Climate change impacts on interactions between wheat, aphids, and arbuscular mycorrhizal fungi

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Abstract

Agricultural land occupies 38% of the Earth's terrestrial surface, making it the planet's greatest land use type. Consequently, on-farm practices have the potential to deplete natural resources and impact the environment. The need to reduce agricultural dependence on finite and ecologically damaging fertilisers and pesticides, while also increasing yields to meet rising demand, is pressing. Using associations formed between crops and arbuscular mycorrhizal (AM) fungi may represent a means by which this can be achieved. These symbiotic soil fungi can enhance plant nutrient uptake and protect crops against environmental stressors, such as those posed by climate change and insect pests. However, outcomes of plant-AM symbioses can be highly variable. Despite this, how abiotic and biotic factors govern crop-mycorrhizal functionality is poorly understood.

Here, I studied the effect of atmospheric [CO_2], a source of carbon (C) for plants, and phloem-feeding aphids, an external plant C sink, on wheat growth responses to an AM fungus (*Rhizophagus irregularis*). Elevated [CO_2] (eCO_2), in-line with climate change predictions, was not found to impact wheat-AM symbioses. Bird cherry-oat aphids (*Rhopalosiphum padi*), which are major pests of cereals, also had little effect on wheat growth responses to AM colonisation. Using radioactive and stable isotopes (^{33}P, ^{14}C, and ^{15}N), I explored the effect of eCO_2 and aphids on C-for-nutrient exchange. Aphids reduced plant C supply to the AM fungus, as theorised, but fungal-acquired P and N uptake by wheat was upheld. Increasing plant C source strength at eCO_2 did not recover plant C transfer to the AM fungus. My findings suggest resource exchange in AM symbioses may not be regulated reciprocally, as is disputed, and/or that biotic drivers are greater determinants of C-for-nutrient exchange than abiotic ones. Lastly, to provide context to my results I used a naturally-occurring AM fungal community in arable soil to investigate how wheat yields are impacted at ambient and elevated [CO_2] and in the presence and absence of aphids. Colonisation by AM fungi from this native community negatively impacted yield, regardless of interacting abiotic or biotic factors.

This research has provided the first insights into the effect of competing plant C sources and sinks on wheat growth responses to AM fungi, and their impact on resource exchange between plants and AM fungi more broadly. Future studies should investigate these effects in different AM fungal-plant-aphid systems, and using a variety of insect herbivores with different feeding approaches.

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Abbreviations

[CO_2] – atmospheric carbon dioxide concentration

eCO_2 – elevated [CO_2]

PPM – parts per million

RuBP – ribulose-1,5-bisphosphate

AM – arbuscular mycorrhizal

AMF – arbuscular mycorrhizal fungi

Zn – zinc

Fe – iron

P – phosphorus

Al – aluminium

N – nitrogen

NH_3 – ammonium

NO_3 – nitrate

N_2O – nitrous oxide

C – carbon

PAM – peri-arbuscular membrane

Pi – orthophosphate

CMN – common mycorrhizal network

BYDV – barley yellow dwarf virus

MAMPs – microbe-associated molecular patterns

HAMPs – herbivore-associated molecular patterns

SA – salicylic acid

JA – jasmonic acid

ET – ethylene

ABA – abscisic acid

GA – gibberellic acid

SL – strigolactone

VOCs – volatile organic compounds

FACE – free-air carbon dioxide enrichment

Chapter 1 General Introduction

1.1 Global food insecurity

1.1.1 Farmland productivity: past, present, and future trends

‘‘The Green Revolution” of the second-half of the twentieth century drove a 162% increase in agricultural productivity, despite modest growth in the land used to grow crops (Burney et al., 2010). Since 1961, global wheat (*Triticum aestivum* L.) yields increased from 1.1 to 3.4 tonnes per hectare, with 760 million tonnes of grain harvested in 2018 (FAO, 2020a). These trends strengthened food security given the widespread cultivation of wheat (Shewry & Hey, 2015) and its major contribution to human calorie/protein intake (Reynolds & Braun, 2019), and were achieved principally by the production and application of fertilisers and pesticides, alongside advances in plant breeding that produced higher yielding crop varieties (Tilman et al., 2001). In particular, short-straw (or semi-dwarf) cultivars developed in the 1970’s differed from their predecessors, as their stunted phenotypes made them less prone to lodging (i.e. collapse) and increased the allocation of plant resources to the grain, thereby increasing yields (Barraclough et al., 2010).

However, evidence suggests yield increases in wheat have stagnated in 37% of their harvested area since the early- to mid-1990’s, with a further 1% suffering yield collapse (Ray et al., 2012). Regions affected include European countries (e.g. the UK, France, Denmark, and Holland) and other major crop producing nations (e.g. the USA and India) (Brisson et al., 2010; Petersen et al., 2010; Grassini et al., 2013). In the UK, wheat productivity has stalled at 7.5 tonnes per hectare since 1995, despite the potential of new cultivars increasing each year (Knight et al., 2012). These trends are not driven by the diminishing use of fertilisers, as applications have instead risen globally over the last three decades (Lu & Tian, 2017). Rather, yield plateaus have been attributed to multiple by-products of agricultural intensification, such as land degradation, declining soil biodiversity or nutrient status, and soil contamination (Zhang et al., 2018).

Global demand for food production is expected to double by 2050 (Godfray et al., 2010), necessitated by human population growth that is projected to exceed 10.9 billion by 2100 (Gerland et al., 2014), and changing dietary habits (driven by growing affluence) towards more Western diets typified by increased meat and dairy consumption (Pingali, 2007). Moreover, the production of bioethanol from feedstocks like wheat will further increase demand (Mohanty & Swain, 2019).

Meeting these needs will require annual yield increases of 2.4% in cereals (Ray et al., 2013), which must be achieved while reducing the environmental burden of agriculture (Mueller et al., 2012). Failure to do so could threaten global food security, with 820 million people already suffering from malnutrition (WHO, 2019).

1.1.2 Climate change and farming

Human activities, most notably the burning of fossil fuels, have increased carbon dioxide concentrations in the atmosphere ([CO_2]) from a pre-industrial baseline of 280 parts per million (ppm) to over 400 ppm in 2013 (IPCC, 2014). Current rates of [CO_2] increase exceed 2.4 ppm annually, with levels likely to surpass 800 ppm by 2100 (Meinshausen et al. 2011).

Multiple aspects of climate change could negatively impact cereal yields. For instance, [CO_2]-driven high temperatures may cause productivity losses in wheat of 6.4% for every 1°C of warming (Liu et al., 2016), with similar yield penalties expected in maize and rice (Zhao et al., 2017). Warming could also lead to less predictable rainfall, driving faster and more intense droughts (Trenberth et al., 2014; Trnka et al., 2019). However, as well as causing these changes, rising [CO_2] may mitigate against their effects. Despite reducing stomatal conductance (Bernacchi et al., 2007), elevated [CO_2] (eCO_2) increases the availability of CO_2 molecules in leaves for combination with ribulose-1,5-bisphosphate (RuBP) in the active site of the photosynthetic enzyme Rubisco (Ainsworth & Rogers, 2007). Thus, eCO_2 enhances the photosynthetic efficiency of C3 crops like wheat, increasing rates of C assimilation (Stiling & Cornelissen, 2007). As such, elevated [CO_2] can improve wheat growth by 25%, and perhaps alleviate the effects of drought by increasing water use efficiency (Fitzgerald et al., 2016). Less positive effects of eCO_2 are expected for C4 crops, however, owing to their CO_2-concentrating mechanism in the bundle-sheath (Leegood, 2002).

While [CO_2] could positively impact wheat growth, evidence suggests grain amino acid and protein concentrations will be reduced at eCO_2 (Soba et al., 2019), as will levels of zinc (Zn) and iron (Fe) which could compromise human nutrition (Myers et al., 2014). Elevated [CO_2] may also increase pest pressure from insect herbivores like aphids (Sun & Ge, 2011). As a result, wheat losses to insects (Deutsch et al., 2018) and insect-borne plant diseases (Trębicki et al., 2015) may grow as climate change intensifies. Demand for fertilisers and pesticides could rise accordingly, arguably two of the most unsustainable aspects of farming.

1.1.2.1 P fertilisers

Phosphorus (P) is one of the most abundant macronutrients in plants, and plays an important role in plant energy metabolism being an essential constituent of nucleic acids and ATP (Campos et al., 2018). However, plant-available P, which takes the form of inorganic orthophosphate (Pi), is immobile and readily forms insoluble mineral complexes in farm soils with metals like Fe and Aluminium (Al) (Sharma et al., 2013). These precipitated forms cannot be assimilated by plants, meaning the proportion of P in soils that is soluble and available to crops is roughly 0.1%. As such, P is considered one of the most inaccessible elements for plants (Holford, 1997). Organic forms of P (e.g. phytic acid) can account for 20-80% of P in soils (Schachtman et al., 1998), but these must be mineralised by microorganisms before they can be acquired by crops.

To counter the inaccessibility of plant-available P, 17 million tonnes of P-based fertiliser are added to farm soils every year, up 3.8 fold from usage in 1961 (Lu & Tian, 2017). The majority of applied P originates from rock phosphate, a non-renewable resource found mainly in Morocco (Cooper et al., 2011). Estimates based on historical and projected demands for P fertiliser predict global rock phosphate reserves will be diminished in 300-400 years (Gilbert, 2009; Cordell & White, 2011). More alarmingly, specific reserves responsible for over 70% of productivity may be exhausted before the end of this century, resulting in yield shortfalls of 100 million tonnes per annum (Cooper et al., 2011). This, paired with the recovery of applied P by crops being just 20% (Schachtman et al., 1998), makes P fertiliser use inherently unsustainable.

1.1.2.2 N fertilisers

Nitrogen (N) is the only element more abundant in plants than P (Campos et al., 2018), and is one of the most crucial yield-limited factors in food production (de Oliviera Silva et al., 2020). N is essential for plant growth being a key component of amino acids (and thus proteins) and chlorophyll, which is used by plants to absorb light energy for photosynthesis (Evans, 1989). N fertilisers are produced using the energy-intensive Haber-Bosch process powered by fossil fuels (Smith et al., 2020). First developed in the early 1900's, this methane-fed process uses a catalyst to convert N gas in the earth's atmosphere (N_2) into ammonia (NH_3) in a reaction that requires high temperatures (375-475°C) and pressures (50-200 bar) (Vojvodic et al. 2014). Because of this, N fertiliser production accounts for

1% of the world's energy usage (Foster et al. 2018) and 1.2% of global CO_2 emissions, more than any other industrial chemical-producing reaction (Smith et al., 2020). For wheat, NH_3 fertiliser use alone is responsible for 40% of the environmental impact of the wheat-to-bread supply chain (Goucher et al., 2017).

In 2013, 108 million tonnes of N fertiliser was added to soils globally, 9.5 times more than in 1961 (Lu & Tian, 2017). However, less than half of all applied N is recovered by crops (de Oliveria Silva et al., 2020). Runoff and leaching of N fertiliser as nitrate (NO_3^-) contaminates drinking water and causes eutrophication in coastal ecosystems. Excessive algal growth in turn drives hypoxia that threatens aquatic species (Good & Beatty, 2011). NH_3 fertiliser use also emits nitrous oxide (N_2O) into the Earth's atmosphere (Reay et al., 2012), a greenhouse gas with a global warming potential 296 times that of CO_2. Consequently, means must be found to reduce N fertilise usage if climate change thresholds, such as 1.5°C warming, are not to be exceeded (IPCC, 2018).

1.1.2.3 Pesticides (insecticides)

Over 2 million tonnes of pesticides are used on farms each year, of which insecticides account for 30% (Sharma et al., 2019). Many novel insecticides were developed between the 1960's and 1980's (Aktar et al., 2009), most of which work by targeting the nervous system of insects. Pyrethroids and neonicotinoids, two key insecticidal groups, interrupt neurotransmission by affecting voltage-sensitive sodium channels, thereby over-stimulating neurons which causes the death of the pest (Hirata, 2016). Insecticides cost growers 8 billion US dollars each year in the USA alone (Foster et al., 2014), but despite their usage, insects remain responsible for annual yield losses of 40.1 and 78.1 million tonnes in wheat and maize globally (Deutsch et al., 2018).

The use of insecticides in farming is troublesome for many reasons. Firstly, if not applied sufficiently then insecticides can improve pest performance (Rix et al., 2016; Sial et al., 2018), thereby increasing herbivore pressure in a mechanism termed hormesis. Secondly, due to the intensity with which chemicals are applied or the types of compounds used (Zuo et al., 2016), insects like cereal aphids have developed resistance to insecticides (Chen et al., 2007; Wang et al., 2018). Lastly, most pesticides impact non-target organisms like pollinators and natural enemies of herbivores (Henry et al., 2012; Hopwood et al., 2013), thereby reducing the ecosystem services they provide (Chagnon et al., 2015). Many

insecticides have been banned in Europe as a result (Van Doorn & de Vos, 2013), including some neonicotinoids which make up 20% of the global agro-chemical market (Gupta et al., 2019). As with fertilisers, pesticides also contaminate groundwater sources and negatively impact human health (Kim et al., 2017). Thus, more sustainable means of managing insect pests are needed.

1.1.3 Sustainable intensification

"Sustainable intensification" has been used to define low-input practices that may enable yield increases while also reducing the negative externalities of farming (Godfray et al., 2010). Conservation agriculture is one such strategy, used primarily in Southern Asia (Jat et al., 2020) and Africa (Corbeels et al., 2013). Principles like reduced tillage, crop rotations, and the retention of crop residues are key to this approach, which could improve soil health (i.e. structure and biodiversity) (Giller et al., 2015). The substitution of pesticides for insect-resistant cultivars or biological control agents may too increase sustainability. Cause for optimism is evidence that modern wheat cultivars may perform optimally under these reduced-input management practices (Voss-Fels et al., 2019). This is despite commercial crop breeding having targeted yield improvements under high inputs of chemical fertilizers and pesticides (Barraclough et al., 2010), and focussed almost entirely on aerial components of crops rather than below-ground processes (Voss-Fels et al., 2018). Such systems would afford the potential to utilise the (often) beneficial associations formed between crops and soil-borne microorganisms, known as arbuscular mycorrhizal (AM) fungi. However, moving to such systems requires greater understanding of how crops and AM fungi interact under rising [CO_2], given breeding may have reduced the adaptability of wheat to climate change (Kahiluoto et al., 2019), and how insect pests impact these associations (Frew & Price, 2019).

1.2 Could AM fungi provide a route to sustainable farming?

Symbioses formed between plants arbuscular mycorrhizal fungi are common, occurring in roots or rhizoids of up to 80% of terrestrial plants (Smith & Read, 2010) across the land plant phylogeny (Hoysted et al., 2018a). These associations are ancient; fossils from Scotland's Rhynie chert (Remy et al., 1994) and the Guttenburg Formation in Wisconsin (Redecker et al., 2000) date the symbiosis at >460 million years old. This evolutionary stability hints at the mutual benefit derived by both partners. Plants associating with AM fungi, which belong to the

phylum Glomeromycotina (Schüβler et al., 2001), gain multiple benefits, including enhanced access to soil nutrients like P, N, and other trace elements (Tamayo et al., 2014). Benefits AM fungi confer to host plants may also be non-nutritional (see section 1.2.3). In exchange, plants supply AM fungi with 4-20% of their net photosynthate (Cotton, 2018), with organic carbon (C) compounds transferred to mycorrhizas in the form of sugars and/or fatty acids (Luginbuehl et al., 2017).

1.2.1 Nutritional benefits

Mycorrhizal plants have two pathways by which they can take up soil nutrients (Figure 1.1a). The first, termed the non-mycorrhizal (or direct) pathway, is the same as that used by non-colonised plants, with high-affinity transport proteins assimilating soil nutrients in the root epidermis or root hair cells (Rausch & Bucher, 2002). The second, termed the mycorrhizal pathway, involves the uptake of nutrients by plants via their fungal partners.

Being obligately biotrophic (Smith & Read, 2010), AM fungi are wholly dependent on plants to meet their C requirements (Roth & Pasckowski, 2017). In the soil, AM fungi form extra-radical hyphae that proliferate in nutrient-rich patches, reaching densities of up to 20.5 metres per gram of soil (Pepe et al., 2018). These hyphal networks enable the capture of nutrients like P. Being highly immobile, uptake of P by plants results in the development of depletion zones around the root. AM fungal hyphae extend beyond this region (up to 15 centimetres from the plant; Jansa et al., 2003) granting mycorrhizal hosts access to greater volumes of soil and thus larger nutrient pools than their non-AM counterparts. As a result, AM plants can acquire <90% of their P via root mutualists (Smith & Read, 2010), and considerable quantities of other nutrients. Evidence suggests AM fungi may too mediate N uptake in inorganic (i.e. NO_3^- or NH_4^+) and organic forms (i.e. amino acids and proteins), the latter following their chemical breakdown from organic matter (Hodge & Fitter, 2010). As such, AM fungal contributions to plant N supply may exceed 20% (Leigh et al., 2009). The ecological relevance of this was once questioned (Helgason & Fitter, 2009; Smith & Smith, 2011a), but AM fungal-mediated plant N uptake can increase plant biomass (Thirkell et al., 2016).

P and N are translocated to the root as polyphosphate and arginine (Hodge, 2018). In the root, AM symbioses are typified by the formation of arbuscules in the root cortical cells (Figure 1.1b). This “diagnostic” structure (Fitter, 2006) forms when intra-radical hyphae penetrate the cell wall and divide, producing branched

arrangements surrounded by a peri-arbuscular membrane (PAM). Consisting of two domains, this interface provides the site of resource exchange (Luginbuehl & Oldroyd, 2017); AM-specific transport proteins have been described for P (Javot et al., 2007) and N (Guether et al., 2009), together with monosaccharaide transporters (*MST2*) that span the PAM and cell cytoplasm (Helber et al., 2011). *MST2* localisation has also been recorded along intra-radical hyphae, suggesting these structures too partake in resource exchange. Until recently, plant C supply was thought to be based purely on the transfer of hexoses, with sugars loaded into the phloem in source tissues (i.e. leaves) and transported to sink tissues (i.e. mycorrhizal roots) (Rennie & Turgeon, 2009). However, evidence suggests lipids also represent a key C source by which plants maintain mycorrhization (Jiang et al., 2017). Which lipids are involved, and the proportion of sugars and/or lipids that make up plant C transfer to AM fungi, is as yet unclear (Keymer et al., 2017).

1.2.2 Nutrient exchange

Plants and AM fungi have the potential to select their partners (Noë & Kiers, 2018); plants may associate with up to 20 AM fungal species at the same time, and each fungus can colonise roots of several plants concurrently, establishing common mycorrhizal networks (CMN) that link hosts below ground. Given the evolutionary persistence of plant-AM symbioses, it is presumed that both partners can identify “cheaters”, these being symbionts that derive benefits from the association while conferring little benefit in return (Smith & Smith, 2015). Although it is unknown how, evidence indicates plants can discriminate between AM fungal taxa, “sanctioning” poor mutualists that provide few nutrients with reduced plant C supply (Bever et al., 2009). The regulatory mechanisms that underpin resource exchange are unresolved, however (Walder & van der Heijden, 2015; Kiers et al., 2016). The ‘biological markets’ framework (Noe & Hammerstein, 1995) asserts that carbon-for-nutrient exchange may be likened to human economics, in which commodities (i.e. resources) are exchanged and have values that vary between plant-AM fungal pairings and under different conditions (Noë & Kiers, 2018). In support of this is evidence that plant uptake of fungal-acquired nutrients may relate directly to plant C transfer to AM fungi (Hammer et al., 2011). By using monoxenic root-organ cultures, Kiers et al., (2011) found that greater plant C allocation induced P transfer from an AM fungus (*Rhizophagus irregularis*, formerly *Glomus intraradices*), with similar trends seen for N (Fellbaum et al., 2012). Likewise, greater nutrient uptake by the AM fungus triggered increased

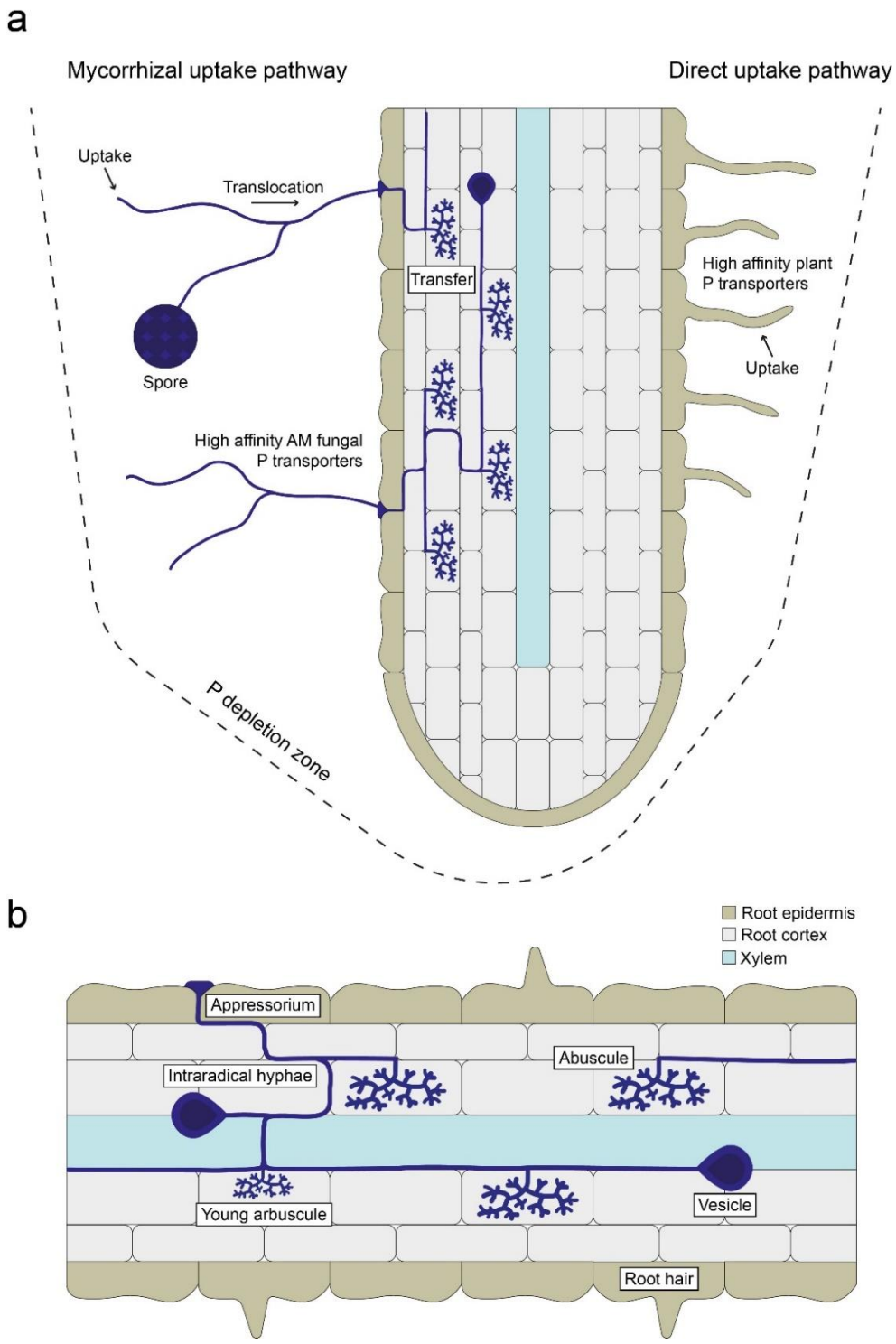

Figure 1.1: Root-external and root-internal colonisation by AM fungi. (a) Mycorrhizal and direct uptake pathways. Direct uptake results in regions of P depletion forming near the root. AM fungal hyphae can grow up to 100 times longer than root hairs (Grønlund et al., 2013), and enter soil microspores inaccessible to the plant. Redrawn from Smith & Read (2010). (b) Simplified anatomy of AM fungi in a longitudinal root section. Extra-radical hyphae enter the root through the epidermis and divide into arbuscules in the cortex. Turnover of arbuscules occurs in 72 hours (Kobae & Hata, 2010). Vesicles form later in colonisation and are fungal lipid stores. Redrawn from Brundrett et al., (2000).

plant C supply in return (Kiers et al., 2011). While such experiments have been criticised for lacking the biological relevance of soil-based systems (Smith & Smith, 2015), their findings are partly supported by studies which do; by altering the plant C source strength of neighbouring hosts through shading, preferential supply of P (Fellbaum et al., 2014) and N (Fellbaum et al., 2014; Weremijewicz et al., 2016) was recorded by a CMN to 'higher quality' plants (i.e. sunlit hosts more capable of providing AM fungi with plant C). Similarly, in growing mature plants next to seedlings, greater fungal-mediated P uptake was observed in large hosts of greater plant C source strength (Merrild et al., 2013).

Despite these examples, reciprocal resource exchange is unlikely to be universal in AM symbioses. For instance, asymmetrical carbon-for-nutrient exchange has been recorded based on the identity of plants rather than plant C supply; in spite of providing little photosynthate, flax (*Linum usitatissimum*) acquired over 90% of mineral nutrients afford by a CMN, while sorghum (*Sorghum bicolor*) supplied the majority of plant C but received minimal nutritional reward in return (Walder et al., 2012). For this reason, and others reviewed by Walder & van der Heijden (2015), the acceptance of resource exchange as being tightly coupled in plant-AM symbioses may be premature (Smith & Smith, 2013). Further research is needed into how abiotic and biotic factors (individually and together) impact C-for-nutrient exchange in crops before reciprocal rewarding can be supported.

1.2.3 Non-nutritional benefits

While the capacity of AM fungi to improve plant nutrient uptake has been the focus of most research into plant-AM symbioses, arbuscular mycorrhizal fungi also promote sustainability through non-nutritional means, being termed 'agro-ecosystem engineers' by some as a result (Cameron, 2010). Extra-radical hyphae of AM fungi can improve the structure of soils by physically binding soil aggregates together (Leifheit et al., 2014; Lehmann et al., 2017). This, in turn, may reduce rates of nutrient leaching from soils, as well as fluxes of N_2O into the atmosphere (Bender et al., 2015). AM fungi can also alleviate symptoms of hypersalinity (Evelin et al., 2009), heavy metal toxicity (Ferrol et al., 2016), and drought stress in plants (Chitarra et al., 2016), in the latter instance maybe due to improved soil water-stable aggregation (Piotrowski et al., 2004) or by reducing stomatal conductance and thus evapotranspiration (Augé et al., 2015).

Protection conferred by AM fungi also extends to biotic pressures, including plant pathogens (Sikes et al., 2009) and insect herbivores (Koricheva et al., 2019). This is achieved via the intricate coordination of signals between partners at different stages of colonisation (Cameron et al., 2013). Prior to root infection, plants detect AM fungi by recognising fungal microbe-associated molecular patterns (MAMPs). This induces plant defence responses typical of attack from biotrophic pathogens, resulting in below-ground increases of salicylic acid (SA). SA pathways are effective against biotrophs as they control outcomes like programmed cell death in plants (Jung et al., 2012). This immune response moderates the extent of AM colonisation (Herrera-Medina et al., 2003), but is supressed by AM fungi in order to facilitate their own infection (Kloppholz et al., 2011). This is achieved by the secretion of *SP7*, a fungal protein that downregulates pathogensis related-transcription factors in the plant nucleus. The promotion of jasmonic acid (JA) is typical of later stages of AM infection owing to cross-talk between pathways (Jung et al., 2012), with absisic acid (ABA) and ethylene (ET) are also thought to play a role in plant-AM signalling (Hause et al., 2007). AM colonisation is thus thought to “prime" plant defences, enabling stronger and faster immune responses after subsequent exposure to biotic stress (Martinez-Medina et al., 2016).

1.2.4 AM fungi in agriculture

Despite increasing interest in exploiting AM fungi in order to reduce fertiliser and pesticide usage (Thirkell et al., 2017), most intensive farming practices negatively affect AM fungal ecology. Physical disturbance of soils through tillage is perhaps the most powerful selection pressure influencing mycorrhizal fungi (Verbruggen & Kiers, 2010), as it disrupts hyphae in the plough layer thereby reducing plant C supply. Other management approaches like monoculture, fallow periods, fertilization, and fungicide application may also threaten AM fungi (Helgason et al., 1998). As such, AM fungal abundance and species richness typically declines with increasing land use intensity (Oehl et al., 2003). AM fungal community composition may also be affected (Jansa et al., 2002; Borriello et al., 2012), with modern practices favouring taxa that prioritise reproduction (i.e. sporulation) thereby selecting for a less beneficial assemblage (Verbrugen & Kiers, 2010). Because of this, sustainable methods like minimum tillage may increase AM function with respect to root colonisation (Bowles et al., 2016) and plant nutrient uptake (Köhl et al., 2014).

1.2.5 Functional variability: impact of atmospheric [CO_2]

Crucially, outcomes of mycorrhizal symbioses are not always beneficial for plants. In growing 10 plant species with 10 AM fungal isolates, Klironomos (2003) found plant growth responses to AM fungi ranged from positive to negative, concluding that the identities of both organisms determined the extent and degree to which plant growth was promoted. This functional variability is not limited to wild plant-AM symbioses; negative, neutral, and positive growth responses to mycorrhizal colonisation have been reported in cereals, including wheat (Hetrick et al., 1992; Ellouze et al., 2016). As a result, AM associations are thought to exist along a parasitic-mutualistic continuum (Johnson et al., 1997). Where AM symbioses lie on this spectrum may be determined by the degree to which plant growth is limited by soil nutrients and/or the availability of plant C for AM fungi (Johnson, 2010). As such, abiotic factors that determine plant C source strength, like shading (Johnson et al., 2015) and [CO_2] (Johnson et al., 2005), may heighten functional differences. eCO_2 may mitigate plant growth depressions caused by AM fungi by reducing the C "costs" associated with supporting them (see section 1.1.2). Alternatively, eCO_2 may increase plant C transfer to AM fungi (Drigo et al., 2010; Field et al., 2012), which could strengthen the mutualism by improving fungal nutrient capture if regulated reciprocally, or as a result of increased AM fungal biomass in roots and soils at eCO_2 (Dong et al., 2018). However, knowledge regarding wheat-AM responses to [CO_2] is lacking. Thus, greater understanding of which environmental factors influence symbiotic outcomes (and how) is needed before AM fungi can be promoted as a sustainable solution for future productivity (Ryan & Graham, 2018).

