. (2007) estimated glomalin's total carbon constituents as 20 – 30%, with nitrogen in the range of 3 – 5% by weight. Further constituents of glomalin include hydrogen (4 – 6%), oxygen (33 – 49%), phosphorus (0.03 – 0.1%) and iron (0.04 – 8.8%) (Prasad et al., 2018). Cameron et al. (2008) gives glomalin a soil half life between 40 and 45 years, potentially indicating glomalin is a suitable long term carbon store within soils (Wright and Upadhyaya, 1996). The distribution of glomalin through a soil profile correlates with AM fungal biomass (Rivera-Becerril et al., 2017). Wang et al. (2017) explored the distribution of soil carbon through an undisturbed soil profile and were able to show soil carbon reduced with greater depth of soil samples up to one metre. Wang et al. (2017) were also able to show that quantified soil glomalin remained consistent throughout soil samples to the same depth, however, based on ratio analysis between glomalin and soil carbon, glomalin contributed more to overall soil carbon in samples taken at a greater depth in the soil profile. This comes about from reductions in other forms of organic carbon, also showing to what extent glomalin and AM fungi can be present in soils. As AM fungi are aerobic biotrophs, oxygen and a host are required for AM fungal life cycles (Section 1.1). Through a the established biotrophic host interactions, oxygen and photosynthate are exchanged for AM fungal acquired soil nutrients across root cortical fungal arbuscules. Approximately 20% of total plant carbohydrates are provided to AM fungi via host interactions (Asmelash et al., 2016), this aids in the survival of AM fungi at deeper layers in the soil profile (Wang et al., 2017) allowing such AM fungi to survive and contribute to soil properties.

The role of AM fungi in carbon sequestration is heavily dependent on the nutrient requirement and symbiosis with the host plant (Treseder and Allen, 2000). Increases in nutrient requirement and higher quantities of photosynthetic carbohydrates being provided to AM fungi via arbuscules, the site of nutrient exchange within root cortical cells (Schnept et al., 2008; Wilkes et al., 2019), thus increasing the amount of atmospheric carbon fixed by the host plant and included in the soil's carbon pool by AM fungi. The study by Violi et al. (2007) found increases in nutrient requirements and the host plant growth rate further increased the production of glomalin, in particular from *Rhizophagus intraradicies*.

Through mechanical disturbance of soils, to a maximum depth of 30cm (20cm in the UK (AHDB, 2020)) with a mouldboard plough, fungal mycelia are physically damaged and fragmented (Lambardo et al., 2019) leading to a reduction in macroaggregates (Zheng et al., 2018). Additionally, CT treatments expose previously aggregate protected carbon allowing it to be metabolically digested by the soil microbiome (Guo et al., 2016) and increasing carbon dioxide emissions, resulting in an overall carbon loss from CT managed soils (Silva-Olaya et al., 2013). Silva-Olaya et al. (2013) estimates an approximate 80% of accumulated carbon over a year's crop growth could be lost in a single pass of a plough in the application of tillage. Conservational tillage, such as zero till (ZT), is a potential land management practice that does not employ soil invasive practices (Schwab et al., 2002) and can provide protection to AM fungal mycelia.

3.2 Experimental approach

The experimental protocols utilised for the investigation of the effect of tillage towards the relationship between AM fungi, glomalin and WSA are detailed in Chapter 2 and summarised in Table 3.1.

Selected sampling fields, determined from similarities between crop histories, soil texture, crop type, fertiliser type and application rates, were subject to sampling from a regular 50m sampling grid within the cropping area. Soils were extracted at each sampling point to a maximum depth of 40cm from a single soil core using a Dutch auger at 4 sampling periods over the growing year between September 2018 and August 2019. Sampled soils were divided into 10cm increments for gradient analysis through an upper soil profile and to

transverse a plough pan if present in CT soils. Control samples were additionally taken within field boundaries of their respective site, however, were taken from outside the cropping area in field headlands that were in permanent grassland. Meteorological conditions for the sampling sites were obtained from Rothamsted's e-RA database.

The experimental method was designed to produce and record comparatives between applied tillage treatments on a field scale through several approaches. Firstly, sampled soils were analysed for their glomalin and WSA correlations between tillage treatments along with measured soil textures and sampling depth. Secondly, quantified soil glomalin was compared with SOM for the assessment of glomalin's contribution to SOM between tillage treatments. Thirdly, glomalin and ergosterol were compared in wheat root samples to determine the abundance of AM fungi in both lab and field grown wheat plants. Fourthly, soil pH in both lab and field samples were assessed for the growth influences towards AM fungi and quantifiable soil glomalin. A further experimental approach investigated the presence and quantity of soil nitrates, potassium and phosphates in regard to the production of glomalin. Finally, effects of temperature on the growth and abundance of AM fungi, long with produced glomalin, were investigated both from sampled field soils and lab grown samples.

Table 3.1 – A summary of the experimental protocols employed for the investigation of the effects of tillage towards the relationship between AM fungi, glomalin and water stable aggregates (WSA).

Experimental approach	Description of experimental approach	Experiment location	Section
Field sampling	Gather arable soils from CT and ZT treatments for comparatives between the land management practices	Field	2.1
Soil texture	Confirm the soil texture given in farmer's records via soil texture triangle	Laboratory	2.2.1
Moisture quantification	Oven dry soils	Laboratory	2.2.3
Organic matter by loss on ignition	Heat soils in muffle furnace to 400°C for 18 hours	Laboratory	2.2.4
Nitrogen, phosphate and potassium (NPK) testing	Measure nitrogen (pre made HACK 339 testing kit), potassium and phosphate in arable soils	Laboratory	2.2.6
Water Stable Aggregates	Separate micro and macroaggregates from sampled soils of each tillage treatment	Laboratory	2.2.7
pH	To determine the relationship between pH and correlate soil pH with ergosterol and glomalin quantities	Laboratory	2.2.8
Bulk density	Mass of sampled soils of known volume	Laboratory	2.2.9
Ergosterol HPLC to determine ergosterol	Measure the total amount of live fungi within soils at each sample depth and location	Laboratory	2.3.3
Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein as an indicator of soil AM fungi population	Laboratory	2.3.5 and 2.3.6

3.3 Results

3.3.1 Field zones

Meteorological data for each sampling site is provided in Appendix 8.6. In brief, the mean annual rainfall for both sites was 712.3mm with a minimum and maximum air temperature -6 to 36°C over the sampling year (September 2018 to August 2019). Minimum and maximum temperatures for soils under grassland and bare soils were measured to be 3 to 20°C and 1 to 23°C respectively for top soils (<10cm). Soil texture analysis of both sites indicated the CT site was homogenous as a sandy loam texture, whilst the ZT sample site was seen to have produced 4 distinct soil textures across the site. Comparisons between CT and ZT sampled soils were made via the direct comparison of sandy loam soils only. Further comparisons were able to be drawn between the 4 distinct soil textures within the boundaries of the ZT sampling site only.

Fields were able to be divided into smaller areas for comparison by defined regions of pH, as seen in Figure 3.1. Figure 3.1 shows the CT field of Farm A divided into 4 unique pH regions at a sampling depth of 10cm (Figure 3.1a), down to a single region at a 40cm sampling depth (Figure 3.1d). The ZT field of Farm B produced 2 distinct regions based on measured pH at a sampling depth of 10cm (Figure 3.4a), down to a single pH region at 40cm sampling depths (Figure 3.4d). There were significant differences ($P < 0.00001$, df: 5,30, F value: 12.93, F critical: 2.53, single factor ANOVA) between similar regional bands of pH range which were dependent on cultivation practice (CT and ZT) in September 2018, with CT producing pH values above and below the reported optimal pH value. Statistical analysis of other biotic and abiotic attributes, such as organic matter and soil moisture, did not produce ANOVA significance between established pH regions given in Figure 3.1 and can be seen in Appendix 8.13. ANOVA testing for the same biotic and abiotic attributes for the remainder of the sampling year are additionally provided in Appendix 8.13.

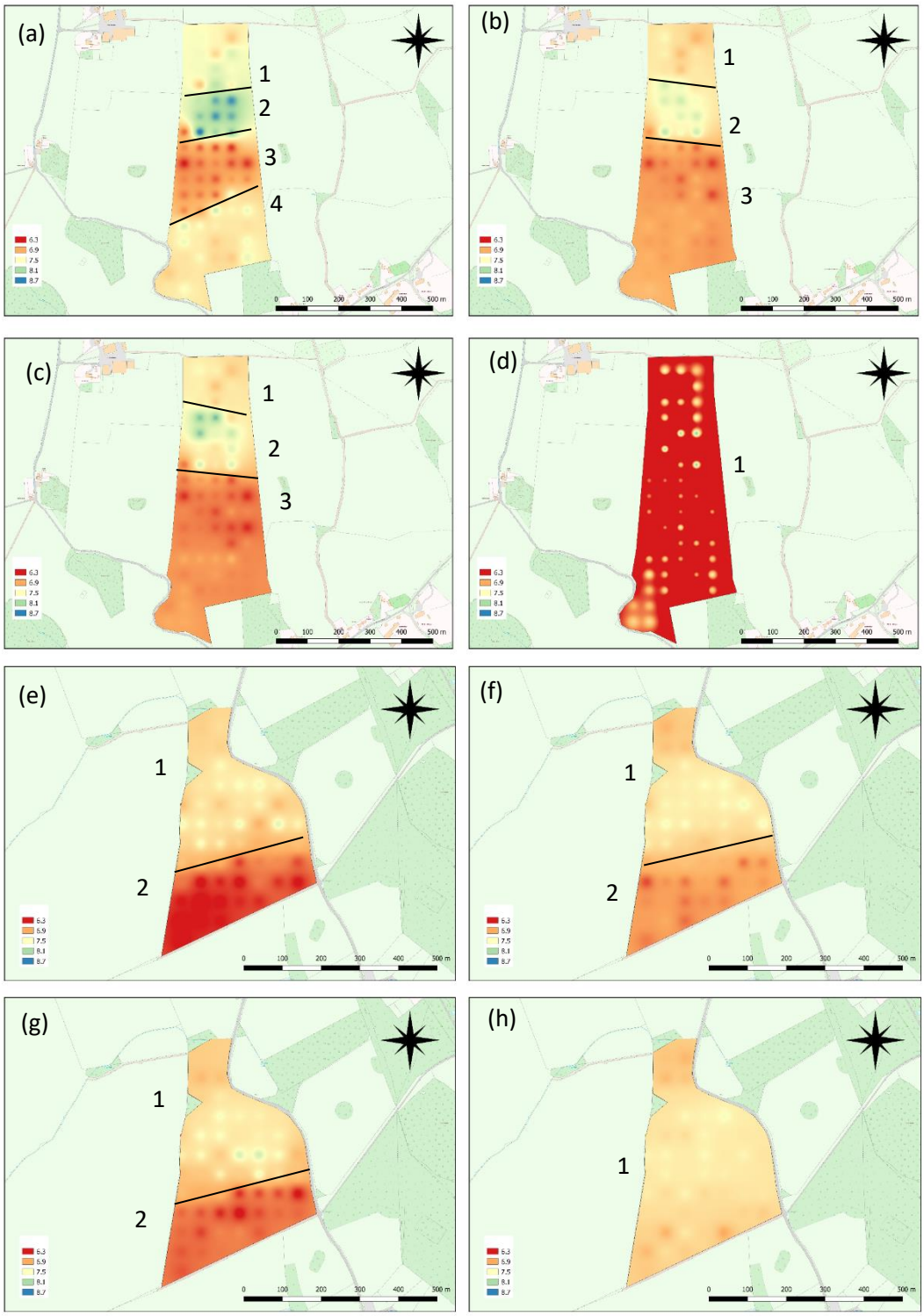

Figure 3.1 – Interpolation maps showing pH on two fields one with conventional tillage (CT) (a - d) and zero tillage (ZT) (e - h) at four sampling depths 10 cm (a and e), 20 cm (b and f), 30 cm (c and g), 40 cm (e and h). CT sampled soils were able to show 4 distinct pH regions (7.5, 8.7, 6.3 and 6.9 for regions 1 – 4 respectively) at 10cm (a), 3 regions (pH 6.9 7.5 and 6.3 for regions 1 – 3 respectively) at 20cm (b), 3 regions at (pH 6.9, 7.7 and 6.3 for regions 1 – 3 respectively) 30cm (c) and a single whole field region (pH 6.3) at 40cm (d). The sampled soils from the ZT sample site were able to indicate 2 distinct pH regions (pH 6.9 and 6.3 for regions 1 and 2 respectively) at 10, 20 and 30cm sampling depth (e – g) and a single whole field pH region (pH 6.3) at 40cm sampling depths (h).

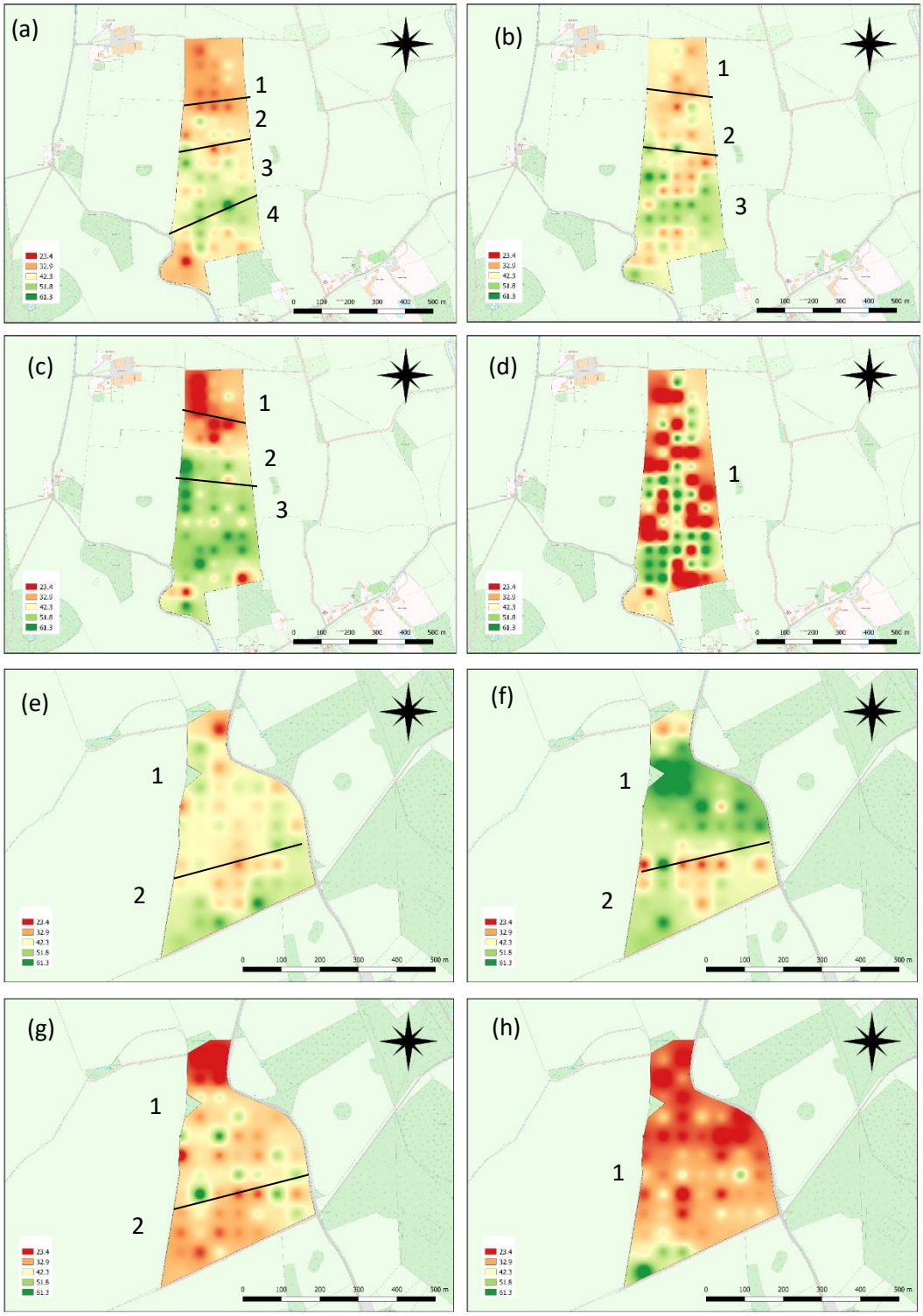

Figure 3.2 - Interpolation maps of the conventional tillage (CT) (a – d) and zero tillage (ZT) (e – h) farm during September 2018 sampling showing total glomalin (g $0.1m^{-3}$) values for 4 sampling depth: (a and e) 10cm, (b and f) 20cm, (c and g) 30cm and (d and h) 40cm, with distinct field pH regions transposed in both CT and ZT sites.

Figure 3.3 - Interpolation maps of the conventional tillage (CT) (a – d) and zero tillage (ZT) (e – h) farm during September 2018 sampling showing the percentage of WSA for 4 sampling depth: (a and e) 10cm, (b and f) 20cm, (c and g) 30cm and (d and h) 40cm, with distinct field pH regions transposed in both CT and ZT sites.

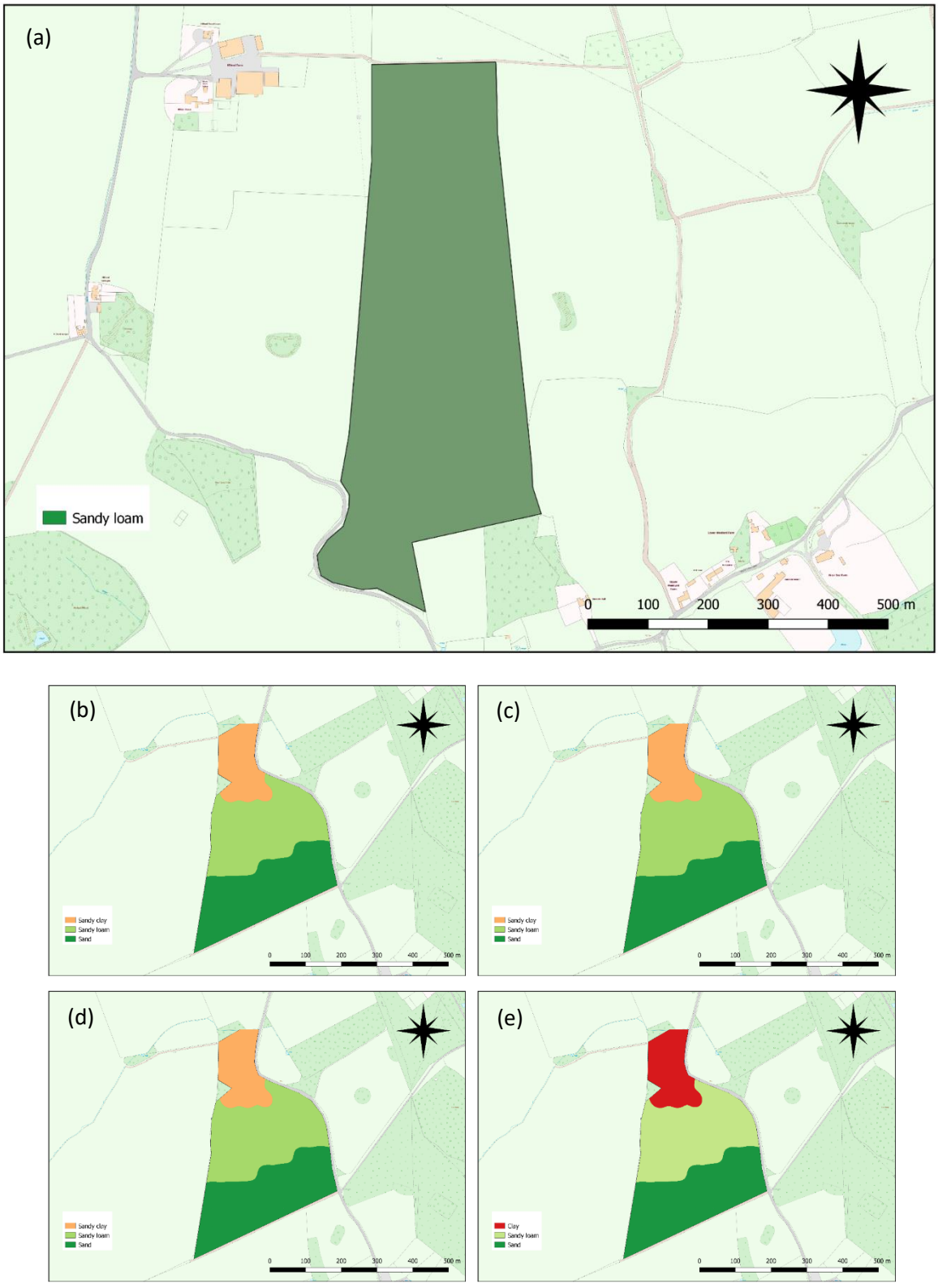

Figure 3.4 - Interpolation maps of the conventional tillage (CT) (a) and zero tillage (ZT) (b – e) farm during September 2018 sampling soil texture at 4 different depths: (b) 10cm, (c) 20cm, (d) 30cm and (e) 40cm. Soil texture for CT soils was measured as sandy loam throughout all sampling depth analysis, whilst ZT soils were noted to consist of sandy clay, sandy loam and sand for the first 3 sampling depths (10, 20 and 30cm) and clay, sandy loam and sand soil textures at 40cm sampling depth.

3.3.3 Differences and distributions between Water Stable Aggregates of CT and ZT treated soils

Applications of tillage, compared with tillage absent management (ZT), was seen to produce a reduction in overall percentage WSA (Figure 3.5). Pre and post presence of a crop, i.e. pre cultivation/sowing and post harvest, noted more comparable WSA percentages. Pre cultivation/sowing sampling (Figure 3.5a) for WSA in both tillage treatments indicated significant distribution of soil aggregates ($P < 0.00001$, df: 3,243, F value: 228.64, F critical: 2.64, single factor ANOVA). WSA trends at each subsequent sampling period were further seen across samples within the same tillage treatment ($P < 0.00001$, df: 3,256, F value: 288.84, F critical: 2.64, single factor ANOVA), with ZT soils consistently quantified with increased soil aggregation. Differences in WSA between tillage treatment throughout the sampling year were noted to be significant ($P < 0.00001$, df:109, t.stat: -30.98, unequal variance T test).

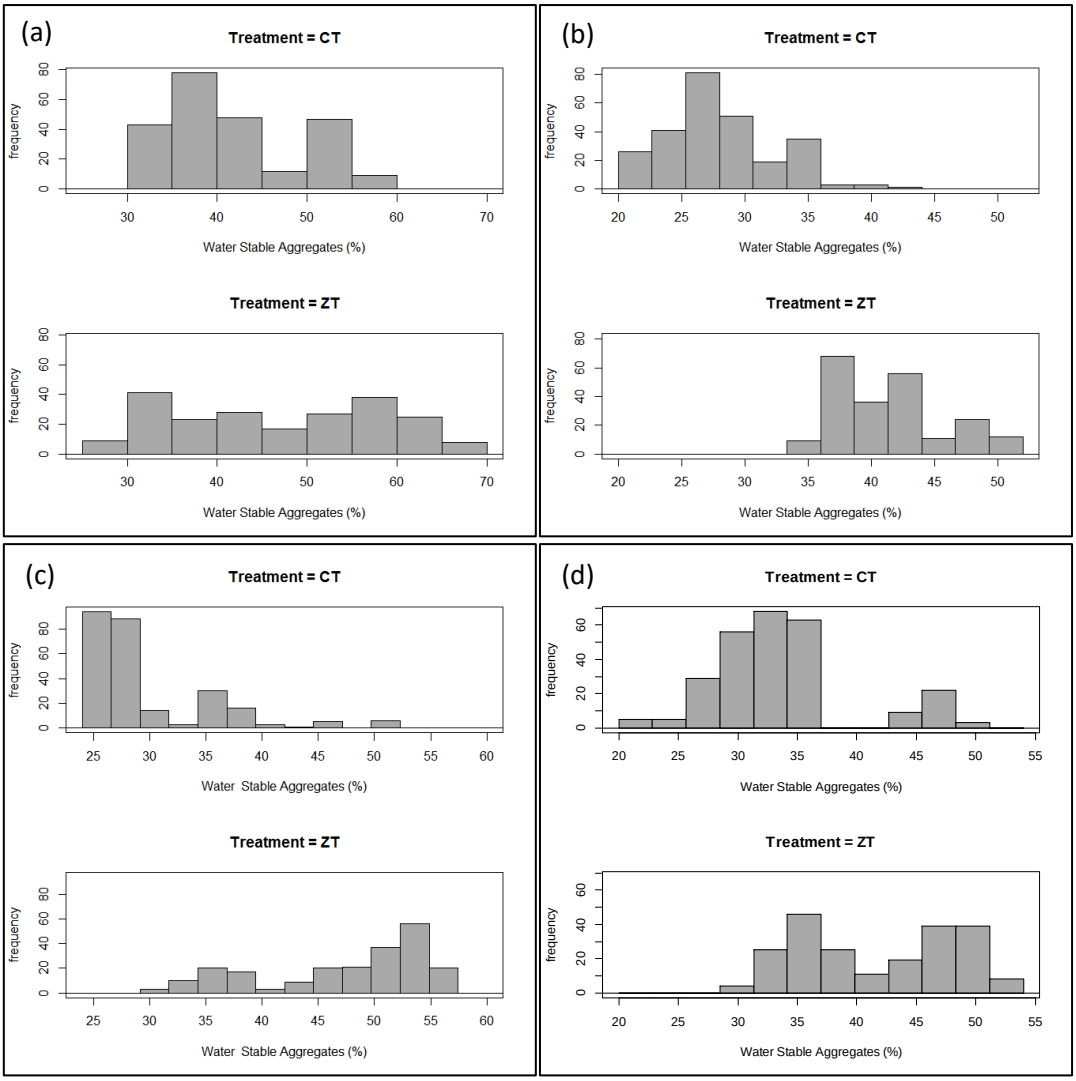

Figure 3.5 – The percentage of water stable aggregates (WSA) for each sampling period expressed as frequency of percentage WSA quantified in the respective tillage treatment: (a) September 2018 (pre cultivation – conventional tillage (CT) (n = 238 per sampling period overall), pre sowing – zero tillage (ZT) (n = 216 per sampling period overall)), (b) February 2019 (3 months post sowing), (c) May 2019 (pre harvest) and (d) August 2019 (pre cultivation – CT, pre sowing – ZT). Sampled soils were collected across each sampling site for their respective sampling period.

3.3.4 Relationship between glomalin and WSA of CT and ZT treated soils

Figure 3.6 displays a scatter plot for the relationship between WSA and soil glomalin for each of the representative tillage treatments. ZT soils were noted to have produced a consistent positive correlation (Pearson's correlation: 0.95, r^2: 0.90) between glomalin ($P < 0.00001$, df: 3,860, F value: 60.15, F critical: 2.62, single factor ANOVA) and WSA, not always present in soils managed under CT. CT control (CTC) samples were seen to be similar in positive correlation to those of ZT and ZT control (ZTC) after September 2018 ($P < 0.00001$, df: 3,76, F value: 12.27, F critical: 2.72, single factor ANOVA, n = 80), the period of seed sowing and cultivation, but not for CT soils within the tilled area. Pearson's correlations, of CT soils, increased post cultivation (September 2018) (Table 3.1) to the end of the sampling year.

Comparisons between tillage treatments and control samples for glomalin concentrations, indicated the application of tillage to arable soils reduced glomalin concentrations, i.e. between CT and ZT, and between CT and CTC, ($P = 0.01$, df:33, t.stat: 1.69, paired unequal variance T test, n = 40). The reductions of glomalin from tillage is reinforced by no significant differences observed between treatments absent of tillage, i.e. between ZT, CTC and ZTC, ($P = 0.16$, df: 37, t.stat: 2.03, paired unequal variance T test, n = 432).

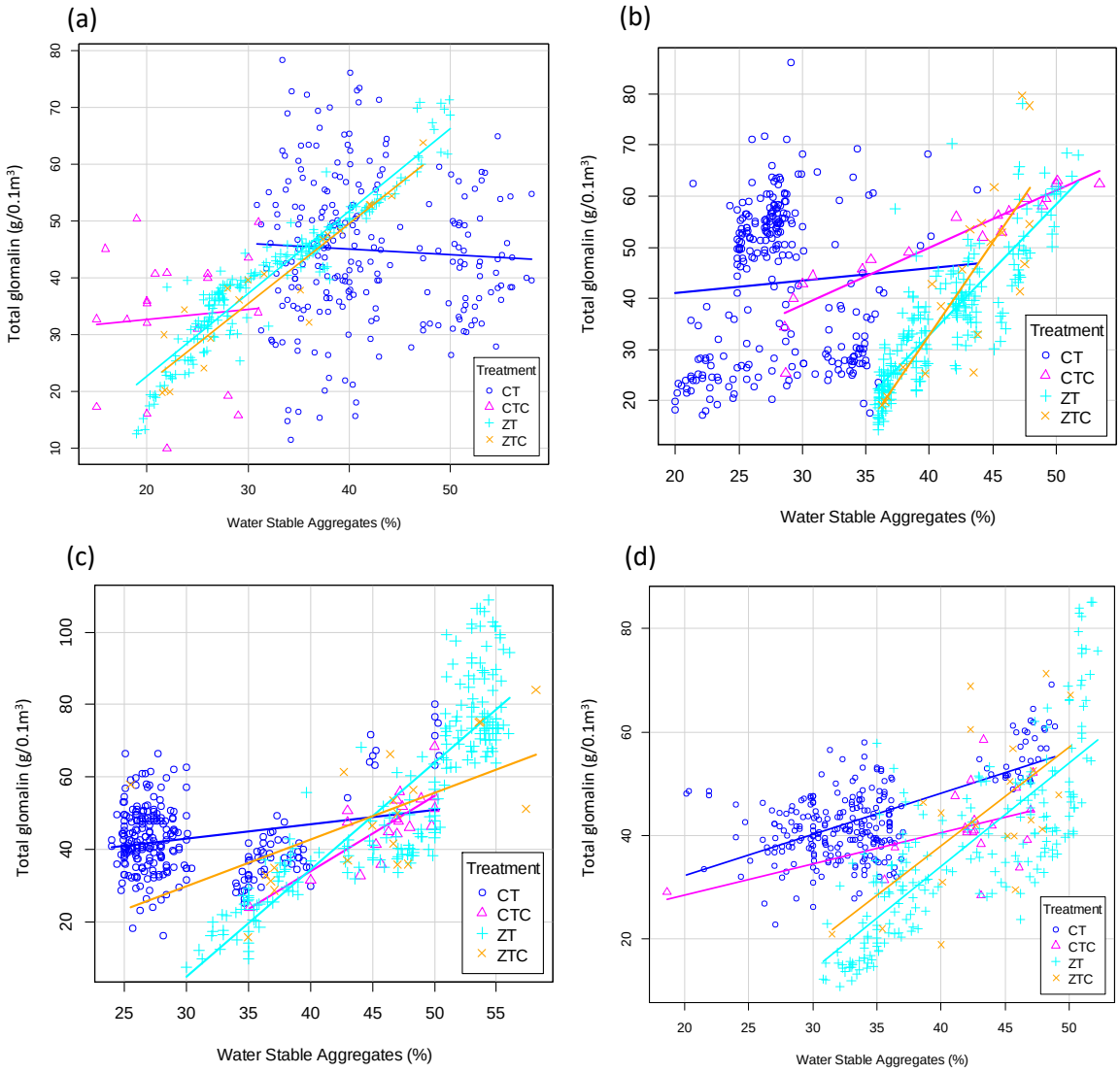

Figure 3.6 – Correlation between total glomalin and WSA over a 12 month field study of 4 sampling periods between conventional tillage (CT) and zero tillage (ZT) with control samples (CTC and ZTC): (a) pre-cultivation/sowing (September 2018), (b) 3 months post sowing (February 2019), (c) pre harvest (May 2019), (d) pre cultivation/sowing (August 2019). Control samples (CTC and ZTC) were taken from areas within the field boundary of their respective sample site and were not under the influence of the applied tillage treatment to the main areas of the cropped fried.

WSA of each treatment was found to be significant for each sampling period and throughout the sampling year ($P < 0.00001$, df: 3,2034, F value: 35.26, F critical: 2.62, single factor ANOVA, n = 2038). Comparisons between tillage treatment yielded significance between CT and ZT $P < 0.00001$, df: 339, t.stat: 1.65, paired unequal variance T test, n = 1878). WSA comparisons between CTC and CT were seen equally as significant, as well between ZT and ZTC.

Table 3.2 – Pearson correlations, and R^2 values, between water stable aggregates and total glomalin concentrations between tillage treatment (conventional tillage (CT), zero tillage (ZT), conventional tillage control (CTC) and zero tillage control (ZTC)) for the sampling year of September 2018 to August 2019.

Sampling time	Tillage treatment	Pearson's correlation	R^2
Pre cultivation/sowing (Figure 3.6a)	CT	-0.05	0.00
	ZT	0.95	0.90
	CTC	0.08	0.01
	ZTC	0.93	0.86
Post cultivation/sowing (Figure 3.6b)	CT	0.07	0.00
	ZT	0.85	0.73
	CTC	0.93	0.87
	ZTC	0.80	0.64
Pre harvest (Figure 3.6c)	CT	0.22	0.05
	ZT	0.87	0.76
	CTC	0.77	0.60
	ZTC	0.60	0.35
Pre cultivation/sowing (Figure 3.6d)	CT	0.61	0.37
	ZT	0.79	0.62
	CTC	0.48	0.61
	ZTC	0.59	0.79

3.3.4.1 Influence of soil depth on the relationship between glomalin and WSA in CT soils

Further analysis to Figure 3.6, investigating soil depth characteristics and tillage on the relationship between WSA and glomalin (Figure 3.7), were able to provide indications of a positive correlation in soils sampled below the zone of tillage (40cm) ($P < 0.00001$, df: 67, t.stat: -6.21, paired unequal variance T test, compared to top soil, <10cm) (Pearson's correlation: 0.52, r^2: 0.27). However, soils within the zone of tillage (10 – 30cm) did not produce a positive trend to the same degree (Table 3.2).

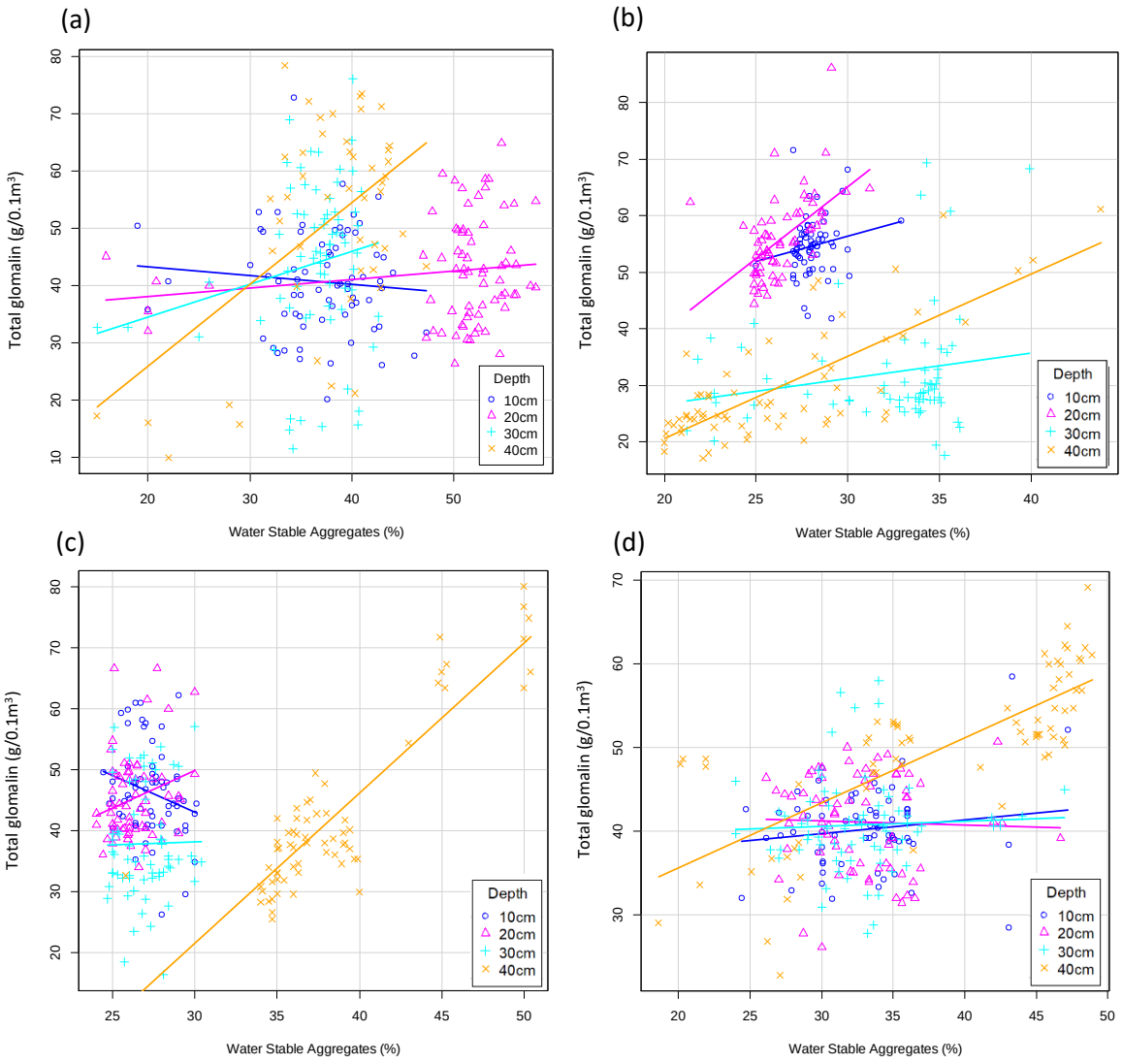

Figure 3.7 - Correlation between total glomalin and WSA over a 12 month field study of 4 sampling periods in conventional tillage (CT) soils in (a) pre-cultivation/sowing (September 2018), (b) 3 months post sowing (February 2019), (c) pre harvest (May 2019), (d) pre cultivation/sowing (August 2019).

Table 3.3 - Pearson correlations, and R^2 values, between water stable aggregates and total glomalin concentrations of sampling depths for conventional tillage (CT) samples for the sampling year of September 2018 to August 2019.

Sampling time	Depth (cm)	Pearson's correlation	R^2
Pre cultivation/sowing (Figure 3.7a)	10	-0.09	0.01
	20	0.14	0.02
	30	0.19	0.04
	40	0.52	0.27
Post cultivation/sowing (Figure 3.7b)	10	0.18	0.03
	20	0.56	0.31
	30	0.19	0.04
	40	0.76	0.58
Pre harvest (Figure 3.7c)	10	-0.23	0.05
	20	0.24	0.06
	30	0.02	0.00
	40	0.89	0.78
Pre cultivation/sowing (Figure 3.7d)	10	0.10	0.01
	20	0.08	0.01
	30	0.01	0.00
	40	0.76	0.58

3.3.4.2 Influences of soil depth on the relationship of glomalin and WSA in ZT treated soils

From sampling depth analysis, there was a strong correlation between glomalin ($P < 0.00001$, df: 3,208, F value: 23.50, F critical: 2.65, single factor ANOVA) levels and WSA ($P < 0.00001$, df: 104, t.stat: 5.97, paired unequal variance T test) (Pearson's correlation: 0.92, r^2: 0.85) at all sampling depths across the whole sampling year (Figure 3.8). Soils sampled to a depth of 30cm equate to the zone of tillage in CT soils. With the absence of tillage in ZT, the positive relationship between glomalin and WSA is maintained.

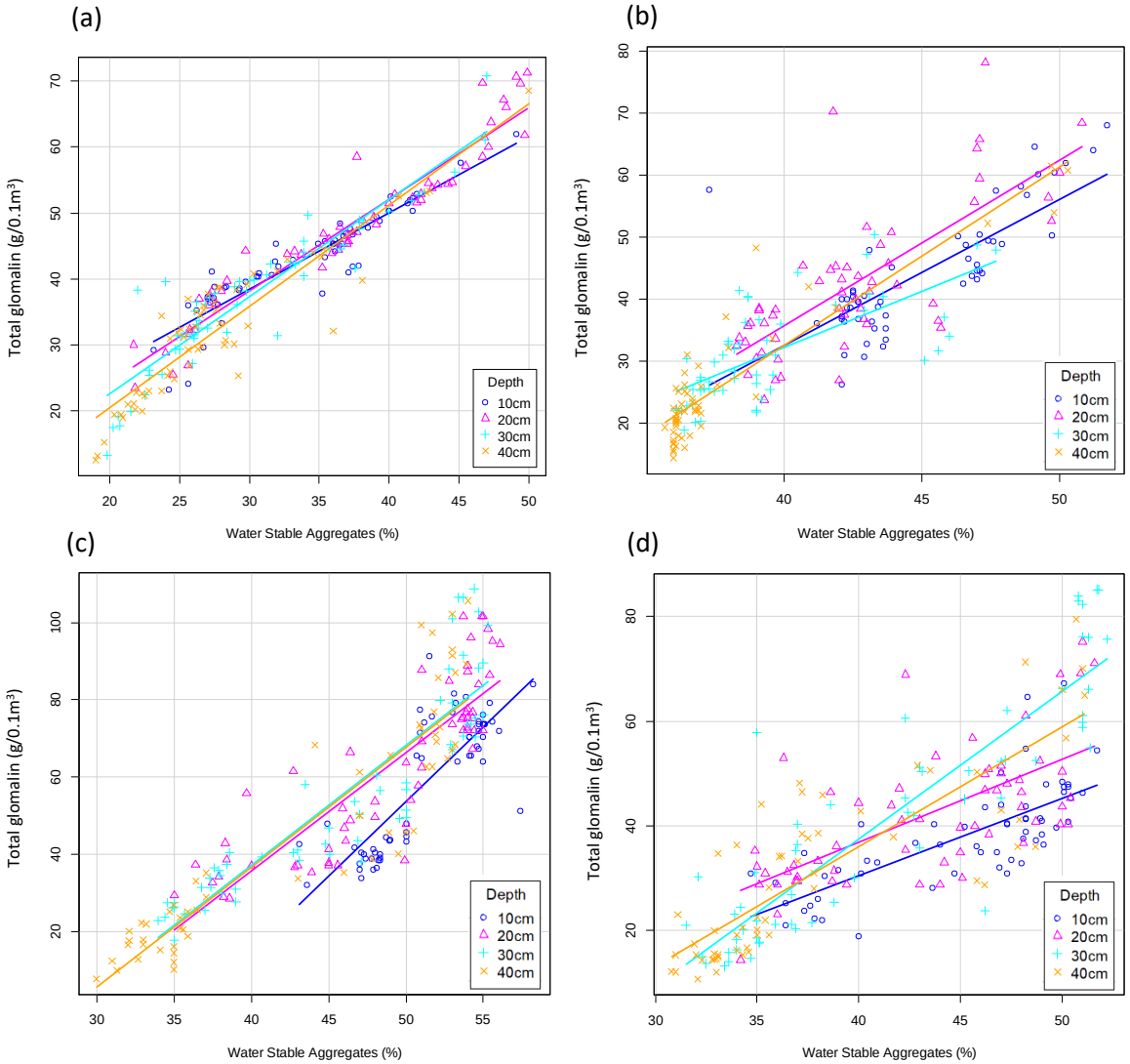

Figure 3.8 - Correlation between total glomalin and WSA over a 12 month field study of 4 sampling periods ((a) pre cultivation, (b) 3 months post cultivation, (c) pre harvest, (d) 1 month pre cultivation) in zero tillage (ZT) soils at 4 sampling depths: 10cm, 20cm, 30cm and 40cm.

Table 3.4 - Pearson correlations, and R^2 values, between water stable aggregates and total glomalin concentrations of sampling depths for zero tillage (ZT) samples for the sampling year of September 2018 to August 2019.

Sampling time	Depth (cm)	Pearson's correlation	R^2
Pre cultivation/sowing (Figure 3.8a)	10	0.92	0.85
	20	0.96	0.92
	30	0.93	0.87
	40	0.91	0.83
Post cultivation/sowing (Figure 3.8b)	10	0.73	0.54
	20	0.76	0.57
	30	0.72	0.52
	40	0.90	0.81
Pre harvest (Figure 3.8c)	10	0.82	0.67
	20	0.86	0.74
	30	0.84	0.71
	40	0.91	0.83
Pre cultivation/sowing (Figure 3.8d)	10	0.74	0.55
	20	0.66	0.43
	30	0.89	0.79
	40	0.83	0.69

3.3.4.3 Influences of soil texture on the relationship of glomalin and WSA in ZT soils

ZT soils were measured to have 4 distinct soil textures (Figure 3.4b - e), whereas CT soils were homogenously sandy loam (Figure 3.4a). Applying soil texture characteristics to the glomalin and WSA relationship previously seen, were able to show sandy and sandy loam soils produced the greatest range of soil glomalin and WSA ($P < 0.001$, df: 3,212, F value: 4.18, F critical: 2.65, single factor ANOVA) (Figure 3.9). Towards the end of the growing year, pre harvest, previously noted positive glomalin and WSA relationships were seen to reduce and become less positive (Pearson's correlation: 0.12, r^2: 0.01).

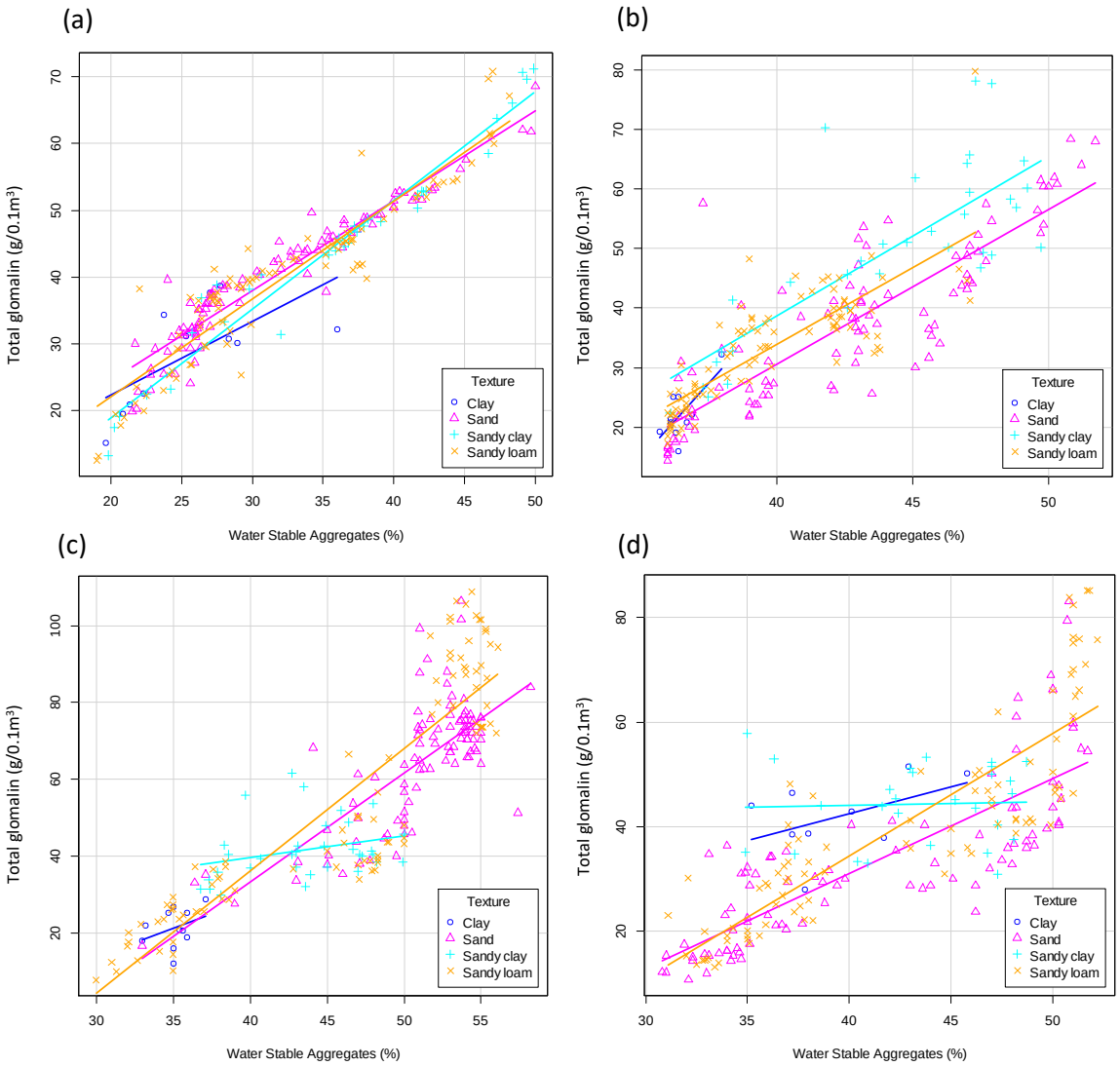

Figure 3.9 - Correlation between total glomalin and WSA over a 12 month field study of 4 sampling periods ((a) pre cultivation, (b) 3 months post cultivation, (c) pre harvest, (d) 1 month pre cultivation) in zero tillage (ZT) soils from 4 soil textures: clay, sand, sandy clay, sandy loam.

Table 3.5 - Pearson correlations, and R^2 values, for zero tillage (ZT) soil textures: clay, sand, sandy clay and sandy loam throughout the sampling year September 2018 to August 2019.

Sampling time	Depth (cm)	Pearson's correlation	R^2
Pre cultivation/sowing (Figure 3.9a)	Clay	0.76	0.57
	Sand	0.77	0.60
	Sandy clay	0.98	0.95
	Sandy loam	0.85	0.73
Post cultivation/sowing (Figure 3.9b)	Clay	0.75	0.56
	Sand	0.87	0.76
	Sandy clay	0.81	0.65
	Sandy loam	0.83	0.68
Pre harvest (Figure 3.9c)	Clay	0.12	0.01
	Sand	0.75	0.56
	Sandy clay	0.36	0.13
	Sandy loam	0.90	0.81
Pre cultivation/sowing (Figure 3.9d)	Clay	0.48	0.23
	Sand	0.81	0.65
	Sandy clay	0.03	0.00
	Sandy loam	0.86	0.74

Soil bulk densities of the ZT treated site differed throughout the field (Figure 3.10), as well as through sampling depth ($P < 0.00001$, df: 3,212, F value: 24.3, F critical: 6.25, single factor ANOVA), with a large proportion of soils being of sand or sandy loam texture (Figure 3.4).

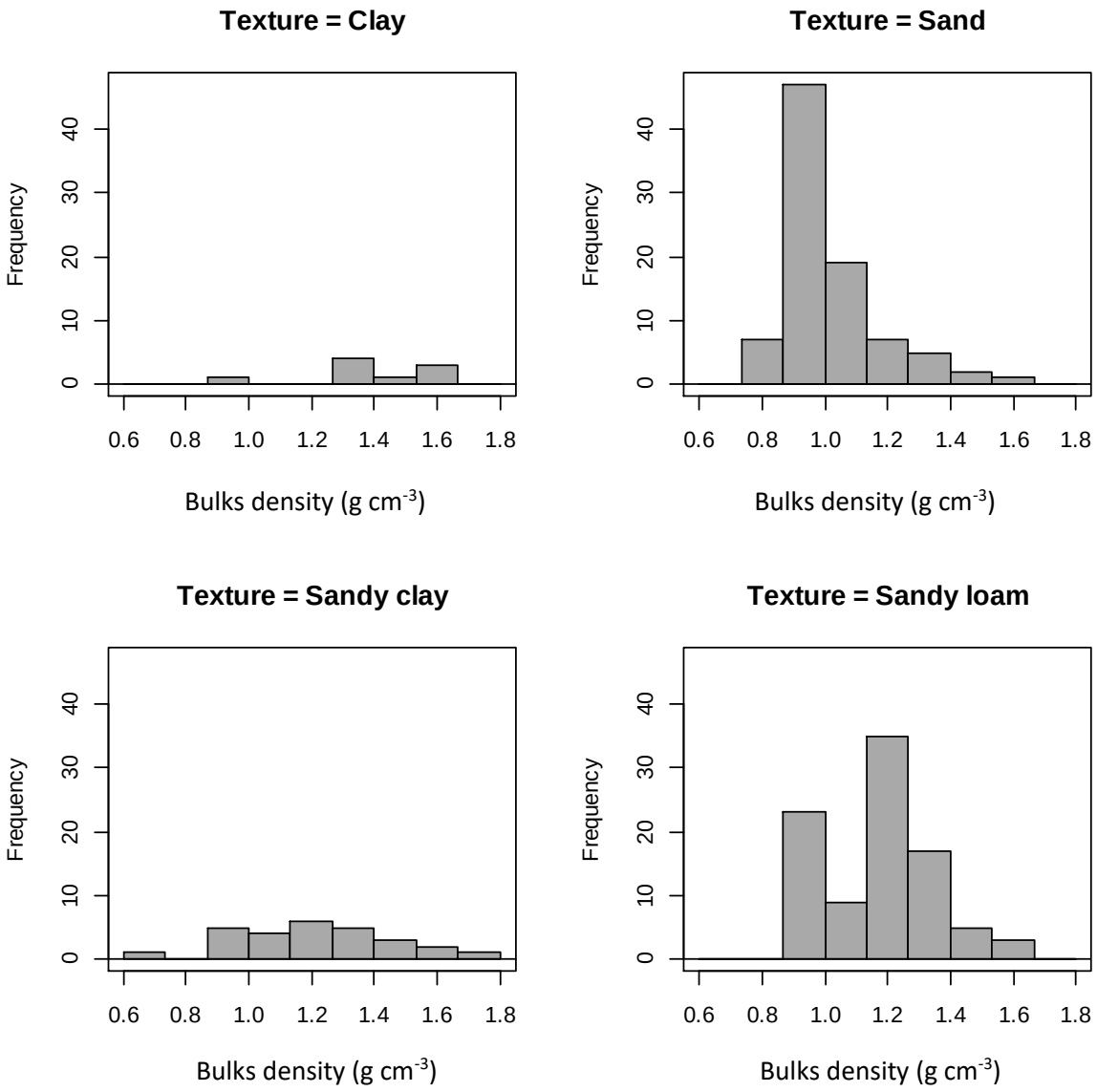

Figure 3.10 - Distribution of bulk densities between soil textures of zero tillage (ZT) treated soils sampled February 2019 (n = 216) with ANOVA significance ($P < 0.00001$) between bulk densities of different soil textures, with frequency (number of samples corresponding to bulk density) expressed as number of samples.

3.3.5 Glomalin and WSA over 12 month study period

Over the 12 month study period, soil glomalin concentrations in CT soils (Figure 3.11) were seen to increase in the top 20cm of soils to a period corresponding to increases in wheat root mass (February 2019) ($P < 0.00001$, df: 3,256, F value: 66.59, F critical: 2.64, single factor ANOVA). Conversely, glomalin concentrations in sampled soils of 30 and 40cm were seen to reduce at the same period as the glomalin increase of the top 20cm soils. Towards the end of the growing year, closer to harvest, glomalin within the top 20cm of soils reduced again to mirror glomalin concentrations similar to those at the beginning of the growing year, pre-cultivation.

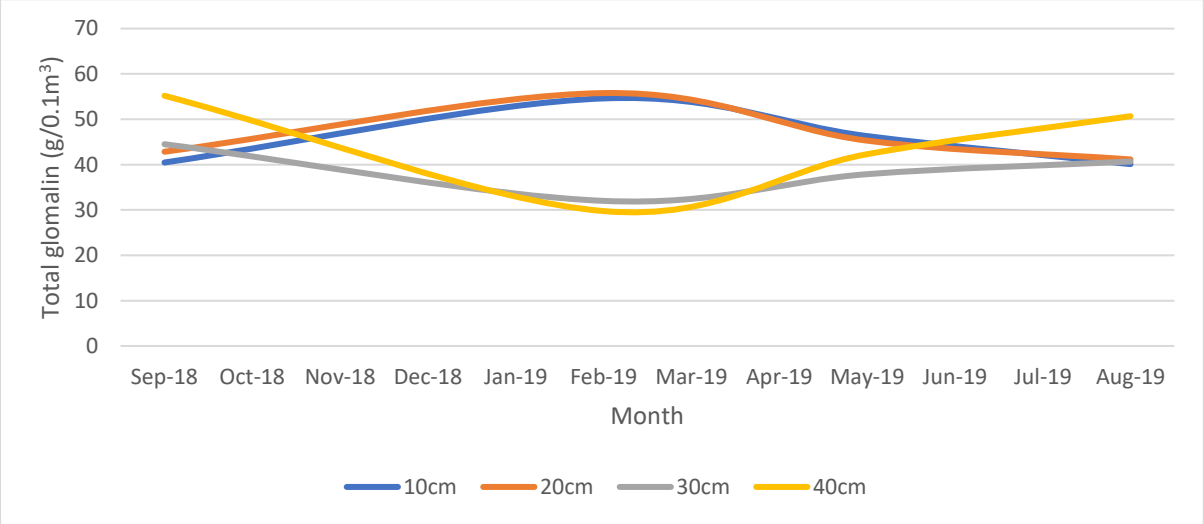

Figure 3.11 – Variance in total glomalin within conventional till (CT) managed soils for the 12 month sampling period for each respective sampling depth

Over the same 12 month period, ZT soil glomalin (Figure 3.12) followed a similar increase at all sampling depths, irrespective of sample depth, with the greatest soil glomalin concentration measured in May 2019 (pre harvest).

Statistical testing between tillage treatments produced T test significance for each sampling depth across the 12 month study period ($P < 0.0001$, df: 83, t.stat: 7.15, paired unequal variance T test) for total glomalin, with exception of 40cm depth samples from pre harvest (May 2019) that showed no significance between tillage treatments ($P = 0.26$, df: 71, t.stat: -1.13, paired unequal variance T test).

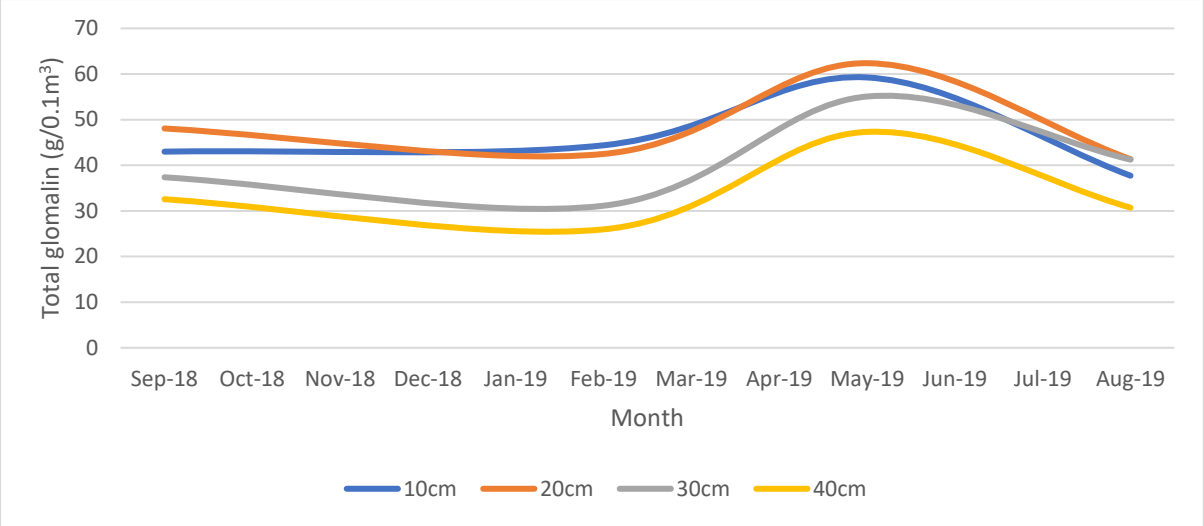

Figure 3.12 - Variance in total glomalin within zero till (ZT) managed soils for the 12 month sampling period for each respective sampling depth

WSA of CT soils over the 12 month study period (Figure 3.13) were seen to decrease after the initial receipt of tillage in September 2018 ($P < 0.00001$, df: 3,256, F value: 231.27, F critical: 2.64, single factor ANOVA) to a greatest reduction in WSA between February and March 2019. WSA for soils sampled at 40cm were recorded to sharply increase and maintain their new degree of aggregation for the remainder of the sampling year. Marginal increases in WSA percentage were noted in soils sampled to a depth of 30cm from February 2019 to the end of the sampling year. These marginal increases were not to the same degree as increased aggregation seen in soils from 40cm depth.

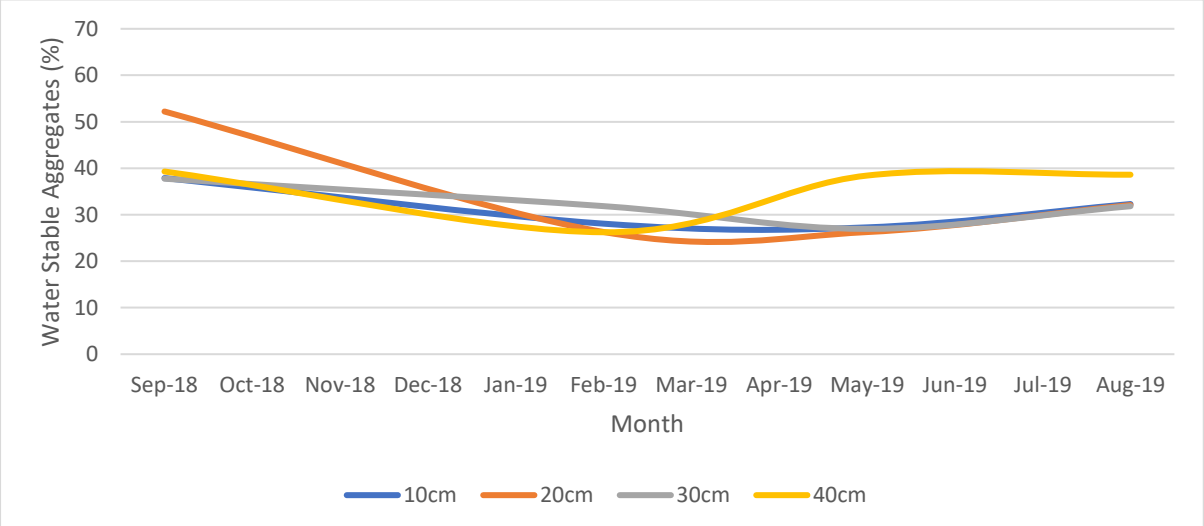

Figure 3.13 - Variance in Water Stable Aggregates within conventional till (CT) managed soils for the 12 month sampling period for each respective sampling depth

Initial WSA of ZT soils in September 2018 (Figure 3.14) were equally distributed throughout the soil depths. Increases in aggregation were recorded for 20 and 30cm depth soil samples, with a maximum increase in May 2019 before all sampling depths reduced marginally towards the end of the sampling year (August 2019). Top soil (<10cm) and 40cm samples were seen to be of greatest WSA percentage in September 2018 and were seen to have reduced in February 2019, achieving the same low WSA percentage again in August 2019 after a slight increase in May 2019.

WSA of soils at all sampling depths in ZT management were seen to be higher than that of CT managed soils ($P < 0.0001$, df: 68, t.stat: -48.06, paired unequal variance T test), with exception of 40cm depth samples from August 2019 ($P = 0.39$, df: 112, t.stat: 0.87, paired unequal variance T test).

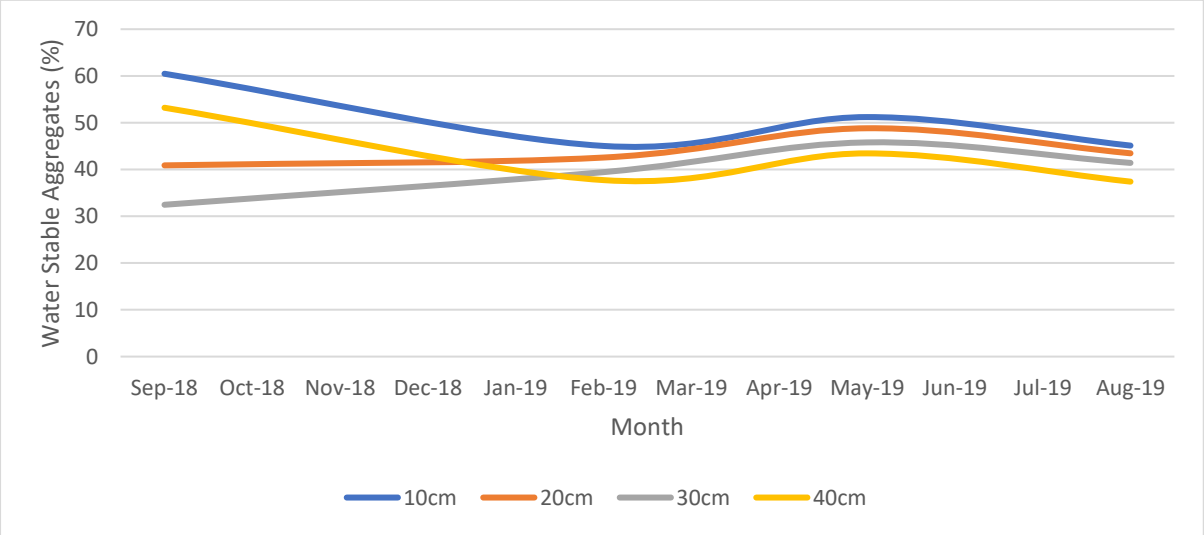

Figure 3.14 - Variance in Water Stable Aggregates within zero till (ZT) managed soils for the 12 month sampling period for each respective sampling depth

3.3.6 Breakdown of organic matter and glomalin between applied tillage practices

Soil organic matter (SOM) was consistently recorded to be of greater percentage in ZT soils than that of CT soils throughout the whole sampling year (Figure 3.15) ($P < 0.00001$, df: 492, t stat: 18.55, post hoc 2 tailed paired T test). SOM, in irrespective of tillage treatment, was seen to reduce throughout the year ($P < 0.00001$, df: 3,1094, F value: 33.73, F critical: 2.61, single factor ANOVA).

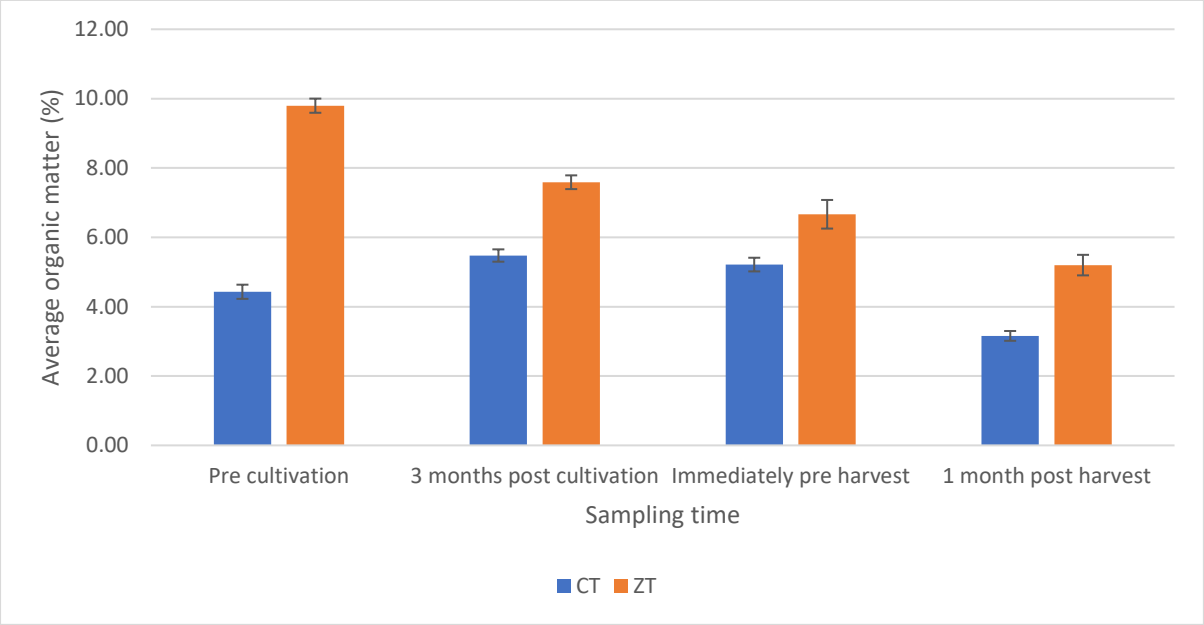

Figure 3.15 – Average (Conventional tillage (CT), n = 1120; Zero tillage (ZT), n = 944) organic matter percentage for each tillage treatment across a 12 month sampling year at 4 regular intervals. Error bars constructed from SEM.

Soil depth was seen to have implication for quantified organic matter in both tillage treatments ($P < 0.00001$, df: 3,256, F value: 51.90, F critical: 2.65, single factor ANOVA). Top soils (<10cm) and soils sampled at 40cm, outside the CT zone of tillage, were measured greatly significant ($P < 0.00001$, df: 102, t.stat: 6.62, unequal variance T test) compared to other sampling depths ($P = 0,28$, df: 114, t.stat: 0.58, unequal variance T test).

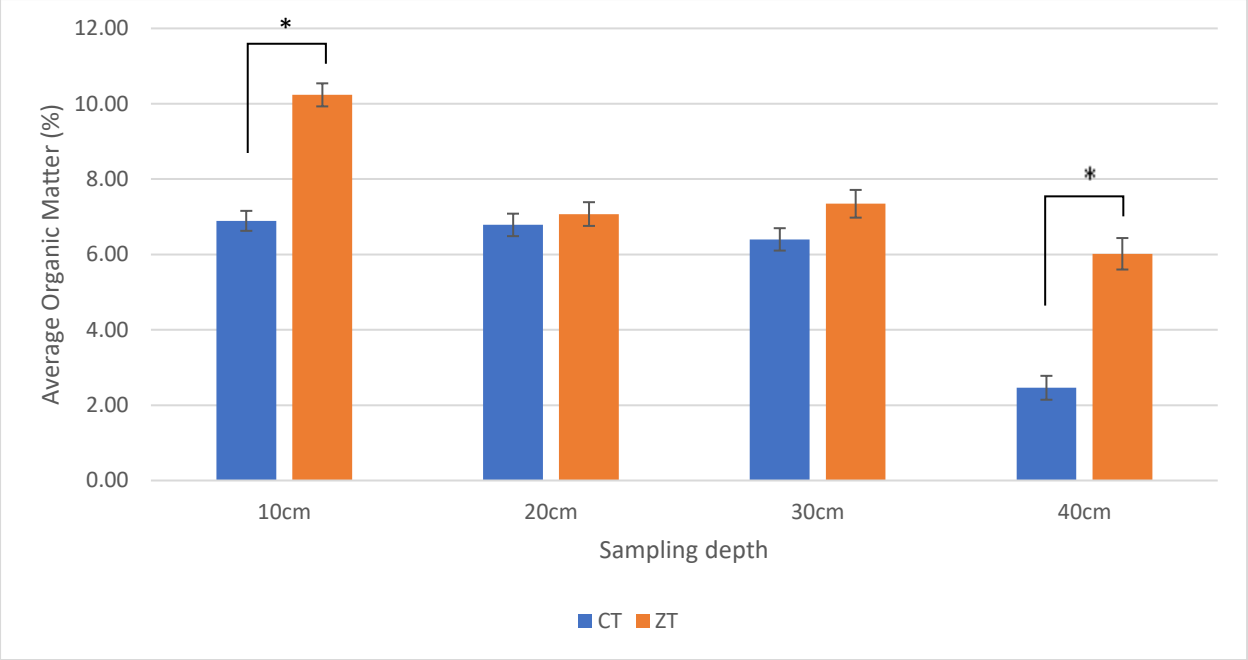

Figure 3.16 – Average (n = 65 per depth – CT: n = 53 per depth – ZT) percentage organic matter for conventional tillage (CT) and zero tillage (ZT) samples soils from a period 3 months post cultivation (February 2019), no statistical differences were observed for pre harvest samples (data not shown). Greatest significance was observed between tillage treatments at 10cm and 40cm sampling depth (*). Error bars constructed from SEM.

Total organic matter is displayed in Figure 3.17 with the contribution of glomalin post cultivation/seed sowing (September 2018) and pre harvest (May 2019) increasing in ZT soils ($P < 0.00001$, df: 104, t.stat: -6.84, paired unequal variance T test). Glomalin of CT soils for the same period was not observed to have altered to the same extent as that of ZT soils ($P = 0.01$, df: 395, t.stat: 2.51, paired unequal variance T test). The increases in glomalin corresponded to a simultaneous increase in crop root development, as seen by Figure 3.11.

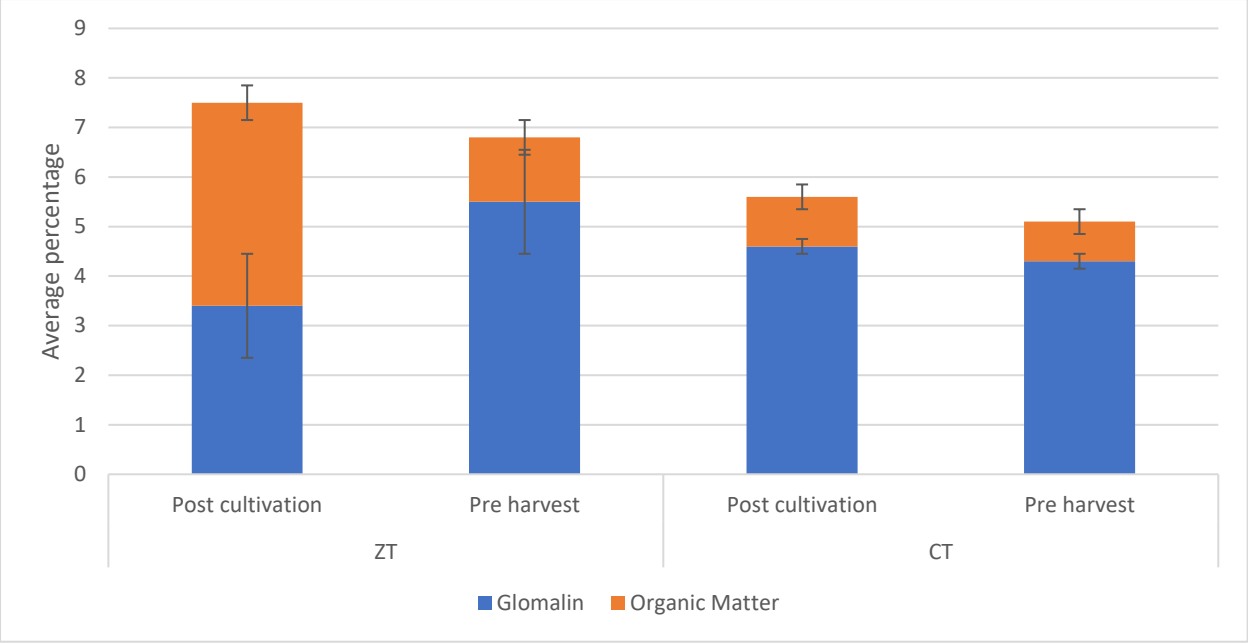

Figure 3.17 – Overall percentage organic with breakdown of glomalin's contribution to average (n = 1408) organic matter in sandy loam texture soils only of conventional tillage (CT) and zero tillage (ZT) between post cultivation (February 2019) and pre harvest (May 2019). Selected sampling periods are displayed due to the presence of wheat root systems as crucial stages in the AM fungal life cycle. Error bars constructed from SEM.

3.3.7 Field sampled and controlled growth determinations of root ergosterol and glomalin from winter wheat

Zulu winter wheat was grown over 22 weeks under controlled conditions and produced a positive relationship with root associated glomalin ($P < 0.00001$, df: 11,18, F value: 14.47, F critical: 2.37, single factor ANOVA) (Figure 3.18).

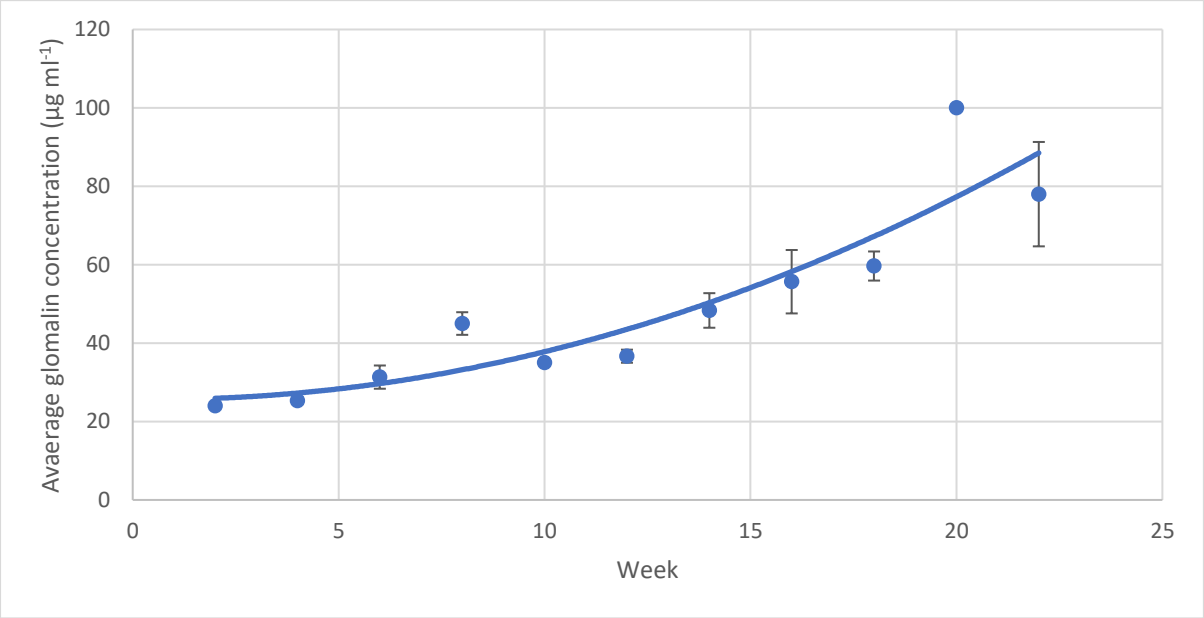

Figure 3.18 – Average (n = 55) root glomalin of controlled growth of Zulu wheat over 22 weeks. Error bars constructed from SEM (r^2 – 1.0)

A scatter plot of root dry mass and average glomalin produced a positive relationship (Figure 3.19) (Pearson's correlation: 0.79, r^2: 0.62).