1.3 Insect herbivores

Insect pests are ubiquitous in all terrestrial habitats (Harrington et al., 2007), with their diversity estimated at <1 million species (Futuyama & Agrawal, 2009). The host ranges of insects can vary considerably (Ali & Agrawal, 2012). Certain pests, termed monophages, are restricted to feeding from plants within one genus, while others (oligophages) target multiple plant species in one family. Insects feeding on plants from many botanical families are considered polyphages or generalists. Insect herbivores also differ in terms of their feeding approach; pests that chew or mine photosynthetic tissues cause extensive physical damage to plants, while phloem feeders, like aphids, siphon sap from plant vascular tissues.

1.3.1 Aphids

Aphids of the superfamily Aphidoidea are a diverse group of approximately 5,000 species in the Hemiptera (Fereres & Moreno, 2009). Aphids are small (>10 mm), phytophagous insects that attack above- and below-ground plant parts, using piercing-sucking mouthparts called stylets to imbibe plant phloem. On stems and leaves, aphid stylets probe intercellularly towards vascular tissues (Figure 1.2) and feed on sugars, amino acids, macroelements (e.g. P and K), microelements (e.g. Fe and Zn), and secondary metabolites contained in sap (Dinant et al., 2010). High sugar concentrations in plant phloem requires osmoregulation to overcome differences in the osmotic pressure between sap and the insect's bodily fluids, meaning aphids also periodically feed from the xylem to avoid dehydration (Sun et al., 2016). Phloem is typically deficient in 9 of the 20 essential amino acids required to form proteins (Douglas, 2006). Thus, aphids rely on endosymbioses with *Buchnera* to reconstitute non-essential amino acids in sap into forms that can be used for insect growth and development (Feng et al., 2019). Because of this, aphids are sensitive to the concentration and composition of phloem-borne amino acids (Ponder et al., 2000; Karley et al., 2002), which are the main form in which N is transported *in planta* (Lalonde et al., 2004).

1.3.2 Significance as crop pests

Although species-rich, only a small sub-group of roughly 100 aphid species are pests of crops (van Emden & Harrington, 2017). The mechanisms by which aphids cause crop failure can be direct and indirect. Yield losses to aphids can result from the direct removal of phloem (i.e. plant C) from the host, with some adult aphids siphoning their own body weight in translocate each day (Dixon, 2012). At high abundances, aphids may therefore represent a significant external plant C sink (Donovan et al., 2013), and slow the delivery of carbohydrates and proteins to other sink tissues in the plant (Aqueel & Leather, 2011). Aphids also affect grain yields by transmitting plant viruses, acting as vectors for more than half of all plant viruses spread by insects (Fereres & Moreno, 2009). Due to their non-destructive feeding mode, phloem feeders provide viruses with living cells required for reproduction (Goggin, 2007). Bird cherry-oat aphids (*Rhopalosiphum padi*) are vectors of barley yellow dwarf virus (BYDV), which is transmitted from infected grasses to cereals (Leather et al., 1989). Longer periods of sap ingestion may increase the efficiency of BYDV transmission (Fereres & Moreno, 2009),

which causes leaf necrosis and stunted wheat growth (Riedell et al., 2003). Yield losses in wheat associated with the spread of BYDV by *R. padi* range from 20-80%, exceeding those of 35-40% from direct feeding (Zeb et al., 2016). Together, cereal-feeding aphids are responsible for annual productivity losses in UK valued at £120 million (Loxdale et al., 2017). Economic thresholds have been defined for cereals like wheat (Kieckhefer et al., 1995) and sorghum (Ragsdale et al., 2007), describing densities and durations of exposure that cause yield loss, enabling farmers to take steps to mitigate this.

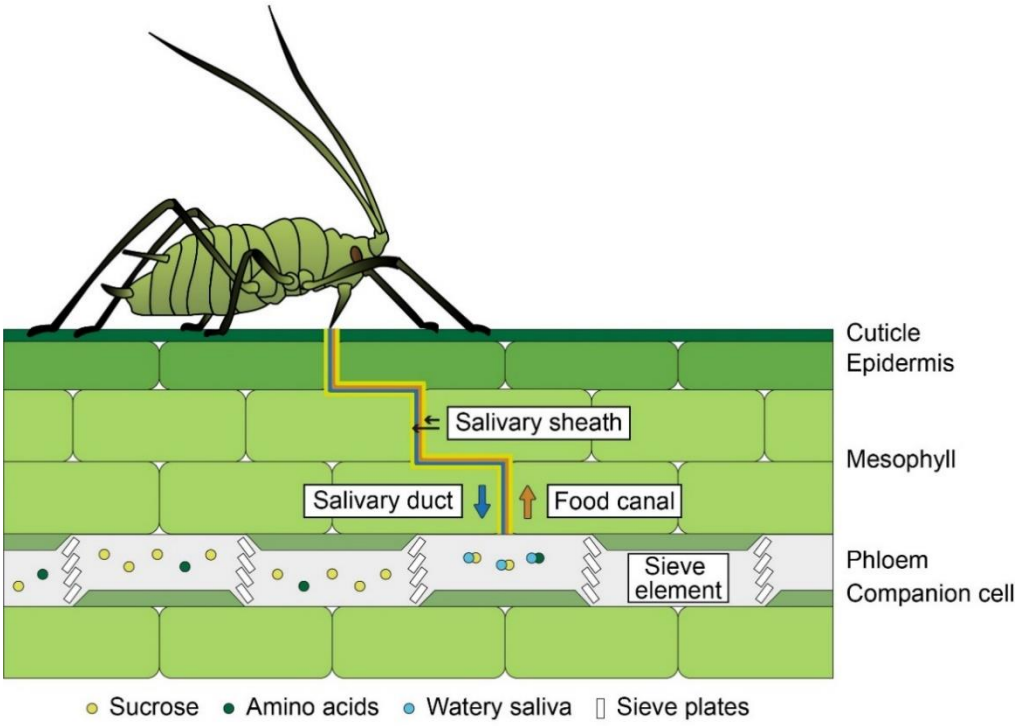

Figure 1.2: The feeding approach used by aphids. Phloem feeders prolong feeding bouts through the secretion of salivary products (Goggin, 2007) that inhibit the blockage of plant vascular tissues (Tjallingii, 2006). As aphid stylets move through the leaf towards the phloem, gelling saliva is secreted from salivary glands, which solidifies and forms a sheath. This salivary sheath minimises damage to plant cells, and prevents an influx of Ca+ ions from the apoplast into the phloem which would otherwise occlude sieve plates and prevent the movement of sap between sieve elements (Will et al., 2007). Watery saliva is secreted from the salivary duct into the phloem which mixes with plant sap and is ingested via the food canal. Redrawn from Nalam et al., (2018).

1.4 Multi-trophic interactions: AM fungal-plant-aphid systems

Over the past three decades the understanding has grown that, despite their spatial separation, above-ground processes can impact those below-ground and

vice versa (Bardgett, 2018). Pioneering studies, like Gange & West (1994) and Gehring & Whitham (1994), showed that AM fungi and insect pests can indirectly impact each other's fitness through numerous mechanisms mediated by their shared plant host (Hartley & Gange, 2009). The means by which insects affect mycorrhizal colonisation (and perhaps function) are termed 'top-down' impacts. Conversely, how AM fungi affect the fitness and behaviour of insect pests like aphids are termed 'bottom-up' impacts (see section 1.4.2).

1.4.1 'Top-down' impacts

As per eCO_2, insect pests may affect plant-mycorrhizal interactions by altering the C source strength of target plants, and ultimately plant C transfer to AM fungi. As well as directly siphoning phloem (Douglas, 2006), aphids may limit plant C availability for AM fungi by reducing rates of photosynthesis (Macedo et al., 2003; 2009), altering root exudate profiles (Hoysted et al., 2018b; Cabral et al., 2018), and/or by activating plant defences. While less destructive than chewers, aphid stylets briefly puncture epidermal and mesophyll cells during probing (Tjallingii, 2006). This, or recognition of chemical cues in aphid saliva (Will et al., 2013), can elicit SA and ABA-transduction pathways in plants (Donovan et al., 2012; Kerchev et al., 2013). As such, the 'carbon-limitation hypothesis' posits that above-ground herbivores may compete with AM fungi for photosynthate (Gehring & Whitham, 1994; 2002), with the degree of AM colonisation being used as a proxy for plant C transfer. Multi-trophic studies concerning AM fungal-plant-aphid interactions have found positive, neutral, and negative effects of aphid feeding on AM colonisation (Table 1.1). Notably, aphid exposure reduced AM infection of broad bean (*Vicia faba* L.) by 20% (Babikova et al., 2014a), while negative (-37%) and positive (+56%) outcomes were recorded in *Asclepias* species (Meier & Hunter 2018). Incidences in which insects increase AM colonisation may be due to plants sequestering C below ground and thus away from pest (Holland et al., 1997; Schwachtje et al., 2006; Babst et al., 2008), or because aphid honeydew represents an additional plant C source for soil microbes (Milcu et al., 2015).

If plants and AM fungi exchange resources reciprocally (see section 1.2.2) then changes in plant C supply following aphid herbivory may be expected to affect fungal-acquired plant nutrient uptake. Reduced plant C supply may compromise the ability of AM fungi to capture soil nutrients, resulting in a less positive symbiosis. However, this remains a significant knowledge gap as no study to date

Table 1.1: Summary of studies investigating the indirect effect of aphids on AM colonisation.

Aphid species	Specificity	Plant species	AM fungal species	Effect on AM fungus	Publication
Acyrthosiphon pisum	Specialist	*Vicia faba*	Mixed community	Negative	Babikova et al., (2014a)
Acyrthosiphon pisum	Specialist	*Vicia faba*	Mixed community	Negative	Babikova et al., (2014b)
Aphis asclepiadis	Specialist	*Asclepias syriaca*	Mixed community	Neutral	Vannette & Hunter (2014)
Aphis fabae	Generalist	*Vicia faba*	*Rhizophagus irregularis*	Neutral	Cabral et al., (2018)
Acyrthosiphon pisum	Specialist	*Medicago truncatula*	*Rhizophagus irregularis*	Neutral	Maurya et al., (2018)
Aphis nerii	Generalist	*Asclepias curassavica*	*Funneliformis mosseae*	Negative	Meier & Hunter (2018b)
Aphis nerii	Generalist	*Asclepias latifolia*	*Funneliformis mosseae*	Neutral	Meier & Hunter (2018b)
Aphis nerii	Generalist	*Asclepias syriaca*	*Funneliformis mosseae*	Negative	Meier & Hunter (2018b)
Aphis nerii	Generalist	*Asclepias incarnata*	*Funneliformis mosseae*	Positive	Meier & Hunter (2018b)
Sitobion avenae	Specialist	*Hordeum vulgare*	Mixed community	Neutral [a]	Wilkinson et al., (2019)
Aphis nerii	Generalist	*Asclepias incarnata*	*Funneliformis mosseae*	Neutral	Meier & Hunter (2019)
Aphis nerii	Generalist	*Asclepias curassavica*	*Funneliformis mosseae*	Neutral	Meier & Hunter (2019)
Acyrthosiphon pisum	Specialist	*Medicago sativa*	*Rhizophagus irregularis*	Neutral	Li et al., (2019)

[a] Neutral effect of aphids also reported for extraradical hyphal lengths and AM fungal community composition.

has directly quantified the effect of aphids on resource exchange between plants and AM fungi. As such, whether biotic interactions with insect pests alter carbon-for-nutrient exchanges in crops, and in turn the positioning of plant-AM symbioses on the parasitism-mutualism continuum, is unclear.

1.4.2 'Bottom-up' impacts

Early experiments into how AM fungi indirectly impact the performance of insects recorded a dichotomy of responses based upon the feeding mode and specificity of the herbivore (Gange & West, 1994). A pattern emerged in which chewing or leaf mining generalists were adversely affected by AM fungi, while specialists or phloem-feeders like aphids appeared to benefit from mycorrhization (Koricheva et al., 2009). However, just 14% of multi-trophic studies addressed interactions between AM fungi and sap-sucking Hemipteran herbivores (Harley & Gange, 2009). As the number of experiments has grown, so too has variability in their conclusions; AM colonisation can have negative, neutral, and positive effects on aphid performance (Table 1.2). The direction and extent of these outcomes may be determined by the genotype of the fungus (Abdelkarim et al., 2011), plant (Tomczak & Müller, 2018), and insect (Rasmussen et al., 2017). Contrasting results in different AM fungal-plant-aphid systems may also depend on the mechanism by which AM fungi impact aphids, which can include improving plant nutrient status (Harley & Gange, 2009), modifying host-plant anatomy (Simon et al., 2017; Garzo et al., 2018), and the priming of plant defences against herbivores (see section 1.2.3). AM fungi may also induce changes in the aerial chemicals elicited by plants called volatile organic compounds (VOCs), thereby altering plant attractiveness to natural enemies of pests (Guerrieri et al., 2004). These mechanisms may operate concurrently with contrasting outcomes for aphid herbivores (Volpe et al., 2018). Critically, despite increasing interest in AM fungal-plant-aphid interactions, how wheat mediates relations between AM fungi and cereal aphids like *R. padi* is poorly understood. Research into this tri-partite interaction is therefore urgently required.

1.5 Impact of dual plant C sources and sinks on AM symbioses

Elevated [CO_2] has the potential to mitigate against the C drain imposed on wheat by pests of cereals like aphids, and restore plant-AM functioning (Figure 1.3). However, the external plant C sink that aphids represent may be stronger at eCO_2, given the abundance of wheat-feeding aphids may increase with increasing

Table 1.2: Summary of studies investigating the effect of AM colonisation on aphid performance.

Aphid species	Specificity	Plant species	AM fungal species	Effect on aphid	Publication
Myzus persicae	Generalist	*Plantago lanceolata*	Mixed community	Positive	Gange & West (1994)
Myzus ascalonicus	Generalist	*Plantago lanceolata*	*Glomus intraradices*	Positive	Gange et al., (1999)
Myzus persicae	Generalist	*Plantago lanceolata*	*Glomus intraradices*	Positive	Gange et al., (1999)
Cryptomyzus ribis	Specialist	*Stachys sylvatica*	*Glomus fasciculatum*	Positive	Gange et al., (2002)
Myzus persicae	Generalist	*Stachys sylvatica*	*Glomus fasciculatum*	Positive	Gange et al., (2002)
Chaitophorous populicola	Specialist	*Populus angustifolia* x *P. fremontii*	Mixed community	Negative	Gehring & Whitham (2002)
Macrosiphum euphorbiae	Generalist	*Lycopersicon esculentum*	*Glomus mosseae*	Negative	Guerrieri et al., (2004)
Myzus persicae	Generalist	*Lolium perenne*	*Glomus intraradices*	Neutral	Wurst et al., (2004)
Rhopalosiphum padi	Specialist	*Phleum pratense*	*Glomus intraradices*	Negative	Hempel et al., (2009)
Rhopalosiphum padi	Specialist	*Phleum pratense*	*Glomus mosseae*	Negative	Hempel et al., (2009)
Rhopalosiphum padi	Specialist	*Triticum aestivum*	*Glomus intraradices*	Neutral	Abdelkarim et al., (2011)
Rhopalosiphum padi	Specialist	*Triticum aestivum*	*Gigaspora margarita*	Negative	Abdelkarim et al., (2011)
Aulacorthum solani	Generalist	*Glycine max*	*Gigaspora margarita*	Positive	Ueda et al., (2013)
Acyrthosiphon pisum	Specialist	*Vicia faba*	Mixed community	Variable [a]	Babikova et al., (2014a)
Acyrthosiphon pisum	Specialist	*Vicia faba*	Mixed community	Positive	Babikova et al., (2014b)
Macrosiphum euphorbiae	Generalist	*Lycopersicon esculentum*	Mixed community	Neutral	Colella et al., (2014)
Aphis gossypii	Generalist	*Trifolium repens*	*Glomus mosseae*	Neutral	Grabmaier et al., (2014)

Table 1.2: Continued

Aphid species	Specificity	Plant species	AM fungal species	Effect on aphid	Publication
Aphis craccivora	Generalist	*Trifolium repens*	*Glomus mosseae*	Neutral	Grabmaier et al., (2014)
Rhopalosiphum padi	Specialist	*Hordeum vulgare*	Mixed community	Neutral	Williams et al., (2014)
Macrosiphum euphorbiae	Generalist	*Solanum tuberosum* STN	Mixed community	Neutral	Bennett et al., (2016)
Macrosiphum euphorbiae	Generalist	*Solanum tuberosum* TBR	Mixed community	Neutral	Bennett et al., (2016)
Macrosiphum euphorbiae	Generalist	*Solanum berthaultii*	Mixed community	Neutral	Bennett et al., (2016)
Macrosiphum euphorbiae	Generalist	*Solanum polyadenum*	Mixed community	Neutral	Bennett et al., (2016)
Myzus persicae	Generalist	*Plantago lanceolata*	*Rhizophagus irregularis*	Variable	Tomczak & Müller (2017)
Macrosiphum euphorbiae	Generalist	*Solanum tuberosum*	Mixed community	Neutral	Karley et al., (2017)
Macrosiphum euphorbiae	Generalist	*Solanum berthaulti*	Mixed community	Neutral	Karley et al., (2017)
Sitobion avenae	Specialist	*Triticum aestivum*	Mixed community	Positive	Simon et al., (2017)
Sitobion avenae	Specialist	*Triticum monococcum*	Mixed community	Positive	Simon et al., (2017)
Aphis nerii	Specialist	*Asclepias* spp.	*Funneliformis mosseae*	Variable [b]	Meier & Hunter (2018a)
Acyrthosiphon pisum	Specialist	*Medicago truncatula*	*Rhizophagus irregularis*	Positive	Maurya et al., (2018)
Myzus persicae	Generalist	Plantago lanceolata	*Rhizophagus irregularis*	Variable [b]	Tomczak & Müller (2018)
Myzus persicae	Generalist	*Poa annua*	*Rhizophagus irregularis*	Negative	Tomczak & Müller (2018)
Acyrthosiphon pisum	Specialist	*Medicago truncatula*	*Rhizophagus irregularis*	Negative	Garzo et al., (2018)

[a] Impact of AM fungi on aphids was dependent on timing of arrival (i.e. whether plants were colonised by AM fungi before aphids or vice-versa).

[b] Multiple aphid life-history characteristics recorded, with some responding positively and some negatively to AM colonisation.

[CO_2] (Chen et al., 2004; Sun et al., 2009a; Wang et al., 2018; Vassiliadis et al., 2018). Reasons or this are two-fold. Firstly, stomatal conductance declines at eCO_2 (Bernacchi et al., 2007) which improves the water status of plants. This may, in turn, enable aphids to prolong phloem feeding bouts while avoiding desiccation, as longer durations of xylem ingestion (see section 1.3.1) have been seen at eCO_2 thereby reducing the osmolarity of the aphid's haemolymph (Sun et al., 2015; Sun et al., 2016). Another impact of stomatal closure at eCO_2 is that leaf temperatures can rise by up to 2°C, thereby accelerating aphid growth rates (O'neill et al., 2011). Secondly, higher aphid abundances at eCO_2 may relate to the suppression of JA- and ET-dependent defences at elevated [CO_2] (Guo et al., 2014a; Sun et al., 2018), which reduces plant resistance against aphids (Guo et al., 2014b). Lastly, and independently of the effect of eCO_2 on aphid abundance, aphids may partake in compensatory feeding at eCO_2 in order to counter lower N (i.e. amino acid) concentrations in the phloem (Sun et al., 2009b). Evidence in support of this comes from honeydew deposits, which may be used as a substitute for the volume of plant C imbibed; eCO_2 drove a three-fold increase in honeydew production by cowpea aphids (*Aphis craccivora*) on *Medicago sativa* (Kremer et al., 2018), with similar findings reported in other plant-aphid systems (Sun et al., 2009b, although see Boullis et al., 2018).

1.6 Project Aims

The primary aim of this thesis is to investigate the how interacting abiotic ([CO_2]) and biotic (aphids) environmental factors affect the functionality of wheat-AM associations. Four key questions are explored:

Key questions:

1. Does eCO_2, a plant C source, alter the lifetime fitness benefits afforded to wheat by arbuscular mycorrhizal fungi?
2. Do aphids, an external biotic plant C sink, lead to less nutritionally mutualistic wheat-mycorrhizal symbioses?
3. Does an aphid-induced loss of plant C impact carbon-for-nutrient exchange between wheat and an AM fungus, and is this mitigated by eCO_2?
4. Do the effects of eCO_2 and aphids on wheat-mycorrhizal symbioses hold true when using a mixed AM fungal assemblage collected from arable soils?

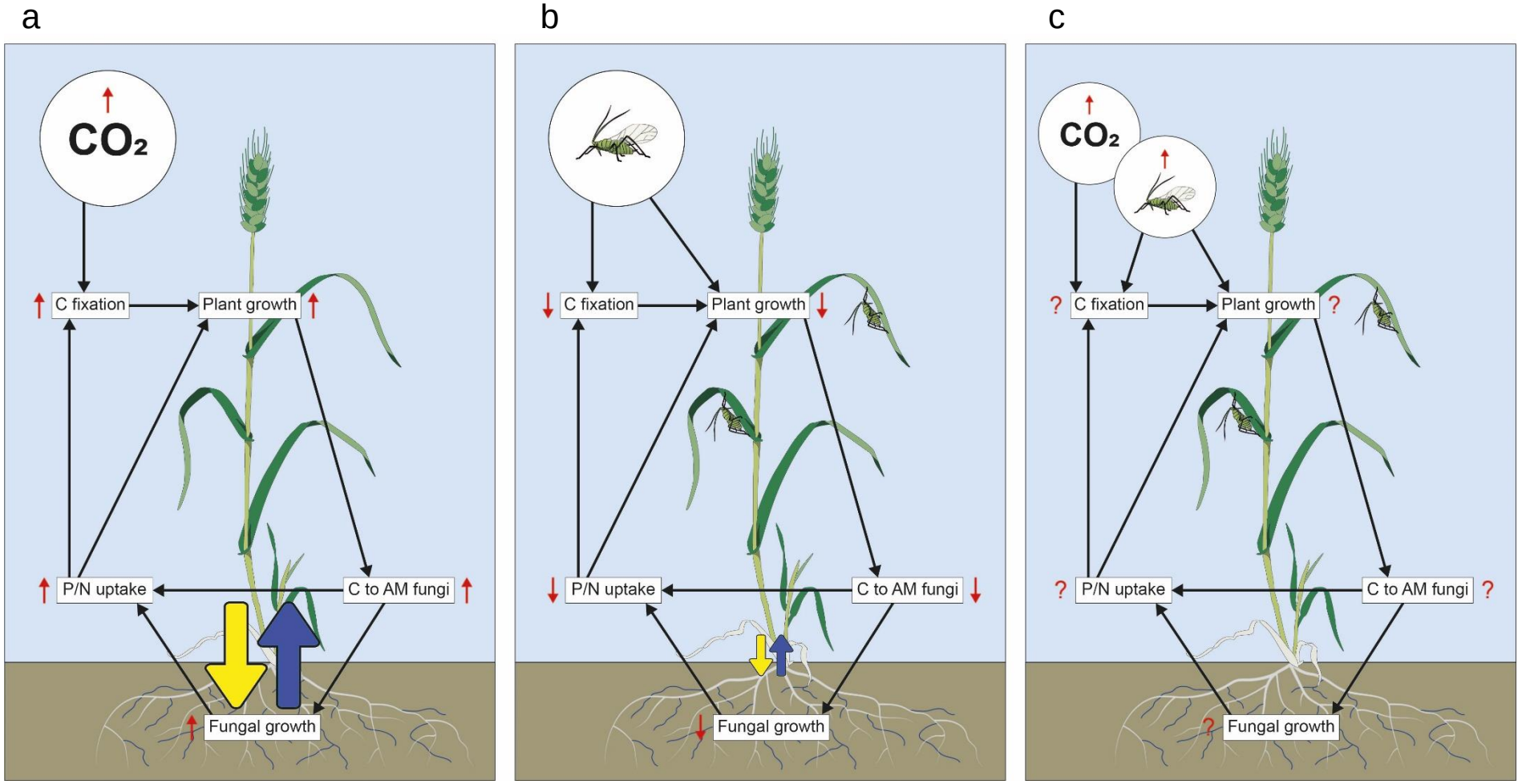

Figure 1.3: Summary of the effects of [CO_2] and aphids individually and together on wheat-AM function. Yellow and blue arrows denote C-for-nutrient exchange. (a) eCO_2 increases rates of photosynthesis and the source strength of wheat for plant C, thereby increasing plant C supply to AM fungi. This may lead to greater fungal-acquired plant nutrient uptake, either directly if resource exchange is tightly linked or as a result of increased fungal growth. This could increase the strength of the mutualism through positive feedback. (b) Aphids siphon phloem from plants and as such represent external plant C sinks. Aphids may compete with AM fungi for C, which could reduce AM infection leading to a less highly functioning symbiosis through negative feedback. (c) Whether eCO_2 offsets the C sink strength of aphids is unknown, as aphid abundances and feeding rates may rise at eCO_2.

Chapter 2 Lifetime fitness benefits of AM colonisation for wheat under contrasting atmospheric [CO_2]

2.1 Introduction

Cereals such as wheat have long been thought to be poorly responsive to AM fungi (Hetrick et al., 1992; 1993), either due of their fine/fibrous rooting systems (Yang et al., 2015) or the development of modern cultivars adept at acquiring nutrients directly from fertilisers (Tawaraya, 2003), thereby reducing their dependence on AM fungi (Martín-Robles et al., 2018). However, studies have shown that mycorrhizal fungi may improve wheat nutrient uptake (Lehmann & Rillig, 2015; Ercoli et al., 2017) and grain yield (Pelligrino et al., 2015; Zhang et al., 2018). Thus, the potential exists to exploit AM symbioses in farming in order to reduce reliance on fertilisers (Thirkell et al., 2017).

However, growth responses of plants to AM fungi can be genotype and/or species-specific (Hoeksema et al., 2010), with neutral and negative effects of AM colonisation recorded in wheat (Ellouze et al., 2016), maize (Sawers et al., 2017), and sorghum (Watts-Williams et al., 2019a). Reasons for these inconsistent effects are unclear. Negative growth responses may result from an inactive fungal uptake pathway (see Chapter 1 section 1.2.1), with AM fungi providing little nutritional benefit while also representing a C “cost” for the plant (Smith & Smith, 2011a). That said, reduced plant growth has been recorded even when AM fungi contribute to plant nutrient uptake (Smith et al., 2003; Li et al., 2006). The failure of mycorrhizal fungi to compensate for a downregulation of the direct uptake pathway could also inhibit plant growth; AM colonisation may reduce P transporter gene expression in the root (Smith & Smith, 2011a), as well as fine root lengths (Lazarevic et al., 2018) and root hair densities (Sun & Tang, 2013). Lastly, a “trade imbalance” in which plant C supply offsets mycorrhizal-acquired nutrient uptake could depress plant growth. That said, negative growth responses have been recorded in wheat even when colonisation is low, suggesting little plant C transfer (Grace et al., 2009).

Whatever the cause, genotypic and environmental factors may determine outcomes of plant-AM symbioses. Plant responsiveness varies between fungal taxa (Munkvold et al., 2004), plant species (Klironomos, 2003), and crop cultivars (Hetrick et al., 1993; Ellouze et al., 2016), in the latter instance perhaps due to differences in release date (Zhu et al., 2001), fungal pathogen resistance, or root architecture (Smith & Smith, 2011a). Environmental factors that impact C, P, and N dynamics may also determine

how plants respond to AM fungi, as outlined in Chapter 1 section 1.2.5. Soil nutrient status impacts plant responsiveness to AM fungi (Johnson, 2010). Mutualistic interactions are typical in P-limited substrates, as mycorrhizal fungi can exchange excess P for plant C (although see Tran et al., 2020). This is also true if N availability is high; N levels are tightly linked with photosynthetic rates, thereby increasing plant C supply to AM fungi resulting in a stronger mutualism (Johnson, 2010). In contrast, commensalism or parasitism may be likely under high soil P, as plants can meet their P requirements without forming AM symbioses. Negative outcomes can also occur in N-limited soils (Johnson et al., 2015) because AM fungi may compete with hosts for N resources (Hodge & Fitter, 2010). Other abiotic factors like shading (Johnson et al., 2015) and drought (Sendek et al., 2019) may impact plant growth responses to AM fungi. As such, a more complete grasp of which abiotic drivers impact the function of wheat-AM associations is required before AM fungi can be fully advocated in farming (Ryan & Graham, 2018).

How rising [CO_2] impacts wheat-AM symbioses is unclear (Becklin et al., 2017). Greater availability of plant C resources at eCO_2 may counter the “costs” of hosting AM fungi, thereby easing plant growth depressions (Johnson et al., 2005). On the other hand, eCO_2 may increase the proportion of plant C allocated to AM fungi by over 20% (Drigo et al., 2010; 2013; Field et al., 2012). Increases in AM colonisation of roots and soils have been attributed this (Dong et al., 2018), being seen in wheat (Zhu et al., 2016) and other grasses (Jakobsen et al., 2016). A systemic signalling cascade may also be involved; eCO_2 can induce strigolactone (SL) biosynthesis following hydrogen peroxide-induced auxin production (Zhou et al., 2019), which can in turn trigger AM fungal spore germination and hyphal branching (Akiyama et al., 2005) and help maintain the symbiosis (Foo et al., 2013). Greater fungal abundance at eCO_2 as a result may improve the capacity of AM fungi to supply wheat with soil nutrients (Zhu et al., 2016), driving a stronger mutualism through positive feedback (Fitter et al., 2000). However, positive effects of eCO_2 on AM infection are not always recorded (Staddon & Fitter, 1998). Hence, AM fungal-acquired plant nutrient uptake may be unresponsive to rising [CO_2] (Gavito et al., 2002; Jakobsen et al., 2016; Thirkell et al., 2019), with parasitism even occurring due to competition for P/N between bigger plants and larger hyphal networks (Alberton et al., 2005). Because of this, a study on 14 plant species found eCO_2 both increased and decreased plant growth responses

to AM fungi (Johnson et al., 2005). By studying how eCO_2 affects wheat responsiveness to AM colonisation across a range of cultivars, key traits for optimal mutualistic outcomes under future [CO_2] scenarios may be found (Meinshausen et al., 2011), which can be used in breeding programmes.

In addition to the effect of [CO_2], plant growth responses to AM fungi may be contingent on timing (Smith & Smith, 2011). Growth depressions following AM colonisation have been recorded at different time points in different crops (Watts-Williams et al., 2019b), with symbiotic outcomes also shifting during crop development; by growing wheat for 6 weeks and until yield with *Glomus intraradices* (formerly *R. irregularis*), Li et al., (2005) found AM colonisation negatively affected wheat growth at the first harvest, but that this effect disappeared by maturity. Such temporal dynamism may be driven by the varying demand for nutrients during crop growth (AHDB, 2018), for example when assimilates are re-allocated above ground during grain filling and ripening (Shrewy, 2009). Alternatively, the extent of colonisation may determine how AM fungi impact plant growth (Treseder, 2013). In order to improve our conception of how crops respond to AM colonisation at eCO_2, the impact of AM fungi on wheat growth must be evaluated at multiple time points, such as during periods of peak nutrient demand (i.e. stem elongation, GS30-GS40) and at yield (Zadoks et al., 1974).

This experiment investigates the impact of atmospheric [CO_2] on the growth and nutritional responses of three wheat cultivars to colonisation by an AM fungus (*R. irregularis*). cvs. Avalon, Cadenza, and Skyfall were grown under contrasting [CO_2] reflecting present-day levels (440 ppm, aCO_2) and conditions projected for the end of the century (800 ppm, eCO_2) (IPCC, 2014). Cultivars differed in their release dates, being a mixture of older (cvs. Avalon and Cadenza) and more modern varieties (cv. Skyfall). cvs. Avalon and Cadenza also differed in their canopy traits (i.e. crop height: Griffiths et al., 2012; heading date: Martinez et al., 2020) and disease resistance (Bass et al., 2006; Gardiner et al., 2020). The impact of the AM fungus on wheat growth was quantified 8 weeks after planting after a preliminary experiment established successful colonisation then, and that GS30 had begun. Plant growth responses were also investigated at yield, enabling an assessment of the lifetime fitness benefits provided by *R. irregularis*. If growth responses to the AM fungus were the same 8 weeks after planting as at yield then future experiments would use the earlier time point.

2.2 Key questions and hypotheses

- Does colonisation of wheat by an AM fungus improve wheat growth and nutrient status at aCO_2, and does this differ between cultivars?
  - Hypothesis 1: Plants are hypothesised to exhibit improved nutrient uptake and growth when colonised by *R. irregularis*. The direction and extent of plant growth responses to the AM fungus may vary between cultivar as recorded previously (Hetrick et al., 1992; 1993). More modern cultivars, like cv. Skyfall, may be less responsive to AM fungi than older ones (Zhu et al., 2001; Martín-Robles et al., 2018). On the other hand, cv. Cadenza may respond less positively to AM colonisation being resistant against biotrophic pathogens (Gardiner et al., 2020).
- Does eCO_2 increase AM fungal abundance in roots and soils of wheat?
  - Hypothesis 2: eCO_2 is hypothesised to increase % root length colonisation and extra-radical hyphal lengths of the AM fungus, in-line with previous findings in wheat (Zhu et al., 2016) and grasses (Jakobsen et al. 2016). This is owing to greater plant C supply to AM fungi at eCO_2 (Drigo et al., 2010; Field et al., 2012). However, studies using the same wheat cultivars recorded no effect of eCO_2 on AM colonisation by a mixed community (Thirkell et al., 2019; Elliott et al., 2020).
- Does eCO_2 impact wheat growth and nutritional responses to AM colonisation?
  - Hypothesis 3: Wheat growth responses to the AM fungus are hypothesised to be more positive at eCO_2. This is because greater fungal biomass in roots and soils of wheat at eCO_2 (see Hypothesis 2) may increase plant uptake of fungal-acquired nutrients, which could in turn improve plant growth and the strength of the symbiosis through positive feedback (Fitter et al., 2000). This effect may differ between cultivars, as variable effects of [CO_2] have been recorded on AM responsiveness between plant genotypes (Johnson et al., 2005).
- Does the time of harvest impact wheat growth responses to the AM fungus?
  - Hypothesis 4: Wheat growth responses to the AM fungus are hypothesised to differ at different time points (Smith & Smith, 2011b). AM responsiveness is expected to be more positive at yield, as recorded previously in wheat (Li et al., 2005). This may be due to altered physiological functioning during different growth stages, or varying degrees of AM colonisation, with greater fungal abundance eliciting more positive outcomes in certain hosts (Treseder, 2013).

2.3 Materials and Methods

2.3.1 Plant material

Seeds of the wheat cvs. Avalon, Cadenza, and Skyfall were supplied by RAGT Seeds Ltd.. cvs. Avalon and Cadenza were chosen given their use as parental lines for the UK bread wheat reference population, established by the Wheat Genetic Improvement Network in 2003 (Ma et al., 2015). These varieties were described in 1991 and 1993, and were crossed because of their phenotypic differences. cv. Avalon possesses the vernalisation response gene *Vrn-A1b* (Griffiths et al., 2009) and the gibberellin-insensitive reduced height gene *Rht-D1b* (Griffiths et al., 2012), while cv. Cadenza carries the dominant alleles of both. cv. Cadenza also possesses multiple desirable resistance genes, granting protection against mosaic disease (*Sbm1*: Bass et al., 2006) and yellow rust disease (*Yr7*: Gardiner et al., 2020). The original cv. Avalon x cv. Cadenza mapping population produced over 200 double haploid lines, which was expanded to over 900 in 2009. This mapping population has been used to study the genetic loci controlling a variety of wheat traits in order to aid breeding efforts. These include grain yield (Ma et al., 2015; Farré et al., 2016), plant height (Griffiths et al., 2012), heading time (Griffiths et al., 2009; Martinez et al., 2020), root characteristics (Bai et al., 2013), and resistance to plant pathogens (Bass et al., 2006).

cv. Skyfall was chosen being a relatively young variety developed using marker-assisted selection in 2012, being a cross of cvs. Hurricane and ‘C4148’ (Allen-Stevens, 2019). It was released to growers in 2014, and has since appeared on every Recommended List published by the Agriculture and Horticulture Development Board (AHDB, 2020). It has also been classified as a Group 1 variety by the National Association of British & Irish Millers (NABIM, 2020). cv. Skyfall is currently the most widely grown cultivar of wheat in the UK because of its high yield, disease resistance, and suitability to the UK climate (RAGT, 2018).

Seeds of plants to be harvested after 8 weeks were surface-sterilised inside a desiccator for 3 hours, using chlorine gas liberated from 100 mL sodium hypochlorite with 3 mL concentrated HCl. Seeds were germinated in 9 cm Petri dishes at 20˚C for 5 days on sterile filter paper moistened with 4 mL autoclaved dH_2O. 24 seedlings of each cultivar (72 plants, n=6) were planted in 4.5” pots, in substrate consisting of a sand: perlite mix (3:1) which had been sterilised at 121˚C for 45 minutes. Seeds of plants to be grown to yield were sterilised using a 10% sodium hypochlorite solution

for 20 minutes, washed with dH_2O five times, and left to swell in dH_2O in the dark overnight. 20 seedlings of cvs. Cadenza and Skyfall (40 plants, n=5) were germinated and potted up as described for plants harvested after 8 weeks. cv. Avalon was not grown to yield as it possess the recessive allele for the vernalisation response gene *Vrn-A1* meaning cold treatments are required to trigger flowering (Griffiths et al., 2009).

2.3.2 Fungal material

Plants in the '+ AMF' treatment were inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Schenck & Smith, 2009). This fungal species was the first to have its 153-Mb haploid genome sequenced in 2013 (Tisserant et al., 2013), and is widely distributed on an intercontinental scale (Savary et al., 2018). *R. irregularis* is considered to be a generalist as it associates with most plants, including poorly mycorrhizal species. For this reason, *R. irregularis* is the most widely used species in commercial inocula (Rosikiewicz et al., 2017), despite already being present in most arable soils regardless of management type (Oehl et al., 2010). This AM fungal strain has previously been shown to engage in cooperative resource exchange in sterile (Kiers et al., 2011; Fellbaum et al., 2012) and non-sterile systems (Fellbaum et al., 2014). Monoxenic cultures were grown in a 22˚C incubator on transformed hairy carrot root (*Daucus carota*) and Phytagel™ MSR media (Declerck et al., 2005). For plants harvested at 8 weeks, the AM fungal inoculum was produced by blending ten plates of *R. irregularis* aseptically with 150 mL sterile dH_2O. Spore counts were conducted in triplicate using 100 µL of inoculum and a microscope, and 15 mL of inoculum containing 21,450 spores was mixed evenly into the substrate. Remaining replicates, hereafter referred to as the '- AMF' treatment, received the same volume of inoculum that had been autoclaved at 121˚C for 30 minutes. Root clearing and staining (see section 2.3.5) confirmed that fungal structures were absent from roots of - AMF plants.

For plants to be grown to yield, ten *R. irregularis* Petri dishes were blended with 150 mL sterilised dH_2O. 15 mL of inoculum consisting of 11,200 spores was mixed uniformly into the substrate of + AMF replicates. Plants within the - AMF treatment were inoculated with an identical volume of twice-sterilised inoculum (121˚C for 30 minutes). Roots of plants to be grown to yield were sampled using a 10mm core borer 8 weeks after planting. Root clearing and staining (see section 2.3.5) confirmed that wheat in the - AMF and + AMF treatments were non-mycorrhizal and mycorrhizal, respectively. Removed substrate was replaced with sterilised sand.

2.3.3 Growth conditions

Plants were grown in controlled environment chambers in the Centre for Plant Sciences at the University of Leeds, at 440 ppm ('aCO_2') or 800 ppm ('eCO_2') atmospheric [CO_2]. Growth conditions were maintained at 20˚C and 70% relative humidity (RH) during a 16-hour day-time cycle, with an average light intensity of 220 µmol m^{-2} s^{-1}. Environmental conditions during the 8-hour night-time cycle were 15˚C and 70% RH. Plants were rotated between cabinets once each month to control for any cabinet effects, and watered when required. Plants were fed once weekly with 30 mL low-P (40%) nitrate-type Long Ashton Solution (LAS) (Smith et al., 1983), and watered with tap water when necessary. Substrate surfaces were covered in 3 mm high density polyethylene (HDPE) granules to prevent algae growth and reduce water loss. Water was gradually withheld from plants grown to yield beyond 15 weeks until all above-ground plant material had dried.

2.3.4 Harvest procedure

One set of plants (72 plants, n=6) was harvested 8 weeks after planting and a second set (60 plants, n=5) was harvested at yield (approximately 18 weeks after planting). At harvest, plants were carefully removed from pots and substrate was loosened from the roots. 10-15 g of substrate was collected from each replicate for fungal hyphal length quantification, and stored in zip-lock bags at 5˚C (see section 2.3.6). Remaining substrate attached to roots was washed off with water and roots were dried using paper towels. Above-ground and below-ground material was separated, and shoot, root, and grain biomass (fresh weights, FW) were recorded using a 3-digit digital scale. Root systems of plants harvested at both time points were divided in two, with half being preserved in 50% ethanol (EtOH, v/v) at 5˚C and later used to quantify AM colonisation (see section 2.3.5). Remaining root sub-samples were re-weighed before being freeze-dried with shoot and grain material for 72 hours. Shoot, root, and grain biomass (dry weights, DW) were recorded using a 5-digit digital scale. Total root biomass and root: shoot ratios were calculated as follows:

Equation 1

$$\text{Total root biomass} = \text{Total root FW} \times \left(\frac{\text{Sub-sample root DW}}{\text{Sub-sample root FW}}\right)$$

Equation 2

$$\text{Root: shoot ratio} = \frac{\text{Total root DW}}{\text{Total shoot DW}}$$

2.3.5 AM colonisation

AM colonisation was quantified following root clearing and staining. Roots were loaded into histology cassettes and suspended in pre-heated 10% KOH (w/v) at 80˚C for 40 minutes. Roots were then washed with dH_2O, and root-internal fungal structures were stained with ink and vinegar solution (5% Pelikan Brilliant Black, 5% acetic acid, 90% dH_2O) for 20 minutes (Figure 2.1; Vierheilig et al., 1998). Roots were de-stained in 1% acetic acid for at least 2 hours, and mounted on microscope slides using polyvinyl lacto-glycerol (16.6 g polyvinyl alcohol powder, 10 mL glycerol, 100 mL lactic acid, 100 mL dH_2O), and left to set over-night at 60˚C. AM colonisation was assessed using the gridline intersection methodology using a hairline eyepiece graticule (McGonigle et al., 1990). 150 fields of view were observed per plant across 20 root fragments at 400x magnification. % root length colonisation, % arbuscules, and % vesicles were quantified using equations from Brundrett et al., (2000).

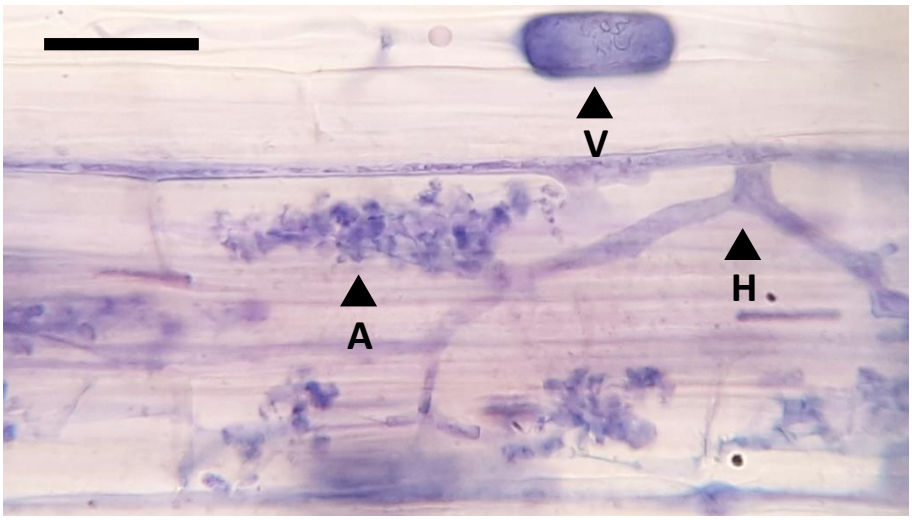

Figure 2.1: A stained section of wheat root colonised by the AM fungus *Rhzophagus irregularis*. Characteristic root-internal fungal structures are labelled, including arbuscules (A), vesicles (V), and intracellular hyphae (H). Bar, 60 µm.

2.3.6 AM hyphal lengths

Hyphae were extracted from soils and lengths quantified using the gridline-intersection methodology. Approximately 4-5 g of substrate was weighed twice using a 3-digit digital balance. One replicate was oven dried at 60˚C for 72 hours, and re-weighed. The second replicate was placed in a large beaker for hyphal extraction, and stirred

with 500 mL dH_2O for 5 minutes. 200 mL was decanted into a second beaker, and stirred for a further 30 seconds. 10 mL was extracted using a syringe, and filtered through two 0.45 µm membrane filters in equal volumes (i.e. 5 ml) using a vacuum pump. Hyphae were stained with Trypan Blue solution (0.4 g Trypan Blue stain, 20% phenol, 20% lactic acid, 20% dH_2O, 40% glycerol), and hyphal intersections were counted at 100x magnification using 50 fields of view against a 10 x 10 grid eyepiece graticule (Tennant, 1975). Hyphal lengths per gram of soil (mg g^{-1}) were calculated using equations from Brundrett et al., (2000).

2.3.7 Plant P determination

Freeze-dried plant material was homogenised using an IKA® mill, and 30-40mg of shoot and root samples weighed in triplicate into acid-washed test tubes (1% HCl). 1 mL concentrated sulphuric acid (H_2SO_4) was pipetted into each tube, which were fitted with cold fingers and left overnight. Samples, and blanks containing no plant material, were then digested at 365˚C for 15 minutes using a digest block. 100 µL of hydrogen peroxide (H_2O_2) was added to cooled samples, which were returned to the block until clear. Digests were diluted to 10 mL with dH_2O, and total P within resulting solutions quantified using colourimetry, following an adapted method from Murphy & Riley (1962) and John (1970). 0.15 mL, 0.2 mL, and 0.5 mL of grain, shoot, and root digest samples were added to separate cuvettes, with 0.5 mL ammonium molybdate and antimony potassium tartrate reagent, 0.2 mL 0.1 M ascorbic acid, and 0.2 mL 3.44M sodium hydroxide (NaOH). Solutions were made up to 3.8mL with dH_2O, and the optical density of samples recorded at 822 nm after 45 minutes development using a spectrophotometer. The P concentration (ppm) of each digest solution was calculated using a standard curve of known [P]. This was produced using a 10 ppm standard P solution containing 44.55 mg sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) dissolved in 1 L dH_2O. The optical density of P standards ranging from 0 to 5 ppm were recorded, and the absorbance of each digest sample was converted into P (ppm) using the equation of the trendline on the standard curve.

2.3.8 Plant N determination

20-30 mg of plant material was weighed into acid-washed test tubes (1% HCl) with 1.1 mL ‘mixed digestion reagent’. The digestion reagent contained 0.21 g selenium powder and 7 g lithium sulphate dissolved in a solution of sulphuric acid (210 mL) and

hydrogen peroxide (175 mL). Cold fingers were placed in tubes and left overnight, before being digested at 365^{o}C for up to 60 minutes until clear. Digest solutions were diluted to 6.25 mL with dH_2O, and total N was determined as described by Thirkell et al., (2016). 15 µL, 20 µL, and 50 µL, of grain, shoot, and root digest solutions were pipetted into cuvettes with equal volumes of 3.44 M sodium hydroxide (NaOH), 1mL of 'solution A', 0.25 mL 'solution B', and made up to 3.8 mL with dH_2O. Solution A contained 10 g trisodium citrate dihydrate, 8.5 g salicylic acid, 2.5 g NaOH, and 0.1 g sodium nitroferricyanide dihydrate dissolved in 250 mL dH_2O. Solution B contained 2.5 g NaIH and 0.2 g sodium dichloroisocyanurate, similarly made up to 250 mL dH_2O. Absorbances were recorded following spectrophotometry at 650 nm after 30 minutes development. The N concentration (ppm) of each digest sample was determined using a standard curve of known [N]. This was produced using a 10 ppm standard N solution containing 38.17 mg ammonium chloride (NH_4Cl). The optical density of N standards ranging from 0 to 20 ppm were recorded, and the absorbance of each digest sample was converted into N (ppm) using the equation of the trendline on the standard curve.

2.3.9 Statistical analysis

All data analyses were performed using R Studio v1.1.453. Data were tested for normality and homogeneity of variances using standard residuals vs fitted and normal Q-Q plots, with Kruskall-Wallis, skewness, and kurtosis tests used when necessary. The effects of AMF, [CO_2], cultivar, and their interactions on shoot and grain biomass, shoot P and [P], root [P], shoot N and [N], grain P and [P], grain N and [N], and grain number were determined using three-way analysis of variance (ANOVA) with additional post hoc Tukey honest significant difference (HSD) tests. Root biomass, root: shoot ratios, total root P, and root N and [N] were Log_{10} transformed and then analysed using the same generalised linear model (GLM). The effect of cultivar, [CO_2], and their interaction on extra-radical hyphal lengths were determined using two-way ANOVA with additional post hoc Tukey HSD tests. % root length colonisation and % arbuscules were Log_{10} transformed and examined similarly. Spearman's rank correlation coefficients were used to analyse the association between AM colonisation and shoot [P]/[N] (at 8 weeks) and grain [P]/[N] and yield (at the later harvest), to determine whether plant nutrient status and growth was a function of the extent of AM infection, in a test of hypotheses one, three, and four. All values reported are means ± standard error (SE). All figures were produced using GraphPad Prism v8.2.0.

2.4 Results

2.4.1 8-week harvest

2.4.1.1 AM colonisation

All wheat plants grown in the '+ AMF' treatment were colonised by the AM fungus *Rhizophagus irregularis*. % root length colonisation was low 8 weeks after planting, ranging from just 3% to 19% (Figure 2.2a). AM colonisation was reduced at eCO_2 (Table 2.1), particularly in plants of cvs. Cadenza (aCO_2: 11.6 ± 2.0 %; eCO_2: 7.5 ± 1.9%) and Avalon (aCO_2: 6.3 ± 0.9 %; eCO_2: 4.3 ± 0.4 %). % root length colonisation also differed between cultivars (Table 2.1), being higher on average in roots of cv. Cadenza (9.5 ± 1.4 %) than those of cvs. Skyfall (6.3 ± 0.8 %) or Avalon (5.3 ± 0.6 %).

Similar tends were recorded for % arbuscules (Figure 2.2b), as arbusucle frequencies were reduced at eCO_2 (Table 2.1). % arbuscules also varied between cultivars (Table 2.1), being most abundant in roots of cv. Cadenza and least abundant in roots of cv. Avalon. No vesicular structures (see Figure 2.1) were found in wheat roots harvested after 8 weeks growth, these being AM fungal structures used primarily for lipid storage.

[CO_2] had no effect on extra-radical hyphal lengths supported by wheat roots (Figure 2.2c; Table 2.1). Instead, hyphal lengths varied between cultivars, being greatest in soils of cv. Skyfall (0.99 ± 0.07 m g^{-1}) and lowest in cv. Avalon (0.72 ± 0.06 m g^{-1}).

Table 2.1: Summary of two-way ANOVA results investigating the effect of [CO_2], cultivar, and their interaction on AM colonisation of wheat 8 weeks after planting. Significant p-values are in bold (n=6).

Factor	**% root length colonisation**			**% arbuscules**			**Hyphal lengths**		
	F	*df*	p	F	*df*	p	F	*df*	p
[CO_2]	6.12	1,29	**0.020**	5.62	1,29	**0.025**	0.13	1,28	0.721
Cultivar	4.65	2,29	**0.018**	12.83	2,29	**<0.001**	4.25	2,28	**0.024**
[CO_2]*Cultivar	0.50	2,29	0.612	0.19	2,29	0.827	1.30	2,28	0.288

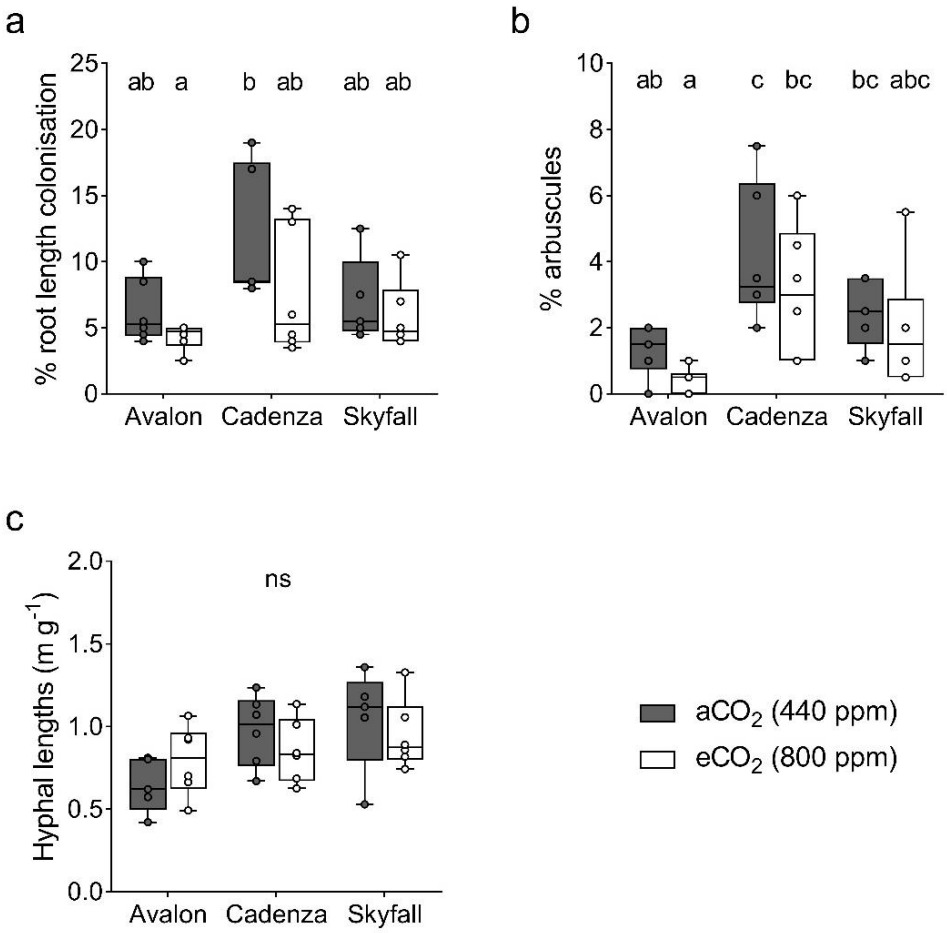

Figure 2.2: AM fungal abundance in roots and soils of 8-week wheat grown at ambient and elevated [CO_2]. (a) % root length colonisation; (b) % arbuscules; (c) extra-radical hyphal lengths. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* and grown in an otherwise sterilised sand: perlite mix (3:1) at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Box plots range from the first to the third quartile. Middle lines signify median values (n=6), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

2.4.1.2 Plant biomass

Wheat plants associated with *R. irregularis* achieved the same shoot biomass at 8 weeks as plants which were not (Figure 2.3a; Table 2.2). This was true across all three cultivars. In contrast, eCO_2 increased shoot biomass, but to a greater extent for plants of cv. Avalon (+35%) than cvs. Cadenza (+17%) and Skyfall (+13%). Consequently, a significant interaction was recorded between cultivar and [CO_2] treatment (Table 2.2). On average, shoot biomass was greatest in cv. Cadenza (aCO_2: 1.59 ± 0.04 g; eCO_2:

1.86 ± 0.02 g), followed by cv. Skyfall (aCO_2: 1.51 ± 0.01 g; eCO_2: 1.71 ± 0.02 g) and then cv. Avalon (aCO_2: 1.13 ± 0.02 g; eCO_2: 1.52 ± 0.03 g).

Root biomass of wheat was affected by AMF treatment (Figure 2.3b; Table 2.2). Roots of '+ AMF' plants were smaller than those of '- AMF' plants in cvs. Avalon (-17 %) and Cadenza (-17 %), although not cv. Skyfall. In contrast, root biomass was unaffected by [CO_2] (Table 2.2). Root biomass also differed between cultivar (Table 2.2), with roots of cv. Avalon (- AMF: 1.69 ± 0.09 g; + AMF: 1.46 + 0.09 g) being larger than those of cv. Cadenza (- AMF: 1.37 ± 0.07 g; + AMF: 1.19 ± 0.08 g) and cv. Skyfall (- AMF: 1.32 ± 0.05 g; + AMF: 1.32 ± 0.08 g). Despite the cultivar-specific effect of AM colonisation on root biomass, no interaction between factors was recorded.

Root: shoot ratios of wheat plants differed significantly between AMF treatments (Figure 2.3c; Table 2.2), being lower in '+ AMF' replicates reflecting their smaller root biomass. A strong effect of [CO_2] was also recorded on wheat root: shoot ratios (Table 2.2), which were reduced at eCO_2 owing to the positive effect of atmospheric [CO_2] on shoot biomass. Lastly, root: shoot ratios differed between each cultivar (Table 2.2), being greater on average in cv. Avalon (1.23 ± 0.07) than cvs. Skyfall (0.82 ± 0.03) and Cadenza (0.75 ± 0.03).

Table 2.2: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on plant growth of wheat 8 weeks after planting. Significant p-values are in bold (n=6).

Factor	**Shoot biomass**			**Root biomass**			**Root: shoot ratio**		
	F	*df*	p	F	*df*	p	F	*df*	p
AMF	0.05	1,57	0.827	5.01	1,56	**0.029**	4.52	1,56	**0.038**
[CO_2]	209.96	1,57	**<0.001**	0.00	1,56	0.999	16.69	1,56	**<0.001**
Cultivar	147.01	2,57	**<0.001**	7.68	2,56	**0.001**	36.57	2,56	**<0.001**
AMF*[CO_2]	0.06	1,57	0.803	0.13	1,56	0.717	0.06	1,56	0.807
AMF*Cultivar	0.24	2.57	0.788	1.11	2.56	0.338	0.77	2.56	0.470
[CO_2]*Cultivar	7.97	2,57	**0.001**	0.46	2,56	0.636	2.43	2,56	0.097
AMF*[CO_2]*Cultivar	0.45	2,57	0.638	0.97	2,56	0.384	0.52	2,56	0.600

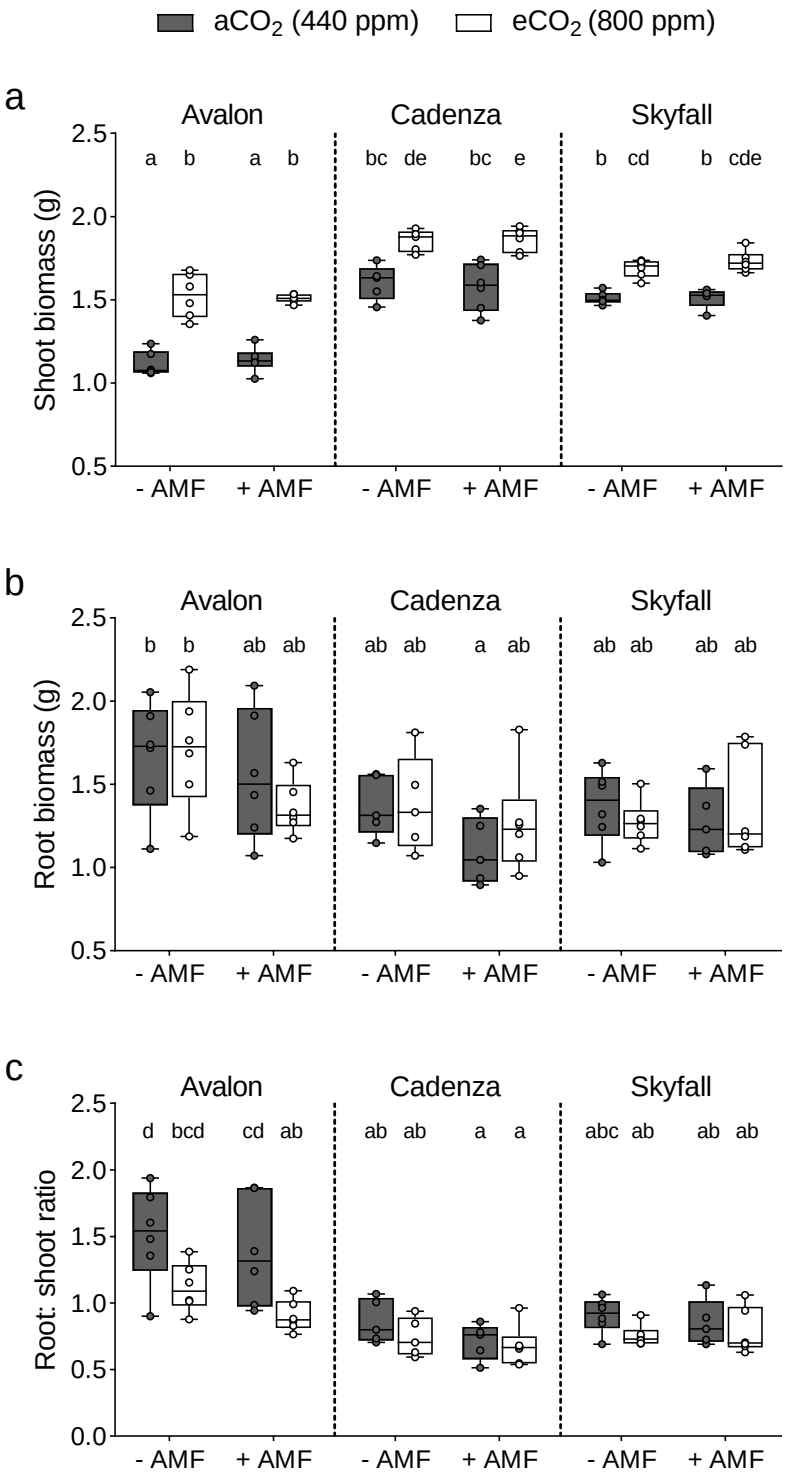

Figure 2.3: Biomass of 8-week non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Shoot biomass; (b) Root biomass; (c) Root: shoot ratio. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=6), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests).