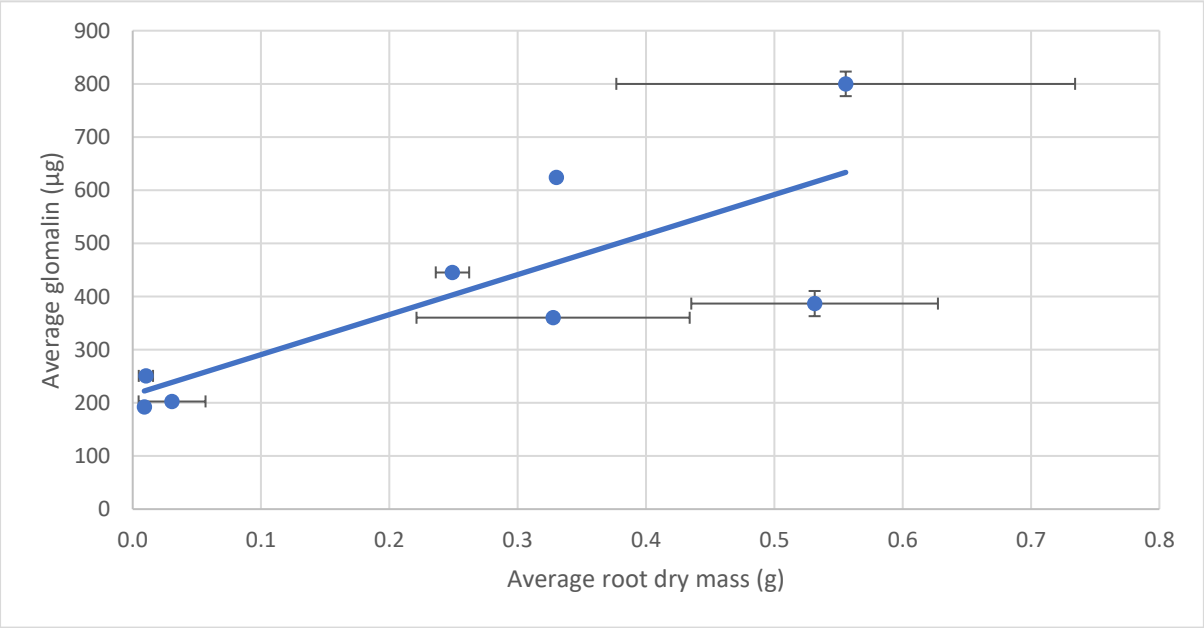

Figure 3.19 – Average (n = 40) controlled growth of Zulu wheat root dry mass and average root associated glomalin producing a Pearson's coefficient of 0.79. Error bars constructed from SEM (r^2 – 0.62)

Roots sampled in Feb 2019 and May 2019 both contained 95% moisture content. Both average glomalin ($P < 0.00001$, df: 11,18, F value: 14.47, F critical: 2.37, single factor ANOVA) and average root dry mass ($P = 0.01$, df: 11,18, F value: 3.29, F critical: 2.37, single factor ANOVA) (Figure 3.20) were noted to be significantly different between sampling periods of February 2019 and May 2019. Glomalin remained comparable between tillage treatments ($P = 0.74$, df: 8, t.stat: 0.38, paired unequal T test) across both sampling periods displayed in Figure 3.20. However, root dry mass was noted to be greater from sampled CT soils than ZT samples ($P < 0.01$, df: 6, t.stat: -3.71, paired unequal T test).

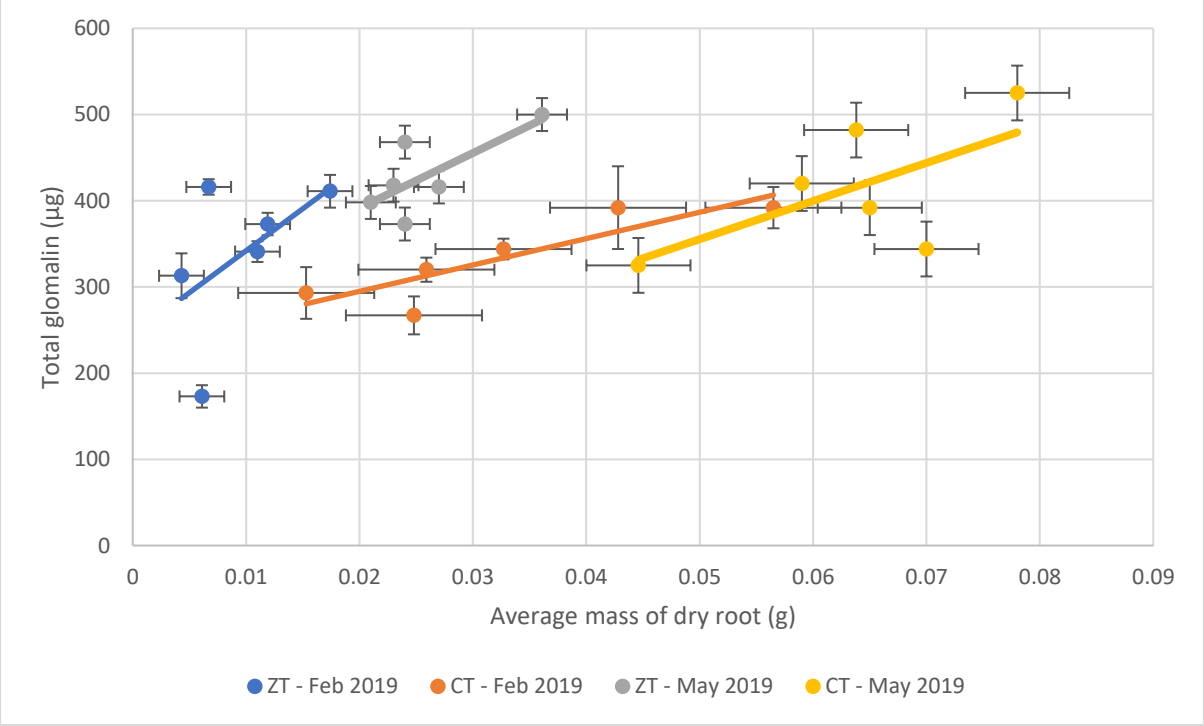

Figure 3.20 - Average (n = 3) root dry mass and root glomalin from zero tillage (ZT) and conventional tillage (CT) sampled fields, with Pearson's coefficients of 0.52 and 0.88 respectively, 3 months post cultivation (February 2019) (ZT, $r^2 - 0.27$) (CT, $r^2 - 0.77$). Correlations of 0.74 and 0.64 were measured for ZT and CT respectively for the sampling period pre cultivation (May 2019) (ZT, $r^2 - 0.54$) (CT, $r^2 - 0.41$).

Root dry mass of lab grown wheat root dry mass (Figure 3.19) and ZT root dry mass for February and May 2019 (Figure 3.20) ($P = 0.02$, df: 12, t.stat: 2.09, paired equal variance T test). Further significance was noted between lab grown wheat root dry mass and root dry mass of CT sampled wheat ($P = 0.01$, df: 12, t.stat: 2.49, paired equal variance T test). No significance was observed between quantified glomalin of lab grown wheat (Figure 3.19) and both CT and ZT sampled wheat (Figure 3.20) ($P = 0.41$, df: 12, t.stat: -0.24, paired equal variance T test).

A negative relationship was observed between rhizosphere ergosterol and glomalin of field sampled wheat roots in February and May 2019 for each respective tillage treatment. Between sampling months, ergosterol was noted to be comparable with the previous wheat rhizospheres, however, glomalin was recorded to have increased greater in CT soils ($P < 0.01$, df: 8, t.stat: -3.42, paired unequal T test) than a corresponding increase in ZT wheat rhizospheres ($P = 0.41$, df: 10, t.stat: -0.87, paired unequal T test).

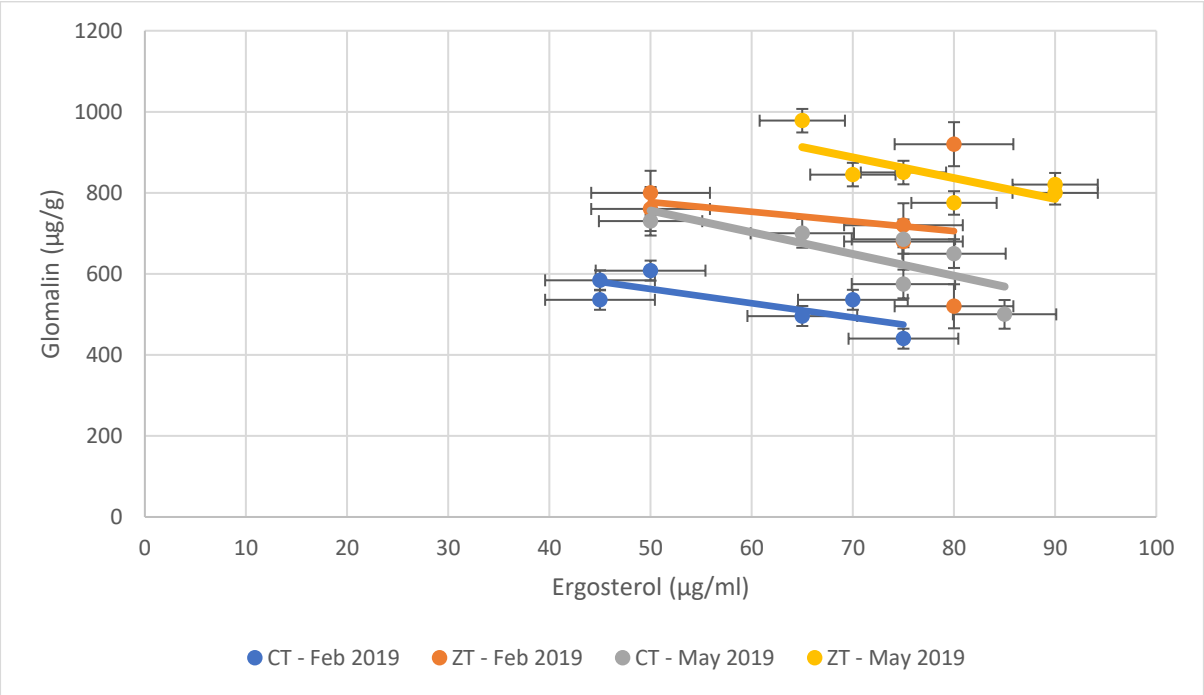

Figure 3.21 – Glomalin and ergosterol comparisons within the rhizosphere of cultivated winter wheat of conventional tillage (CT) and zero tillage (ZT) sites, 3 months post-sowing (February 2019), producing a Pearson's coefficient of -0.77 and -0.25 respectively. (CT, r^2 – 0.60) (ZT, r^2 – 0.07). CT and ZT correlations were noted to be -0.69 and 0.81 respectively (CT, r^2 – 0.48) (ZT, r^2 – 0.65) pre cultivation (May 2019).

Examination of field sampled wheat rhizoplanes indicated ergosterol and glomalin were highly comparable with no notable differences between tillage treatment (P = 0.24, df: 10, t.stat: -1.25, paired unequal T test). However, between sampling months, rhizoplane ergosterol was recorded to have increased for each tillage treatment (P < 0.001, df: 10, t.stat: 2.23, paired unequal T test).

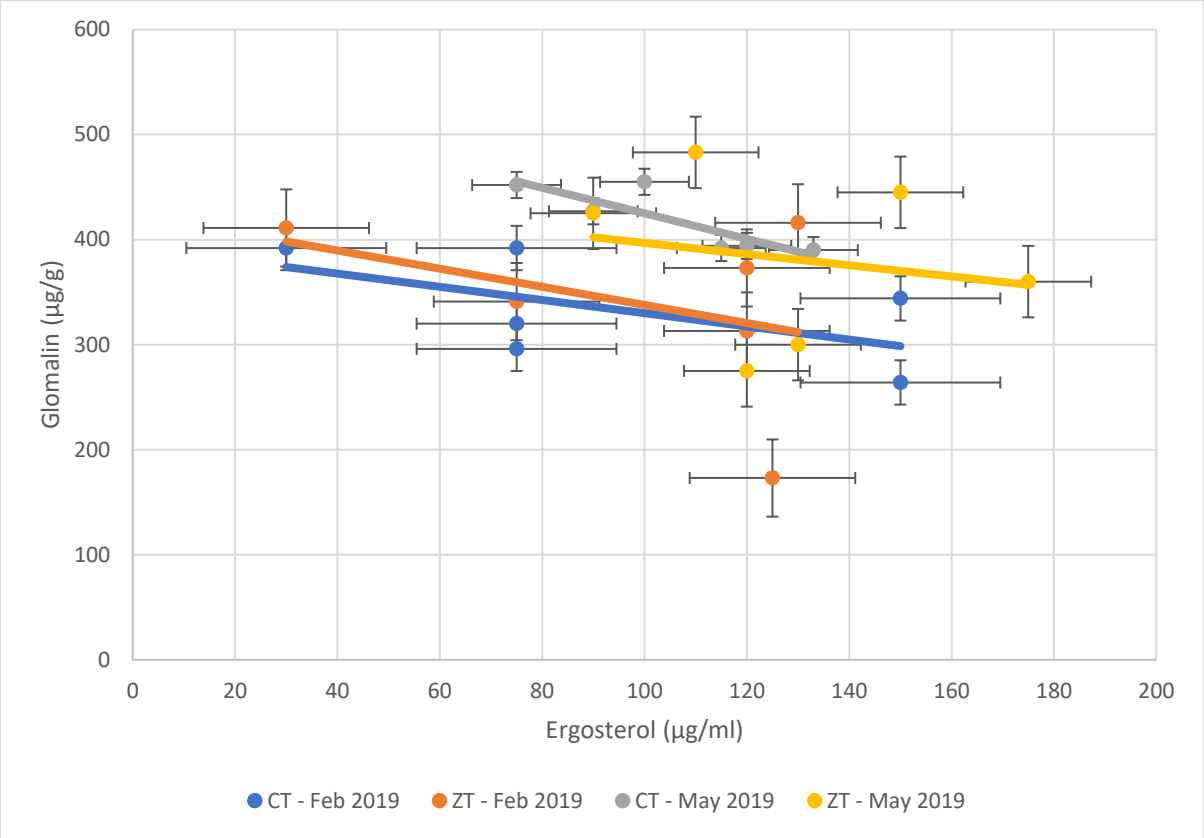

Figure 3.22 – Glomalin and ergosterol comparisons of the roots of cultivated winter wheat of conventional tillage (CT) and zero tillage (ZT) sites 3 months post-sowing (February 2019), producing a Pearson's coefficient of -0.58 and -0.38 respectively. (CT, r^2 – 0.77) (ZT, r^2 – 0.94). Where as Pearson's coefficient produced -0.84 and -0.19 for CT and ZT respectively 1 month pre cultivation (May 2019) (CT, r^2 – 0.72) (ZT, r^2 – 0.04).

3.3.8 AM fungal growth over a controlled pH range in determination of biomass, via ergosterol, and glomalin production

AM fungal biomass, from ergosterol, correlated more positively in pH 6 and 7 adjusted soils compared with other studied pH values (Figure 3.23). Glomalin and ergosterol were significantly different across pH values ($P < 0.00001$, df: 5,30, F value: 12.93, F critical: 2.53, single factor ANOVA). Comparatives between pH 6 and 7 produced no notable differences ($P = 0.71$, df: 10, t.stat: 2.23, unequal variance T test).

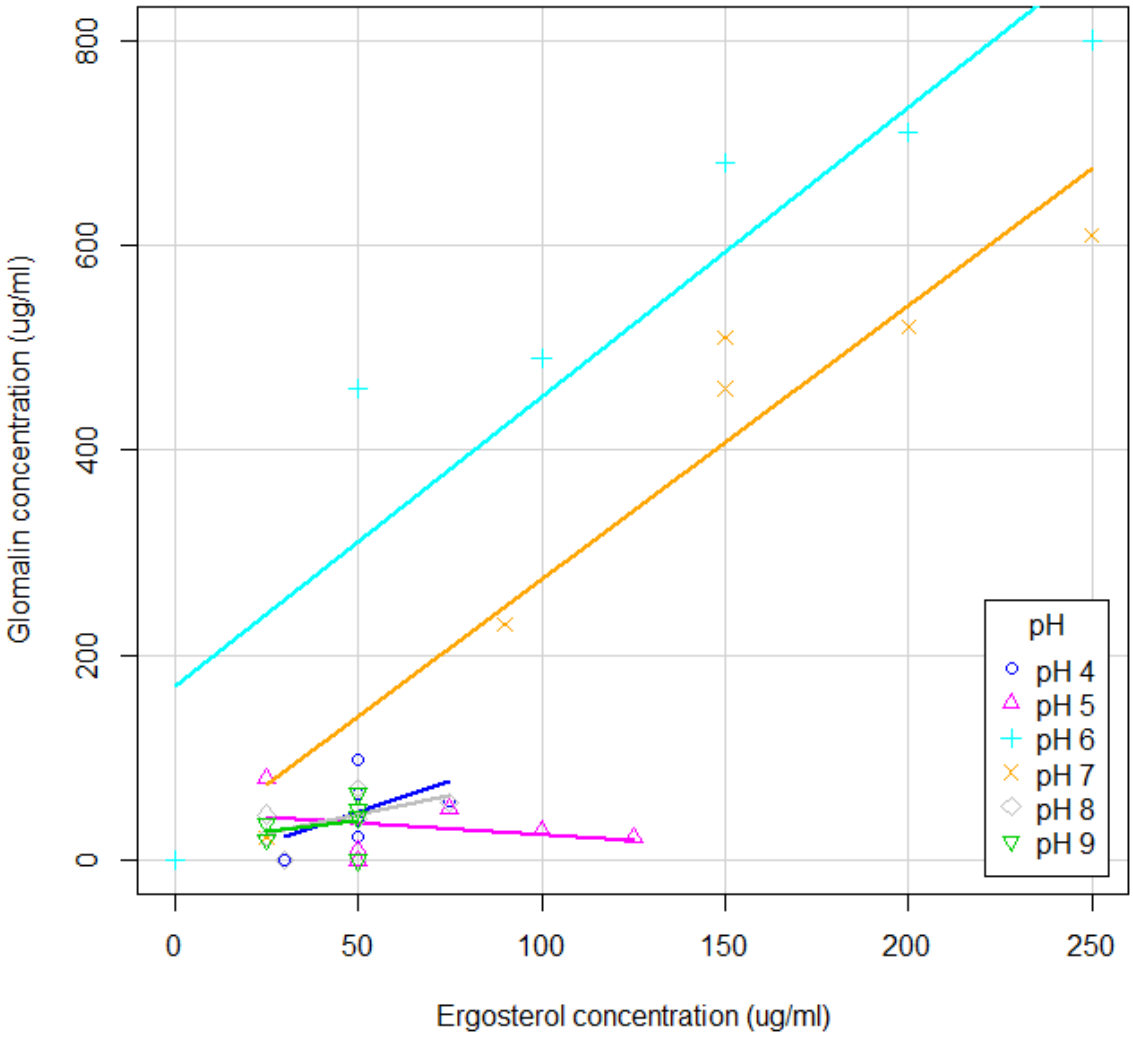

Figure 3.23 – Growth of AM fungus (*R. intraradicices*) in pH adjusted top soils showing increases in glomalin and ergosterol ($P < 0.00001$ – ergosterol, $P < 0.00001$ – glomalin) at pH 6 (Pearson's correlation: 0.92, r^2 – 0.84) and 7 (Pearson's correlation: 0.96, r^2 – 0.92), with proportional increases in glomalin compared with ergosterol indicating glomalin may be used as a biomass indicator for AM fungi.

3.3.9 Comparison of the correlation between glomalin and soil nutrients between tillage treatment

A comparison between glomalin, soil potassium and phosphates did not produce any statistical significance correlation indicating the existence of a relationship within arable soils at the time of study.

Soil nitrates generally produced a positive relationship with glomalin in all sampled soils (Pearson's correlation: 0.31, r^2: 0.09) with the exception of CT soils that gave indications of a negative relationship (Pearson's correlation: -0.04, r^2: 0.00). All tillage treatments, with the exception of CTC samples (P = 0.09, df: 28, t.stat: 1.76, paired unequal variance T test), were seen to increase nitrate concentrations (P < 0.01, df: 39, t.stat: 2.63, paired unequal variance T test).

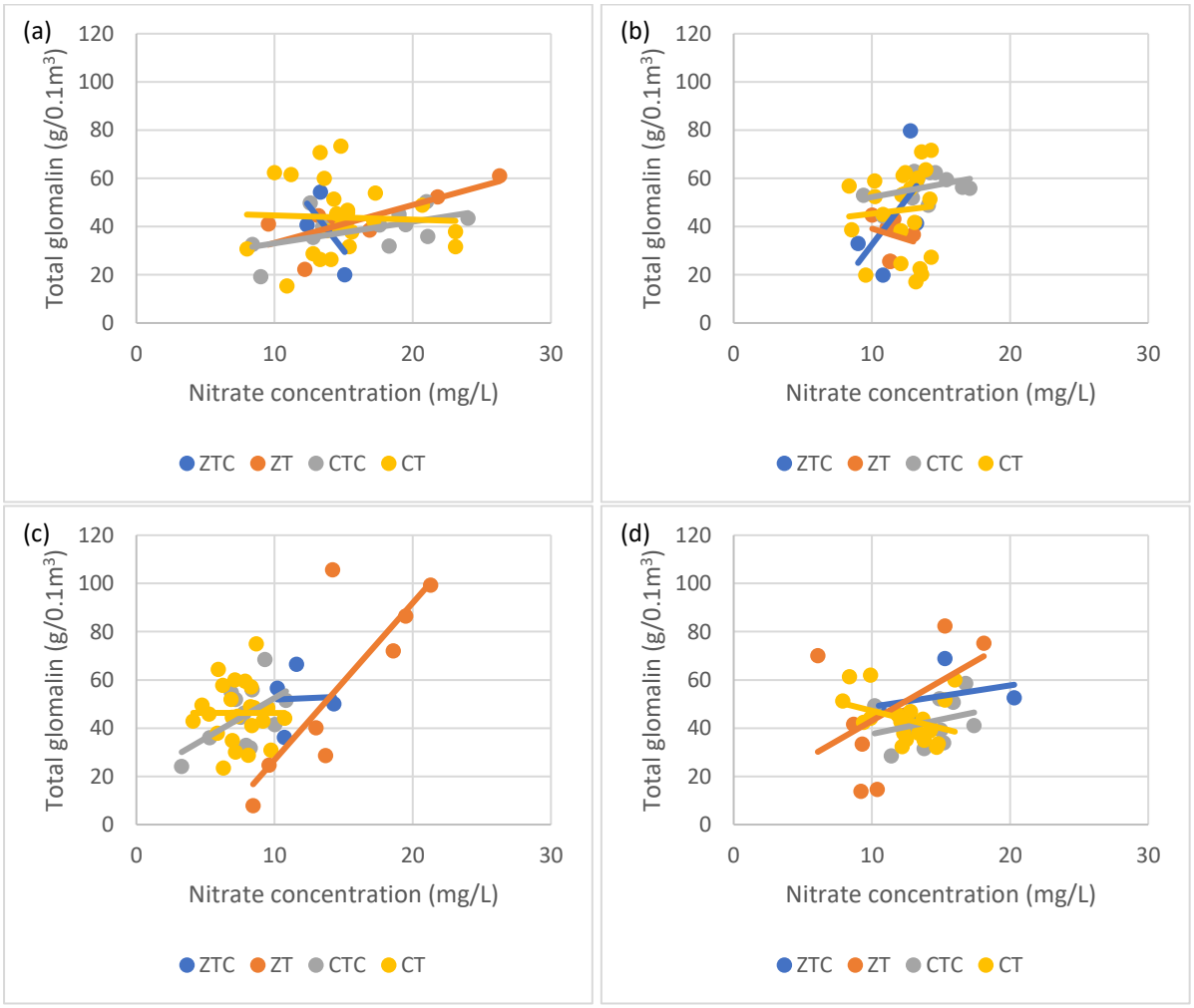

Figure 3.24 – Correlation between soil nitrates and total glomalin for 4 sampling periods of the growing season between conventional tillage (CT) and zero tillage (ZT) managed sandy loam soils: (a) pre cultivation, (b) 3 months post cultivation, (c) pre harvest and (d) pre cultivation 2019.

Table 3.6 – Pearson's correlation, and R^2 values, between nitrate and total glomalin correlations in conventional tillage (CT) and zero tillage (ZT) soils with respective control samples (Conventional tillage control (CTC) and Zero tillage control (ZTC)) over the sampling year September 2018 to August 2019.

Sampling time	**Depth (cm)**	**Pearson's correlation**	**R^2**
Pre cultivation/sowing (Figure 3.24a)	CT	-0.04	0.00
	ZT	0.31	0.09
	CTC	0.43	0.19
	ZTC	-0.26	0.07
Post cultivation/sowing (Figure 3.24b)	CT	-0.07	0.01
	ZT	-0.19	0.04
	CTC	0.52	0.27
	ZTC	0.62	0.04
Pre harvest (Figure 3.24c)	CT	0.00	0.00
	ZT	0.47	0.22
	CTC	0.50	0.25
	ZTC	-0.48	0.22
Pre cultivation/sowing (Figure 3.24d)	CT	-0.39	0.15
	ZT	0.48	0.23
	CTC	0.22	0.05
	ZTC	0.22	0.05

Average nitrate concentrations over 12 months for soil textures, observed from ZT soils, produced little variance (P = 0.19, df: 3,92, F value: 1.61, F critical: 2.70, single factor ANOVA) (Figure 3.25). However, sandy clay soils were measured to produce greater concentrations of average soil nitrates closely followed by sandy loam soils.

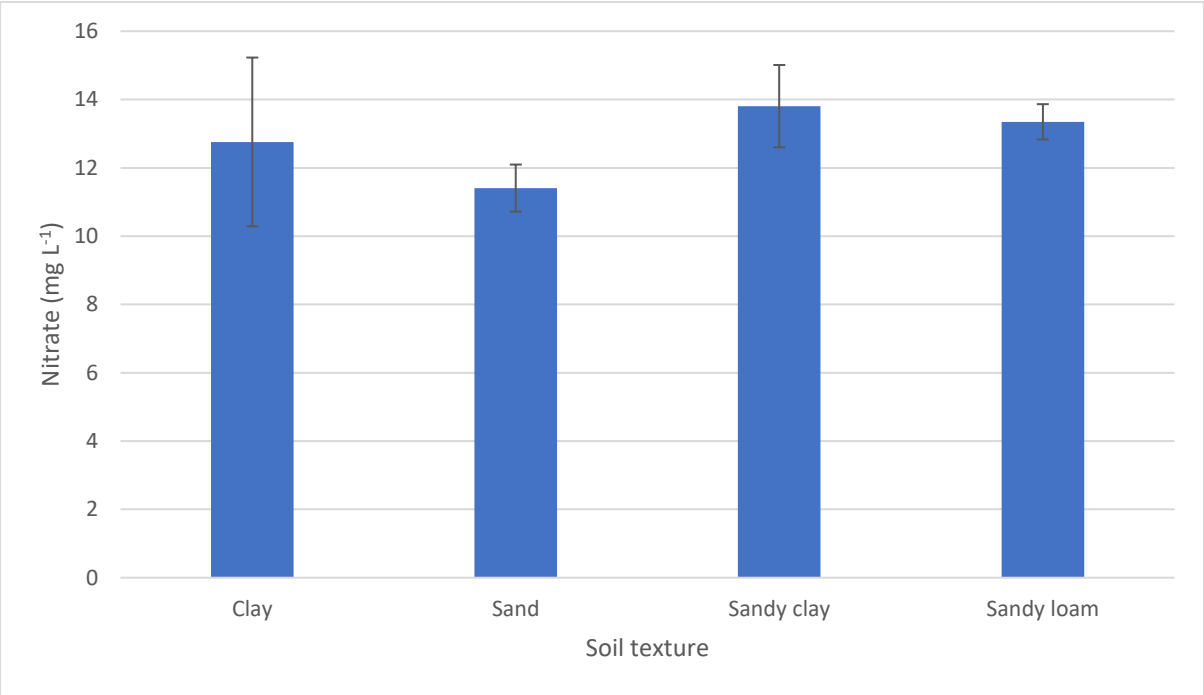

Figure 3.25 – Average (n = 96) soil nitrate concentrations of all depths in zero tillage (ZT) soils over 12 month sampling period per identified soil texture.

3.3.10 The effect of soil temperature on the quantified biomass of AM fungi

Field ergosterol quantities for each tillage treatment were observed to decrease after initial sampling in September 2018 (Figure 3.26). Soils subject to greatest disturbance, i.e. CT, produced the decreased greatest in soil ergosterol ($P < 0.00001$, F value: 20.59, df: 3,48, F critical: 2.79, single factor ANOVA) over the sampling year. Soil temperatures, under field conditions, did not have an influence on the quantity of soil ergosterol.

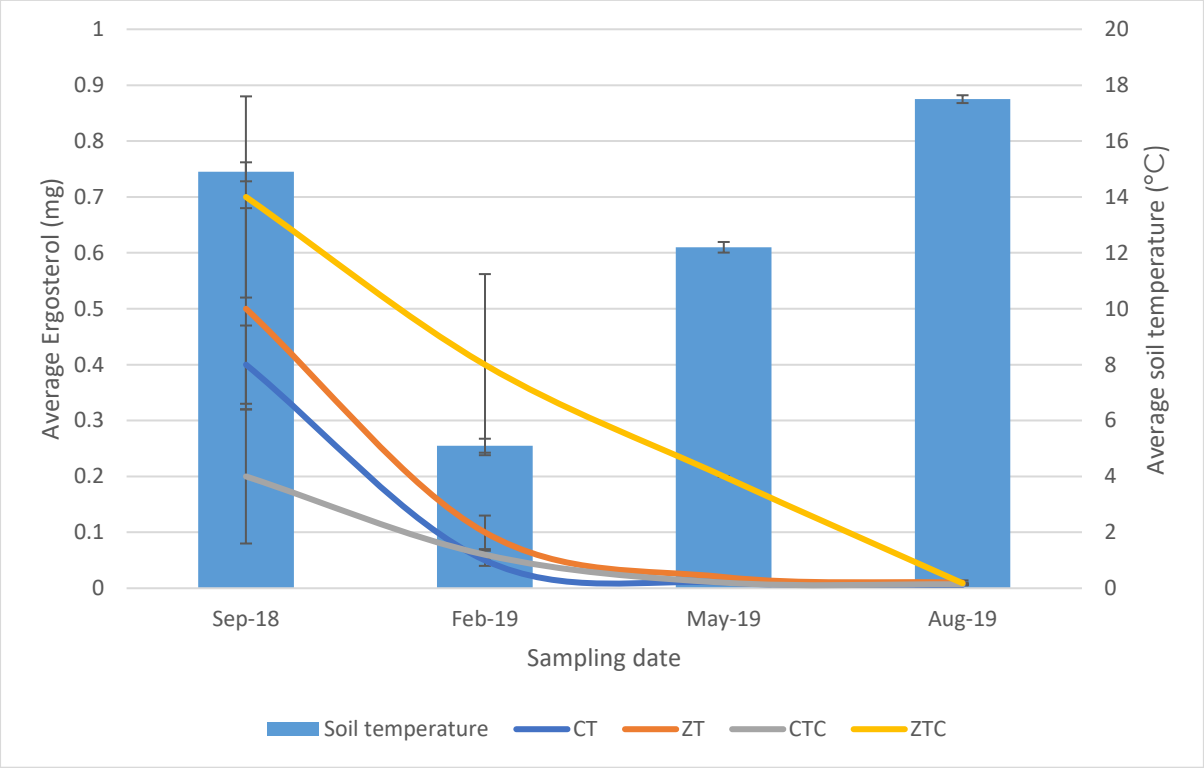

Figure 3.26 – Measured average (Conventional tillage (CT), n = 96; Zero tillage (ZT), n = 69; Conventional tillage control (CTC), n = 80; Zero tillage control (ZTC), n = 80) ergosterol concentration of sampled soils from CT and ZT sites over a the sampling year (pre cultivation – September 2018; 3 months post cultivation – February 2019; pre harvest – May 2019; 1 month post harvest – August 2019).

Further analysis of the influences of soil temperature on quantifiable ergosterol was able to show a positive relationship between the attributes in both CT (Pearson's correlation: 0.26, r^2: 0.07) and ZT (Pearson's correlation: 0.26, r^2: 0.53) tillage treatments (Figure 3.27). ZT managed soils produced an overall increased amount of ergosterol ($P < 0.001$, df: 58, t.stat: -3.45, paired unequal variance T test) when compared against soils managed under CT.

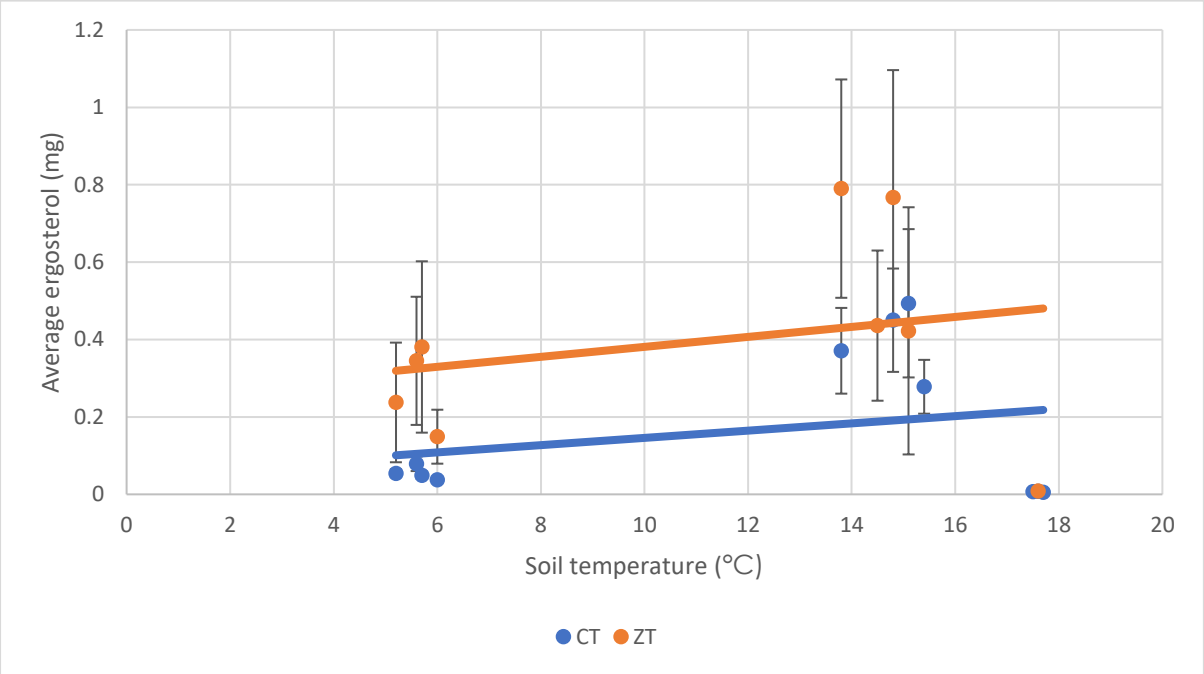

Figure 3.27 – Average (Conventional tillage (CT), n = 96; Zero tillage (ZT), n = 69) soil ergosterol across soil temperature for the whole sampling year. Pearson coefficients were measured for CT (0.26, r^2 – 0.07) and ZT (0.26, r^2 – 0.53)

HPLC quantification chromatographs (Figures 3.28 and 3.29 for CT and ZT respectively) were able to indicate the abundance of soil AM fungi from each respective sample site at all sampling depths, with ZT soils able to maintain an increased abundance in AM fungi when compared with soils managed under CT treatments. Ergosterol standard, ran for retention time comparison, is displayed in Appendix 8.14.

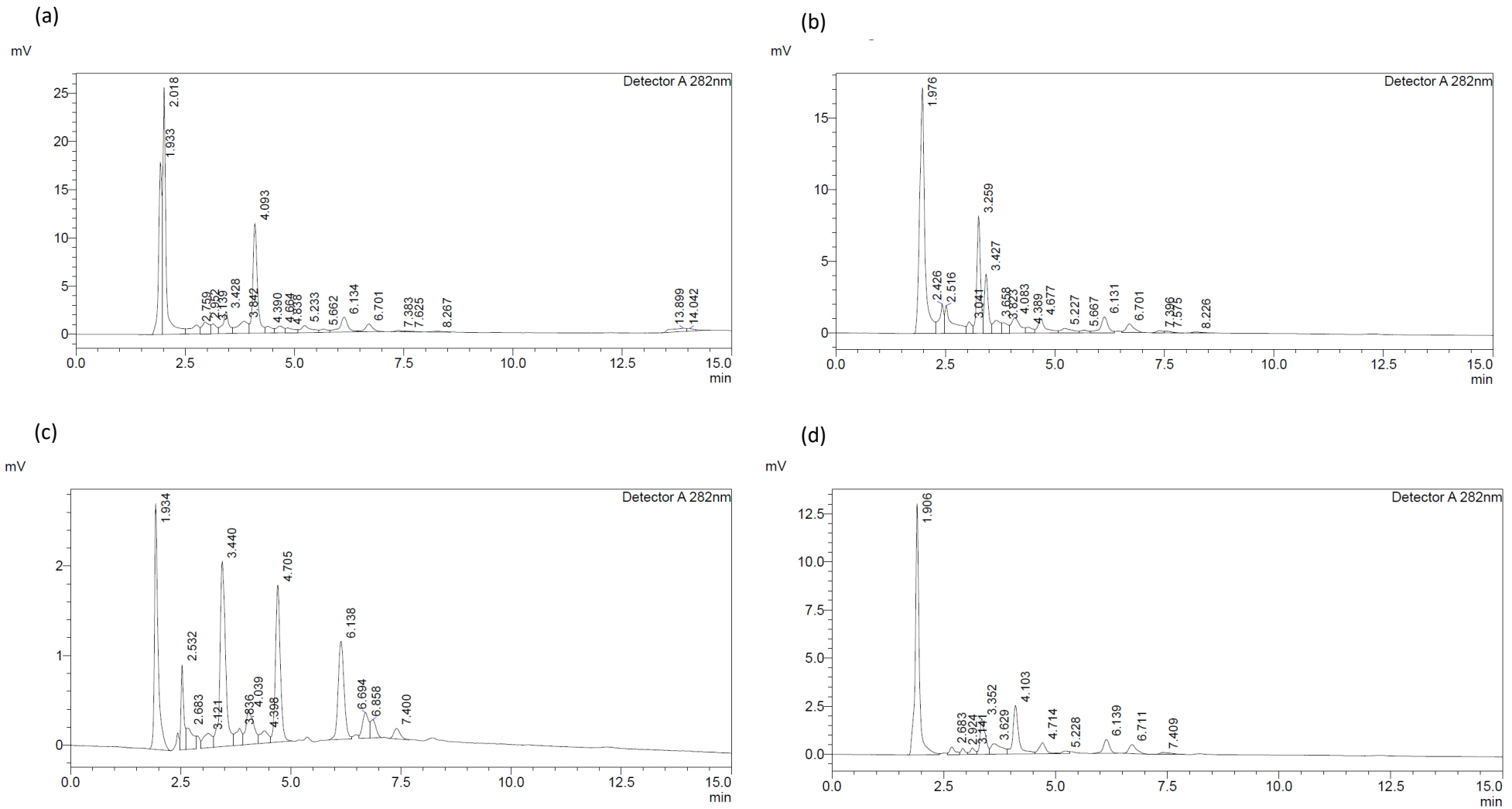

Figure 3.28 - HPLC chromatographs of ergosterol (retention time: 8.4 minutes) from bulk soils, measured at 282nm, of conventional tillage (CT) managed soils pre-sowing (September 2018) at (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm sampling depths

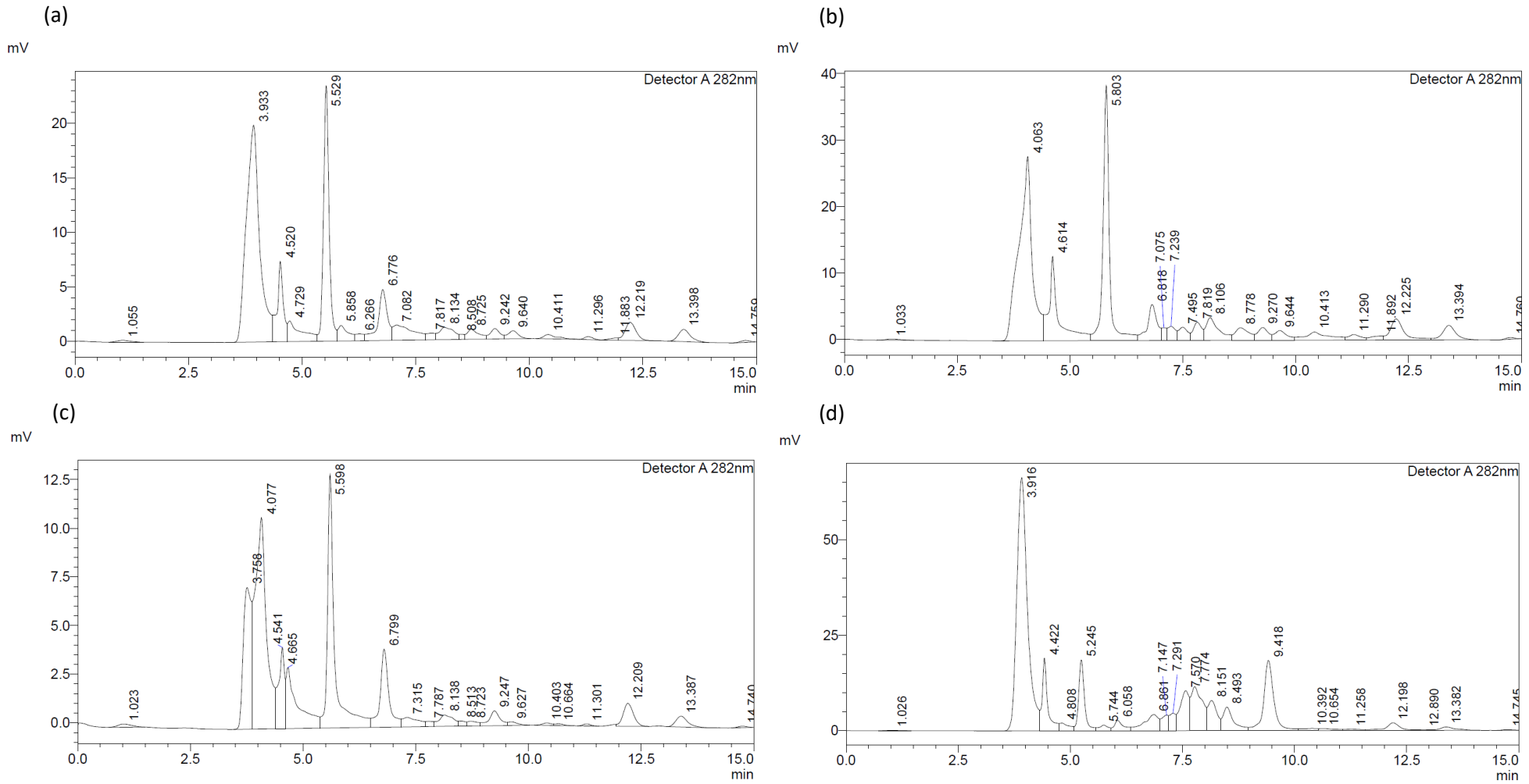

Figure 3.29 – HPLC chromatographs of ergosterol (retention time: 8.4 minutes) in bulk, measured at 282nm, soils of zero tillage (ZT) managed soils pre-sowing (September 2018) at (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm sampling depths

3.3.10.1 The effects of temperature on the biomass of AM fungi and production of glomalin from lab controlled protocols

Controlled growth of isolated AM fungi indicated an optimum growth temperature of 25°C, with no recorded growth over 25°C as quantified via ergosterol HPLC methodologies. Glomalin was also measured over the same controlled growth temperatures and was not quantifiable at temperatures over 25°C.

Fungal biomass over a known temperature (Pearson's correlation: 0.97, r^2, 0.95) growth range did not produce any significance (P = 0.75, df: 4,20, F value: 0.48, F critical: 2.87, single factor ANOVA). A Pearson's Chi squared test indicated significance (P < 0.00001) between quantified ergosterol and growth temperature.

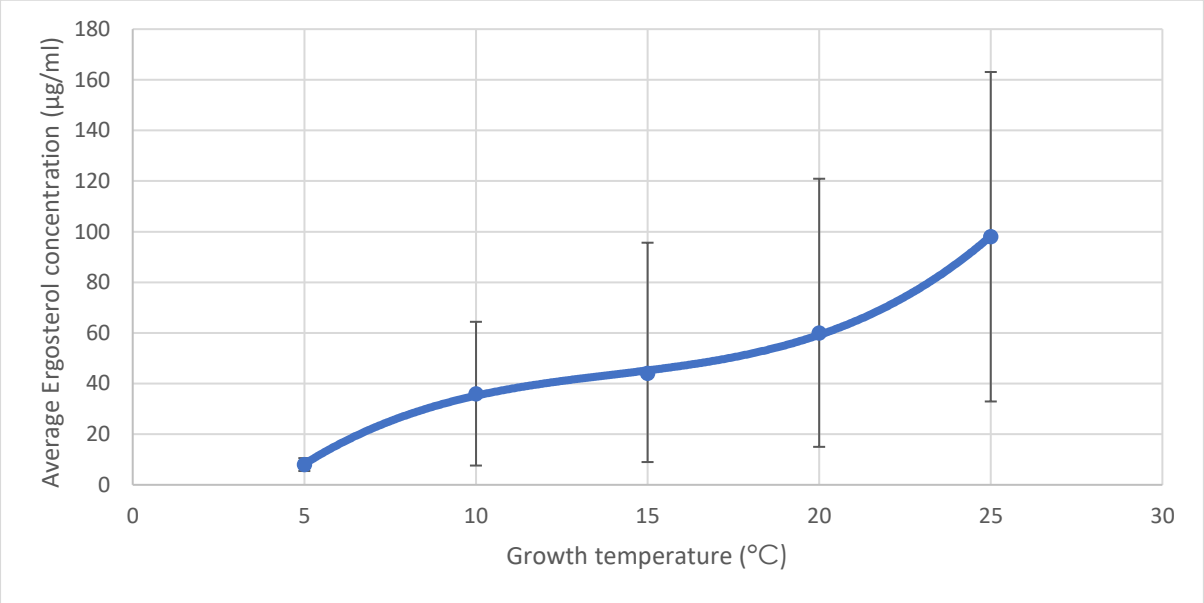

Figure 3.30 – Average (n = 25) ergosterol concentration from isolated AM fungi, sampled from areas of permanent grassland and isolated on a nutrient media, were incubated over a known temperature range (Pearson's coeffect 0.97, r^2 0.95). No growth was recorded at temperatures above 25°C. Error bars from SEM.

Average glomalin concentrations over a known growth temperate range produced a positive relationship (Pearson's correlation: 0.79, r^2: 0.62). A Pearson's Chi squared test indicated significance (P < 0.00001) between quantified glomalin and growth temperature.

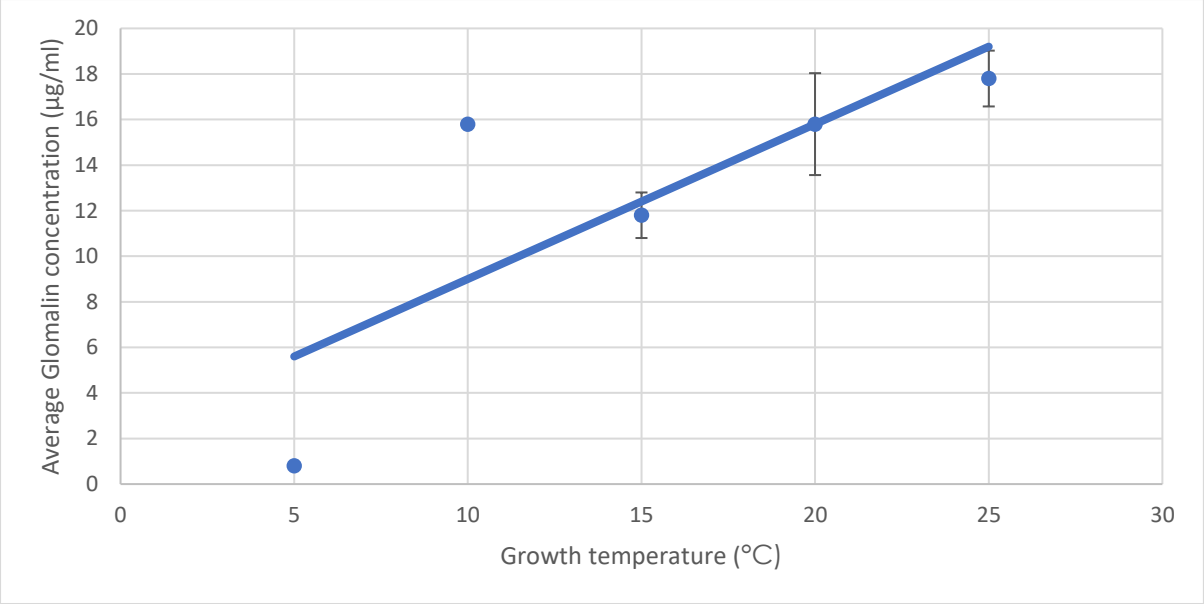

Figure 3.31 - Average (n = 25) glomalin from isolated AM fungi, sampled from areas of permanent grassland and isolated on a nutrient media, were incubated over a known temperature range (Pearson's coeffect 0.79, r^2 0.62). Error bars from SEM.

3.4 Discussion

Glomalin is a crucial compound within soils for two main reasons. Firstly, glomalin contains an estimated 30 – 40% carbon and 3 – 5% nitrogen making it an excellent carbon and nitrogen storage molecule (Lovelock et al., 2004). Secondly, glomalin has adhesive properties towards soil microaggregates (<250µm) improving soil structure and reducing erosion (Wright and Upadhyaya 1996). Land management by CT physically destroys the adhesive properties of glomalin resulting in soils becoming more susceptible to wind and water erosion (Vaidya et al., 2011). There strong positive relationship between glomalin and WSA in ZT soils which was not found in CT soils (Figure 3.6). This indicates improvements to soil structure associated with conservational land management practices. These findings correspond in part with those of Lombardo et al. (2019) who showed reductions in glomalin and aggregate stability from the application of tillage. While this study also notes a reduction in aggregate stability in CT it finds that glomalin levels fluctuate temporally in both the CT and ZT systems. Soil glomalin levels are significantly higher in the ZT system during late spring, when the crop root network and the potential for AM fungi – crop symbiosis is at its maximum. Conventional tillage damages mycelial networks, reducing AM fungal biomass. Glomalin is produced by AM fungi as a structural support molecule along mycelia (Driver et al., 2005) to aid in the growth through pore spaces (Wright et al., 1996). Soil inversion reduces AM fungal abundance which in turn reduces glomalin production, decreases aggregate stability and increases the risk of soil erosion. In contrast, the lack of soil inversion of ZT conserves the AM fungal mycelial networks and allows increased glomalin production. Not only does this increase aggregate stability but also results in higher soil carbon from that contained within the glomalin. When comparing four different soil textures, the positive relationship between WSA and glomalin (Figure 3.9) is maintained for ZT. This suggests ZT land management practices prove beneficial for AM fungi over a broad range of soil types and over large areas of agricultural land. It is noted however, that soils in the CT treatment exhibited a slight positive increase in soil glomalin over a period of seven months post cultivation. This is likely the result of AM fungi producing more glomalin and increasing in biomass after their initial disturbance from cultivation. Overall glomalin concentrations were still reduced in CT compared to ZT soils. Curaqueo et al. (2010) found that total hyphal length was reduced in CT managed soils (3.5m g^{-1}) compared to ZT soils (5m g^{-1}) supporting hyphal damage as described by Lambardo et al. (2019). Additionally, the

length of active hyphae in CT soils was less (0.5 m g^{-1}, 14.3% of total hyphal length), in comparison to ZT managed soils (1.0m g^{-1}, 20% of total hyphal length). Curaqueo et al. (2010) further showed that an increase in active hyphae enhanced soil glomalin and was further correlated to increases in WSA in ZT soils. This was not evident in CT soils. Finding presented in the current chapter supports Curaqueo et al. (2010) with a higher positive Pearson's correlation present (0.85 Figure 3.6b, and 0.87 Figure 3.6c for ZT soils) between glomalin and WSA in the undisturbed ZT soils. The Pearson's correlation coefficient between WSA and total glomalin in the CT treatment increased slightly, from 0.07 to 0.13, for the period post cultivation to pre-harvest. This suggests that any regeneration of AM fungi mycelial networks in an annually cultivated crop are marginal.

3.3.1 The relationship between glomalin and WSA between soil textures

Soil texture is a key attribute in maintaining a greater aggregate stability (Nciizah and Wakindiki, 2014). The ZT managed field had four distinct soil textures: sand, sandy loam sandy clay and clay. All four textures demonstrated a positive correlation between WSA and total glomalin in the ZT treatment. From Figure 3.9, sandy soils had the greatest positive correlation of all the soil textures, suggesting that this soil texture benefits the most from ZT with respect to maintaining and / or increasing glomalin and aggregate stability. Increases in glomalin were quantified in sandy soils by both the Bradford assay and Equation 2, ranging between 20 – 100µg ml^{-1} and 14.4 – 68.4g 0.1m^{-3} respectively. Berruit et al. (2016) report that soil aeration is favourable to the growth of AM fungi. From the bulk density distribution, Figure 3.10, sandy soils have a marginally lower density than sandy loam soils. This, coupled with the greater increase in glomalin, may indicate that the aeration of sandy soils is higher compared to the other soil textures supporting the conclusions of Chang et al. (2015). These authors conclude that because sandy soils are comprised of larger pore spaces relative to other soil textures there is a greater proportion of air contained within the soil profile, producing an aerobic environment. Such soils are also more free draining and less likely to have elevated water filled pore spaces which again, will decrease the likelihood that anaerobic soil conditions will be created. Overall, it supports the notion that soil aeration is favourable to AM fungi (Berruit et al., 2016) and additionally explains the greater increase in glomalin quantified from this soil texture. An increase in soil compaction coupled with a decline in soil aeration in ZT treatments have been noted by several studies

(Calonego et al., 2017; Nunes et al. 2015; Teodor et al., 2011) compared to CT managed soils (Busari et al., 2015). Whilst ZT practices may have long term benefits for AM fungi due to reduced physical disturbance and preventing damage to hyphal networks, the practice does not necessarily create an optimal soil environment.

All soil types, except clay soils, had a similar range in WSA (35.7 – 51.7%). Clay soils tend to have a greater underlying aggregate stability compared to, for example, sandy soils. This is attributed to the physical properties of the soil itself, namely a higher cation exchange capacity (CEC) (Arthur et al., 2020) rather than the quantity of glomalin within the soil. Kobierski et al. (2018) also report a higher CEC in clay soils but this was further correlated with increases in soil organic matter. However, comparing the studies of both Wang et al. (2017) and Kobierski et al. (2018) with the data presented here, the field sampled clay soils had quantifiably less WSA (36%) and glomalin (0.2% of total soil mass) than the clay soils reported in the other two studies (WSA, 44%; glomalin 2.2% of total soil mass). The glomalin in clay soils was lower than the other soil textures, as shown in Figure 3.9, suggesting a corresponding reduction in AM fungal abundance. Clay soils are vulnerable to compaction, especially when wet (Nunes et al., 2015). Elevated levels of soil compaction creates a sub-optimal anaerobic soil environment detrimental to AM fungi growth (Berruit et al., 2016) and hence glomalin production. It is likely that the WSAs observed for this soil texture are a product mainly of the CEC inherent in the clay soil texture rather than due to the levels of glomalin. It does however highlight a potential negative associated with ZT systems, ZT on clay soils especially.

3.4.1.2 Influences of tillage towards the glomalin and WSA relationship in sandy loam soils

Both the ZT and CT treatments had sandy loam soils within the field. Removing soil texture as a variable and comparing the two tillage regimes for this soil texture only reveals a decline in the correlation between WSA and total glomalin in the CT treatment. This suggests the physical application of a soil inversion system has significant negative implications for soil stability. Zheng et al. (2019) highlight a reduction in soil aggregate stability of 34% from the application of CT management practices. Zheng et al. (2019) additionally showed an increase in organic matter content with the employment of a ZT management system, corresponding to that of Figure 3.16. Figure 3.6b shows glomalin for

both CT and ZT during the first sampling period, post cultivation, were comparable, 17.1 - 86.1g $0.1m^{-3}$ and 14.4 – 78.1g $0.1m^{-3}$ respectively. This is likely due to the biotrophic nature of AM fungi. During post cultivation sampling, root systems were still developing and not able to host an abundant AM fungal community. This is supported by the increase in glomalin in Figure 3.6c, 16.3 - 80.1g $0.1m^{-3}$ and 7.7 – 108.8g $0.1m^{-3}$ for CT and ZT respectively. The increase of 30.7g $0.1m^{-3}$ observed in ZT soils between the February and May sampling periods is attributed to mycelial networks remaining complete in the absence of soil inversion. This means an abundance of AM fungi are available for glomalin production and carbon sequestration as soon as host plant root development occurs. This is not the case with CT managed soils. Mycelial networks are damaged or destroyed from soil inversion causing a lag phase in the development of plant-fungal associations due to the biotrophic nature of AM fungi. This is supported by the reduction in levels of glomalin quantified between post cultivation and pre harvest, -6.0 g $0.1m^{-3}$. Interestingly, a decline in glomalin occurs in the undisturbed layers of the CT treatment (30 – 40cm) and the ZT treatment between September 2018 and February 2019 before increasing simultaneously with crop root growth during the spring. In contrast the increase in glomalin in the 0 – 20cm layer of the CT treatment during February is within the zone of physical disturbance, possibly due to the deposition of glomalin within soils upon death of the AM fungi (Driver et al., 2005). The lower correlation between glomalin and WSA in CT but not ZT (Figure 3.6) would support this assertion. Glomalin then declines rapidly in the 0 – 20cm zone of CT during the three month period immediately afterwards despite crop root growth being at its most prolific. Root symbiosis evidently declines in this part of the soil profile despite the available plant root material being at its optimum. Glomalin is highest in the upper soil profile of the ZT treatment, especially during May when it peaks. The disturbance of soil due to cultivation of the 0 – 20cm in the CT treatment during September reduces the glomalin concentration in this zone and in the CT treatment overall. At the end of August 2019 both the CT and ZT crops have been harvested decreasing the available living root material with which the AM fungi may form symbiosis. Whilst the glomalin values are relatively similar between CT and ZT at this stage in the cropping cycle, the associated WSA for each tillage treatment are not. There is a clear increase in stability in ZT managed soils further corroborating Zheng et al. (2019). Hajabbasi and Hemmat (2000) studied changes in aggregate stability and aggregate size between three tillage systems: CT, ZT and

mouldboard plough with disking. They conclude that ZT management practices increased both soil aggregate stability and the size of soil aggregates. The data presented in Figure 3.6 shows reduced WSA in CT soils supports these findings further. Hajabbasi and Hemmat (2000) were additionally able to show that reductions in organic matter produce correspondingly weaker soil aggregates. A reduction in the overall organic matter and WSA in CT managed soils was also identified here. The work by Haddaway et al. (2016) also describes the loss of soil carbon, a component of soil organic matter, through the implementation of CT management practices.

ZT enhanced soil glomalin relative to CT in sandy loam soils. The practice evidently improves AM fungal populations and therefore the associated benefits provided to the crop grown coupled with the potential for soil carbon storage attributed to the glomalin molecule. While reducing physical disturbance has a corresponding benefit, the environment created is not entirely optimal. Although the hyphal networks are maintained through limiting the physical disturbance of the soils this may be compromised in part due to the elevated risk of anaerobic soil conditions. Minimising soil compaction and anaerobic soil conditions in ZT systems is a key requirement in order to fully maximise the benefit to be derived from AM fungi. Wang et al. (2017) and Berruit et al. (2016) both conclude that glomalin was a proportionally greater contributor to soil carbon in deeper parts of the soil profile relative to the total percentage contribution to soil organic matter. Bradley et al. (2005) note the contribution of organic matter to be greatest in the upper soil profile. In order to maximise soil carbon sequestration, the higher organic matter reported in the upper soil profile of ZT systems requires full integration with an increase in soil carbon in the lower soil profile provided by glomalin. Critically it may offer a solution to the observations of Powlson et al. (2014) that ZT does not increase soil carbon due to an increase in organic matter content overall but merely redistributes it within the soil profile i.e. it increases in the upper profile while declining in the lower profile. This is hypothesised to be in part due to the lack of incorporation of crop residues into the lower soil profile in the absence of tillage (Krauss et al., 2017). Enhancing the abundance of AM fungi and the associated production of glomalin in the lower soil profile in ZT systems would potentially counter this issue i.e. carbon within organic matter will increase in the upper soil profile while simultaneously the carbon derived from glomalin is enhanced in the lower. The role of AM

fungi in augmenting soil carbon cannot therefore be underestimated. Improved soil aeration in lower parts of the soil profile and the avoidance of soil compaction, a potential risk in ZT systems, is key particularly in the small particulate clay soils. The enhancement of soil organic matter is cited by Reicosky (2003) but as noted by Krauss et al. (2017) this may be difficult in ZT systems due to not being able to incorporate crop residues. The manipulation of crop rotations to include root crops with the capacity to penetrate compacted soils (Jabro and Allen, 2018) coupled with low wheel pressure controlled traffic farming (Antille et al., 2019; Millington, 2019) may offer potential solutions. Carbon sequestration and the associated benefits for climate change mitigation will be enhanced, both through an increase in the organic matter content (Smith et al., 2020) but also from greater quantities of the carbon containing glomalin molecule. The relationship between root crops and AM fungi, and the impact on soil glomalin due to the inclusion of such crops within an arable rotation requires further investigation.

The range in the quantity of organic matter is narrow for both CT and ZT treated soils for each sampling period, 1 – 12% and 3 – 15% for CT and ZT respectively. The range for WSA is larger within ZT soils, 35.7 – 51.7%, exhibiting a positive correlation (0.85 Pearson's coefficient) for each of the sampling periods assessed. As previously described by many authors, CT reduces soil carbon through the inversion and subsequent aeration of the soil, resulting in the oxidisation of carbon to CO_2 (Haddaway et al., 2017; Zhao et al., 2017). This would appear to be the case for CT soils analysed here, as both soil carbon (Figure 3.16) and WSA were higher in the soils of the ZT treatment compared to that of CT. Carbon is also a constituent of glomalin (Lovelock et al., 2004). The lower quantities of glomalin and soil carbon in the CT soils has a number of implications. Firstly, the soil carbon store attributed to glomalin is reduced, reducing its value in terms of climate change mitigation (Vlcek and Pohanka, 2020). Further, the value glomalin has in preventing negative impacts, such as soil erosion through an increase in WSA, is diminished. The decline in glomalin overall suggests that its production, and therefore source (AM fungi), has also been negatively impacted. AM fungi have an important role in crop systems by improving nutrient acquisition (Ingraffia et al., 2019), aggregate stabilisation via mycelial networks (Driver et al., 2005) and long term carbon storage from the production of glomalin, with additional improvements to WSA. Conventional tillage is potentially detrimental to these three key soil functions.

3.4.2 Root and rhizosphere AM fungi

Rhizosphere soils are high in organic root exudates, photosynthate and microbial diversity. This region of soils has been studied for many years for its complex myriad of plant-microbe associations and community dynamics (Lei et al., 2018). AM fungi-host relationship can sequester carbon to glomalin which has aggregate stabilising properties through aggregate adhesion (Wright et al. 1996; Jia et al. 2018). Walley et al. (2014) investigated glomalin in rhizosphere soils in regards to carbon and nitrogen nutrition as well as carbon sequestration. Walley et al. (2014) concluded rhizosphere glomalin was a crucial contribution to carbon sequestration and a key source to improve overall soil aggregation. Therefore, increases in rhizosphere glomalin would have a greater impact in the restoration of soil aggregation in CT treated soils than ZT soils, seen as glomalin reduction in CT treated soils in Figure 3.11 and 3.12. Figure 3.20 shows a highly comparable range of glomalin between both tillage treatments across each sampling month. Variation comes about from root dry mass, which varied due to root length with ZT root systems being much shorter (average 27cm (n = 18), post cultivation) compared with CT roots (average 43cm (n = 18) post cultivation). This additionally supports the measured attributes of fungal abundance and biomass in CT sampled soils being much reduced compared to ZT. However, root length increases in the presence of a low abundance of AM fungi is not an area reported by literature. Present findings would suggest, in combination with the work of Stolan et al. (2016) and Pepe et al. (2018), a reduction in AM fungal mycelia that would increase root system surface area in soils and be able to exchange nutrients across arbuscules which would result in a reduction of plant allocated resources to root growth and development.

Negative correlations, between glomalin and ergosterol from rhizosphere soils on sampled wheat in Figure 3.21, can be used to suggest a comparable abundance of fungi in the rhizospheres of both CT and ZT sampled wheat. However, differences in glomalin between both tillage treatments and sampling months vary in the rhizosphere. As glomalin is unique to AM fungi (Wright et al., 1996; Rillig et al., 2002, Lovelock et al., 2004, Driver et al., 2005; Bendini et al., 2009; Adeleke, 2010; Prassad et al., 2018), it can be used as an indicator of abundance from its association with AM fungal mycelia. From this, ZT soils have more abundant AM fungi with wheat rhizospheres compared to CT wheat rhizospheres. Interestingly, the close similarity between CT rhizospheres in May 2019 and post cultivation

ZT rhizospheres potentially suggests a regenerative property with AM fungi, from quantified glomalin. To take this further, Figure 4.13 of Section 4.2, from a controlled glasshouse experiment, shows the recovery of AM fungal arbuscules 12 months after tillage was applied and absent from the next annual tilling treatment. This may provide indicators that CT soils may increase in glomalin annually until a following tillage treatment is applied to soils, reducing glomalin back to a reduced quantity (Figure 4.13). Furthermore, if tillage was not applied annually to CT managed soils, Figure 4.13 may be used to provide an early indication that glomalin concentrations will increase to a more comparable quantity to ZT managed soils further supporting a building body of evidence that CT treatments are detrimental to soil quality (Apesteguia et al., 2017) as opposed to ZT managed soils and the maintenance of soil quality (Busari et al., 2015).

Root ergosterol quantification not only includes ectomycorrhizal fungi, but likely to include fungal biomass from intracellular arbuscules. This is from the corrosive nature of the ergosterol extraction process, which has similarities to the initial stages of arbuscular staining of environmentally sampled roots. In regards to arbuscular staining, the corrosion by potassium hydroxide (Section 2.3.4) is designed to hydrolyse pectin between plant cells prior to FAA fixation (Section 2.3.4) to then allow arbuscular stain to penetrate and interact with the target structures. Ergosterol extraction via potassium hydroxide is designed to aid in the initial breakdown of fungal cells in preparation for ergosterol extraction. With root tissues, potassium hydroxide will damage the plant cell exposing other fungal structures that are then processed through ergosterol extraction, resulting in an inflated quantification of fungal biomass with root tissues and the rhizoplane. Indications of Figure 3.21 and 3.24 show CT and ZT root samples producing very similar and comparable root and rhizoplane glomalin and ergosterol respectively. This suggests that there may only be marginal differences between tillage treatment regarding AM fungi in the rhizoplane. This is further supported by Figure 4.13 showing a marginal increase to intracellular root cortical arbuscules of AM fungi in ZT sampled wheat. This gives rise to a potential similarity between CT and ZT tillage practices and may be a strong candidate for CT soil regeneration towards soil quantities seen and studies in ZT soils. However, literature is unable to comment on AM fungi in the rhizoplane and differences between tillage treatments. Schreiner et al. (1997) produces investigations into rhizoplane mycorrhizae with conclusions of the increase to

WSA achieved through their presence. Schreiner et al. (1997) used an experimental protocol under controlled conditions and field investigations into rhizoplane mycorrhizae have only been carried out via molecular means into fungi diversity (Singh et al., 2008) also without the impact of a tillage treatment. This implies a need for further investigation as Schreiner et al. (1997), only in part, supports the notion of a potential CT soil regeneration in WSA towards levels quantified in ZT treated soils.

3.4.3 Glomalin and carbon sequestration

Glomalin has the potential to sequester carbon into soil (Walley et al., 2014). interestingly, Wang et al. (2020) investigated soil glomalin in poplar woodland in relation to glomalin's overall contribution to soil quality. Wang et al. (2020) gave glomalin as 1.8 to 2.0 times higher in the upper 40cm of sampled soils compared with soils sampled between 40cm to 1m from the soil surface. However, Wang et al. (2020) used a modified glomalin extraction protocol that did not give sufficient heat treatment for the denaturation of all other proteins within the soil sample. The method used here and described in Section 2.3.5, maintains a temperature of 121°C for 60 minutes to ensure only glomalin remains. Glomalin has similarities to heat shock protein and will not denature under the given extraction conditions (Section 2.3.5). Wang et al. (2020) only maintained the constant 121°C for 30 minutes and altered the soil to extraction solution ratio from 1:8 to 1:4. Jonas et al. (2008) investigated soil to extraction solution ratios and found little differences in quantifiable glomalin if the ratio between soils and extraction solution was maintained at 1:8. Jonas et al. (2008) found unpredictable variances when the ratio between soil and extraction solution was altered away from a 1:8 ratio. Following this, a 1:4 soil to extraction solution ratio employed by Wang et al. (2020) would not produce consistent results. Furthermore, Wang et al. (2020) used 4,000 rpm centrifugation after heat treatment. Centrifugation of 4,000 rpm is not sufficient to pellet cellular debris from soil organisms or other fine particulates of organic matter that may be transferred in the supernatant to spectroscopy as described by Wright and Upadhyaya (1996). From the results of the glomalin analysis performed in Section 2.3.5. centrifugation of 4,000 rpm will give unreliable absorbance data and contribute to an artificially inflated glomalin quantification from suspended organic debris. This potential source of error has been removed by the protocols adopted in this study.

3.4.4 Effects of temperature on AM fungal biomass

AM fungi, like all organisms, have an optimal growth temperature. Gavito et al. (2005) studied temperature ranges on phosphate transfer from soils to host plants and concluded that *Glomus intraradices* has an optimal growth temperature between 18 and 30°C. Zhu et al. (2017) further explores the effects of a varying temperature range on AM fungi and are able to show the reduction in host plant root colonisation from AM fungi at lower temperatures. This supports data shown in Figure 3.27 indicating reductions in soil temperature are associated with reductions in soil ergosterol, as a measure of fungal biomass. Figure 3.26 also indicates a reduction in ergosterol with reduced soil temperature during February 2019 sampling, a point in plant development typically associated with AM fungal colonisation (Fuzy et al. 2015; Bernaola et al. 2018). Presented data cannot give a direct suggestion of optimal AM fungal colonisation with stages in crop development, and as there are few reports in the literature remains an area requiring further investigation. However, as root systems were present in February 2019 sampling, ZT managed soils produced an increase (Figure 4.1) in AM fungal symbiosis compared to CT managed soils as quantified by root arbuscules. Whilst the optimal stage of AM fungal colonisation is yet to be determined, ZT soil management practices have produced improvements to AM fungal symbiosis (Figure 4.13) at each assessment opportunity. Furthermore, Figure 5.1 shows the greatest arbuscule increase of 1072 between weeks 1 and 2 of the control sample. Whilst this experiment was performed under controlled conditions and in purchased soils, it may be used to suggest that first 2 weeks of initial wheat growth are a crucial stage for AM fungal symbiosis to occur. This experimental set up requires further investigation in field soils to ascertain a more precise time period of optimal AM fungal associations with a host crop under environmental conditions.

Changes in temperature away from optimal growth temperature alter mycelial physiology (Kilpelainem et al., 2020). As mycelial growth instigates root colonisation, as shown in Figure 1.2 Section 1.0, temperature related alterations to mycelial physiology results in inhibited mycelial growth, growth pattern and phenology (Gavito et al. 2005; Zhu et al. 2017; Campant et al. 2010). As soils gradually warm, mycelial growth is stimulated (Hawkes et al., 2008). This provides explanation to Figure 3.27 showing increased fungal biomass with soil temperatures between 12 and 16°C. However, Figure 3.26 indicates a continued reduction in ergosterol over subsequent sampling with noted increases to soil

temperature. However, soil temperature is not the only variable impacting quantified soil ergosterol. As explored in Section 4.3.1., the application of glyphosate likely has a strong negative impact on the total quantity of AM fungi, thereby producing a measurable reduction in soil ergosterol concentrations. Furthermore, soil compaction is likely to influence the amount of quantifiable ergosterol in soils through the reduction of soil oxygen penetration in more compact soils. As AM fungi are aerobic, reduced soil oxygen is detrimental to their sustained growth. However, host-fungal interactions play a role in the provision of AM fungal survival at greater depths in soils where oxygen may be a limiting resource via root provided oxygen (Kapoor and Singh, 2017; Zhang et al., 2014).

Figure 3.27 provides a positive trend between soil temperature and ergosterol between both tillage treatments, whilst still indicating that ZT treated soils maintain an increased abundance of soil fungi. Coupling this with the increased quantities of glomalin (Figures 3.11 and 3.12), this could lead to confusions of increased AM fungal abundance in ZT soils. Furthermore, another potential explanation for the reduced quantities of ergosterol seen in Figure 3.26 for May and August 2019 may come from the reductions in viable root tissues for AM fungi to associated with. As wheat reaches maturity, root tissues begin to senesce. As AM fungi are biotrophic, this negatively impacts their life cycle (Section 1.1.1) and reduces their total abundance. As given by Hawkes et al. (2008) and supported by Figures 3.27 and 4.29, increases in temperature produce increased quantifiable ergosterol linked with fungi biomass. Development of the microbiome may be having a strong influence to the abundance of AM fungi as described by Xiae et al. (2007) through the development of biocontrol properties of *B. subtilis* against fungal pathogens (further explored in Section 5.3). Under laboratory conditions, both ergosterol and glomalin increased with temperature. Differences between laboratory controlled growth and field quantified ergosterol, as well as glomalin, may be due to agrichemicals applied to soils. Hage-Ahmed et al (2019) explores the range of implications pesticide application have towards AM fungi, noting a range of growth responses from AM fungi including increased or reduced overall biomass. Factors having a string influence towards the total AM fungal biomass include soil type, microbiome constituents and the range of metabolic activities against an applied pesticide as well as the overall physico-chemical properties of pesticides (Hage-Ahmed et al., 2019). Under lab conditions, AM fungi were grown in the absence of agrichemicals and over a range of growth temperatures that covered soil temperatures.

Therefore, the differences of applied agrichemicals between field and laboratory grown AM fungi provides support towards the notions implicated by Hage-Ahmed et al. (2019). It is note worthy that the investigations of agrichemicals, pesticides in particular, produce varying results with AM fungi as between different pesticides and from the same pesticides studied (Rivera-Becerril et al., 2017). This suggests that investigations into the effects of particular agrichemicals on AM fungi are still greatly under investigation with implications as yet not clear.

Gavito et al. (2005) gave AM fungal optimal growth temperatures between 18 and 30°C, however, at temperatures above 25°C no growth was quantifiable (Figure 3.30). From data given in Figures 3.30 and 3.31, a suggested maximum optimal temperature of 25 to 28°C and a minimum optimal temperature of 13 to 15°C can be given. The results presented here modifies the suggested temperature range from Gavito et al. (2005) to 13 to 28°C for AM fungal optimum growth. Further, Figure 3.26 shows soil temperature influences on average ergosterol between sampled bulk soils for both tillage treatments. Temperature fluctuations have growth limiting implications towards overall AM fungal biomass as presented in laboratory controlled temperature investigations in Figure 3.30. As shown by Figure 3.27, an increase in soil temperature produced an increase in the total quantifiable ergosterol. However, Figure 3.26 did not show such an increase to ergosterol with increased soil temperature. A likely cause of this difference comes about from the presence of root systems and biotrophic interactions between host and AM fungi. Furthermore, the application of glyphosate in ZT managed soils has long term effects and explored further in Section 4.3.1. Additionally, Figure 3.27 reinforces the notion that tillage damages fungal abundance, quantified via ergosterol, as opposed to a conservation method of soil management such as ZT, as CT treated soils consistently produced reduced quantities of soil ergosterol.

3.4.5 Influences of soil pH towards glomalin quantities

From field measured data, distinct regions of pH within both sampled sites were able to be determined. For each field a zone within the top soil (<10cm depth) provided optimal growth pH for AM fungi of 6.3 (Rousk et al., 2010; Herrrera-Peraza et al., 2011). Furthermore, a region of AM fungal optimal pH was additionally quantified at each sampling depth to a maximum depth of 30cm in CT (Figure 3.1a-c) and ZT soils (Figure 3.5e-g)

additionally to a maximum sampling depth of 30cm for a single region. Both sites in September 2018 did not produce optimal AM fungal pH at sampling depths of 40cm. Singh et al (2008) produced rhizosphere investigations into AM fungal diversity and abundance whilst producing a conclusion that soil pH had no influence in the overall abundance of AM fungi. However, Singh et al. (2008) did not take into account the buffering effects of the bacterial constituents of the microbiome as explored by Ratzke et al. (2018), Jones et al. (2009) and Rousk et al. (2010), producing data rationale of pH sensitivities by AM fungi as presented in Figure 3.23.

Ergosterol is used by several studies (Olsson et al. 2003; Yang et al. 2015; Rodrigues, 2018) as a biomarker in the membrane of fungi, giving indications of total fungal biomass. Glomalin, however, is specific to an estimated 80% of G*lomeromycetes*, the family of AM fungi (Wright et al., 1996). Therefore, glomalin may be used, alongside ergosterol, to give total fungal biomass indications and further indication AM fungal biomass. This is further supported by Figure 3.23 of laboratory grown AM fungi (*R. intraradicies*) over a pH range of 4 to 9 revealing greatest biomass, by ergosterol and glomalin, is produced at pH 6. This has additionally been reported by Lui et al. (2020) and Van Aarle et al. (2002). The majority of each field in September 2018 (pre cultivation) was measured to be at a pH away from the AM fungal optimal, and is accompanied by the reduced glomalin quantified at 40cm sampling depths (Figures 3.8 and 3.9).

Later sampling in February 2019 for both CT and ZT sites (Appendix 8.7), were predominately quantified away from AM fungal optimal growth pH. However, the greatest differences arise from Figure 3.1a and b with Figure 3.2a and b showing a majority of >60g $0.1m^{-3}$ total glomalin in February 2019 compared with an average 40g $0.1m^{-3}$ total glomalin in September 2018. This also indicates a peak in glomalin concentration at this sampling period. Whilst the pH is not AM fungal optimal, the increases in quantified glomalin is likely due to an increase in AM fungal growth from abiotic associations with developing root systems of winter wheat. This suggests a temporal delay in the effects of soil pH on the growth and development of AM fungi, which is seen as a reduction in glomalin at later sampling. This is supported by the reduction in glomalin measured in May 2019 (pre harvest) (Appendix 8.9), which is associated with a soil pH closer to AM fungal optimal than February 2019.