2.4.1.3 Plant P

Shoot P was affected by the three-way interaction between AMF, [CO_2], and cultivar at 8 weeks (Figure 2.4a; Table 2.3). eCO_2 increased shoot P for cvs. Avalon (- AMF: +23%; + AMF: +16%) and Skyfall (- AMF: +8%; + AMF: +9%), but to a greater extent in the former cultivar. In contrast, the effect of eCO_2 on shoot P of cv. Cadenza differed between AMF treatments, being neutral for '- AMF' plants and positive for '+ AMF' ones (+24%). cv. Cadenza achieved highest shoot P on average (3.19 ± 0.08 mg), followed by cvs. Skyfall (2.89 ± 0.03 mg) and Avalon (2.66 ± 0.06 mg).

Shoot P concentrations ([P]) were also affected by the interaction between all three factors after 8 weeks growth (Figure 2.4b; Table 2.3). eCO_2 reduced shoot [P] across both AMF treatments for cvs. Avalon (- AMF: -9%; + AMF: -13%) and Skyfall (- AMF: -4%; + AMF: -4%). The effect of eCO_2 on shoot [P] of cv. Cadenza depended on the mycorrhizal status of plants, being reduced in '- AMF' plants (-13%) but increased in '+ AMF' ones (+5%). On average, shoot [P] was higher in cv. Avalon (2.03 ± 0.04 mg g^{-1}) than cvs. Cadenza (1.85 ± 0.03 mg g^{-1}) and Skyfall (1.79 ± 0.01 mg g^{-1}).

Root P of wheat was unaffected by AMF treatment at the 8-week harvest (Figure 2.4c; Table 2.3). Instead, a significant interaction between [CO_2] and cultivar was recorded on root P (Table 2.3). eCO_2 increased root P across all three cultivars, but to a greater degree in plants of cv. Cadenza (+47%) than cvs. Avalon (+20%) and Skyfall (+3%). On average, higher root P was recorded in cv. Avalon plants (1.45 ± 0.06 mg) than cvs. Skyfall (0.91 ± 0.04 mg) and Cadenza (0.62 ± 0.04 mg)

Root P concentrations ([P]) were affected by the interaction between AMF, [CO_2], and cultivar at 8 weeks (Figure 2.4d; Table 2.3). Root [P] was increased at eCO_2, but the magnitude of this effect varied between AMF treatments for cvs. Avalon (- AMF: +6%; + AMF: +47%) and Cadenza (- AMF: +63%; + AMF: +11%). For cv. Skyfall, eCO_2 increased root [P] of '- AMF' plants (+23%) but reduced those of '+ AMF' plants (-10%). On average, root [P] was greater in cv. Avalon (0.94 ± 0.04 mg g^{-1}) than cvs. Skyfall (0.69 ± 0.03 mg g^{-1}) and Cadenza (0.49 ± 0.03 mg g^{-1}).

2.4.1.4 Plant N

Wheat plants colonised by *R. irregularis* achieved the same shoot N at the 8-week harvest as plants not inoculated with the AM fungus (Figure 2.5a; Table 2.4). Likewise, no effect of [CO_2] was recorded on shoot N. Instead, shoot N differed significantly between cultivars (Table 2.4), being greater in cvs. Cadenza (11.2 ± 0.16 mg) and Skyfall (10.9 ± 0.18 mg) than cv. Avalon (8.31 ± 0.09 mg).

Shoot N concentrations ([N]) were also not affected by AMF (Figure 2.5b; Table 2.4). Rather, a significant interaction between [CO_2] and cultivar was recorded (Table 2.4). Shoot [N] was reduced at eCO_2, but to a greater extent in cv. Avalon (-31%) than cvs. Cadenza (-18%) or Skyfall (-10%), reflecting changes in shoot biomass.

Root N was equivalent in '- AMF' and '+ AMF' plants of each cultivar at 8 weeks (Figure 2.5c; Table 2.4). A significant interaction between [CO_2] and cultivar was observed for root N (Table 2.4). Root N was increased at eCO_2, particularly in cv. Cadenza (+35%), but to a lesser degree in cvs. Skyfall (+7%) and Avalon (+3%). On average, root N was greater in cv. Avalon (6.17 ± 0.14 mg) than cvs. Skyfall (3.22 ± 0.08 mg) and Cadenza (2.99 ± 0.19 mg).

Root N concentrations ([N]) were affected by the interaction of AMF, [CO_2], and cultivar after 8 weeks (Figure 2.5d; Table 2.4). The effect of eCO_2 on root [N] depended on AMF treatment and cultivar; eCO_2 increased root [N] in '- AMF' to a greater extent than in '+ AMF' plants for cvs. Cadenza (- AMF: +50%; + AMF: +14%) and Skyfall (- AMF: +14%; + AMF: +3%). The opposite was true of cv. Avalon (- AMF: -3%; + AMF: +20%). On average, root [N] was greatest in cv. Avalon (4.06 ± 0.17 mg g^{-1}) and lower in cvs. Cadenza (2.42 ± 0.16 mg g^{-1}) and Skyfall (2.49 ± 0.1 mg g^{-1}).

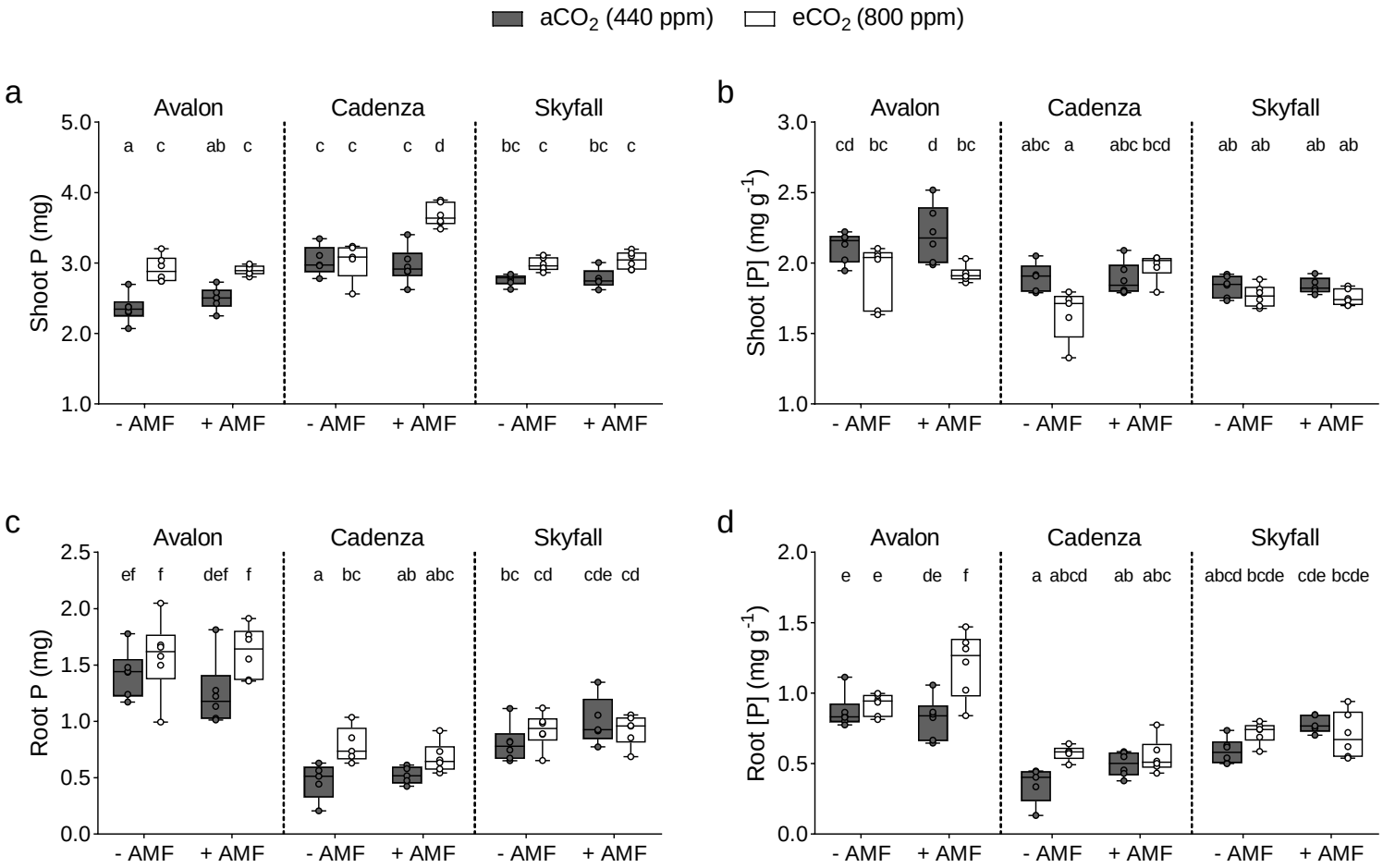

Figure 2.4: P uptake by 8-week non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Shoot P; (b) Shoot [P]; (c) Root P; (d) Root [P]. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=6), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests).

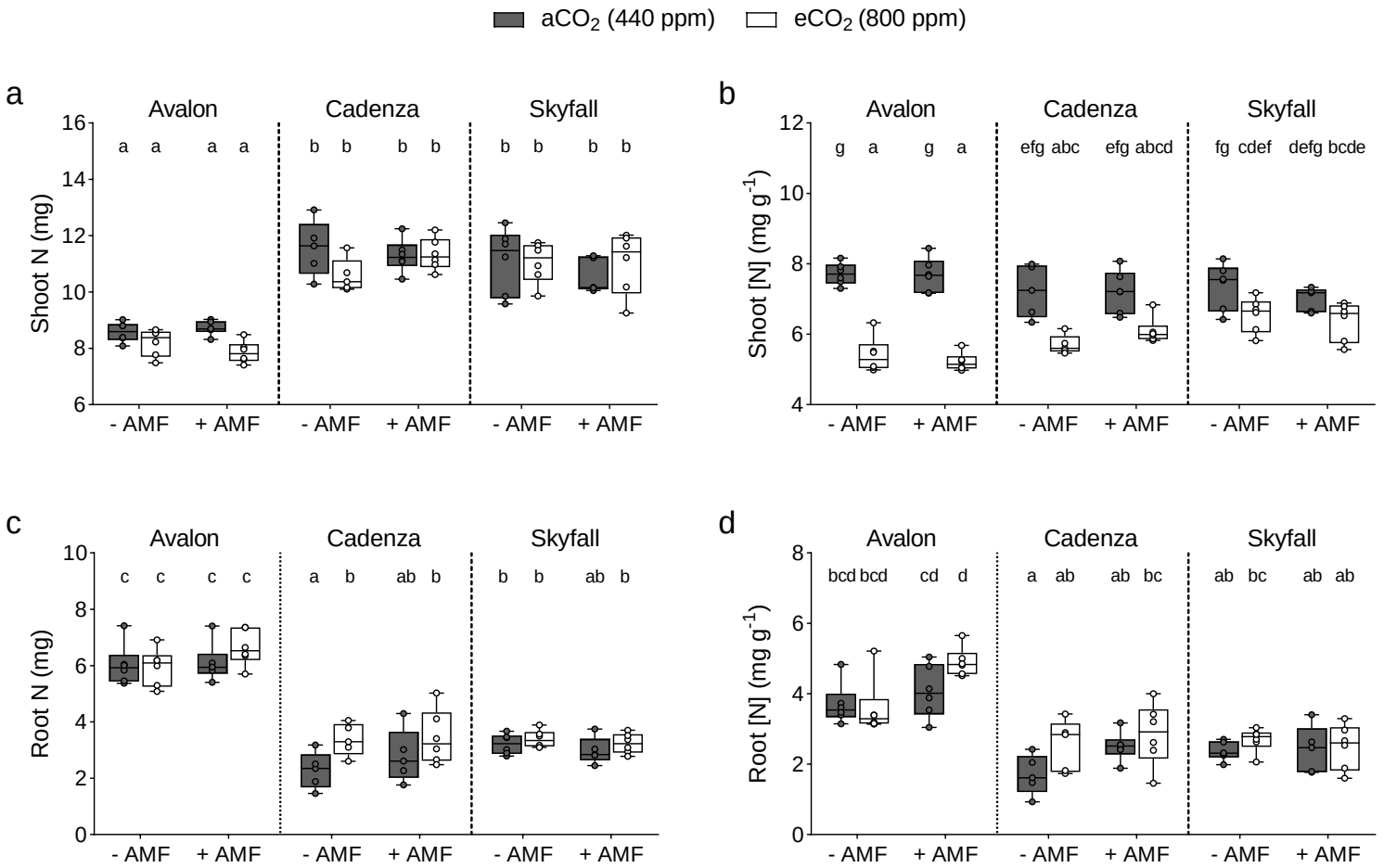

Figure 2.5: N uptake by 8-week non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Shoot N; (b) Shoot [N]; (c) Root N; (d) Root [N]. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=6), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests).

Table 2.3: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on P uptake by wheat 8 weeks after planting. Significant p-values are in bold (n=6).

Factor	**Shoot P**			**Shoot [P]**			**Root P**			**Root [P]**		
	F	*df*	P	F	*df*	p	F	*df*	p	F	*df*	p
AMF	8.91	1,57	**0.004**	4.91	1,57	**0.031**	0.11	1,56	0.738	7.99	1,57	**0.006**
[CO_2]	78.79	1,57	**<0.001**	17.72	1,57	**<0.001**	15.10	1,56	**<0.001**	18.15	1,57	**<0.001**
Cultivar	52.81	2,57	**<0.001**	23.92	2,57	**<0.001**	96.75	2,56	**<0.001**	76.36	2,57	**<0.001**
AMF*[CO_2]	5.08	1,57	**0.028**	1.48	1,57	0.228	0.98	1,56	0.326	0.01	1,57	0.910
AMF*Cultivar	3.65	2.57	**0.032**	2.69	2.57	0.076	0.86	2.56	0.427	0.30	2.57	0.739
[CO_2]*Cultivar	3.01	2,57	0.057	3.33	2,57	**0.043**	3.91	2,56	**0.026**	3.23	2,57	0.047
AMF*[CO_2]*Cultivar	9.92	2,57	**<0.001**	4.84	2,57	**0.011**	2.13	2,56	0.128	8.58	2,57	**0.001**

Table 2.4: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on N uptake by wheat 8 weeks after planting. Significant p-values are in bold (n=6).

Factor	**Shoot N**			**Shoot [N]**			**Root N**			**Root [N]**		
	F	*df*	P	F	*df*	p	F	*df*	p	F	*df*	p
AMF	0.05	1,57	0.822	0.24	1,57	0.624	0.55	1,56	0.461	6.17	1,57	**0.016**
[CO_2]	2.89	1,57	0.095	159.28	1,57	**<0.001**	9.85	1,56	**0.003**	5.49	1,57	**0.023**
Cultivar	118.12	2,57	**<0.001**	2.59	2,57	0.084	124.63	2,56	**<0.001**	40.04	2,57	**<0.001**
AMF*[CO_2]	0.96	1,57	0.331	0.34	1,57	0.564	0.02	1,56	0.879	0.22	1,57	0.640
AMF*Cultivar	0.79	2.57	0.457	1.24	2.57	0.297	1.27	2.56	0.289	3.14	2.57	0.051
[CO_2]*Cultivar	1.96	2,57	0.150	17.34	2,57	**<0.001**	4.19	2,56	**0.020**	0.94	2,57	0.396
AMF*[CO_2]*Cultivar	1.74	2,57	0.185	0.52	2,57	0.600	0.96	2,56	0.390	2.18	2,57	0.123

2.4.1.5 Correlations

In order to study whether AM colonisation impacted shoot nutrient status of wheat 8 weeks after planting, Spearman's rank correlation coefficients were performed on root-internal and root-external colonisation and shoot P and N concentrations. % root length colonisation of wheat at 8 weeks was not related with shoot [P] (Figure 2.6a) or [N] (Figure 2.6b). Likewise, no association was recorded between extra-radical hyphal lengths and shoot [P] (Figure 2.6c) or [N] (Figure 2.6d).

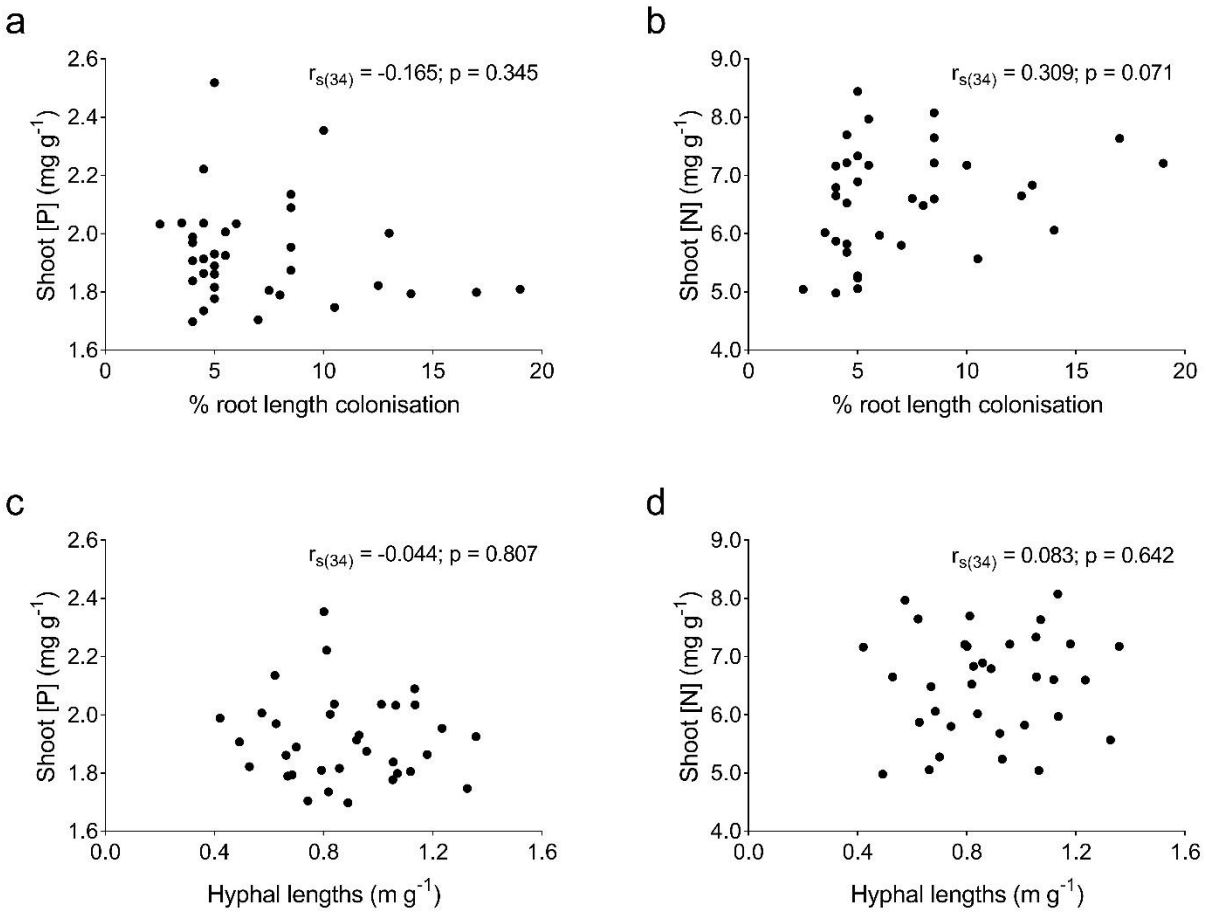

Figure 2.6: Correlations between shoot nutrient status and AM fungal abundance in roots and soils of 8-week wheat. (a) Shoot [P] vs % root length colonisation; (b) Shoot [N] vs % root length colonisation; (c) Shoot [P] vs extra-radical hyphal lengths; (d) Shoot [N] vs extrar-adical hyphal lengths. All data pooled across wheat cultivars (Avalon, Cadenza, and Skyfall) and [CO_2] treatments (aCO_2 and eCO_2). Correlations were tested using Spearman's rank correlation coefficients.

2.4.2 Yield harvest

2.4.2.1 AM colonisation

AM colonisation of wheat ranged from 65% to 98%. No effect of [CO_2] or cultivar was recorded on % root length colonisation or % arbuscules (Figure 2.7a&b; Table 2.5). Unlike at 8 weeks, vesicles were abundant in wheat roots (Figure 2.7c). A significant interaction was recorded between [CO_2] and cultivar on % vesicles (Table 2.5), with eCO_2 reducing vesicle frequencies in cv. Skyfall roots (aCO_2: 36%; eCO_2: 28%) but increasing them in roots of cv. Cadenza (aCO_2: 22%; eCO_2: 30%)

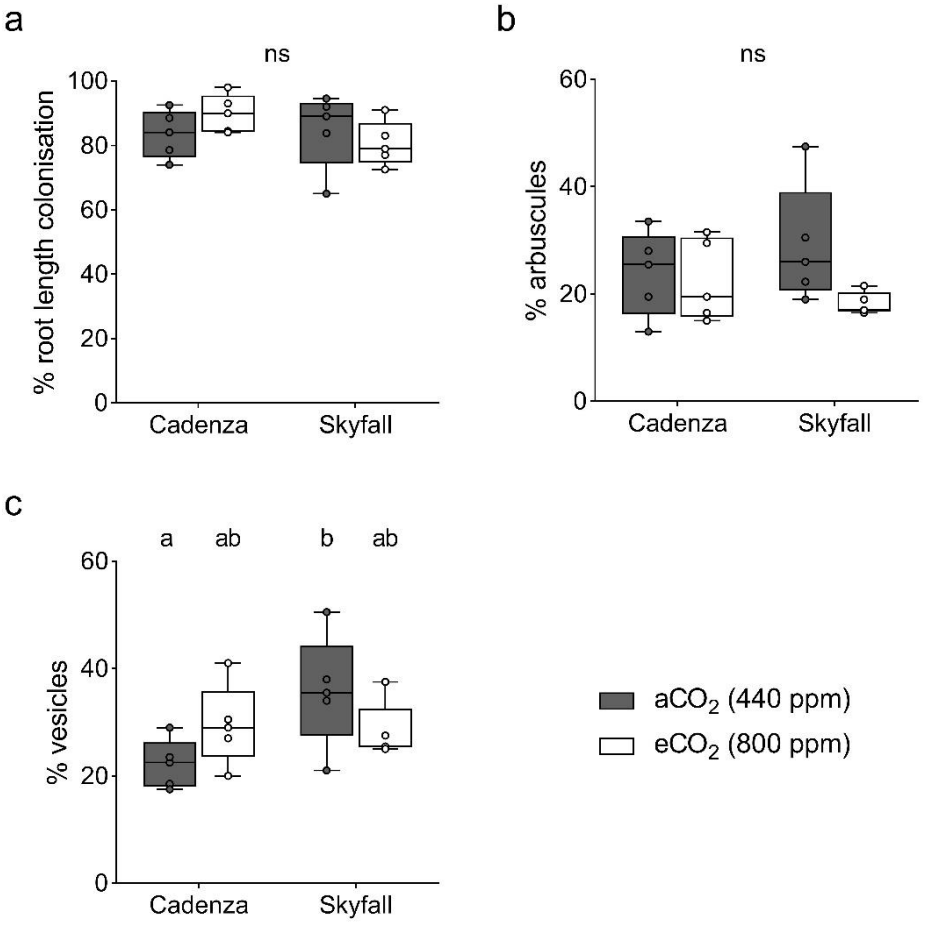

Figure 2.7: AM fungal abundance in roots of wheat grown to yield at ambient and elevated [CO_2]. (a) % root length colonisation; (b) % arbuscules; (c) % vesicles. cvs. Cadenza and Skyfall were inoculated with *R. irregularis* and grown in an otherwise sterilised sand: perlite mix (3:1) at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=5), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

Table 2.5: Summary of two-way ANOVA results investigating the effect of [CO_2], cultivar, and their interaction on AM colonisation of roots of wheat grown to yield. Significant p-values are in bold (n=5).

Factor	% root length colonisation			% arbuscules			% vesicles		
	F	*df*	p	F	*df*	p	F	*df*	p
[CO_2]	0.07	1,16	0.788	3.00	1,16	0.103	0.01	1,16	0.964
Cultivar	1.16	1,16	0.297	0.01	1,16	0.925	3.48	1,16	0.080
[CO_2]*Cultivar	2.08	1,16	0.168	1.68	1,16	0.213	5.11	1,16	**0.038**

2.4.2.2 Grain number and biomass

AMF treatment had no effect the number of grain produced by wheat plants of either cultivar (Figure 2.8a; Table 2.6). Instead, grain number was significantly affected by atmospheric [CO_2], as eCO_2 increased grain number for plants of cv. Cadenza (+18%) and cv. Skyfall (+10%) regardless of AMF treatment. The number of grain produced per plant also differed significantly between cultivar (Table 2.6), averaging 37.8 ± 0.7 for plants of cv. Skyfall and 30.9 ± 0.9 for plants of cv. Cadenza.

Similar patterns were recorded for grain biomass (Figure 2.11b). No effect of AM colonisation of wheat was detected on grain biomass of either cultivar (Table 2.6). Rather, there was a significant effect of [CO_2] on grain biomass, as eCO_2 significantly increased grain biomass of cv. Cadenza (+22%) and cv. Skyfall (+11%) irrespective of AMF treatment. Cultivar type also impacted grain biomass (Table 2.6), which was greater for cv. Skyfall (1.76 ± 0.03 g) than cv. Cadenza (1.45 ± 0.04 g).

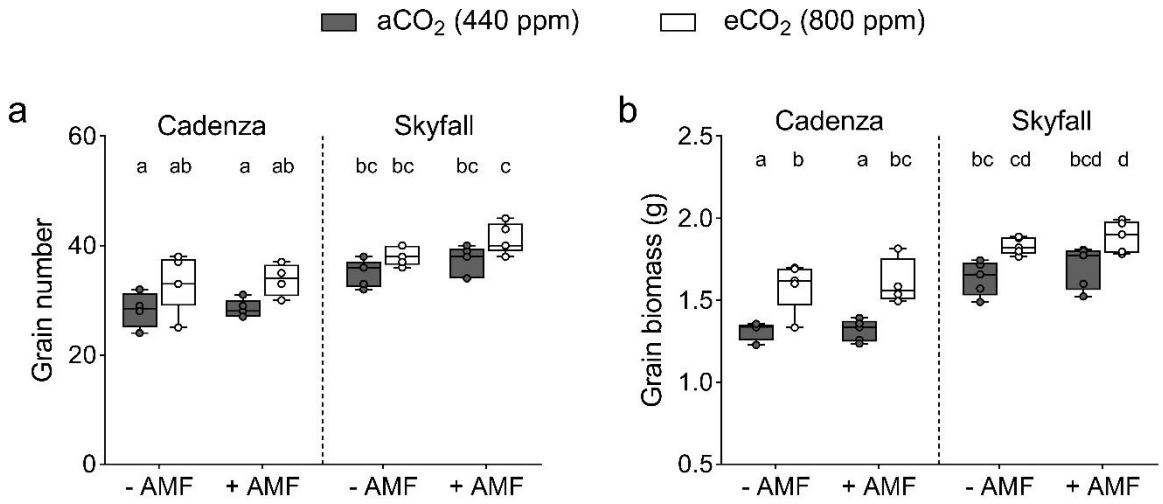

Figure 2.8: Yield of non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Grain number; (b) Grain biomass. cvs. Cadenza and Skyfall were inoculated with the AM fungus *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=5) and whiskers extend to minimum and maximum data points (closed or open markers). Different letters indicate significant differences between means (where $p < 0.05$, Tukey HSD tests).

Table 2.6: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on grain yield of wheat. Significant p-values are in bold (n=5).

Factor	Grain number			Grain biomass		
	F	*df*	p	F	*df*	p
AMF	2.16	1,30	0.152	1.00	1,30	0.325
[CO_2]	19.77	1,30	**<0.001**	46.27	1,30	**<0.001**
Cultivar	50.06	1,30	**<0.001**	79.66	1,30	**<0.001**
AMF*[CO_2]	0.13	1,30	0.718	0.00	1,30	0.955
AMF*Cultivar	1.19	1,30	0.285	0.55	1,30	0.465
[CO_2]*Cultivar	0.64	1,30	0.430	1.92	1,30	0.176
AMF*[CO_2]*Cultivar	0.02	1,30	0.880	0.03	1,30	0.859

2.4.2.3 Grain P

Grain P was affected by the three-way interaction between AMF, [CO_2], and cultivar (Figure 2.9a; Table 2.7). eCO_2 significantly increased grain P for cv. Cadenza plants in both AMF treatments (- AMF: +17%; + AMF: +11%). This was not the case for cv. Skyfall, as eCO_2 increased grain P in '- AMF' plants but reduced grain P in '+ AMF' ones (-AMF: +17%; + AMF: -7%). Grain P was did not vary between cultivars (Table 2.7), averaging 5.26 ± 0.09 mg for cv. Skyfall and 5.14 ± 0.10 mg for cv. Cadenza.