3.4.6 Soil nutrients and glomalin concentrations

Current literature is unable to suggest any correlation between soil potassium and phosphates with glomalin concentrations. Data presented in section 3.2.8 was also unable to find a correlation between glomalin and the two soil nutrients, therefore excluding it as an influencing factor. However, as Figure 3.24 depicts, a relationship between glomalin and soil nitrates does occur. Bukovska et al. (2016) was able to show an increase in AM fungal mycelia with increases in soil nitrogen contents. However, Bukovska et al. (2016) concluded select bacterial constituents of the soil microbiome, including *Nitrospira* spp. from molecular analytical methods, produced nitrogen compounds then utilised by AM fungi, of which do not possess enzymatic pathways for the utilisation of soil nitrogen themselves. Wang et al. (2018a) conducted a 2 year field and plot study investigating the relationship between AM fungi, host plants and soil nitrogen. Wang et al. (2018a) produced data indicating AM fungi and host plant were in strong competition for soil nitrogen and applied benomyl to inhibit AM fungal root colonisation. From the application of benomyl, plant tissues were quantified higher in nitrogen contents than plants with AM fungal colonisation present. However, the metabolic pathways AM fungal acquired nitrogen enters was not explored by either Bukovska et al. (2016) or Wang et al. (2018). Data presented by Lovelock et al. (2004) suggests that glomalin accounts for an estimated 5% of soil nitrogen within the first 10cm of top soil. This may provide a potential metabolic pathway in which AM fungal acquired soil nitrogen is utilised by the fungus. However, literature is currently unable to comment on this and remains an area requiring investigation, with a number of nitrogen and AM fungal studies investigating AM fungal mycelial length and/or biomass rather than the production of glomalin, a known nitrogen containing molecule produced by AM fungi (Lovelock et al., 2004).

Nitrate concentrations shown in Figure 3.24 present similar ranges between tillage treatment (CT and ZT), as well as their corresponding controls (CTC and ZTC). This indicates that the physical application of tillage has the greatest influence, as nitrate fertiliser applications were highly comparable between sample sites as provided by the respective land managers. While this is true, Figure 3.31c of pre harvest, shows an increased range of nitrates in ZT which is not seen in other tillage treatments of the same period. Following the corresponding periods shows a corresponding increase in glomalin (Figure 3.6c) with an

increased range in nitrates (Figure 3.24c). This could potentially suggest the methodology applied for nitrate quantification may include glomalin bound nitrogen.

Throughout the 12 month study period, ZT Pearson coefficients between glomalin and nitrates varied marginally with the exception of Figure 3.24b for 3 month post cultivation sampling. This may be resultant from a delayed growth response from AM fungi around the establishing winter wheat root structures. Due to the leguminous crop previously cultivated within the same field, residual nitrates from the legume-Rhizobacter relationship reduce the quantity of intracellular root arbuscules and vesicles (Azcon et al., 2011). As the symbiosis between AM fungi and host plant are a fundamental aspect of the AM fungal lifecycle, the negative correlation seen in ZT samples between total glomalin and soil nitrates of Figure 3.24b could be due to the non favourable conditions, such as soil pH and soil compaction (Nawaz et al., 2012), within the AM fungal lifecycle. This is further supported by the control samples within the same field, but outside the cropping and fertiliser areas having a positive correlation.

In contract to ZT soils, CT samples were consistently seen to produce negative, or very little, correlations between total glomalin and soil nitrates. Control CT samples were within the same field boundary and were in highly similar conditions as CT samples, with the only difference coming about from a lack tillage applied to control samples and did not produce negative correlations between total glomalin and soil nitrates. This aids to enforce the notions that applied tillage has negative connotations for soil quality. Additionally, glomalin was seen to average around 40 g/0.1m^3 in CT managed soils throughout the sampling year, whilst other soils absent of tillage all averaged about or equal to this., with exception of ZT in Figure 3.24b as previously described.

The Pearson's coefficient of each tillage types could be used in conjunction with the N_2O emissions of Sigh et al. (2013). Positive correlations between glomalin and soil nitrates show an increase to nitrates, contributed by glomalin. However, negative correlations indicate a loss of nitrogen from soils. In conjunction with Figure 3.24, applied tillage is influencing the loss of nitrogen from soils. Work by Bramorski et al (2015) additionally indicates nitrogen loss from tilled soils via increases in soil erosion. Such erosion links to glomalin's ability to adhere soil aggregates.

3.4.7 Summary of the influences of tillage towards soil glomalin

Glomalin is repeatedly seen to be reduced in CT soils compared with ZT soils. Following the suggestions from Driver et al. (2005) that glomalin is deposited within soils upon fungal death, and the report of tillage damaging mycelia and reducing fungal abundance, this would lead thinking towards CT soils having an increased amount of glomalin compared to ZT managed soils. However, all data presented in Section 3.2 shows that this is not the case and glomalin is always lower in CT managed soils. Presented data supports the notion of applied tillage reducing fungal abundance but does not support the glomalin deposition from fungal death. In actuality, data indicates increases in fungal biomass correspond with greater concentrations of soil glomalin without the death of the fungus.

4.0 Effects of tillage on root cortical arbuscules of AM fungi

The current experimental chapter aims at testing the hypothesis that different tillage practices will affect AM fungal arbuscular development, through the following aims:

1. To investigate the influence of CT and ZT treatments on the establishment of intracellular AM fungal arbuscules and the influence thereof on wheat root length.
2. To investigate the effects of different nutrient sources in a controlled system from field sampled soils with the application of nutrient fertilisers.
3. To investigate the distribution of root exudates, as organic acids, between tillage treatments.
4. To test whether the application of glyphosate in ZT treated soils has a negative impact on AM fungal colonization of wheat roots.
5. Compare the effects of tillage and glyphosate on the established symbiosis of AM fungi with a host plant, with the hypothesis that the application glyphosate in ZT management has a negative impact on AM fungal root colonisation.

4.1 Introduction

Initial colonisation of the rhizosphere by microorganisms is facilitated via root exudates deposited into soil spaces. AM Fungi respond to strigolactone which is exuded from the roots and encourages mycelial growth towards the host roots (Figure 1.1 Section 1.1.1) in the initial stages of pre-symbiosis. Strigolactone is a compound originally associated with Striga a parasitic plant (Genre et al., 2013) and shown to be important in the colonisation of the plant root by AM Fungi (Kretzschmar et al., 2012). As reported by Malbreil et al. (2014), AM fungi have a large arsenal of enzymes that provide and improve the root invasion ability prior to the establishment of root cortical intracellular structures, such as arbuscules. AM fungal arbuscules come in two forms, Paris and Arum (Armstrong et al., 2002). Paris type arbuscules form coil-like structures, whilst Arum type arbuscules form in or around living root cells in a tightly folded form, described as “tree-like” (Alexopolus et al., 2004). Around the arbuscular structure is the peri-arbuscular membrane (PAM) that was formed from the invagination of the plant cell plasma membrane (Pamiske, 2008). These membranes are the site of nutrient transfer between the fungus and host plant. Host plant specificity determines the type AM fungal arbuscule formed with arum type arbuscules common in arable crops (Karuppiah et al., 2019). *Rhizophagus intraradices* has been studied using

microscopy and shown to form arum type arbuscules through the invagination of the host cell membrane (Alecopolus et al., 2004).Many fungal pathogens excrete mycotoxins or cellulase to aid in the invasion of plant cells. However, genome investigations of *R. intraradicies* did not reveal mycotoxin or cellulase production for plant cell invasion, , via infection-like pathway, for the instigation of AM fungal-host symbiosis (Tisserant et al., 2013).

In order for AM fungi to form a symbiosis with a host plant plant cell walls require modification. Studies by Tisserant et al. (2013), Chen et al. (2018) and Hao et al. (2019) have indicated that AM fungi do not possess genes that would allow fungal induced modification of cell walls. However, in the presence of a colonising AM fungus, plant cell wall remodelling genes have been studied and noted to be expressed in greater quantities (Guether et al., 2009), suggesting that intracellular fungal component establishment comes about from plant invitations, via chemical signals such as strigolactone (Besserer et al., 2006). Due to the absence of AM fungal genes for the production of cell wall degrading enzymes, pathogenic and damage associated molecular signals indicative of plant cell wall damage (Screparti et al., 2016), are not produced and do not illicit an immune response from the host plant (Perez-de-Luque et al., 2017).

While plants are able to utilise their nutrients directly from the soil, plant-microbe interactions are able to provide soil bound nutrients previously unavailable to the host plant (Cruz and Ishii, 2012). AM fungal mycelia are able to access smaller pore spaces between soil particulates and aggregates and increase their growth towards a source of nutrients (Tibbett, 2000; Cavagnaro et al, 2005; Drinkwater et al., 2017). Through the increase in hyphal to root surface area, larger quantities of soil bound nutrients are metabolised by AM fungi, which are then provided to the host plant via large arbuscular surface areas. This aspect of the plant-microbe relationship is particularly useful in the case of nutrient poor or depleted soils (Berendsen et al., 2012), or soils high in bound immobile nutrients such as ammonium and phosphate (Drinkwater et al., 2017). Johnson et al. (1997) reasoned that arable crops may not produce symbiotic relationships with AM fungi as nutrients are provided through the industrialisation of fertilisers, and any such symbiosis would be detrimental to the host plant. Later, Hodge (2004) concluded that AM fungi increase the

recovery of nitrogen from plant debris and decomposing material but was unable to produce a rationale as to the change in rates of organic matter decomposition.

Atmospheric carbon dioxide is bound into an organically useable substrate through photosynthesis. Via the phloem, photosynthetic carbohydrates are transported to root tips and apical stems required for plant growth. Within roots, photosynthetic carbohydrates are cleaved to produce glucose and fructose monomers (Giaquanta, 1983). The breakdown of photosynthetic sucrose is required due to AM fungi not possessing sucrose-cleaving enzymes (Schubert et al., 2004; Schaarschmidt et al., 2006; Wang et al., 2006). During the initial stages of host invasion, AM fungi induce the host plant to express sucrose-cleaving enzymes (Schaarschmidt et al., 2006). Whilst these carbohydrates can be utilised for root tip growth, an estimated 20% of photosynthetic carbohydrates are provided to symbiotic AM fungi (Bago et al., 2000) via intracellular arbuscules. Doidy et al. (2012) found AM fungal symbiosis increased with increased of root carbohydrate and is connected to further increases in carbon sinks. The carbon provided via the host plant is required by the extraradiating hyphae for continued fungal growth which aids nutrient utilisation and water acquisition, producing increased formation of fungal spores (Bago et al., 2003). Early studies (Beilby and Kidby, 1980; Jabajihare, 1988; Becard et al., 1991) indicated the breakdown and conversion of carbohydrates to fatty acids led to an increase in AM fungal vesicles which store lipids (Bago et al., 2003). AM fungi have a preference towards the metabolism of fatty acid lipids within the main fungal body directl contact with the host plant (Trepanier et al., 2005). The storage of lipids in this manner allows for lipid conversion to hexose carbohydrates in extraradial hyphae (Bago et al., 2003), where carbohydrate metabolism is in higher demand.

Within an arable setting, strigolactone signalling may originate from several host plants in close proximity to the AM fungal mycelia (Malbreil et al., 2014). This potentially results in many host plants being connected via the same AM fungal mycelial network. In undisturbed soil AM fungal mycelial networks have been found to increase the size and number of host plant connections over time (Bennett et al., 2013). The application of tillage has previously been shown to reduce AM fungal abundance and the ability of AM fungi to colonise host plant roots due to the damage of the AM fungal mycelia through the

application of tillage, as quantified by soil ergosterol (Lu et al., 2018; Millie-Lindblom and Tranvij, 2003).

The broad-spectrum herbicide glyphosate, is favoured as a weed control measure in zero till (ZT) cropping systems. Such systems do not have the capacity to implement weed control via soil inversion as is the case in 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 (Palme and Nagy, 2008) while tyrosine derived metabolites are critical for plant survival (Xu et al., 2019). Critically, both the Shikimate pathway and EPSP synthase are found in most fungi and bacteria in addition to plants (Helander et al 2018, Helander et al. 2012). Therefore, the impact of glyphosate application on the soil microbiome requires evaluation.

4.2 Approach

The experimental protocols utilised for investigation of the effect of tillage towards established root cortical AM fungal establishments are detailed in Chapter 2 and also summarised in Table 4.1.

This approach utilises sampled soils, collected previously (Section 2.1) from each respective tillage treatment (CT and ZT), analysed under controlled laboratory and glasshouse conditions. This was employed as an experimental justification between fields to assess the degree of influence resultant of tillage without the variables of meteorological conditions to address the aims given for this chapter.

Table 4.1 - A summary of the experimental protocols employed to investigate the effects of tillage on root cortical AM fungi.

Experimental approach	Description of experimental approach	Experiment location	Section
Fungal identification	Identify soil isolated fungi	Laboratory	2.3.2
Ergosterol HPLC	Measure the total amount of live fungi within soils	Laboratory	2.3.3
Root staining with Sheaffer blue	Quantify intracellular mycorrhizal fungal structures in root cortical cells	Laboratory	2.3.4
Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein	Laboratory	2.3.5 and 2.3.6
***In vitro* glomalin production to soil pH**	Quantity soil glomalin and fungal biomass over a soil pH range	Laboratory and growth cabinet	2.3.3, 2.3.5 2.3.6 and 2.3.7
Fungal influence of root length	AM fungal influence on root length from established AM fungal-host relationship between different nutrient sources	Growth cabinet	2.3.8
Root exudate HPLC	Rhizodeposited compounds from winter wheat into rhizosphere soils	Laboratory	2.3.9
Glyphosate	Growth inhibition on non target organisms	Glasshouse	2.4.4
Controlled growth conditions	Maintain a constant environment for the sustained growth of winter wheat	Glasshouse and growth cabinet	2.4.1

4.3 Results

4.3.1 Comparisons between Conventional and Zero tillage on the number of intracellular AM fungal root cortical arbuscules of winter wheat roots from

Examination of AM fungal wheat root arbuscules were seen to be consistently higher in ZT managed soils than in CT treated soils (P = 0.01, df: 10, t.stat: -2.41, paired unequal variance T test) (Figure 4.1). Quantified root arbuscules were noted to have increased in both tillage treatments during spring (May 2019) sampling, but the differences were not statistically significant (P = 0.16, df: 7, t stat: -1.05, paired unequal variance T test). Comparisons between sampling periods of the same tillage treatment, i.e. post cultivation and pre harvest in CT, was not observed to indicate any significant increase in AM fungal arbuscules per centimetre of root tissue (P = 0.26, df: 7, t.stat: -0.67, paired unequal variance T test).

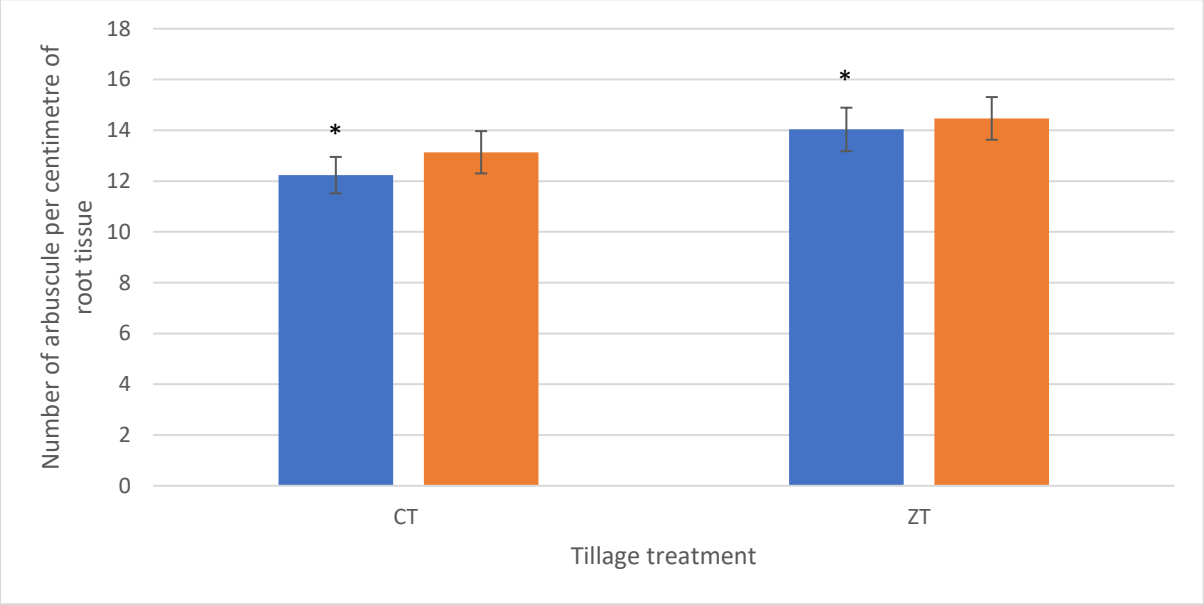

Figure 4.1 – Average (n = 120) stained intracellular AM fungal arbuscules at post cultivation (blue) and pre-harvest (orange) between CT and ZT managed soils. Significance was observed between indicated (*) data (P = 0.01. df: 10, t stat: -2.41, paired unequal variance T test).

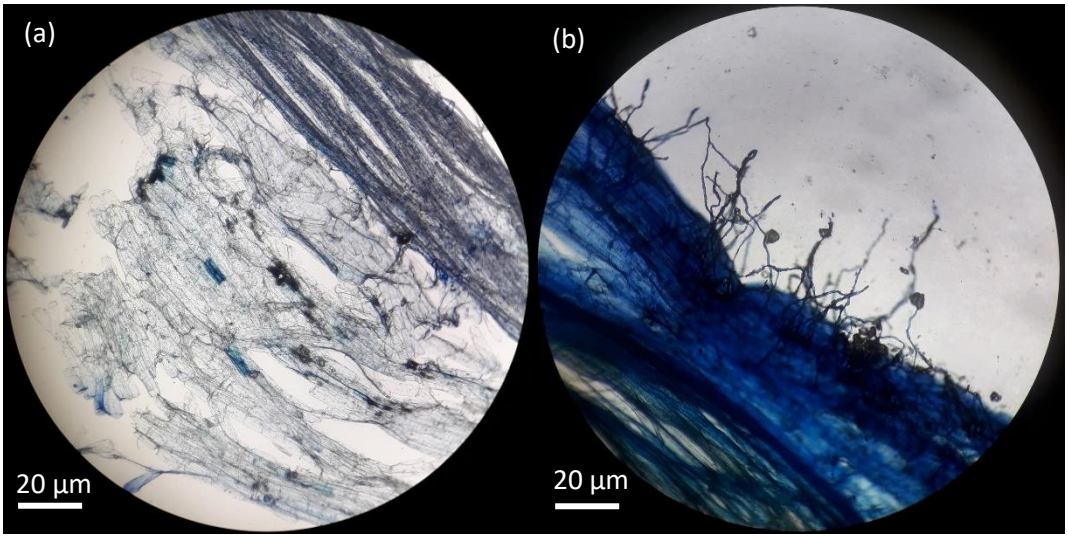

Figure 4.2 – Random samples of 1cm root sections of winter wheat stained with Sheaffer® blue, sampled from (a) ZT and (b) CT soils 3 months post cultivation (February 2019). Imaged with a Samsung Galaxy Android 3J camera smartphone at a magnification of x40

4.3.2 In vitro effect of AMF on wheat root growth under different nutrient regimes

The largest response in root length to AM fungi was observed in the absence of nutrient application ($P = 0.04$, df: 2,33, F value: 3.50, F critical: 3.28, single factor ANOVA). Where AM fungi were not present, the application of nutrients reduced root length ($P < 0.0001$, df: 2,33, F value: 12.92, F critical: 3.28, single factor ANOVA).

Between the presence and absence of an AM fungal inoculant, with respective nutrient source, no measurable significance was quantified (Figure 4.4 and 4.5) via statistical methods. However, root lengths in the presence of a nutrient source (Figure 4.5) presented as having a notable effect on the reduction of overall root length, with the exception of roots grown in the absence of any nutrient source.

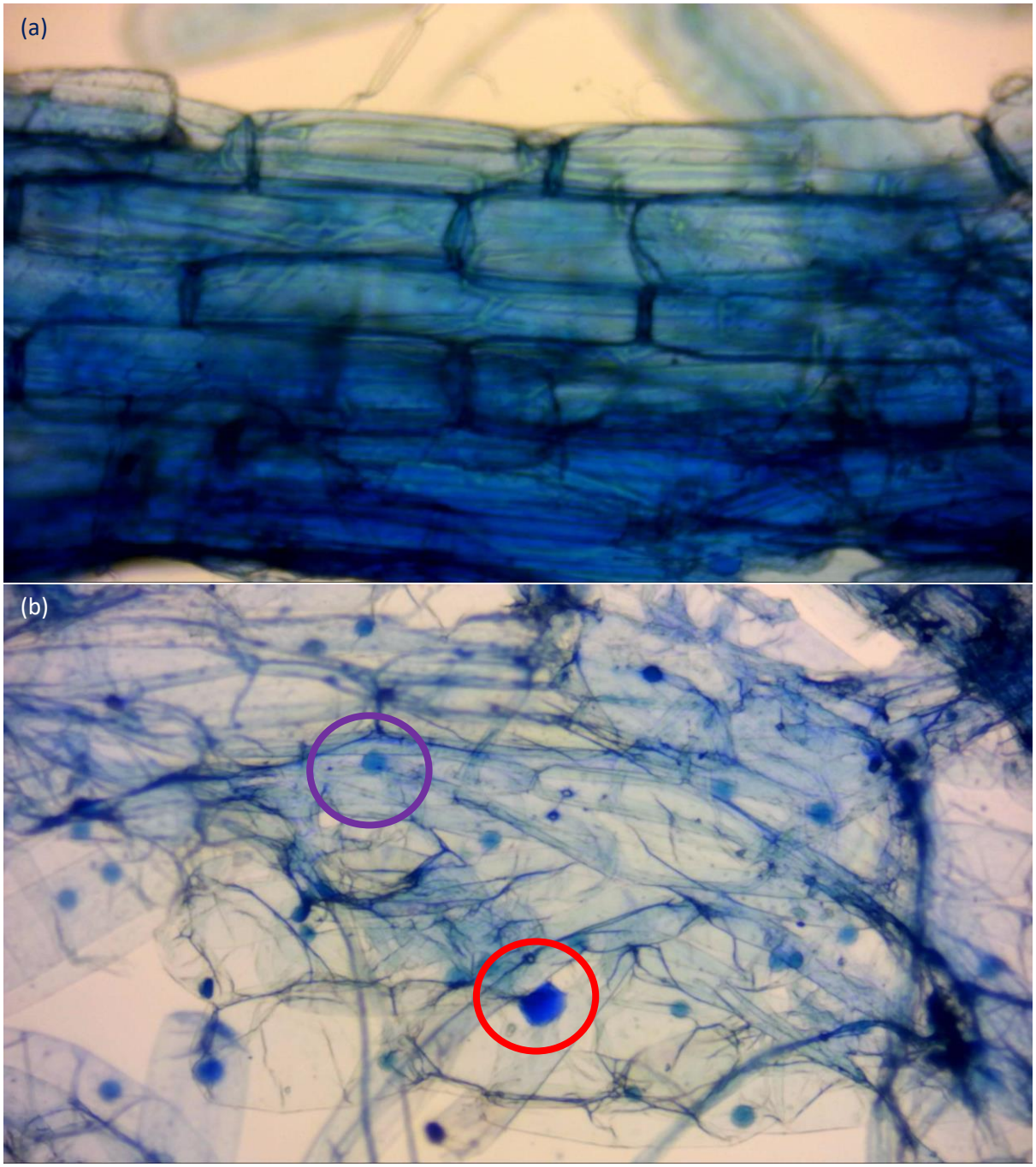

Figure 4.3 – Sheaffer® blue stained (a) sterile Zulu wheat root at a x100 magnification of a Vickers® compound microscope and (b) a non sterile lab control Zulu wheat root (Wilkes et al., 2019) showing a stained vesicle (purple) and arbuscule (red) at a magnification of x100 of a Vickers® compound microscope imaged with a Bresser® HD microscope camera.

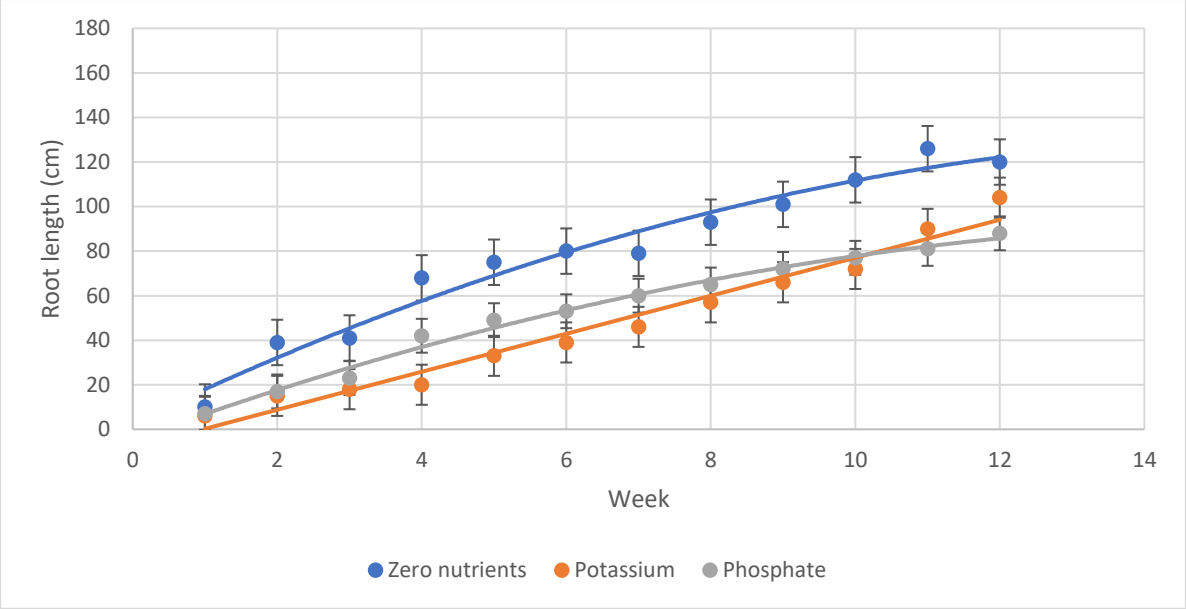

Figure 4.4 – Average (n = 180 overall) lab controlled growth of Zulu winter wheat without fungal inoculum on different nutrient media for a total period of 12 weeks. Error bars constructed from SEM.

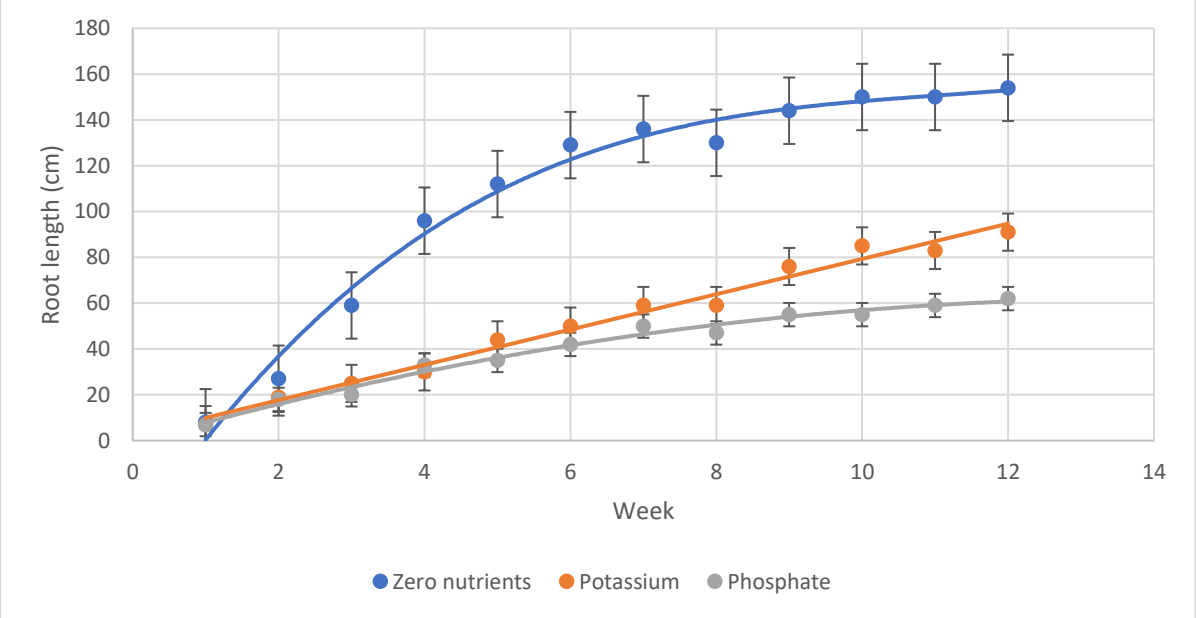

Figure 4.5 – Average (n = 180 overall) lab controlled growth of Zulu winter wheat with fungal inoculum on different nutrient media for a total period of 12 weeks. Error bars constructed from SEM

Zulu winter wheat, grown in non-sterilised soils, with varying amounts of respective nutrients to mirror that of fertiliser types applied to the study farm sites, produced differences in the quantity of AM fungal arbuscules per 1cm root sections (Figure 4.6), and total root length (Figure 4.7), between nutrient types and their respective quantities. Over a 6 week period, the quantity of root cortical arbuscules was noted to vary between the amounts of fertiliser ($P < 0.0001$, df: 12,52, F value: 47.54, F critical: 1.94, single factor ANOVA), with sodium dihydrogen phosphate producing the greatest increase in arbuscules per 1cm root section ($P < 0.01$, df: 8, t.stat: -3.21, paired equal variance T test). Urea however, had a negative influence on AM fungal arbuscule number ($P < 0.001$, df: 8, t.stat: 6.23, paired equal variance T test). Similarly, root length (Figure 4.7) varied between types of fertiliser applied ($P < 0.0001$, df: 12,52, F value: 129.44, F critical: 1.94, single factor ANOVA). An application of 1.0 g sodium dihydrogen phosphate was optimal for AM fungal arbuscule number (Figure 4.6) but not root length (Figure 4.7). Pearson correlations between root length and root arbuscules at week 6 (Figure 4.8 and Table 4.2) presented a correlation of -0.30 (r^2 0.09) in the control samples suggesting AM fungal associations with host root systems have a strong implication for total root length. Furthermore, a negative correlation with the quantity of urea at week 6 (-0.90, R^2 0.81 at 1.5g) (Table 4.2), indicative of an increase in arbuscules per centimetre of root tissue with no overall increase in root length, shows select amounts of particular fertiliser treatments can decrease AM fungal host root associations.

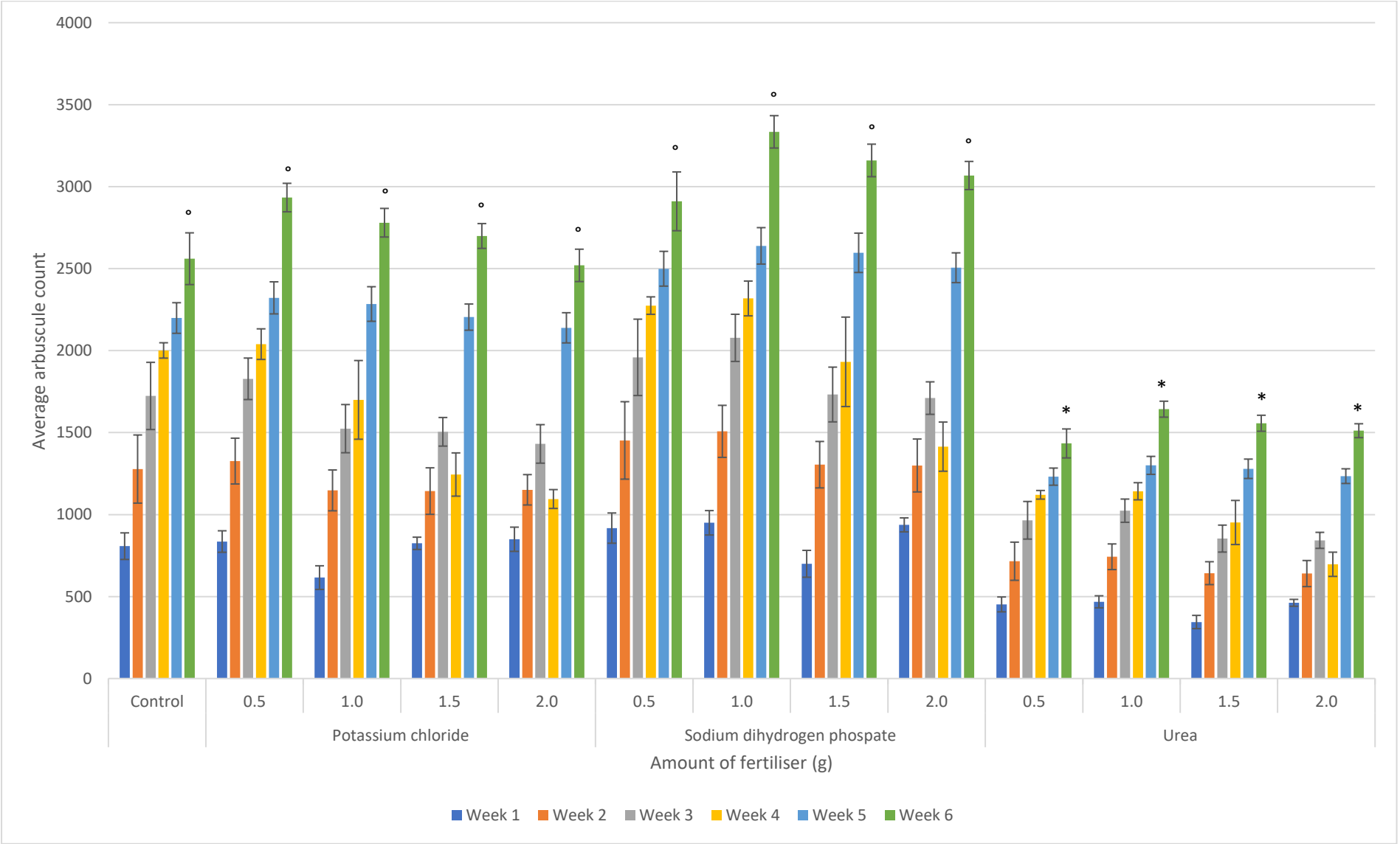

Figure 4.6 – Average arbuscule count from sampled root sections (n = 390 overall) from the application of 3 nutrient sources (potassium chloride, sodium dihydrogen phosphate, urea), with each nutrient source producing an increase in quantifiable AM fungal symbiosis at a respective quantity of nutrients. ANOVA significance ($P < 0.0001$) was noted at week 6 from all nutrient types. Error bars were constructed from SEM. Bonferroni factor indications of significance came from urea treatments (*), with no indications that other fertiliser treatments contributed to ANOVA significance (°).

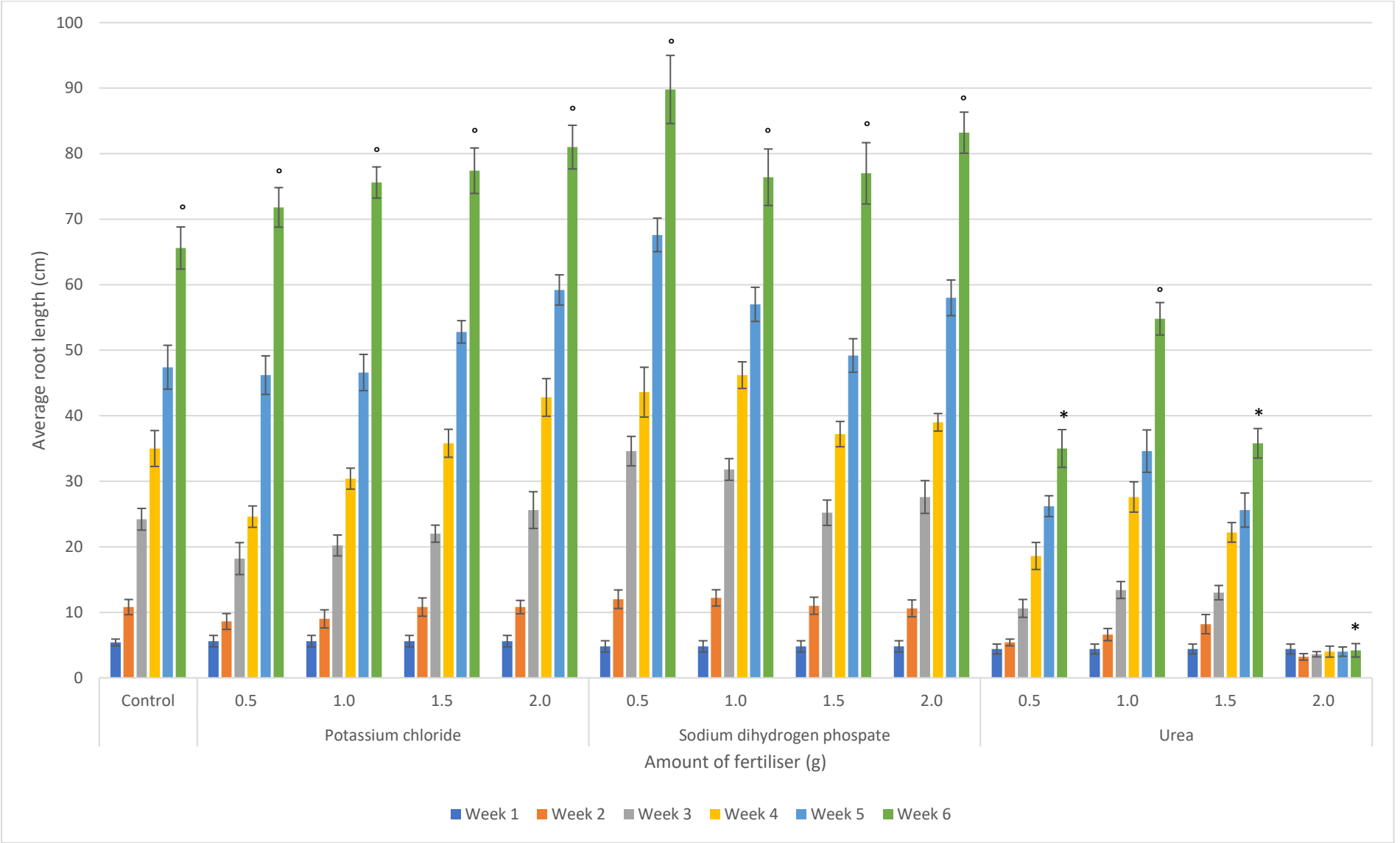

Figure 4.7 - Average root length from sampled Zulu wheat (n = 390 overall) from the application of 3 nutrient sources (potassium chloride, sodium dihydrogen phosphate, urea), with each nutrient source producing an increase in root length at an optimal quantity of respective nutrients. ANOVA significance ($P < 0.0001$) was noted at week 6 from all nutrient types. Error bars were constructed from SEM. Bonferroni factor indications of significance came from urea treatments (*), with no indications that other fertiliser treatments contributed to ANOVA significance (°).

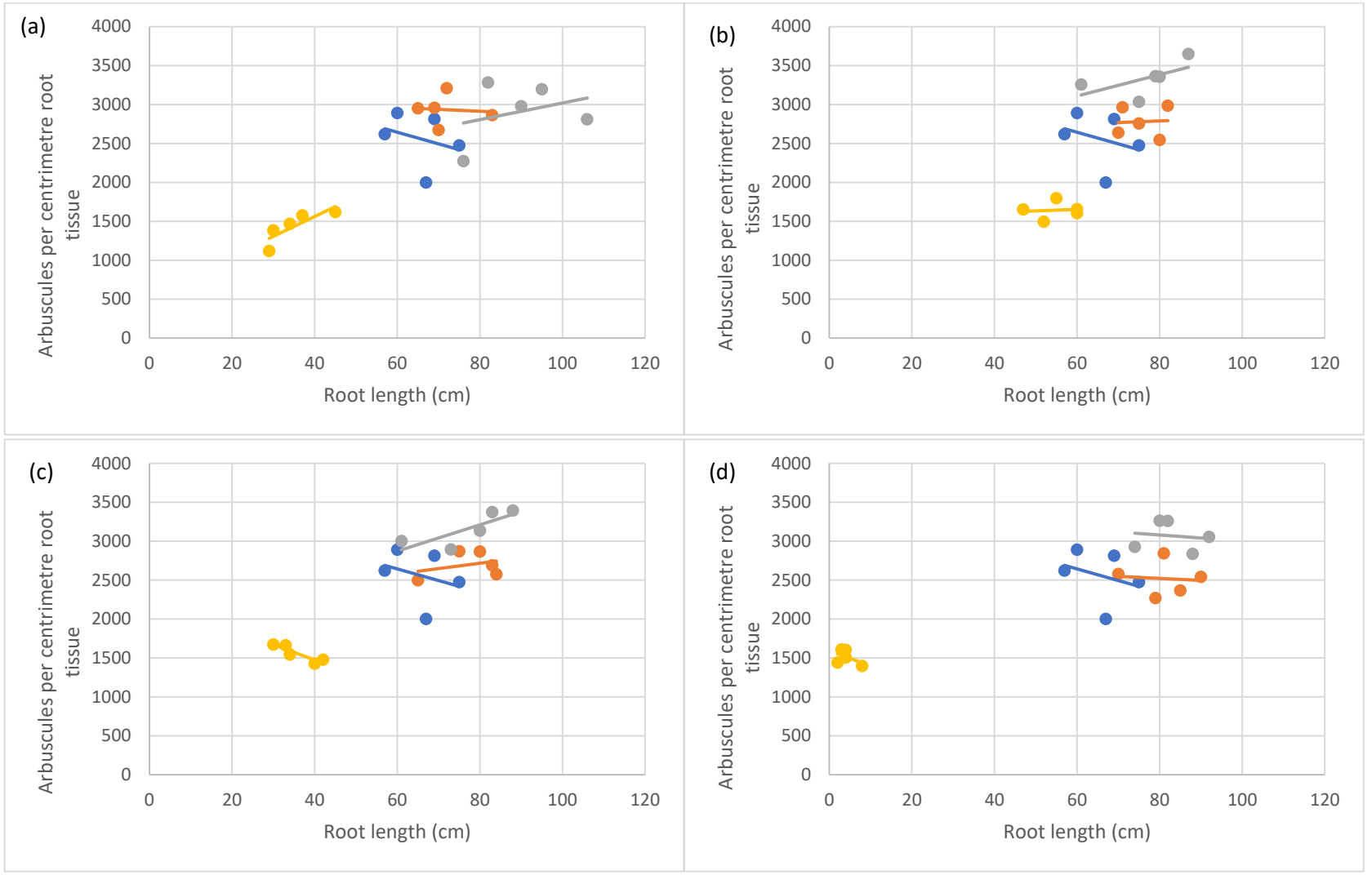

Figure 4.8 – Correlations between root length and quantified AM fungal root arbuscules in the presence of different fertiliser treatments; Potassium chloride (orange), Sodium hydrogen phosphate (grey), Urea (yellow) and no fertiliser application (control – blue), for sampling at week 6 for the controlled growth of Zulu winter wheat. Fertiliser treatments were applied at different quantities per 300cm^3 of growth media; (a) 0.5g, (b) 1.0g, (c) 1.5g and (d) 2.0g.

Table 4.2 – Pearson's correlation and R^2 values for correlated arbuscule counts and root lengths between fertiliser applications.

Fertiliser	Amount of fertiliser (g)	Pearson's correlation	R^2
No fertiliser	0.00	-0.30	0.09
Potassium chloride	0.50	-0.08	0.01
	1.00	0.06	0.00
	1.50	0.32	0.10
	2.00	-0.09	0.01
Sodium hydrogen phosphate	0.50	0.31	0.10
	1.00	0.60	0.36
	1.50	0.80	0.64
	2.00	-0.14	0.02
Urea	0.50	0.83	0.69
	1.00	0.10	0.01
	1.50	-0.90	0.81
	2.00	-0.48	0.23

Figure 4.9 - Zulu winter wheat development after 6 weeks in controlled glasshouse conditions given urea in varying quantities. (a) 0g (control), (b) 0.5g, (c) 1.0g, (d) 1.5g and (e) 2.0g. Urea was applied once during initial experimental set up.

The number of arbuscules present at weeks 15 and 30 of stained winter wheat root sections, grown in controlled glasshouse conditions in soil extracted from CT and ZT fields, produced greatest quantified arbuscules in ZT managed soils ($P < 0.01$, df: 5, t.stat: -4.00, paired unequal T test) for both sampling weeks (Figure 4.10), showing CT soils constantly producing reduced quantities of root arbuscular structures. However, CT stained roots in Figure 4.13 quantified greater AM fungal structures than wheat from field samples grown 12 months previous (Figure 4.1) ($P < 0.001$, df: 4, t.stat: 5.22, unequal variance T test). Additionally, the absence of glyphosate applications also produced greater quantifiable AM fungal root structures when compared to field sampled wheat roots 12 months previous to control growth wheat samples ($P < 0.001$, df: 4, t.stat: 5.47, unequal variance T test) of Figures 4.1 and 4.12 with a 41.6 fold increase at week 30.

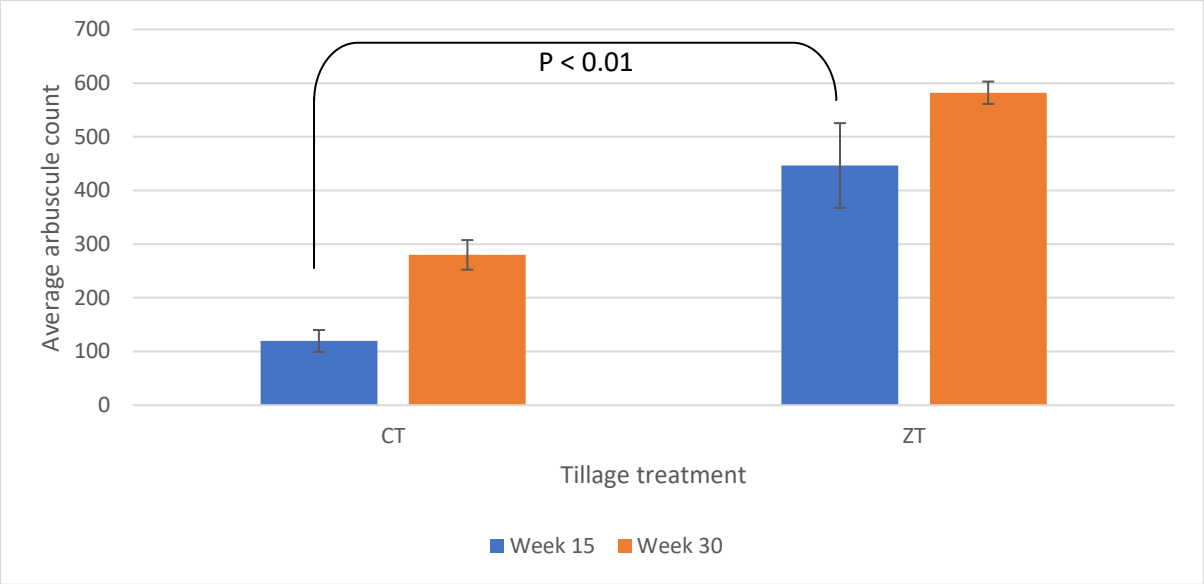

Figure 4.10 – Average (n = 80 overall) stained intracellular AM fungal arbuscules from glasshouse winter wheat (Zulu variety) after 30 weeks. Significance was measured between tillage treatments ($P < 0.01$). Error bars are constructed from SEM.

4.3.3 Root exudates

Organic acid root exudate HPLC largely did not show the indicated organic acids. Areas under each peak are indicative of relative abundance of each substance responsible for the associated peak.

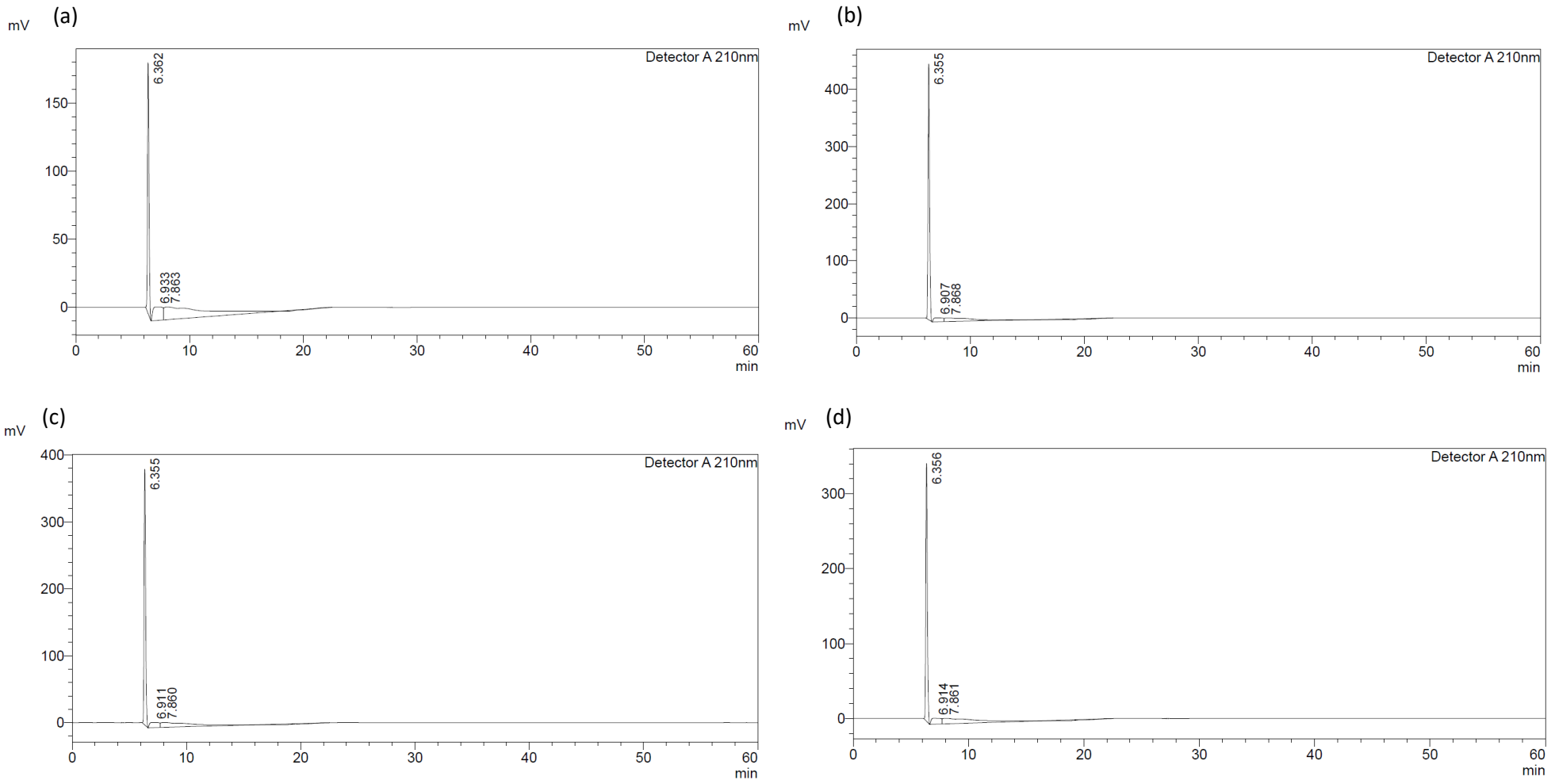

Figure 4.11 - HPLC chromatographs of root exudates present in bulk soils, measured at 210nm, of CT managed soils pre-sowing (September 2018) at (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm sampling depths. Methanol and sulphuric acid eluent producing a retention time of 6.3 minutes.

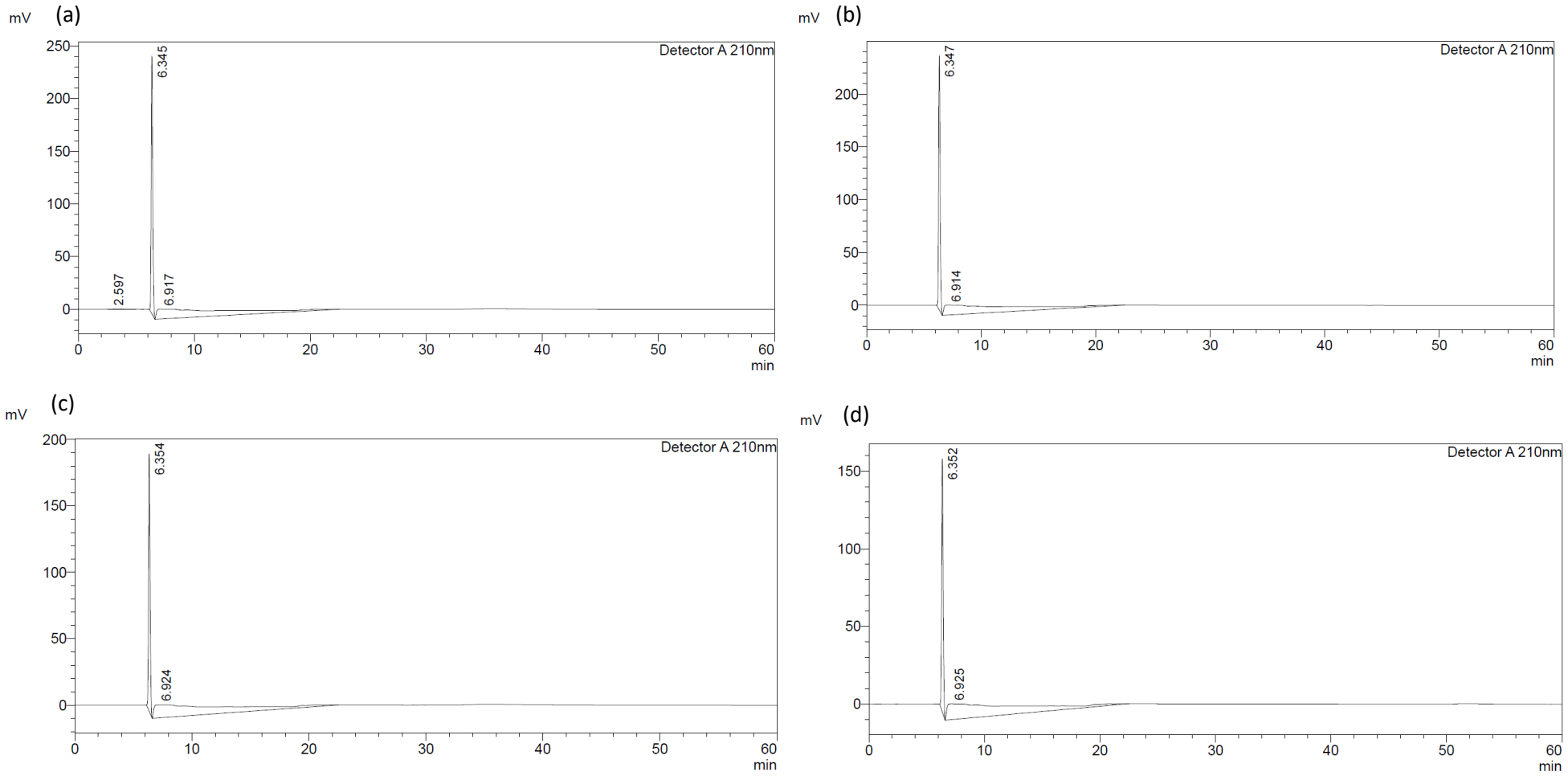

Figure 4.12 – HPLC chromatographs of root exudates present in bulk soils, measured at 210nm, of ZT managed soils pre-sowing (September 2018) at (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm sampling depths. Methanol eluent producing a retention time of 6.3 minutes.

4.3.4 Glyphosate applications to sampled field soils

The application of glyphosate to both CT and ZT soils was seen to inhibit growth of AM fungi, as measured by ergosterol, at all concentrations applied. Lower glyphosate concentrations were noted to allow fungal biomass to increase after several days. However, fungal growth was reduced in CT soils (Figure 4.13), a soil managed through practices absent of the use of glyphosate, with all glyphosate applications of 50g L^{-1} active ingredient and above ($P = 0.03$, df: 4, t.stat: 2.44, paired unequal variance T test). Glyphosate concentrations of 10g L^{-1} did not show reductions to the same degree of fungal biomass ($P = 0.12$, df: 7, t.stat: 1.31, paired unequal variance T test).

Fungal biomass in ZT soil (Figure 4.13), soils typically managed with the application of glyphosate, were additionally able to show reductions of fungal biomass with all glyphosate applications of 10g L^{-1} and above ($P = 0.02$, df: 4, t.stat: 2.89, paired unequal variance T test). However, glyphosate concentrations of 10g L^{-1} did not show reduction of fungal biomass to the same extent ($P = 0.12$, df: 7, t.stat: 1.35, paired unequal variance T test) as applications above 10g L^{-1}, but was seen to have marginally reduced biomass compared to control samples (in absence of glyphosate).

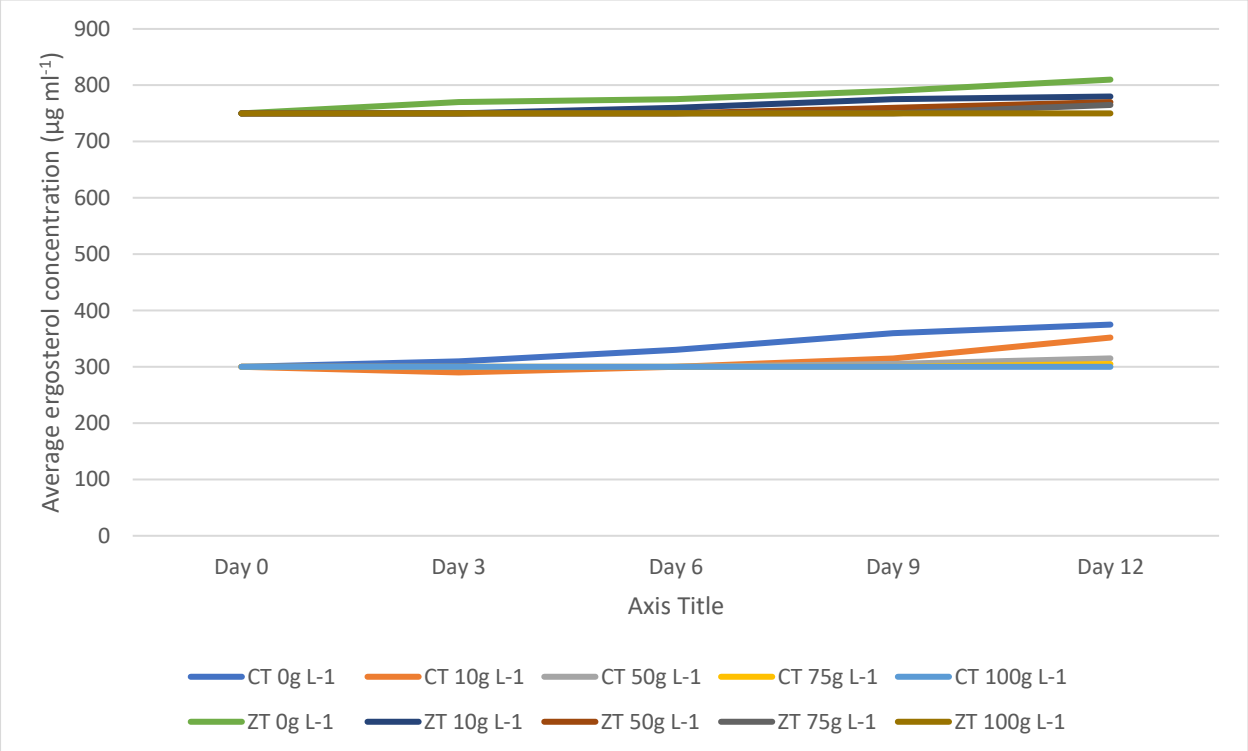

Figure 4.13 – Average (n = 50 overall) ergosterol concentrations from a single glyphosate application to CT and ZT soils over a 12 day period. Significant reductions to ergosterol concentrations ($P < 0.00001$) were observed for both tillage treatments, CT and ZT. Error bars were constructed from SEM.

4.4 Discussion

4.4.1 Effects of tillage towards AM fungal symbiosis

Soils managed via CT produced marginally lower fungal arbuscules per centimetre of root tissue than ZT managed soils with a negligible increase observed when sampled again later in the year ($P = 0.01$). Quantified arbuscules increased by 17% from post cultivation to pre harvest in CT soils, whilst quantified ZT arbuscules for the same period increased by 4%. The lower number of fungal root structures in the CT root tissues from previously sampled field wheat roots do, however, show additional radiating mycelia from the rhizoplane compared to the ZT treatment. Studies by Lu et al. (2019) and Baltruschat et al. (2019) additionally saw similar phenomena. There is a significant increase in the quantifiable fungal structures associated with AM fungi-root symbiosis per centimetre of root tissue in the absence of tillage. It concurs with the previous findings of Sun et al. (2018), Mathew et al. (2012) and Frey et al. (1999) that tillage is detrimental to fungal communities. Indeed Brito et al. (2012) showed a 40% reduction in AM fungi through operational taxonomic units (OTU) due to soil inversion from CT treatments compared with ZT management. The reduction in fungal abundance, measured via ergosterol as a biomass indicator, means that fewer living fungi are present within soils. This then reduces nutrient availability for the host plant as well as reducing the diversity and abundance of symbiotic fungi due to interference with its life cycle. Lu et al. (2018) studied the diversity and abundance of AM fungi between CT and ZT treatments and was able to conclude that CT managed soils had significantly reduced fungal diversity coupled with significantly reduced abundance of Glomus spp., a well reported genera of AM fungi, and was further associated with reductions in soil aggregate stability from the damage to fungal mycelia resultant of CT treatments. However, CT arbuscule counts from Figure 4.10 indicate AM fungal recovery is possible when soils do not receive such invasive soil management practice or do not receive the next annual tillage treatment, corresponding to the time taken for the potential regeneration of AM fungal biomass post-cultivation. This was made possible by glasshouse experimental approaches removing the following annual tillage application i.e. increased the time elapsed since tillage to above 12 months in the CT treatment soil. The number of AM fungal arbuscules increased nearly 10-fold due to the elimination of this one tillage operation. An increase in plant-

fungal symbiosis is, therefore, possible and within a relatively short timeframe in the absence of annual tillage.

4.4.2 Effects of AM fungal presence and nutrient type on total length of wheat roots

The provision of nutrients to a sterile (Figure 4.4) and AM fungal inoculated (Figure 4.5) root system on Zulu variety winter wheat indicate reductions in root length when AM fungi form symbiosis with a host in the presence of potassium and phosphate nutrient sources. Finch et al. (2017) grew wheat and blackgrass (*Alopecurus myosuroides Huds*) upon Murashige-Skoog media after surface sterilising seeds. The use of Murashige-Skoog media provides additional nutrients to the developing plant, unlike the presented experimental data in Figures 4.4 and 4.5 which provided no nutrients or a select type of nutrient. The difference in approach between Finch et al. (2017) and the present investigation has the potential to reduce the requirement of AM fungal established relationships from the employment of Murashige-Skoog media, whilst the use of a nutrient absent media encourages AM fungal symbiosis on the host wheat.

Chen et al (2018) explored the beneficial effects of AM fungi towards the mitigation of plant stress, drought and nutrient acquisition in nutrient reduced soils. Chen et al. (2018) further gives rationale to the reduction in host root lengths with the increases in AM fungal colonisation. Root length was reduced by 15 and 30cm where AM fungi was absent compared to inoculated treatments with potassium or phosphate applied respectively. However, in the case of the nutrient absent media, root length increased in the presence of an AM fungal inoculum. This is likely due to the exploratory nature of the root system in search of a nutrient source (Giehl and Wiren, 2014). Bouan et al. (2019) produced experimental results that suggested plant roots require more chemically bound energy, in the form of photosynthetic carbohydrates for growth, development and exploration of soils in order to search for a nutrient source. This reinforces the increased root length quantified in wheat grown on nutrient absent media in Figure 4.5.

Nutrient exchange and transfer from environment to plant has been an area of interest within arable agriculture for some time. The early work of George et al. (1992) was able to report the acquisition of soil potassium by AM fungi and supplied to the host plant. Analysis of mycelia and host roots indicated the ability of potassium transport along a mycelia network (Olosson et al., 2008). Between presence and absence of AM fungal

inoculum for potassium nutrient media of Figures 4.4 and 4.5, little difference in root length is observed at week 12 (15cm). However, a greater rate of root growth was noticed from wheat grown in the presence of a fungal inoculum (Figure 4.5). With fungal presence, root length was reduced by 10cm. This conforms to literature (Bago et al. 2003; Wright 2005; Zhang et al. 2017; Stoain et al. 2019) following the theory that potassium is fungal acquired and provided to the plant, resulting in a reduction of planted expend resources for the development of a large root system in the search of such nutrients. Statistical tests showed no significance between root length of potassium nutrients treatments of Figures 4.4 and 4.5. Whereas a significant decrease was observed between nutrient absent and potassium nutrient root length in the presence of fungi ($P = 0.003$) and in the absence of fungi ($P = 0.03$). This suggests Zulu wheat is able to assimilate a greater quantity of nutrients from an established symbiosis with AM fungi.

The presence of phosphate within the growth media had the greatest reduction in root length when comparing between presence and absence of fungi. This leads thinking in the direction of phosphate acquisition of AM fungi being greater than that of a sterile root system (Figure 4.4), as well as AM fungi transferring phosphate nutrients more readily than other forms of nutrient. Yang et al. (2012) studied phosphate transport to a host plant via intracellular arbuscular structures and found, as part of the established plant-fungus symbiosis, a symbiotic phosphate transporter was expressed exclusively in plant cells containing arbuscules. Of all benefits of a plant-fungus relationship, phosphate provision has been reported to be one of the key aspects of the relationship (Vigneron et al., 2018). Presented findings are able to conform with literature that the establishment of an AM fungal relationship with a host plant facilities improved nutrient uptake, whilst additionally showing how root length is effected by the presence of an AM fungal presence.

4.4.3 The influence of tillage on identifiable root exudates

Establishment and continued communication, via chemotaxis within the rhizosphere, is mediated through release of organic acids, amino acids and carbohydrates from root structures (Yuan et al., 2015). The organic acids presented in Appendix 8.14 were selected from literature searches giving clear indications that cereal crops produced this array of organic acid exudates. Chromatographs of CT and ZT soils, Figures 4.6 and 4.7 respectively, produced no comparable peaks for the retention times of those presented in Appendix 8.14.

No organic acids were detected from either bulk soils or rhizosphere soils (2mm clearance zone) for the 12 month sampling period. From further literature searches, it was determined the lack of peak in the chromatograms was not due to the applied methodology as this also produced the organic acid standards for retention time comparison (Appendix 8.14). The lack of detectable organic acids was found to be from the sample. Literature suggests organic acid exudates, along with other forms of root exudation, are produced in low quantities and quickly metabolised by the microbiome as a nutrient source once signalling had been complete. Koo et al. (2005) suggests organic acid composition within *Zea mays.*L equates for an approximate 1% of total root dry mass with concentrations of 10 – 20mMol L^{-1}, with carbohydrates quantified as 90mMol L^{-1}. Whilst this allows for the quantification of total organic acid exudates, individual acids were not accounted for. Kawasaki et al. (2018) further investigated root exudation from wheat and barley cereal crops over a 30 day period under sterile hydroponic conditions. Kawasaki et al. (2018) further states that investigations into root exudates are difficult due to their rapid metabolism by the soil microbiome. Such metabolites account for an estimated 11% of plant acquired carbons and are in the form of sloughed off root cells, volatile organic compounds as well as high and low molecular weight compounds such as sugars, amino acids and phenolics (Jones et al., 2009). Following the reasoning given by Kawasaki et al. (2018) field sampled bulk and rhizosphere soils are not likely to allow the detection of root exudates due to the already established microbiome and root-rhizosphere community. The work by Yuan et al. (2015) additionally suggests oxalic acid, an organic acid, is an important exudate targeted towards bacterial suppression of fungal pathogens. This was later confirmed by Palmieri et al. (2019) finding oxalic acid exudation increased fungal pathogen protection by 30 – 70%. However, oxalic acid is quickly metabolised by oxalotrophic bacteria. This would provide reasoning for the non-detection of oxalic acid in Figures 4.6 and 4.7. This also would suggest tillage is not a direct factor in differences of detectable organic acids, rather the soil microbiome. Studies have indicated tillage can alter the soil microbiome communities.

4.4.4 Glyphosate

4.4.4.1 The influence of glyphosate on quantifiable AM fungal root arbuscules as a measure of fungal-host symbiosis

The arbuscule counts (an indicator of AM fungi-root symbiosis) of wheat grown in field soils of Farm B practicing ZT, where glyphosate was applied, showed no significant increase i.e. no increase in fungal-root symbiosis between sampling three months post-cultivation and one month pre-harvest stage. Jiang et al. (2011) observe a reduction in soil fungal biomass due to the application of tillage. The lower arbuscule count in the CT treatment is likely to be a result of soil inversion three months earlier. By reducing the total biomass of AM fungi, symbiosis is reduced. Winter wheat root samples, grown under ZT field conditions (Figure 4.1), grew in soil that had received a glyphosate application during initial cultivation, 2 weeks after pre cultivations samples were taken. From land manager's records, glyphosate was applied at a concentration of 360g L^{-1}, active ingredient, at a rate of 5 litres per hectare. Soil extracted for use in the glasshouse experiment was collected post harvest and one month before a further glyphosate application and drilling of the following crop were due to commence. The residual glyphosate level in the extracted soils will have declined during the period immediately after sowing the winter wheat to the time of extraction post-harvest, between of 2 – 147 days in soils (Monsanto 2015; Lewis et al., 2016), in ZT soils. Both field and glasshouse soils received the same type and quantity of fertiliser and this can be eliminated as a potential variable. ZT soils used for glasshouse experiments received no additional application of glyphosate, whereas the ZT field received its annual glyphosate application pre crop sowing. The average arbuscule count in the roots of Zulu winter wheat grown in the glasshouse was significantly greater in ZT soils relative to the CT treatment. This is due to the absence of, firstly, the soil inversion before soil was extracted from the field, and secondly the absence of the glyphosate application in the glasshouse unlike the soils remaining in the field. This supports, in part, the conclusions of Zaller et al (2014) who found that the application of glyphosate reduced AM fungal colonization and symbiosis by 40%. The removal of glyphosate application as shown in the ZT glasshouse treatment increased AM fungal symbiosis by 3,126%. Although this study agrees broadly with the findings of Zaller et al (2014), further study into the difference between reductions in AM fungal root colonisation from glyphosate inhibition is required. Furthermore, continued investigation is required to ascertain whether glyphosate impact

towards host plants has a greater reduction to overall AM fungal abundance, an indirect effect, compared with direct AM fungal growth inhibition from glyphosate. Both studies use comparable staining methodologies, Sheaffer® ink as opposed to trypan blue, except Zaller et al (2014) use Sheaffer® black as opposed to Sheaffer® blue ink used in the quantification of AM fungi in this investigation, as described in Section 2.3.4. 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 used by Zaller et al. (2014) is not sufficient to allow stain penetration of root tissues. A staining time of three minutes allows for a more thorough stain penetration. Furthermore, the use of a fixative solution allows for 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 the target structures to be viewed and quantified. The lower percent reduction of arbuscules observed by Zaller et al (2014) may be due to a less effective staining protocol. The negative impact of glyphosate on AM fungi may have been underestimated.

This supports the hypothesis that the application of glyphosate in ZT managed land reduces the root colonisation effects of AM fungi.

4.4.4.2 Influences of glyphosate towards fungal biomass as quantified by soil ergosterol

Bulk soil ergosterol, used as an indicator of AM fungal biomass, increased in the ZT non-cultivated control (ZTC) relative to the ZT soil treatment in the absence of a glyphosate application (Figure 3.26 Section 3.3.10). 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 (Helander et al 2018; Helander et al. 2012; Palme and Nagy, 2008; Xu et al., 2019). The ZTC soils are representative of soils void of inversion, aeration, disturbance or chemical treatments. Between both tillage types sampled, CT and ZT, glyphosate was the only chemical applied that was different between the sites. This was confirmed by records obtained from land managers. Ergosterol levels quantified pre-drilling and glyphosate application were higher than the remainder of the sampling year. In samples taken in the ZT treatment following a glyphosate application three months post cultivation

the quantity of ergosterol decreased by a factor of five, from 0.5mg to 0.1mg per 0.1 m^3. Although a decrease was observed in the ZTC soil samples, that did not receive a glyphosate treatment, ergosterol was reduced by a factor of 1.75 (Figure 3.26 Section 3.3.10). This is likely the result of environmental and meteorological conditions, such as soil and air temperature as described by Pietikainen et al. (2005), producing growth conditions moving away from the AM fungal optimum (Begum et al., 2019). Both the ZT and ZTC soils are within the same field boundary and are not likely to produce differences from meteorological conditions, potentially further justifying the effects of glyphosate.

Glyphosate is the main variable for both post cultivation and pre-harvest phases of the crop cycle. The similar quantities of ergosterol in both the ZT and ZTC immediately post-harvest is due to the removal of the crop and decline in living plant tissue. By reduction of living viable plant tissue, the biotrophic nature of AM fungi cannot continue and would produce a measurable decrease in soil ergosterol (Mille-Lindblom et al., 2004).

The application of glyphosate to ZT soil in the glasshouse produced reduced quantities of AM fungal ergosterol. However, this was performed using bare soils indicating the direct growth inhibition of glyphosate to AM fungi. A further investigation is required for the implication of glyphosate in the removal of a host plant and the resulting implication towards the AM fungal community. Ergosterol increased over each three day period where glyphosate was not applied in glasshouse experimentation, as described in Section 2.3.5. Ergosterol is reduced even at lower concentrations of glyphosate, 10g L^{-1}, with no increase evident until after six days as opposed to three. There is no increase in ergosterol after 12 days at application rates of 100g L^{-1} or above. A field application rate is equivalent to 75g L^{-1} per 50g soil, where a small increase in ergosterol is noted after day 12. From the perspective of the AM fungal life cycle, an increase in fungal biomass should have been measured across the sampling year due to increases in root mass along with increases in soil and air temperature moving towards the AM fungal optimal (Gavito et al., 2005). The arbuscule count in ZT field extracted root samples was not significantly different to the CT treatment pre-harvest and 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 has potentially profound reductions on fungal biomass, growth and symbiosis establishment (Halender et al., 2018; Zaller et al., 2014; Schonbrunn et al., 2001).

4.4.4.3 Effects of glyphosate as an AM fungal growth inhibition

Glyphosate concentrations above 100g L^{-1} produced no additional growth of AM fungi over 12 days after glyphosate was applied. A similar pattern was observed where glyphosate was applied to CT soil samples in the glasshouse although the initial ergosterol content of the soil was over 50% lower than the ZT soil. Under optimal conditions, ergosterol would take an estimated 90 days in the CT soils to equal level of the initial day 0 of ZT ergosterol due to the initial disturbance and damage to AM fungal mycelial, reducing overall AM fungal biomass, from the previous application of cultivation. Field conditions are however rarely optimal for AM fungal growth (Heinemeyer and Fitter, 2004) and the potential recovery time would be expected to be longer. A further tillage application associated with the following crop would have occurred by this time, reducing AM fungal abundance once again.

Although ergosterol in ZT soils was higher than in CT soils, the rate of fungal growth over 12 days was comparable (60µg ml^{-1} ergosterol) for both tillage treatments. The application of glyphosate to CT soils, a tillage treatment that does not require glyphosate (Okada et al., 2019) and in which the associated AM fungi have not been pre-exposed to this herbicide and its mode of action, lend further support to the inference that glyphosate inhibits fungal growth from similarities between AM fungal physiological enzymes and EPSP synthase. Fungal growth was evident after 12 days at glyphosate application rates of between 0g L^{-1} and 75g L^{-1}.

4.4.5 Implications of tillage and glyphosate on AM fungal symbiosis

The application of CT and the use of glyphosate in ZT, against the overall biomass of AM fungi within arable soils, are able to show reductions from both practices. In glasshouse experimentation, applications of CT and glyphosate from ZT were not present. By the removal of a further annual application of both CT and glyphosate from ZT practices, an increase to AM fungal abundance was quantified by established symbiosis with a host plant (Figure 4.10), a 20 and 41.57 fold increase for CT and ZT respectively. Sosa-Hernandez et al. (2019) described AM fungi as most abundant within top soils (<10cm), the region of a soil profile disturbed greatest in CT managed soils and the area of soils in contact with glyphosate. From experimental rationale in Figure 4.13 indications of a glyphosate

application of 75g L^{-1} allows AM fungi to begin increasing in abundance within 12 days after initial glyphosate application. The studied ZT site applied glyphosate at 360g L^{-1}. From data shown in Figure 4.13, a potential change to the concentration of applied glyphosate for weed control in ZT managed soils may be more advantageous than higher concentration applications. Such implications may offset the benefits of sustainable conservational ZT treatments. This is an area requiring further investigation to ascertain the degree of weed control and to potentially suggest a change in ZT management procedure.

CT managed soils have been shown to reduce AM fungal abundance (Figure 3.26 Section 3.3.10). Whilst the reduction in AM fungal abundance in CT can be comparable to the reduction in ZT, resultant of glyphosate, CT practices reduced AM fungal abundance to a greater extent than ZT. The depth to which soil disturbance is achieved in CT practices has larger scale implications towards the reductions of AM fungi and their associations with a host crop via root interactions (Figure 4.1) via the dilution of AM fungal mycelia through a soil profile. A potential mitigation comes about from reduced tillage (RT) practices that only disturb top soils. However, this is also the region of soils that is greatly abundant in AM fungi (Sosa-Hernandez et a., 2019). This is a further area that requires further investigation to balance land management practices to conserve AM fungi whilst achieving the desired degree of weed control.

5.0 Interactions between AM Fungi and rhizobacteria, and their effects on plant growth

The present chapter will test the hypothesis that selected rhizobacteria will affect AM fungi differently in CT & ZT fields and affect plant growth through the following objectives:

1. Do different species of rhizosphere bacteria influence AM fungal colonisation of the root of winter wheat
2. Is the relationship between selected rhizosphere bacteria and AM fungi time dependant
3. Is plant growth, quantified via tiller length, tiller numbers and root length, influenced by the level of AM fungal root colonisation as quantified by the formation of root arbuscules.
4. Does applied tillage practices have an influence on select rhizobacteria and AM fungi on wheat growth development
5. Measure persistence of select rhizobacteria and AM fungi inoculant for wheat growth throughout/ between the growing seasons

5.1 Introduction

The rhizosphere has a diverse microbiome (Lei et al., 2018) that includes mycorrhizal fungi and rhizosphere bacteria, many of which are unculturable and have only been identified through microbial sequencing studies. Recent research has identified bacteria that are associated with AM fungi having been shown to be of benefit to mycorrhizal fungi (mycorrhizal helper bacteria (MHB)) (Deveau and Labbe, 2016). MHB have been reported to have a high specificity towards a species of mycorrhizal fungi leading to species specific enhancement while other fungal species in close proximity are inhibited (Frey-Klett et al., 2007). Mycorrhizal helper bacteria influence mycorrhizal growth and development in species specific mechanisms producing a spectrum of resultant effects from growth enhancement to growth inhibition. Mycorrhizal growth promotion, via MHB, increases fungal-plant symbiosis through promoted extensions of mycelia (Frey-Klett et al., 2007). A protective property of MHB is apparent when mycorrhizal mycelia proliferate towards root structures and during root colonisation (Rigamonte et al., 2010). This protective and improved mycorrhizal growth property of MHB has been studied in field trials in low input agroforestry (Schrey et al., 2005; Labbe et al., 2014). Whilst mycorrhizal associations in agroforestry are typically ectomycorrhizal, the similarities of MHB effects on

ectomycorrhizal are comparable to AM fungi via mycelial growth, branching and pre symbiotic mechanisms (Figure 1.1) (Rigamonte et al., 2010). Carvalho et al. (2010) explored MHB associations with AM fungi in agroforestry further and was able to conclude MHB have been an influential resource in the establishment of mycorrhizal symbiosis with many host plants and an area that requires detailed study. This is later echoed by Geoffory et al. (2017).