Grain P concentration ([P]) was affected by the interaction between AMF and [CO_2] (Figure 2.9b; Table 2.7). eCO_2 reduced grain [P] of '+ AMF' plants to a greater extent than '- AMF' plants for cv. Cadenza (- AMF: -3%; + AMF: -9%) and cv. Skyfall (- AMF: +3%; + AMF: -16%). Grain [P] also differed between cultivar (Table 2.7), being higher on average in cv. Cadenza (3.55 ± 0.05 mg g^{-1}) than cv. Skyfall (2.99 ± 0.05 mg g^{-1}).

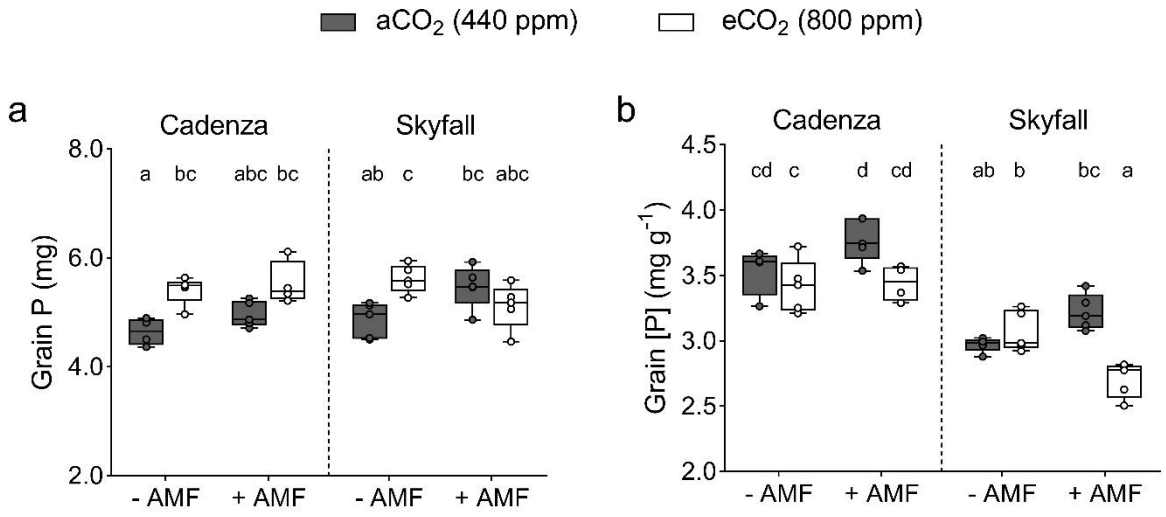

Figure 2.9: Grain P status of non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Grain P; (b) Grain [P]. cvs. Cadenza and Skyfall were inoculated with the AM fungus *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=5), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests).

Table 2.7: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on grain P status of wheat. Significant p-values are in bold (n=5).

Factor	**Grain P**			**Grain [P]**		
	F	*df*	p	F	*df*	p
AMF	1.28	1,30	0.268	0.44	1,30	0.514
[CO_2]	15.24	1,30	**<0.001**	20.18	1,30	**<0.001**
Cultivar	1.38	1,30	0.249	127.26	1,30	**<0.001**
AMF*[CO_2]	10.59	1,30	**0.003**	18.44	1,30	**<0.001**
AMF*Cultivar	0.60	1,30	0.445	3.59	1,30	0.068
[CO_2]*Cultivar	4.69	1,30	**0.038**	0.09	1,30	0.772
AMF*[CO_2]*Cultivar	4.71	1,30	**0.038**	4.04	1,30	0.054

2.4.2.4 Grain N

A significant interaction between AMF and [CO_2] was also recoded for grain N (Figure 2.10a; Table 2.8). Wheat grown at eCO_2 achieved higher grain N than at aCO_2, but this was only true for '- AMF' plants of cvs. Cadenza (- AMF: +6%; + AMF: +0%) and Skyfall (- AMF: +5%; + AMF: -5%). Grain N also differed between cultivars (Table 2.8), being higher on average in plants of cv. Skyfall (29.58 ± 0.39 mg) than cv. Cadenza (27.44 ± 0.34 mg).

When expressed as a concentration, grain [N] was unaffected by AMF (Figure 2.10b; Table 2.8). Instead, a strong effect of [CO_2] was recorded, as eCO_2 reduced on grain [N] for cv. Cadenza (-15%) and cv. Skyfall (-11%) regardless of AMF treatment, reflecting the effect of [CO_2] on grain biomass. Grain [N] also varied between cultivar (Table 2.8), being higher in plants of cv. Cadenza plants (19.06 ± 0.49 mg g^{-1}) than those of Skyfall (16.85 ± 0.33 mg g^{-1}). No interactions were recorded between factors.

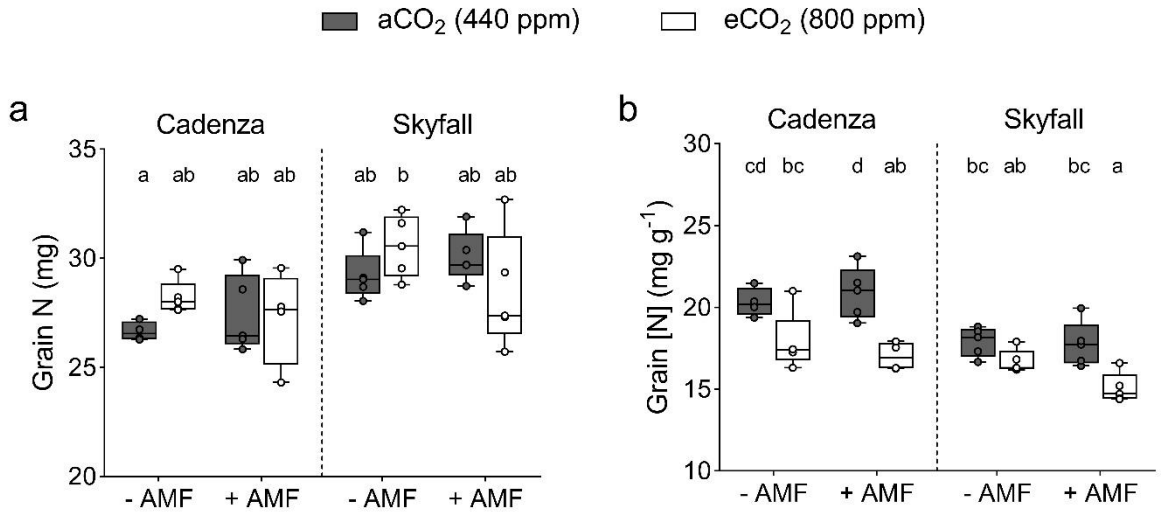

Figure 2.10: Grain N status of non-mycorrhizal and mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Grain P; (b) Grain [P]. cvs. Cadenza and Skyfall were inoculated with the AM fungus *R. irregularis* (+ AMF) or a sterilised control inoculum (- AMF) and grown at aCO_2 (440 ppm, grey boxes) or eCO_2 (800 ppm, white boxes). Boxes range from the first to the third quartile. Middle lines signify median values (n=5), and whiskers extend to minimum and maximum data points (closed or open markers). Different letters indicate significant differences between means (where $p < 0.05$, Tukey HSD tests).

Table 2.8: Summary of three-way ANOVA results investigating the effect of AMF, [CO_2], cultivar, and their interactions on grain N status of wheat. Significant p-values are in bold (n=5).

Factor	Grain N			Grain [N]		
	F	*df*	p	F	*df*	p
AMF	0.50	1,30	0.485	1.62	1,30	0.213
[CO_2]	0.28	1,30	0.600	40.78	1,30	**<0.001**
Cultivar	16.86	1,30	**<0.001**	31.41	1,30	**<0.001**
AMF*[CO_2]	5.01	1,30	**0.033**	3.46	1,30	0.073
AMF*Cultivar	0.25	1,30	0.618	0.84	1,30	0.368
[CO_2]*Cultivar	0.62	1,30	0.437	2.51	1,30	0.123
AMF*[CO_2]*Cultivar	0.36	1,30	0.556	0.00	1,30	0.985

2.4.2.5 Correlations

To investigate whether the extent of AM colonisation determined grain yield and nutrient concentrations of mycorrhizal plants at the yield, Spearman's rank correlation coefficients were performed between % root length colonisation and grain number, grain biomass, grain [P] and grain [N]. No correlation was detected between % root length colonisation and the number of grain produced by each plant (Figure 2.11a), nor grain biomass (Figure 2.11b). % root length colonisation was also not related to grain [P] (Figure 2.11c) or grain [N] (Figure 2.11d).

Similarly, the frequency of arbuscules in wheat roots was unrelated to grain number ($r_{s[19]}$ = -0.200; p = 0.411) and grain biomass ($r_{s[19]}$ = -0.133; p = 0.588) (Figures not shown). % arbuscules was also not correlated with grain [P] ($r_{s[19]}$ = 0.331; p = 0.166) or grain [N] ($r_{s[19]}$ = 0.220; p = 0.336) at yield (Figures not shown).

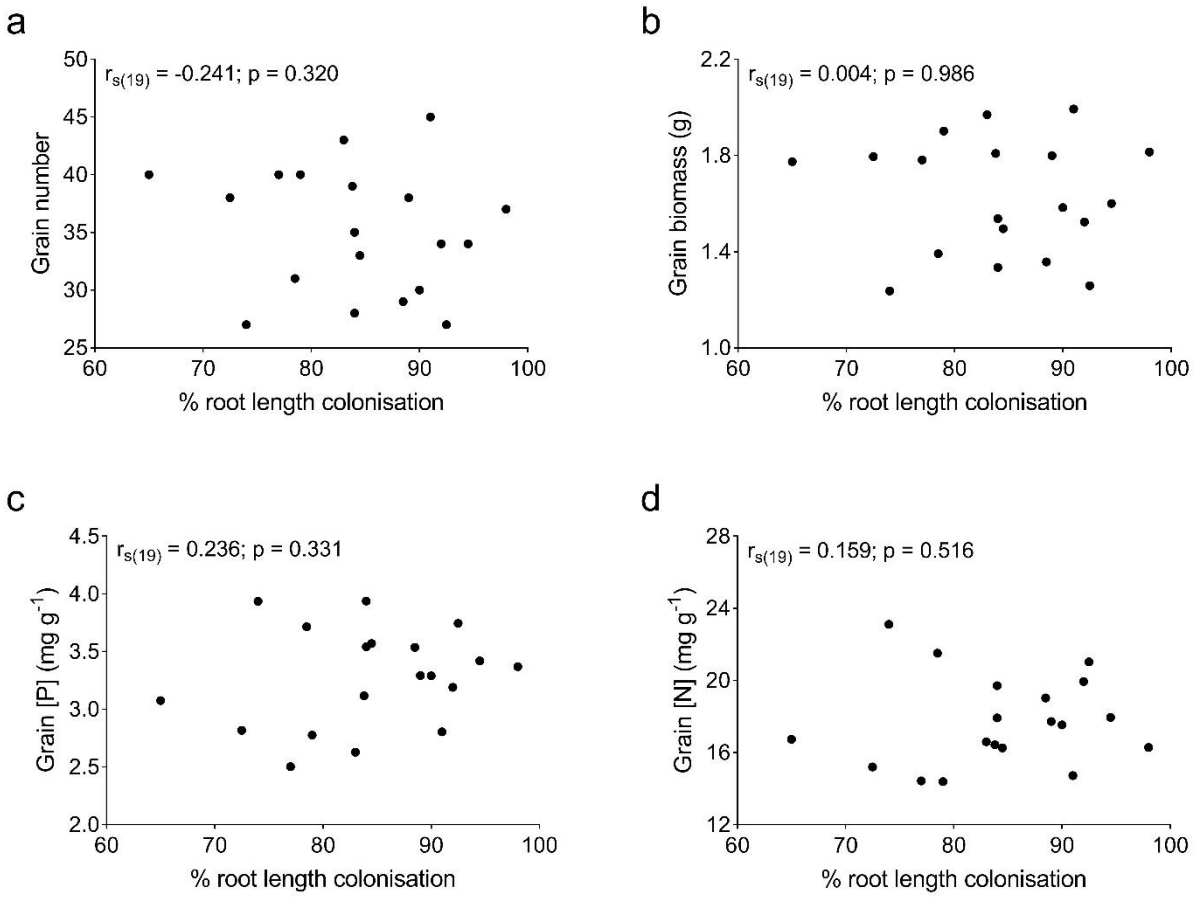

Figure 2.11: Correlations between grain characteristics and AM fungal abundance in roots of wheat grown to yield. (a) Grain number vs % root length colonisation; (b) Grain biomass vs % root length colonisation; (c) Grain [P] vs % root length colonisation; (d) Grain [N] vs % root length colonisation. All data pooled across wheat cultivars (cvs. Cadenza and Skyfall) and [CO_2] treatments (aCO_2 and eCO_2). Correlations were tested using Spearman's rank correlation coefficients.

2.5 Discussion

Despite interest in exploiting AM fungi in agriculture in order to reduce fertiliser usage, the occurrence of inconsistent plant growth responses in wheat to colonisation by AM fungi remains a significant obstacle preventing their widespread adoption. Evidence suggests genotypic factors may determine wheat responsiveness to AM fungi (Hetrick et al., 1992; Munkvold et al., 2004; Ellouze et al., 2016), as well as the time of harvest (Li et al., 2005). However, whether abiotic factors that alter plant C availability, such as atmospheric [CO_2], impact wheat-AM fungal symbioses is unclear. By growing wheat with and without *Rhizophagus irregularis* for 8 weeks and until yield, the lifetime fitness benefits afforded by AM fungi to wheat were assessed at ambient atmospheric [CO_2] and at CO_2 levels projected for the end of the century (IPCC, 2014).

2.5.1 Ambient atmospheric [CO_2]

2.5.1.1 Wheat growth responses to AM colonisation

Colonisation of wheat by the AM fungus *R. irregularis* did not affect shoot biomass at aCO_2 8 weeks after planting (Figure 2.3a). Similarly, AM colonisation had no effect on grain yield, as grain number and biomass were equivalent across AMF treatments (Figure 2.8). These findings run counter to the first hypothesis, which predicted positive wheat growth responses to AM colonisation (Pelligrino et al., 2015; Zhang et al., 2018). They also counter hypothesis four, that anticipated plant responsiveness to the AM fungus would shift throughout crop development, as recorded before in wheat (Li et al., 2005) and other cereals (Watts-Williams et al., 2019b), due to differences in plant-AM function or changes in the extent of AM colonisation (Treseder, 2013).

Reasons for neutral plant growth responses at aCO_2 may differ between time points. For instance, AM colonisation of wheat was low 8 weeks after planting, ranging from 5-19% at aCO_2 (Figure 2.2a). Similarly, extra-radical hyphal lengths were lower than those recorded previously in cvs. Avalon and Cadenza (Elliott et al., 2020) (Figure 2.2b). AM colonisation of roots and soils is often used as a measure of C supply by plants, given AM fungi are entirely reliant on host photosynthate for the development and regeneration of fungal structures (Roth & Paszkowski, 2017). These observations, alongside the absence of vesicular structures, suggest that plant C allocation to *R. irregularis* was minimal early in the development of the symbiosis. This could mean that the C “cost” associated with supporting the AM fungus was not large enough to

depress plant growth, while the nutritional benefits afforded by *R. irregularis* may have also been insufficient to increase it. However, this explanation is unsatisfactory for multiple reasons. Firstly, the extent of AM colonisation does not always correlate with plant-AM function (Smith et al., 2004; Nagy et al., 2009), meaning low levels of AM infection may not accurately reflect the plant C sink strength of mycorrhizal roots, or the nutritional benefits provided. Secondly, modest levels of AM colonisation do not always result in neutral growth responses, as growth depressions have been recorded in wheat when colonisation is low (Li et al., 2008). For instance, plant biomass of cvs. Newton and Kanzler was halved when inoculated with AM fungi, despite colonisation averaging just 5% and 17%, respectively (Hetrick et al., 1992). Finally, the same logic cannot be used to explain neutral growth responses to the AM fungus at yield, as roots were heavily infected at aCO_2 (Figure 2.7).

An alternative explanation could be that nutritional benefits provided by *R. irregularis* at aCO_2 were offset by a downregulation of the direct uptake pathway, having a net neutral effect on plant growth. Partial evidence for this may be found when looking at the effect of the AM fungus on root biomass at 8 weeks. Cultivar-specific effects of AM colonisation were recorded on root biomass of wheat (Figure 2.3b); AM plants of cvs. Avalon and Cadenza (but not cv. Skyfall) had lower root biomass than those grown without the fungus, which would likely have compromised direct nutrient uptake across the root. Despite this, shoot [P] and [N] were broadly speaking the same in '- AMF' and '+ AMF' plants of these cultivars (Figure 2.4b & 2.5b), suggesting that total nutrient capture (i.e. the sum of plant- and fungal-acquired uptake) was equivalent in mycorrhizal and non-mycorrhizal wheat. This outcome could indicate a functional AM uptake pathway for P and N, which compensated for the reduced functionality of the direct pathway. Such a conclusion could only be validated by assessing transporter gene expression in the root (Smith & Smith, 2011a), or by using isotope tracers (Chapter 4). Mycorrhizal-acquired ^{33}P and ^{15}N uptake has been recorded recently in wheat (Thirkell et al., 2019; Elliott et al., 2020), as well as in other cereals (Sawers et al., 2017), and can occur in the absence of positive growth responses to AM fungi (Smith et al., 2003). Intriguingly, the effect of AM colonisation on root biomass contrasts previous work which recorded either neutral (Thirkell et al., 2019) or positive effects of AM fungi on root growth of spring and winter wheat (Pérez et al., 2016; Zhu et al., 2016). Variable outcomes may be due to the use of different isolates between

studies (de León et al., 2020), or environmental conditions such as P and N availability. Future work may want to study this root biomass response across a range of AM fungal treatments and soil nutrient concentrations, and also consider the effect of AM fungi on finer-scale aspects of wheat root morphology, such as branching (Lazarević et al., 2018) and root hair densities (Sun & Tang, 2013).

Lastly, the neutral effect of AM colonisation on wheat growth at aCO_2 may be a consequence of the year of release of each cultivar. To date, wheat breeding efforts have focussed primarily on enhancing yield-related traits (e.g. thousand grain weight, grain number, grain width etc.) and other above ground characteristics such as crop height and heading date (Martinez et al., 2020). These traits have been selected for under optimal growth conditions, and may have inadvertently constrained wheat root characteristics via linkage drag (Voss-Fells et al., 2017). Because of this, more modern varieties are thought to possess smaller roots (Den Herder et al., 2010) and lower root: shoot ratios (Siddique et al.,1990), and perhaps exhibit reduced responsiveness to AM fungi. For instance, Hetrick et al., (1992) found that "new" cultivars were less reliant on AM fungi than ancestral/old wheat varieties, with similar observations made by Zhang et al., (2018). However, in both instances "new" cultivars were varieties released after 1950. Even though cvs. Avalon, Cadenza, and Skyfall range in their release date by two decades, all were developed post 1950 making them all highly bred according to this classification. This perhaps explains their unresponsiveness to AM colonisation at both time points, and why no variation was recorded in plant growth responses between cultivars, which was predicted as part of the first hypothesis.

2.5.2 Elevated atmospheric [CO_2]

2.5.2.1 Impact of eCO_2 on wheat growth and nutrient status

eCO_2 increased shoot biomass of wheat 8 weeks after planting (Figure 2.3a), and grain yield at maturity (Figure 2.8) in-line with values reported previously in the field (Dong et al., 2018). This stimulatory effect was the same in mycorrhizal and non-mycorrhizal plants at both time points. This makes sense, given AM colonisation had little effect on shoot [P] and [N] (Figure 2.4b & 2.5b) or grain [P] and [N] (Figure 2.9b & 2.10b), and the nutrient status of plants plays a key to role in determining plant growth responses to eCO_2 (Pandey et al., 2015). The reason for this is that as plant biomass increases at eCO_2 (Ainsworth & Rogers, 2007), the demand for mineral

nutrients also grows. Therefore, rates of photosynthesis may become P- for N-limited at eCO_2, tempering the CO_2 fertilisation effect (Cavagnaro et al., 2011). This dynamic may explain differences between cultivars in the extent to which eCO_2 promoted plant growth at both harvests. Growth stimulation by eCO_2 at 8 weeks was greatest in cv. Avalon, which had highest shoot and root [P] (Figure 2.4b & 2.4d). Similarly, the positive effect of eCO_2 on grain yield was more marked in cv. Cadenza, which achieved highest grain [P] and [N] (Figure 2.9b & 2.10b). Alternatively, the year of cultivar release may again explain these cultivar-specific effects. Previous work has found that shoot biomass of older wheat cultivars responded more positively to eCO_2 than newer ones (Manderscheid & Weigel, 1997). This compliments the findings presented here, as growth stimulation by eCO_2 at both harvests was highest in the oldest cultivars. An explanation for this may be that selection pressures imposed by commercial crop breeding programmes have reduced the capacity of European wheat to adapt to climatic change (Kahiluoto et al., 2019).

In contrast, no stimulatory effect of eCO_2 was recorded on root biomass of wheat (Figure 2.3b), supporting prior findings in different cultivars (Zhu et al., 2016). While this could be an artefact of growing plants in pots and thereby artificially restricting their rooting volume (Poorter et al., 2012), root lengths of wheat may also be unresponsive to eCO_2 in the field (Pacholski et al., 2015). These finding indicates that as C availability increases, wheat C resources are not allocated below ground but are instead retained/invested in the shoot, which is reflected in reduced root: shoot ratios at eCO_2 (Figure 2.3c). Why this occurs may also relate to the characteristics for which modern wheat cultivars are bred. Varieties with extensive roots may be considered “C-inefficient” by crop breeders if fertiliser applications mean nutrients are abundant and accessible (Thirkell et al., 2019). Thus, elite wheat lines tend to invest a smaller fraction of plant biomass below ground than older cultivars (Siddique et al., 1990). This finding raises the question of how other environmental factors that impact plant C source strengths, like biotic interactions with insect herbivores, affect wheat C dynamics (Chapters 3 and 4).

eCO_2 reduced shoot [N] 8 weeks are planting (Figure 2.5b), but to a greater extent in cv. Avalon than cvs. Cadenza and Skyfall reflecting the cultivar-specific effect of eCO_2 on shoot biomass. eCO_2 also reduced grain [N] at yield (Figure 2.10b). Such responses are well characterised in wheat grown at eCO_2 (Broberg et al., 2017).

Termed the ‘nitrogen dilution effect’, enhanced rates of photosynthesis at eCO_2 increases the carbon-to-nitrogen ratio (C:N) of plant tissues (Stiling & Cornelissen, 2007). Reduced grain [N] at eCO_2 may also coincided with lower grain protein and free amino acid levels (Soba et al., 2019). Not only does this reduce the nutritional quality of C3 grains (Myers et al., 2014), but it may also impact plant-insect interactions. Greater C:N ratios at eCO_2 can induce compensatory feeding in insects like aphids, causing pests to consume more phloem in order to assimilate equivalent quantities of N as at aCO_2 (Sun & Ge, 2011). This mechanism is important to consider when studying wheat-aphid interactions at eCO_2, as aphids may limit plant C availability to a greater extent, particularly if growth rates are increased (Chapters 4 and 5).

2.5.2.2 Impact of eCO_2 on AM colonisation

% root length colonisation of wheat was reduced at eCO_2 8 weeks after planting, with this effect being consistent across all cultivars (Figure 2.2a). This was despite eCO_2 increasing shoot growth at this time point, and therefore the source strength of wheat for plant C. Similar trends were recorded for % arbuscules at eCO_2 (Figure 2.2b), these being the diagnostic structure of the symbiosis (Fitter, 2006) across which the majority of nutrient exchange is thought to occur (Luginbuehl & Oldroyd, 2017). These findings counter the second hypothesis, which predicted eCO_2 would increase AM colonisation (Alberton et al., 2005; Dong et al., 2018) as recorded in wheat (Zhu et al., 2016) and other grasses (Jakobsen et al., 2016).

The full range of AM colonisation responses to eCO_2 have been recorded previously in plants, including positive, neutral, and negative effects, although the latter are less common (Staddon & Fitter, 1998; Cavagnaro et al., 2011). Because of this, reasons for their occurrence are uncertain. Lower % root length colonisation at eCO_2 cannot be ascribed to differences in root biomass, as roots of wheat were unaffected by atmospheric [CO_2] (Figure 2.3b). Instead, reduced AM colonisation may suggest plant C supply to *R. irregularis* was lower at eCO_2 than at aCO_2, contrasting previous work (Drigo et al., 2010; Field et al., 2012). Why this might be is unclear, but it may be linked to patterns in plant C partitioning outlined above in modern wheat cultivars. In order to test this, AM colonisation responses to eCO_2 should be studied across a range of ancestral, old, and new wheat lines. Alternatively, lower % root length colonisation at eCO_2 could be due to the low nutrient status of the substrate. eCO_2 reduced shoot [P] and [N], which may have limited rates of photosynthesis as P is a key component of

ATP and roughly ¾ of plant N is invested in the chloroplast (Johnson, 2010). Therefore, plant nutrition at eCO_2 may have reduced plant C availability, and in turn the amount of C supplied to the AM fungus. Indeed, the effect of eCO_2 on AM colonisation has previously been shown to be dependent on soil nutrient status (Klironomos et al., 1996; Jakobsen et al., 2016). AM colonisation responses to eCO_2 can be complex, therefore, with atmospheric [CO_2] not determining the extent of AM infection in isolation. Future studies ought to investigate the interactive effect of eCO_2 and soil P/N status on AM colonisation of wheat, as carried out recently in a C4 cereal crop (Watts-Williams et al., 2019c).

% root length colonisation of wheat was unaffected by eCO_2 at yield (Figure 2.7a). This could suggest that over the course of the symbiosis the amount of plant C allocated to AM fungi was equivalent at aCO_2 and eCO_2. However, as indicated above, the degree to which plant roots are colonised by AM fungi is not always tightly linked with nutrient exchange (Smith & Read, 2010). Sophisticated isotopic tracers could be used to validate this. By employing this labelling approach, Thirkell et al., (2019) recorded no effect of eCO_2 on plant C supply by modern wheat cultivars to a mixed AM fungal community. This raises the question as to how responsive modern cultivars might be to other environmental factors that impact plant C availability, for instance biotic interactions with insect herbivores that reduce the source strength of wheat for plant C (Chapter 3 and 4).

2.5.2.3 Impact of eCO_2 on plant growth responses to AM fungi

Wheat growth responses to AM colonisation were the same at eCO_2 as they were at aCO_2. This result counters hypothesis three, which predicted more positive plant growth responses to the AM fungus at CO_2 levels projected for 2100 (IPCC, 2014). This was predicated on eCO_2 increasing AM fungal biomass and thus fungal-acquired plant nutrient uptake, which would in turn drive plant growth (and nutrient demand) leading to a stronger mutualism (Fitter et al., 2000).

Extra-radical hyphal lengths may be better indicators of mycorrhizal-mediated nutrient uptake in plants than root-internal colonisation. This is because hyphae grow beyond nutrient depletion zones that surround plant roots, and can increase the below-ground absorptive surface area of plants 40-fold (Pepe et al., 2018). For instance, Sawers et al., (2017) found that P uptake by *R. irregularis* was positively correlated with hyphal

lengths in six maize lines (*Zea mays* L.). Likewise, hyphal lengths of two *Glomus* species were positive associated with fungal P uptake in cucumber (*Cucumis sativus* L.) (Munkvold et al., 2004). Here, eCO_2 did not increase extra-radical hyphal lengths of wheat 8 weeks after planting (Figure 2.2c), likely for the same reasons discussed above for % root length colonisation. This finding may suggest that the nutritional benefits provided to wheat by the AM fungus were equivalent at aCO_2 and eCO_2, thereby explaining the similar growth responses to AM colonisation recorded under contrasting [CO_2]. In support of this, extra-radical hyphal lengths were found not to be correlated with shoot [P] or [N] (Figure 2.6c & 2.6d), although it should be noted that this is the sum of both plant- and fungal-acquired P and N uptake, with isotopic tracers and AM fungal-only soil compartments being needed to distinguish between pathways. Similar correlations could not be made at yield, as water had been withheld from plants beyond week 15 meaning extra-radical hyphae had likely dried. Moreover, plant C supply to AM fungi, which maintains hyphal growth, probably ceased during grain filling and ripening. Consistent with this were the high frequencies of vesicles within roots of plants at yield (Figure 2.7c). Vesicles are fungal lipid storage organs, and are widely thought to form when plant C supply to AM fungi declines, for instance after exposure to biotic stress like herbivory (Wearn & Gange, 2007) (Chapter 3).

Although Johnson et al., (2005) documented positive and negative effects of eCO_2 on plant responsiveness to AM fungi, neutral effects were by far the most common outcome, being recorded in 11 of the 14 pairings studied. Clearly, further research is needed into the drivers of these variable outcomes. Fungal genotype may be an important factor, as eCO_2 tended to increase the beneficial effects of Gigasporaceae species to a greater extent than *Glomus* species (Johnson et al., 2005). The study of wheat growth responses at eCO_2 to AM colonisation by AM fungi native to arable soils is therefore necessary (Chapter 5).

2.5.3 Cultivar differences

Cultivars differed in the degree to which their roots were colonised by AM fungi 8 weeks after planting, with cv. Cadenza supporting greater fungal biomass than cvs. Skyfall and Avalon. This finding may indicate that plant C supply to the AM fungus was greater in cv. Cadenza early in the symbiosis. That said, recent studies found no differences in plant C transfer to AM fungi in these cultivars (Thirkell et al., 2019; Elliott et al., 2020). Alternatively, genetic factors may drive variability in AM receptivity

between cultivars, with the quantitative trait loci (QTL) involved found localised to chromosomes 3A, 4A, and 7A (Lehnert et al., 2017). AM colonisation did not differ between varieties at yield, however, highlighting the importance of assessing plant-AM symbioses across multiple time points.