Mycorrhizal spores may require the presence of MHB to germinate (Kavier and Germida, 2003). This is thought to be due to the production of the carbohydrate raffinose, of which the developing spores utilises as a carbon source (Hilderbrant et al., 2006). Later, Lecomte et al. (2011) described isolates of *Proteobacteria* and *Firmicutes* inhabited the surface of mycorrhizal spores, forming close relationships and appeared to be important during germination. Long et al. (2018) produced results of mycelia associated bacteria via culture dependant methodologies in which 12 genera of *Proteobacteria* were identified, including *Rhizobium* and *Pseudomonas*, 8 genera of *Actinobacteria,* including *Streptomyces* and *Mycobacterium*, as well as 3 genera of *Firmicutes*, including *Bacillus, Brevibacillus* and *Paenibacillus*. Furthermore, some bacterial taxa have been found to live in the cytoplasm of mycorrhiza as endobacteria (Bonfante and Anca, 2009) in numbers up to 250,000 bacterial cells (Iffis et al., 2014). *Candidatus Glomeribacter gigasporarum* is a known endobacterium associated with *Glomus margarita* (Jargeat et al., 2014). Upon axenic culture of *Candidatus Glomeribacter gigasporarum*, no bacteria were detectable suggesting that endobacteria are difficult to culture and are likely obligate biotrophs in a similar manner as AM fungi are biotrophic with its host plant. Due to endobacteria having biotrophic requirements towards AM fungi, and AM fungi having biotrophic requirements towards a host plant, a tripartite relationship has been suggested (Bonfante and Anca, 2009; Iffis et al., 2014), and presents a more accurate representation of the established relationship between microbe and host plant (Akyol et al., 2019; Johansson et al., 2004) due to the strong influences soil bacteria play in the enhancement of mycelial growth and development. Therefore, the types of interactions that occur between AM fungi and bacteria are likely to be an underestimate of the true nature of these types of interactions. Additionally, the interactions and growth enhancement of MHB towards AM fungal mycelia is still poorly understood requiring further investigation (Battini et al., 2017; Akyol et al., 2019). The mechanisms by which MHB

enhance AM Fungi is largely unknown but may involve the acquisition of phosphorus. Phosphate solubilising bacteria (PSB) (Ordoñez et al., 2016) are able to breakdown phosphate stored in rock material, which can be metabolised further by AM fungi to produce phosphate utilisable by a host plant (Gamalero et al., 2004; Miransari, 2011). Interactions between AM fungal mycelia and some soil bacteria may or may not be of benefit to a host plant, either acting as a plant pathogen or indeed supressing plant pathogens (Table 5.1).

Table 5.1 - Examples of the type of interaction between AM fungal and bacterial species along with their associated pant species

Interaction type	AM Fungi	Bacterial/Fungal species	Plant	Reference
Growth promotion	*Funneliformis mosseae*	*Bacillus sonoensis*	*Solanum lycopersicum; Capsicum annum*	Desai et al (2019), Rabab (2014)
	Rhizophagus intraradicies	*Bacillus polymyxa*	*Stevia rebaudiana*	Vafadar et al. (2014)
	Glomus deserticola	*Bacillus pumilus; Bacillus licheniformis*	*Medicago sativa*	Medina et al. (2003), Nanjundappa et al. (2019)
	Rhizophagus intraradicies; Funneliformis mosseae	*Pseudomonas putida; Pseudomonas fluorescens; Paenibacillus brasilensis*	*Triticum aestivum* L.	Perez-de-Luque et al. (2017), Jaderlund et al. (2008)
	Funneliformis mosseae	*Paraburkholderia graminis*	*Solanum lycopersicum*	Caradonia et al. (2019)
Pathogen suppression	*Rhizophagus intraradicies*	*Fusarium oxysporum*	*Allium cepa; Setaria glauca*	Sikes (2010)
	Gigaspora margarita	*Fusarium oxysporum; Rosellina necatrix; Rhizoctonia solani; Pythium ultimum*	Laboratory controlled axenic culture	Cruz and Ishii (2012)
	Rhizophagus intraradicies	*Botrytis Cinerea*	*Chrysanthemum*	Frey-Klett et al. (2011)
Nutrient acquisition	*Rhizophagus intraradicies*	*Agrobacterium rhizogenes*	*Solanum tuberosum*	Ordoñez et al. (2016)
	Gigaspora margarita; *Funneliformis mosseae; Rhizophagus irregulare*; *G. clarum*; *G. deserticola*; *G. monosporum*; *G. brasilianum; G. aggregatum*		*Vicia faba; Triticum aestivum* L.	Ingraffia et al. (2019)

	Rhizophagus intraradicies	*Paenibacillus Validus*	Laboratory controlled axenic culture	Deveau et al. (2010)

The exact abundance, diversity and roles of MHB are still unclear and requires further investigation, with indication that MHB and mycorrhizal interactions are dependent on the soil microbiome (Svenningsten et al., 2018) as influenced by soil chemical and physical properties such as pH and soil stability. MHB induced mycelial growth increases the probability of mycorrhizal fungi encountering a root system which directly correlates to an increase in mycorrhizal-host interactions (Labbé et al., 2014). However, in vitro studies have not always been able to replicate the MHB and mycelial interactions in environmental samples, therefore, not providing the correlation between MHB and mycelial growth noted from environmental samples (Labbe et al, 2014; Zhao et al., 2014; Deveau and Labbe, 2016). Further study is required for the relationship between MHB and mycorrhiza in early mycelial growth and development. Deveau et al. (2010) produced in vitro evidence that early mycelial growth requires thiamine (vitamin B1), evidence of which is provided in environmental conditions, as well as the presence of *Paenibacillus Validus* in symbiosis with *Rhizophagus intraradices* that provided AM fungi with sufficient thiamine.

It is clear from literature that there is a multitrophic community of microbial interactions involving the plant, its AM fungal symbiont and rhizosphere bacteria. The role of selected AM fungi and selected rhizosphere bacteria on promoting plant growth is well established. However, the role of MHB and their interaction with AM fungi is poorly understood.

5.2 Experimental approach

The experimental protocols utilised to determine the Interactions between AM fungi and rhizobacteria, and their effects on plant growth are detailed in Chapter 2 and summarised in Table 5.2. This approach utilises field sampled soils, collected previously (Section 2.1) from each respective tillage treatment (CT and ZT), analysed under controlled laboratory and glasshouse conditions to determine glomalin quantities as an indicator AM fungi biomass (Secrion 2.3.5 and 2.3.6), root arbuscule count as an indicator of symbiosis (Section 2.3.4) and ergosterol to determine fungal biomass (Section 2.3.3). ITS sequencing of isolated AM fungi (Section 2.3.2). Furthermore, the impact on the soil microbiota of tillage in the absence of climatic variables were assessed.

Table 5.2 - A summary of the experimental protocols employed to investigate interactions between AM fungi and rhizobacteria, with studied resultant effects towards wheat biomass.

Experimental approach	Description of experimental approach	Experiment location	Section
Fungal identification	Identify soil isolated fungi	Laboratory	2.3.2
Ergosterol HPLC	Measure the total amount of live fungi within soils	Laboratory	2.3.3
Root staining with Sheaffer blue	Quantify intracellular mycorrhizal fungal structures in root cortical cells	Laboratory	2.3.4
Glomalin assay and total glomalin	Quantify AM fungal produced glycoprotein	Laboratory	2.3.5 and 2.3.6
Rhizobacterial organisms	Known rhizobacteria from a commercially available mixture	Laboratory	2.3.10
Identification of *Bacilli* spp. rhizobacteria	API identification of *Bacilli* spp. from Section 2.3.10	Laboratory	2.3.11
qPCR of rhizobacteria and AM fungi	Presence and persistence of selected rhizobacteria and AM fungi in sampled soils	Laboratory	2.3.12
Glomalin and root mass	Glomalin as a proxy indicator of AM fungal biomass associated with wheat roots	Laboratory	2.4.2
Nutrient applications and root arbuscules	Influence of nutrient type, as fertiliser applications, with AM fungal root cortical cells	Glasshouse	2.4.3 and 2.3.4
Rhizobacteria and glomalin	Rhizobacterial influences on AM fungal biomass determined via glomalin quantification	Glasshouse	2.4.5
Rhizobacterial and root arbuscules	Rhizobacterial influences on AM fungal-host interactions	Glasshouse	2.4.6
Rhizobacteria and ergosterol	Rhizobacterial influences on AM fungal biomass determined via ergosterol quantification	Glasshouse	2.4.7
Rhizobacterial and AM fungal inoculant to Zulu variety winter wheat	Inoculant induced AM fungal-host association effects on wheat biomass between CT and ZT	Glasshouse	2.4.8

Methods employed for the determination of the Interactions between AM fungi and rhizobacteria, and their effects on plant growth, produced clear indications of increased wheat biomass and AM fungal-host associations in the presence of selected *Bacilli* spp. rhizobacteria.

5.3 Results

5.3.1 The effect of selected *Bacillus* spp. on AM fungal arbuscule development within wheat roots

Selected *Bacillus* spp. rhizobacteria, as isolated and identified in Sections 2.3.10 and 2.3.11,were seen to have positive, *B. amyloliquefaciens*, negative, *B. subtilis,* and neutral, *B. pumilis,* effects on the density of AM fungal arbuscules within a 1cm section of root tissue is shown in Figure 5.1 and Figure 5.2. ($P < 0.0001$, df: 3,16, F value: 11.55, F critical: 3.24, single factor ANOVA). *B. subtilis,* applied to developing Zulu winter wheat, was able to reduce the quantities of root arbuscules of AM fungi ($P < 0.0001$, df: 7, t.stat: 6.69, unequal variance T test) whereas, the application of *B. amyloliquefaciens* were noted to have increased the overall quantities of AM fungi arbuscules ($P < 0.01$, df: 8, t.stat: -3.02, uneneed qual variance T test). *B. pumilis,* however, was not seen to have induced any alterations to quantifiable root arbuscules, indicative of a neutral interaction.

During week one, irrespective of the bacterial treatment there were no observable differences but after that post hoc 2 tailed, paired T test of equal variance indicated significance from ANOVA tests at week 6. The Bonferroni correction factor indicated TRUE corrections for *Bacillus subtilis* and *Bacillus amyloliquefaciens*, and a FALSE correction for *Bacillus pumilis* inoculation in comparison with the control sample. However, as indicated by Figure 5.1, *B. subtilis* and *B. amyloliquefaciens* produced TRUE corrections indicating these 2 *Bacilli* spp. had greater influence on root arbuscule quantities when compared with control samples, whilst *B. pumilis* was seen to have neutral effects on observable AM fungal root arbuscules.

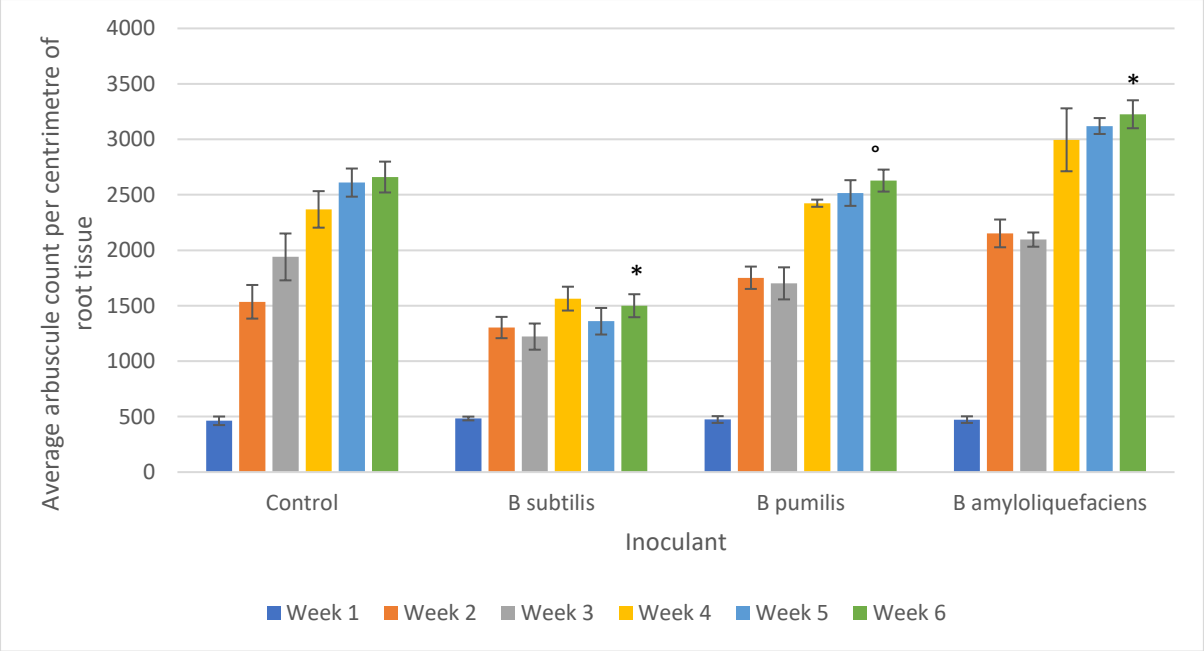

Figure 5.1 – Average (n = 270 overall) arbuscule count for inoculated Zulu variety winter wheat over 6 weeks, producing ANOVA significance (P < 0.0001). TRUE Bonferroni corrections are represented by (*), whilst FLASE Bonferroni corrections are presented as (°). Error bars constructed from SEM.

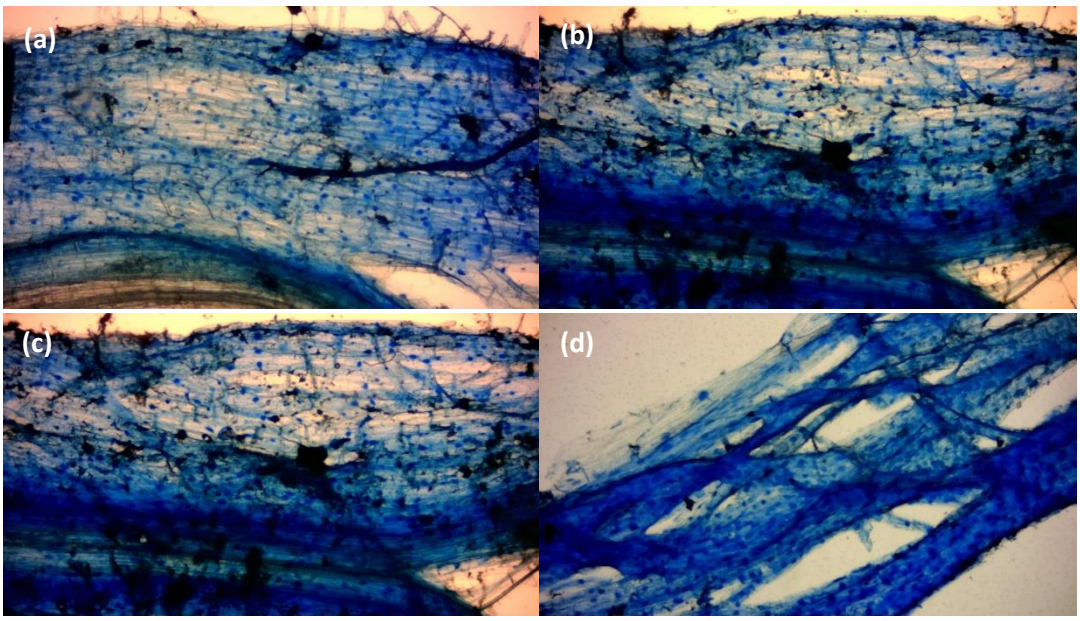

Figure 5.2 – *Sheaffer® blue stained Zulu variety winter wheat from (a) control, (b)* B.subtilis*, (c)* B.pumilis *and (d)* B.amyloliquefaciens *at 2 weeks post germination, taken using an Apex microscope at a total magnification of x100 and a Bresser® HD microscope camera.*

Root length of Zulu winter wheat was measured between the different inoculations of the selected *Bacillus* spp. rhizobacteria (P < 0.00001, df: 3,16, F value: 21.73, F critical: 3.29, single factor ANOVA) (Figure 5.3). *B. subtilis* was observed to have the greatest increase to root length (P < 0.0001, df: 8, t.stat: -5.87, unequal variance T test), whilst *B. pumilis* and *B.*

amyloliquefaciens were both measured as having a neutral effect to root length of Zulu winter wheat, as confirmed by a Bonferroni correction FALSE for *Bacillus pumilis* and *Bacillus amyloliquefaciens* and TRUE for *Bacillus subtilis* in comparison with the control sample.

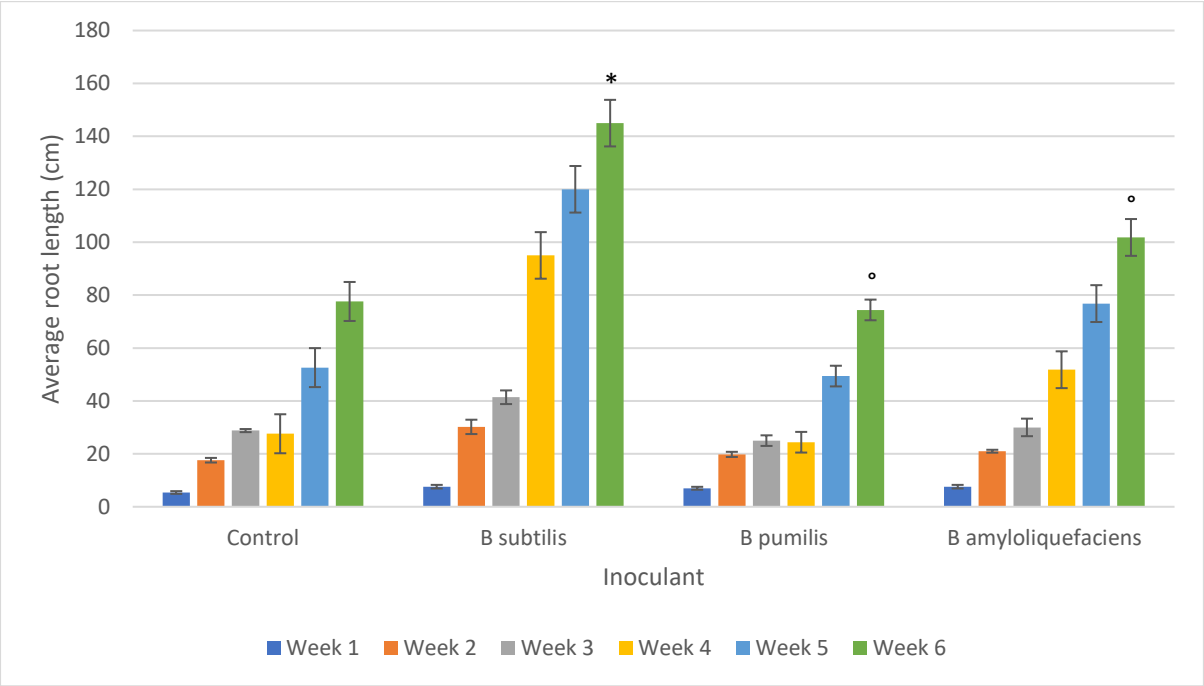

Figure 5.3 - Average (n = 270 overall) root length for select rhizosphere *Bacillus* spp. inoculated Zulu variety winter wheat over 6 weeks, producing ANOVA significance ($P < 0.00001$). TRUE Bonferroni corrections are represented by (*), whilst FLASE Bonferroni corrections are presented as (°). Error bars constructed from SEM.

Total number of tillers was measured to increase greatest with *B. amyloliquefaciens* over the 6 weeks of inoculation period ($P < 0.006$, df: 6, t.stat: -3.54, unequal variance T test). *B. subtilis* and *B. pumilis* were both observed to have not had an influence on the number of wheat tillers compared to the control sample. Bonferroni correction factor indicated TRUE for *B. amyloliquefaciens*, and a FALSE correlation for *B. pumilis* and *B. subtilis* inoculation in comparison with the control sample.

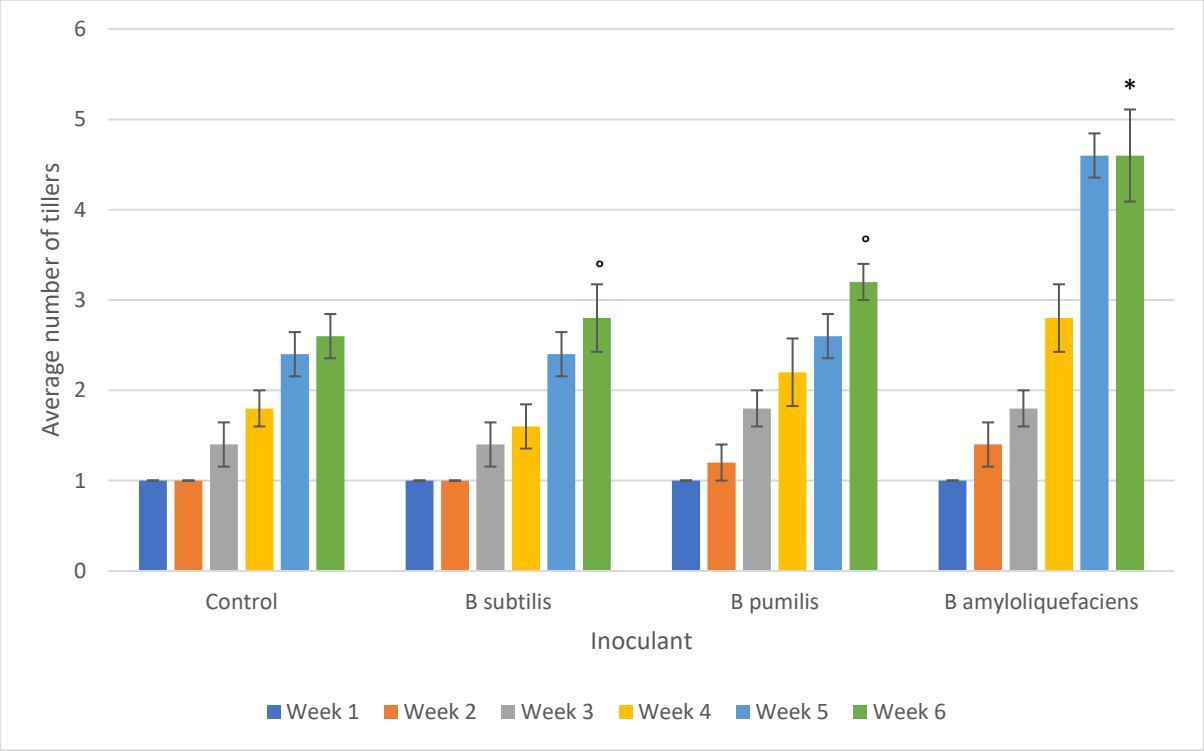

Figure 5.4 - Average (n = 270 overall) number of tillers per plant for select rhizosphere *Bacillus* spp. inoculated Zulu variety winter wheat over 6 weeks, producing ANOVA significance ($P < 0.005$). TRUE Bonferroni corrections are represented by (*), whilst FLASE Bonferroni corrections are presented as (°) Error bars constructed from SEM.

5.3.2 Influence of select *Bacillus* spp. and AM fungi on quantifiable soil glomalin between CT and ZT tillage regimes

Bare soils sampled from ZT (Figure 5.5) and CT (Figure 5.6) were inoculated with selected *Bacillus* spp. and *R. intraradicies*, as identified from ITS sequencing in Section 2.3.2. Positive and/or negative variances in quantified glomalin were compared against control samples with no inoculation. ZT soils, were noted to have produced an alteration to overall glomalin concentrations over the 6 week study period (Figure 5.5 ($P < 0.00001$, df: 4,30, F value: 15.80, F critical: 2.69, single factor ANOVA)), with the greatest differences in glomalin concentration seen to increase most when *B. amyloliquefaciens* was applied ($P < 0.00001$, df: 8, t.stat: -9.48, unequal variance T test) and reductions to glomalin from *B. subtilis* ($P < 0.00001$, df: 7, t.stat: 7.29, unequal variance T test). Inoculants of *B. pumilis* and *R. intraradicies* did not indicate any significant increase from control samples ($P = 0.62$, df: 8, t.stat: -0.48, paired unequal variance T test).

Post hoc 2 tailed, paired T test of equal variance indicated significance from ANOVA tests at week 6. The Bonferroni correction factor indicated TRUE corrections for all *Bacillus* spp. and a FALSE correction for AM fungal inoculation in comparison with the control sample.

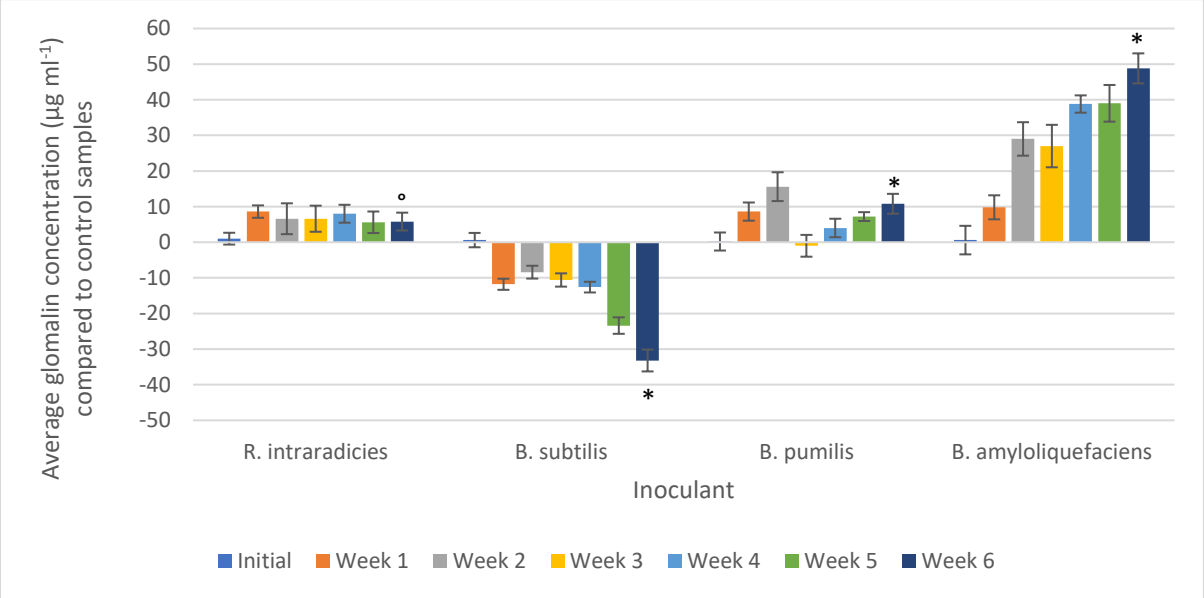

Figure 5.5 – Average (n = 175 overall) difference in glomalin concentration between inoculated and non-inoculated control from sampled sandy loam top soil (<10cm) from ZT managed site over 6 weeks with weekly inoculant of select rhizosphere *Bacillus* spp. in controlled glasshouse conditions. TRUE Bonferroni corrections are represented by (*), whilst FLASE Bonferroni corrections are presented as (°). Error bars constructed from SEM.

Select *Bacillus* spp. and *R. intraradicies* applications to CT soils additionally produced greatest differences in glomalin concentration from applied *B. amyloliquefaciens* ($P < 0.0001$, df: 8, t.stat: -6.04, unequal variance T test) and reductions from *B. subtilis* ($P < 0.00001$, df: 6, t.stat: 8.42, unequal variance T test), with *R. intraradices* and *B. pumilis* having neutral effects ($P = 0.59$, df: 7, t.stat: -0.56, paired unequal variance T test).

The Bonferroni correction factor indicated TRUE correlations for *B. subtilis* and *B. amyloliquefaciens* and a FALSE correction for AM fungal and *B. pumilis* inoculations in comparison with the control sample.

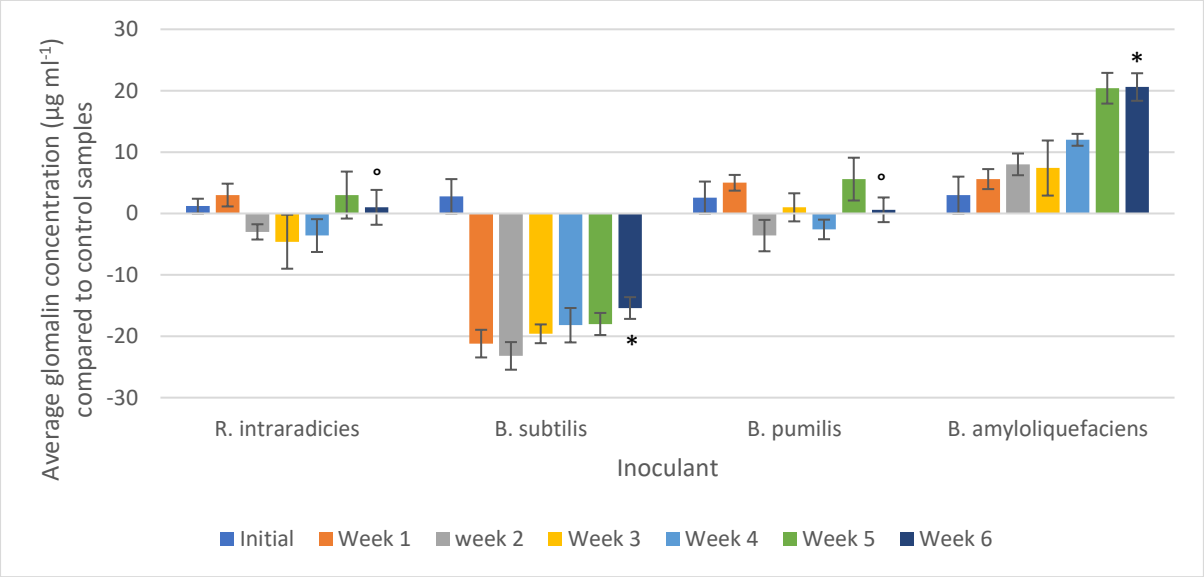

Figure 5.6 – Average (n = 175 overall) difference in glomalin concentration between inoculated and non-inoculated control from sampled sandy loam top soil (<10cm) from CT managed site over 6 weeks with weekly inoculant of select rhizosphere *Bacillus* spp. in controlled glasshouse conditions. TRUE Bonferroni corrections are represented by (*), whilst FLASE Bonferroni corrections are presented as (°). Error bars constructed from SEM.

Inoculant applications between tillage treatments (Figures 5.5 and 5.6) also produced significance between glomalin concentrations for all samples at week 6 (Table 5.3).

Table 5.*3 – 2 tail, unequal variance T tests between tillage treatments of Figure 5.5 and 5.6 for each inoculant.*

Inoculant	**P value**	**Degrees of freedom**	**T stat**
Control	<0.00001	8	9.84
R. intraradices	<0.00001	11	9.60
B. subtilis	<0.001	12	3.12
B. pumilis	<0.0001	8	6.08
B. amyloliquefaciens	<0.001	8	3.85

5.3.3 Effect of select *Bacillus* spp. and AM fungi on wheat growth and root development over a 30 week period between field sampled soils under controlled conditions, sampled at weeks 15 and 30

5.3.3.1 Week 15 samples

The number of tillers was generally lower in the CT treatments than ZT treated soils except for those plants to which *B. amyloliquefaciens* had been inoculated ($P < 0.00001$, df: 7,32, F value: 9.38, F critical: 2.31, single factor ANOVA). This was also the case for ZT inoculated soils ($P < 0.00001$, df: 7,32, F value: 6.57, F critical: 2.31, single factor ANOVA) (Figure 5.7). Post hoc T tests showed *B. amyloliquefaciens* inoculum produce the greatest increase to overall tiller numbers ($P < 0.0001$, df: 8, t.stat: -3.89, paired equal variance T test) in ZT soils, whilst greatest significant increase to tiller numbers in CT soils came about from AM fungal inoculum alone and in combination with *B. subtilis* ($P < 0.0001$, df: 8, t.stat: -6.67, paired equal variance T test).

The influence of the inoculations, in relation to tiller length as above ground biomass, was seen to increase overall in CT sampled soils ($P = 0.03$, df: 7,32, F value: 5.57, F critical: 2.31, single factor ANOVA) at week 15 sampling, as did ZT inoculated soils ($P = 0.01$, df: 7,32, F value: 6.57, F critical: 3.13, single factor ANOVA) (Figure 5.8). Post hoc T tests showed *B. pumilis* along with AM fungal and *B. subtilis* co-inoculations, AM fungi and *B. amyloliquefaciens* co-inoculations produce the greatest increase to overall tiller length ($P < 0.01$, df: 8, t.stat: -3.54, paired equal variance T test) in ZT soils, whilst greatest significant increase to tiller numbers in CT soils came about from *B. subtilis* and *B. pumilis* inoculums alone ($P < 0.01$, df: 8, t.stat: -5.15, paired equal variance T test).

ZT soils, however, were seen to have a greater overall root length ($P < 0.00001$, df: 7,32, F value: 10.86, F critical: 2.31, single factor ANOVA) (Figure 5.9) when compared to CT soils for all inoculations ($P < 0.00001$, df: 7,32, F value: 13.88, F critical: 2.31, single factor ANOVA), with the exception of the application of AM fungi alone. Greatest differences were noted between control samples (receiving no inoculum) of each tillage treatment, whilst *B. subtilis* inoculations increased root length, ($P < 0.01$, df:8, t.stat: -5.01, paired equal variance T test) and AM fungi applications decreased overall root length ($P < 0.01$, df:8, t.stat: 2.98, paired equal variance T test. Post hoc T testing indicated significance from *B. amyloliquefaciens, B. subtilis,* AM fungal inoculations alone and AM fungi with *B. pumilis* as well as AM fungi with *B. amyloliquefaciens* co-inoculations ($P < 0.01$, df:8, t.stat: 3.21, paired equal variance T test) in CT soils.

Quantified root arbuscules (Figure 5.10) in CT soils were generally lower per centrimetre of root tissue than ZT soils, however, all inoculations in CT soils were noted to have produced variances in measured AM fungal arbuscules compared to the control samples ($P < 0.00001$, df: 7,32, F value: 6.45, F critical: 2.31, single factor ANOVA), with *B. amyloliquefaciens* producing the greatest quantity of arbuscules when inoculated to soils alone and in combination with AM fungi. Post hoc T testing showed inoculations with *B. subtilis* alone and in co-inoculation with AM fungi had a significant impact on AM fungal symbiosis ($P < 0.01$, df: 8, t.stat: 4.16, paired equal variance T test). Bonferroni factors also indicated that *B. subtilis* and *B. subtilis* with AM fungi inoculations contributed more to ANOVA significance than other inoculations. Inoculations in ZT samples produced were also seen to produce the greatest number of root arbuscules with *B. amyloliquefaciens* both alone and in combination with AM fungi ($P < 0.00001$, df: 7,32, F value: 12.57, F critical: 2.31, single factor ANOVA). Post hoc T tests showed *B. subtilis* and *B. subtilis* with AM fungi inoculations to ZT soils had significant implications towards AM fungal root cortical arbuscules ($P < 0.001$, df: 8, t.stat: 5.96, paired equal variance T test). Bonferroni factors gave indications that *B. subtilis* and *B. subtilis* with AM fungi inoculations had the greatest contribution to ANOVA significance.

Week 15 sampled soils were further analysed in relation to glomalin concentrations between inoculants. CT glomalin varied by a small degree and inoculants were not seen to have had an influence on quantified glomalin. ZT soils, however, were noted to have produced a greater degree of variance between inoculants when compared to CT soils and were significantly different ($P < 0.001$, df: 7,32, F value: 12.57, F critical: 2.31, single factor ANOVA) with inoculations of *B. amyloliquefaciens* having the greatest increase to quantified glomalin producing a 1.84 fold increase compared to CT samples.

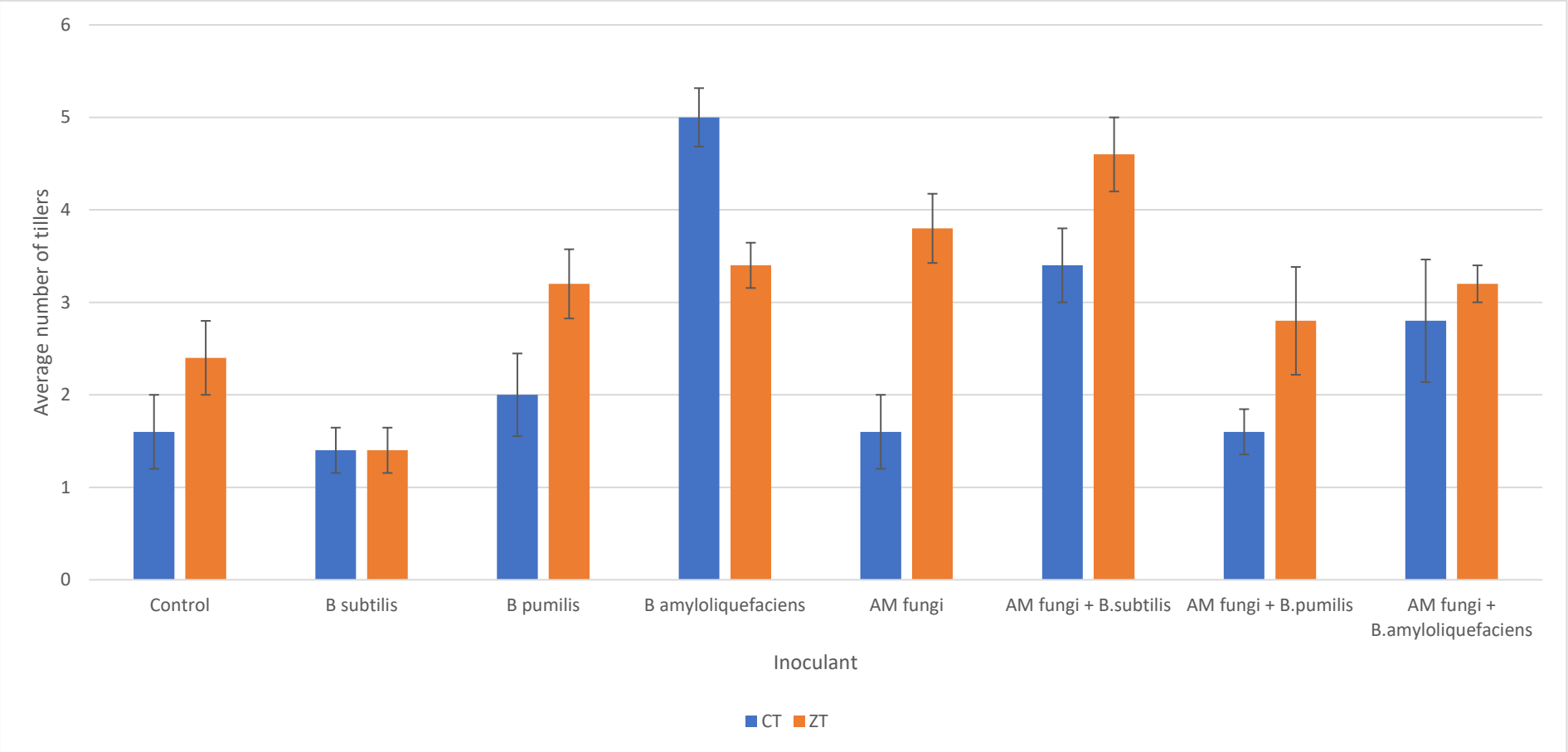

Figure 5.7 - Average (n = 80 per tillage treatment) number of tillers from glasshouse controlled growth of Zulu variety winter wheat at 15 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp.and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed significance for CT ($P < 0.00001$) and ZT samples ($P < 0.00001$). Error bars constructed from SEM.

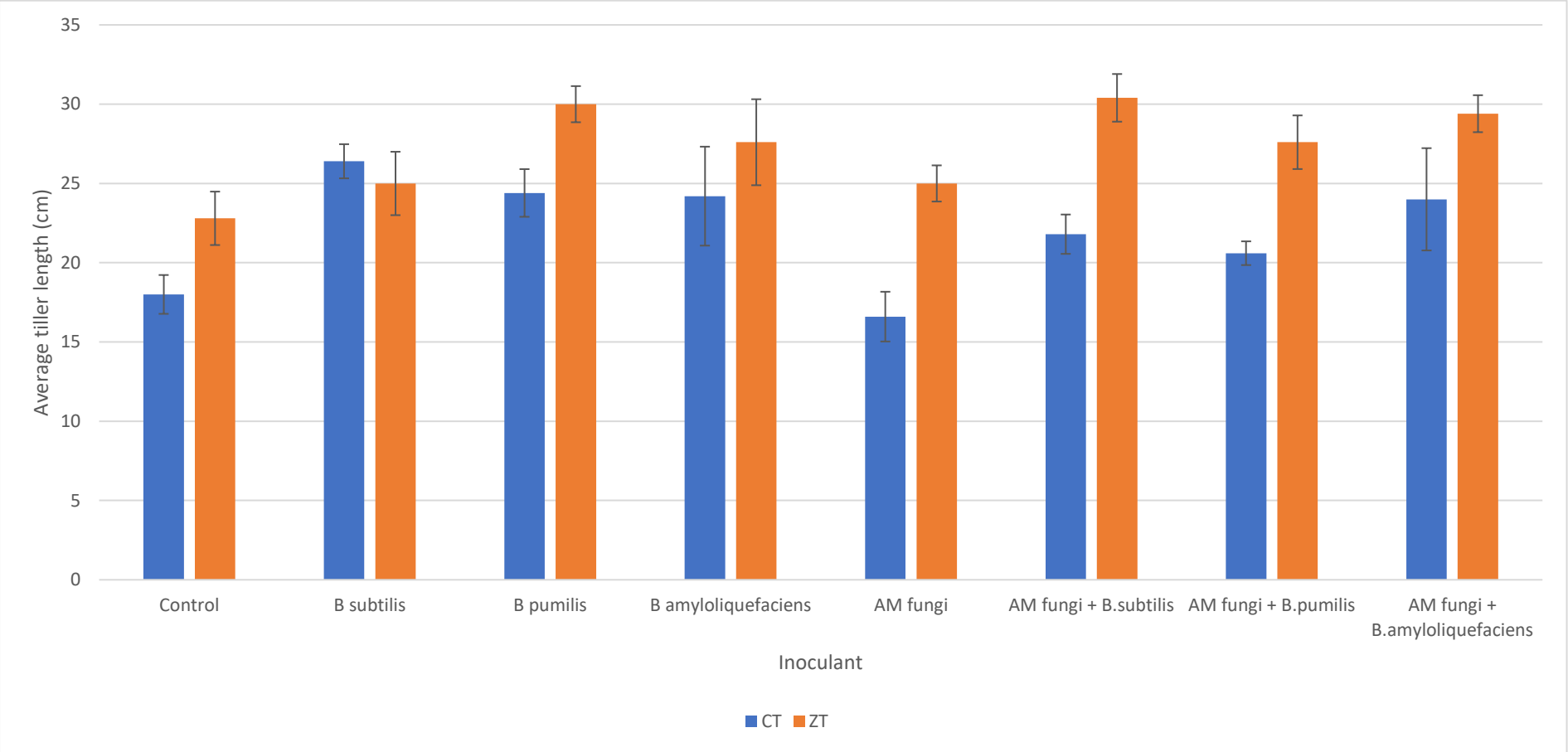

Figure 5.8 - Average (n = 80 per tillage treatment) tiller length from glasshouse controlled growth of Zulu variety winter wheat at 15 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp. and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT (P = 0.01) and ZT samples (P = 0.03). Error bars constructed from SEM.

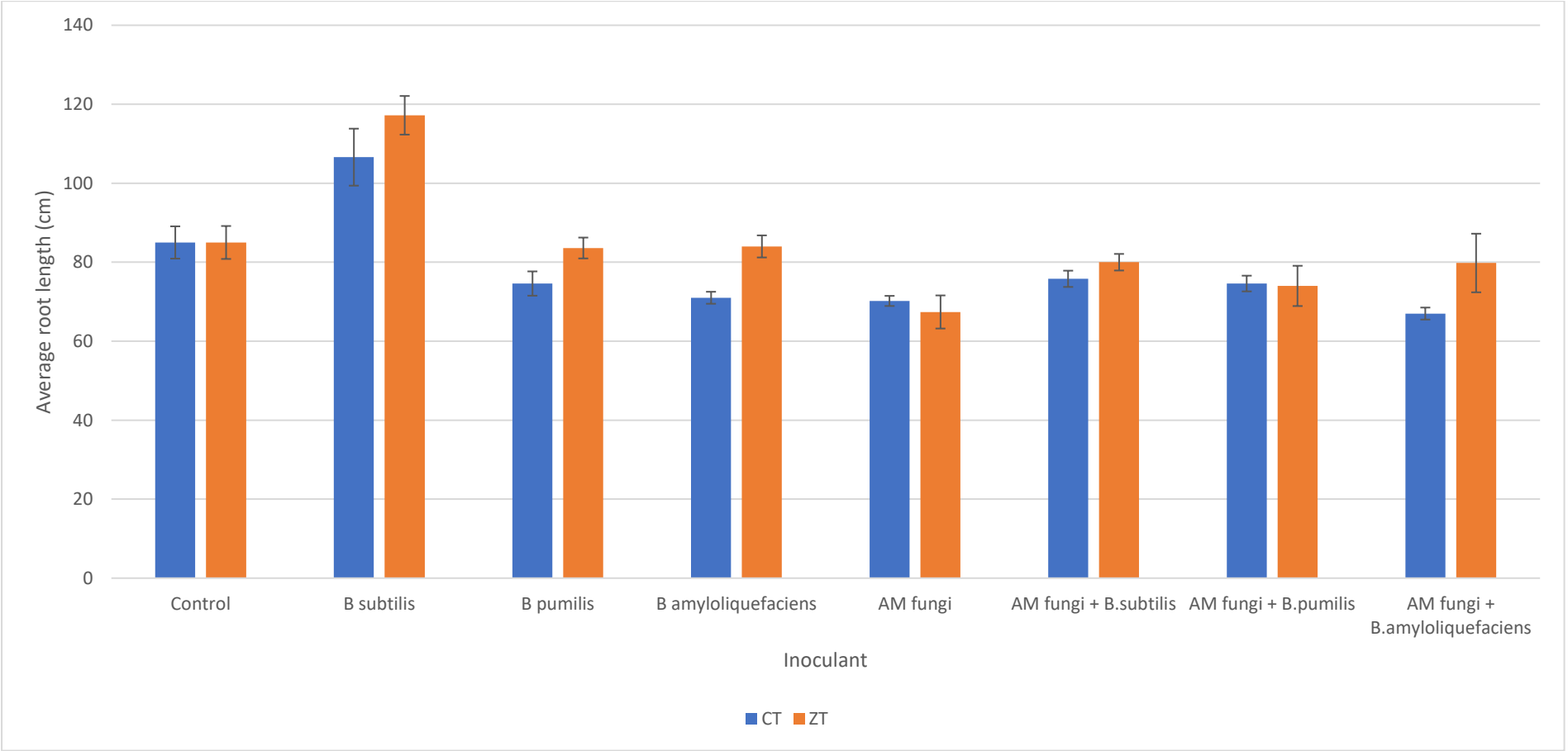

Figure 5.9 - Average (n = 80 per tillage treatment) root length from glasshouse controlled growth of Zulu variety winter wheat at 15 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp.and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT ($P < 0.00001$) and ZT samples ($P < 0.00001$). Error bars constructed from SEM.

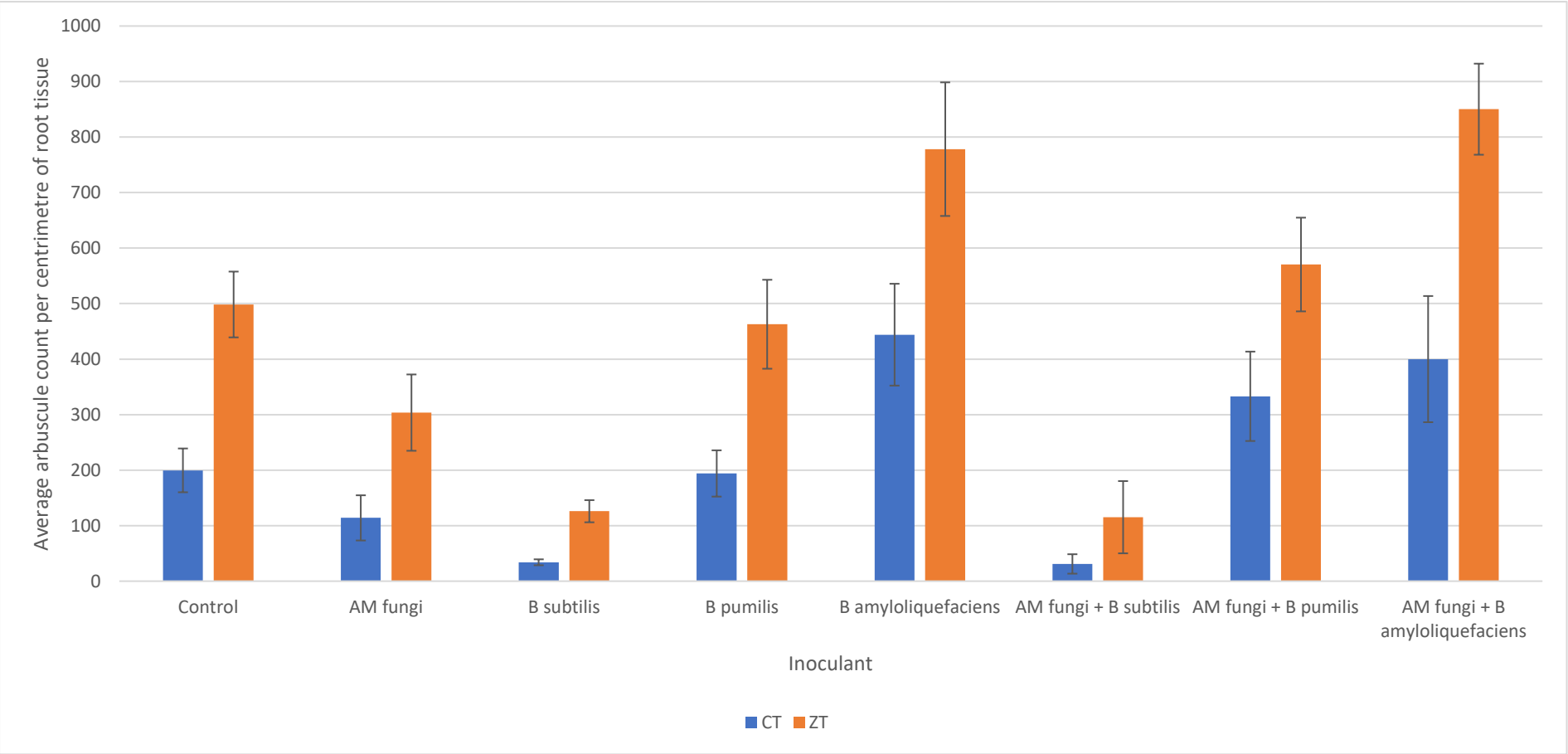

Figure 5.10 - Average (n = 80 per tillage treatment) root arbuscules per 1cm root sections from field collected soil of CT ($P < 0.00001$, ANOVA) and ZT ($P < 0.00001$, ANOVA) treatments with Zulu winter wheat grown under controlled glasshouse conditions. AM fungi and select select rhizosphere *Bacillus* spp.were applied to the soil surface on a weekly basis. Error bars constructed from SEM.

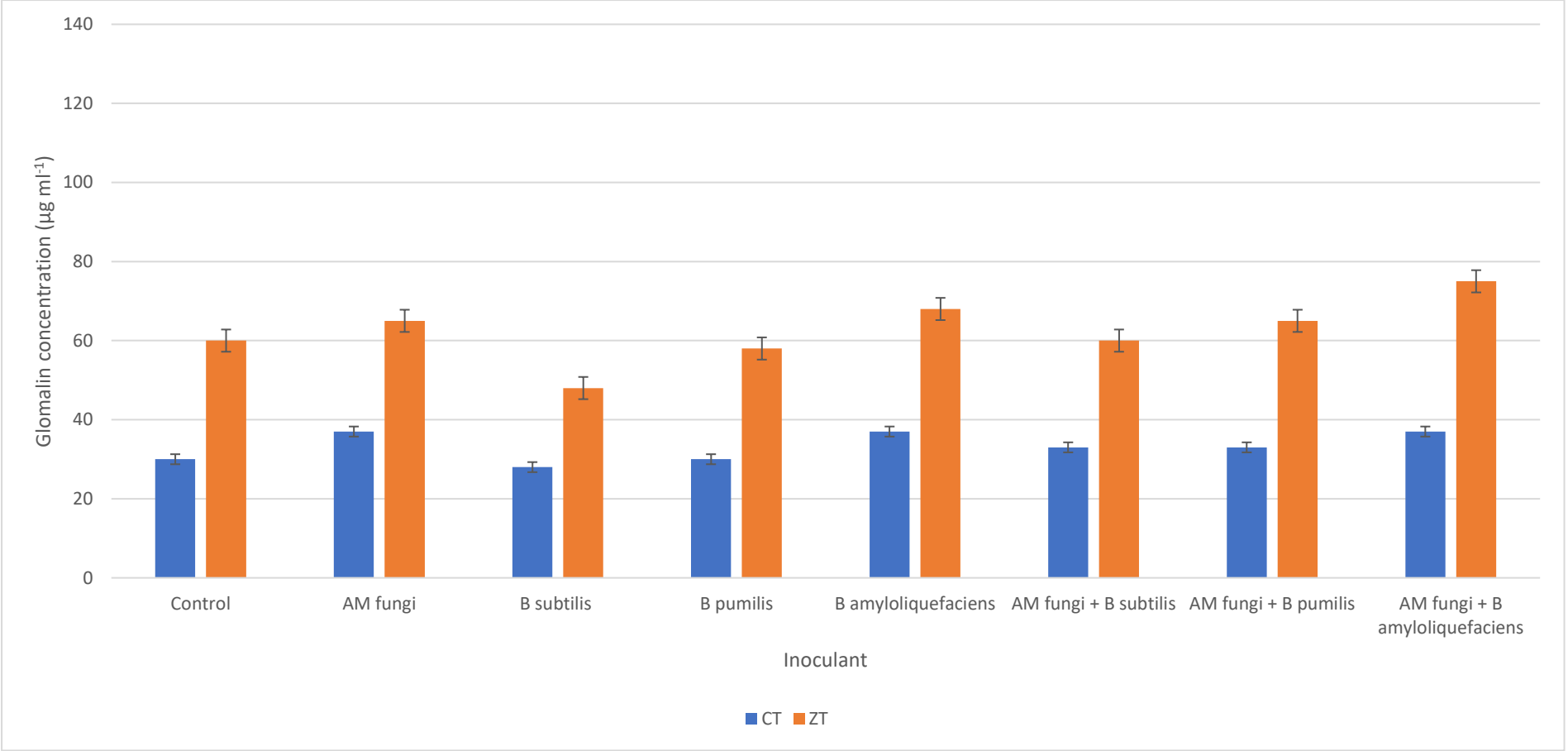

Figure 5.11 – Average (n = 80 per tillage treatment) glomalin concentration of field sampled soils from CT ($P < 0.00001$, ANOVA) and ZT ($P < 0.00001$, ANOVA) treatments with the growth of Zulu winter wheat grown under controlled glasshouse conditions. AM fungi (*R. intraradicies*) and select *Bacillus* spp. were applied to the soil surface on a weekly basis. Error bars constructed from SEM.

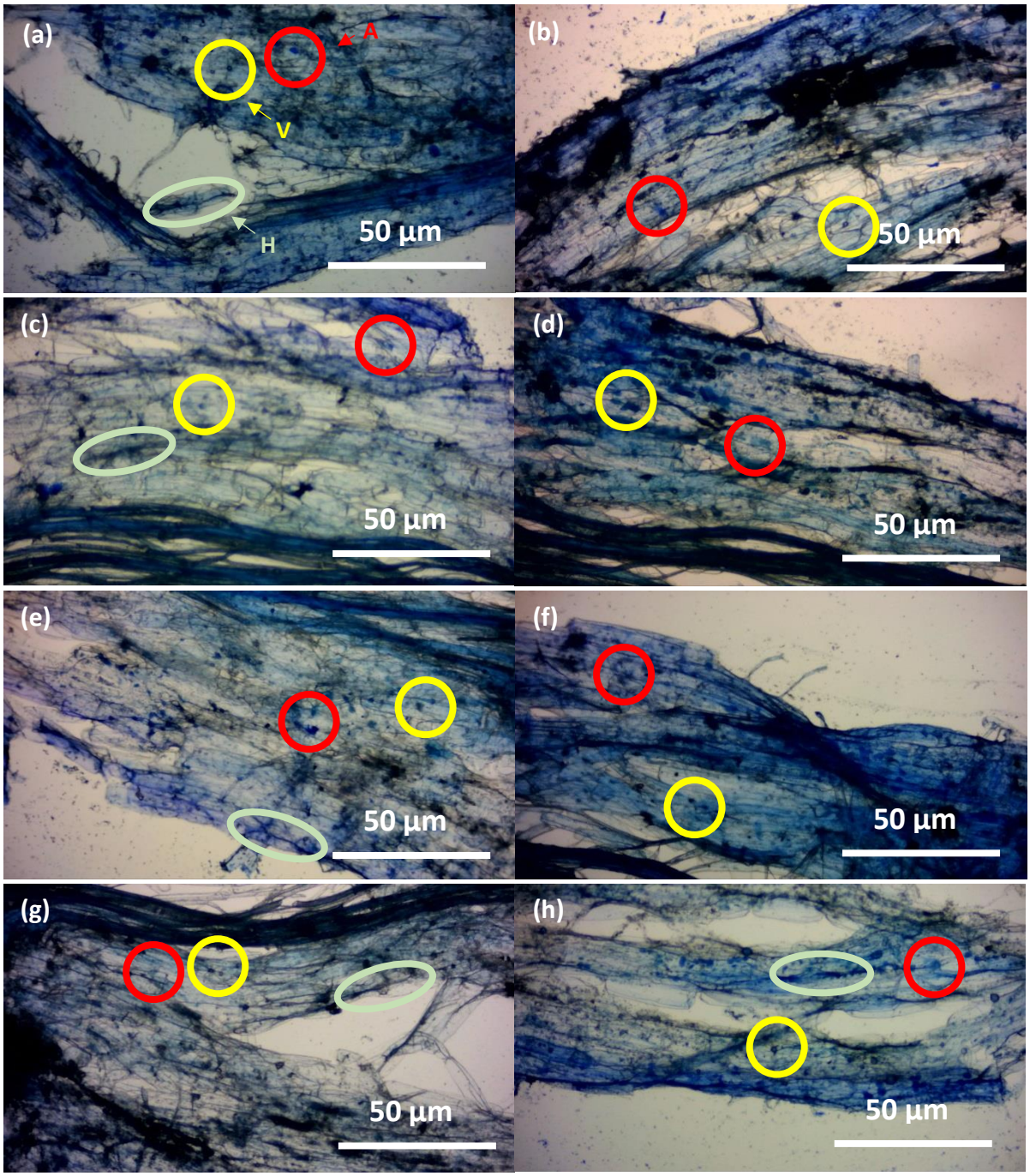

Figure 5.12 – Images of Sheaffer® blue stained root sections from Zulu wheat grown in collected ZT soils at 15 weeks of growth. A – arbuscule, V – vesicle, H – intraradiating hyphae. (a) control, (b) AM fungi (*R. intraradicies*), (c) *B. subtills,* (d) *B. amyloliquefaciens,* (e) *B. pumilus*, (f) AM fungi and *B. subtills,* (g) AM fungi and *B. amyloliquefaciens*, (h) AM fungi and *B. pumilus*, taken using an Apex microscope at a total magnification of x100 and a Bresser® HD microscope camera.

Figure 5.13 – Zulu wheat tiller heights at week 15 grown in soils collected form ZT (a) and CT (b) managed sites with a weekly inoculation of AM fungi. Appendix 8.15 presents tiller heights from different inoculations.

5.3.3.1.1 Correlated influences of selected *Bacillus* spp. and AM fungi on measured tiller and root lengths of wheat between CT and ZT field sampled soils under controlled growth conditions sampled at week 15

The relationship between arbuscules in 1 cm root segments and root lengths of the different treatments can be seen in Figure 5.14. This figure shows that AM fungal root arbuscules were negatively correlated with root length of sampled Zulu winter wheat. This is indicating an decrease in arbuscular density with increased root length. ZT soils were consistently seen to produce greater quantities of root arbuscules across all inoculants ($P < 0.00001$, df: 7,32, F value: 12.53, F critical: 2.31, single factor ANOVA). Statistical significance was also observed between root length across all inoculations ($P < 0.0001$, df: 7,32, F value: 10.86, F critical: 2.31, single factor ANOVA). Soils inoculated with *B. amyloliquefaciens* were observed to produce increased amounts of observable arbuscules per 1cm root section. CT soils, however, consistently produced a reduced quantity of arbuscules between inoculants ($P < 0.00001$, df: 7,32, F value: 6.45, F critical: 2.31, single factor ANOVA) and increases to root length

A positive correlation was observed between arbuscular quantities in root tissues and tiller length for both CT ($P < 0.00001$, df: 7,32. F value: 9.38, F critical: 2.31, single factor ANOVA) and ZT ($P < 0.00001$, df: 7,32. F value: 6.57, F critical: 2.31, single factor ANOVA) for all inoculants showing adding to an increase to above ground biomass.

Tiller numbers were additionally seen to be positively correlated with arbuscule quantities in root tissue (Figure 5.16), with ZT ($P < 0.00001$, df: 7,32. F value: 6.57, F critical: 2.31, single factor ANOVA) sampled wheat consistently producing a greater quantity of tillers per plant across all inoculants compared with CT ($P < 0.00001$, df: 7,32. F value: 9.38, F critical: 2.31, single factor ANOVA) sampled wheat.

Whole plant responses to inoculants were produced for each of the tillage treated soils through calculating the ratios 1) of arbuscule numbers per 1cm root : root length, and 2) through the ratio of tiller length : tiller number (Figure 5.16). Ratio distribution of the arbuscules per 1cm root to root length in ZT were seen to be greatly different between applied inoculants ($P < 0.00001$, df: 7,32, F value: 15.19, F critical: 2.31, single factor ANOVA)

as well as CT soils ($P < 0.00001$, df:7,32, F value: 7.70, F critical: 2.31). Ratios between tiller length and tiller numbers (Figure 5.17) were further seen to be significantly different between applied inoculants in ZT ($P < 0.0001$, df: 7,32, F value: 5.09, F critical: 2.31, single factor ANOVA) and CT ($P < 0.00001$, df: 7,32, F value: 16.33, F critical: 2.31, single factor ANOVA). Results are able to show overall increases to wheat biomass from inoculations of *B. amyloliquefaciens* and reductions to overall biomass from *B. subtilus* inoculants.

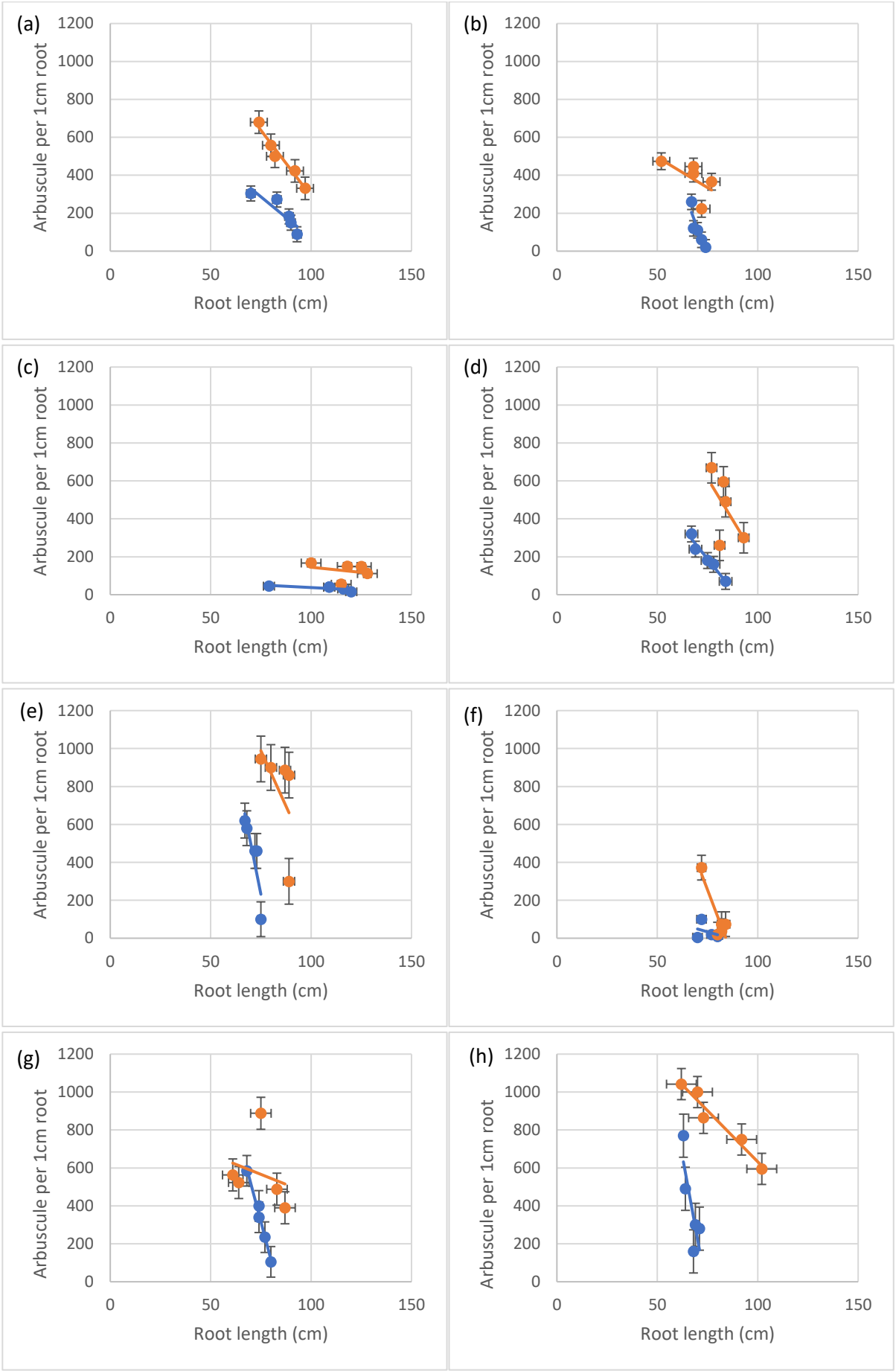

Figure 5.14 – Zulu wheat at week 15 indicating negative correlations between root length in ZT (orange) ($P < 0.00001$, df: 7,32, F value: 10.86, F critical: 2.31, Single factor ANOVA) and CT (blue) ($P < 0.00001$, df: 7, 32, F value: 13.88, F critical: 2.31, Single factor ANOVA) with average (n = 400) root cortical arbuscules in ZT ($P < 0.00001$, df: 7,32, F value: 12.80, F critical: 2.31, Single factor ANOVA) and CT ($P < 0.00001$, df: 7,32, F value: 6.45, F critical: 2.31, Single factor ANOVA). Within ZT soil samples, (a) control

samples received no inoculant (Pearson's correlation: -0.98, r^2 0.96). (b) AM fungi, Pearson's correlation: -0.61, r^2 0.37. (c) *B.subtilis*, Pearson's correlation: -0.27, r^2 0.07. (d) *B.pumilis*, Pearson's correlation: -0.58, r^2 0.33. (e) *B.amyloliquefaciens*, Pearson's correlation: -0.54, r^2 0.29. (f) AM fungi and *B.subtilis*, Pearson's correlation: -0.90, r^2 0.81. (g) AM fungi and *B.pumilis*, Pearson's correlation: -0.26, r^2 0.07. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: -0.97, r^2 0.95. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: -0.90, r^2 0.81). (b) AM fungi, Pearson's correlation: -0.90, r^2 0.80. (c) B.subtilis, Pearson's correlation: -0.74, r^2 0.54. (d) B.pumilis, Pearson's correlation: -0.98, r^2 0.95. (e) B.amyloliquefaciens, Pearson's correlation: -0.88, r^2 0.77. (f) AM fungi and B.subtilis, Pearson's correlation: -0.36, r^2 0.13. (g) AM fungi and B.pumilis, Pearson's correlation: -0.99, r^2 0.98. (h) AM fungi and B.amyloliquefaciens, Pearson's correlation: -0.83, r^2 0.68.

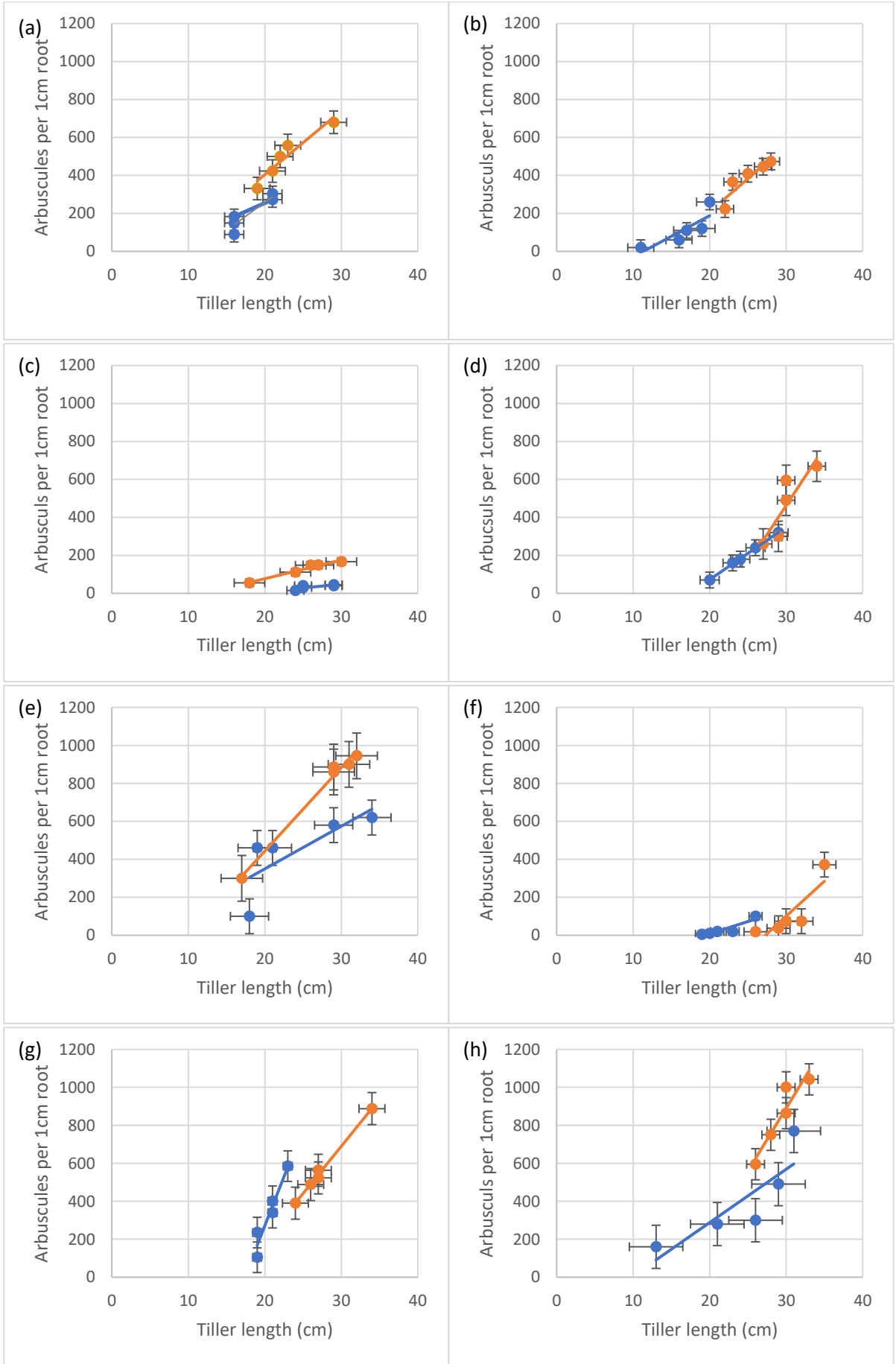

Figure 5.15 - Zulu wheat at week 15 indicating positive correlations between tiller length in ZT (orange) (P = 0.03, df: 7,32, F value: 2.67, F critifcal: 3.21, Single factor ANOVA) and CT (blue) (P = 0.01, df: 7.32, F value: 3.12, F critical: 2.31, Single factor ANOVA) with average (n = 400) root cortical arbuscules in ZT (P < 0.00001, df: 7,32, F value: 12.80, F critical: 2.31, Single factor ANOVA) and CT (P < 0.00001, df: 7,32, F value: 6.45, F critical: 2.31, Single factor ANOVA). Within ZT soil samples, (a) control samples received no

inoculant (Pearson's correlation: 0.96, r^2 0.91). (b) AM fungi, Pearson's correlation: 0.91, r^2 0.93. (c) *B.subtilis*, Pearson's correlation: 0.98, r^2 0.97. (d) *B.pumilis*, Pearson's correlation: 0.87, r^2 0.76. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.99, r^2 0.99. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.85, r^2 0.72. (g) AM fungi and *B.pumilis*, Pearson's correlation: 1.00, r^2 0.99. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.94, r^2 0.88. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.89, r^2 0.79). (b) AM fungi, Pearson's correlation: 0.92, r^2 0.84. (c) *B.subtilis*, Pearson's correlation: 0.65, r^2 0.42. (d) *B.pumilis*, Pearson's correlation: 0.89, r^2 0.78. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.90, r^2 0.80. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.52, r^2 0.27. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.83, r^2 0.68. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.95, r^2 0.90.

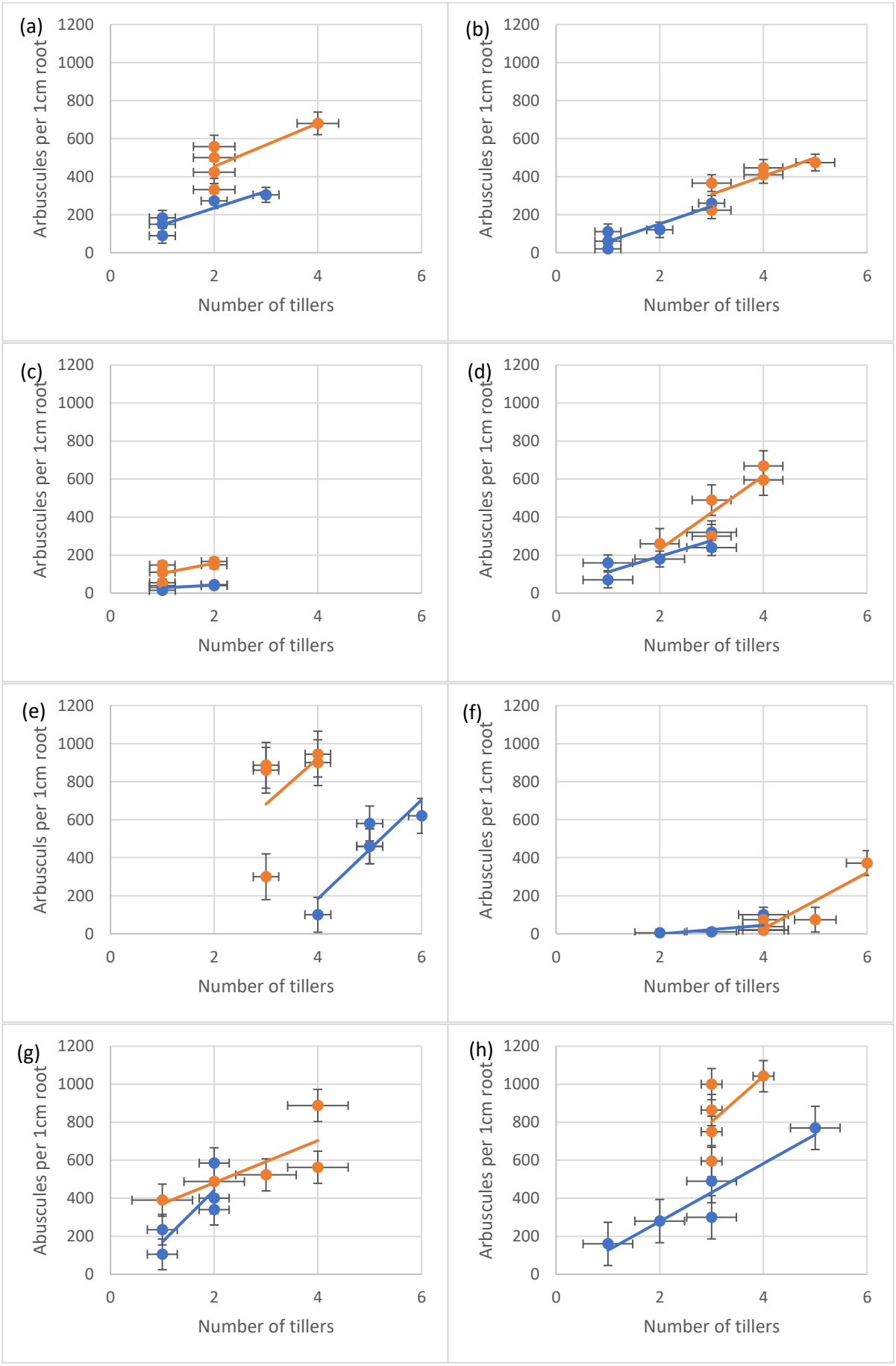

Figure 5.16 - Zulu wheat at week 15 indicating positive correlations between number of tiller numbers in ZT (orange) ($P < 0.00001$, df: 7,32, F value: 6.57, F critical: 2.31, Single factor ANOVA) and CT (blue) numbers ($P < 0.00001$, df: 7,32, F value: 9.38, F critical: 9.38, F critical: 2.31, Single factor ANOVA), with average (n = 400) root cortical arbuscules in ZT ($P < 0.00001$, df: 7,32, F value:

12.80, F critical: 2.31, Single factor ANOVA) and CT ($P < 0.00001$, df: 7,32, F value: 6.45, F critical: 2.31, Single factor ANOVA). Within ZT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.77, r^2 0.59). (b) AM fungi, Pearson's correlation: 0.81, r^2 0.66. (c) *B.subtilis*, Pearson's correlation: 0.65, r^2 0.43. (d) *B.pumilis*, Pearson's correlation: 0.90, r^2 0.81. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.49, r^2 0.24. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.91, r^2 0.83. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.76, r^2 0.59. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.58, r^2 0.34. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.92, r^2 0.84). (b) AM fungi, Pearson's correlation: 0.84, r^2 0.70. (c) *B.subtilis*, Pearson's correlation: 0.76, r^2 0.57 (d) *B.pumilis*, Pearson's correlation: 1.00, r^2 1.00. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.76, r^2 0.58. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.91, r^2 0.83. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.96, r^2 0.92. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.85, r^2 0.72.