Wheat cvs. Avalon, Cadenza, and Skyfall also differed in their growth habits. cv. Avalon had shorter stems but more tillers and leaves than plants of cvs. Cadenza and Skyfall 8 weeks after planting (data not shown). This was anticipated given cv. Avalon carries the reduced height semi-dwarfing gene *Rht-D1b* mapped to chromosome 4D (Griffiths et al., 2012). Larger root biomass of this cultivar compared to cvs. Cadenza and Skyfall may also be attributed this recessive allele (Wojciechowski et al., 2009). Differences between cultivars in nutrient status at 8 weeks may have been driven by slight differences in growth stage; cv. Avalon was beginning stem elongation (GS30), while cvs. Cadenza and Skyfall had progressed onto booting (GS40) (Zadoks et al., 1974). Greater shoot [P] and [N] of cvs. Cadenza and Skyfall may therefore be because nutrient uptake peaks during construction phases (AHDB, 2018). At yield, grain number and weight were highest in cv. Skyfall, which is broadly considered one of the best performing varieties of wheat in the UK (RAGT, 2018).

2.6 Conclusions

This study investigated the growth responses of three modern wheat cultivars to colonisation by the AM fungus *Rhizophagus irregularis*. Plant responsiveness to AM colonisation was assessed at ambient and elevated atmospheric [CO_2], and at two time points of interest. Here, AM colonisation did not promote wheat growth at aCO_2 either 8 weeks after planting or at yield. This was true across all three modern cultivars, reaffirming the need for commercial breeding programmes to consider wheat responsiveness to AM fungi when selecting germplasm for the development of new elite lines. Despite an increase in plant C source strength at eCO_2, wheat growth responses to AM fungi were unchanged. This finding could suggest that other environmental factors that alter plant C availability, such as biotic interactions between crops and insect herbivores, may also not impact wheat responsiveness to AM fungi, although this requires investigation (Chapter 3). The finding that AM colonisation of wheat was reduced at eCO_2 early in development but not at yield highlights the complex and variable effects of [CO_2] on AM fungal abundance in roots and soils of plants. Partial evidence pointed towards a functional mycorrhizal uptake pathway for

plants at aCO_2 and eCO_2; despite AM colonisation reducing root biomass of cvs. Avalon and Cadenza, mycorrhizal plants of theses cultivars achieved broadly speaking equivalent shoot P and N concentrations. The introduction of isotopic tracers to mycorrhizal-only regions of substrate would be needed to validate this, however (Chapter 4). The finding that AM colonisation had a neutral effect on wheat growth when recorded 8 week after planting and at yield suggests growth responses of these three cultivars to *R. irregularis* was not contingent on host-plant growth stage. Because of this, and the successful colonisation of plants after 8 weeks growth, experiments presented in Chapters 3 and 4 of this thesis will utilise the earlier time point.

Chapter 3 Does aphid herbivory impact wheat growth and nutritional responses to an AM fungus?

3.1 Introduction

AM fungi have been widely touted as ‘sustainable saviours’ within future agro-ecosystems (Thirkell et al., 2017), given their capacity to enhance plant nutrient uptake in exchange for plant C (Smith & Read, 2010). However, as highlighted in Chapter 2, outcomes of wheat-AM symbioses are not always positive. Abiotic factors that alter the availability of plant C resources, such as atmospheric [CO_2] (Johnson et al., 2005) and shading (Johnson et al., 2015), can sometimes determine the direction and extent to which AM fungi impact plant growth. It follows, therefore, that biotic factors that alter plant C availability, such as interactions between plants and insect herbivores, also have the potential to affect how plants respond to colonisation by AM fungi.

Despite an increase of 80% in global pesticide usage since 1990 (FAO, 2020b), insect herbivores remain a threat to production in all agricultural systems. Phloem feeding insects like aphids target almost all crops including wheat (Blackman & Eastop, 2000), and use piercing-sucking mouthparts known as stylets to siphon C-rich phloem from plant vascular tissues (Douglas, 2006). Rates of sap assimilation per aphid have been quantified following stylectomy at up to 3 µl per hour (Donovan et al., 2013), meaning large infestation can represent a considerable drain on plant C resources. Aphids may increase feeding rates by manipulating plant C partitioning (Girousse et al., 2003; 2005), with some studies recording increased plant C export towards aphid exposed leaves (Kaplan et al., 2011). The impact of aphids on the C budget of plants is not limited to the removal of sap, however. Plant recognition of aphids through mechanical or chemical cues (Erb et al., 2012) may increase phytohormone levels in hosts (Ali & Agrawal, 2011), and/or the accumulation secondary metabolites like benzoxazinoids in cereals (Ahmad et al., 2011) resulting in a trade-off between growth and immunity (van Butselaar & van den Ackerveken, 2020). Evidence suggests aphid feeding may too limit photosynthesis in crops (Macedo et al., 2003; 2009), perhaps caused by reduced stomatal conductance (Sun et al., 2015), slower rates of ribulose-1,5-bisphosphate (RuBP) renewal, or reduced Rubisco activity (Pierson et al., 2011). That said, examples exist of increased photosynthetic activity in aphid infested plants (Kucharik et al., 2016), which could benefit insects by increasing plant C availability.

Given AM fungi represent sinks for plant C by obtaining 4-20% of newly-fixed photosynthate (Cotton, 2018), the 'carbon-limitation hypothesis' suggests that the removal or redirection of plant C by aphids away from AM fungi could negatively impact plant-mycorrhizal associations (Gehring & Whitham, 1994; 2002; Barto & Rillig, 2010). Reduced plant C allocation to AM fungi owing to sink competition in aphid exposed plants (Larson & Whitham, 1997) could compromise the ability of AM fungi to maintain root-internal structures required for resource exchange. In support of this hypothesis, aphids have been shown to reduce AM colonisation in broad bean (*V. faba* L.) by 20% (Babikova et al., 2014a), and by over 25% in two *Asclepias* species (Meier & Hunter, 2018). This could in turn limit the ability of AM fungi to supply plants with nutrients, thereby reducing plant growth (and nutrient demand) leading to a less mutualistic symbiosis through negative feedback. As such, growth responses of target plants to AM colonisation may be less positive in the presence of chewing insect herbivores (Bennett & Bever, 2007), suggesting a less highly functioning association. To my knowledge, such effects have not been studied in AM fungal-crop-aphid systems.

However, how aphids effect AM infection varies between AM fungal-plant-aphid combination, with neutral and positive impacts also reported (see Chapter 1 section 1.4.1; Table 1.1). The genotype of organisms at all trophic levels may drive this variability. Aphids likely differ in the extent to which they limit plant C availability, as feeding rates vary between species (Puterka et al., 2017). Similarly, phloem chemistry (i.e. amino acid concentrations and composition) can differ between plant species, which in turn may influence rates of aphid sap assimilation (Taylor et al., 2012). This could predispose certain plants to C limitation, as aphids compensate for poor quality diets by siphoning more phloem (and therefore plant C) in order to meet their nutritional needs. Lastly, AM fungi vary in their plant C requirements (Lendenmann et al., 2011), as well as the efficiency with which they acquire C from their hosts (Cotton et al., 2015). As such, AM fungi with greater C needs are more likely to be disrupted by an aphid-induced C drain. To date, only one study has investigated whether aphids alter AM colonisation of roots and soils of cereals, with Wilkinson et al., (2019) recording no effect in barley (*Hordeum vulgare* L.). The same may expected in wheat, as their close taxonomic relationship in the Gramineae (Poaceae) means they share similar phloem chemistries (Gregersen et al., 2008), and are targeted by the same specialist aphids (Blackman & Eastop, 2000). That said, contrasting colonisation responses to aphids

have been recorded even in closely related plants (Meier & Hunter, 2018), meaning equivalent experiments are now required in wheat. The bird cherry-oat aphid (*Rhopalosiphum padi*) is a cereal-feeding aphid responsible for yield losses in wheat of 35-40% from direct feeding effects, and 20-80% from plant virus transmission (Zeb et al., 2016). These impacts may be greater if aphid siphoning of plant C also reduces the functional efficiency of wheat-AM symbioses. As per Chapter 2, by investigating how aphids affect plant responsiveness to an AM fungus across multiple wheat cultivars, important characteristics for benefitial mutualistic outcomes following aphid infestation may be identified, which could be used by crop breeders.

Considering these plant-mediated interactions from the opposite direction, colonisation of plant roots by AM fungi may alter plant resistance to insect herbivores. Resistance to insects can be divided into three categories, these being antibiosis, antixenosis, and tolerance (Girvin et al., 2017). Antibiosis resistance impacts the survivorship, fecundity, and, development of pests, while antixenosis resistance determines host plant preference. Lastly, tolerance resistance refers to the ability of plants to withstand herbivore damage and/or compensate for it. AM colonisation may impact all three facets of plant resistance. AM fungi can “prime” plant defence-signalling pathways against insects (i.e. antibiosis: Cameron et al., 2013), and alter plant attractiveness to pests (and their natural enemies) by changing plant volatile emissions (i.e. antixenosis: Guerrieri et al., 2004). Finally, by increasing plant resource availability, AM fungi may improve the capacity of plants to maintain or increase plant biomass when challenged by herbivores (i.e. tolerance: Bennett et al., 2005), and stimulate re-growth after their removal (Kula et al., 2005). Tolerance is an especially desirable component of pest resistance, as it is less costly for plants than chemical defence (Tao et al., 2016), and does not impose selection pressures on insects thereby reducing the chance of virulence developing (Crespo-Herrera et al., 2014). Improved tolerance against pests may benefit AM fungi, as it could permit the continued supply of C from host plants.

Through impacting plant resistance AM fungi may reduce aphid abundance on shared plant hosts, although neutral and positive effects have also been recorded (see Chapter 1 section 1.4.2; Table 1.2), perhaps driven by the improved nutrient status (Hartley & Gange, 2009) or altered leaf physiology (Simon et al., 2017) of mycorrhizal hosts. The only previous study into how the generalist AM fungus *Rhizophagus irregularis* affects wheat-*R. padi* interactions recorded no change in aphid abundance

(Abdelkarim et al., 2011), but only did so in one wheat cultivar (cv. Pioneer '26R22'). Given the significance of plant genotype in mediating AM fungal-plant-aphid interactions (Meier & Hunter, 2018), research into how AM colonisation affects *R. padi* abundance is now required across multiple cultivars. Furthermore, while tolerance of wheat to bird cherry-oat aphids has been studied before (Razmjou et al., 2012; Zeb et al., 2016; Girvin et al., 2017), the effect of AM fungi on wheat tolerance of *R. padi* is unknown. Given all three components of herbivore resistance are present in resistant plants to varying degrees (Crespo-Herrera et al., 2014), understanding how AM colonisation impacts tolerance in wheat could also help identify candidate varieties for future research into the effect of AM fungi on antibiosis and antixenosis resistance.

Here, I investigated the impact of aphids on the growth and nutritional responses of the three wheat cultivars to colonisation by an AM fungus (*R. irregularis*). cvs. Avalon, Cadenza, and Skyfall were grown in the presence or absence of bird cherry-oat aphids (*R. padi*), maybe the principal pest of cereals in temperate agricultural systems (Blackman & Eastop, 2017). *R. padi* aphids cost UK growers £120 million each year in yield shortfalls (Loxdale et al., 2017), and may have developed resistant to certain insecticide classes (e.g. orthophoshatases: Chen et al., 2007; neonicotinoids: Wang et al., 2018). The effect of AM colonisation on aphid abundance and plant tolerance of aphid herbivory was also assessed, defined as the plant's ability to maintain or increase shoot biomass in spite of aphid exposure (Girvin et al., 2017). Although genetic regions associated with tolerance to *R. padi* aphids have been identified in wheat localised to chromosomes 5AL and 5BL (Crespo-Herrera et al., 2014), these three cultivars are not known to be resistant against aphids.

3.2 Key questions and hypotheses

- Do aphids effect AM colonisation of wheat, and is this effect equal across cultivars?
  - Hypothesis 1: Aphids are hypothesised to reduce AM fungal biomass in roots and soils of plants, as seen in other AM fungal-plant-aphid interactions (Babikova et al., 2014a). This is owing to the removal/redirection of plant C away from the AM fungus (Gehring & Whitham, 1994; 2002). However, Wilkinson et al., (2019) reported no effect of aphids on AM colonisation of barley, a fellow cereal crop, meaning the same may be expected in wheat. The direction and degree to which aphids impact AM colonisation is hypothesised to differ between cultivars, as described in other closely related species (Meier & Hunter, 2018).
- Does AM colonisation impact aphid abundance and is this effect cultivar-specific?
  - Hypothesis 2: Colonisation of wheat by *R. irregularis* is not hypothesised to impact aphid abundance, as seen previously (Abdelkarim et al., 2011). However, fungal-induced priming of plant defences may reduce aphid numbers (Cameron et al., 2013). Then again, an increase in plant nutrient status following AM colonisation may increase pest abundance (Hartley & Gange, 2009). The effect of the AM fungus on aphids is not hypothesised to differ between cultivars, as the chosen varieties are not known to carry resistance.
- Do aphids impact wheat growth and nutritional responses to AM fungi?
  - Hypothesis 3: Wheat growth responses to the AM fungus are hypothesised to less positive when exposed to aphids than when not, as seen in other AM fungal-plant-insect systems (Bennett & Bever, 2007). This could be due to lower AM colonisation in roots and soils of aphid infested plants (see Hypothesis 2), which may reduce fungal-acquired plant nutrient uptake. This could, in turn, limit plant growth and drive a more parasitic symbiosis through negative feedback.
- Does AM colonisation increase wheat tolerance against aphid herbivory?
  - Hypothesis 4: If plants exposed to aphids accumulate shoot biomass to a similar or greater extent as plants that are not then they are considered to be tolerant of the pest (Girvin et al., 2017). In the absence of the AM fungus, aphids are hypothesised to reduce shoot biomass (Zeb et al., 2016). AM colonisation is predicted to increase wheat tolerance of aphids, given tolerance can be linked to plant resource availability (Maschinski & Whitham, 1989). This could manifest in less negative or positive biomass responses to aphids in AM hosts.

3.3 Materials and Methods

3.3.1 Plant material

Seeds of the wheat cvs. Avalon, Cadenza, and Skyfall were provided by RAGT Seeds Ltd. Despite quantitative trait loci for *R. padi* tolerance being identified in wheat (Crespo-Herrera et al., 2014), these cultivars are not known to carry alleles conferring resistance. As such, all varieties were assumed to be equally susceptible to the cereal-feeding herbivore used in this experiment (see section 3.3.4).

Seeds were sterilised using Cl_2 gas for 3 hours (see Chapter 2 section 2.3.1). Sterilised seeds were germinated at 20˚C for 7 days on autoclaved filter paper that was dampened with 4 mL sterilised dH_2O. 20 seedlings of each cultivar (60 plants, n=5) were transplanted into 4.5" pots containing substrate consisting of 3 parts sand to 1 part perlite, which had been sterilised at 121˚C for 45 minutes.

3.3.2 Fungal material

Plants within the mycorrhizal treatment ('+ AMF') were inoculated with the AM fungus *Rhizophagus irregularis* (Schenck & Smith, 2009) that had been cultured as described in Chapter 2 section 2.3.2. The inoculum was created by homogenising nine petri-dishes of *R. irregularis* with 150 mL autoclaved distilled H_2O using a food processor. 15 mL of inoculum containing 23,500 spores was mixed throughout the substrate of each + AMF replicate. Remaining plants, hereafter called the '- AMF' treatment, were inoculated with the same volume of inoculum that was autoclaved at 121˚C for 30 minutes. Root clearing and staining at harvest confirmed that no fungal structures were present in roots of - AMF plants.

3.3.3 Growth conditions

60 plants were grown in 6 insect rearing cages (47.5 cm^3) in semi-controlled glasshouse conditions at the University of Leeds. Temperature was kept at 20˚C during a 16:8 hour light: dark cycle. Natural daylight was supplemented with LED lighting and automatic blinds, which provided an average light intensity of 190 µmol m^{-2} s^{-1} at canopy level inside each nylon mesh-lined cage. Plants were fed once each week with 30 mL of 40% nitrate-type Long Ashton solution, and watered when necessary. Pot surfaces were covered with HDPE pellets (see Chapter 2 section 2.3.3).

3.3.4 Insect material and culture

Bird cherry-oat aphids (*Rhopalosiphum padi*) were provided by Dr. Tom Pope at Harper Adams University (Figure 3.1). The primary hosts of *R. padi* aphids are *Prunus padus* and *P. virginiana* in Europe and North America, respectively (Blackman & Eastop, 2000), on which the aphid over-winters and has its sexual phase. Eggs hatch in the spring winged forms migrate to their secondary hosts, these being members of the Gramineae (or Poaceae), using visual (Nottingham et al., 1991) and olfactory cues (Quiroz & Niemeyer, 1998). *R. padi* aphids feed on wheat during all stages of growth (CABI, 2020), but phloem siphoning does not cause chlorosis of the leaf and as such is considered to be cryptic (Crespo-Herrera et al., 2014).

BYDV-free aphid cultures were maintained on wheat plants of cvs. Avalon, Cadenza, and Skyfall grown in composted soil inside insect rearing cages. Growth conditions for aphid cultures were the same as those for experimental plants (see section 3.3.3).

Figure 3.1: Bird cherry-oat aphids (*Rhopalosiphum padi*) of mixed life-cycle stages feeding on winter wheat. *R. padi* aphids are oval shaped and typically dark-green in colour, with red areas around the cornicles (Blackman & Eastop, 2000). During their non-sexual phase, *R. padi* aphids reproduce rapidly through parthenogenesis, giving birth to live offspring that develop quickly through nymphal instars into adults. As such, population sizes of *R. padi* aphids can double in 2 days under optimal conditions, with each adult female (right) producing 3 nymphs per day (60 throughout their life) (Taheri et al., 2010). Bar, 1 mm.

3.3.5 Aphid exposure

After experimental plants had grown for 8 weeks, 30 wingless aphids of mixed life-cycle stages were added to half of all - AMF and + AMF plants (30 plants, n=5), hereafter termed the '+ aphids' treatment. Aphids were carefully transplanted from culture plants onto '+ aphid' replicates, with 10 aphids placed using a paint brush on three leaves on the main tiller of each plant. '- AMF' and '+ AMF' plants of each cultivar within the '+ aphid' treatment were grown for a further 2 weeks in separate insect rearing cages. This prevented aphids from switching from mycorrhizal to non-mycorrhizal plants, and enabled the assessment of how AM colonisation impacted final aphid abundance and aphid load (see section 3.3.6). '- AMF' and '+ AMF' plants of each cultivar not exposed to *Rhopalosiphum padi* (termed the '- aphid' treatment) were also grown for a further 2 weeks (30 plants, n=5), but in the same insect rearing cages due to space constraints and the number of available cages.

3.3.6 Harvest procedure and plant P and N determination

At the end of the 2-week aphid exposure period, final aphid abundance per plant was recorded and aphids were removed. Plants were extracted from their pots and substrate was dislodged from the roots. A known volume of substrate was collected from each pot and stored at 5 ˚C. This was used to calculate extra-radical hyphal lengths of the fungus (see Chapter 2 section 2.3.6). Roots were washed with water to remove any remaining substrate and dried using paper towels. Shoots and roots were separated using a scalpel, and root biomass (FW) measurements were recorded using a 3-digit digital scale. Roots were split equally in two, with half being stored in 50 % EtOH (v/v) at 5 ˚C. These roots were cleared and stained less than 48 hours later, and % root length colonisation by the AM fungus was determine using the gridline intersection methodology (see Chapter 2 section 2.3.5). Remaining roots were weighed again and freeze-dried with shoot material for 3 days. Shoot and root biomass (DW) were then measured using a 5-digit digital scale, and total root biomass and root-to-shoot ratios were calculated (see Chapter 2 section 2.3.4). Aphid load on each aphid exposed plant was also calculated using Equation 13 (Petermann et al., 2010):

Equation 13 $\text{Aphid load } \left(\text{Ng}^{-1}\right) = \frac{\text{Final aphid abundance}}{\text{Shoot biomass (DW)}}$

3.3.7 Statistical analyses

All analyses of data were conducted using R Studio v1.1.453. Data were tested to ensure assumptions of normality and homogeneity of variances were met using typical residuals vs fitted and normal Q-Q plots, with supplementary Kruskall-Wallis, skewness, and kurtosis tests performed when required. The effects of AMF, cultivar, and their interaction on final aphid abundance and aphid load were tested using a generalised linear model (GLM) with additional post hoc Tukey HSD tests. Given the phenotypic differences reported between cultivars in Chapter 2, and to control for any variation in final aphid abundance (and therefore herbivore pressure) between varieties, the effects of AMF, aphids, and their interaction on plant growth (i.e. shoot biomass, root biomass, and root: ratio) and nutrition (i.e. shoot P and [P], root P and [P], shoot N and [N], and root N and [N]) were determined within each cultivar using a GLM with additional post hoc Tukey HSD tests. Data were Log_{10} transformed when test assumptions could not be met, as indicated in the footings of Tables 3.3-3.5. The effect of aphids on % root length colonisation, % arbuscules, % vesicles, and extra-radical hyphal lengths were determined for cvs. Avalon, Cadenza, and Skyfall using Student's t tests. Percentage data were arcsine square root transformed when test assumptions could not be met, as also indicated in the footings of Table 3.2. Spearman rank-test correlation analyses were conducted between final aphid abundance or aphid load and shoot P/[P] and N/[N], in order to investigate the relationship between plant nutrient status and aphid performance, in a test of part of hypothesis two. All figures were produced using GraphPad Prism v8.2.0.

3.4 Results

3.4.1 Aphids

AMF had no effect of final bird cherry-oat aphid abundance (Figure 3.2a; Table 3.1). However, aphid abundance differed significantly between cultivar, being greatest on plants of cv. Avalon (223 ± 18), followed by cv. Cadenza (121 ± 16) and cv. Skyfall (67 ± 6). Identical patterns were recorded for aphid load, this being the number of aphids per gram of shoot biomass. Aphid loads were unaffected by AMF (Figure 3.2b; Table 3.1), but varied significantly between cultivar, again being greatest on plants of cv. Avalon (102 ± 9 Ng^{-1}), then cv. Cadenza (54 ± 7 Ng^{-1}) and cv. Skyfall (36 ± 4 Ng^{-1}).

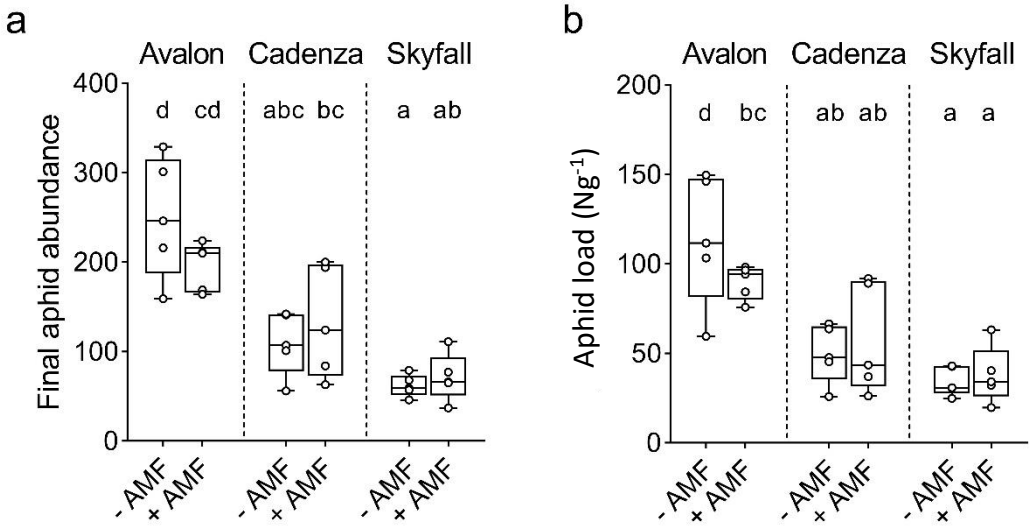

Figure 3.2: Aphid performance on non-mycorrhizal and mycorrhizal wheat. (a) Final aphid abundance; (b) Aphid load. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised inoculum (- AMF) and exposed to bird cherry-oat aphids (*R. padi*) for 14 days after 8 weeks growth. Boxes extend from the 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to minimum and maximum data points (closed or open markers, n=5). Different letters indicate significant differences between treatment means (where $p < 0.05$, Tukey HSD tests).

Table 3.1: Summary of two-way ANOVA results investigating the effect of AMF, cultivar, and their interaction on aphid performance on wheat. Significant p-values are in bold (n=5).

Factor	**Final aphid abundance**			**Aphid load**		
	F	*df*	p	F	*df*	p
AMF	0.01	1,24	0.949	0.03	1,24	0.869
Cultivar	31.94	2,24	**<0.001**	20.51	2,24	**<0.001**
AMF*Cultivar	0.86	2,24	0.435	0.40	2,24	0.675

3.4.2 AM colonisation

All wheat plants in the '+ AMF' treatment were colonised by the AM fungus *Rhizophagus irregularis*. For cv. Avalon, there was no effect of aphid treatment on % root length colonisation (Figure 3.3a; Table 3.2), which averaged 45.2 ± 5.2 %. Likewise, aphid treatment did not impact % arbuscules or % vesicles (Table 3.2). Extra-radical hyphal lengths in soils of cv. Avalon were also unaffected by aphids (Figure 3.3b; Table 3.2), averaging 1.29 ± 0.14 mg^{-1}, the lowest of all three cultivars.

Similar patterns were recorded for AM colonisation of cv. Cadenza. Aphids did not affect % root length colonisation (Figure 3.3c; Table 3.2), which averaged 39.6 ± 4.7 %. % arbuscules was, however, significantly higher in roots of cv. Cadenza exposed to aphids than those not (- aphid: 4.9 ± 0.7 %; + aphid: 8.7 ± 1.3 %) (Table 3.2). % vesicles was unaffected by aphids (Table 3.2), as were hyphal lengths which were the highest of all cultivars at 2.07 ± 0.11 mg^{-1} (Figure 3.3d; Table 3.2).

Aphids had no effect on % root length colonisation of plants of cv. Skyfall (Figure 3.3e; Table 32), which averaged 24.1 ± 3.1 %, lower than that of cvs. Avalon and Cadenza. % arbuscules and % vesicles were also unaffected by aphid treatment (Table 3.2). Extra-radical hyphal lengths supported by roots of cv. Skyfall were also equivalent in '- aphid' and '+ aphid' plants (Figure 3.3f; Table 3.2), averaging 1.43 ± 0.09 m g^{-1}.

Table 3.2: Summary of Student t-test results investigating the effect of aphids on AM colonisation of thee wheat cultivars. Significant p-values are in bold (n=5).

Variable	**cv. Avalon**			**cv. Cadenza**			**cv. Skyfall**		
	t	*df*	p	t	*df*	p	t	*df*	p
% total RLC	0.32	5.75	0.757	1.43	7.85	0.190	0.79	7.67	0.453
% arbuscules	1.02	4.47	0.358	2.58	6.06	**0.041**	-0.02	6.22	0.988
% vesicles	0.31[a]	7.16[a]	0.766[a]	0.13	7.56	0.897	-0.13	5.67	0.898
Hyphal lengths	0.89	5.63	0.412	1.50	7.95	0.173	0.06	7.12	0.950

[a] Arcsine square root transformed.

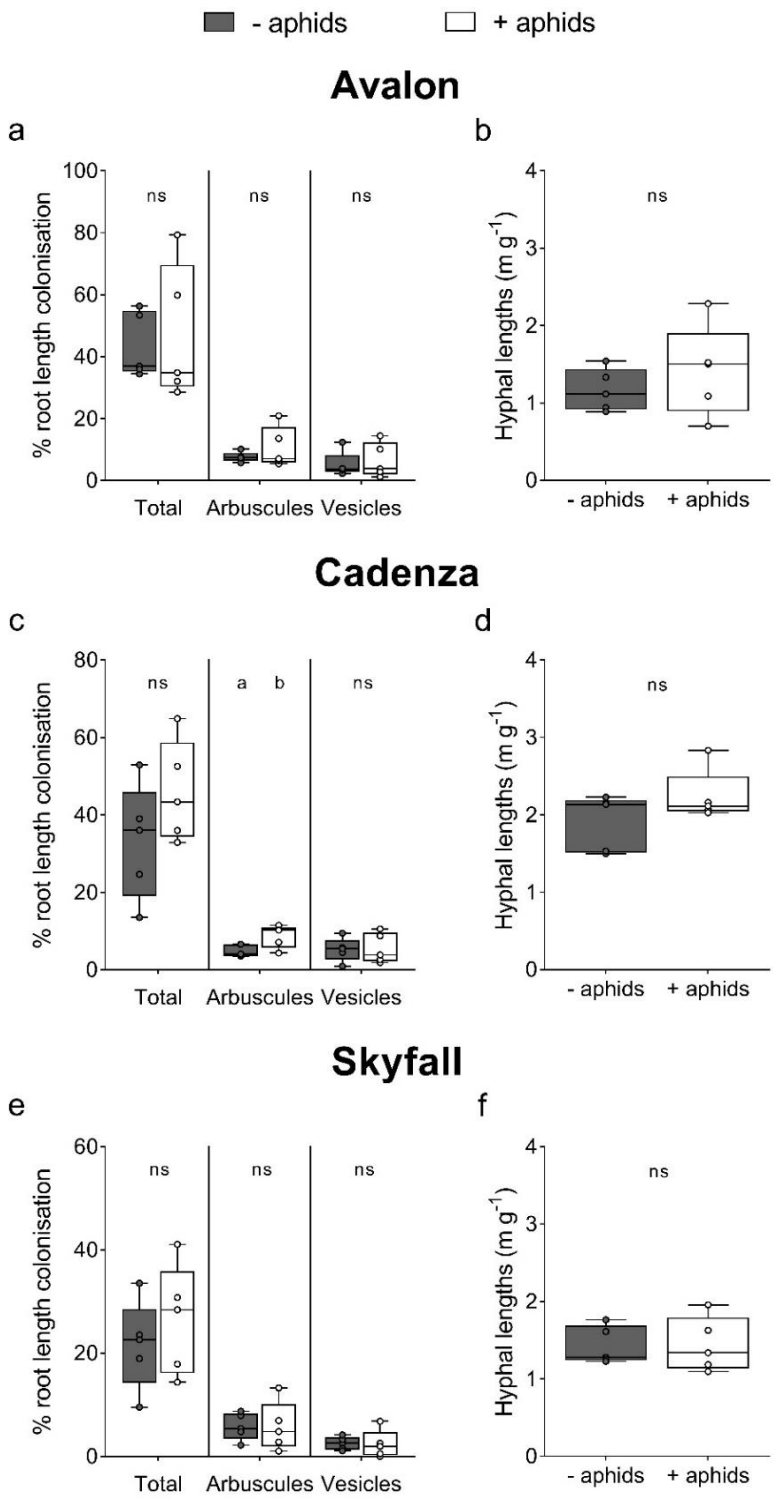

Figure 3.3: AM fungal abundance in roots and soils of wheat grown in the presence and absence of aphids. (a, c, e) AM colonisation; (b, d, f) Extra-radical hyphal lengths. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* and exposed (+ aphids, white boxes) or not (- aphids, grey boxes) to bird cherry-oat aphids (*R. padi*) for 14 days. Boxes extend from the 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to minimum and maximum data points (closed or open markers, n=5). Different letters indicate significant differences between means (where $p < 0.05$). 'ns' indicates no differences.