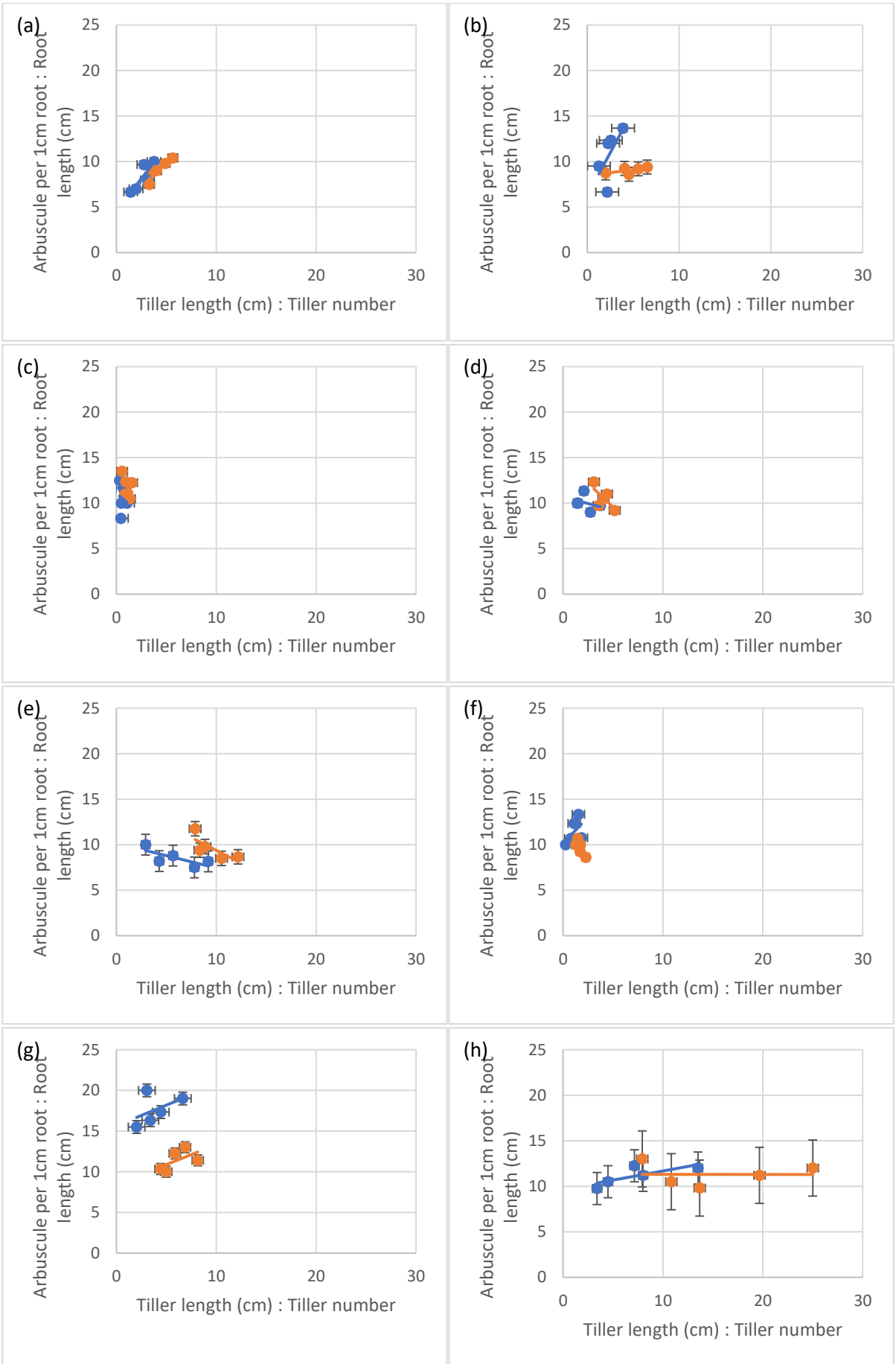

Figure 5.17 - Correlations of the ratio between arbuscules per 1cm root and root length, with the ratio between tiller length and tiller number tiller length in Zulu wheat at week 15 in ZT (orange) and CT (blue) with average (n = 400) root cortical arbuscules in

ZT and CT. Within ZT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.92, r^2 0.86). (b) AM fungi, Pearson's correlation: 0.64, r^2 0.41. (c) *B.subtilis*, Pearson's correlation: -0.47, r^2 0.24. (d) *B.pumilis*, Pearson's correlation: -0.70, r^2 0.49. (e) *B.amyloliquefaciens*, Pearson's correlation: -0.76, r^2 0.58. (f) AM fungi and *B.subtilis*, Pearson's correlation: -0.82, r^2 0.68. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.58, r^2 0.34. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.00, r^2 0.00. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.85, r^2 0.73). (b) AM fungi, Pearson's correlation: 0.64, r^2 0.41. (c) *B.subtilis*, Pearson's correlation: -0.17, r^2 0.03. (d) *B.pumilis*, Pearson's correlation: -0.33, r^2 0.11. (e) *B.amyloliquefaciens*, Pearson's correlation: -0.74, r^2 0.54. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.52, r^2 0.27. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.43, r^2 0.23. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.76, r^2 0.58.

5.3.3.2 Week 30 samples

The responses of Zulu wheat sampled at week 30 can be seen in Figure 5.18 and the results continued to indicate inoculants influencing tiller numbers in CT ($P < 0.00001$, df: 7,32, F value: 10.93, F critical: 2.31, single factor ANOVA) and ZT ($P < 0.01$, df: 7,32, F value: 3.30, F critical: 2.31, single factor ANOVA) soils. None of the inoculants produced a greater improvement to overall tiller numbers, but produced comparably different tiller numbers compared to control samples ($P < 0.01$, df: 8, t.stat: -3.13, paired equal variance T test) in ZT soils, whilst the greatest degree of increase to tiller numbers in CT soils came about from *B. amyloliquefaciens* inoculum alone ($P < 0.0001$, df: 8, t.stat: -4.02, paired equal variance T test).

Measurements of tiller length can be seen in Figure 5.19 and was further seen to maintain its degree of increase in CT ($P < 0.002$, df: 7,32, F value: 4.08, F critical: 2.31, single factor ANOVA) and ZT inoculated soils ($P = 0.05$, df: 7,32, F value: 2.21, F critical: 3.13, single factor ANOVA) (Figure 5.19). No improvement was observed to overall tiller length from inoculations compared with the control ($P = 0.15$, df: 8, t.stat: -1.61, paired equal variance T test) in ZT soils, whilst greatest increase to tiller numbers in CT soils came about from *B. amyloliquefaciens* inoculums alone ($P < 0.01$, df: 8, t.stat: -3.83, paired equal variance T test).

Root length (Figure 5.20) from ZT inoculated soils were seen to be greatest in *B. subtilis*, whilst inoculations of AM fungi and *B. amyloliquefaciens* were both measured to reduce overall root length ($P < 0.00001$, df: 7,32, F value: 33.44, F critical: 2.31, single factor ANOVA). Combined inoculations in ZT soils saw a further decrease in root length with AM fungi and *B. amyloliquefaciens*. Combinations of AM fungi and *B. subtilis* were seen to reduce root length in both tillage treatments compared to lone inoculations of each organisms and compared to the control samples (Figure 5.20) ($P < 0.00001$, df:8, t.stat: 6.79, paired equal variance T test).

Quantified root arbuscules (Figure 5.21) in CT soils was influenced by inoculated organisms ($P < 0.00001$, df: 7,32, F value: 15.05, F critical: 2.31, single factor ANOVA) for applications of AM fungi and *Bacilli* spp. *B. subtilis* and *B. amyloliquefaciens* alone and co-inoculation AM fungi with *B. subtilis* had the greatest influence on root arbuscules, with measurable decreases from all *B. subtilis* applications and increases from all *B. amyloliquefaciens* ($P < 0.01$, df: 8, t.stat: 4.23, paired equal variance T test) (0.93 fold increase). Bonferroni factors also indicated that *B. amyloliquefaciens* and *B. subtilis*, as well as *B. subtilis* with AM fungi inoculations, produced the greatest degree of influence compared to control samples.

Inoculations in ZT samples (Figure 5.21) also was able to show inoculated organisms influenced AM fungal root arbuscules ($P < 0.00001$, df: 7,32, F value: 48.48, F critical: 2.31, single factor ANOVA). All inoculations to ZT soils, with the exception of *B. pumilis* alone and in combination, had implications towards AM fungal root cortical arbuscules ($P < 0.00001$, df: 8, t.stat: -9.58, paired equal variance T test), with *B. subtilis* having the greatest decrease in quantifiable arbuscules and *B. amyloliquefaciens* having the greatest degree of increase (1.67 fold increase).

Quantified glomalin at week 30 (Figure 5.22) between inoculants of each CT ($P < 0.00001$, df: 7,32, F value: 16.33, F critical: 2.31, single factor ANOVA) and ZT ($P < < 0.0001$, df: 7,32, F value: 5.09, F critical: 2.31, single factor ANOVA) soils showed greater variance than previously observed at week 15, with ZT soils producing an average 2.30 fold increase in soil glomalin concentrations compared to CT soils

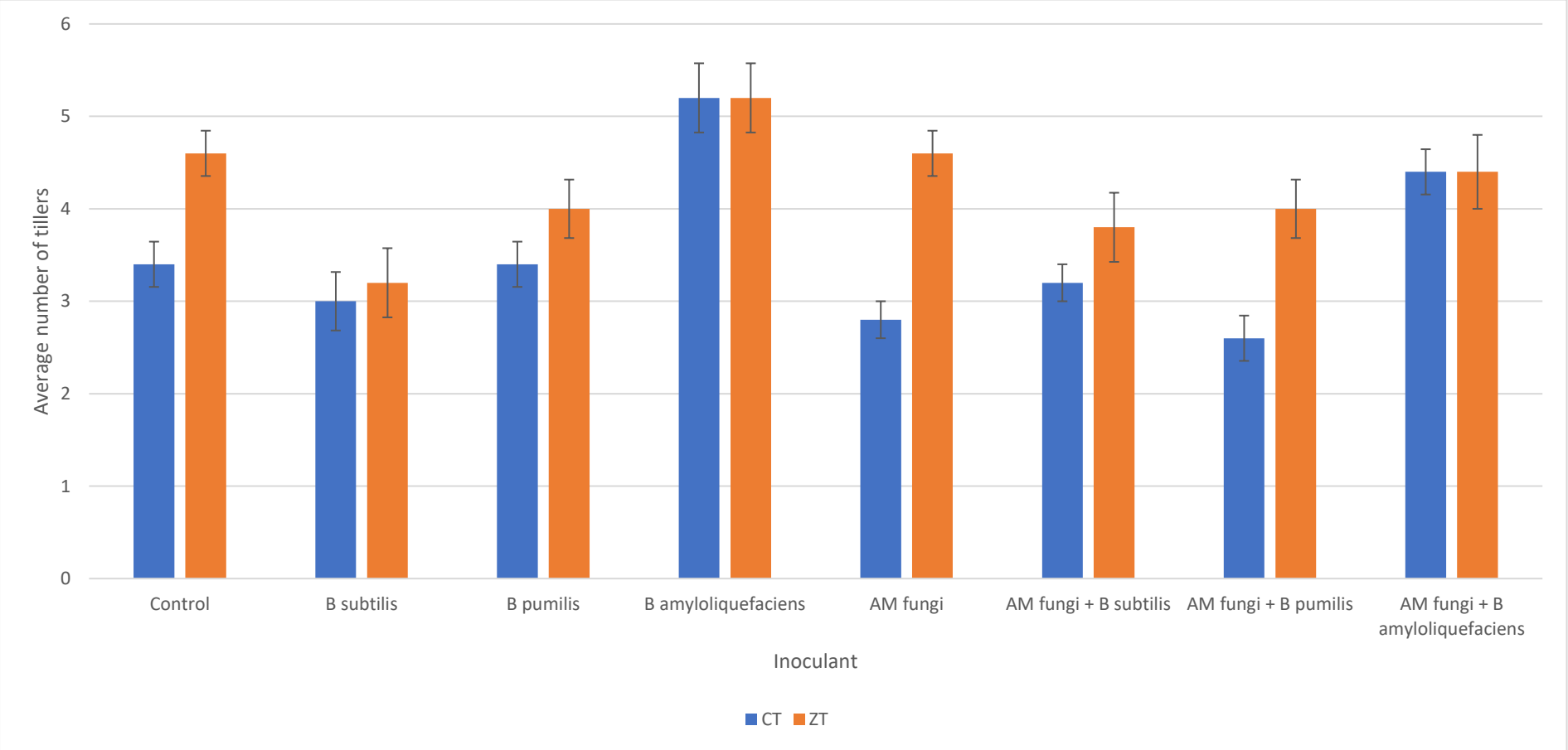

Figure 5.18 - Average (n = 80 per tillage treatment) number of tillers from glasshouse controlled growth of Zulu variety winter wheat at 30 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp. and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT ($P < 0.00001$) and ZT samples ($P < 0.01$). Error bars constructed from SEM.

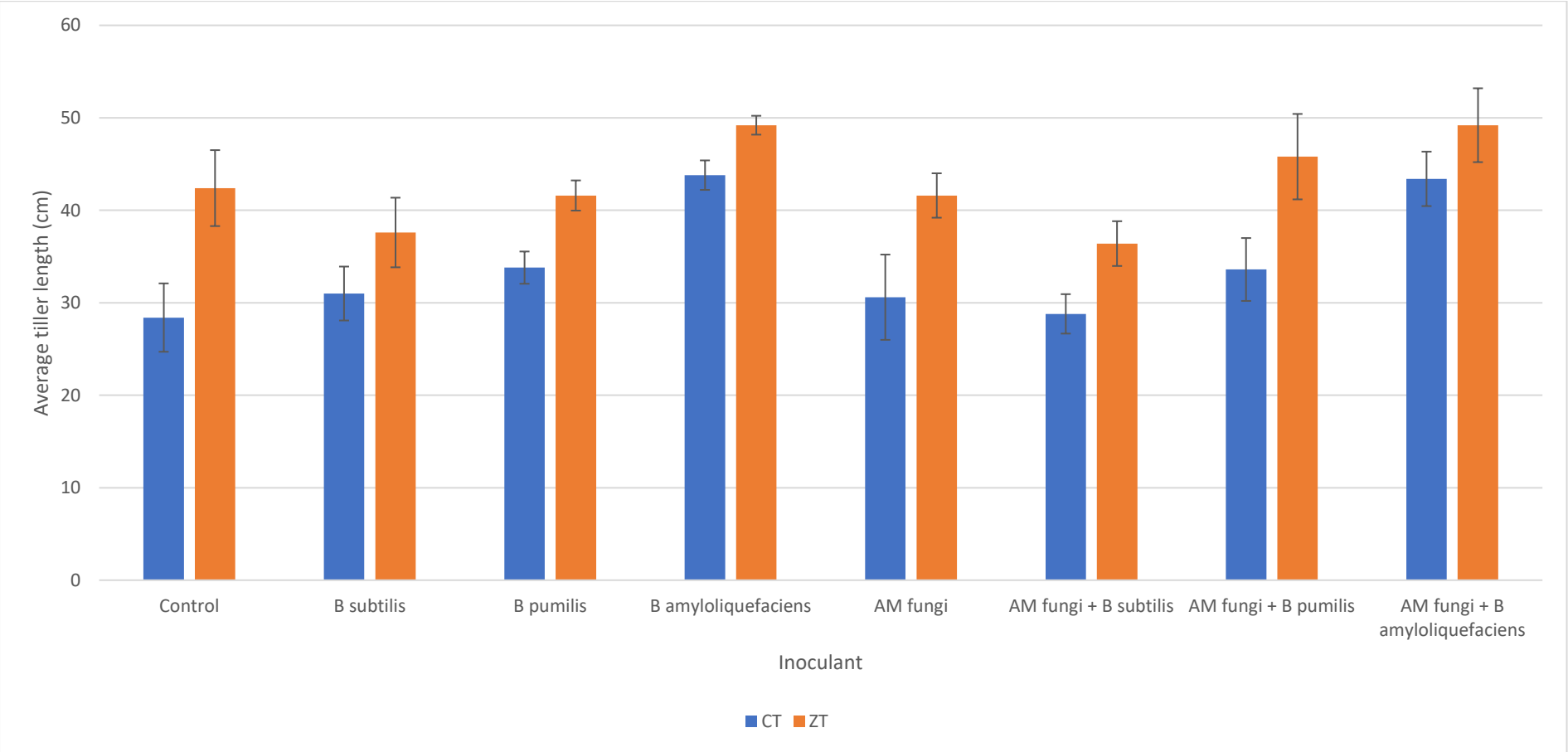

Figure 5.19 - Average (n = 80 per tillage treatment) tiller length from glasshouse controlled growth of Zulu variety winter wheat at 30 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp. and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT ($P < 0.002$) and ZT samples ($P = 0.05$). Error bars constructed from SEM.

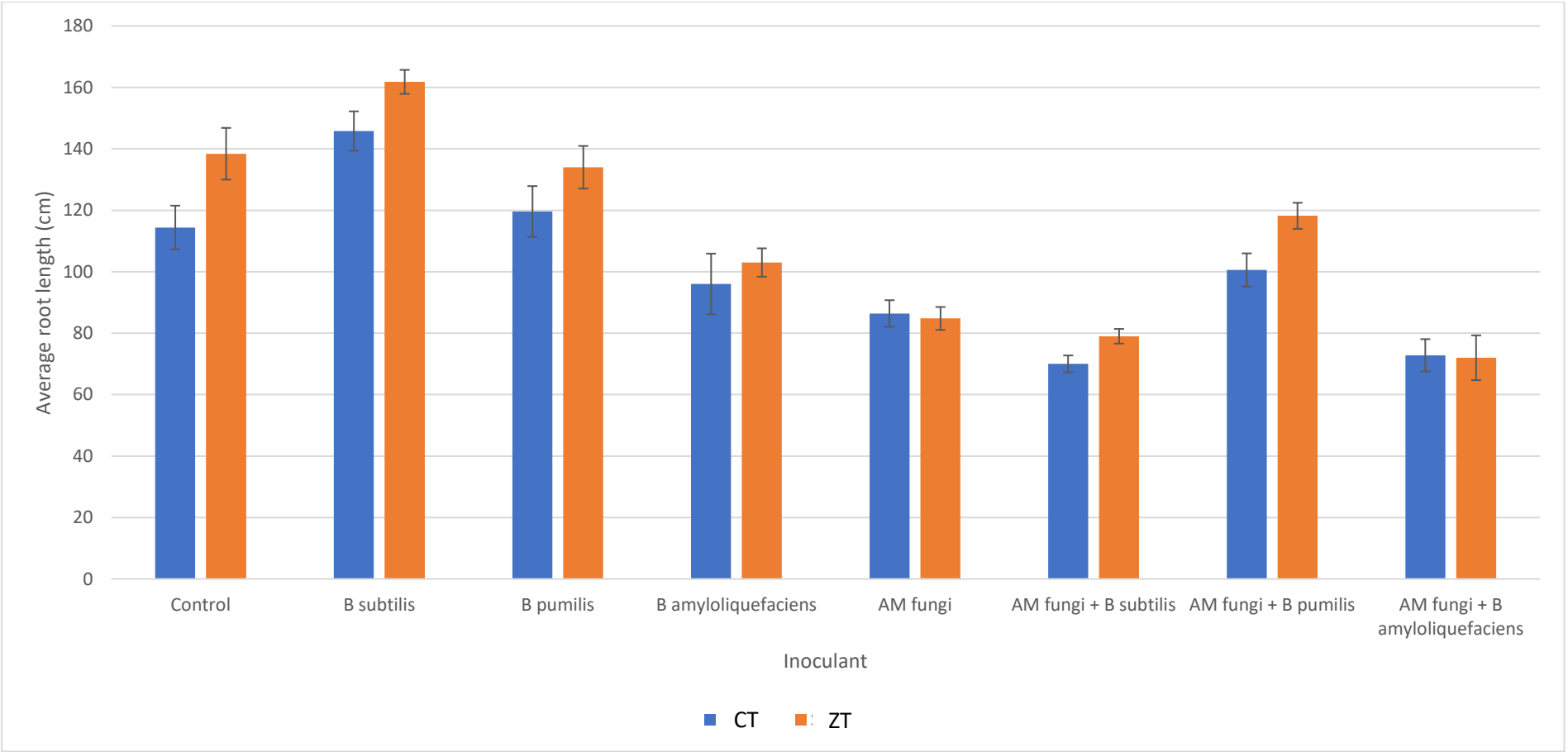

Figure 5.20 - Average (n = 80 per tillage treatment) root length from glasshouse controlled growth of Zulu variety winter wheat at 30 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp. and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT ($P < 0.00001$) and ZT samples ($P < 0.00001$). Error bars constructed from SEM.

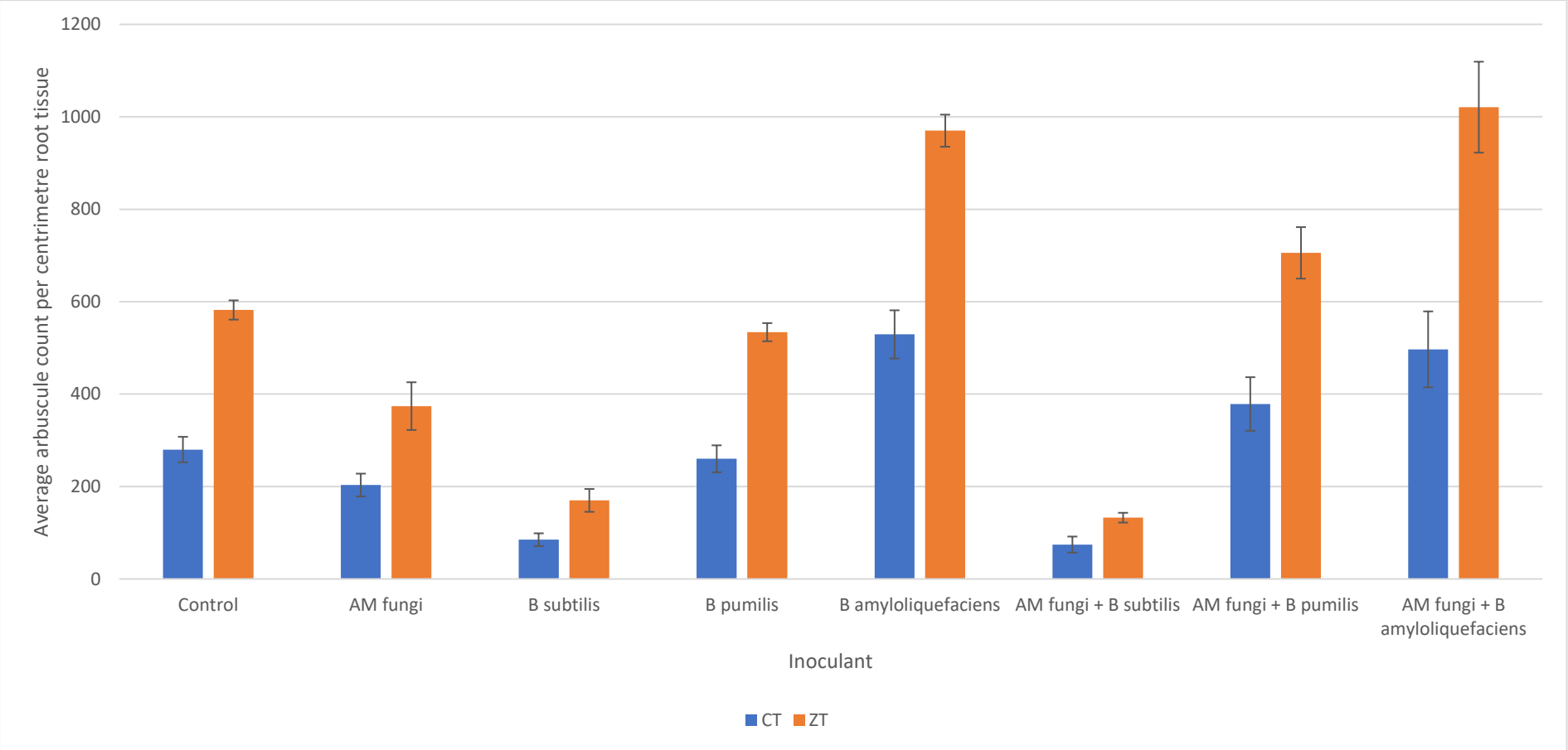

Figure 5.21 - Average (n = 80 per tillage treatment) root arbuscules from glasshouse controlled growth of Zulu variety winter wheat at 30 weeks post germination with their associated weekly select rhizosphere *Bacillus* spp. and AM fungal applications. Tiller numbers between microbial inoculum and control samples showed ANOVA significance for CT ($P < 0.00001$) and ZT samples ($P < 0.00001$). Error bars constructed from SEM.

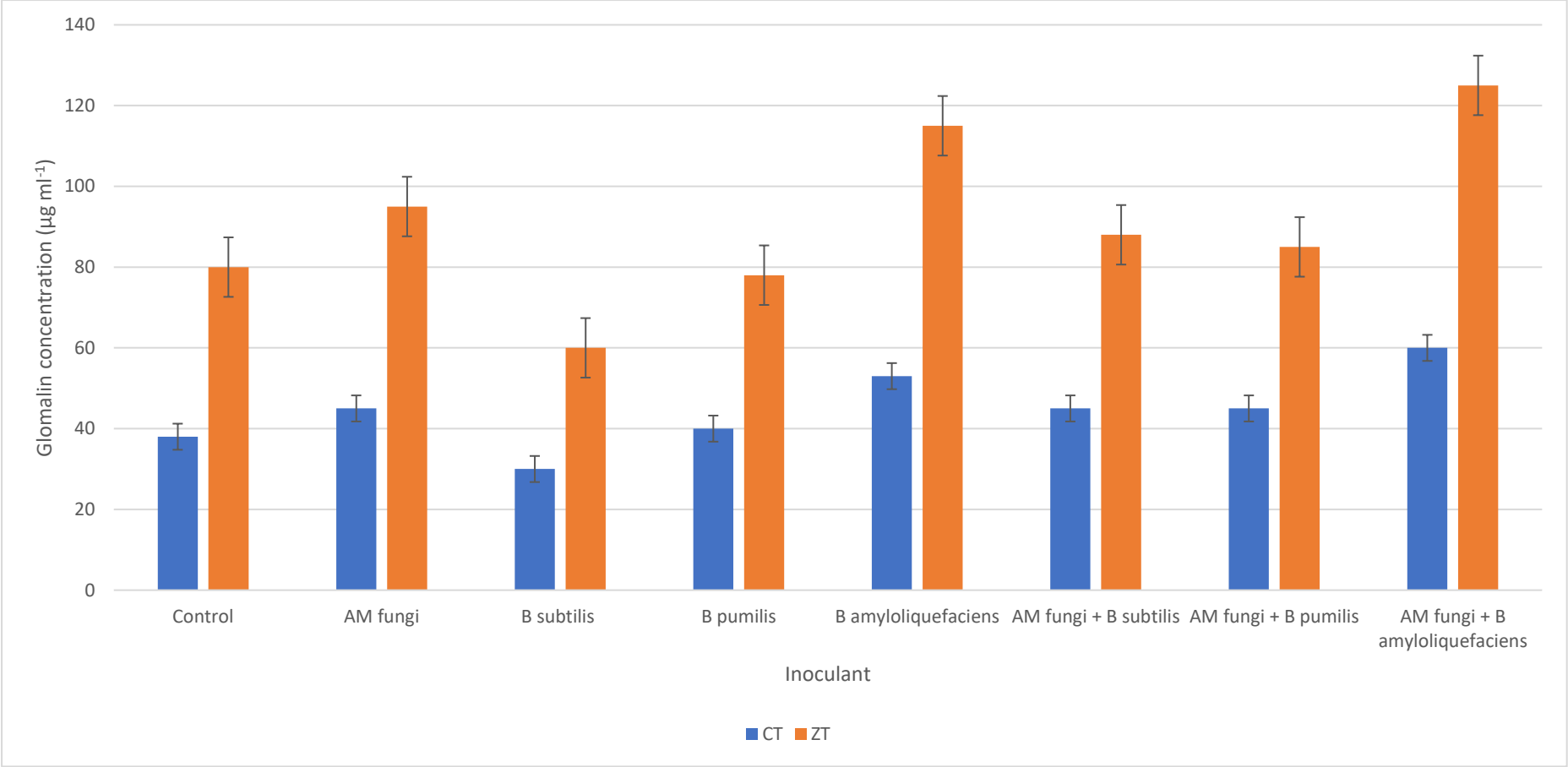

Figure 5.22 - Average (n = 80 per tillage treatment) glomalin concentration of field sampled soils from CT ($P < 0.00001$, ANOVA) and ZT ($P < 0.00001$, ANOVA) treatments with the growth of Zulu winter wheat grown under controlled glasshouse conditions. AM fungi (*R. intraradicies*) and select *Bacillus* spp. were applied to the soil surface on a weekly basis. Error bars constructed from SEM.

5.3.3.2.1 Correlated influences of selected *Bacillus* spp. and AM fungi on measured tiller and root lengths of wheat between CT and ZT field sampled soils under controlled growth conditions sampled at week 30

Corrleations between AM fungal root arbsucules and wheat biomass indicators such as rooth length, tiller length and tiller numbers, were seen to produce similar correlations and can be seen in Appendix 8.16.

Root length ($P < 0.0001$, df: 7,32, F value: 33.72, F critical: 2.31, single factor ANOVA) was neagtively correlated with arbuscule quantiifacations ($P < 0.00001$, df: 7,32, F value: 48.56, F critical: 2.31, single factor ANOVA) in both tillage sampled soils.

Positive correlations were seen between average arbuscular numbers and tiller length across all inoculants; however, no significance was noted ($P = 0.06$, df: 7,32, F value: 2.57, F critical: 2.31, single factor ANOVA).

5.3.4 qPCR identification of inoculum persistence of select rhizosphere bacteria and AM fungi inoculated soils from differently tilled sites.

qPCR analysis for the determination of inoculant persistence in inoculated soils of each tillage treatment was unable to show a presence of *B. subtilis, B. pumilus* and *R. intraradicies* (AM fungi). However, *B. amyloliquefaciens* was consistently present in samples for both weeks 15 (Figures 5.23 to 5.26) and 30 (Figures 5.27 to 5.30) as indicated by qPCR curves for the respected tillage treatments and inoculum combinations. Presence of *B. amyloliquefaciens*, from qPCR curves, is associated with the increased AM fungal arbuscules count (arbuscules per 1cm root section) in Figures 5.10 and 5.21 for inoculated soils in Sections 5.2.3.1 and 5.2.3.2.

qPCR of *B. amyloliquefaciens* inoculated Zulu wheat at week 15 grown in CT sampled soils under glasshouse house conditions, was not noted to have produced increases in the persistence of *B. amyloliquefaciens* between all inoculants ($P = 0.96$, df: 7, 472, F value: 0.27, F critical: 2.03, single factor ANOVA).

Wheat grown, at week 15, in ZT sampled produced saw a persistent presence of *B. amyloliquefaciens* between all inoculants ($P < 0.00001$, df: 7,472, F value: 28.16, F critical: 2.03, single factor ANOVA).

Zulu wheat at week 30, grown in CT sampled soils in glasshouse house conditions produced a persistent presence of *B. amyloliquefaciens*. However, no statistical differences were observed between inoculants ($P = 0.99$, df: 7, 472, F value: 0.18, F critical: 2.03, single factor ANOVA).

Wheat grown at week 30 (Figure 5.28) in ZT sampled produced a measurable presence of *B. amyloliquefaciens* in all samples between all inoculations ($P < 0.00001$, df: 7,472, F value: 5.32, F critical: 2.03, single factor ANOVA). Soils inoculated with *B. amyloliquefaciens* produced a reduced long term presence of *B. amyloliquefaciens* compared with other inoculants.

Table 5.4 – Copy numbers of *B.amyloloiquefaciens* qPCR samples taken from Zulu winter wheat grown in controlled glasshouse conditions at 15 and 30 weeks in soils taken from zero and conventional tillage sites. Copy numbers for *B.subtilis* , *B.pumilis* and AM fungi (as *R. intraradicies*) are not shown due to no copy numbers being detected in any of the produced samples.

Tillage treatment	Inoculant	Week 15	Week 30
Conventional tillage	Control	$10^{2.5}$	$10^{2.52}$
	B subtilis	$10^{2.52}$	$10^{2.49}$
	B pumilis	$10^{2.5}$	$10^{2.52}$
	B amyloliquefaciens	$10^{1.5}$	$10^{2.52}$
	AM fungi	$10^{2.5}$	$10^{2.52}$
	B subtilis + AM fungi	$10^{2.52}$	$10^{2.52}$
	B pumilis + AM fungi	$10^{2.5}$	$10^{2.49}$
	B amyloliquefaciens + AM fungi	$10^{2.52}$	$10^{2.52}$
Zero tillage	Control	0	0
	B subtilis	$10^{1.5}$	$10^{2.49}$
	B pumilis	0	$10^{2.5}$
	B amyloliquefaciens	0	$10^{1.5}$
	AM fungi	0	$10^{2.49}$
	B subtilis + AM fungi	0	$10^{2.52}$
	B pumilis + AM fungi	0	$10^{2.5}$
	B amyloliquefaciens + AM fungi	0	$10^{2.5}$

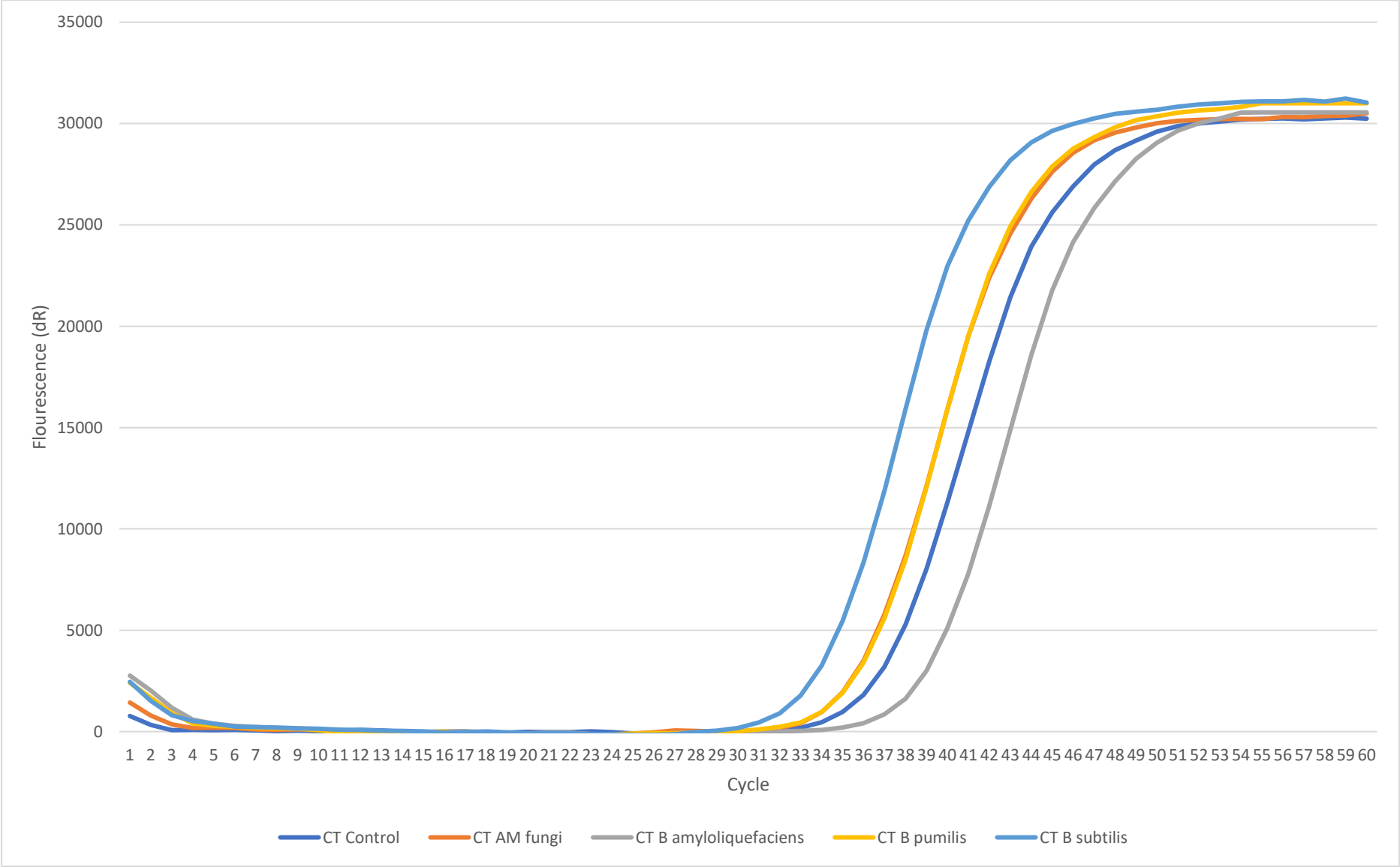

Figure 5.23 - Average (n = 15 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 15 of inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm).

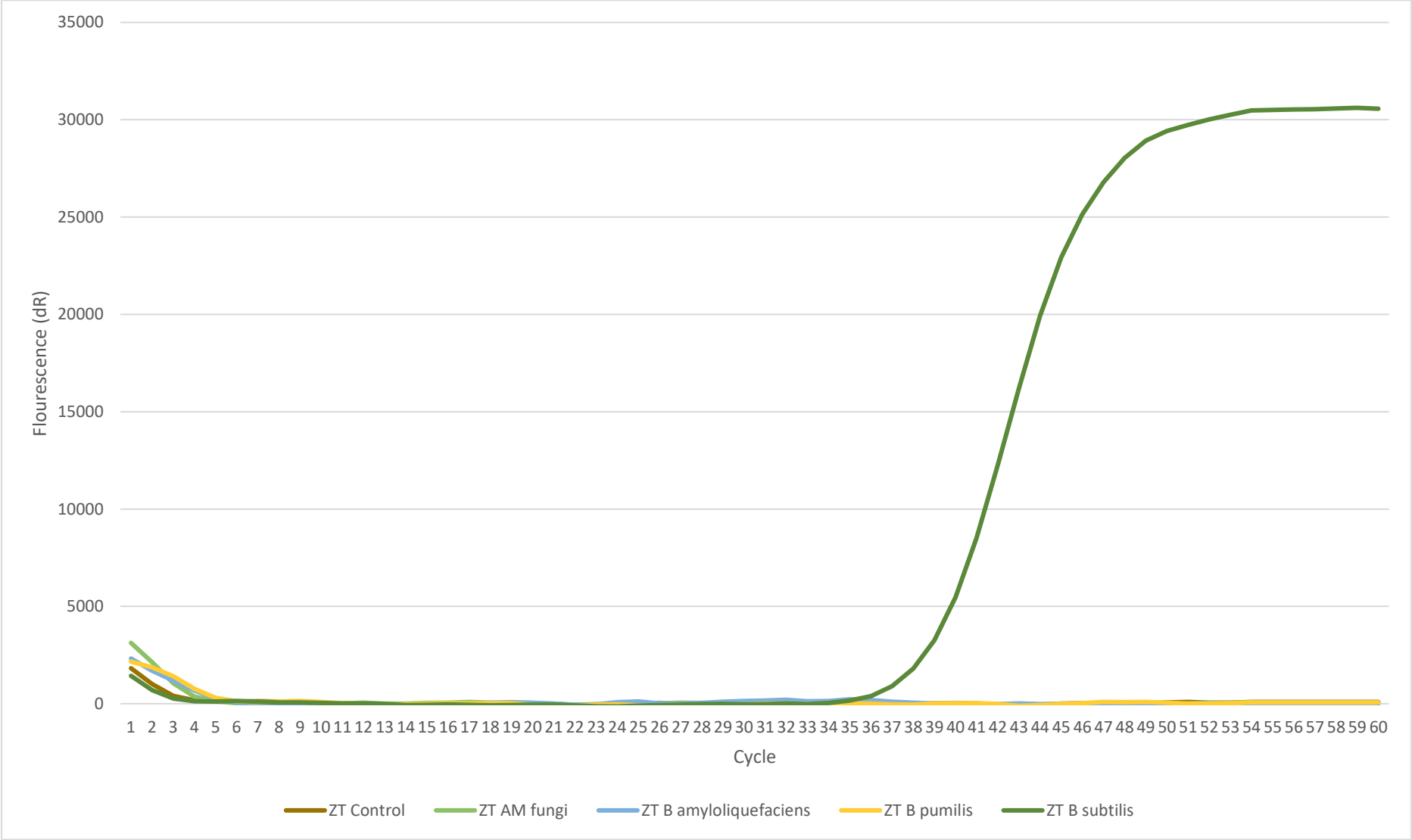

Figure 5.24 - Average (n = 15 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 15 of inoculated soils under glasshouse controlled conditions and wheat growth, sampled from ZT sites post harvest from top soils (<10cm).

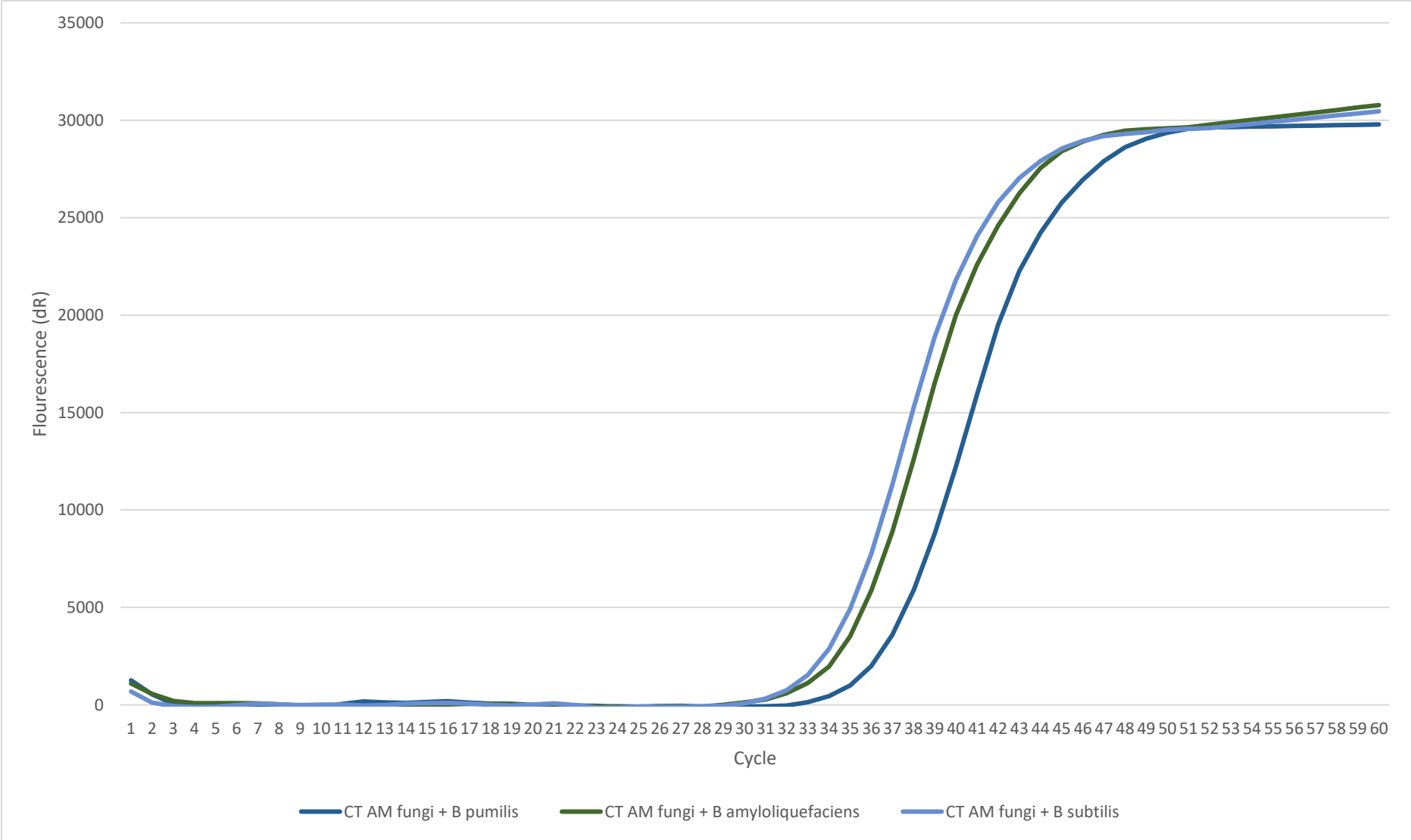

Figure 5.25 - Average (n = 9 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 15 of AM fungi (*R. intraradicies*) and *Bacillus* spp. inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm).

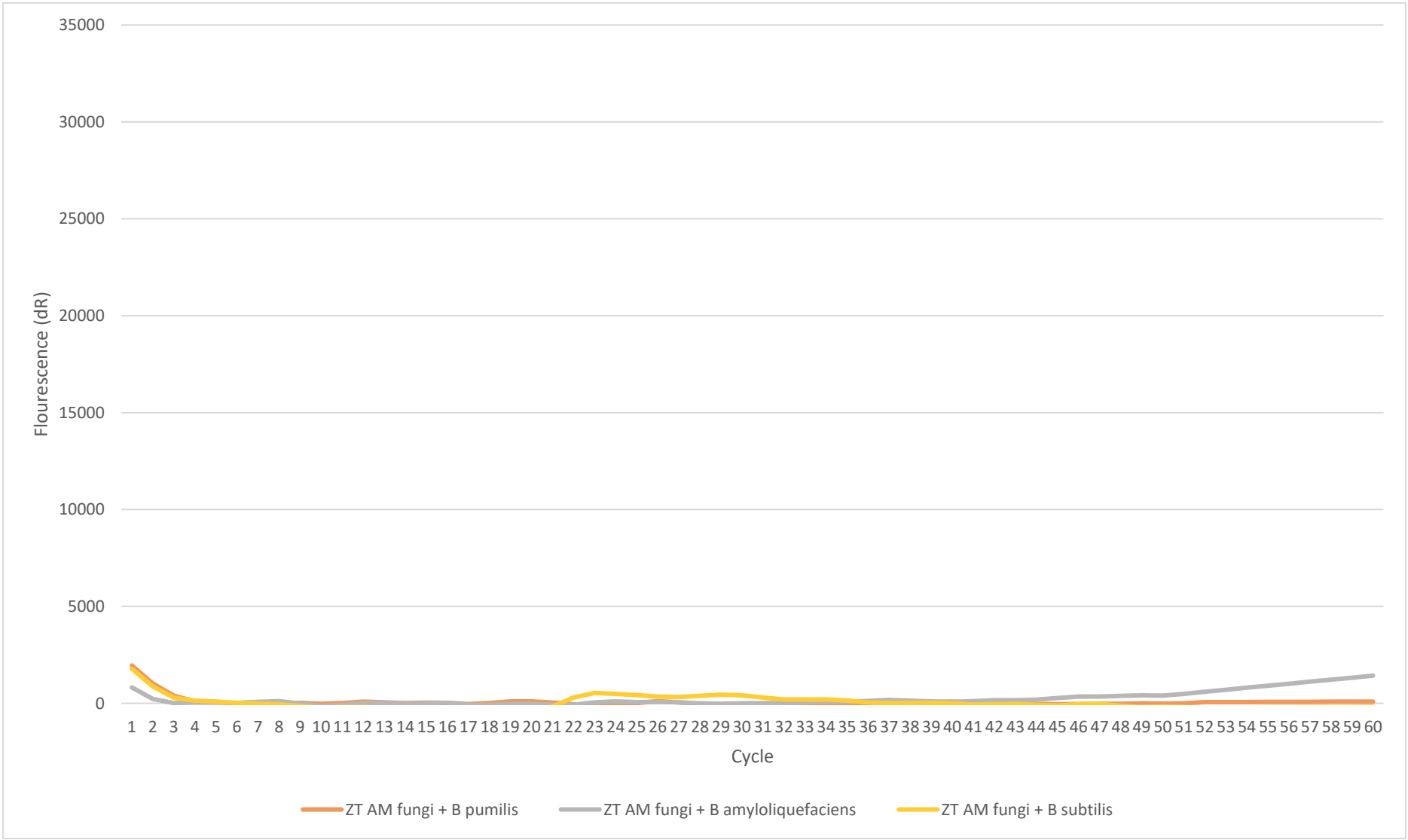

Figure 5.26 - Average (n = 9 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 15 of AM fungi (*R. intraradicies*) and *Bacillus* spp. inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm).

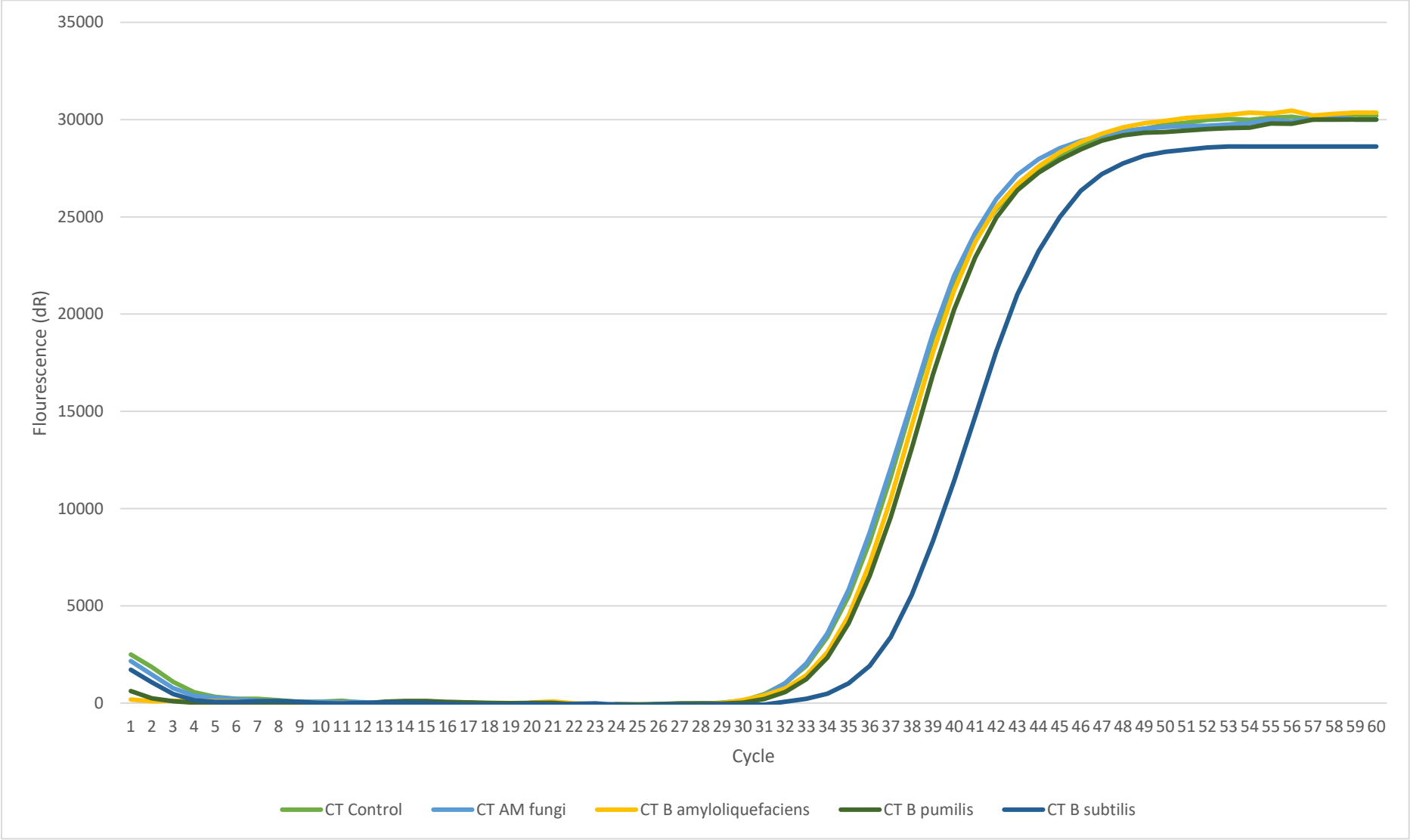

Figure 5.27 - Average (n = 15 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 30 of inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm).

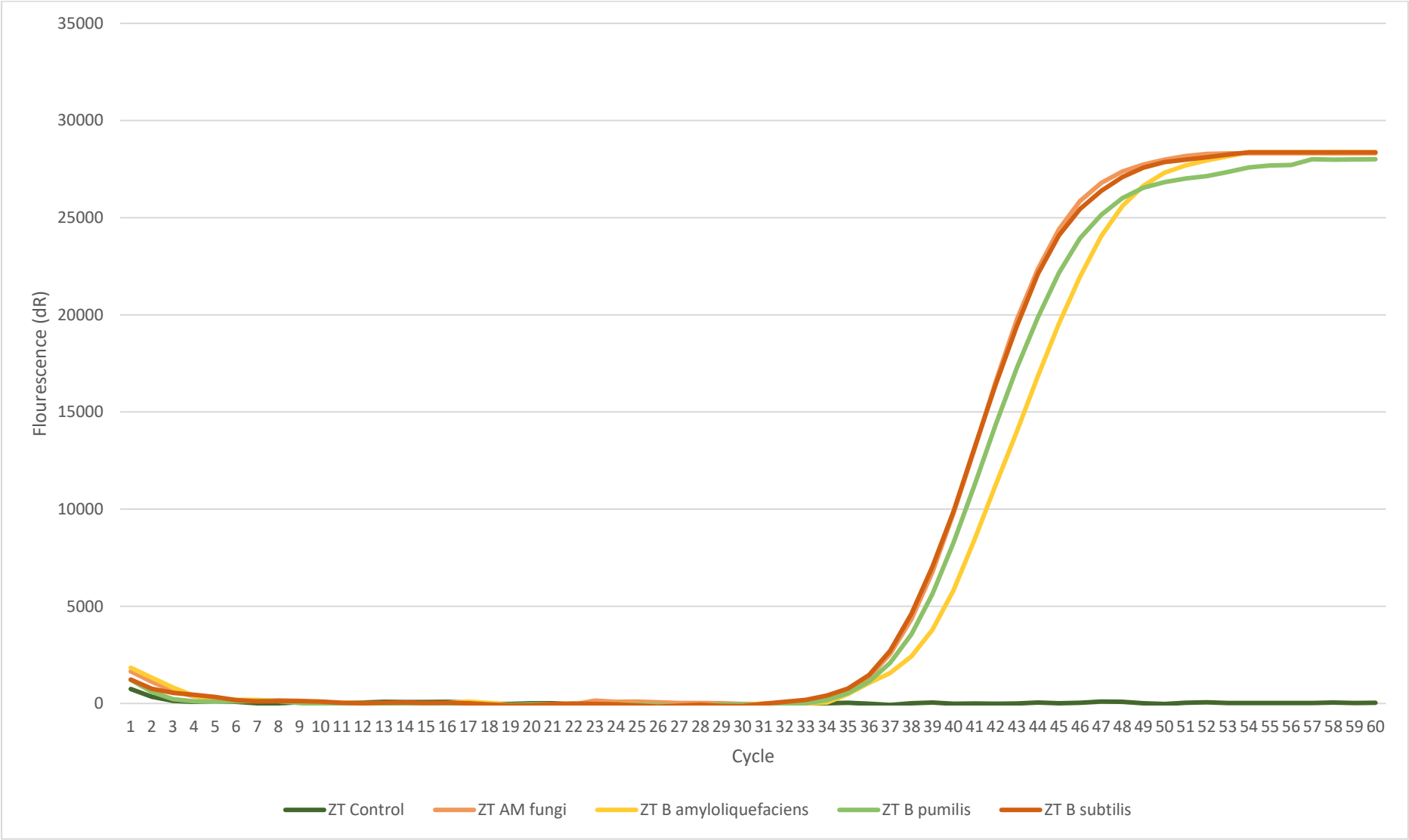

Figure 5.28 - Average (n = 15 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 30 of inoculated soils under glasshouse controlled conditions and wheat growth, sampled from ZT sites post harvest from top soils (<10cm).

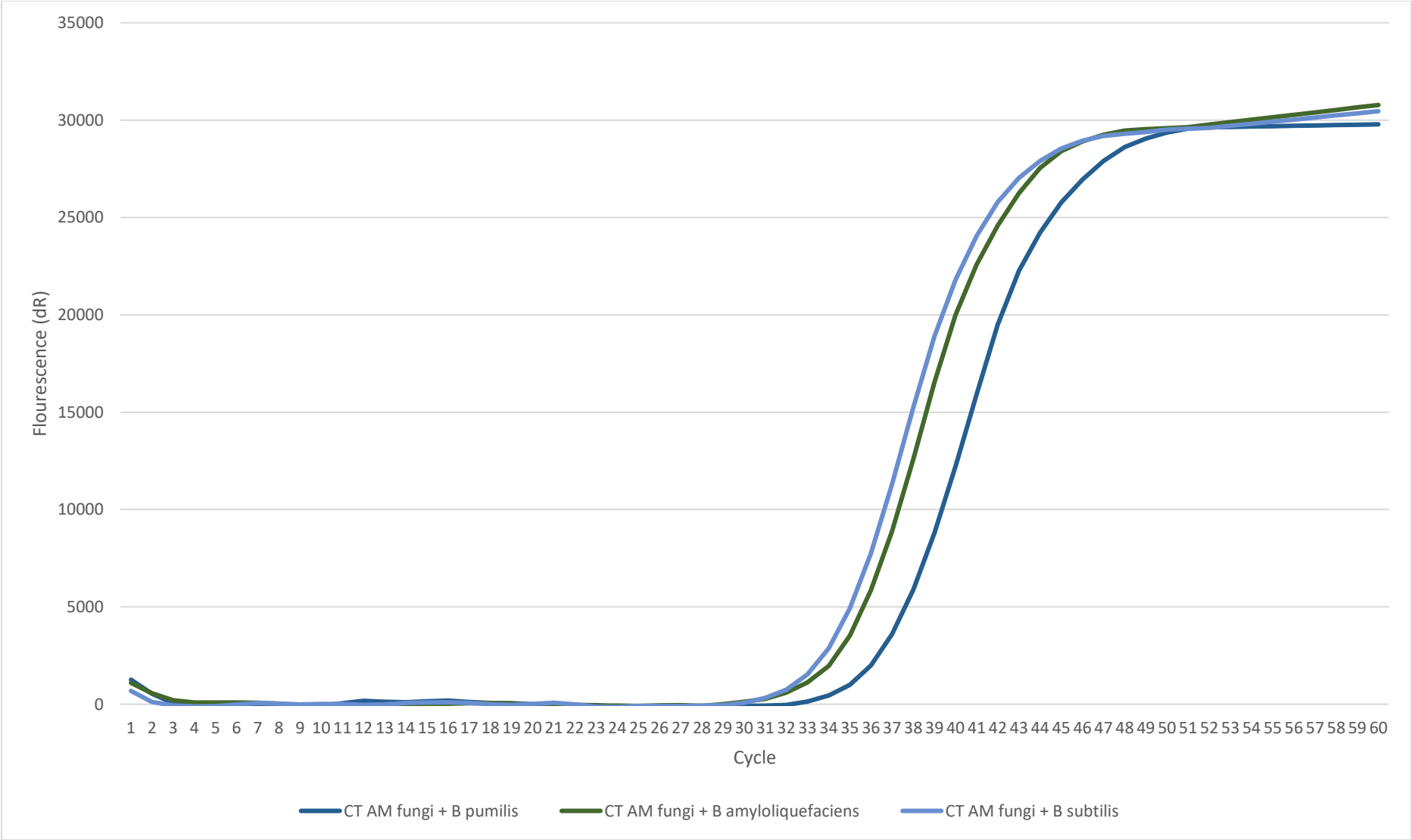

Figure 5.29 - Average (n = 9 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 30 of AM fungi (*R. intraradicies*) and *Bacillus* spp. inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm).

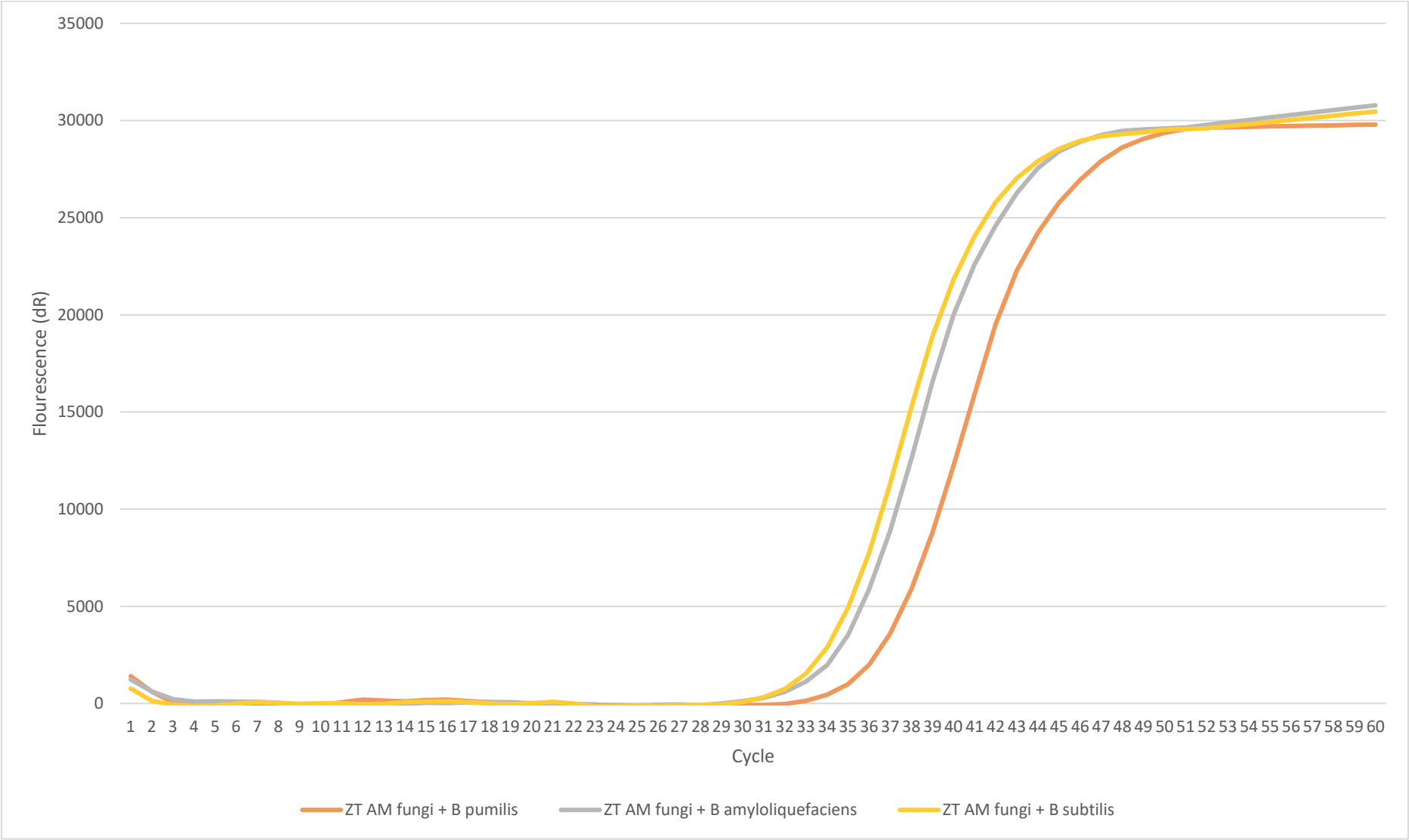

Figure 5.30 - Average (n = 9 overall) qPCR of TaqMan *B. amyloliquefaciens* at week 30 of AM fungi (*R. intraradicies*) and *Bacillus* spp. inoculated soils under glasshouse controlled conditions and wheat growth, sampled from CT sites post harvest from top soils (<10cm)

5.4 Discussion

5.4.1 Select *Bacillus* spp. on the influence of AM fungal on wheat roots and soil glomalin concentrations

The impact of zero and conventional tillage on the relationship between three species of rhizobacteria and AM fungi has been evaluated using two indicators of AM fungal growth: arbuscule number and quantity of soil glomalin. A further two indicators, wheat plant root length and tiller number, quantify potential secondary impacts on crop growth. Different responses are observed between AM fungi and each of the three rhizobacteria species. A positive relationship exists with *B. amyloliquefaciences*, as indicated by an increase in arbuscule count and glomalin production, and tiller number. The influence of *B. pumilis* appears negligible, while that of *B. subtilis* is suppressive to AM fungal growth. The species specific nature of the relationship between the three rhizobacteria and AM fungi was not impacted by tillage regime. These results have important implications for the supplementary application of rhizobacteria to soils for crop growth enhancement, especially using mixtures that contain all three rhizobacteria species.

The application of *B. amyloliquefaciences* increased soil glomalin concentrations over a six week period in both tillage treatments, CT and ZT. Glomalin was significantly higher ($P < 0.00001$) at week six compared with the initial samples in both treatments, with higher glomalin concentrations in the ZT treatment (111ug ml^{-1}) compared to CT soil (66ug ml^{-1}). Existing literature regarding the association of *B. amyloliquefaciences* and the production of soil glomalin from AM fungi is sparse. Glomalin is attributed to AM fungi (Franzluebbers et al. 2000; Lovelock et al. 2004; Driver et al. 2005; Janos et al. 2008; Bendini et al. 2009; Jai et al. 2018; Sharifi et al. 2018; Lambardo et al. 2019).Any increase in glomalin indicates an increase in AM fungal biomass since glomalin functions as a structural support molecule to facilitate the growth and penetration of fungal mycelia within soils (Driver et al., 2005). Xie et al. (2018) also report an increase in AM fungal symbiosis due to the presence of *B. amyloliquefaciences* using other plant species (*Lotus corniculatus*, *Thymus serpyllum* and *Trifolium repens)* instead of wheat. Whilst their study did not use soil glomalin as an indicator of AM fungal growth, their findings were comparable with *B. amyloliquefaciences* applications noted to increase AM fungal biomass due to its rhizobacterial MHB properties.

An increase in plant root exudation and communication between the plant and fungus was evident (Huang et al., 2014). The exact physiological pathway of this interaction is still unknown (Canarini et al., 2019), however, AM fungi are reported to benefit from the presence of *B. amyloliquefaciences* (Yuan et al., 2015). The significant increase of glomalin in soils from both tillage treatments reported in the current chapter further supports this conclusion. This chapter also identifies that the full benefit of *B. amyloliquefaciences* application is realised in combination with soil management conducive to the enhancement of AM fungi, such as ZT. A further indication of the positive effect on AM fungal growth attributed to *B. amyloliquefaciences* is the increase in the number of root arbuscules.

Arbuscules facilitate the transfer of soil nutrients acquired by AM fungal mycelia to the plant roots (Kobae et al., 2016), as well as further enhancing glomalin (Figure 5.5) due to increasing the fungal biomass. The greater proliferation of AM fungi root symbiotic structures increases the plant root surface area, allowing for less plant derived resources required for root growth and development to assimilate the same quantity of nutrients. Additional benefits are realised by the soil, including increased microaggregate stability and carbon sequestration (Ostle et al., 2009; Wilson et al., 2009). The direct effect of rhizobacteria on soil aggregate stability is unknown. An increase in plant biomass from the inoculation of soils with select *Bacilli* spp. is reported by Nanjundappa et al. (2019). Application of the Bonferroni factor to the data and *Bacilli* spp. reported in Figure 5.1 finds that *B. amyloliquefaciences* has the largest positive impact on vegetative plant growth as indicated by a significant increase in tiller number. Although *B. pumilis* is reported by Chakraborty et al. (2011) to have the potential to enhance plant growth, it was not the conclusion based on the data reported here. No interaction with AM fungi, positive or negative, was apparent. Further, although several authors report a positive relationship between rhizobacteria and AM fungi (Alam et al., 2011; Heinemeyer et al., 2019; Rahman et al., 2010) and its subsequent benefit on plant development, the relationship of *B. subtilis* with AM fungi appeared to be antagonistic.

Soil glomalin declined in both ZT and CT immediately after the first application of *B. subtilis* suggesting a rapid inhibition of AM fungal growth. A smaller number of quantifiable arbuscules were also noted. The quantity of soil glomalin was significantly lower after six

weeks in both treatments. The root length of the inoculated winter wheat plants increased (Figure 5.3), however there was no significant effect on tiller number (Figure 5.4). Many authors report the impact of rhizobacteria on plant growth only, they do not report on the nature of the interaction between the bacteria and AM fungi directly. Rahman et al. (2011) note that the simultaneous application of *B. subtilus* and AM fungi to basil (*Ocimum basilicum*) under artificially induced saline stress increased plant height, branch number, fresh and dry weight, percent oil content and yield. The influence of *B. subtilus* on AM fungal root colonisation is not considered, rendering it difficult to distinguish which species, the bacteria or the fungi, is having the dominant effect on plant growth. In response to the application of *B. subtilis* to marigold (*Tagetes erecta*), Flores et al. (2007) report a combination of improvements to cosmetic (flower colour enhancement) and selected plant growth characteristics (increased inflorescence number, flower fresh weight). The promotion of plant growth is also noted by Awasthi et al. (2011) for sweet wormwood (*Artemisia annua*), for rose geranium (*Pelargonium graveolens*) by Alam et al. (2011) and cucumber (*Cucumis sativus*) by Rabab (2014). Although root length increased in the current experiment, this is not necessarily indicative of a beneficial plant response. Since the decrease in glomalin indicates a decrease in AM fungi, the increase in root length is potentially a mechanism instigated by the plant to compensate for diminished AM fungal arbuscular density, as a measure of symbiosis, (as indicated by lower glomalin concentration and a decrease in arbuscule count) and a corresponding decline in soil nutrient assimilation efficiency. The wheat plant allocates greater resources to root development in order to compensate for the loss of nutrients derived from the symbiotic relationship with AM fungi. The increase in growth of one particular component of the plant does not, in this case, confer an associated beneficial impact to be attributed to the inoculation of the soil with a rhizobacteria. Requena et al. (1996) report enhanced mycorrhizal root colonisation of the legume *Anthyllis cytisoides* when naturally occurring rhizobacteria (*Rhizobium spp* and *Rhizobacterium*) were applied in combination with the AM fungi *G. intraradices.* The opposite effect was observed when applied with *G. coronatum* instead of *G. intraradices*. Inoculation with *G. coronatum* and *Rhizobium* only however, enhanced mycorrhizal root colonisation. The nature of the impact was related to individual species of AM fungi and species specific combinations of rhizobacteria. Requena et al. (1996) also comment on the variability of rhizobacteria behaviour as a function of host plant species and soil

environmental conditions. Xiao et al. (2007) offer a further explanation, that the reduction of glomalin and quantifiable arbuscules due to *B. subtilis* may be a result of its plant pathogenic fungal biocontrol properties that extend to selected beneficial AM fungi also.

Xiao et al. (2007) note a 20% reduction in maize (*Zea mays* L.) root colonisation by the AM fungus *G. etunicatum* in response to the application of *B. subtilis*. *Bacillus subtilis* is able to reduce fungal pathogens by producing volatile compounds and antifungal lipopeptides which act as growth inhibitors (Liu et al., 2005). This is however possibly strain specific (Xiao et al., 2007) which may in part explain the conflicting findings of other authors such as Rabab (2014). Data presented in Section 5.3 is additionally able to indicate that applications of *B. subtilis* reduced AM fungal root colonisation, falling in line with the explanations provided by Xiao et al. (2007) of fungal inhibition from *B. subtilis*. Requena et al. (1996) cite a further potential variable, the total rhizobacterial population, as a key factor. Where the number of rhizobacteria are high in a given volume of soil, the negative impact observed may be due to increased competitiveness with other components of the soil microbiota or due to a direct pathogenic effect as demonstrated by Xiao et al. (2007). Although the inhibition of fungal pathogens is beneficial to a developing crop, the inhibition of spore germination and hyphal development of AM fungi is a negative secondary impact. The decline in mean arbuscule count (Figure 5.1) lends further support to a fungal inhibitory mechanism due to *B. subtilis*, corroborating Liu et al. (2009) and Xiao et al. (2007). The method of soil management and the use of tillage does not appear to influence the magnitude of the negative effect of *B. subtillis* on AM fungi and soil glomalin concentration (Figures 5.5 and 5.6). In reference to the conclusions of Requena et al. (1996) if appropriate numbers of *B. subtillis* were present such that the antagonistic impact reported by Xiao et al. (2007) becomes aligned with the benefits reported in other studies (Alam et al., 2011; Awasthi et al., 2011; Flores et al., 2007; Rabab., 2014), opportunities to enhance its biocontrol potential while preserving the benefits of enhanced AM fungi colonisation may exist. Future work will be required to determine optimal numbers.

The species-specific nature of the rhizobacteria – AM fungi interaction has been highlighted by a number of authors including Thirkell et al. (2017) and Nanjundappa et al.

(2019). In a study of AM fungal community succession in agricultural land, Roy et al. (2017) using Illumina sequencing of ribosomal DNA (rDNA) identify species of AM fungi from seven families, mainly Glomeraceae, Diversisporaceae and Claroideoglomeraceae. They note a change in community composition in response to the time elapsed since the previous cultivation. By this rationale, the species composition of ZT and CT soils may also differ. Although the response to each of the three rhizobacteria species was similar, this suggests tillage induced differences to the select rhizobacteria was not necessarily the case. Future work would benefit from establishing precisely which species of AM fungi were present, and whether the interaction with the three select rhizobacteria species varies with different AM fungi, crop plant species or quantity of rhizobacteria present in the plant rhizosphere. If differences in AM fungi community structure and species are identified between tillage management practices, appropriate rhizobacteria species mixtures may be tailored to given combinations of agricultural management and crop plant species.

5.4.2 Influence of select rhizosphere *Bacillus* spp. and AM fungi on wheat growth and AM fungal root associations

Literature has described an increase to plant and crop biomass from application of select rhizosphere *Bacillus* spp. with specific reference to rhizobacteria species that are able to fix nitrogen, produce soluble phosphate and inhibit pathogens (Li et al. 2020). Hashem et al. (2019) review the influences of *Bacilli* spp., with particular reference to *Bacillus subtilis*, having antibiosis activities towards several organisms within a microbiome to aid in fungal pathogen suppression. Tilak et al. (2006) inoculated leguminous crops with *B. subtilis* and quantified root nodules from *Rhizobacter*. Tilak et al. (2006) produced experimental rationale that *B. subtilis* increased the amount of *Rhizobacter* and increased the quantity of root nodules. Further to this, Tilak et al. (2006) was also able to show an overall increase in nitrogen fixation indirectly from *B. subtilis*. Figures 5.7 and 5.8 indicate applications of select rhizosphere *Bacillus* spp., and AM fungi, have implications towards the number of wheat tillers and tiller length, a positive indicator of plant growth, after 15 weeks (of GS22 wheat growth stage at crop tillering (Lancashire et al., 1991))of select microbe inoculants, and again at week 30 (Figures 5.16 and 5.17). Between the tillage types, CT and ZT, *B. amyloliquefaciens* was noted to significantly ($P < 0.00001$) increase the number of tillers and tiller length compared to a non inoculated control sample. In contrast, *B. subtilis* was seen

to tiller number declined in both treatments although the decrease was less pronounced in CT soils. Zhang et al. (2014) demonstrated *B. subtilis* increased crop biomass when inoculating wheat. Root length (Figure 5.9) and tiller length (Figure 5.8) both produce support for the increases in crop biomass from *B. subtilis* inoculations when compared to a non inoculated control. This suggests that *B. subtilis* can be used to improve crop biomass but not increase crop yields through the reduction in tiller numbers. Overall, this may not be a beneficial inoculant. Later sampling at week 30, GS39 wheat growth stage with notable ear formation (Lancashire et al., 1991), increases to tiller length was reduced in ZT compared with the corresponding control whilst *B. subtilis* increases to crop biomass in CT were noted to be slightly greater when compared with a corresponding tillage control. Root length, however, continued to be longer than that of control samples. The data presented at week 15 and 30 sampling of Zulu wheat would lead thinking towards crop biomass increases in young and developing crops due to inoculation. Wheat from Figure 5.3 was grown in the same controlled condition with Zulu wheat also, however, Figure 5.3 allows the early development of wheat root length to be considered. Week 2 measurements of Figure 5.3 shows increases in root length compared with the control, non inoculated, samples. Data would suggest *B. subtilis* begins to exert biomass increases, especially to root length, within the first week of crop development and has a diminished influence towards week 30. Literature is currently not able to provide a timeline for the optimal periods in crop development for select rhizosphere *Bacillus* spp. or rhizobacteria applications. It stands to reason that further study should be undertaken to ascertain biomass alterations from time dependant inoculation of *B. subtilis*. This may additionally be highly specific to a crop species and variety. However, the current chapter provides data that is able to suggest greatest increase to tiller numbers from *B. amyloliquefaciens* inoculations to soils containing Zulu wheat is achieved by week 6 of weekly rhizobacterial inoculations.

In regards to the interactions between *B. subtilis* and AM fungi, Figure 5.10 shows the lowest number of intracellular AM fungal arbuscules from wheat inoculated with *B. subtilis*, with a corresponding increase in root length (Figure 5.9). This produces a reduced density of arbuscules which is further associated with reductions in AM fungal biomass. Soils taken from ZT treated soils produced increases in quantifiable arbuscules in comparison to CT soils. Whilst both soils, in glasshouse conditions, received the same inoculum of *B. subtilis*, the soils would have differing microbiomes from presence or absence of tillage (Lei

et al., 2018; Brito et al., 2012). As previously mentioned, the work by Xiao et al. (2007) used *B. subtilis* as a biocontrol agent towards fungal pathogens and noted a reduction in overall fungal biomass. This supports the data presented in figure 5.1. This potentially indicates that the increases in crop biomass through the reduction of symbiotically associated AM fungi, associated with the *B. subtilis* inoculations, are not as beneficial to crop development as previously thought. By negatively influencing the plant-AM fungal symbiotic relationship, nutrient acquisition is diminished and limited to the volume of soil the roots, and limited present AM fungi, have penetrated. To counter this, Radhakishnan et al. (2017) reviewed data and studies showing *B. subtilis* aids in the management of environmental stress and secretion of metabolites (aromatic, glutamic and aspartic amino acids) to promote plant growth and inhibit plant pathogen infection (allelochemicals). Further, indole-3-acetic acid, gibberellic acid and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production from *B. subtilis* facilitate the regulation of intracellular phytohormones to furthering the mediation of plant environmental stress (Glick, 2014). Figure 3.26 and 3.27, of Section 3.2, shows a consistent reduction in soil ergosterol in CT treated soils compared with ZT. Biocontrol properties of *B. subtilis* will have a greater impact in ZT soils due to the increase in AM fungi, incurring a greater reduction of viable AM fungi to establish host-fungal symbiosis. In regards to CT, the same effects to viable AM fungi from *B. subtilis* biocontrol are not seen to the same extent, this is due to the soil inversion induced reductions to fungal abundance, Figures 3.26 and 3.27 of Section 3.2. This would lead thinking towards a competition between AM fungi and *B. subtilis* within the rhizosphere, with specific competition for a host-microbe relationship in order to gain photosynthetic carbohydrates. To facilitate plant pathogenic mitigation, *B. subtilis* has been studied to produce a range of cell-wall-degrading substances including chitosanase, protease, cellulase, glucanase, lipopeptides and hydrogen cyanide (Elshakh et al., 2016). With this in mind, CT and ZT soils sampled and AM fungal arbuscules quantified in Figures 5.9, 5.10 and 5.20 are indicative of *B. subtilis* induced AM fungal inhibition. Continued study is required to ascertain the degree of soil physical and chemical characteristics, such as soil carbon and aggregate ability that AM fungi contribute towards via glomalin production and deposition, from the use of *B. subtilis* as either a biofertiliser or plant pathogen inhibitor.

Fertiliser applications can be mitigated as a contributing factor between both types of tilled soils as both farm sites utilised highly comparable types and quantities of fertiliser. *B. subtilis* has been studied for its biocontrol properties against fungal pathogens (Xiao et al. 2007, Lastochkina et al. 2019) with notable reductions from AM fungi abundance through *B. subtilis* production of bio-active substances. Lastochkina et al. (2019) reported *B. subtilis* as having a very strong competition with other constituents of the microbiome for resources and space, producing a strong community control influence. The data presented here also found that *B. subtilis* reduced AM fungal abundance significantly in both CT and ZT, as indicated by arbuscule count.

The interaction between *B. subtilis*, AM fungi and host plants are an example of a tripartite association (Nanjundappa et al. 2019). In contrast to *B. subtilis*, *B. amyloliquefaciens* produced a significant ($P < 0.00001$) increase in the number of tillers, especially from CT soils, and a 2.25 fold increase to quantifiable root cortical AM fungal arbuscules in CT soils at week 15 and a 1.56 fold increase in ZT samples at the same sampling time. Quantified arbuscules in wheat root grown in CT soils had the greatest increase in arbuscule numbers from incoculants due to lower numbers of arbuscules present in non inoculated control samples, suggesting a differing microbiome between tillage treatments (Berendsen et al., 2012). Additionally, lower density of root arbuscules in CT control samples contributed to the proportional increase in arbuscules compared with *B. amyloliquefaciens* inoculated samples. At week 30, CT arbuscules were noted to be 1.83 times higher than control samples, whilst ZT arbuscules were 1.66 times higher than controls. The lower proportional increase in ZT samples compared to CT samples is, in part, due to the increased number of arbuscule in ZT control samples compared with the *B. amyloliquefaciens* inoculated samples. The measured increases to AM fungal arbuscules continued to produce a negative correlation with root length, further showing AM fungal contributions to root surface area and reductions in plant resource expenditure for root growth and development.

Root length decreased in response to inoculation with *B. amyloliquefaciens* at both 15 and 30 weeks. Simultaneously, the density of root cortical arbuscules increased indicating AM fungal symbiosis was enhanced from *B. amyloliquefaciens* inoculations. This validates the notion further that AM fungi are contributing to root functions and reducing

plant expenditure of resources for root development. Continued evidence for this comes from an increased number of tillers (Figure 5.7) in both tillage treatments at weeks 15 and week 30. A positive relationship with *B. amyloliquefaciens* is reported in other studies. Xie et al. (2018) investigated the inoculation of serval plant species including, *Fragaria vesca, Lotus corniculatus and Viola tricolor*. Xie et al. (2018) produce data indicating the inoculation of each plant species from *B. amyloliquefaciens* resulted in the increase of AM fungal arbuscules by an average 11.6% compared to control samples. Whilst this result concurs with data presented in Figure 5.10 and 5.22, direct comparisons are difficult as the plant species studied are not the same. However, this may indicate that many species have similar responses to AM fungal inoculations. Xie et al. (2018) found an average 11.6% increase of arbuscules, whilst data from week 15 (Figure 5.10) produced an increase of 56.1% in root arbuscules in ZT soils and 122.4% increase in CT soils from *B. amyloliquefaciens* inoculmn alone. Combnied inoculmn of *B. amyloliquefaciens* and AM fungi produce arbuscular increases of 70.6% and 100.4% for ZT and CT respectively at week 15. Arbuscules increased at week 30 (Figure 5.20) at 66.8% and 89.0% for ZT and CT treatment respectively from *B. amyloliquefaciens* inoculum alone. Combined inoculants further indicated increases of 75.4% and 77.4% for ZT and CT respectively. Karuppiah et al. (2019) inoculated wheat with *B. amyloliquefaciens* and were able to present findings of increased BLR1/BLR2 and NADPH oxidase gene expression resulting in increases to overall crop biomass and metabolism, however, they did not report on the individual increases of each gene independently suggesting the reported study had limitations. Following the work of Karuppiah et al. (2019), improved gene regulation peri arbuscular membrane formation in wheat may be a key contributor to the increases in arbuscules relative to the control samples in this study.

Walley et al. (2013) explored the inoculations of soils with rhizobacteria to increase overall quantified glomalin in soils via interactions with AM fungi. As is shown in Figure 5.11 of week 15 samples, soils of a ZT origin consistently produced increased concentrations of glomalin compared with CT soils. This further shows the direct influence of tillage against a soil microbiome having detrimental implications. Walley et al. (2013) was able to provide experimental rationale, using wheat, that glomalin concentrations increased in the presence of a rhizobacterial-AM fungal interaction state. This supports the increase in glomalin observed in this study. Furthermore, the previously seen inoculations of *B.*

amyloliquefaciens having a positive interaction with AM fungal, and leading to the increase in above ground wheat biomass, was able to increase the amount of quantifiable glomalin in the sampled soils. This would suggest, along with the increases in root arbuscules, that a strong positive interaction between *B. amyloliquefaciens* and AM fungi can be advantageous to the growth and development of winter wheat. In contrast, *B. subtilis* was seen to reduce the overall concentration of soil glomalin, suggesting a negative interaction in the production of the glycoprotein. This conforms to the view of Cruz and Ishii (2012) that *B.subtilis* as a fungal biocontrol agent impacts negatively on AM Fungi . Following the work of Wallet et al. (2013) and their quantification of rhizobacterial-AM fungal associations in the attempt to sequester a greater amount of soil carbon, an area that is mentioned as requiring further investigation to ascertain a particular species of rhizobacteria that would assist carbon sequestration, the presented data in Figures 5.11 and 5.22 is able to indicate a greater degree of carbon may have been sequestered from the application of *B. amyloliquefaciens,* producing an increase of 1.84 in ZT soils compared with CT soils at week 15 and a 2.30 fold increase in ZT compared with CT at week 30. This implies a temporal aspect to MHB assisted carbon sequestration that has not been reported by previous literature. Further to Walley et al. (2013), the work of Padmavathi et al. (2015) additionally investigates the effects of rhizobacterial inoculation and AM fungal associations. However, Padmavathi et al. (2015) are not able to show a significant difference between inoculated and non inoculated samples in tomato and bell pepper crops, but able to show increases to overall crop biomass from rhizobacterial inoculations.

Bacillus pumilus applications to collected field soils under controlled glasshouse conditions were noted to have improved the average number of wheat tillers in both CT and ZT tillage treatments (Figure 5.7). However, upon post hoc T testing, *B. pumilus* was not measured as producing a statistically significant improvement to the average number of tillers. Bonferroni factors also indicated this upon analysis. Additionally, root length and AM fungal arbuscules did not produce significant improvements to plant growth when compared with a non inoculated control sample. Whilst no statistically significant of *B. pumilus* inoculations was observed in regards to tiller numbers and arbuscules, tiller length was significantly improved for both CT ($P < 0.01$) and ZT ($P < 0.001$) at week 15 providing an increase to above ground plant biomass. This is indicative of a positive relationship between

inoculum and wheat plant. A positive relationship was additionally noted by Yadev et al. (2016). Yadav et al. (2016) investigated co-inoculations of AM fungi, *Glomus* spp., and *B. pumilus* in a similar manner as described in Section 2.4.8. They found increased leaf size, plant height and biomass in *Ocimum basilium*.

The neutral effects of *B. pumilus* towards root length and development have been indicated by previous studies (Yadav et al. 2016, Nanjundappa et al. 2019, Ruiz-Herrer et al. 2015). Interestingly, co-inoculations of *B. pumilus* with an AM fungus have been reported to improve the levels of nitrogen fixation (Ruiz-Herrera et al., 2015) and suggested improved efficiency towards symbiotic relations with a host crop. Hernamdez et al. (2009) applied *B. pumilus* to arable soils in the investigation of nitrogen fixation and were able to show the contribution to soil nitrogen from *B. pumilus* in the fixation of nitrogen to ammonia. Joo et al. (2004) and Sari et al. (2007) explore plant growth promotion from *B. pumilus* in wheat finding plant biomass was increased. Sari et al. (2007) additionally found *B. pumilus* improved disease resistance to *Graeumannomyces gramminis* in wheat. With Mulvaney et al. (2009) describing the requirement to carefully match nitrogen fertilisers to the nitrogen requirements of cereal crops, the selective application of *B. pumilus* as a biofertiliser may be utilised to off-set the use of chemical fertilisers. Further to this, soils that have been shown to be readily depleted of nitrogen, such as sandy soils (Hernandez et al., 2009), could be inoculated with *B. pumilus*. This is an area that requires further study. ..

Co-inoculation treatment of AM fungi and *B. pumilus* at week 15 provide improvements to root arbuscules, indicative of AM fungi. Improvements were marginal when directly compared with *B. pumilus* inoculum alone in ZT soils. Greatest improvement came about from CT soils compared against non-inoculated samples and *B. pumilus* only inoculum. This may lend support to the notion that *B. pumilus* improves AM fungal nutrient exchange efficiency rather than direct plant benefits, however, this is still an area of study yet to provide definitive conclusions. Anuroopa and Bagyaraj (2017) produced data that would indicate a potential for the increase in nutrient exchange via AM fungi via the measurement of nitrogen in different plant parts using *Withania somnifera*, potentially justifying the notion that *B. pumilus* aids in the efficiency of nutrient exchange.

Positive correlations between tiller length and root arbuscules of single inoculants can be seen in the data presented in this study. Co-inoculations of AM fungi and *Bacilli* spp.

improved tiller numbers and maintained a positive relationship with root arbuscules having a significant increase ($P < 0.001$) compared to control samples in both tillage treatments. This was also the case for tiller numbers and root length. Differences in both wheat biomass and root arbuscules were significantly different ($P < 0.0001$) between tillage treatments.

Due to soil inversion, top soils in CT treatments are homogenised (Lombardo et al., 2019) and the microbiomes become very similar at depths towards the plough pan due to aeration (Hajabbasi et al., 2000). This creates more notable differences with ZT practices that are not aerated due to the absence of soil inversion. The diversity of the microbiome has been documented in the literature and is still under investigation (Sanders and Frossard, 2003; Wright et al., 2011; Kabir, 2005; Lombardo et al., 2019). Garbeva et al. (2003) explored the community abundance of *Bacilii* spp. under different arable land managements, permanent pasture and pasture-crop rotations, from top soil (<10cm) and reported PCR sequenced samples having an approximate 95% *Bacilli* spp. present of total community numbers. Garbeva et al. (2003) reported the predominatnt *Bacilli* spp. to be *B. pumilis, B. mycoides* and *B. megaterium*. Garbeva et al. (2003) did not report the tillage practices used between sample sites, but suggesting land used for long term arable production had increased numbers of detectable *Bacilli* spp. Wang et al. (2016) explored the differences in microbiome dynamics between CT and ZT practices in Northern China and was able to report that the distribution of *Bacilli* spp. was more uniform in CT managed soils, especially in zones of tillage. Wang et al. (2016) further identified the same abundance of *Bacilli* spp., 85% of total PCR sequences, in ZT as CT. However, the distribution of *Bacilli* spp. was less dilute and homogenous in ZT soils. Further, Hou et al. (2017) was able to demonstrate the presence and abundance of soil *Bacilli* spp. correlated with soil organic matter and demonstrated that *Bacilli* spp. are a component of the decomposition cycle. From this, CT and ZT soils utilised in the methodologies of Section 2.0 showed ZT soils were measured to have higher soil organic matter, Section 3.2. This may provide a potential explanation to the response in *Bacilli* spp. inoculations to CT sampled soils used for glasshouse experimentation and qPCR analysis. Reduced organic matter in CT soils could limit the abundance of soil *Bacilli* spp. and applied inoculation for data in Section 5.2. This may be a possible explanation to the differences observed in the presence of *B. amyloliquefaciens* in CT soils at Week 15 (Table 5.3) as opposed to ZT soils.

Win et al. (2019) explored the use of *B. pumilus* on rice crop yields and overall biomass. Whilst this study looked at the development of rice rather than wheat, other studies have noticed similarities in the established relationships between AM fungi, *B. pumilus* and a host plant (Yang et al., 2012; Chakraborty et al., 2011; Sari et al., 2007). Studies (Jiang et al., 2011; Elshakh et al., 2016; Win et al., 2019) have reported similarities in the function of *Bacilli* spp. in paddy fields and arable soil through proteolytic and saprophytic mechanisms. Win et al. (2019) applied *B. pumilus* as a biofertiliser and successfully quantified an increase in crop height, tiller length and nutrient uptake. Similar to that of Figures 5.7 and 5.8, *B. pumilus* increased the number of tillers in rice as well as their height compared with a control. Whilst AM fungal root colonisation and symbiosis was not the primary point of investigation by Win et al. (2019), they were able to comment on improved nutrient uptake transfer to a host crop. However, Masood et al. (2019) did not concur with AM fungal mediated nutrient transfer improvements during co-inoculants with *B. pumilus*. Masood et al. (2019) did however, show improved tillering and increased crop height from *B. pumilus* as a biofertiliser to rape seed. This further reinforces the notion that improvements to plant height and tiller numbers from *B. pumilus* inoculum is not species linked, and has wider applications.

The benefits of AM fungi in field sampled soils can be seen from Figures 5.14 to 5.16 at week 15 and Figures 5.21 to 5.23 at week 30 provides evidence of the effects AM fungi have towards growth and biomass of developing wheat. The negative correlations for both ZT and CT (Figure 5.14), related to root length, are clear indications that AM fungal symbiosis reduces the requirement of plant resource expenditure on root development. This is achieved through the development of a large surface area produced from AM fungal mycorrhizosphere for water and nutrient acquisition (Lombardo et al., 2019). Between Figures 5.13 and 5.16 shows an average range of arbuscules in CT (10 – 775) much reduced compared with ZT (27 – 1030). This can be used as an early indication that CT soils have a reduced abundance of AM fungi than ZT. As explored by Wang et al. (2017) and described in Section 3.2, physical manipulations of soil are likely the cause of the reduction in AM fungal abundance.

Stoian et al. (2019) studied mycorrhizal root development and concluded that AM fungal symbiosis with plant species reduced overall root length with a growing relationship with the symbiotic fungi, supporting similar findings in the present study. Zhang et al. (2019)

published a broad study utilising seven crop types (wheat, maize, rice, barley, sorghum, millet, oats) in England, China, India, North America, Australia and Central Asia. Zhang et al. (2019) produced data that came from either field inoculated or laboratory inoculated crop samples. Their results were comparable with data of AM fungal inoculation presented in Section 5.2.2 on field sampled soils that showed a significant ($P < 0.00001$) increase in tiller numbers in ZT treatment. A drawback of the study by Zhang et al. (2019) was not accounting for types of tillage. Paddy fields in China were cultivated via CT, whereas some regions in North America employed ZT. Tillage practice again differed in participating farms in England, practicing CT, and some reduced tillage equivalents in regions of Central Asia.

Saia et al. (2015) performed a similar experimental protocol to that in Section 2.4.8 with inoculations of AM fungi and select rhizosphere *Bacillus* spp. Saia et al. (2015) applied AM fungi (6 species of *Glomus* including a species now referred to as *R. intraradicies*) and rhizobacteria species (12 *Bacilli* spp. including *B. amyloliefaciens*) alone, and in combination, in field based investigations at 1.55g of inoculum per square metre over a total plot area of 7.6m by 6.0m in Mediterranean soils. AM fungal inoculants contained 25 spores per species per gram, whilst select rhizosphere *Bacillus* spp. inoculants consisted of one billion cfu per gram. The protocol in Section 2.4.8 applied a greater quantity of AM fungi (0.5g ml^{-1} at 2ml per 200g soil) and only a single species of AM fungi *R. intraradicies*. From the inoculation of AM fungi alone, Saia et al. (2015) reported increases to plant biomass above ground with reduced root length accompanied by increased root cortical arbuscules as an indication of AM fungal colonisation. This agrees with data presented in Section 5.2.2. However, Saia et al. (2015) assessed AM fungal colonisation via the employment of trypan blue. As described in a separate study by Wilkes et al. (2019), trypan blue is not a suitable stain for the assessment of root cortical symbiotic AM fungi and reduces overall quantification of arbuscules as the site of nutrient transfer. Sheaffer® blue ink to improve overall AM fungal arbuscular quantification. From this, Saia et al. (2015) may have been able to show more significantly AM fungal relationships with wheat in Mediterranean soils than first observed with trypan blue. Whilst data presented in Section 5.2.3 focuses on inoculations related to wheat biomass and AM fungal arbuscular densities as a marker of symbiosis, Saia et al. (2015) focused on wheat gene expression resultant of inoculations. qPCR analysis of phosphate transporter gene *Pht1* and *Pht2* increased by 86% and 49% respectively in

combined inoculation of AM fungi and rhizobacteria. AM fungal colonisation inducing, to an extent, the up regulation of *Pht1*, inserting a phosphate transporter channel in the peri arbuscular membrane, shown in Figure 1.3 (Section 1.2.1). Karandashov and Bucher (2005) utilised the increased regulation of *Pht1* as a potential marker to assess the level of AM fungal symbiosis. However, results were too variable to give an accurate depiction of the extent to which AM fungi had associated with a host plant. Karandashov and Bucher (2005) were able to indicate that rhizobacteria within the rhizosphere had additionally contributed to the increase in *Pht1* and *Pht2* gene expression. Saia et al. (2015) does not indicate which rhizobacteria were combined with AM fungi to produce this increase in phosphate transporter genes. With AM fungal inoculum alone, phosphate transporter genes expression was increased by 50%. (Saia et al. 2015). The authors do not present the proportional split between *Pht1* and *Pht2* genes.

The application of tillage alters the microbiome of soils (Wang et al., 2016) leading to a reduction in diversity in CT treated soils, whilst ZT treatments conserve the soil microbiome and maintain diversity (Wang et al., 2017). Piazza et al. (2019) demonstrated the recovery of the microbiome from a movement away from CT towards ZT and was able to conclude fungal diversity increased with a change in soil management. Castro-Sowinski et al. (2007) reviewed soil rhizobacteria inoculation and explored the community dynamics surrounding microbial abundance. Castro-Sowinski et al. (2007) produced conclusions that would suggest the reduced numbers of qPCR identified *B. amyloliquefaciens* in Week 15 CT soils inoculated with *B. amyloliquefaciens* (Table 5.3) is due to antimicrobial production from other constituent organisms in the microbiome as a biocontrol mechanism. This may also be a potential explanation for the lack of *B. amyloliquefaciens* identified in ZT soils at Week 15 (Figure 5.26 and Table 5.3), with the exception of ZT soils inoculated with *B. subtilis* that did identify *B. amyloliquefaciens* present in small numbers ($10^{1.5}$ qPCR copies). This is the same qPCR copy number for Week 15 CT soils inoculated with *B. amyloliquefaciens*. Such a similarity may reinforce the notion of antimicrobial biocontrol against *B. amyloliquefacines* when applied to soils in large quantities.

Reduced copy numbers in CT soils at Week 30 in samples, inoculated with *B. subtilis,* may be marginally reduced due to the biocontrol effects of *B. subtilis* as previously described. Xie et al. (2018) describes the relationship between AM fungi, with particular

reference to *R. intraradicies,* and *B. amyloliquefaciens*. Following from Xie et al. (2018), the reduced numbers of qPCR identified *B. amyloliquefaciens* from *B. subtilis* inoculations in Week 30 CT soils may be due to the reduction in AM fungi that was further seen in the reduction of root arbuscules (Figure 5.20). ZT soils at Week 30 began to show similarities to Week 15 CT inoculated soils. This delay in *B. amyloliquefaciens* numbers in the microbiome is likely due to the community dynamics and biocontrol mechanisms of a diverse microbiome Castro-Sowinski et al. (2007).

Data presented in Section 5.2 could be used to select microbe inoculation combinations for winter wheat. It is noteworthy that the data in Section 5.2 was produced using the Zulu variety of winter wheat and measured effects of any microbial inoculant combinations may require adaption for other varieties of wheat. qPCR analysis indicated *B. amyloliquefaciens* had a more persistent presence in both CT and ZT sampled glasshouse experimentation soils compared to that of the *B pumilis, B. subtilis* and AM fungi (*R. intraradicies*). However, CT soils maintained a consistent and persistent population of *B. amyloliquefacines* for both week 15 and 30 sampling compared with ZT soils that only indicated the presence of *B. amyloliquefaciens* at week 30. Week 15 of ZT, however, did not indicate a presence on any of the inoculated *Bacilli* spp. or AM fungi with the exception of wheat plants that were inoculated with *B. subtills* producing a detectable population of *B. amyloliquefaciens* whilst not producing detectable *B. subtilis.* Data in Section 5.2 indicates inoculants of *B. amyloliquefaciens* improved AM fungal symbiosis through the increase in root arbuscule density. In turn, increases in fungal arbuscules improves nutrient exchange between plant and fungus (Wang et al., 2017). While *B. amyloliquefaciens* was seen to reduce root length *B. subtilis* did the opposite and increased root length (Figures 5.3, 5.9 and 5.21). As previously discussed, the reduction of root length from *B. amyloliquefaciens* is accompanied by the increase in AM fungal arbuscules in stained root tissues. Week 15 produced negative correlations between root length and root arbuscules. A combination of AM fungi and *B. amyloliquefaciens*, or, to a lesser degree, *B. amyloliquefaciens* inoculation in isolation, confer the greatest benefit to plant growth. Furthermore, the data presented here indicates the combined inoculation of *B. amyloliquefaciens* and AM fungi is beneficial to both CT and ZT treated soils, but the greatest effects of increased AM fungal symbiosis, as quantified by root arbuscules, is in ZT managed soils.

6.0 Summary

Tillage is utilised in the preparation of land for the sowing and cultivation of a crop. Several methods of land preparation may be employed by land managers to achieve this, including conventional and zero tillage. Whilst CT has a larger requirement of agricultural machinery than ZT, CT practices have been well documented (Gai et al., 2015) to manage emergent weeds and burry crop debris without the need for additional chemical applications to soils. In the case of ZT managed soils, glyphosate is used to reduce weed emergence prior to the germination of a developing crop (Druille et al., 2013). Through the removal of emergent weeds and the preparation of a seed bed via CT, soils are homogenised and aerated to a degree that would not be seen in ZT managed soils as a non-invasive and reduced disturbance approach (Gai et al., 2015). Consequently, an advantage of CT managed soils comes from increased soil porosity, looser soils, and incorporation of organic matter such as solid fertiliser, e.g. manure (Moussa-Machraoui et al., 2010). However, this cannot be said for the employment of ZT managed soils. ZT practices have been studied to reduce soil erosion, soil compaction at depths corresponding to that of a plough pan in CT (AHDB, 2020) and maintain a higher degree of soil moisture from crop residue remaining (Bott et al., 2011).

CT practices have been reported to have profound implications and alterations towards the soil microbiome (Castro-Sowinski et al., 2007), whereas a more conservational tillage regime, such as ZT, has been reported to maintain an increased level of biodiversity and select species abundancies (Piazza et al., 2019). Part of such a maintained diversity does include plant pathogenic organisms such as *Fusarium* spp., *Tapesia* spp., *Alternaria* spp. and *Stemphylium* spp. in the case of wheat ear blight infections (Watkins 2004; Sari et al. 2007; Chalkley 2010; Zhang et al. 2014). The incubation effects of the remaining crop residue from ZT is not seen in CT due to the incorporation of crop residue into top soils (Ghimire et al., 2017). AM fungi form a group of soil dwelling organisms with plant and soil beneficial characteristics that are damaged by the employment of CT management. Many reported studies have focused on AM fungi within undisturbed soils and been able to show positive relationships between AM fungi and their environment, for example the increase to soil aggregation from the production of glomalin (Kobierski et al., 2018; Lui et al., 2018; Zheng et al., 2018). ZT soils are typically similar in respect to dramatically less soil disturbance (Busari et al., 2015) when compared with CT and fit within the reported relationships between AM fungi, glomalin and soil aggregation correlations. It is, however, the application of CT and

invasive disruptive soil management that has been less studied and reported by literature that requires investigation (Lu et al., 2018; Kabir, 2005; Galvez et al., 2001; Brito et al., 2012).

This study provided evidence that a migration away from the employment of an invasive management regime to one of less invasive and more sustainable regime is of interest to the maintenance of AM fungi, a key component of reduced soil degradation leading to soil stability and more sustainable crop production. Additionally, the adoption of a zero till practice results in the reduction of fuel usage and equipment requirements for the upkeep of a developing crop through the growing seasons (USDA, 2017). Furthermore, this study has provided evidence that various techniques, measurement of glomalin and ergosterol for example, relate to fungal biomass and can be used as easily quantified alternatives to measuring AM fungi directly. Current literature has not been able to provide evidence that glomalin can be used as a biomass indicator for AM fungi. Data presented in Chapter 3 is able to provide rationale for the implementation of glomalin as a biomass indicator of AM fungi, in conjunction with the quantification of ergosterol, building upon the work of Driver et al. (2005) that glomalin is a component of AM fungal mycelia as a structural support molecule. This study further provides evidence that there is a marked reduction of AM fungi in field soils managed through a CT regime in comparison to ZT. ZT managed soils have maintained an abundance of AM fungal biomass. This has further implications for soil carbon, aggregate stability and nutrient availability to developing crops.

Soil aggregation, a factor that strongly influences soil degradation and erosion, was seen to be diminished in CT soils with marginal recovery over the growing season. ZT soils, due to their less invasive management practices, were seen to maintain a higher degree of soil aggregation. Whilst the correlated relationship between glomalin and soil aggregation has been well established in undisturbed soils, the data presented here has been able to show the application of a CT management practice, compared to ZT, has reduced the correlated relationship between glomalin and soil aggregation. As such, this decreases the cohesive properties of the soil has resulted in increased soil degradation, a consequence of the increased effects of wind and watererosion (Vaidya et al., 2011). Over the sampling year, glomalin was quantified to a similar concentration in CT as that of ZT, however, soil aggregation was not seen to be increased. This may be due to a diminished ability for glomalin's adhesive properties to bind microaggregates further presenting another potential

implication invasive land management. Further evidence of CT induced damages to AM fungal produced glomalin can be seen from depth analysis of the soil aggregation and glomalin relationship indicated a positive correlation at soil depths below the zone of tillage (40cm). This supports the notation that the adoption and employment of an invasive land management practice, such as that of CT, damages the relationship between glomalin and soil aggregation.

Reports in the literature has previously been able to show soils managed under ZT have increased quantities of organic matter compared to CT managed soils (Curaqueo et al., 2010). Data presented in the Chapter 3 has been able to show glomalin's contribution to organic matter, being of greater contribution in ZT soils than CT soils. Whilst the ZT study site produced 4 distinct soil textures, all measured textures maintained greater organic matter and glomalin, as well as their relationships with soil aggregation, when directly compared with CT management practices.

Investigations of crop roots were additionally able to show the consequences of the reduction to AM fungi resultant of CT practices through the increase in root dry mass of sampled wheat. The increased range of root dry mass of wheat grown in CT soils indicates fewer AM fungal associations, as explored further in Chapter 4. Mycelia of AM fungi increase the surface area of host plant root systems (Gimsing et al., 2004), allowing the host plant to utilise resource expenditure on other areas of crop growth and development other than that of the root system (Schweinsberg-Mickan et al., 2010). This again was further studied and presented in Chapter 5.

In regard to the effects of tillage towards AM fungal-host symbiosis, the presented data draws conclusions indicating ZT managed soils have a consistently higher level of AM fungal associations as quantified via root cortical arbuscules.Root length, influenced by levels of AM fungal-host relationships, alter in the presence of differing nutrients in both a sterile environment and that of soils. This corresponds with previous reports (Armstrong and Peterson 2002; Bago et al. 2003; Balzergue et al. 2013). Furthermore, AM fungal arbuscules were seen to have an optimal quantity of applied nutrient for each potassium, phosphate and urea. The corresponding quantity of nutrients was seen to have produced

reduced root length, further supporting the previous notion that increases in AM fungal symbiosis reduces the plant's requirement to produce larger root systems as the AM fungal mycelia increase the overall surface area of host plant roots.

Investigations into root exudates were disappointingly unsuccessful with no exuded organic acid and/or simple carbohydrates detected from controlled cultivation of winter wheat in both sterile and soil growth media. The reasons for these difficulties are obscure but the literature suggested that this is through the diverse metabolic pathways that are constituents of the soil microbiome (Garcia et al., 2001) utilising the exudated compounds as primary metabolites (Badri and Vivanco, 2009).

Interestingly, sampled soils 12 months after the initial cultivation and seed sowing in September 2018, and before the following annual tillage and sowing applications, soils were able to produce an increase in AM fungal arbuscules when in the presence of winter wheat. This suggests that there is potential for CT managed soils to potentially recover and regenerate their AM fungal constituents in as little time as 12 months. This was not, however, the same levels of AM fungi overserved in ZT soils which was further increased in the absence of a glyphosate application.

The use of glyphosate in ZT land management practices was seen to have inhibitory effects on AM fungal biomass. When glyphosate was applied in a range (0 – 350 gL^{-1}) of concentration to CT and ZT top soils, similarities were noted in recovery time from the initial application of glyphosate. This confirms the notion that glyphosate is inhibitory to AM fungi, and the consequences of this should be studied further. However, from the data presented, glyphosate inhibited AM fungi could have consequential implications for the production of glomalin, sequestration of soil carbon and aggregate stability whilst also having reduced mycelial mass, leading to lower amounts of macroaggregate formation and increasing soil erosion. This suggests that ZT land management practices may not be as conservational as first thought.

Controlled growth experimentation of Zulu variety winter wheat, grown in purchased soils, were inoculated with *Bacillus subtilis*, *B. pumilis* or *B. amyloliquefaciens* provided clear evidence that *B. subtilis* had negative effects on the establishment of AM fungal symbiosis with host wheat producing a decrease in quantifiable root arbuscules and

an increase in root length. This, again, further supports the notion that increases in AM fungal root arbuscules decrease the total root length of the host plant by assisting in the total surface area of root systems via branching fungal mycelia. Furthermore, *B. subtilis* was associated with an overall reduction in soil glomalin, which continues to indicate the overall reduction of AM fungal biomass. In contrast to *B. subtilis*, *B. amyloloiquefaciens* was seen to produce an increase to AM fungal root structures, reduce root length and increase the quantified amount of soil glomalin. Soils inoculated for the assessment of glomalin quantities from the interactions of AM fungi and inoculated select *Bacillus* sp. rhizobacteria were sampled from each type of tillage sample site. Similarities in the trend of glomalin concentrations from inoculated bacteria were noted for each species of inoculant. Differences, however, were seen between soils from each tillage treated sites indicating the applications of tillage, or the mitigation there of, influences the overall soil microbiome (Busari et al., 2015; Curaqueo et al., 2011). Such a difference in the initial soil samples additionally produced variance in the interactions between inoculant and AM fungi, as well as the inoculation of further AM fungi. *B. pumilis* was noted to have neutral effects towards AM fungi and their association with a host plant when compared against control samples.

The growth of Zulu wheat in sampled soils from each of the two tillage types were produced for a period of 30 weeks and were able to show interactions between select *Bacillus* spp. rhizobacteria, AM fungi and host plant. Experimental data reflects that of the controlled growth in purchased soils, indicating that *B. subtilis* reduced AM fungal root structures and thereby AM fungi associations with the host crop. Combination inoculants were further able to show the greater interactions between rhizobacteria-AM fungi-host crop. The ratio of root to shoot was able to present the implications of overall crop biomass alterations from rhizobacteria-AM fungi interaction. The employment of qPCR determined *B. amyloliquefaciens* remained in inoculated soils for greater periods compared with other inoculants, implying a temporal relationship between inoculant, AM fungi and host crop.

The data provided in this thesis suggest

(1) that the correlation between glomalin and WSA is maintained under ZT managed soils and diminished under employed CT soil treatments

(2) the zone of tillage is more susceptible to the reduction in quantified glomalin, WSA associations

(3) AM fungal biomass, as measured via ergosterol, is reduced under employed CT treated soils

(4) glomalin has greater contributions to organic matter in ZT managed soils compared with CT soils

(5) fungal abundance in ZT soils produces shorter wheat roots coupled with an increase in quantifiable AM fungal root structures

(6) inoculations of select rhizobacteria to soils of different tillages elicits differing biomass alterations to winter wheat

(7) *B. amyloliquefaciens* produces greatest increase to AM fungal abundance, as measured via intracellular root structures, and results in reduced root length of wheat,

(8) glyphosate applied as part of ZT regime can have a detrimental implications towards AM fungi as CT management systems

(9) the usage of glomalin and ergosterol for quantification of soil AM fungal abundance as a substitution technique for other fungal quantification methodologies.

Further investigations are merited for the understanding of the temporal delay between applications of tillage in the damage of AM fungal mycelia and the increase in soil glomalin quantified later in the sampling year. This may suggest a progressive loss in AM fungal biomass, glomalin and soil aggregation rather than a simultaneous destruction of their established abundance and relationships as depicted by literature. Also, the implications of glyphosate towards the inhibition of AM fungi and established host symbiosis requires deeper investigation as the presented data in the present study provides early indications that applications of glyphosate may be as damaging to AM fungi as invasive soil management.

7.0 References

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8.0 Appendices

8.1 HPLC setup

HPLC equipment set up used for both organic acid root exudate and ergosterol analysis

Table 8.1 – Equipment and programs used for HPLC set up

SYSTEM	MANUFACTURER	ITEM	MODEL NAME/NUMBER	PART NUMBER	SERIAL NUMBER
Shimadzu HPLC	Shimadzu	Reservoir Tray	Prominence	228-45041-91	L20305035075 SL
	Shimadzu	Degassing Unit	Prominence DGU-20A5R	228-45019-43	L20705061245 IX
	Shimadzu	Pump	Prominence LC-20AD	228-45000-28	L20105177262 US
	Shimadzu	Autosampler	Prominence SIL-20AHT	228-4519-38	L20345170452 US
	Shimadzu	UV/Vis Detector	Prominence SPD-20A	228-45003-28	L20135174569 US
	Shimadzu	Column Oven	Prominence CTO-20AC	228-45010-28	L20215175319 US
	Fujitsu	PC	MI4W-D2990	ABN:K1017-V300-1900	YLCN112624
	BenQ	LCD Monitor	GL2250-T	9H.LA2LA.TPU	ETX3D07086019

8.2 Phosphate solutions

Extraction solution

- Weigh 3g ammonium sulphate and dissolve in 500ml deionised water, whilst slowly adding 20ml concentrated sulphuric acid
- Allow solution to call and add deionised water up to a litre volume total

Complex solution

- Weigh 10g ammonium molybdate and dissolve in 500ml deionised water, whilst slowing adding 240ml concentrated sulphuric acid
- Allow the solution to cool and add deionised water up to a litre volume total

Standard curve construction

- Known concentrations of phosphate solution were produced (3, 6, 10, 12, 15, 20, 40, 80, 100, 150, 300ppm)
- 10ml of each known concentration was added to 20ml deionised water and 2ml complex solution
- To each concentration, a spatula of ascorbic acid crystals was added to be in excess
- Samples were heated to boiling over a Bunsen burner, allowed to cool and read via photospectrometry at an absorbance of 650nm

8.3 Potassium solutions

Extraction solutions

- Weigh 15.4g calcium lactate and 7.9g calcium acetate and dissolve in 500ml deionised water whist heating – do not exceed 50°C
- Slowly add 17.9ml glacial acetic acid whilst stirring
- Allow the solution to cool and make up to 1 litre by the addition of deionised water and allow to stir, through magnetic means, for 20 minutes to ensure adequate homogenisation

Standard curve construction

- Known concentrations of potassium chloride solution (1, 5, 10, 50, 75, 100, 150ppm) were produced
- 3ml of each known solution was added to 3ml of 2:1 EDTA and formaldehyde solution with the addition of 1ml 5% w/v sodium tetraphenylborate, and vortexed
- Samples were analysed at 690nm absorbance, using blank extraction solution a reference
- A standard curve was constructed using spectrographic readings and known sample concentrations

8.4 Freeze drying process

Freeze drier equipment and procedure for the preparation of soils for ergosterol analysis.

Equipment:

- MechaTech Systems LSB40 Single Shelf Freeze Dryer Chamber (S/N: F001)
- Edwards RV5 Vacuum Pump (S/N: 099405319)
- Thermo Scientific MicroModulyo Freeze Dryer (S/N: O24U-423963-OU)

Program:
The freeze dryer was run using the following program:

Table 8.2 – Temperature cycling performed for freeze drying process

	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5
Starting Temperature	20°C	-40°C	-30°C	-10°C	20°C
Target Temperature	-40°C	-30°C	-10°C	20°C	20°C
Rate of Temperature Change	1°C/min	1°C/min	1°C/min	1°C/min	None
Vacuum Valve	Closed	Open	Open	Open	Closed
Dwell Time	60 mins	300 mins	300 mins	420 mins	60 mins
Total Segment Time	120 mins	310 mins	320 mins	450 mins	60 mins

Total Freeze Drying Time: 1260 minutes (21 hours).

8.5 Growth media recipe

Nutrient broth (Breckland Scientific) comprised of constituent component as given by Table 8.3. Nutrient agar (Breckland Scientific) comprised of the same constituents with the addition of 15g agar powder.

Table 8.3 – Composition of nutrient broth used for the culturing of *Bacilli* spp. rhizobacteria and fungal isolates as provided by Breckland Scientific.

Ingredients	**Quantity (g/L)**
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
'Lab-Lemco' powder	1.0

8.6 Meteorological data

Soil temperatures were provided from Rothamstead Research and summarised for soil temperatures under grassland (Figure 8.1), temperature under bare soils (Figure 8.2) and for min/max air temperature.

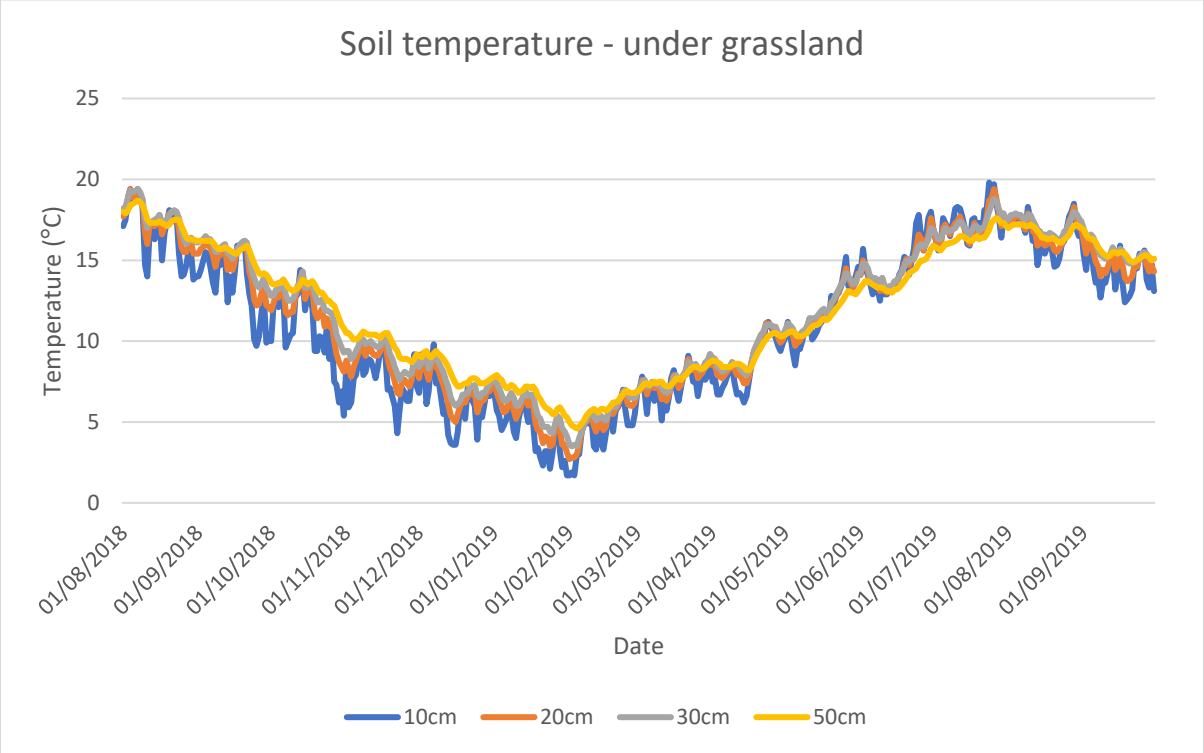

Figure 8.1 – Soil temperature under grassland across the sampling year for Hertfordshire, England, at soil depths of 10cm, 20cm, 30cm and 50cm

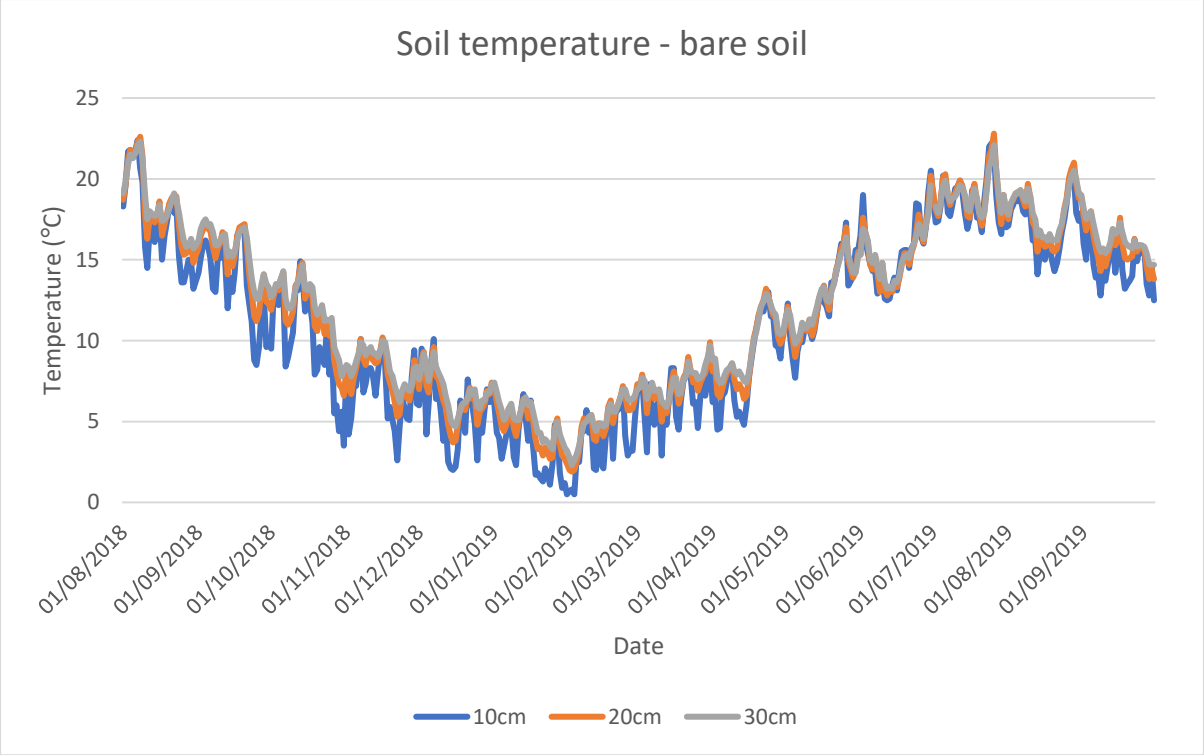

Figure 8.2 – Soil temperature under bare soils across the sampling year for Hertfordshire, England, at soil depths of 10cm, 20cm and 30cm.

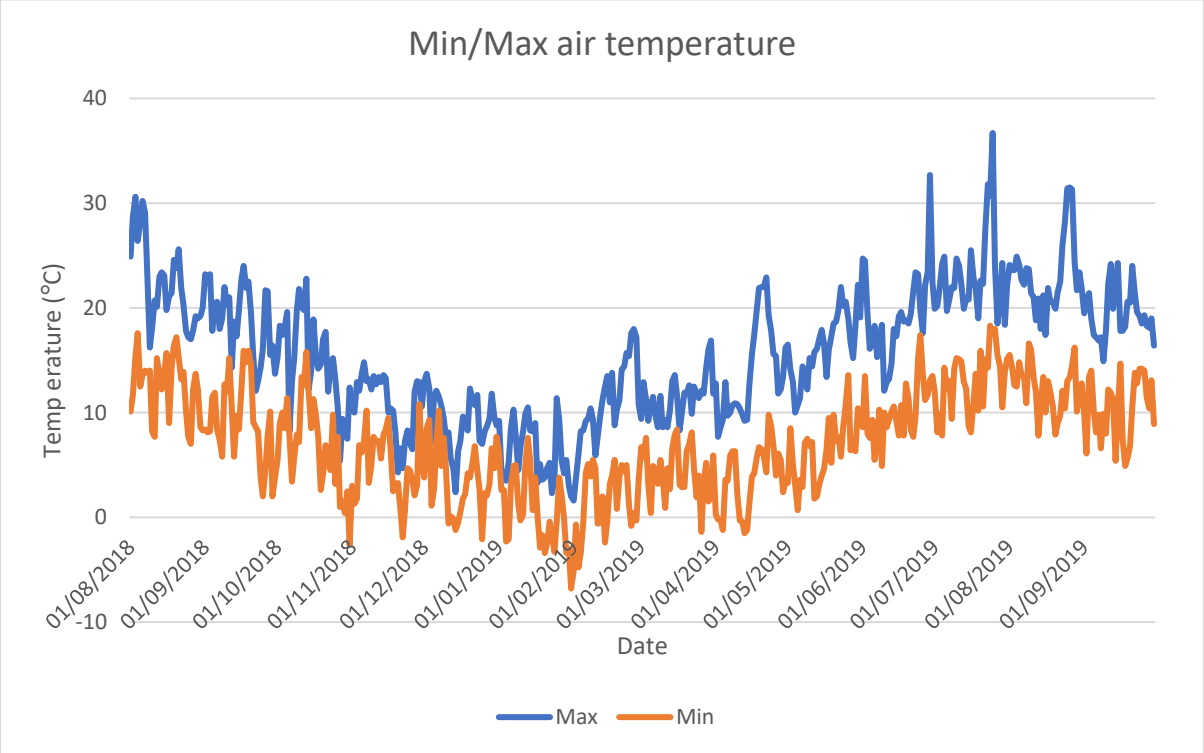

Figure 8.3 – Min/max air temperatures for Hertfordshire, England, across the sampling year

8.7 Post cultivation (February 2019) CT field

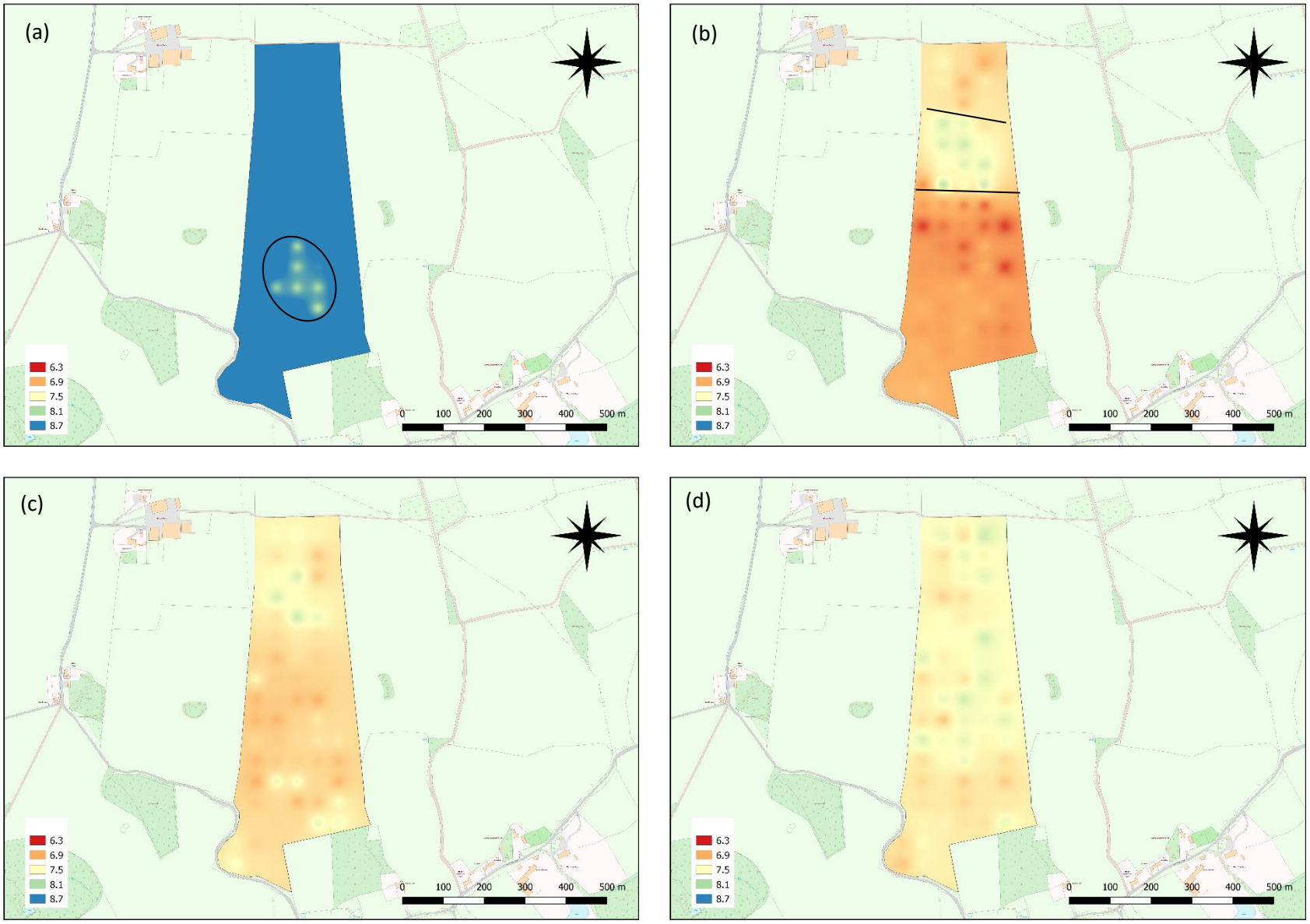

Figure 8.4 – Field measured soil pH for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

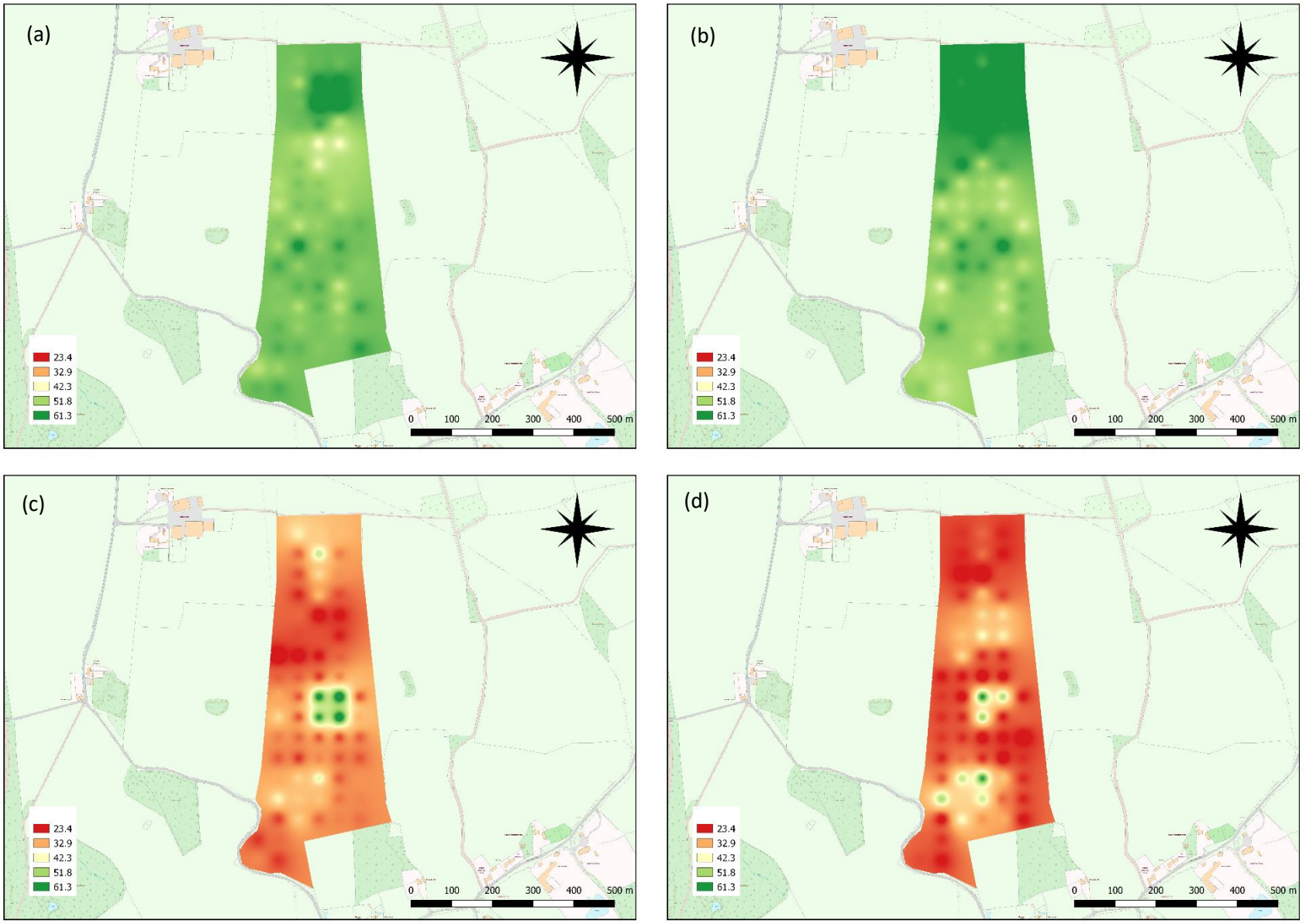

Figure 8.5 - Field measured soil glomalin for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

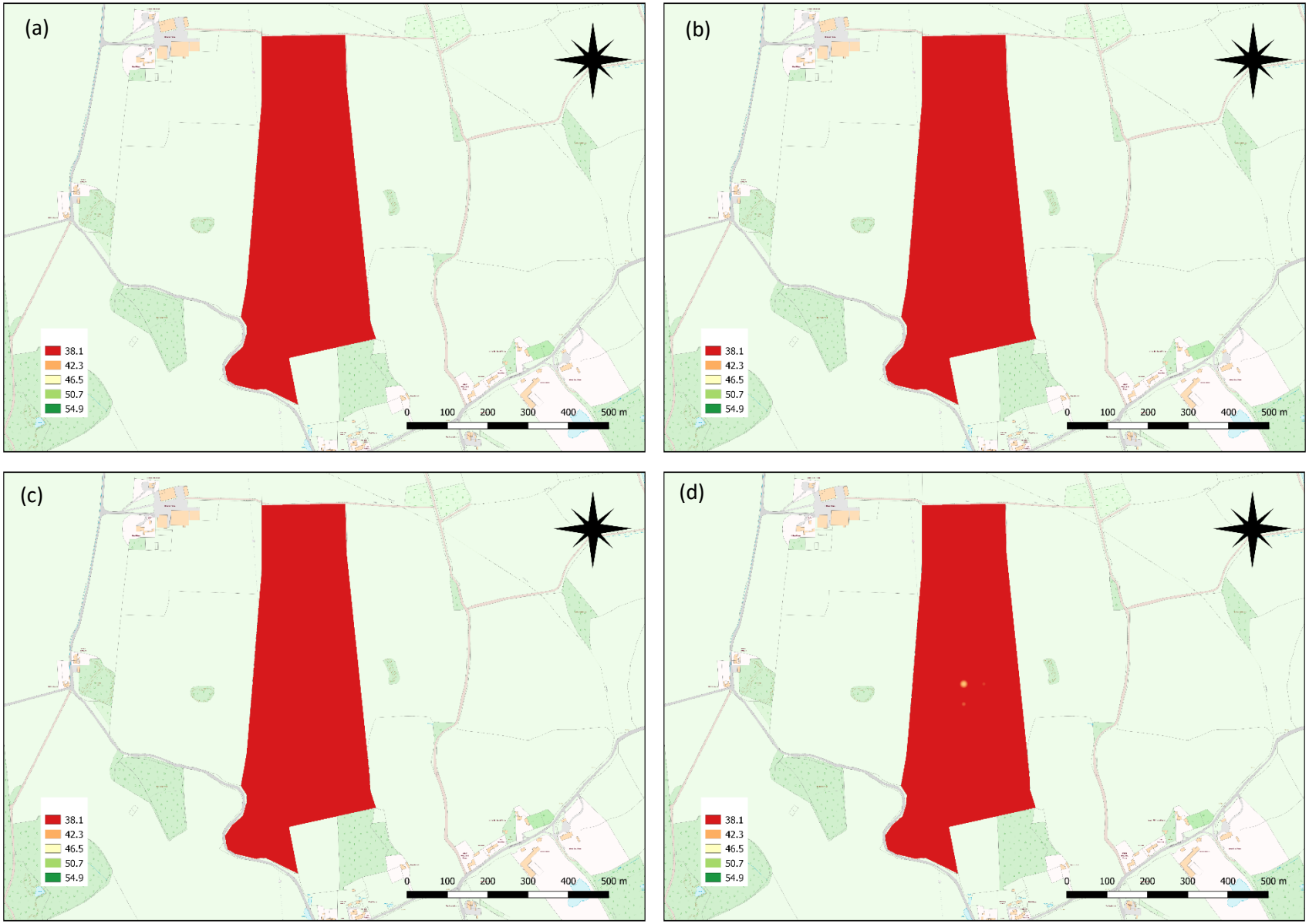

Figure 8.6 - Field measured soil WSA for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

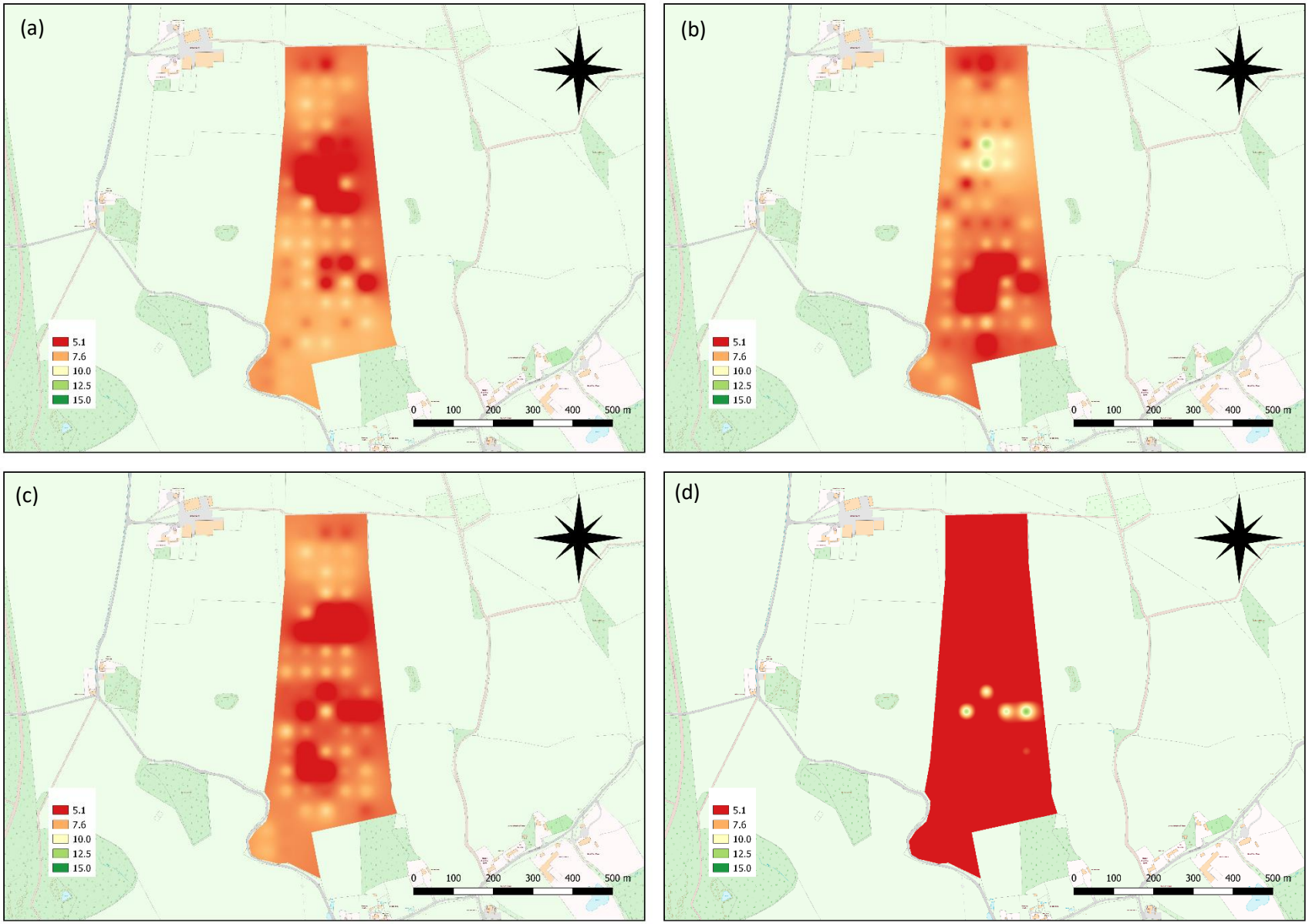

Figure 8.7 - Field measured soil organic matt for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

8.8 Post cultivation (February 2019) ZT field

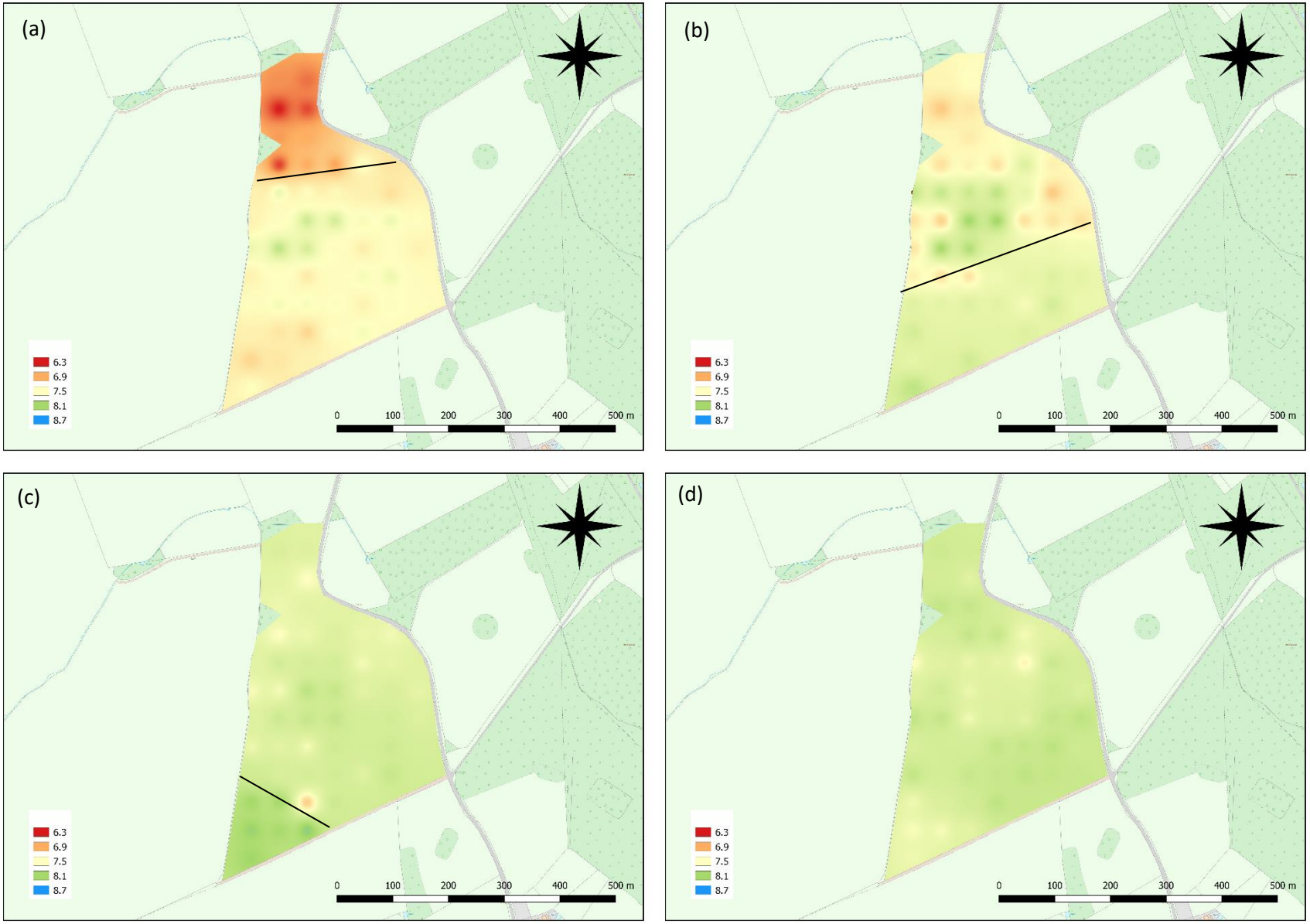

Figure 8.8 - Field measured soil pH for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

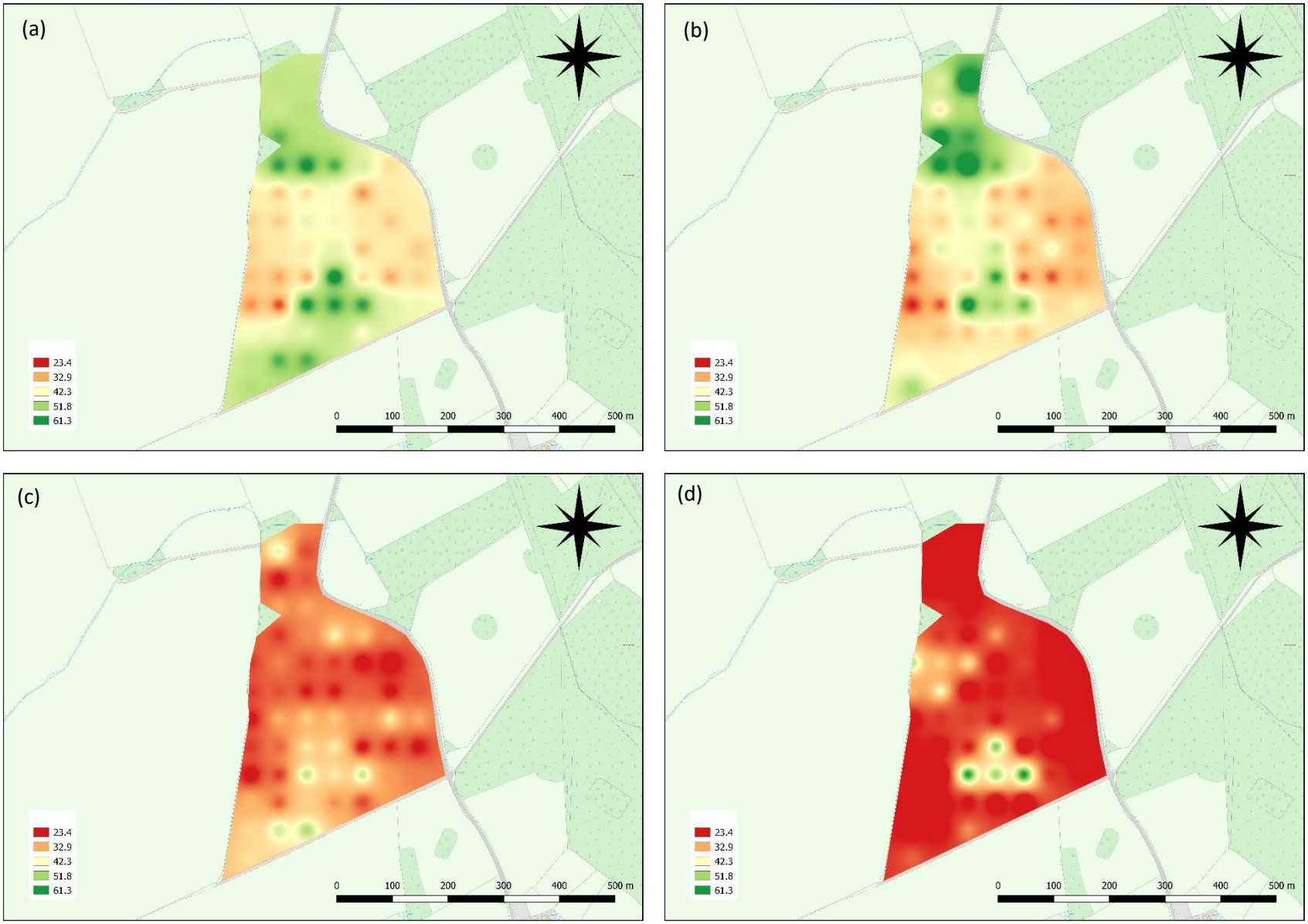

Figure 8.9 - Field measured soil glomalin for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

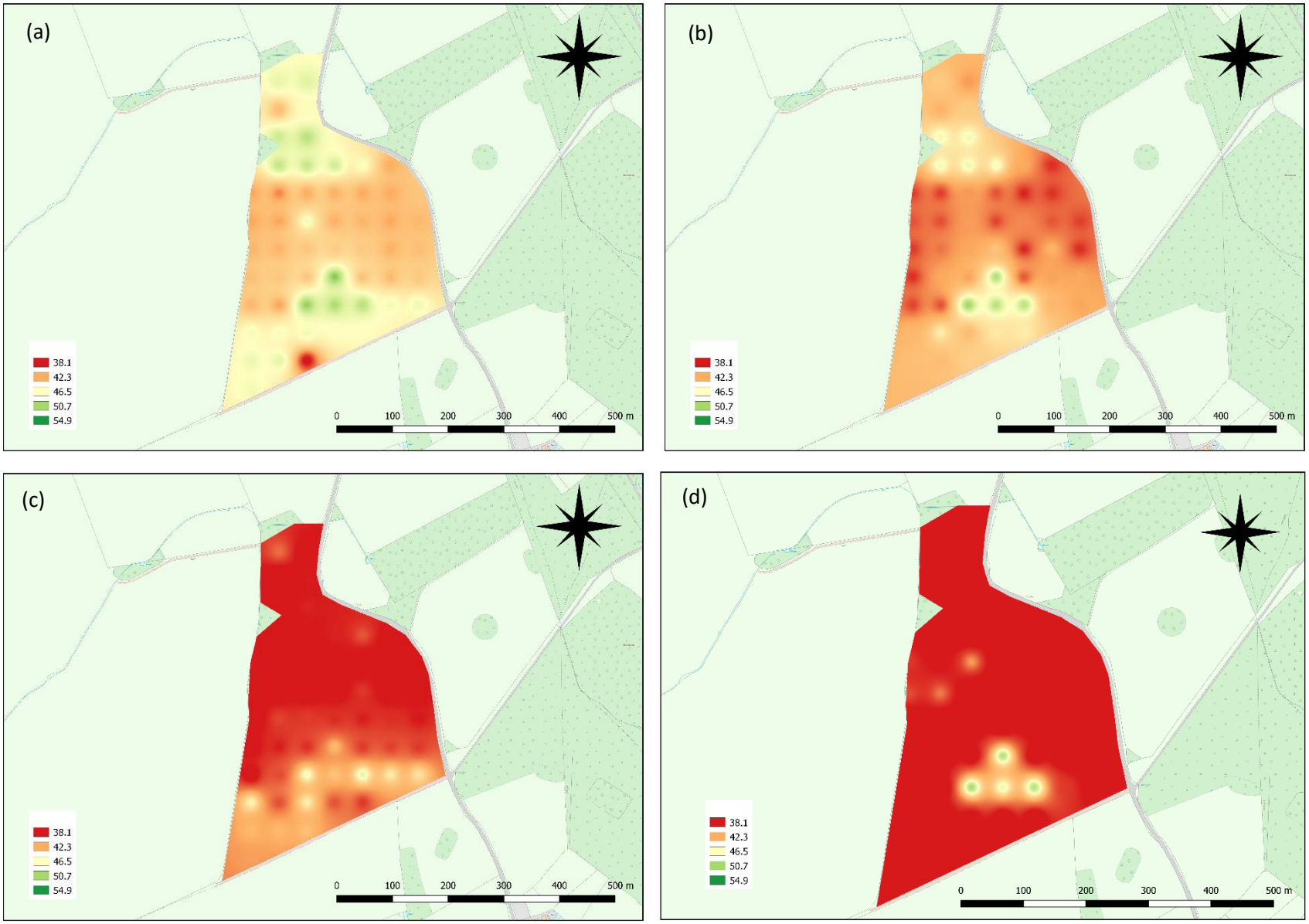

Figure 8.10 - Field measured soil WSA for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

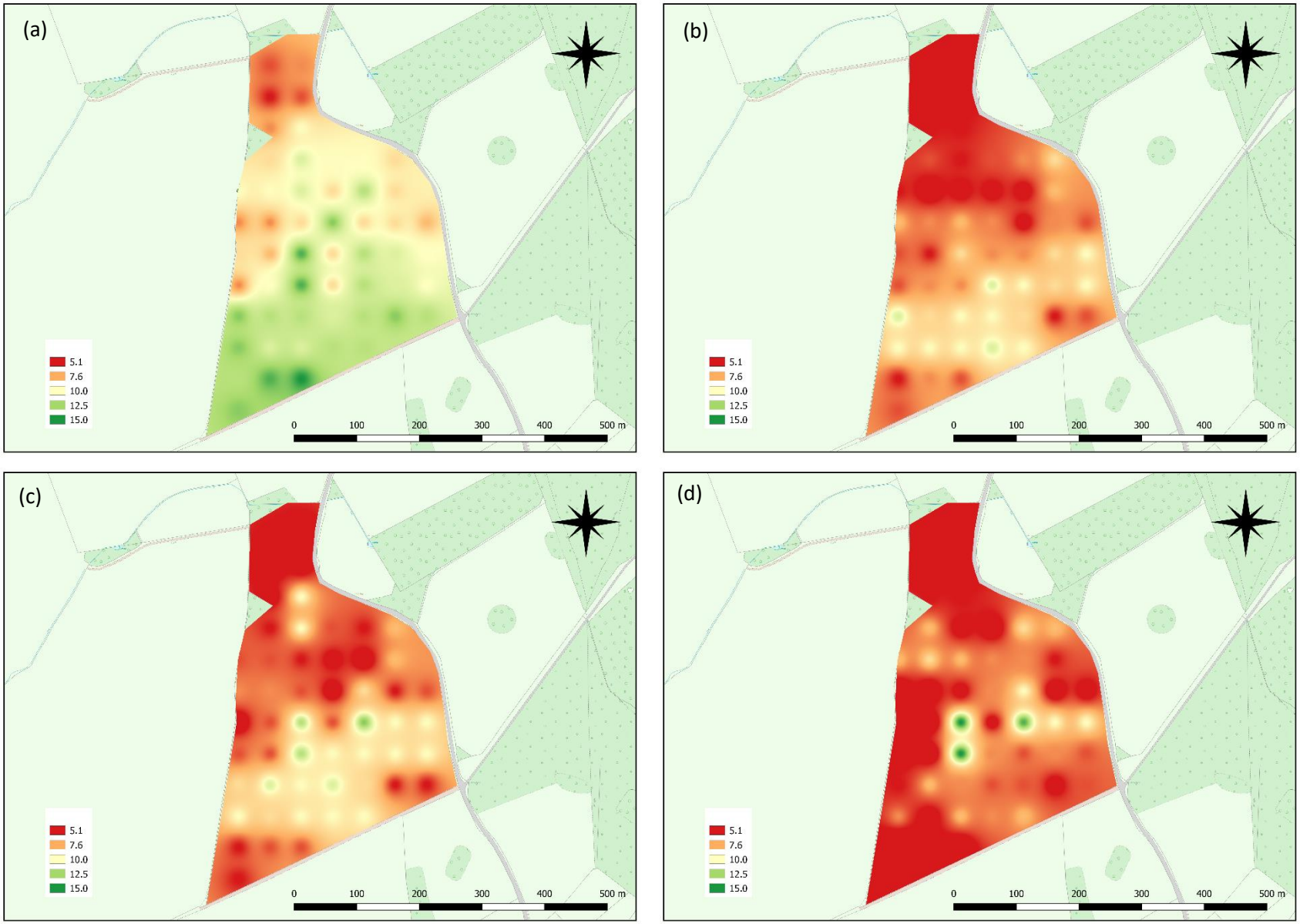

Figure 8.11 - Field measured soil organic matter for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period 3 months post cultivation (February 2019)