3.4.3 Plant growth

Shoot biomass of cv. Avalon was unaffected by AMF treatment (Figure 3.4a; Table 3.3). Instead, shoot biomass was affected by aphids, being greater in '+ aphid' plants than '- aphid' ones (- AMF: +11%; + AMF: +6%). In contrast, root biomass of cv. Avalon was unaffected by aphids (Figure 3.4b; Table 3.3), but differed according to AMF treatment. AMF reduced root biomass of cv. Avalon in both aphid treatments (- aphids: -40%; + aphids: -33%). Root: shoot ratios of cv. Avalon were also significantly affected by AMF (Figure 3.4c; Table 3.3), being reduced in regardless of aphid treatment (- aphids: -43%; + aphids: -30%) reflecting their smaller root biomass. No effect of aphids was recorded on root: shoot ratios of cv. Avalon (Table 3.3).

Shoot biomass of cv. Cadenza was unaffected by AMF or aphids (Figure 3.4d; Table 3.3), averaging 2.27 ± 0.05 g. Instead, root biomass differed across treatments, being significantly affected by the interaction between AMF and aphids (Figure 3.4; Table 3.3). Root biomass of '-AMF' plants was unaffected by aphid treatment (-1%), but aphids increased root biomass in '+ AMF' plants (+49%). Given no effect of AMF or aphids was recorded on shoot biomass of cv. Cadenza, root: shoot ratio trends for cv. Cadenza reflected those of root biomass alone (Figure 3.4f; Table 3.3).

A significant interaction between AMF and aphids was recorded for shoot biomass of cv. Skyfall (Figure 3.4g; Table 3.3). Shoot biomass of '- AMF' plants was unaffected by aphids. However, shoot biomass of '+ AMF' plants was greater in aphid exposed plants than unexposed ones (+14%). Another interaction between AMF and aphids was recorded for root biomass of cv. Skyfall (Figure 3.4h; Table 3.3). Roots of plants not exposed to aphids were greater in the '+ AMF' treatment than '- AMF' treatment (+38%). In contrast, cv. Skyfall plants exposed to aphids had similar root biomass across AMF treatments. An interaction between AMF and aphids was recorded for root: shoot ratios of cv. Skyfall (Figure 3.4i; Table 3.3), with AMF increasing root: shoot ratios in '- aphids' plants (+50%) but not in '+ aphids' plants (-12%).

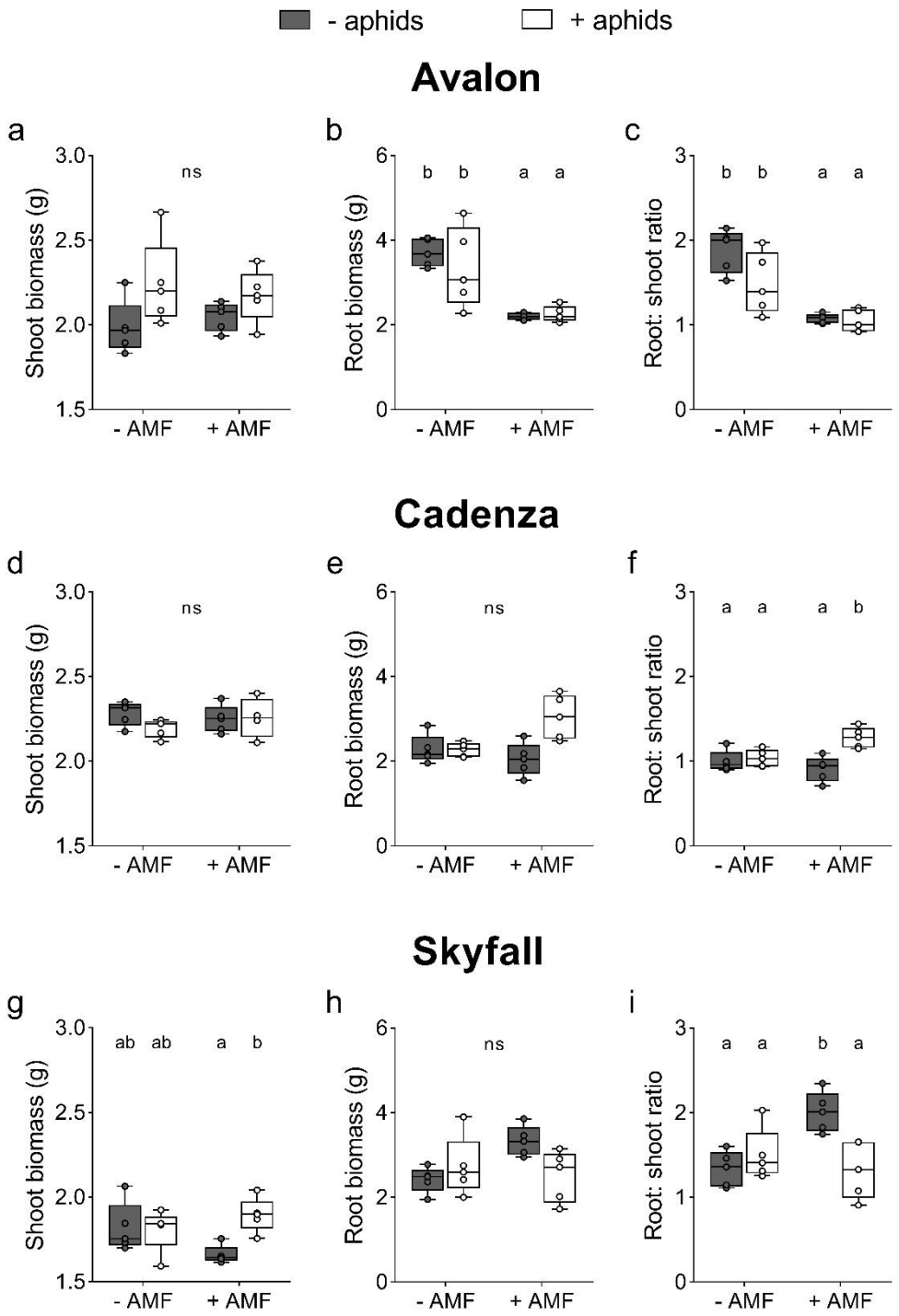

Figure 3.4: Biomass of non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids. (a, d, g) Shoot biomass; (b, e, h) Root biomass; (c, f, i); Root: shoot ratios. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised inoculum (- AMF) and exposed (+ aphids, white boxes) or not (- aphids, grey boxes) to bird cherry-oat aphids (*R. padi*) for 14 days. Boxes extend from the 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to minimum and maximum data points (closed or open markers, n=5). Different letters indicate significant differences between means (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

Table 3.3: Summary of two-way ANOVA results investigating the effect of AMF, aphids, and their interaction on plant biomass of three wheat cultivars. Significant p-vales are in bold (n=5).

Variable	Factor	cv. Avalon			cv. Cadenza			cv. Skyfall		
		F	df	p	F	df	p	F	df	p
Shoot biomass	AMF	0.01	1,16	0.972	0.08	1,15	0.785	0.52	1,16	0.482
	Aphids	5.96	1,16	**0.027**	1.34	1,15	0.265	4.89	1,16	**0.042**
	AMF*Aphids	0.72	1,16	0.408	1.59	1,15	0.226	5.95	1,16	**0.027**
Root biomass	AMF	40.09[a]	1,16[a]	**<0.001**[a]	2.56	1,16	0.129	2.13	1,16	0.164
	Aphids	0.64[a]	1,16[a]	0.434[a]	8.64	1,16	**0.010**	1.20	1,16	0.290
	AMF*Aphids	1.19[a]	1,16[a]	0.292[a]	9.09	1,16	**0.008**	6.02	1,16	**0.026**
Root: shoot ratio	AMF	39.79[a]	1,16[a]	**<0.001**[a]	1.86	1,16	0.191	3.99	1,16	0.063
	Aphids	4.22[a]	1,16[a]	0.057[a]	13.61	1,16	**0.002**	4.37	1,16	0.053
	AMF*Aphids	2.31[a]	1,16[a]	0.148[a]	9.02	1,16	**0.008**	11.67	1,16	**0.004**

[a] Log_{10} transformed.

3.4.4 Plant P

AMF increased shoot P of cv. Avalon (- aphids: +7%; + aphids: +9%) (Figure 3.5a; Table 3.4). Aphids also increased shoot P (- AMF: +10%; + AMF: +6%). Shoot P concentrations ([P]) were unaffected by AMF, aphids, or their interaction (Figure 3.5b; Table 3.4), averaging 1.83 ± 0.04 mg g^{-1}.Root P of cv. Avalon was unchanged by AMF, but was reduced by aphids (- AMF: -25%; + AMF: -28%) (Figure 3.5c; Table 3.4). Similar patterns were seen for root P concentrations ([P]) (Figure 3.5d; Table 3.4).

Shoot P and shoot [P] of cv. Cadenza were not affected by AMF, aphids, or their interaction (Figure 3.5e & 3.5f; Table 3.4), averaging 3.86 ± 0.07 mg and 1.70 ± 0.03 mg g^{-1}, respectively. In contrast, AMF and aphids both increased root P of cv. Cadenza independently (Figure 3.5g; Table 3.4). Root [P] was unaffected by AMF, aphids, or their interaction (Figure 3.5h; Table 3.4), averaging 0.54 ± 0.02 mg g^{-1}.

No effect of AMF was recorded on shoot P of cv. Skyfall (Figure 3.5i; Table 3.4). Instead, aphids reduced shoot P (- AMF: -9%; + AMF: -8%). A significant interaction between AMF and aphids was recorded for shoot [P] (Figure 3.5j; Table 3.4). Aphids reduced shoot [P] in both AMF treatments, but to a greater degree in '+ AMF' plants (-19 %) than in '- AMF' plants (-9%). An interaction between AMF and aphids was also found for root P of cv. Skyfall (Figure 3.5k; Table 3.4), being increased by AMF in '- aphid' plants (+38%) but reduced by AMF in '+ aphid' plants (-8%). However, no effects were recorded for root [P] (Figure 3.5l; Table 3.4), which averaged 0.67 ± 0.03 mg g^{-1}.

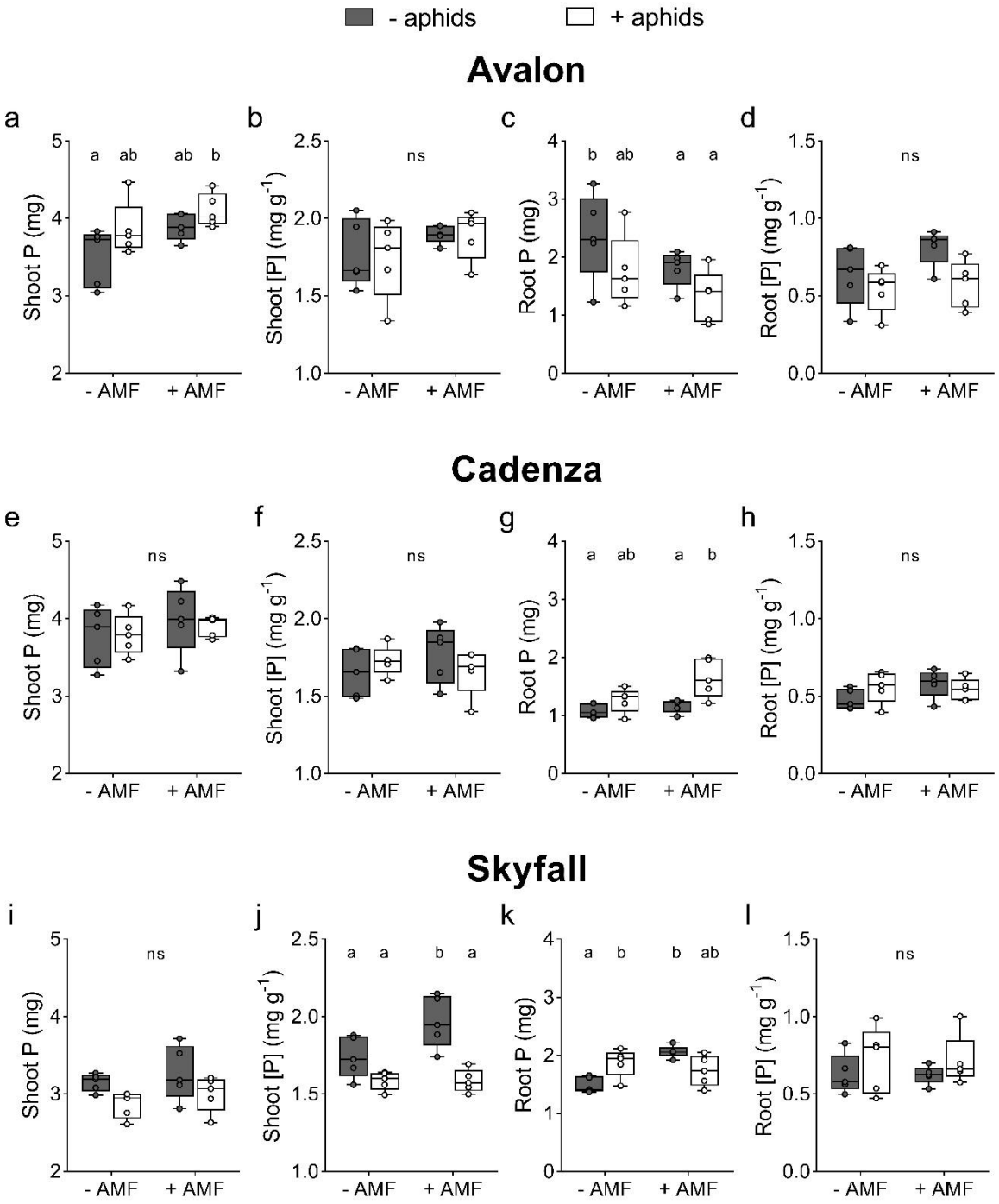

Figure 3.5: P uptake by non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids. (a, e, i) Shoot P; (b, f, j) Shoot [P]; (c, g, k) Root P; (d, h, l) Root [P]. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised inoculum (- AMF) and exposed (+ aphids, white boxes) or not (- aphids, grey boxes) to bird cherry-oat aphids (*R. padi*) for 14 days after 8 week growth. Boxes extend from the 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to minimum and maximum data points (closed or open markers, n=5). Different letters indicate significant differences between means (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

Table 3.4: Summary of two-way ANOVA results investigating the effect of AMF, aphids, and their interaction on P uptake by three wheat cultivars. Significant p-values are in bold (n=5).

Variable	Factor	cv. Avalon			cv. Cadenza			cv. Skyfall		
		F	*df*	p	F	*df*	p	F	*df*	p
Shoot P	AMF	5.81	1,16	**0.028**	1.24	1,16	0.282	1.51	1,16	0.237
	Aphids	4.86	1,16	**0.042**	0.05	1,16	0.831	6.89	1,16	**0.018**
	AMF*	0.33	1,16	0.576	0.14	1,16	0.714	0.01	1,16	0.929
Shoot [P]	AMF	2.81	1,16	0.113	0.15	1,16	0.701	4.30[a]	1,16[a]	0.055[a]
	Aphids	0.04	1,16	0.853	0.09	1,16	0.771	27.09[a]	1,16[a]	**<0.001**[a]
	AMF*	0.02	1,16	0.886	2.03	1,16	0.173	4.47[a]	1,16[a]	**0.050**[a]
Root P	AMF	4.04	1,16	0.062	5.85[a]	1,16[a]	**0.028**[a]	5.85	1,16	**0.028**
	Aphids	4.76	1,16	**0.044**	11.66[a]	1,16[a]	**0.004**[a]	0.10	1,16	0.752
	AMF*	0.05	1,16	0.830	1.71[a]	1,16[a]	0.210[a]	16.01	1,16	**0.001**
Root [P]	AMF	2.31	1,16	0.148	1.35	1,16	0.263	0.01	1,16	0.936
	Aphids	5.91	1,16	**0.027**	0.27	1,16	0.609	1.93	1,16	0.184
	AMF*	1.03	1,16	0.324	2.58	1,16	0.128	0.00	1,16	0.978

[a] Log_{10} transformed.

3.4.5 Plant N

AMF increased shoot N of cv. Avalon regardless of aphid treatment (- aphids: +8%; + aphids: +3%) (Figure 3.6a; Table 3.5). Likewise, aphids increased shoot N in '- AMF' plants (+9%) and '+ AMF' plants (+4%). Shoot N concentrations ([N]) were unaffected by AMF, aphids, or their interaction (Figure 3.6b; Table 3.5), averaging 6.24 ± 0.1 mg g^{-1}. The same was true for root N of cv. Avalon (Figure 3.6c; Table 3.5), but there was a trend for reduced root N in '+ AMF' plants. AMF increased root N concentrations ([N]) equally in both aphid treatments (- aphids: +28%; + aphids: +27%) (Figure 3.6d; Table 3.5).

Shoot N and shoot [N] of cv. Cadenza were not affected by AMF, aphids, or their interaction (Figure 3.6e & 3.6f; Table 3.5), averaging 3.86 ± 0.07 mg and 1.70 ± 0.03 mg g^{-1}, respectively. In contrast, root N of cv. Cadenza was affected by AMF and aphids independently (Figure 3.6g; Table 3.5), being increased in by aphids (- AMF: +39%; + AMF: +54%) and AMF (- aphids: +15%; + aphids: +27%). A significant interaction between AMF and aphids was recorded for root [N] (Figure 3.6h; Table 3.5), as aphid exposure increased root [N] of '- AMF' plants (+40%), but no such effect was recorded for root [N] of '+ AMF' plants (+3%).

No effect of AMF, aphids, or interaction was recorded for shoot N of cv. Skyfall (Figure 3.6i; Table 3.5), which averaged 11.07 ± 0.19 mg g^{-1}. However, a significant interaction between AMF and aphids was recorded for shoot [N] (Figure 3.6j; Table 3.5). Aphid exposure did not affect shoot [N] of '- AMF' plants (-2%), but reduced shoot [N] of '+ AMF' plants (-21%). No effect of AMF, aphids, or interaction was recorded on root N or root [N] of cv. Skyfall (Figure 3.6k & 3.6l, Table 3.5), which averaged 7.65 ± 0.31 mg and 2.92 ± 0.19 mg g^{-1}, respectively.

Table 3.5: Summary of two-way ANOVA results investigating the effect of AMF, aphids, and their interaction on N uptake by three wheat cultivars. Significant p-values are in bold (n=5).

Variable	**Factor**	**cv. Avalon**			**cv. Cadenza**			**cv. Skyfall**		
		F	*df*	p	F	*df*	p	F	*df*	p
Shoot N	AMF	6.45	1,16	**0.022**	1.41	1,16	0.252	0.09	1,16	0.774
	Aphids	9.06	1,16	**0.008**	0.04	1,16	0.847	4.32	1,16	0.054
	AMF*Aphids	1.31	1,16	0.270	0.38	1,16	0.546	1.40	1,16	0.254
Shoot [N]	AMF	2.16	1,16	0.161	0.53	1,16	0.478	1.52	1,16	0.236
	Aphids	0.60	1,16	0.451	0.10	1,16	0.754	20.60	1,16	**<0.001**
	AMF*Aphids	0.04	1,16	0.850	0.01	1,16	0.940	13.62	1,16	**0.002**
Root N	AMF	4.07	1,16	0.061	8.23	1,16	**0.011**	0.85	1,16	0.371
	Aphids	0.15	1,16	0.704	31.12	1,16	**<0.001**	0.37	1,16	0.553
	AMF*Aphids	0.77	1,16	0.393	1.55	1,16	0.230	0.01	1,16	0.907
Root [N]	AMF	6.48	1,16	**0.022**	7.03	1,16	**0.017**	2.40	1,16	0.141
	Aphids	0.05	1,16	0.834	29.78	1,16	**<0.001**	1.39	1,16	0.255
	AMF*Aphids	0.00	1,16	0.965	19.50	1,16	**<0.001**	2.72	1,16	0.118

[a] Log_{10} transformed.

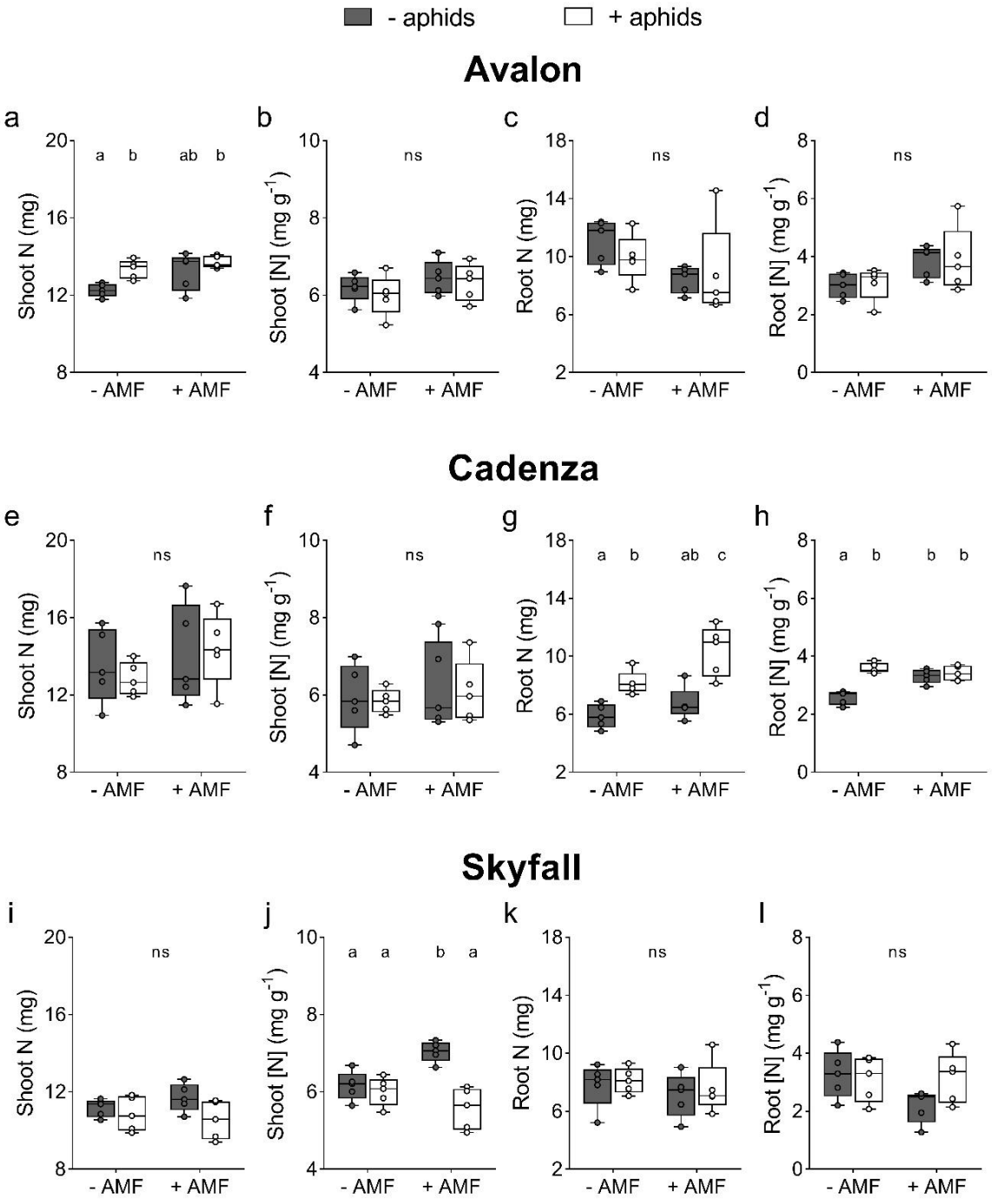

Figure 3.6: N uptake by non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids. (a, e, i) Shoot N; (b, f, j) Shoot [N]; (c, g, k) Root N; (d, h, l) Root [N]. cvs. Avalon, Cadenza, and Skyfall were inoculated with *R. irregularis* (+ AMF) or a sterilised inoculum (- AMF) and exposed (+ aphids, white boxes) or not (- aphids, grey boxes) to bird cherry-oat aphids (*R. padi*) for 14 days after 8 week growth. Boxes extend from 25th to the 75th percentile. Middle lines represent median values, and whiskers extend to the minimum and maximum data points (closed or open markers, n=5). Different letters indicate significant differences between means (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

3.4.6 Correlations

In order to investigate whether the nutritional status of wheat determined aphid performance, Spearman's rank correlation coefficients were carried out on shoot P and [P] and final aphid abundance and aphid load. There was a strong positive correlation between shoot P and final aphid abundance, meaning plants with higher shoot P content supported greater aphid numbers (Figure 3.7a). Likewise, final aphid abundance was positively correlated with shoot [P] (Figure 3.7b). When considering aphid load, plants that achieved greater shoot P (Figure 3.7c) and shoot [P] (Figure 3.7d) similarly supported a larger number of aphids per gram shoot (dry weight).

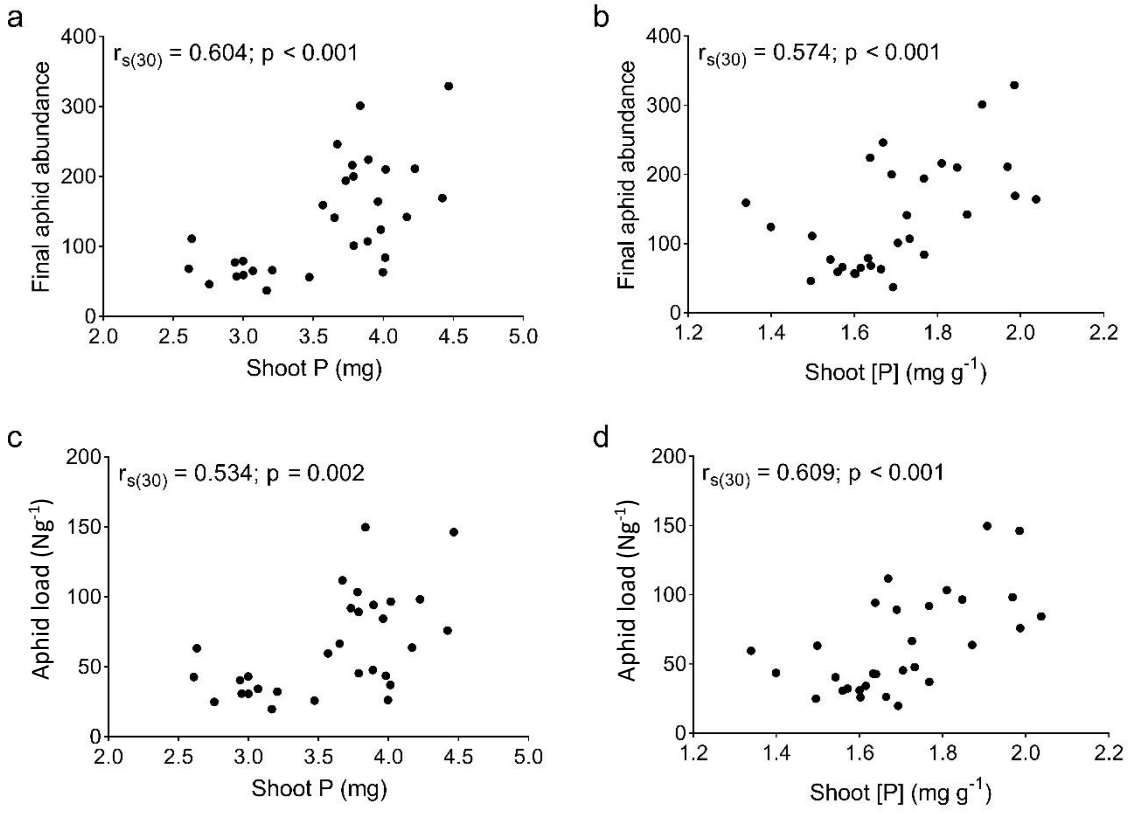

Figure 3.7: Correlations between shoot P status and aphid performance on wheat. (a) Final aphid abundance vs shoot P; (b) Final aphid abundance vs shoot [P]; (c) Aphid load vs shoot P; (d) Aphid load vs shoot [P]. All data pooled across wheat cultivars (cvs. Avalon, Cadenza, and Skyfall) and AMF treatments (- AMF and + AMF). Correlations were tested using Spearman's rank correlation coefficients.

Spearman's correlations were also conducted to evaluate whether shoot N and [N] impacted aphid performance. Total shoot N was positively correlated with final aphid abundance (Figure 3.8a), and aphid load (Figure 3.8c). However, in contrast to shoot [P], aphid performance was less strongly associated with shoot [N]. When expressed as a concentration, no association was recorded between shoot [N] and final aphid abundance (Figure 3.8b) or shoot [N] and aphid load (Figure 3.8d).