8.9 Pre harvest (May 2019) CT field

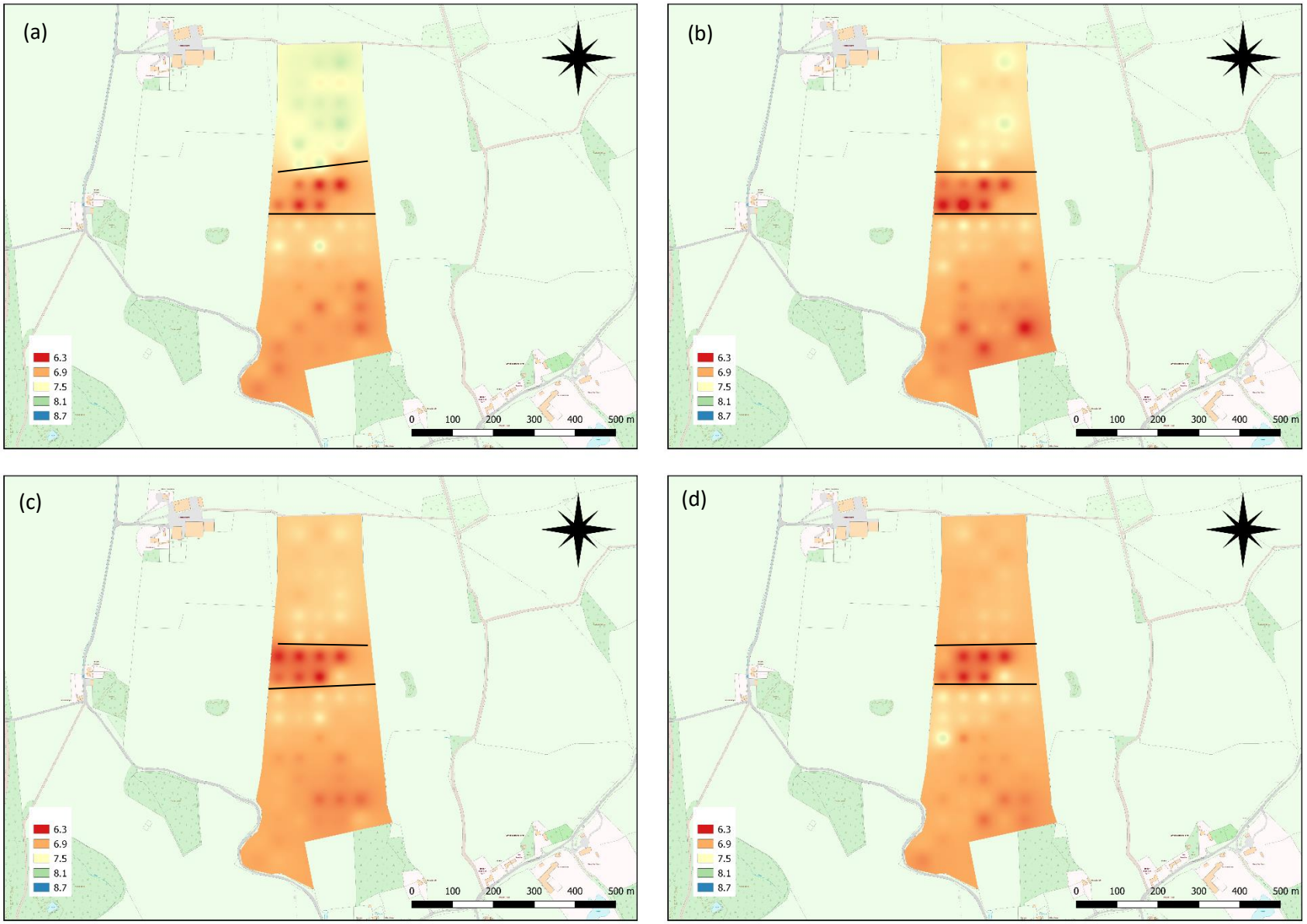

Figure 8.12 - Field measured soil pH for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

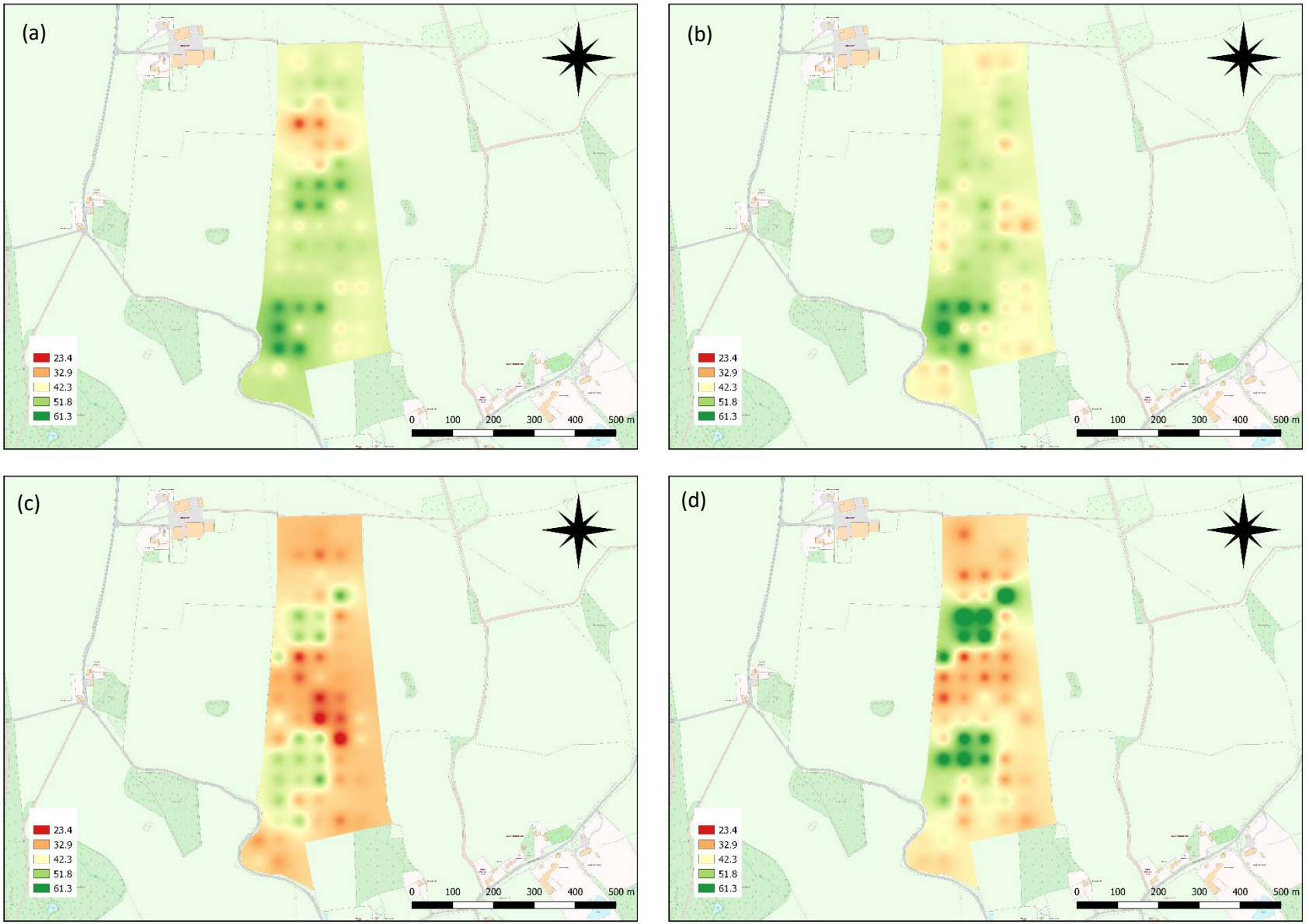

Figure 8.13 - Field measured soil glomalin for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

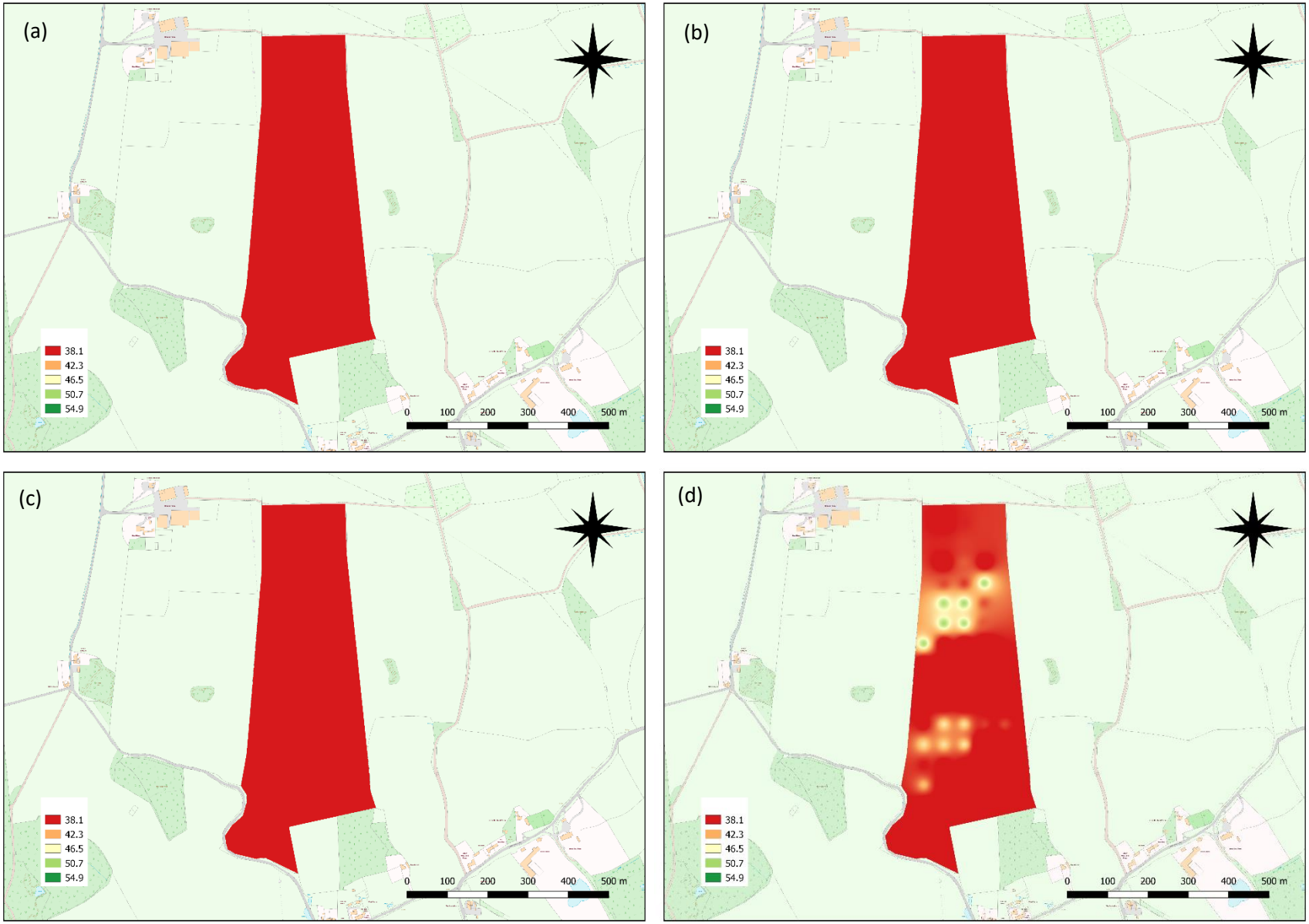

Figure 8.14 - Field measured soil WSA for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

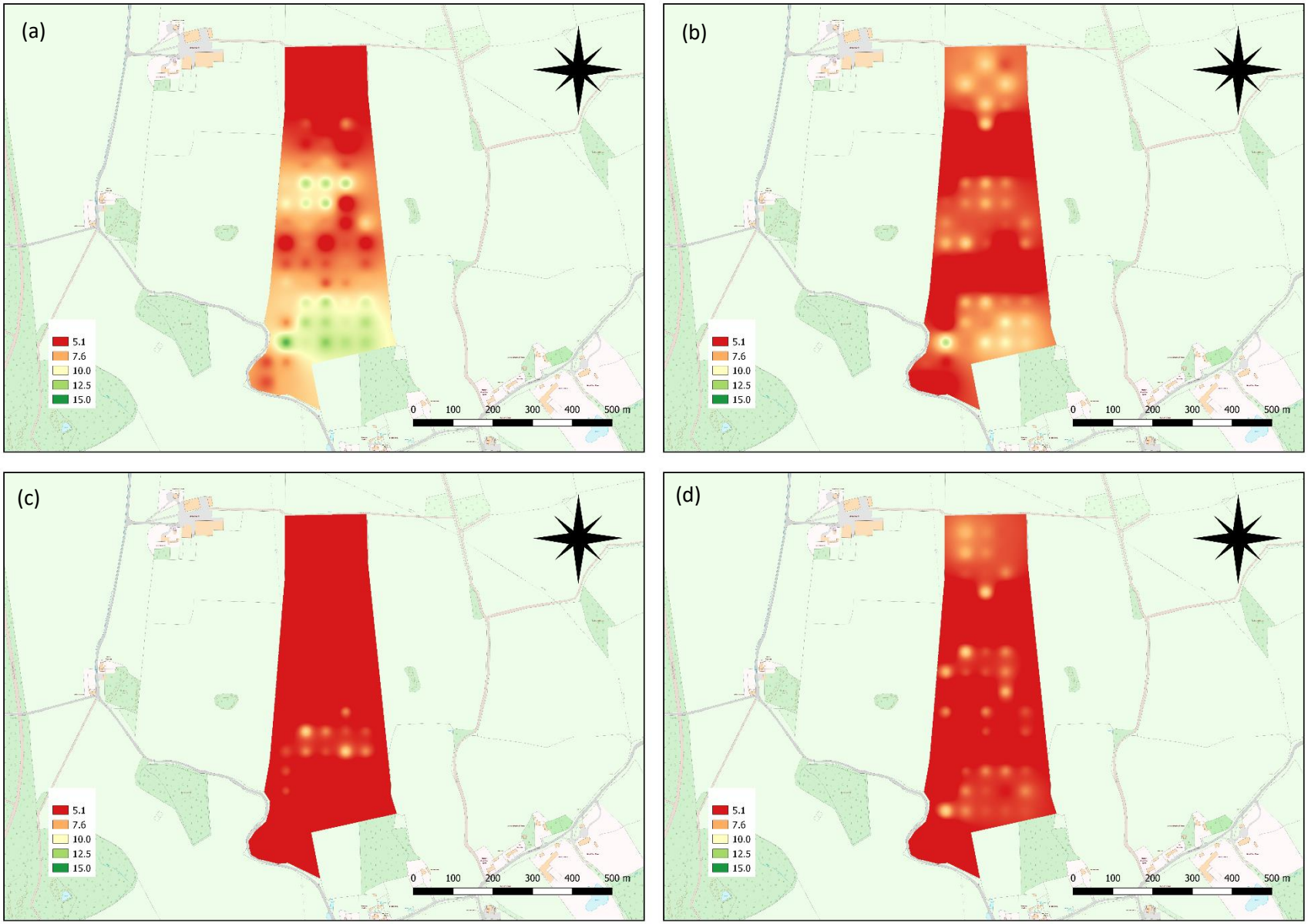

Figure 8.15 - Field measured soil organic matter for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

8.10 Pre harvest (May 2019) ZT field

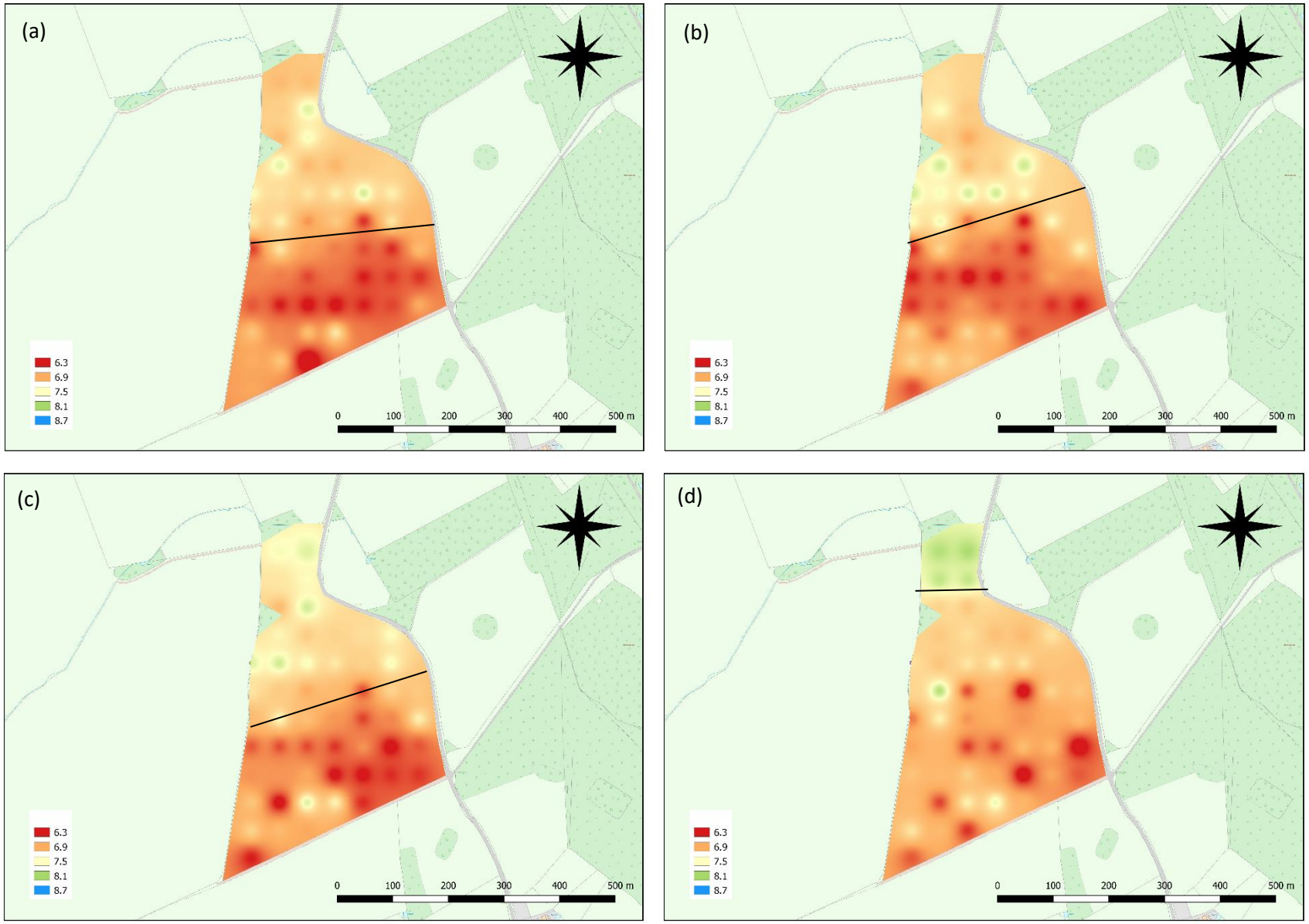

Figure 8.16 - Field measured soil pH for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

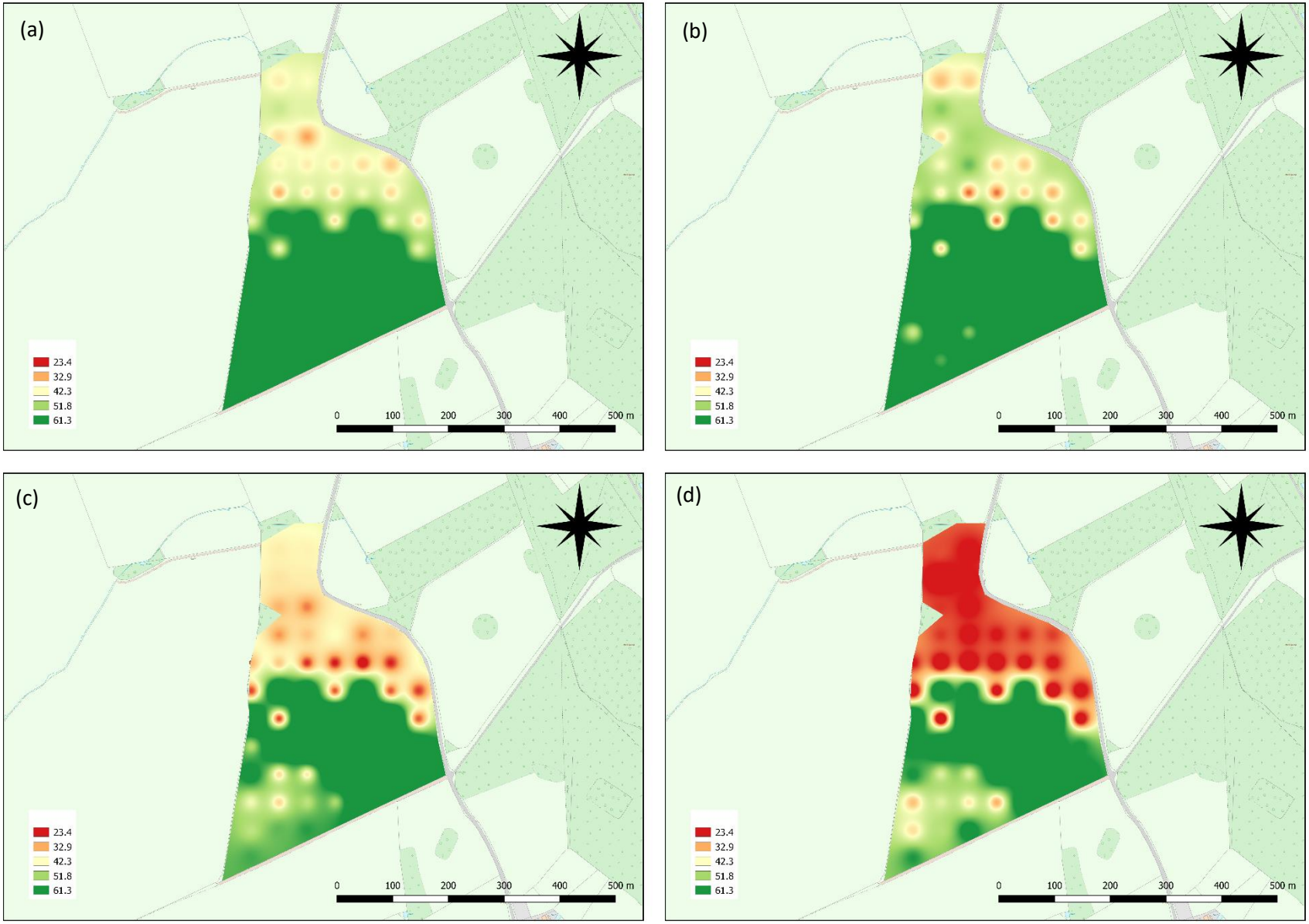

Figure 8.17 - Field measured soil glomalin for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

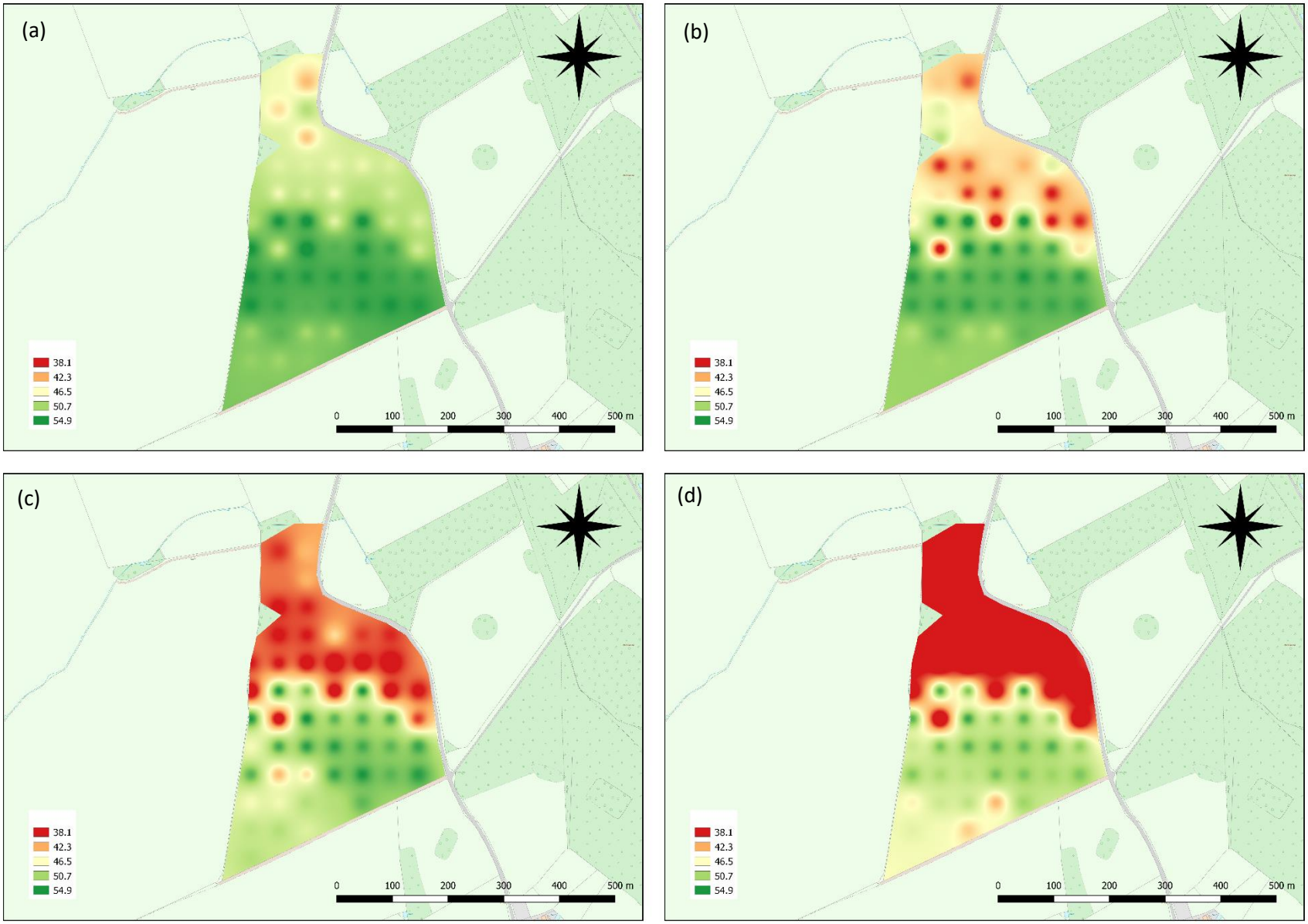

Figure 8.18 - Field measured soil WSA for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

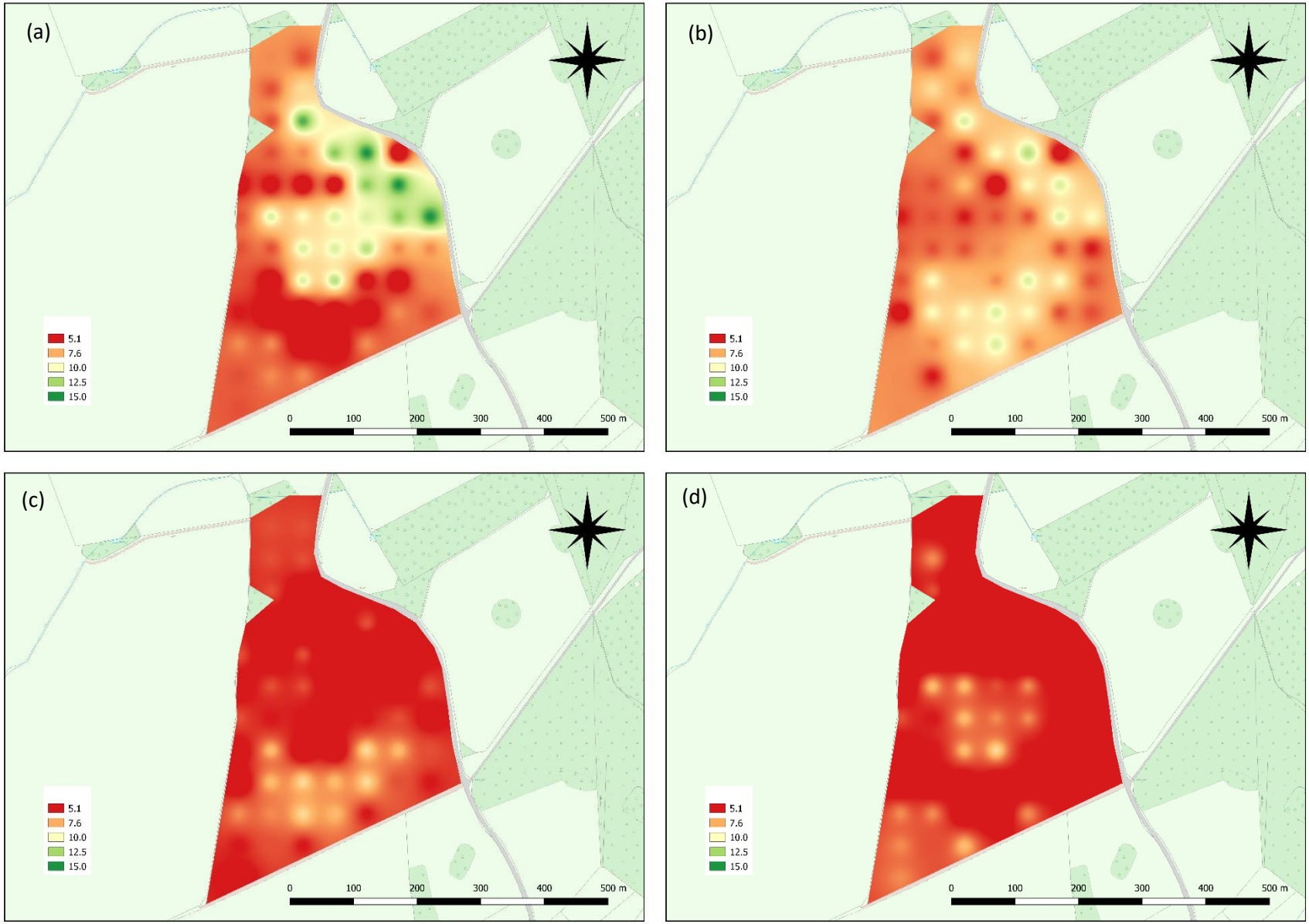

Figure 8.19 - Field measured soil organic matter for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (May 2019)

8.11 Post harvest (August 2019) CT field

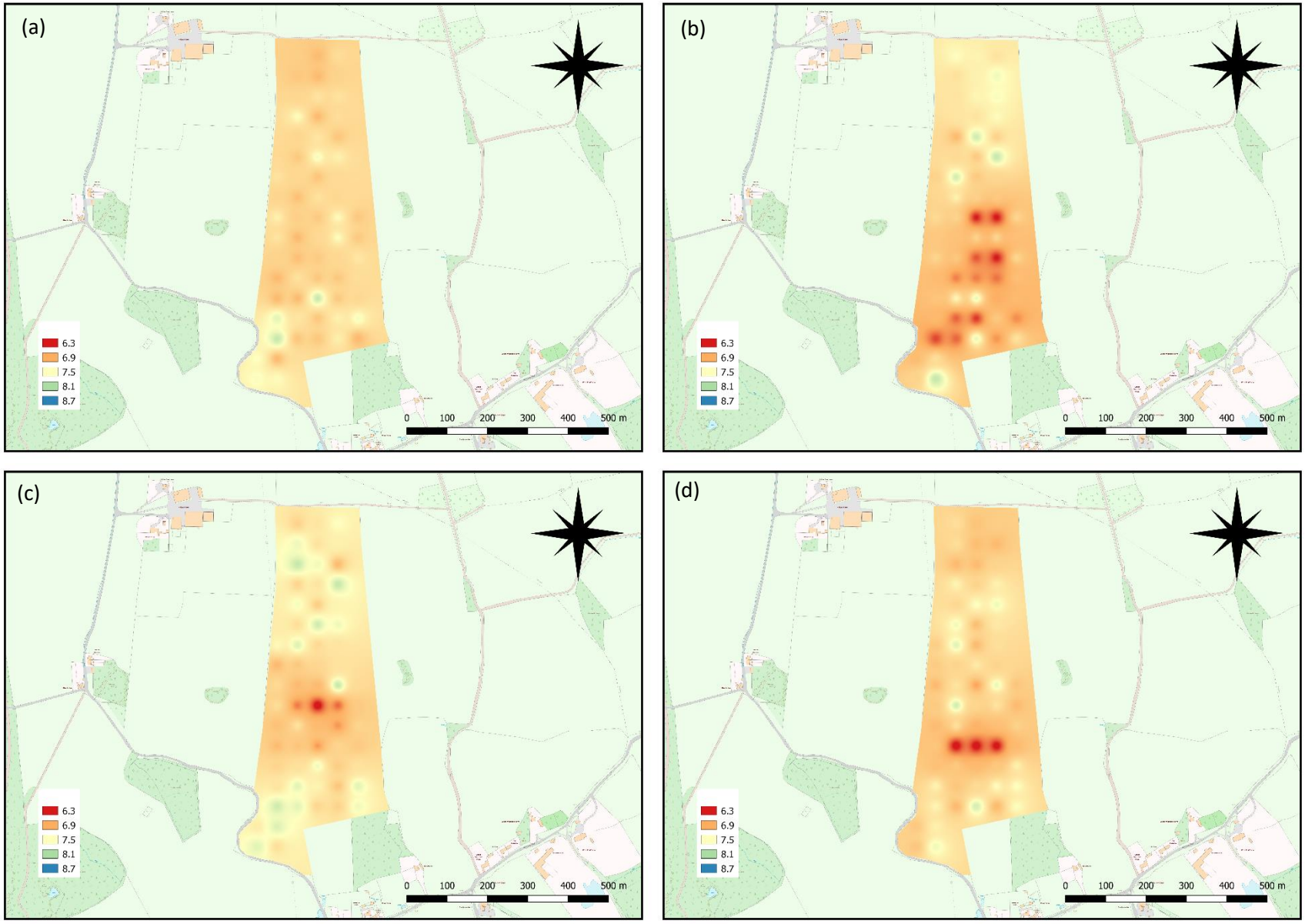

Figure 8.20 - Field measured soil pH for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

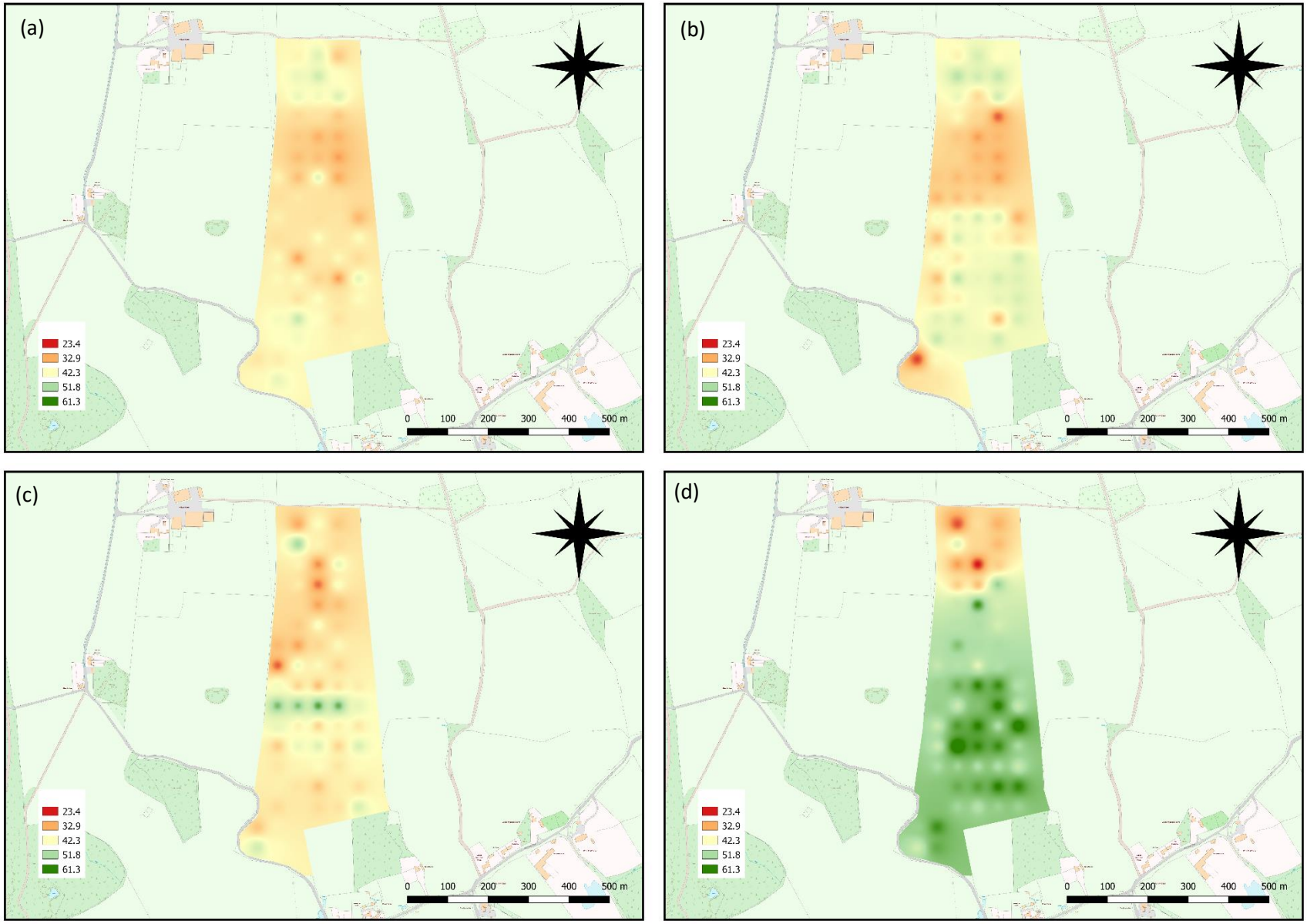

Figure 8.21 - Field measured soil glomalin for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

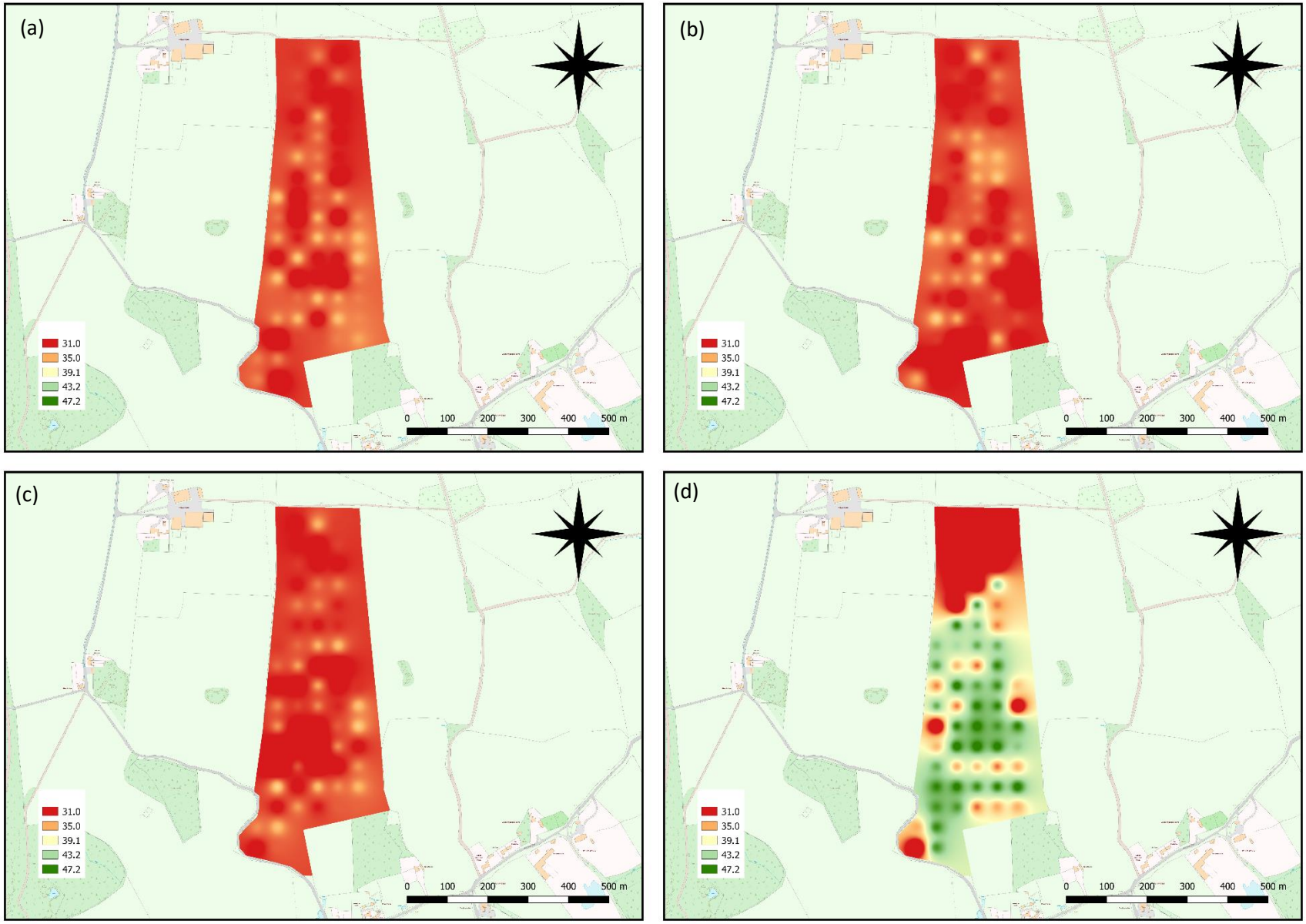

Figure 8.22 - Field measured soil WSA for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

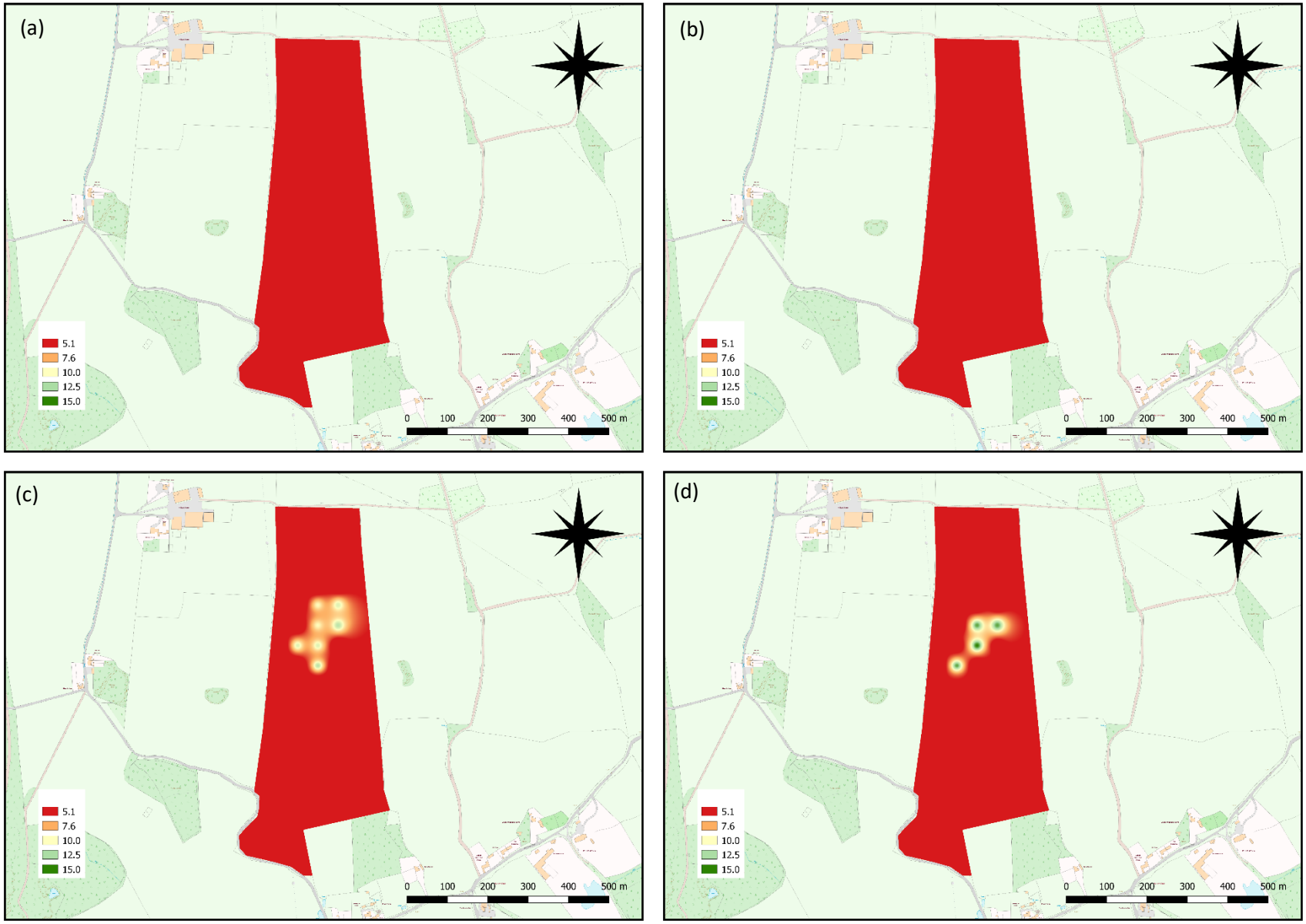

Figure 8.23 - Field measured soil organic matter for CT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

8.12 Post harvest (August 2019) ZT field

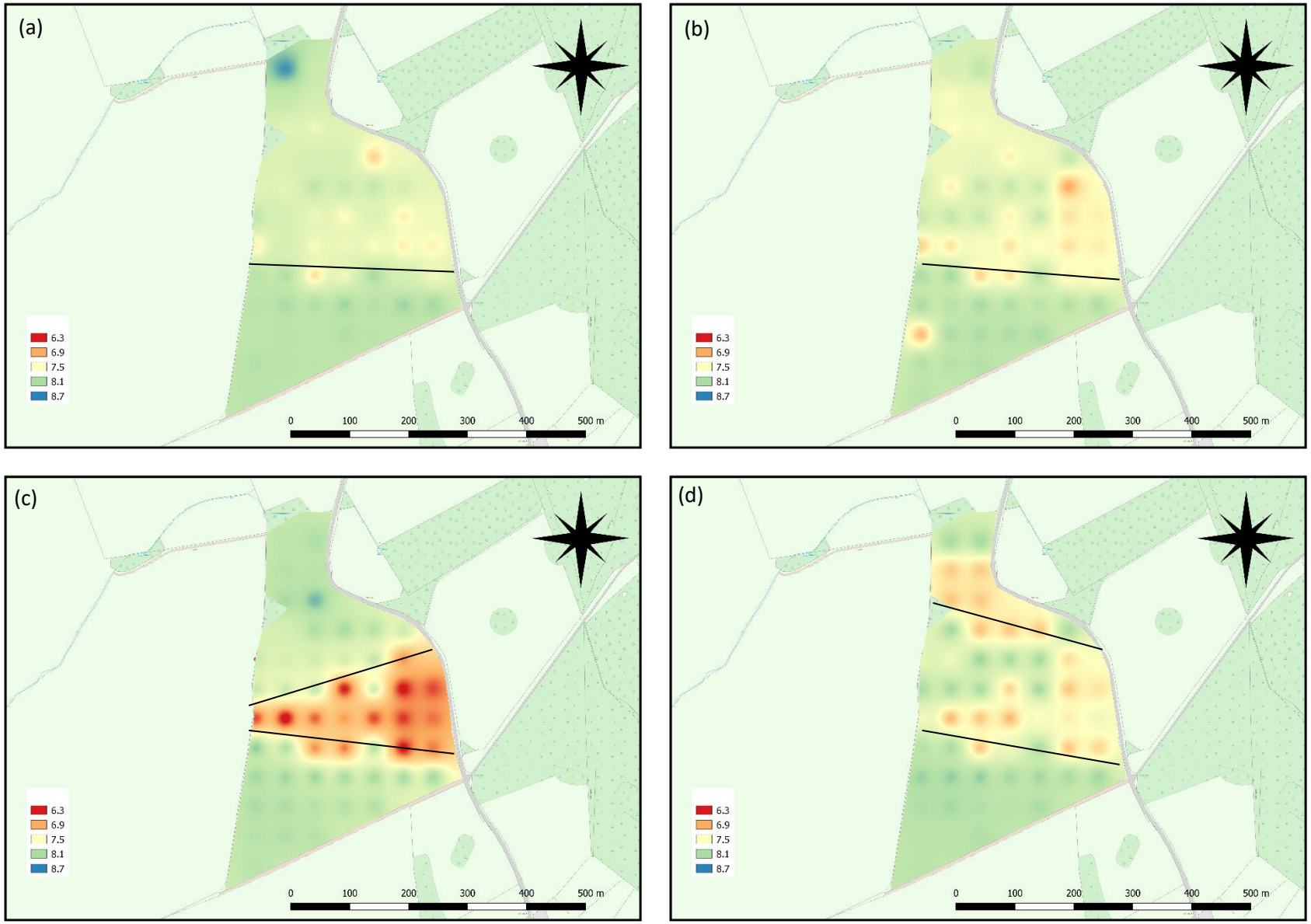

Figure 8.24 – Field measured soil pH for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

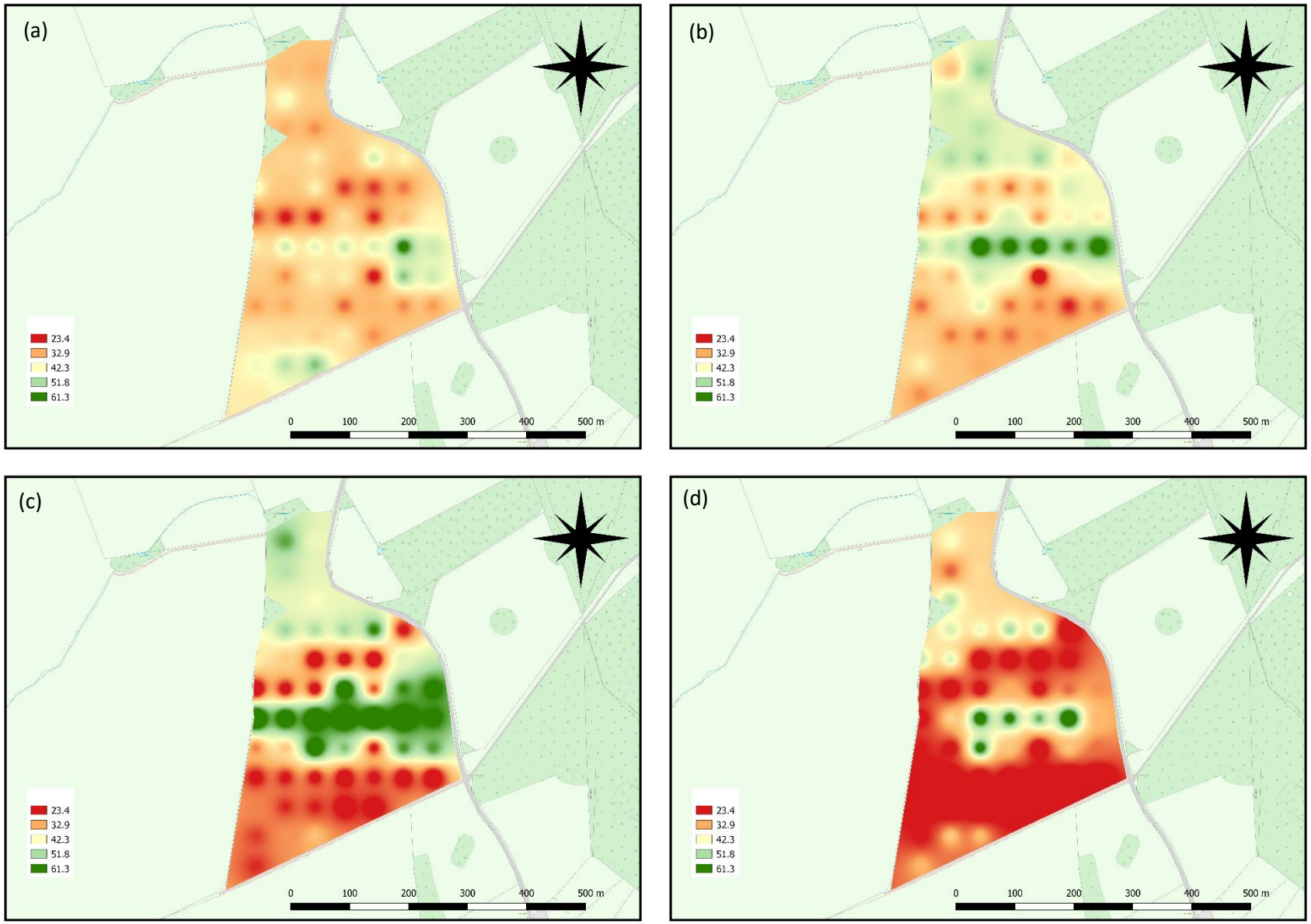

Figure 8.25 – Field measured soil glomalin for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

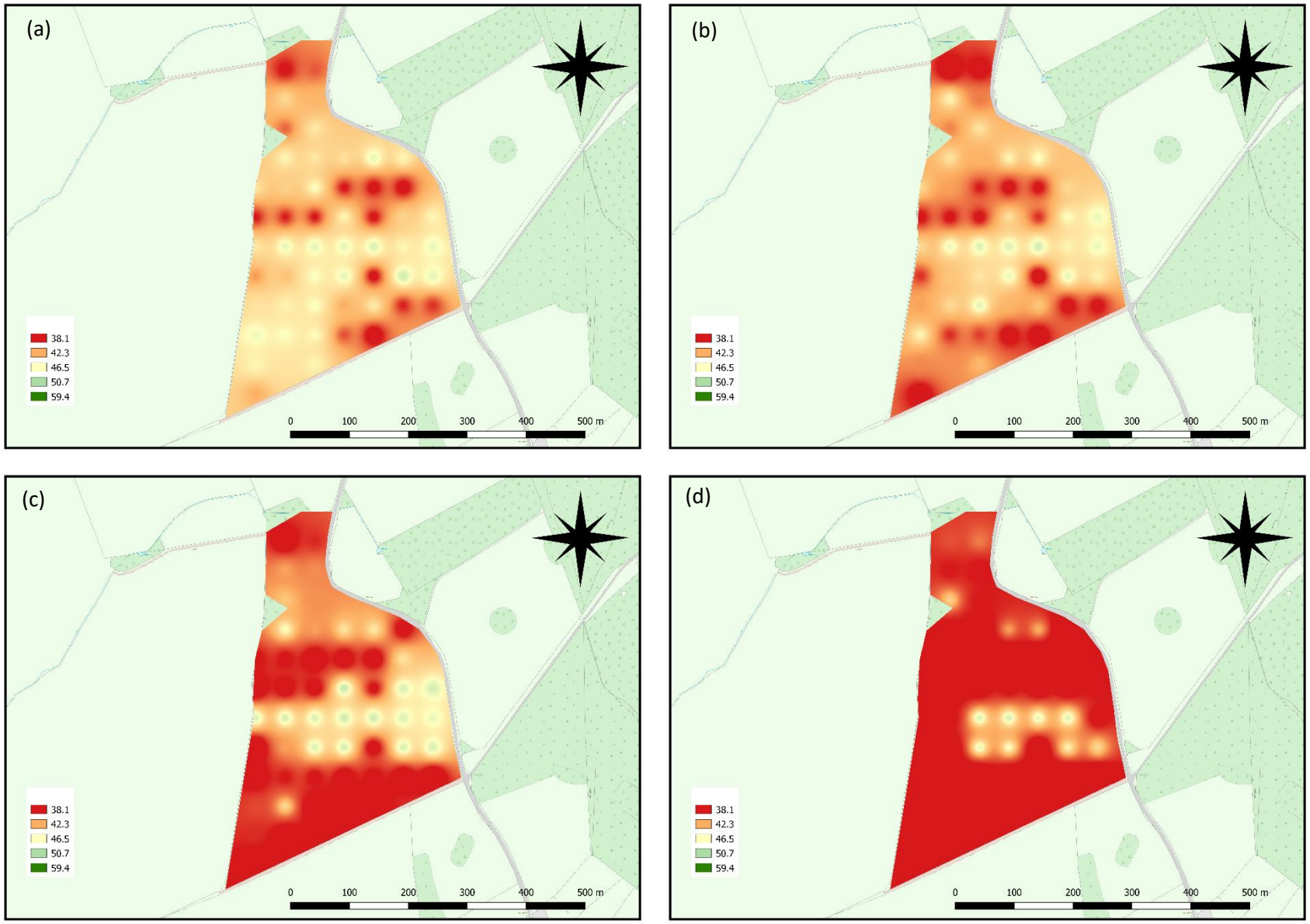

Figure 8.26 - Field measured soil WSA for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

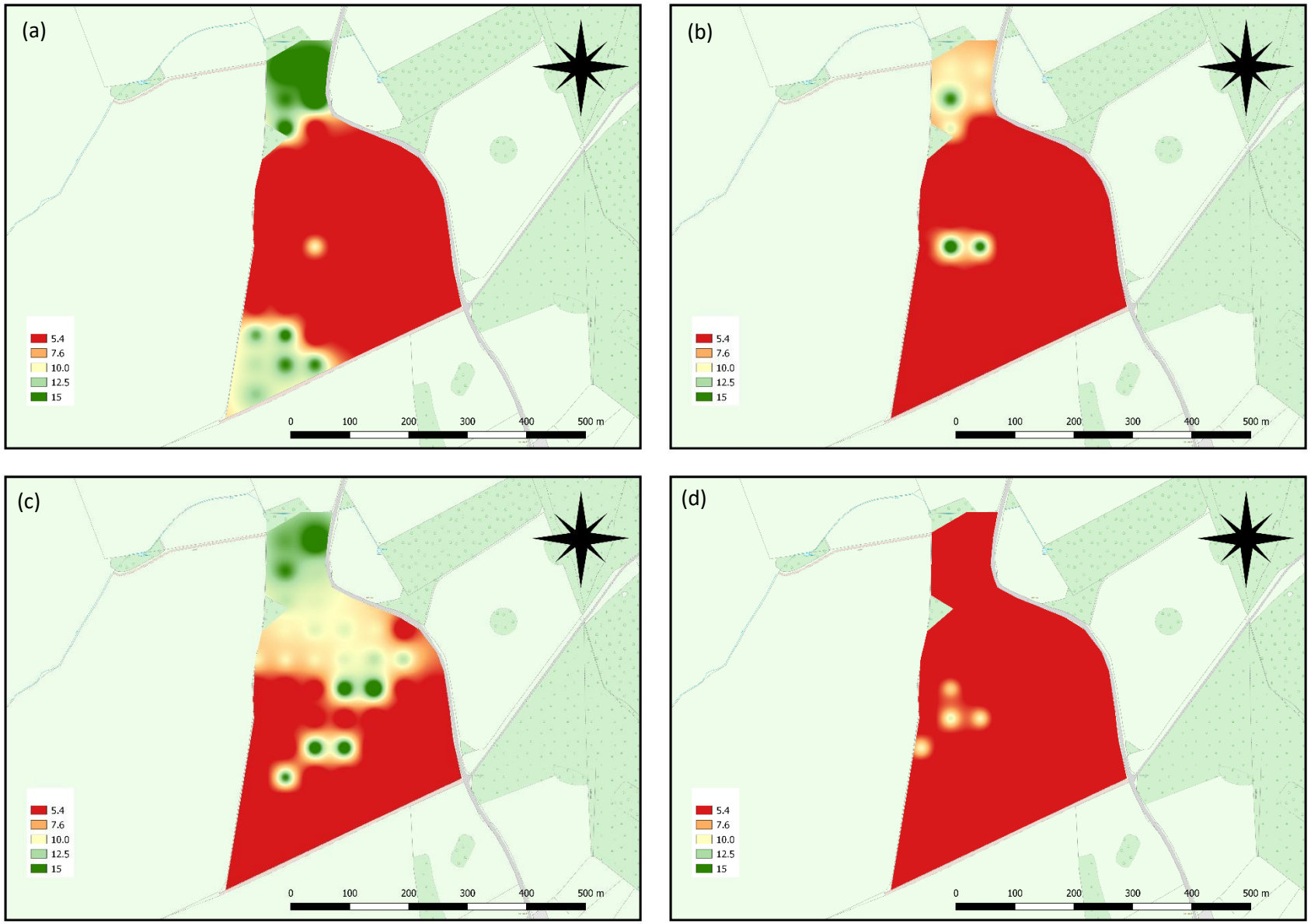

Figure 8.27 - Field measured soil organic matter for ZT managed soils at sampling depths of (a) 10cm, (b) 20cm, (c) 30cm and (d) 40cm at a sampling period pre harvest (August 2019)

8.13 Field ANOVA tables

ANOVA table divided into different pH regions, as depicted by interpolation maps, for attributes such as; pH, WSA, organic matter, moisture, total glomalin and bulk density. ANOVA tables are given for September 2018 (Table 8.4), February 2019 (Table 8.5), May 2019 (Table 8.6) and August 2019 (Table 8.7).

Table 8.4 - Table of ANOVA P values for each measured attribute between CT and ZT fields, for the sampling period pre cultivation (September 2018), for their corresponding sampling depth between the number of measured regions determined by pH, Figure 3.1 and 3.5. Depths that did not produce noticeable bands under pH did not perform during ANOVA and noted as n/a.

Tillage	Depth (cm)	pH	WSA	Organic matter	Moisture	Total glomalin	Bulk density
CT	10	<0.00001	<0.00001	0.05	<0.00001	0.003	<0.00001
	20	<0.00001	0.62	0.22	0.1	0.15	0.005
	30	<0.00001	0.31	0.60	0.24	<0.00001	0.0001
	40	n/a	n/a	n/a	n/a	n/a	n/a
ZT	10	<0.00001	<0.00001	<0.00001	0.11	0.07	0.94
	20	<0.00001	<0.00001	0.04	0.69	0.02	<0.00001
	30	<0.00001	<0.00001	0.03	0.05	0.51	<0.00001
	40	n/a	n/a	n/a	n/a	n/a	n/a

Table 8.5 – ANOVA P values for the pH regions depicted in CT (Figure 8.4) and ZT (Figure 8.8)

Tillage	Depth (cm)	pH	WSA	Organic matter	Moisture	Total glomalin	Bulk density
CT	10	<0.00001	0.4	0.11	0.53	0.65	0.98
	20	<0.00001	<0.00001	0.02	<0.0001	<0.00001	<0.0001
	30	n/a	n/a	n/a	n/a	n/a	n/a
	40	n/a	n/a	n/a	n/a	n/a	n/a
ZT	10	<0.00001	<0.01	<0.00001	0.32	<0.00001	0.68
	20	0.08	0.08	<0.00001	<0.00001	0.12	<0.00001
	30	0.08	0.08	0.08	0.08	0.08	0.08
	40	n/a	n/a	n/a	n/a	n/a	n/a

Table 8.6 - ANOVA P values for the pH regions depicted in CT (Figure 8.12) and ZT (Figure 8.16)

Tillage	Depth (cm)	pH	WSA	Organic matter	Moisture	Total glomalin	Bulk density
CT	10	<0.00001	0.72	<0.00001	<0.00001	<0.001	0.08
	20	<0.00001	0.72	<0.00001	<0.00001	<0.001	0.08
	30	<0.00001	0.72	<0.00001	<0.00001	<0.001	0.08
	40	<0.00001	0.72	<0.00001	<0.00001	<0.001	0.08
ZT	10	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.93
	20	<0.00001	<0.00001	0.02	<0.00001	<0.00001	0.90
	30	<0.00001	<0.00001	<0.001	<0.00001	<0.00001	0.82
	40	n/a	n/a	n/a	n/a	n/a	n/a

Table 8.7 - ANOVA P values for the pH regions depicted in CT (Figure 8.20) and ZT (Figure 8.24)

Tillage	Depth (cm)	pH	WSA	Organic matter	Moisture	Total glomalin	Bulk density
CT	10	n/a	n/a	n/a	n/a	n/a	n/a
	20	n/a	n/a	n/a	n/a	n/a	n/a
	30	n/a	n/a	n/a	n/a	n/a	n/a
	40	n/a	n/a	n/a	n/a	n/a	n/a
ZT	10	0.03	0.12	0.06	<0.001	0.05	0.93
	20	0.03	0.12	0.06	<0.001	0.05	0.93
	30	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001
	40	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001

8.14 HPLC chemical standards chromatographs

Ergosterol was purchased from Sigma, UK, and dissolve in HPLC grade methanol to cover a concentration range of 1 to 1,000 µg ml^{-1}. Ergosterol standard is shown in Figure 8.28.

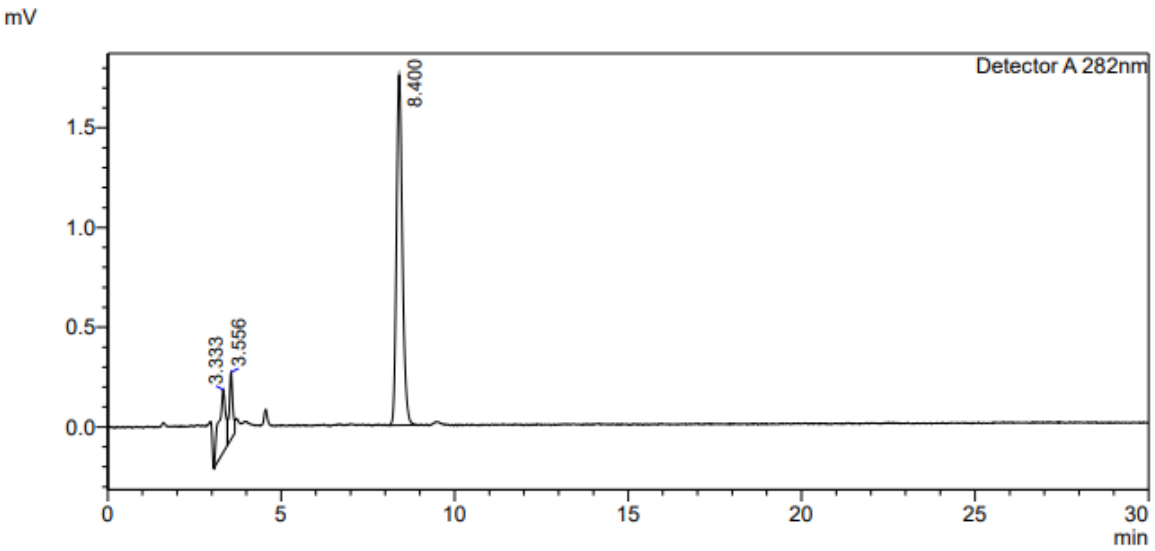

Figure 8.28 – Ergosterol standard from purchased sample (Sigma, UK) in HPLC grade methanol. Retention time: 8.4 minutes

Organic acid standards were run through HPLC for retention time comparisons of field sampled soils. Organic acids assessed were citric acid (Figure 8.29), oxalic acid (Figure 8.30), malic acid (Figure 8.31), succinic acid (Figure 8.32), malonic acid (Figure 8.33) and fumaric acid (Figure 8.34).

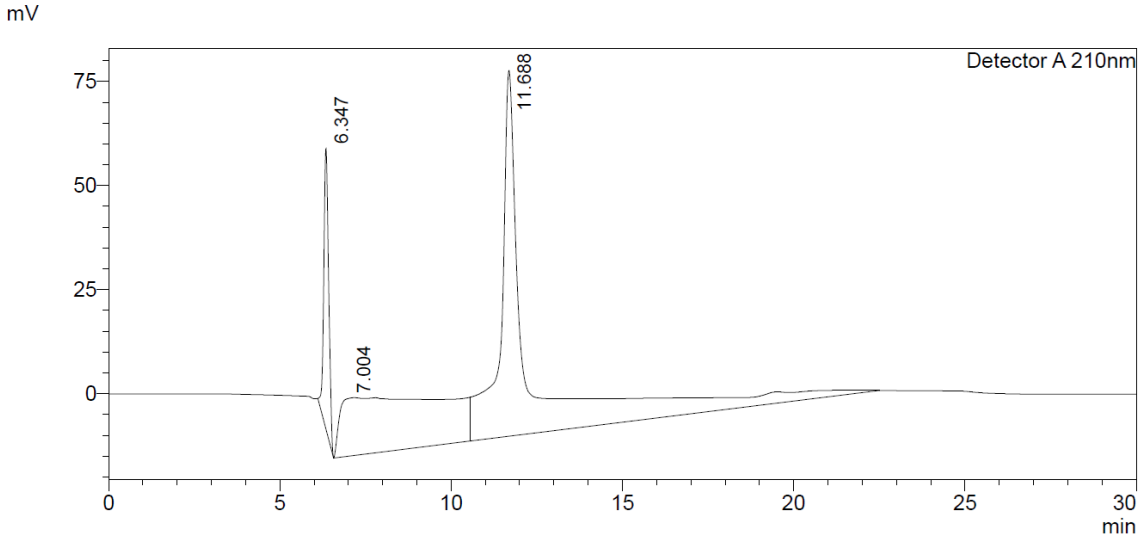

Figure 8.29 – Citric acid. Retention time: 11.7 minutes

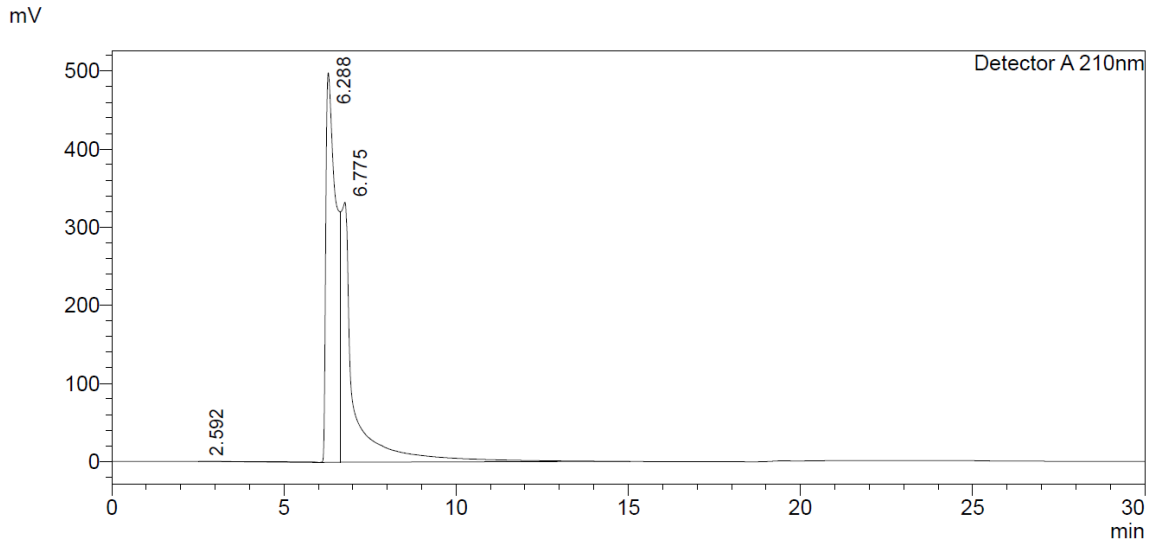

Figure 8.30 – Oxalic acid. Retention time: 6.8 minutes

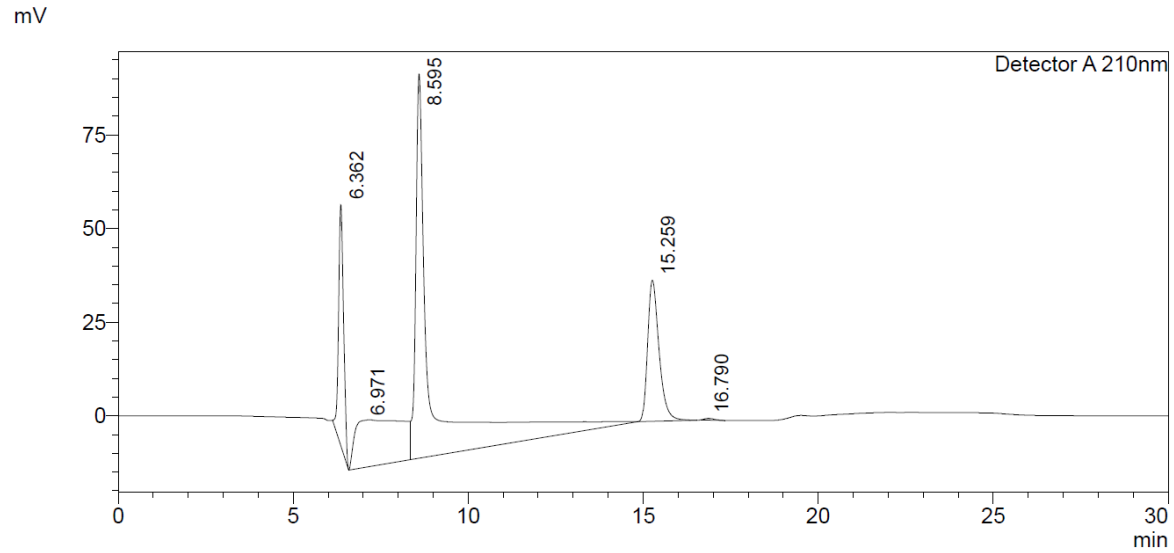

Figure 8.31 – Malic acid. Retention time: 8.6 and 15.3 minutes

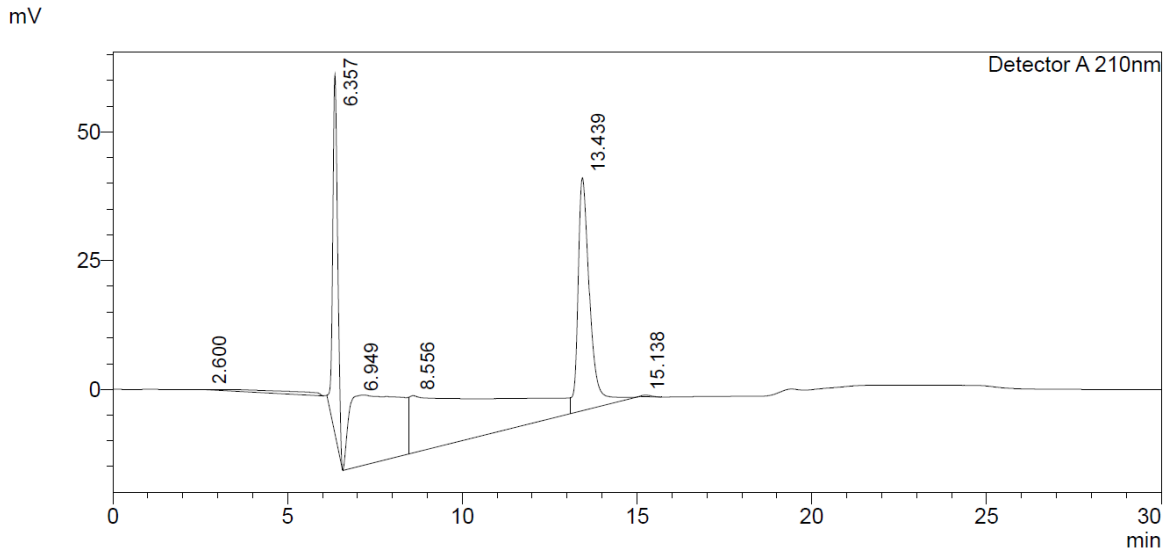

Figure 8.32 – Succinic acid. Retention time: 13.4 minutes

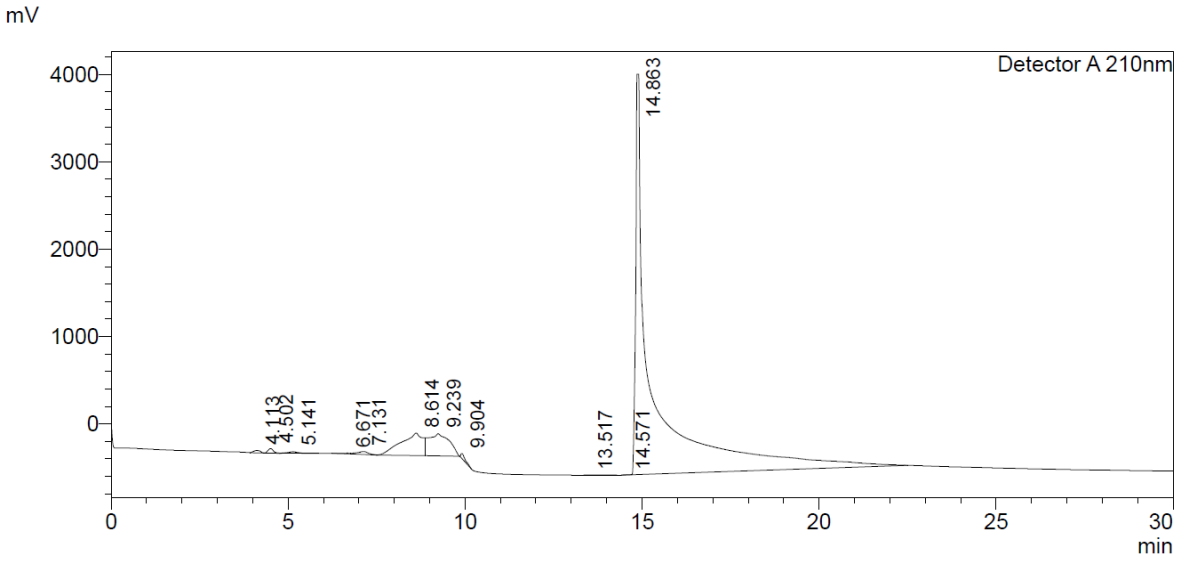

Figure 8.33 – Malonic acid. Retention time: 14.9 minutes

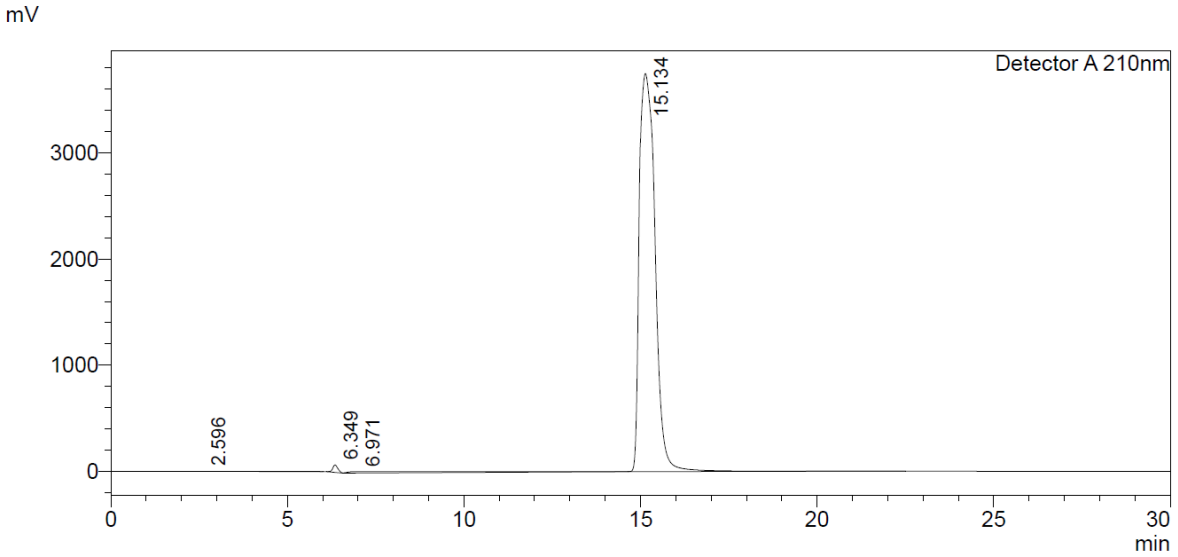

Figure 8.34 – Fumaric acid. Retention time: 15.1 minutes

8.15 Week 15 PGPR Zulu wheat – tillers

Sampled wheat photographed for Chapter 5 tiller length for each inoculant (*B. subtillus, B. pumillus, B. amyloliquefacines* and *R. intraradicies*) for each CT and ZT sampled field soils.

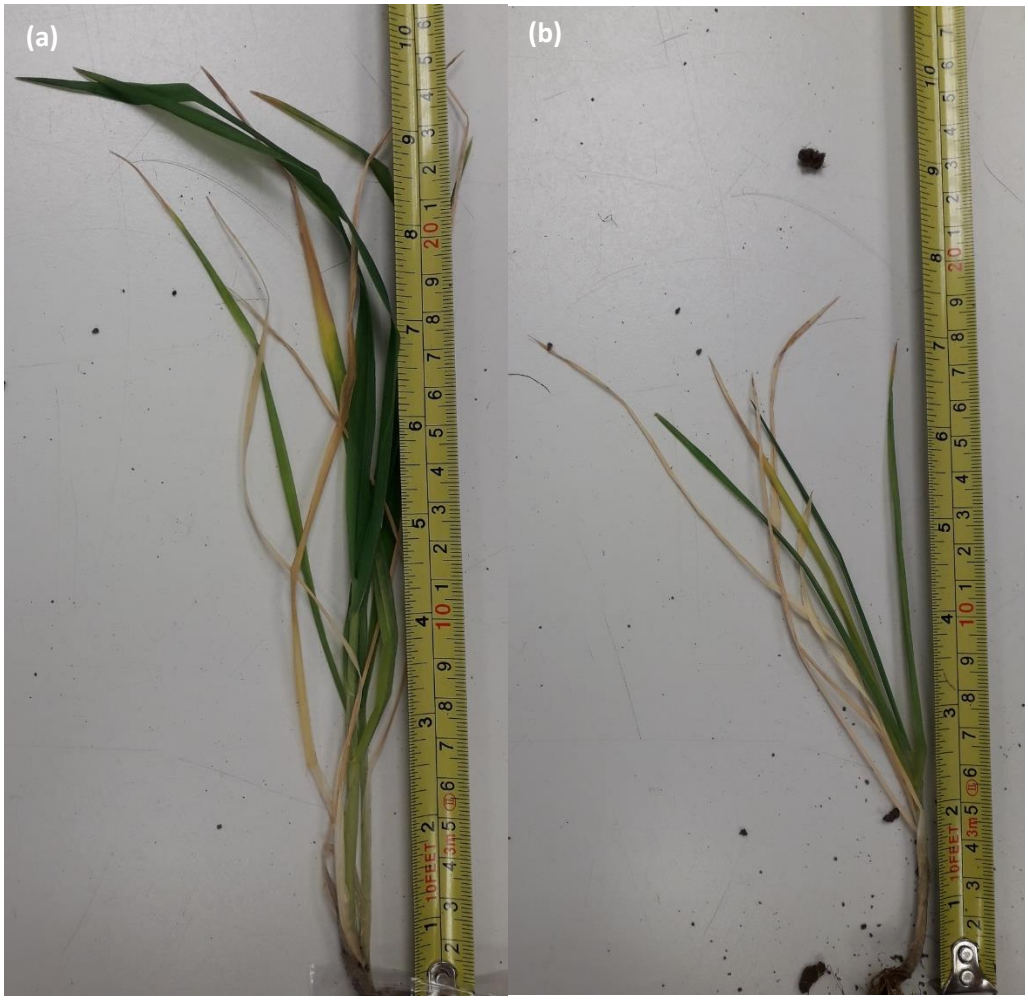

Figure 8.34 – Week 15 sampled wheat from controlled glasshouse conditions for tiller lengths of (a) ZT control and (b) CT control in respective soils from sample sites

Figure 8.35 – Week 15 sampled wheat from controlled glasshouse conditions for tiller length of *B. subtilis* inoculated soils in (a) ZT and (b) CT sampled soils

Figure 8.36 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of *B. pumilus* inoculated soils in (a) ZT and (b) CT sampled soils

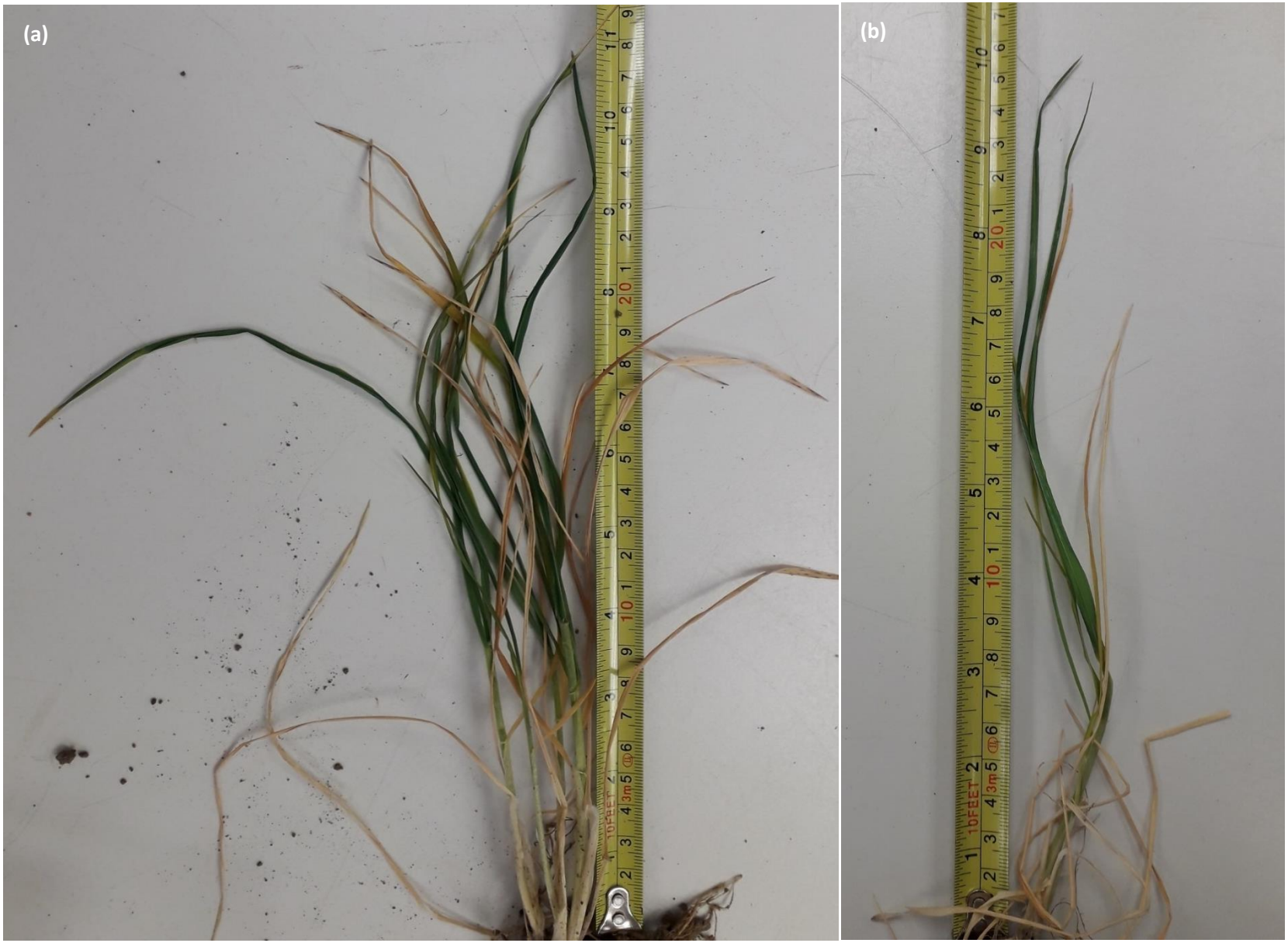

Figure 8.37 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of *B. amyloliquefaciens* inoculated soils in (a) ZT and (b) CT sampled soils

Figure 8.38 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of *R. intraradicies* inoculated soils in (a) ZT and (b) CT sampled soils

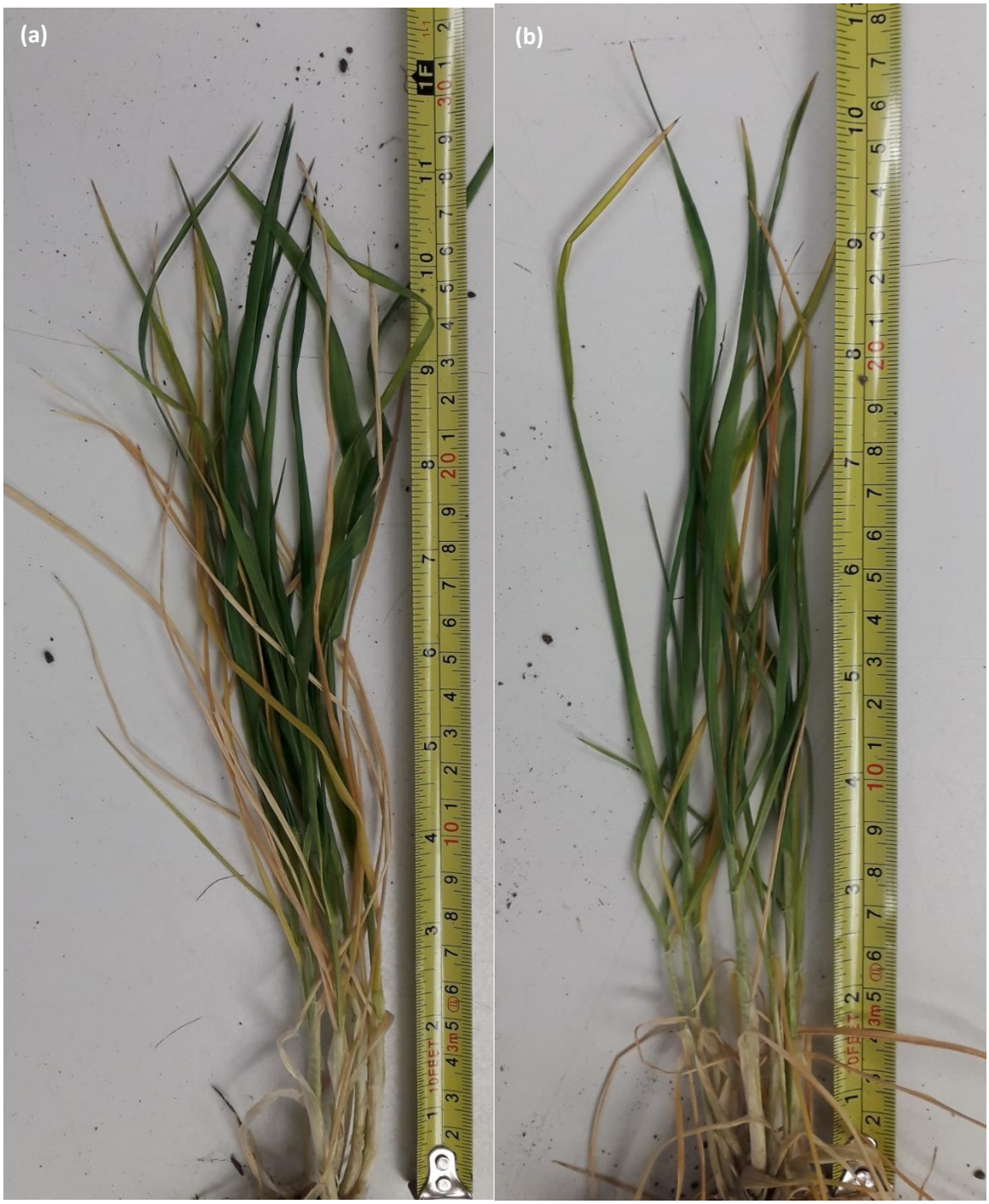

Figure 8.39 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of combined *B. subtills* and *R. intraradicies* inoculated soils in (a) ZT and (b) CT sampled soils

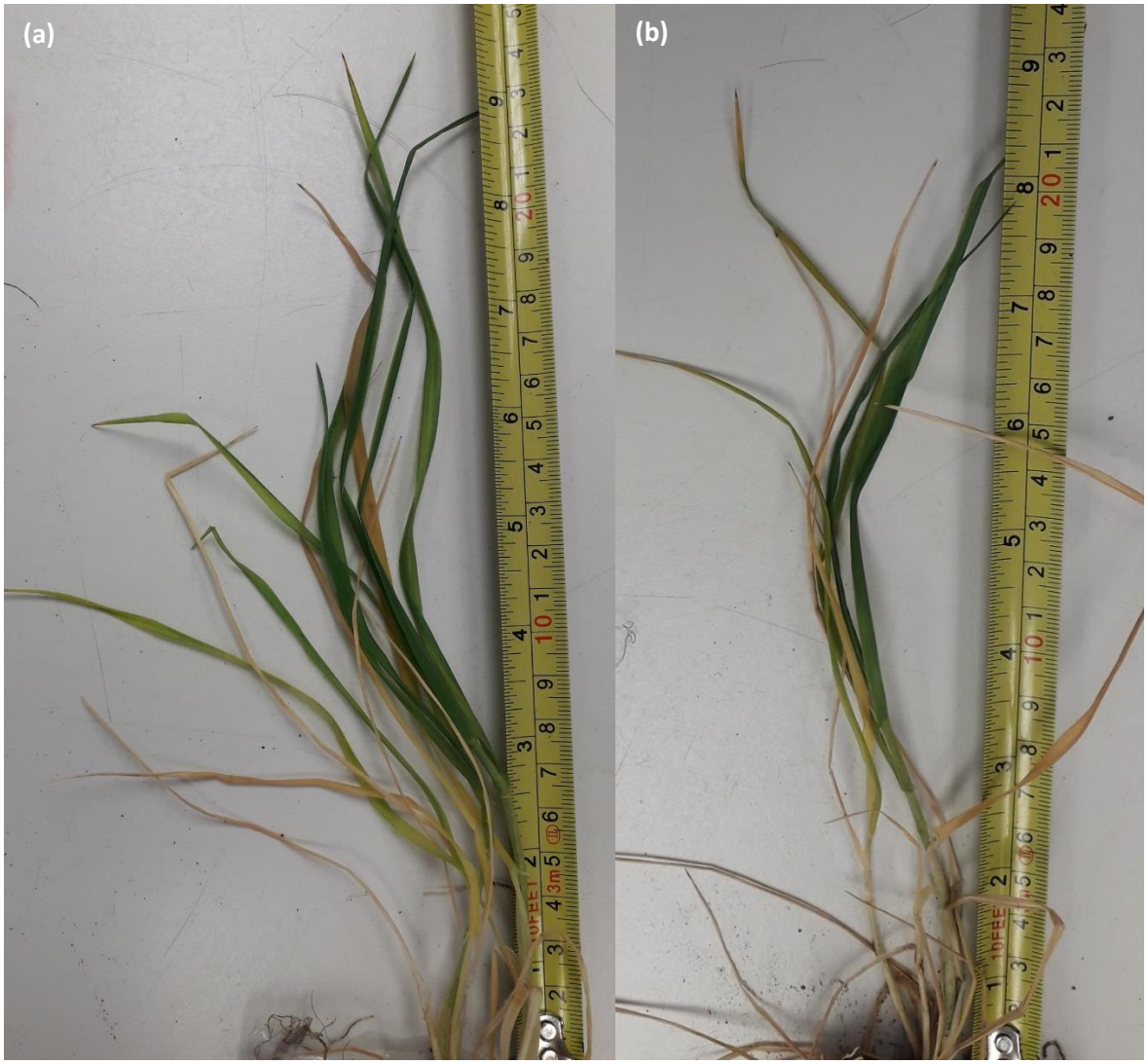

Figure 8.40 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of combined *B. pumilus* and *R. intraradicies* inoculated soils in (a) ZT and (b) CT sampled soils

Figure 8.41 - Week 15 sampled wheat from controlled glasshouse conditions for tiller length of combined *B. amyloliquefaciens* and *R. intradicies* inoculated soils in (a) ZT and (b) CT sampled soils

8.16 Correlated relationships between AM fungi and wheat biomass

Week 30 samples of glasshouse controlled growth of Zulu winter wheat inoculated with *B. subtills, B. pumilis, B. amyloliquefaciens* or *R. intraradices* (AM fungi) on collected soils from either CT or ZT sampling sites.

Correlations between AM fungal arbuscular structures and plant biomass, as measuorange by tiller length, number of tillers and root length, was seen to produce similarities with earlier week 15 sampling. Applications of *B. amyloliquefaciens* increased AM fungal arbsuscles and saw a correlated decrease in root length, increase in tiller length and numbers. This, however, is not the case with *B. subtilis* that increased root length and had limited influence on wheat biomass attributes.

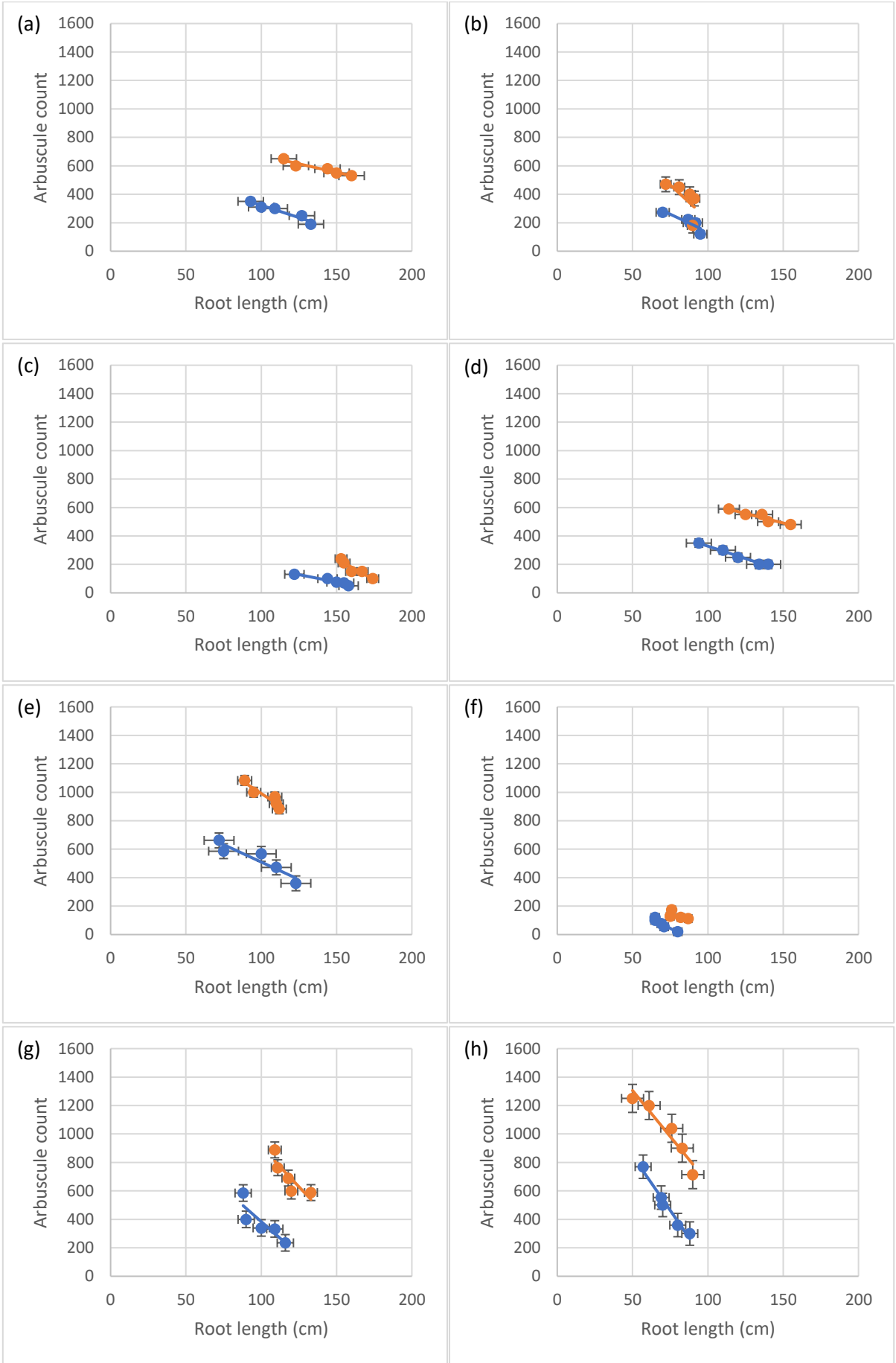

Figure 8.42 - Zulu wheat at week 30 indicating negative correlations between root length in ZT (orange) ($P < 0.00001$, Single factor ANOVA) and CT (blue) ($P < 0.00001$, Single factor ANOVA) with an average (n = 400) root cortical arbuscules ($P < 0.00001$, Single factor ANOVA) in ZT and CT ($P < 0.00001$, Single factor ANOVA). Within ZT soil samples, (a) control samples received no inoculant (Pearson's correlation: -0.96, r^2 0.93). (b) AM fungi, Pearson's correlation: -0.69, r^2 0.45. (c) *B.subtilis*, Pearson's correlation: -0.94, r^2 0.89. (d) *B.pumilis*, Pearson's correlation: -0.94, r^2 0.89. (e) *B.amyloliquefaciens*, Pearson's correlation: -0.93, r^2 0.87. (f) AM fungi and *B.subtilis*, Pearson's correlation: -0.58, r^2 0.34. (g) AM fungi and *B.pumilis*, Pearson's correlation: -0.83, r^2 0.73. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: -0.96, r^2 0.92. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: -0.97, r^2 0.93). (b) AM fungi, Pearson's correlation: -0.87, r^2 0.76. (c) *B.subtilis*, Pearson's correlation: -0.97, r^2 0.93. (d) *B.pumilis*, Pearson's correlation: -0.99, r^2 0.98. (e) *B.amyloliquefaciens*,

Pearson's correlation: -0.93, r^2 0.87. (f) AM fungi and *B.subtilis*, Pearson's correlation: -0.96, r^2 0.92. (g) AM fungi and *B.pumilis*, Pearson's correlation: -0.87, r^2 0.75. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: -0.98, r^2 0.97.

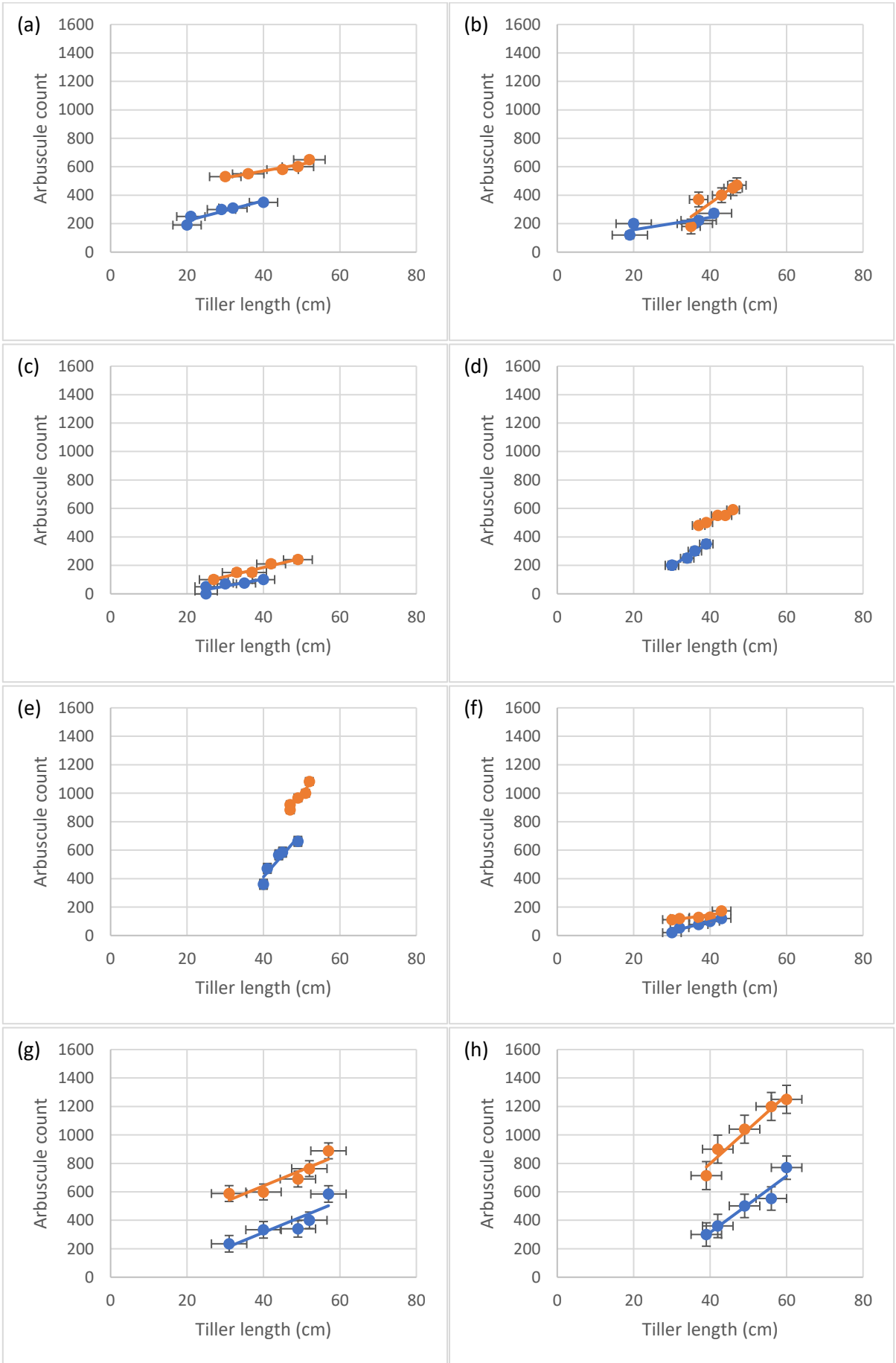

Figure 8.43 - Zulu wheat at week 30 indicating positive correlations between tiller length in ZT (orange) (P = 0.06, Single factor ANOVA) and CT (blue) (P < 0.00001, Single factor ANOVA) with an average (n = 400) root cortical arbuscules (P < 0.00001, Single factor ANOVA) in ZT and CT (P < 0.00001, Single factor ANOVA). Within ZT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.94, r^2 0.89). (b) AM fungi, Pearson's correlation: 0.88, r^2 0.78. (c) *B.subtilis*, Pearson's correlation: 0.98, r^2 0.96. (d) *B.pumilis*, Pearson's correlation: 0.98, r^2

0.96. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.96, r^2 0.92. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.86, r^2 0.74. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.92, r^2 0.84. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.98, r^2 0.96. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.94, r^2 0.88). (b) AM fungi, Pearson's correlation: 0.81, r^2 0.65. (c) *B.subtilis*, Pearson's correlation: 0.96, r^2 0.93. (d) *B.pumilis*, Pearson's correlation: 0.99, r^2 0.99. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.95, r^2 0.89. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.98, r^2 0.96. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.88, r^2 0.96. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.96, r^2 0.95.

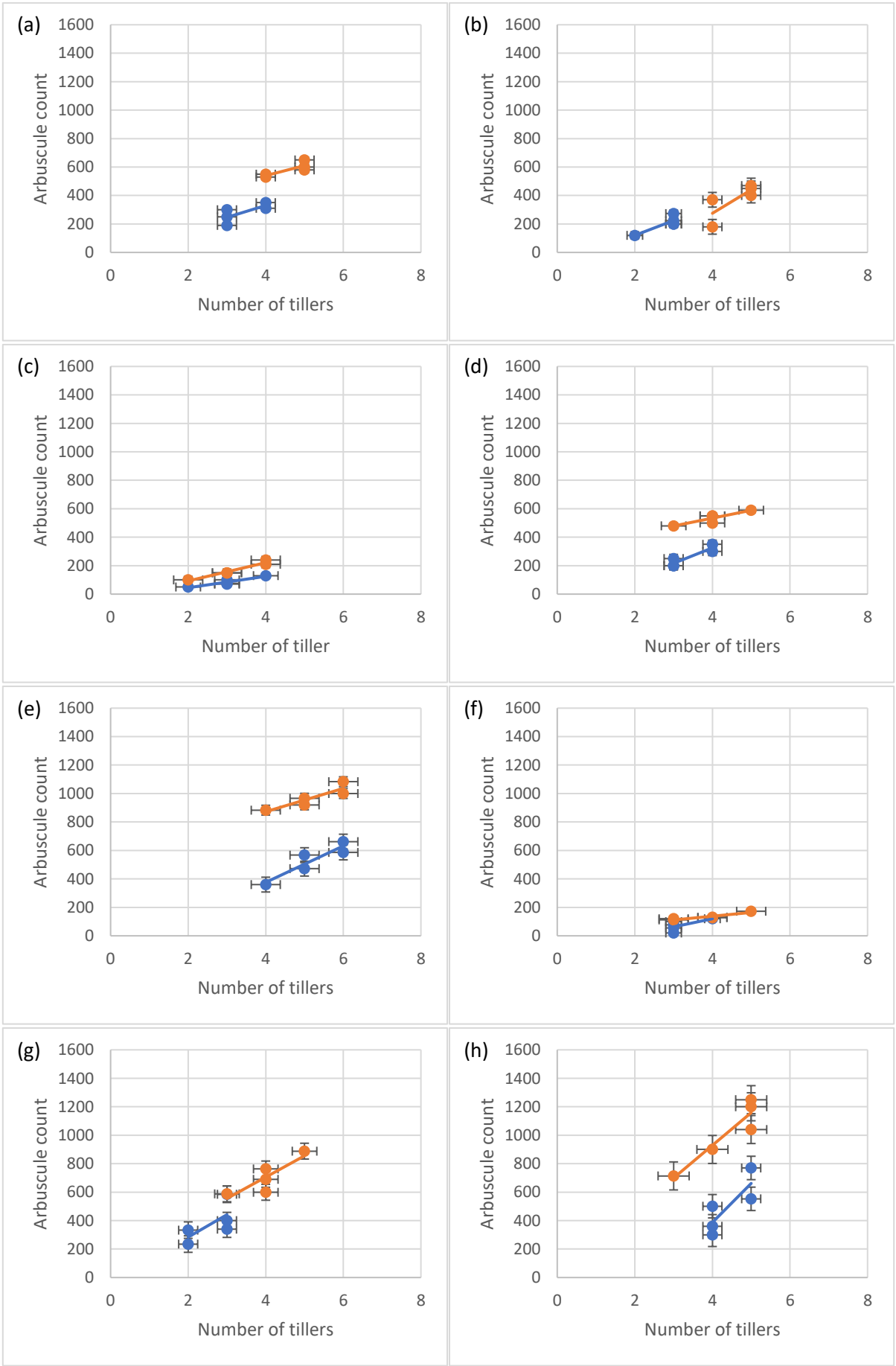

Figure 7.44 - Zulu wheat at week 15 indicating positive correlations between tiller numbers in ZT (orange) (P = 0.01, Single factor ANOVA) and CT (blue) (P = 0.01, Single factor ANOVA) with an average (n = 400) root cortical arbuscules (P < 0.00001, Single factor ANOVA). Within ZT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.82, r^2 0.68). (b) AM fungi, Pearson's correlation: 0.78, r^2 0.61. (c) *B.subtilis*, Pearson's correlation: 0.97, r^2 0.95. (d) *B.pumilis*, Pearson's correlation: 0.89, r^2 0.78. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.89, r^2 0.79. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.93, r^2 0.86. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.85, r^2 0.73. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.93, r^2 0.87. Within CT soil samples, (a) control samples received no inoculant (Pearson's correlation: 0.74, r^2 0.55). (b) AM fungi, Pearson's correlation: 0.84, r^2 0.71. (c) *B.subtilis*, Pearson's correlation: 0.92, r^2 0.84. (d) *B.pumilis*, Pearson's correlation: 0.91, r^2 0.82. (e) *B.amyloliquefaciens*, Pearson's correlation: 0.92, r^2 0.85. (f) AM fungi and *B.subtilis*, Pearson's correlation: 0.65, r^2 0.43. (g) AM fungi and *B.pumilis*, Pearson's correlation: 0.67, r^2 0.44. (h) AM fungi and *B.amyloliquefaciens*, Pearson's correlation: 0.82, r^2 0.67.

8.17 Soil extracted DNA confirmation

Extracted soil DNA was examined under NanoDrop® for confirmation of extracted nucleic acid.

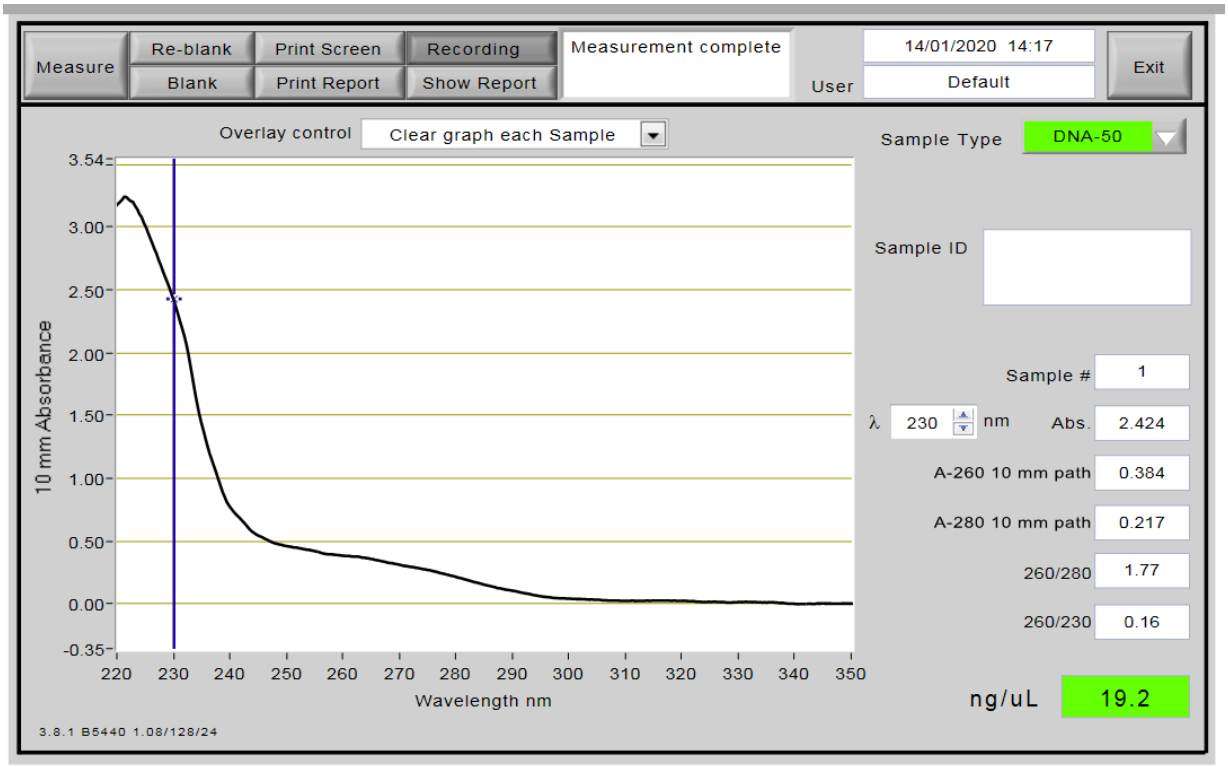

Figure 8.45 – NanoDrop confirmation of the presence of extracted soil DNA

8.18 qPCR thermal cycles

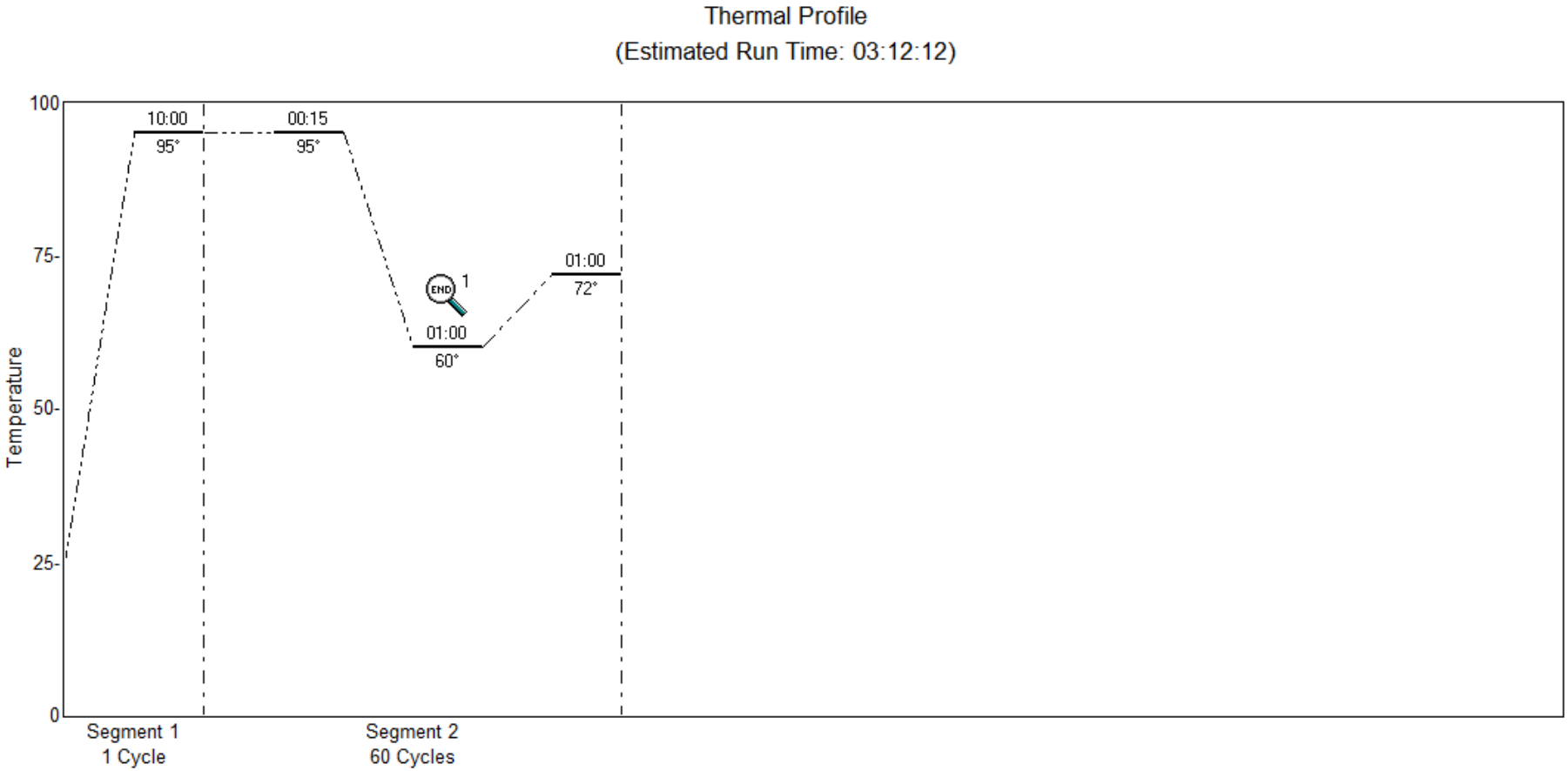

Figure 8.46 - qPCR thermo cycling design

8.19 qPCR standard curve

qPCR standard curve produced from known dilutions of nucleic acid

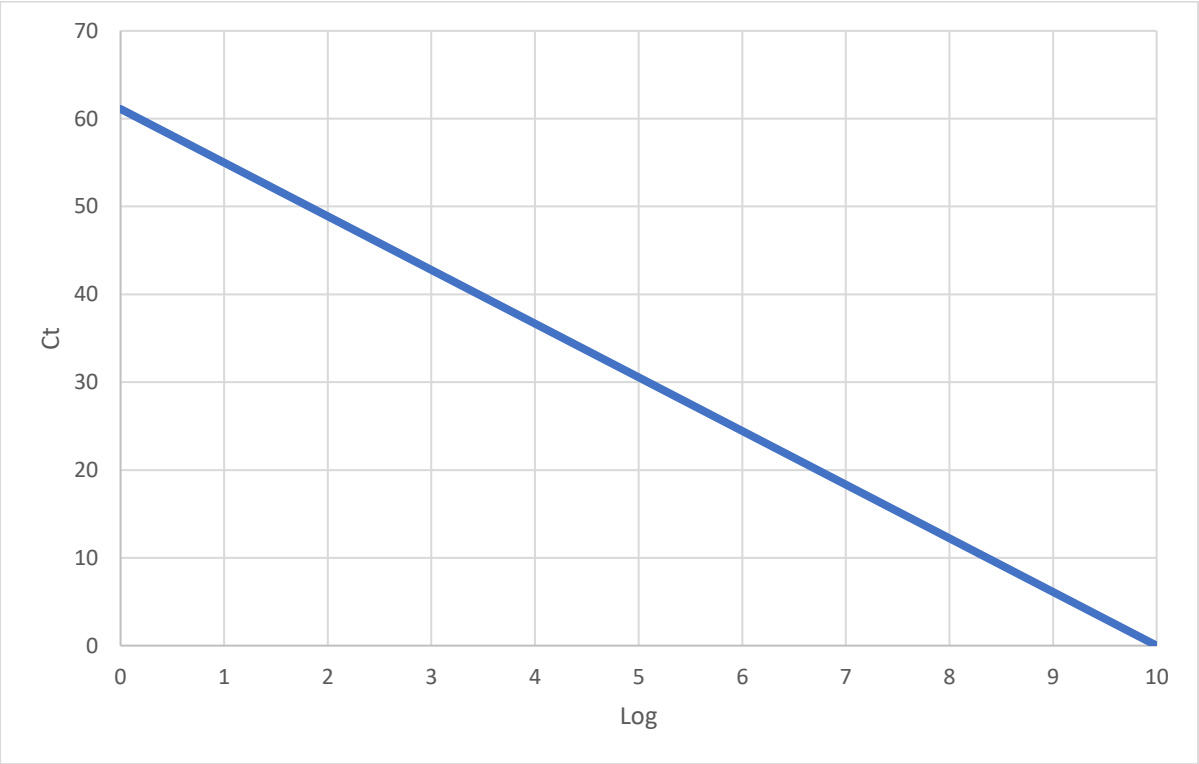

Figure 8.47 – Standard curve for qPCR analysis

9.0 Publications and Posters

ACCESS MICROBIOLOGY

METHOD
Wilkes et al., Access Microbiology
DOI 10.1099/acmi.0.000083

MICROBIOLOGY SOCIETY

MICROBIOLOGY

A comparison of methodologies for the staining and quantification of intracellular components of arbuscular mycorrhizal fungi in the root cortex of two varieties of winter wheat

Thomas I. Wilkes*, Douglas J. Warner, Veronica Edmonds-Brown, Keith G. Davies and Ian Denholm

Abstract

Arbuscular mycorrhizal (AM) fungi are one of the most common fungal organisms to exist in symbiosis with terrestrial plants, facilitating the growth and maintenance of arable crops. Wheat has been studied extensively for AM fungal symbiosis using the carcinogen trypan blue as the identifying stain for fungal components, namely arbuscles, vesicles and hyphal structures. The present study uses Sheaffer blue ink with a lower risk as an alternative to this carcinogenic stain. Justification for this is determined by stained wheat root sections (n=120), with statistically significant increases in the observed abundance of intracellular root cortical fungal structures stained with Sheaffer blue ink compared to trypan blue for both Zulu ($P=0.003$) and Siskin ($P=0.0003$) varieties of winter wheat. This new alternative combines an improved quantification of intracellular fungal components with a lower hazard risk at a lower cost.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are the most common fungal organisms to exist symbiotically with the root structures of vascular plants [1]. It is currently thought that the closely established relationship between plant and fungi contributed to early plant colonization of land [2]. To plants, AM fungi provide increases in nutrients and water through large branching mycelial networks and large surface areas resultant of intracellular components within root cortical cells [3]. In exchange, the plant provides photosynthetic products [4], such as carbohydrates [5]. Within arable farming, this aids the improved use of applied fertilizers, reduction of disease, resistance to salt stress and salinity, improved drought tolerance and improvements to crop quality [6].

Current staining procedures target arbuscules, vesicles and hyphal structures within the root cortex. Staining of target structures is performed for rapid, simple and cost-effective assessment of fungal symbiosis. Using light microscopy, the required skill sets are lower and the procedure can be performed with ease. Lactophenol cotton blue ($C_{20}H_{17}N_2Na_2O_9S_2$) is one of several stains that has been widely utilized for many years [7]. However, a move to the use of trypan blue ($C_{34}H_{28}N_6O_{14}S_4$), originally developed by Phillips and Hayman (1970) [8], has improved the clarity of characteristic AM fungal components [9]. To increase the selectiveness of trypan blue towards fungal root structures, the employment of a formaldehyde fixative solution to preserve plant tissue is required. A comparison of 14 different AM fungal staining methods developed between 1970 and 2014 typically used formaldehyde as a fixative solution to stabilize the plant cells in combination with trypan blue [10]. With the employment of a heat treatment, root fungal structures can be deliberately damaged to allow trypan blue to enter fungal cells. The fixing of plant tissues reduces damage from heat treatment, allowing trypan blue to have increased sensitivity towards intracellular fungal root components. Without formaldehyde, trypan blue would stain all cells in the sample, preventing the differentiation of individual fungal components. By selectively damaging the fungal structures, trypan blue becomes more effective.

Many widely used fungal root stains, including trypan blue, are known carcinogens [11]. As a consequence, many practitioners experienced in the biological staining of fungal root components have been searching for alternative dyes and

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Keywords: AM fungi; trypan blue; Sheaffer blue; wheat; staining.
Abbreviations: AM, Arbuscular Mycorrhizal; PBS, Phosphate Buffered Saline; SB, Sheaffer Blue; TB, Trypan blue.
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stains. Trypan blue, however, is still a widely used stain for root cortical fungal structures. Tsaousis *et al.* [12] investigated the effects of trypan blue on human trabecular cells for time-dependant toxicity. Their study was able to show that damage to living tissues occurred after an exposure time of 60 s. The desire to move away from trypan blue, lactophenol blue and other dyes comes from their potential carcinogenic properties and long-term hazards to human health. It is acknowledged that the fixative formaldehyde is also carcinogenic, but this is removed during the staining process when the samples are washed prior to autoclaving. The focus of this study is the stain itself, which remains in the plant tissue after the process has been completed.

The use of an ink–vinegar stain has been proposed as a safer alternative. The chemical composition of the ink–vinegar stain is not stated by [11, 13], although a key component is vinegar or acetic acid (CH_3COOH). The constituents of commercially available fountain pen ink were reported by [14] as being primarily elemental carbon (48%), high carbon-containing organic compounds (23%), sodium sulphate (16%), calcium sulphate (7%), potassium sulphate (4%) and 1% 'other' (iron sulphate, copper and zinc). This approach to the staining of fungal components was reported previously, two decades ago, by Vierheilig *et al.* [11]. They initially compared the staining of root samples from several species of arable crop, including beans (*Phaseolus vulgaris*), barley (*Hordeum vulgare*), cucumber (*Cucumis sativus*) and wheat (*Triticum aestivum*). Vierheilig *et al.* [11] reported that the stain was of sufficient quality to enable he identification of a difference in root fungal components between the crops studied. The use of an ink–vinegar stain has not, however, gained in popularity in the years that followed the publication of Vierheilig *et al.*'s work [11]. The reasons for this are not entirely clear. Extracellular hyphae from *Rhizoctonia cerealis* inoculation were stated as being observable on wheat roots, but the authors do not present the data in any further detail. Another potential factor that may explain the lack of more wide-scale adoption is that in subsequent years, focus shifted toward the immunological identification of characteristic intracellular root cortical fungal structures [13].

Immunohistochemical (IHC) methodologies are advantageous due to their higher specificity and the reduced damage caused to histological architecture [15]. When processing larger volumes of samples, however, chemical staining is preferred due to higher throughput, ease of use and fewer training requirements for the user. Both approaches confer advantages and disadvantages. Meanwhile, the ink–vinegar stain method originally reported by Vierheilig *et al.* [11] has remained largely ignored. The re-evaluation and further development of this method are therefore overdue.

The present study compares the efficacy of trypan blue and Sheaffer blue ink as stains of intracellular AM fungal components in the root sections of two varieties of winter wheat (Zulu and Siskin). The focus is on image clarity, quantifiable structures (arbuscules and vesicles) and the potential for the substitution of a hazardous staining material with a safer alternative.

Impact Statement

The present study investigates an alternative staining method for root cortical intracellular vesicles and arbuscules of mycorrhizal fungi, as a safer and more robust replacement for carcinogenic azo dye trypan blue stain. Household vinegar and inks used in pens have been trialled and have met with varying degrees of success. However, none have been adopted widely as a simple and cost-effective staining protocol. The present study compared the efficacy of a novel staining method (Sheaffer blue ink) with the more commonly used trypan blue approach. The results suggest that this approach is not only comparable to the trypan blue one, but confers an advantage through improved stain clarity. A move towards an ink–vinegar stain is both safer for human health and more cost-effective.

METHODS

Seed variety

Winter wheat (variety: Siskin), 98% germination rate, with no chemical pretreatment was supplied by KWS UK Ltd. A second winter wheat variety (Zulu) was sourced from a farm in central Hertfordshire as farm-saved seed. The percentage of organic matter of the adjusted soil was confirmed via modified loss on ignition (LOI) methodologies obtained from Myrbo *et al.* [16], using 5 g of adjusted soils heated at 400 °C for 12 h. Soils were adjusted through the addition of J Arthur Bowers multipurpose compost to correspond with he measured percentage of organic matter of field-tested farm topsoil equating to 5%.

Growth conditions

Individual seeds were introduced into 300 g of adjusted, pre-purchased, top soils (J Arthur Bowers) and kept in controlled growth room conditions at 25 °C, 1770 lm and a humidity of 35%.

Sample preparation and staining

Intracellular root arbuscules and vesicles were examined after the roots were left fully submerged in a formaldehyde, acetic acid, alcohol (FAA) and deionized water solution, 10:5:50:35, respectively, for 24 h. Roots were removed and rinsed with deionized water prior to autoclaving. Root systems, containing small quantities of soils, were subject to sonication at 42 kHz for 10 min and rinsed in deiontized water. If small amounts of soils still adhered to root systems, a soft fine paint brush was used to remove debris. Root systems were submerged in 5% hydrochloric acid for 30 min and incubated at 60 °C in a water bath. After cooling to room temperature, root material was sectioned into 1 cm pieces, with adjacent sections

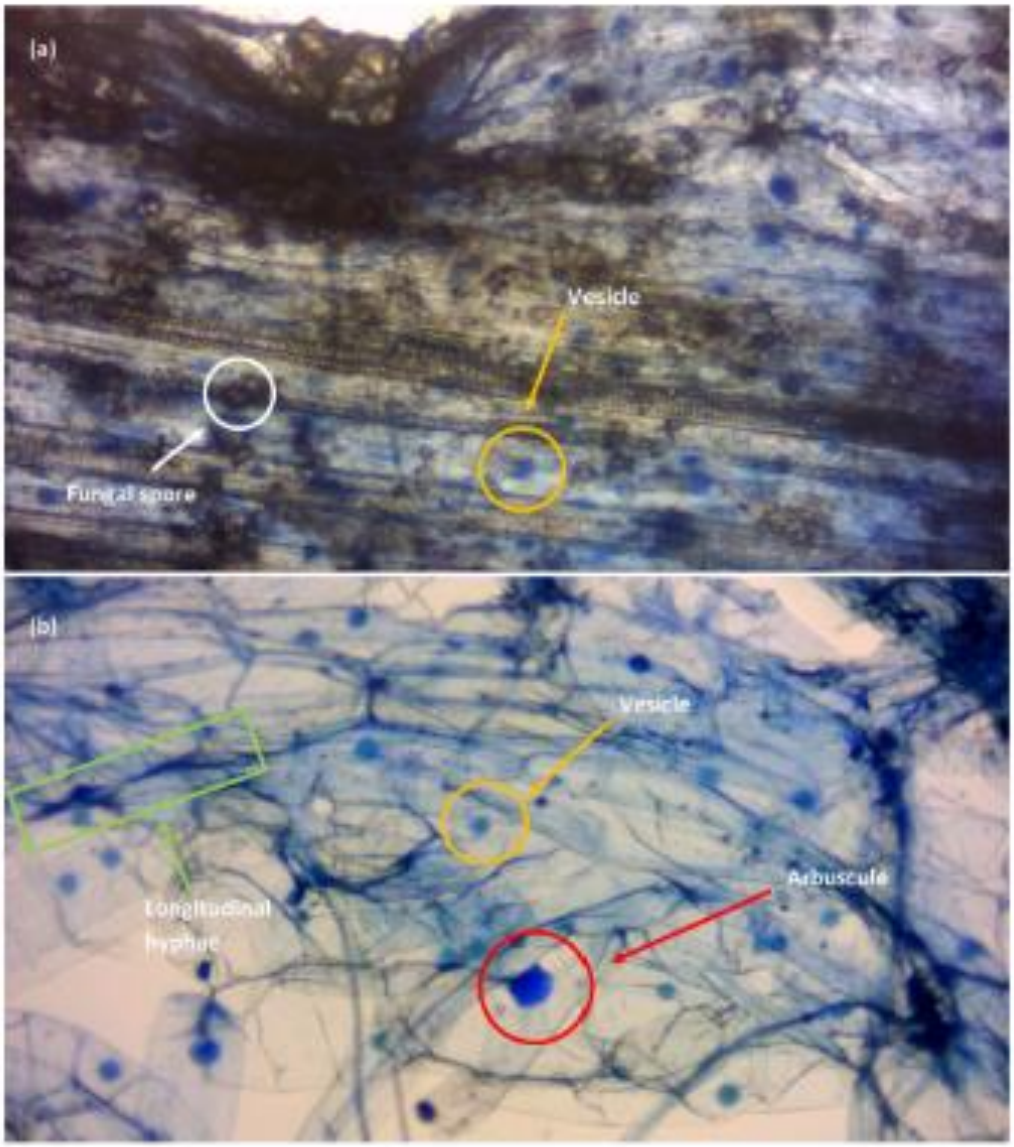

Fig. 1. Week-old Zulu variety wheat stained with (a) 0.4% trypan blue in PBS and (b) 10% Sheaffer blue in 25% acetic acid. Fungal vesicles are clearly stained as defined blue spheres, and fungal spores have not been stained and show as transparent brown circles. In (a) trypan blue in PBS was unable to stain longitudinal and radiating hyphal structures. The larger stained structure of (b) was later identified as an intracellular arbuscule after marginal destaining. Images were recorded using a Bresser HD microscope camera under a total magnification of 100× of a Vickers compound microscope.

subjected to different stains. Five 1 cm root sections were each allowed to stain in 0.4% trypan blue in phosphate-buffered saline (PBS) (Fisher Scientific) and 10% Sheaffer blue ink in 25% glacial acetic acid [11] for 3 min. Samples were produced over a 6-week period from Zulu (n=60) and Siskin (n=60) wheat varieties in controlled growth conditions. The samples were viewed initially at a total magnification of 40× using a Vickers compound microscope. The counting of stained root vesicles and arbuscules was performed at a total magnification of 100×, and fungal components were counted and recorded with the focus on arbuscule and vesicle quantity. Images of samples were taken with a Bresser HD microscope camera.

Statistics

Standard errors and means were calculated from raw data for each week of sample collection. Paired t-tests were employed for null hypothesis testing of differences between trypan blue and Sheaffer blue staining. Statistical significance was determined by P values ≤0.05.

RESULTS

The difference in clarity of the stained root sample between the trypan blue and Sheaffer blue ink approaches can be seen

Table 1. A comparative of trypan blue and Sheaffer blue stains for AM fungal root structures and components from stained samples (n=120), observable under a Vickers compound microscope at a total magnification of 100×

Observable components	Trypan blue	Sheaffer blue
Arbuscules	+/−	+
Vesicles	+	+
Longitudinal hyphae	−	+
Radiating hyphae	−	+
Fungal spores	+	−

in Fig. 1a, b. Root-associated fungal spores are shown as light brown spheres in Fig. 1a. These obscure the image for the accurate counting of vesicles and arbuscules, and risk being included within the final count of stained fungal structures. Fig. 1a, b shows the clarity of sample from 1 cm sections adjacent to each other of the same root of the same plant. The degree to which the clearing of roots was carried out was identical for all samples and can be eliminated as a variable – i.e. this was not the cause of the absence of spores in Fig. 1b.

From the samples examined (n=120), staining with trypan blue did not produce sufficient clarity (i.e. quantifiably observable fungal root components) in comparison to staining with Sheaffer blue for the two varieties of winter wheat investigated. Quantification of fungal spores is possible from the images presented in Fig. 1a. The observational characteristics are summarized in Table 1.

Under testing, the null hypothesis, that there are no measurably significant differences between the staining techniques, was not upheld from a paired t-test for Zulu (degrees of freedom (df)=5, t value=−4.5, P=0.003) and Siskin (df=5, t value=−7.5, P=0.0003) (Fig. 2) varieties of winter wheat. Fig. 2 shows an increase in the difference between fungal components (stained arbuscules) as the age of he root systems increases. The variation in the standard error of the mean (SEM) for those samples stained with trypan blue (Fig. 2) reflects the greater variation in the number of AM fungal root structures identified during each sampling week.

DISCUSSION

The present study has identified a statistically significant difference between staining techniques with respect to observable intracellular root cortical fungal components. The employment of Sheaffer blue ink over trypan blue is more favourable in terms of both improved image clarity and reduction of the user's exposure risk to chemicals with potential long-term health hazards. As an azo stain, trypan blue is a known carcinogen [17, 18]. Sheaffer blue, on the other hand, is a commercially available pen ink that is much safer. This potentially makes it a far more widely accessible and cost-effective technique.

Although subject to scrutiny in previous studies, the use of ink–vinegar as a staining method has not been adopted widely. In 1998, Vierheilig *et al.* [11] investigated a range of coloured inks for the staining of AM fungus-associated root structures. They commented that ink–vinegar staining allowed the observation of extracellular hyphae on wheat roots inoculated with *Rhizoctonia cerealis*, but did not present the data or images to substantiate the comment in any further detail. Vierheilig *et al.* [11] also used different ink colours, for example black, which were possibly not as effective as the blue ink used in the analysis here. Coupled with a simultaneous shift in interest

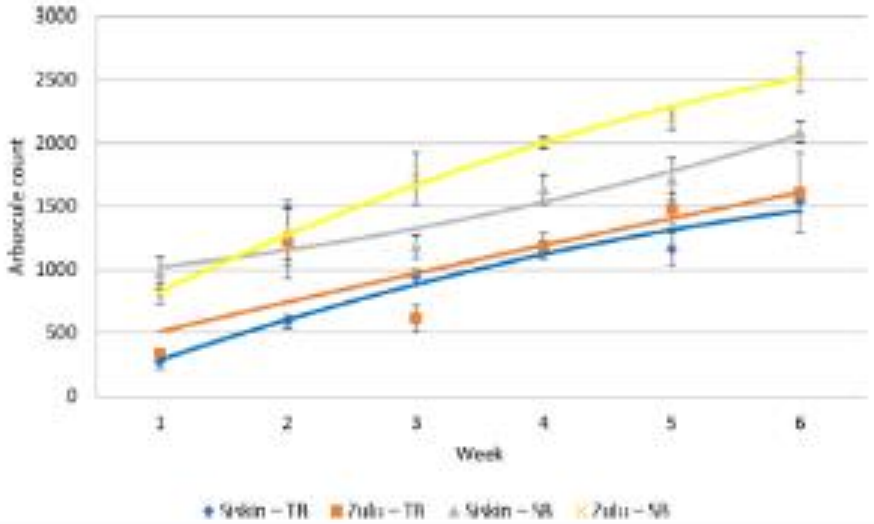

Fig. 2. Mean AM fungal root components (n=5) per week for comparison between AM fungal stains of Zulu and Siskin varieties of winter wheat using Sheaffer blue (SB) and trypan blue (TB) over a period of 6 weeks (n=120). Paired t-tests showed significant differences between the staining techniques for Zulu (P 0.003) and Siskin (P = 0.0003) varieties. The error bars were constructed from the sem.

toward the use of IHC techniques, this may explain the lack of more widescale adoption of the ink–vinegar approach post-1998. Importantly, it contradicts the findings of the current study. Hyphal components were clearly visible in all 60 root samples stained with Sheaffer blue ink. Trypan blue stain, on the other hand, did not provide adequate clarity to allow identification of the same hyphal structures. The clarity of arbuscules and vesicles was hindered using trypan blue due to fungal spores obscuring these structures in the sample. This did not occur when using Sheaffer blue as a stain.

Cottet *et al.* [10] compared 14 different methods of AM fungal staining developed between 1970 and 2014. Each method used a form of fixative solution to stabilize the plant cells, with FAA solution being the most common, while the concentration of trypan blue varied. The microscopy images presented for each method evaluated do not demonstrate image clarity comparable to that in images featuring samples stained with Sheaffer blue (illustrated in Fig. 1b of this study). It is acknowledged that Cottet *et al.* [10] studied the staining of AM fungal arbuscules and vesicles in bryophytes as opposed to the staining of wheat, a monocotyledon angiosperm. The findings of Cottet *et al.* [10], and those of Vierheilig *et al.* [11, 13], who analysed other crop species (namely wheat, barley, beans and cucumber), demonstrate that individual staining methodologies can be applied to a range of plant species. The evaluation of Sheaffer blue as a stain for roots in a broader range of crop types will be investigated in the future.

The clearing of soil materials from roots is an important first step in the preparation of stained samples due to the desired components being potentially obscured by debris. The present study used adjacent root sections. This negates any differences from root clearing. In most cases, roots are cleared with the use of 10% w/v potassium hydroxide [19]. Whilst this method does remove debris and leave root ready to be processed further for staining, the use of potassium hydroxide reduces the structural integrity of the root cells by chemical degradation of the cell wall [19]. As suggested by Dodd *et al.* [20], the employment of 10% w/v potassium hydroxide solution should be reserved for root cells that are highly pigmented. The present study utilized plants grown under controlled conditions and did not produce highly pigmented root structures. The data presented by Vierheilig *et al.* [11, 13], Kobae [19] and Cottet *et al.* [10] used plant materials from environmental sources and saw pigmented root cells. In the case of environmental samples, a potassium hydroxide solution would be suitable. More relevant to the present study, root clearing via sonification was sufficient to achieve a level of debris removal to allow further sample processing and staining quantification. By using sonification, the practitioner is removed from a highly corrosive solution, leading to a lower-hazard procedure.

An area of limitation within the method used arises from the manual counting of stained fungal components and the time input required. Although potentially faster approaches exist for the quantification of fungal structures, employing image analysis software, the programs are only able to scan the field of view for objects that are different to the background image. There is a high risk of misidentification and classification of structures and hence this was not considered to be a sufficiently reliable approach for the purpose of the current study.

Ford and Becker [21] produced data, from a study with Wistar rats, indicting that trypan blue had mutagenic and carcinogenic implications for reticuloendothelial neoplasm (RES), predominantly in liver cells. In later years, Kwok *et al.* [22] investigated he toxicity of trypan blue against retinal pigment epithelium (RPE) in cell culture using three concentrations of trypan blue, and discovered reduced cell viability in those treated with trypan blue. The present study suggests a move away from the use of trypan blue staining in plant root cells. No current literature is able to indicate the toxicity or carcinogenic properties of pen ink, indicating a less hazardous alternative to the widely used trypan blue.

In conclusion, the employment of Sheaffer blue staining allows for the effective, safe and low-cost quantification of fungal components in commercially important plant species such as wheat. The manual handling of slides, whether old or new, comes with reduced long-term health risks when stained with Sheaffer blue as opposed to the carcinogenic azo dye trypan blue. Further, the number of fungal components that obstruct viewing are limited, resulting in a more reliable quantification of the established AM fungal infection and symbiosis within the roots of wheat plants.

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Author contributions
T.W.: experimental design, practical work, data analysis and manuscript writing. Co-authors: project supervision and advice, manuscript editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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An exploratory study into the relationship between soil glomalin concentrations and AM fungal biomass

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Key words: Fungal biomass, glomalin, ergosterol, pH, AM fungi, arable soil

Introduction

- Arbuscular Mycorrhizal (AM) fungi have evolved a strong symbiotic relationship with approximately 80% of all vascular plant families (Schubler et al, 2001).
- Glomalin is a glycoprotein abundant on mycorrhizal hyphae and contributes to total soil carbon, as well as water stable aggregates (Wang et al, 2017).
- Lower pH reduces the soils ability to retain carbon and moves away from optimal growth (pH 6.3) conditions of the AM fungus *Rhizophagus* spp. (Blaszkowski et al, 2008).
- The present study aims at testing the relationship between soil glomalin and ergosterol over the optimal pH range of *Rhizophagus* spp.

Methods

- Top soils, from the University of Hertfordshire's environmental field station, were pH adjusted to a range of 4 – 9 and inoculated with previously isolated *Rhizophagus intraradices* (identified via Blaszkowski et al (2008)).
- Samples were produced weekly over 6 weeks
- Inoculated soils were dried at 40°C and glomalin quantified via a citrate extraction, modified from Wright and Upadhyaya (1996)
- AM fungal biomass was produced following HPLC methodologies from Mille-Lindblom et al (2004)

Results

- Increased glomalin concentrations are seen in pH values 6 and 7 (Figure 1), with increased concentrations of ergosterol seen at the same pH values (Figure 2). This conforms with optimal growth pH of AM fungi.
- pH 5, from Figure 3, visually presents similarities to pH 7. Graphical data from Figure 2 supports similarities between ergosterol from pH 5 and 7.
- Presented data indicates a strong correlation between ergosterol and glomalin concentrations in soils from Pearson's coefficient testing, resulting in 0.92 and 0.96 for pH values 6 and 7 respectively (Figure 4).
- Optimal growth pH of *Rhizophagus intraradices* is between pH 6 and 7 as indicated by Figure 2 ergosterol values and visual representation of Figure 3. Supported by the work of Blaszkowski et al (2008).
- Ergosterol concentrations are significantly reduced at pH 4 and 9 (0.04 and 0.01 respectively). Pearson coefficient is less strong (0.49 and 0.25) compared with optimal pH values.

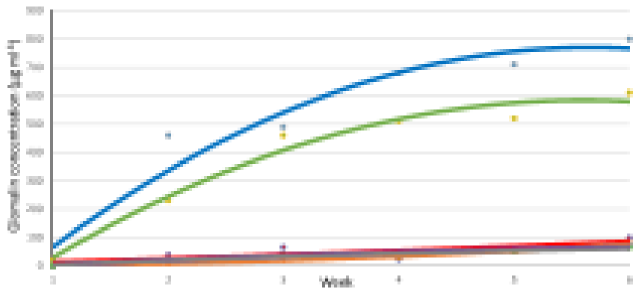

Figure 1 – Average soil glomalin concentration (n=3) measured over a 6 week period for pH values ranging from 4 – 9. Red; pH4. Orange; pH5. Blue; pH6. Green; pH 7. Purple; pH8. Grey; pH9

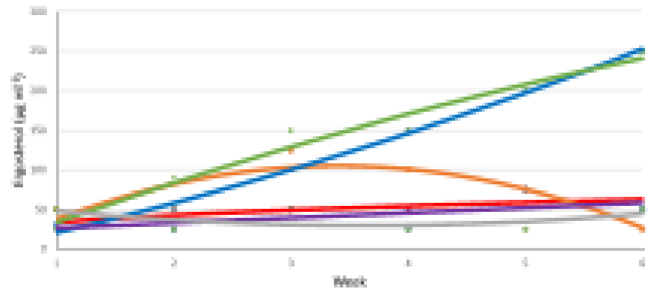

Figure 2 – Extracted soil ergosterol following Mille-Lindblom et al (2004) and identified via UV HPLC for each pH range across 6 weeks of sampling. Red; pH4. Orange; pH5. Blue; pH6. Green; pH 7. Purple; pH8. grey; pH9

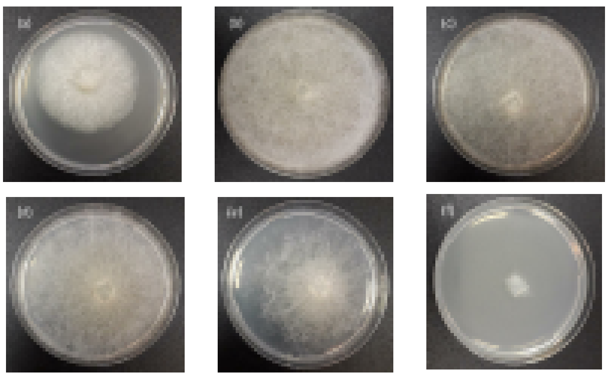

Figure 3 – Visual representation of *Rhizophagus intraradices* grown on pH adjusted solidified nutrient broth for 2 weeks. (a) pH4, (b) pH5; (c) pH6, (d) pH7, (e) pH8, (f) pH9

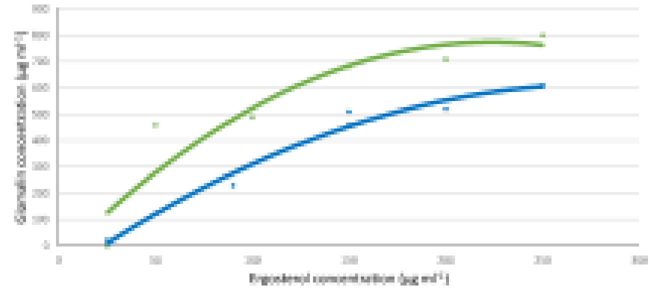

Figure 4 – A positive correlation between glomalin and ergosterol concentrations for pH 6 (green) and pH 7 (blue). Pearson coefficient testing indicates pH 6 and 7 have strong correlations at 0.92 and 0.96 respectively.

Conclusion

- Strong correlations between soil glomalin and fungal ergosterol are present over optimal pH values (6 and 7) of *Rhizophagus intraradices*.
- *R.intraradices* exhibits reductions in glomalin production away from optimal pH range.
- Fungal growth is significantly reduced at pH 9 and 4

Future developments

- Figure 2 shows ergosterol concentrations from pH 6 increasing beyond those concentrations of pH at week 6. Extended study would be beneficial in order to extrapolate continued trend.
- Testing pH values between 6 and 7 would provide greater insights into the relationship between glomalin and ergosterol.

References

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Soil glomalin and the glomalin-nitrate relationship: impact of tillage

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Introduction

- Arbuscular Mycorrhizal (AM) fungi form close symbiotic relationships with an estimated 80% of vascular plants.
- Some inorganic soil nutrients (for example phosphorus) require converting into plant useable compounds via AM fungi (Begum et al., 2019). Nutrients are exchanged at intracellular fungal arbuscules within the root cortex of the host plant (Figure 1). In return AM fungi receive plant derived photosynthetic carbohydrates from the plant-fungi symbiotic relationship.
- Agricultural practices such as tillage have a potentially strong influence on the soil microbiome, and in particular AM fungi (Kabir, 2005).
- Glomalin, a glycoprotein produced by AM fungi, consists of approximately 30 – 40% carbon and has adhesive properties towards water stable soil aggregates (WSAs), thereby increase the stability of the soils and reducing the risk of soil erosion (USDA, 2002).
- Conventional tillage (CT) inverts the soil. During this process AM fungal hyphae and glomalin may be ripped apart, diminishing their adhesive properties and the quantity of soil aggregates, increasing the risk of soil erosion compared to that of a zero tillage (ZT) system.
- The exact nitrogen composition of glomalin is yet to be determined although Schindler et al. (2007) estimate it to be 3 to 5%. In total, it is estimated that glomalin contributes 5% of soil carbon and nitrogen (Lovelock et al., 2004).

Aims

- To evaluate if tillage systems have an impact on soil glomalin levels.
- To investigate the relationship between soil nitrogen levels in relation to the presence of glomalin.

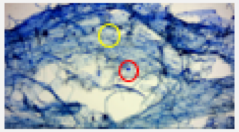
Figure 1 – (Red) AM fungal arbuscule and (Yellow) AM fungal vesicle in the root cortex of 2-4x winter wheat. (Wilkes et al., 2019)

Methods

Field sampling: two farms within central Hertfordshire, practicing no till or conventional till exclusively, were sampled pre-seed drilling and pre harvest from September 2018 to May 2019. Fertiliser applications and timings were obtained from the respective land mangers, and found to be of similar type and timings.

Soil glomalin: quantified through a modified method of Wright and Upadhyaya (1996) involving extended autoclaving (90 minutes at 121°C 15 p.s.i) of 1g field sampled soil in 80ml 5mM trisodium citrate buffer. 1.5ml samples were taken from the autoclaved soil suspension in triplicate and centrifuged at 16,000 x g for 15 minutes. Samples were then subject to the Bradford assay (Termo Fisher), via manufacturer's instructions, and analysed at 595nm photospectrometry.

Soil nitrate levels: determined through a calcium sulphate extraction using 5g dried soil and 0.2g calcium sulphate in 20ml deionised water. After filtration, 1ml of filtrate was added to a nitrate testing kit (HACH®). Quantification was performed according to the manufacturer's instructions.

Water soil aggregates (WSA): determined through wet sieving 100g of dried soil previously allowed to stand in a 1:1 ratio of soil and deionised water. Soils were placed on a 250µm mesh and agitated with tepid water running through the sample for 60 seconds. Soil remaining on the meth as dried and percentage remaining calculated.

Statistical testing: ANOVA and T test performed to ascertain the degree of significance within the data and to test the null hypothesis that "the application of tillage reduces glomalin". Pearson coefficients were used to test correlations between data.

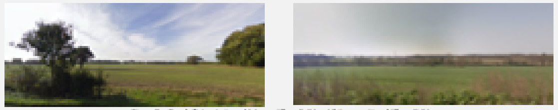
Figure 2 – Google® street view of (a) zero tillage field and (b) conventional tillage field

Results

- Statistical testing ($P < 0.0001$) of the difference in glomalin between tillage types, upheld the null hypothesis, showing reductions in overall glomalin levels in a CT system
- Average glomalin increase of 5.71 g/0.1m^3, between CT and ZT ($P = 0.05$)
- Nitrate levels significantly reduced in CT ($P < 0.00001$) and ZT ($P = 0.02$)

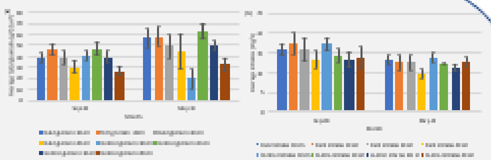
Figure 3 – Seed drilling winter wheat (September 2018) to harvest (May 2019) of a zero till (ZT) sampling site. (a) Average total glomalin between field samples within the cropping area for 4 sample depths (n=6, per depth) and control samples outside the cropping area, within the same field (n=5, per depth). (b) Average nitrates between field samples within the cropping area for 4 sample depths (n=6, per depth) and control samples outside the cropping area, within the same field (n=5, per depth).

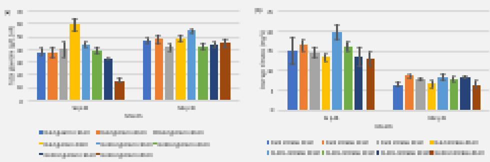
Figure 4 – Seed drilling winter wheat (September 2018) to harvest (May 2019) of a conventional till (CT) sampling site. (a) Average total glomalin between field samples within the cropping area for 4 sample depths (n=6, per depth) and control samples outside the cropping area, within the same field (n=5, per depth). (b) Average nitrates between field samples within the cropping area for 4 sample depths (n=6, per depth) and control samples outside the cropping area, within the same field (n=5, per depth).

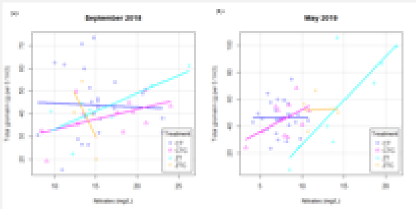

Figure 5 – Glomalin – nitrate correlation for a (a) pre-sowing (September 2018) and (b) immediately pre-harvest (May 2019) between CT (n = 24) and ZT (n = 24) systems, along with control samples of their respective tillage practice (CTC (n = 5) and ZTC (n = 5))

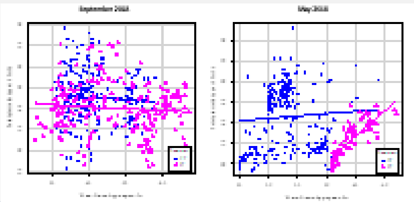
Figure 6 – Correlation between WSA and glomalin (a) pre-sowing (September 2018) and (b) pre-harvest (May 2019) between CT (n = [illegible]) and ZT (n = [illegible]) tillage systems.

- ZT treated soil produced a Pearson's correlation between glomalin and nitrates of 0.31 pre-sowing (Figure 5a) and 0.47 pre-harvest (Figure 5b)
- CT soils indicated little to no correlation between glomalin and nitrates pre-sowing (Figure 4a) (-0.04) and pre-harvest (Figure 5b) (0.00)
- WSA (Figure 6a), of a pre-sowing period, produced significance between tillage systems (glomalin – $P = 0.001$, WSA $P = 0.001$), and produced a Pearson's coefficient of -0.06 and -0.08 for CT and ZT respectively
- WSA (Figure 6b) were significantly reduced ($P < 0.001$) in CT soils compared with ZT soils. Effect of tillage produced a correlation of 0.06 against 0.91 in a tillage absent system (ZT)

Discussion

- The type of tillage has direct connotations for glomalin levels within soils (Sharifi et al., 2018; Singh et al., 2013). Through the null hypothesis, this was upheld giving strong indications that the land management practices, such as CT, reduce glomalin. As glomalin is also a carbon storage molecule, the impact on soil organic carbon will be evaluated in future work.
- CT inverts the soil exposing previously unexposed soil to the atmosphere. This aeration of soils temporarily increases microbial respiration increasing the rate of carbon loss from soils through the emission of carbon dioxide. Glomalin is additionally broken down, in some part, by this process. Coupled with fungal mycelia, undisturbed glomalin maintains an adhesive quality between soil aggregates. Applying tillage physically breaks mycelia, reducing secondary structural support, and breaks apart glomalin's adhesion. Therefore, less glomalin is present within CT soils reducing its soil aggregate stabilising properties. CT reduced the WSA compared to ZT at the end of the growing season immediately pre-harvest (Figure 5b), supporting the conclusions of Singh et al. (2013) that conservation tillage provides more stable soil aggregates and increases soil glomalin levels.
- Glomalin contains nitrogen. As a nitrogen store, it can aid in the reduction of N_2O emissions into the atmosphere via an indirect effect on nitrification (Singh et al., 2013).. Figures 5a and 5b suggest that the application of a CT system reduces glomalin levels compared to ZT, with implications for soil nitrate levels immediately pre-harvest (Figure 5b). This warrants further investigation.
- A negative relationship exists between the quantities of glomalin measured pre-sowing and WSA (Figure 5a). While the current crops were both winter wheat, the previous crops differed, with cereal and legume crop present in the CT and ZT systems respectively. The potential impact of this difference in crop type is to be further investigated in future work.
- In summary the present study concludes that CT reduces glomalin levels and the glomalin to WSA correlation, reducing soil stability and potentially increasing the risk of soil erosion. Initial results indicate a positive correlation between soil nitrate levels and glomalin within ZT managed soils, with little to no correlation observed in CT soils. AM fungi require a nitrogen source to produce glomalin. It stands to reason that increases in soil nitrates, as a fungal substrate, would be utilised by the fungus directly as well as provided to the host plant as part of the symbiotic relationship. The precise relationship between AM fungi, soil glomalin and soil nitrate will be investigated further in future work.