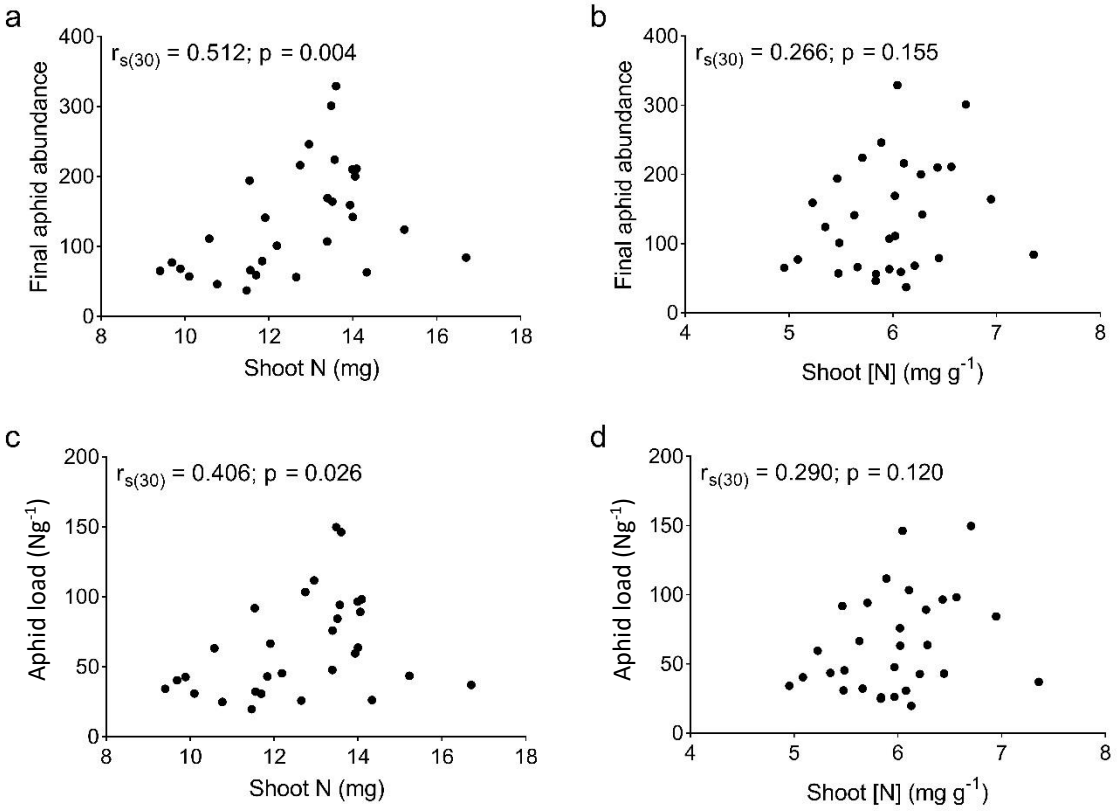

Figure 3.8: Correlations between shoot N status and aphid performance on wheat. (a) Final aphid abundance vs shoot N; (b) Final aphid abundance vs shoot [N]; (c) Aphid load vs shoot N; (d) Aphid load vs shoot [N]. All data pooled across wheat cultivars (cvs. Avalon, Cadenza, and Skyfall) and AMF treatments (- AMF and + AMF). Correlations were tested using Spearman's rank correlation coefficients.

3.5 Discussion

Wheat growth responses to colonisation by AM fungi can range from positive to negative. How biotic interactions, such as those between crops and insect herbivores, impact plant responsiveness to AM fungi has received little attention. Phloem-feeders, such as aphids, can limit plant C resources directly through the removal of phloem, and indirectly by inducing defence-signalling pathways in plants (Ali & Agrawal, 2011) or by altering rates of photosynthesis (Macedo et al., 2003; 2009). However, how an aphid-induced C drain impacts wheat-AM interactions has remained unstudied. Here, colonisation of wheat by the AM fungus *Rhizophagus irregularis* was unaffected by aphid herbivory (*Rhopalosiphum padi*), with plant growth and nutritional responses to mycorrhization also being unchanged in the presence of the specialist cereal-feeding pest. *R. irregularis* had no effect on aphid abundance or load within each cultivar, but seemingly increased the tolerance resistance of cv. Skyfall against herbivory.

3.5.1 Top-down impacts

3.5.1.1 Impact of aphids on AM colonisation

Before now, the impact of aphid feeding on AM colonisation of wheat had not been studied. Here, no effect of herbivory by a specialist cereal-feeding aphid (*R. padi*) was recorded on AM colonisation of roots and soils of wheat (Figure 3.3). This finding challenges the first hypothesis, which suggested AM colonisation would be reduced in aphid exposed plants (Babikova et al., 2014a; 2014b; Meier & Hunter, 2018) owing to sink-competition for plant C resources between aphids and AM fungi (Larson & Whitham, 1997; Barto & Rillig, 2010). Neutral effects of aphid feeding on the AM fungus were detected across all three cultivars, again countering the first hypothesis which anticipated cultivar-specific responses in % root length colonisation to aphids, given the variable outcomes recorded previously in closely related plant species (Meier & Hunter, 2018).

Being obligate biotrophs (Smith & Read, 2010), plant C represents the only means by which AM colonisation is supported. As such, AM fungal biomass in roots and soils of plants may be used as an alternative for plant C transfer to AM fungi. Equivalent levels of AM colonisation across aphid treatments may suggest that plant C supply to the fungus was equivalent (Figure 3.3). Similar frequencies of particular fungal structures, such as arbuscules and vesicles (Figure 3.3b-c), also hints at comparable

physiological functioning. Potential reasons for these observations are three-fold. The first is that the magnitude of the plant C drain imposed by aphids was insufficient to disrupt AM colonisation. This could be because of the size of the infestation or duration of the exposure. To test this hypothesis, future work should manipulate both these factors in order to identify (if possible) thresholds beyond which AM colonisation of wheat is affected. The cumulative aphid-days (CAD) metric may be of use here, as it combines the number of aphids per plant and the length of the exposure into one measure, and has been used to predict yield loss in crops (wheat: Kieckhefer et al., 1995; soybean: Ragsdale et al., 2007). Reduced AM infection of broad bean was recorded when targeted by fewer than 80 aphids (Babikova et al., 2014a), and in *Asclepias* species when exposed for just 6 days (Meier & Hunter, 2018). This suggests that thresholds beyond which AM colonisation is affected vary between AM fungal-plant-aphid combinations. This may be expected, as rates of phloem siphoning can differ between aphid species (Puterka et al., 2017) and fungal C demands also vary between isolates (Lendenmann et al., 2011). These plant C dynamics could explain idiosyncratic outcomes noted in the literature (see Chapter 1 section 1.4.1; Table 1.1).

Secondly, equivalent levels of AM colonisation across aphid treatments could, as per Chapter 2, be a due to the year of cultivar release. Modern wheat cultivars typically invest fewer resources below ground than older varieties, having been bred to produce high yields under optimal growing conditions (Voss-Fels et al., 2017). For this reason, root biomass (Den Herder et al., 2010), root: shoot ratios (Siddique et al., 1990), and plant C supply to AM fungi may be low in modern lines (Thirkell et al., 2019). It is possible, therefore, that wheat plants may not “sanction” their mycorrhizal partners after aphid exposure by reducing fungal biomass, as to so would liberate few additional plant C resources for other processes and perhaps reduce nutrient uptake via the AM pathway. This may also explain equivalent AM fungal abundances in roots and soils of barley when infested or not with English grain aphids (*Sitoban avenae*) (Wilkinson et al., 2019). That said, neutral AM colonisation responses to aphids have also been recorded in non-crop plants (Vannette & Hunter, 2014; Maurya et al., 2018; Meier & Hunter, 2019; Li et al., 2019). To test this mechanism, the effect of aphids on AM infection of wheat should be studied across a range of old and new cultivars.

Finally, equal levels of AM colonisation may be due to issues with the experimental design of the study. Wheat plants exposed to *R. padi* aphids were grown in the same

glasshouse as plants which were not. Plants infested with insects may emit volatile organic compounds (VOC) that repel aphids (Babikova et al., 2013a) and warn nearby plants of herbivore attack (Erb et al., 2015). These long-distance signals can induce hormones in unexposed plants (Heil & Ton, 2008) which in turn may regulate AM colonisation (Pozo et al., 2015). In Chapters 4 and 5 of this thesis, plants exposed or not to aphids will be grown separately in order control for the potential effect of VOCs.

3.5.1.2 Impact of aphids on wheat growth and nutritional responses to AM colonisation

Inoculation of wheat with the AM fungus *R. irregularis* did not promote shoot growth in the absence of aphids (Figure 3.4). This was true across all cultivars. Plant growth responses to AM colonisation were also, broadly speaking, unchanged when exposed to *R. padi* aphids. This finding counters hypothesis three, which predicted wheat-mycorrhizal symbioses would be less mutualistic in the presence of aphids (Bennett & Bever, 2007), owing to reduced AM colonisation and thus the ability of AM fungi to provide plants with soil nutrients. This, in turn, could have limited plant growth (and nutrient demand) leading to a more parasitic interaction.

Examples of neutral and even negative growth responses of wheat to inoculation with AM fungi are common (Hetrick et al., 1992; 1993; Ellouze et al., 2016). Hypotheses as to why they occur are discussed in Chapter 2. Briefly, AM fungi may fail to positively impact plant growth if they provide few nutritional benefits in exchange for plant C received, thereby imposing a net “cost” on plants (Smith & Smith, 2013). That said, over 50% of P uptake by wheat may be absorbed via AM fungi in the absence of positive growth responses (Li et al., 2006). This could be explained by reduced plant P uptake directly via the roots after AM colonisation (Smith & Smith, 2011a). Partial evidence for this may be found in cv. Avalon. In accordance with results presented in Chapter 2, AM colonisation by *R. irregularis* reduced root biomass of this cultivar (Figure 3.4b). Despite this, shoot P and shoot N were greater in AM than in non-AM replicates (Figure 3.5a & 3.6a), with concentrations of both nutrients being equivalent across treatments (Figure 3.5b & Figure 3.6b). Taken together, these findings could suggest a functional AM uptake pathway in cv. Avalon (as recorded recently [Thirkell et al., 2019; Elliott et al., 2020]), which counterbalanced reduced P and N capture via the root. That growth and nutritional responses of cv. Avalon to the AM fungus were

unchanged by aphids perhaps reflects the similar extent of AM colonisation across aphid treatments.

Despite similar levels of AM colonisation in roots of cv. Skyfall, plant growth promotion of cv. Skyfall by the AM fungus was seemingly more positive in the presence of aphids. This finding contrasts that recorded in *Plantago lanceolata* colonised by *Glomus* white, in which the presence of an insect pest appeared to lessen plant growth promotion by the fungus (Bennett & Bever, 2007). Reasons for this contrasting outcome could be two-fold. Firstly, differences may related to fungal genotype, as plants inoculated with *Scutellospora* or *Archaeospora* species responded similarly to AM fungi regardless of insect herbivory (Bennett & Bever, 2007). This is perhaps not surprising, as AM fungi can differ in the extent to which they promote plant growth (Munkvold et al., 2004). Alternatively, contrasting outcomes may be driven by the differing feeding approaches of the pests. Chewing herbivores may represent larger plant C sinks than phloem-feeders, as they reduce plant C availability via the removal of foliar biomass and by reducing photosynthetic rates in undamaged leaves (Zangerl et al., 2002) owing to the disruption of plant vascular tissues. Additionally, their destructive feeding mode may trigger defence-signalling pathways in plants different to those induced by aphids (Ali & Agrawal, 2012), which too may impact photosynthesis (Nabity et al., 2013) and thus the availability of plant C and impact AM colonisation directly (Pozo et al., 2015).

Nonetheless, an effect of aphids was recorded on the nutritional response of cv. Skyfall to AM fungi. In the absence of aphids, shoot [P] and [N] of cv. Skyfall was higher in mycorrhizal plants (Figure 3.5b & 3.6b). The same was not true when plants were exposed to aphids. This could suggest that the nutritional benefits provided by the AM fungus were reduced in the '+ aphid' treatment compared to the '- aphid' treatment, hinting at a loss of symbiotic function. This would be in-line with my third hypothesis, which predicted that fungal symbionts of plants exposed to aphids may be inhibited from foraging for, and/or exchanging, mineral soil nutrients owing to reduced plant C supply. Such an outcome would not be expected from colonisation scoring alone, but the extent of AM colonisation does not necessarily correlate with physiological function (i.e. carbon-for-nutrient exchange) in plant-AM symbioses (Nagy et al., 2009; Sawers et al., 2017). It remains possible, therefore, that aphids impacted fungal-acquired plant nutrient uptake by cv. Skyfall.

However, it was impossible to definitively state whether this increase in the '+ AMF' treatment in the absence of aphids was driven by increased P/N uptake via the mycorrhizal uptake pathway, or directly via the root (see Chapter 1 section 1.2.1). Root: shoot ratios of plants of cv. Skyfall were greater when inoculated with AM fungus in the absence of aphids, suggesting greater below-ground investment by plants, which could have resulted in the increased shoot nutrient concentrations achieved. The use of isotope tracers of P and N would be required to quantify mycorrhizal-acquired nutrient uptake in wheat in the presence and absence of aphids.

3.5.2 Bottom-up impacts

3.5.2.1 Impact of AM colonisation on aphids

No effect of AM colonisation of wheat was recorded on final aphid abundance (Figure 3.2a). The same was true when controlling for shoot biomass, as aphid loads on cvs. Avalon, Cadenza, and Skyfall were also unaffected by AM colonisation (Figure 3.2b). This finding confirms the second hypothesis of this experiment, which predicted *R. padi* abundance would be unchanged by *R. irregularis* based on results of previous work in wheat (cv. Pioneer '26R22') (Abdelkarim et al., 2011). The conformity in findings suggests no effect of wheat genotype on this bottom-up interaction. However, these investigations represent the only the known research into how *R. irregularis* - an almost ubiquitously occurring AM species (Oehl et al., 2010) - impacts possibly the primary pest of wheat globally (Blackman & Eastop, 2017). Further experiments across a wider range of cultivars are required to validate this.

Although colonisation of wheat did not impact the abundance of *R. padi* aphids, positive and negative effects of AM colonisation have been recorded on aphid fitness in other AM fungal-plant-aphid systems (see Chapter 1 section 1.4.2; Table 1.2). There are a number of possible explanations for these contrasting outcomes. The first is that the genotype of the AM fungus may determine how colonisation of a shared host plant impacts aphids. For instance, while *R. irregularis* was found to have no effect on *R. padi* abundance on wheat (Abdelkarim et al., 2011), colonisation by *Gigaspora margarita* reduced aphid numbers. This variation could be driven by differences in the rate at which AM fungi colonise plant roots (Hart & Reader, 2002), as the stage of AM colonisation can determine of how mycorrhizas impact aphid development (Tomczak & Müller, 2017). Also, trade-offs may exist between the nutritional and non-nutritional

benefits provided by AM fungi, with some isolates affecting plant growth and others impacting responses to pests (Bennett & Bever, 2007). More experiments are required to identify which isolates confer the greatest benefits to wheat in terms of controlling *R. padi* abundance. Inoculating field-grown wheat with these strains could sustainably manage aphid populations, and reduce insecticide usage that can increase aphid pest pressure if applied in non-lethal doses (Rix et al., 2016). Additionally, understanding how native AM fungi impact aphid abundance on wheat represents an important research goal. Although modern farming practices may reduce AM fungal species richness (Helgason et al., 1998; Oehl et al., 2003), arable soils can still harbour diverse mycorrhizal communities (Oehl et al., 2017). Given the significance of fungal genotype (Abdelkarim et al., 2011), how AM fungi resident to farm soils affect *R. padi* abundance must be studied (Chapter 5).

Another reason for the variable effects of AM fungi on aphid performance may relate to the traits selected to determine outcomes for the insect. Here, by studying the effect of AM colonisation on aphid abundance only, it is possible that fungal-mediated effects on other aspects of aphid fitness were missed. For instance, AM colonisation has been shown to impact adult aphid weight (Gange et al., 1999; Gange et al., 2002, Meier & Hunter, 2018), relative growth rates (Tomczak & Müller 2017; Tomczak & Müller, 2018), survivorship (Volpe et al., 2018), and aphid feeding behaviours (Simon et al., 2017). Any effect of AM fungi on these characteristics may, over longer periods of exposure, have impacted aphid abundance. Therefore, future studies on this tri-partite interaction ought to address the effect of AM fungi on other aphid traits.

3.5.2.2 Impact of AM fungi on plant tolerance of aphid herbivory

Plant tolerance of insects can be identified if plants exposed to pests amass biomass to the same or greater extent as plants of the same cultivar that are not exposed (Girvin et al., 2017). To this end, all three cultivars could be considered tolerant of *R. padi* aphids, as aphid exposure did not reduce shoot biomass relative to unexposed plants (Figure 3.4). For cvs. Avalon and Skyfall, shoot biomass was greater in the presence of aphids indicating over-compensatory growth (Kula et al., 2015), although this was only true of cv. Skyfall colonised by the AM fungus. This finding confirms the fourth hypothesis, which predicted AM colonisation would increase plant tolerance of aphids.

Biomass loss in wheat following aphid exposure depends on the size of the infestation and growth stage of the crop (Riedell et al., 2003). Exposure to non-viruliferous *R. padi* aphids at levels below those used in this study have been shown to reduce grain yields (Kieckhefer & Gellner, 1992; Kieckhefer et al., 1995). As such, the increase in shoot biomass of cv. Avalon in the presence of aphids is of interest (Figure 3.4a). Higher yields in aphid exposed plants have been documented in other crop-aphid systems, such as soybean challenged by soybean aphids (*Aphis glycines*) (Liere et al., 2015; Kucharik et al., 2016). Although not a universal physiological response, this may occur following an increase in ambient photosynthetic rates after herbivore exposure (Tiffin, 2000). Such an outcome may counteract the external plant C sink of aphids, or meet the increased energy requirements of phloem loading caused by feeding (Hawkins et al., 1987). To date, positive effects of aphids on wheat photosynthesis have not been recorded, with aphids instead lowing net photosynthesis due to reduced stomatal conductance (Macedo et al., 2009). The effect of *R. padi* aphids on gas exchange of cv. Avalon therefore ought to be studied, perhaps across a range of aphid abundances given this photosynthetic response appears to be density-dependent in other cereals (Kucharik et al., 2016). More efficient photosynthesis in '+ aphid' plants of cv. Avalon may in turn explain the equivalent levels of AM colonisation in roots of this variety across aphid treatments (Figure 3.3).

The over-compensatory growth of cv. Skyfall to *R. padi* aphids was only recorded in plants colonised by *R. irregularis* (Figure 3.4g), suggesting AM colonisation enabled this variety to better tolerate aphid feeding. Improved pest tolerance in AM plants has been recorded before (Kula et al., 2005; Bennett & Bever, 2007; Frew et al., 2020), and may relate to the impact of AM fungi on plant nutrient status (Tao et al., 2016). This is because greater plant resource availability increases plant tolerance of herbivory (Maschinski & Whitham, 1989). However, AM colonisation did not increase shoot [P] or [N] of cv. Skyfall (Figure 3.5j & 3.6j), meaning greater tolerance cannot be attributed to fungal nutrient capture. Instead, the over-compensatory growth of AM plants exposed aphids may also relate to physiological changes involved in gas exchange. Positive effects of AM colonisation have been recorded on host-plant photosynthesis (Kaschuk et al., 2009), including in cereals (Zhu et al., 2002), and may occur in the absence of nutritional benefits, being instead stimulated by the plant C sink that AM fungi represent (Gavito et al., 2019). To the best of my knowledge, how

multiple plant C sinks, like insects and AM fungi, impact photosynthetic rates of shared plant hosts has not been studied, but ought to be as a potential mechanism of AM fungal-induced tolerance.

The extent to which AM fungi increase plant tolerance of insects differs with host-plant genotype (Kula et al., 2005). This perhaps explains why AM colonisation of cvs. Avalon and Cadenza did not also increase plant tolerance of aphids beyond that recorded in non-AM hosts. Likewise, how AM colonisation impacts plant tolerance may differ between fungal species (Bennett & Bever, 2007). As discussed above, how native AM fungi impact wheat tolerance of aphids should therefore be investigated (Chapter 5).

3.5.3 Cultivar differences

3.5.3.1 Impact of cultivar on AM fungi

As per the findings presented in Chapter 2, AM colonisation of wheat varied between cultivars. % root length colonisation was highest in roots of cv. Avalon, the oldest variety, and lowest in roots cv. Skyfall, the most modern (Figure 3.3a). This may reinforce the suggestion that AM receptivity is related to the year of cultivar release, with roots of older wheat varieties being more colonised by AM fungi than more modern ones. Intriguingly, extra-radical hyphal lengths were instead greatest in soils of cv. Cadenza (Figure 3.3d), suggesting that *R. irregularis* adopted varying growth strategies when colonising different cultivars of wheat. These findings reiterate conclusions from Chapter 2 in which variation in AM colonisation of roots and soils were also recorded between cultivars, although in different patterns. Different trends may be attributed to growth conditions, given the same wheat genotypes and species of AM fungus was used. Temperature (Cabral et al., 2018), light intensity (Johnson et al., 2015), and the time of harvest could all affect AM colonisation, making cross-study comparisons problematic.

3.5.3.2 Impact of cultivar on aphid abundance

Final abundance of bird cherry-oat aphids varied considerably between wheat cultivars. Aphids were most abundant on cv. Avalon, followed by cvs. Cadenza and Skyfall (Figure 3.2a). Two potential reasons explain this. Firstly, the wheat cultivars used here may vary in their resistance to *R. padi* aphids. Plant resistance can be divided into three categories, these being antibiosis, antixenosis, and tolerance (Girvin et al., 2017). Lower aphid abundance on certain cultivars may indicate antibiosis

resistance, which impacts aphid fecundity, survival, and development. One genetic region associated with antibiosis resistance against *R. padi* aphids (QRp.slu-4BL) has been determined on chromosome 4BL in wheat (Crespo-Herrera et al., 2014). This chromosome also carries genes implicated in benzoxazinoid biosynthesis, which are tryptophan-derived metabolites of cereals (Cotton et al., 2019) that may determine host-plant immunity against aphids (Elek et al., 2009; Ahmad et al., 2011; Meihls et al., 2013). Alternatively, physical defences may be involved. Leaf toughness can defend plants against aphids (Kos et al., 2011), and may vary between cultivars of maize (Maag et al., 2015). Likewise, vascular bundle width can determine aphid susceptibility or resistance in *Triticum monococcum* (Simon et al., 2017). To the best of my knowledge, chemical and physical defences against aphids have not been studied in the three wheat cultivars used here. The cv. Avalon x cv. Cadenza mapping population has been used to investigate wheat resistance to mosaic virus (Bass et al., 2006) and yellow rust (Gardiner et al., 2020), so may be a useful resource for investigating aphid antibiosis resistance. The development of aphid-resistant wheat varieties is desirable, as it could reduce insecticide usage and perhaps be a more practical means of managing aphid populations; not only have *R. padi* aphids developed resistance to certain insecticides (Wang et al., 2018), but other cereal aphids can cause leaf curl in wheat, which may limit the efficacy of insecticidal sprays that are toxic only upon direct contact with insects (Razmjou et al., 2012).

Alternatively, differences in aphid performance between cultivars may be explained by shoot nutrient status. Wheat plants that achieved greater shoot P and [P] supported larger aphid abundance and load (Figure 3.7). Similarly, shoot N was positively associated with aphid abundance and load (Figure 3.8a & 3.8c). This relationship between the nutritional quality of plant foliage and aphid performance has been recorded previously for P and N (Ponder et al., 2000) as well as other macronutrients (Myers & Gratton, 2006). Aphid fitness can thus depend on diet, with *R. padi* aphids on wheat responding positively to the addition of fertilisers (Aqueel & Leather 2011). This observation underlines a potential means by which AM fungi could increase aphid abundance on plants more responsive to AM colonisation (Hartley & Gange, 2009).

3.5.4 Conclusions

This study investigated the effect of aphids on the growth responses of three wheat cultivars to the AM fungus *Rhizophagus irregularis*. AM colonisation of wheat was

unaffected by aphids across all three varieties. Likewise, AM fungi had no impact on final aphid abundance or load. Instead, insect performance was positively associated with shoot nutrient status, particularly plant P. Aphid herbivory did not impact wheat growth responses to the AM fungus, reinforcing conclusions from Chapter 2 which suggested the availability of plant C resources had little effect on the responsiveness of modern wheat to AM fungi. However, AM colonisation did differentially affect shoot [P] and [N] of cv. Skyfall depending on whether plants were exposed to aphids or not. This finding could hint at a change in functional efficiency of the crop-AM symbioses in the presence of aphids. However, separating the effect of aphids on plant- or fungal-acquired P and N uptake was not possible in this study. In order to understand the mechanism underpinning wheat-AM responses to aphids, direct measurements of carbon-for-nutrient exchange are needed (e.g. Thirkell et al., 2019; Elliott et al., 2020) (see Chapter 4). Wheat cultivars differed in their tolerance resistance against aphids, this being their capacity maintain or increase shoot biomass in spite of aphid exposure. For cv. Skyfall, AM plants were seemingly more tolerant of aphids than non-mycorrhizal plants. Wheat tolerance of aphids might also be due to, or mediated by, the redirection of plant C resources and enhanced P and N assimilation from AM fungi (Chapter 4).

Chapter 4 : Aphid feeding drives asymmetry in carbon-for-nutrient exchange between wheat and an arbuscular mycorrhizal fungus

4.1 Introduction

Despite the establishment of a successful symbiosis being a prerequisite for AM fungi to complete their life cycle (Smith & Read, 2010), the proportion of photosynthetically-derived plant C allocated to mycorrhizal fungi varies between systems (Kaschuk et al., 2009). The plant C sink strength of AM roots is seemingly determined by the C needs of the fungus (Lendenmann et al., 2011), the functional group of the plant (Thirkell et al., 2019), and abiotic environmental factors like atmospheric CO_2 concentrations ([CO_2]). Elevated [CO_2] (eCO_2) increases levels of CO_2 molecules in leaves, which when combined with RuBP is catalysed by Rubisco and converted into sugars (Ainsworth & Rogers, 2007). These sugars are ultimately metabolised into lipids (Fan et al., 2019). As such, eCO_2 may increase the availability, and in turn allocation, of organic C compounds from plants to AM fungi (Drigo et al., 2010; 2013; Field et al., 2012). Thus, atmospheric [CO_2], which is predicted to exceed 800 ppm by 2100 (IPCC, 2014), can be a powerful driver impacting the source strength of plant C for AM fungi (see Chapter 2).

The regulation of resource exchange between plants and AM fungi remains unclear. Preferential plant C supply to rewarding symbionts may stabilise the mutualism (Bever et al., 2009; Bever, 2015). Carbon-for-nutrient exchange could be tightly coupled as a result (Hammer et al., 2011); evidence from *in vitro* studies using transformed carrot root (*D. carota*) suggests AM fungi deliver more nutrients to plants which supply most plant C, and that, in a reciprocal manner, plants transfer greater quantities of plant C to mycorrhizal fungi that provide greatest nutrition of P (Kiers et al., 2011) and N (Fellbaum et al., 2012). Nonetheless, these systems are artificial; the absence of shoots inhibits the subsequent movement of nutrients from roots to plant vascular tissues, for instance, as well as above-ground below-ground plant signalling that may regulate future nutrient uptake (Smith & Smith, 2013). Because of this, extrapolating these findings to more complex settings is problematic (Smith & Smith, 2015). Tightly coordinated resource exchange may operate only within a sub-set of plant-AM symbioses, therefore (Walder & van der Heijden (2015). Unequal carbon-for-nutrient exchange has been recorded between a common mycorrhizal network (CMN) and two

host plants based on genotype and not plant C delivery (Walder et al., 2012), for example, perhaps due to the divergent ability of plants to alter plant C supply to non-beneficial AM fungi (Grman, 2012). Likewise, abiotic factors, such as [CO_2], can change the relative "costs" of resources exchanged in the symbiosis. Field et al., (2012) found that the ratio of soil P received by liverworts per unit C allocated to AM fungi increased at eCO_2, due to greater mycorrhizal-mediated P uptake that outweighed the increase in plant C supply in return. In contrast, the symbiotic efficiency of vascular plant-AM symbioses was reduced at eCO_2, owing to a large increase in plant C delivery to AM fungi but no change in fungal supplied P (Field et al., 2012). Despite awareness of the importance of context on resource exchange between plants and AM fungi, the impact of biotic interactions, like those between plants and insects that represent external plant C sinks (Girousse et al., 2003), remain understudied.

Aphids are almost universal in food production systems. As highlighted in Chapter 3, aphid feeding may limit plant C availability for AM fungi through many means (Gehring & Whitham, 2002; Drigo & Rillig, 2010). Aphids use specialised mouthparts known as stylets to feed on the C-heavy phloem of target plants, with mature aphids can imbibing their own body mass in sap every 24 hours (Dixon, 2012). As such, large infestations may substantially drain plant C resources (Donovan et al., 2013). Furthermore, aphids may also limit plant C availability by inducing defence-signalling responses in host plants (Ali & Agrawal, 2012), or by reducing rates of photosynthesis (Macedo et al., 2003; 2009). As such, aphids have been shown to reduce AM colonisation in roots of plants by up to 36% (Babikova et al., 2014a; 2014b; Meier & Hunter, 2018), potentially owing to competition between plant C sinks (Larson & Whitham, 1997). Reduced plant C transfer to AM fungi in aphid exposed plants could impede the ability of the fungus to supply their plant partners with mineral nutrients, particularly if resource exchange is tightly coordinated (Kiers et al., 2011; Hammer et al., 2011). That said, neutral or even positive effects of aphids have been found on AM colonisation (see Chapter 1 section 1.4.1; Table 1.1), in the latter instance perhaps due to the sequestration of C resources below-ground, as seen in other plant-insect systems (Holland et al., 1996).

The extent of AM colonisation in roots of plants, as determined by microscopical methods, does not always correlate with physiological function in plant-AM symbioses. For instance, in plant roots colonised by multiple AM fungi, the relative contribution of each isolate to plant P uptake may be unrelated to the % of root length occupied (Smith

et al., 2004). Likewise, in cereals such as maize, fungal-mediated P uptake may not be linearly related with AM abundance in the root (Sawers et al., 2017). Crucially, what is even less certain is whether changes in AM colonisation induced by biotic and abiotic environmental factors accurately reflect changes in mycorrhizal function. For instance, mycorrhizal-mediated P uptake by tomato was almost completely supressed at high soil P compared to a low P control, despite only a moderate decline in AM colonisation (Nagy et al., 2009). Thus, relying on microscopical methods to infer a less highly functioning symbiosis following aphid herbivory based on the extent of AM colonisation could be misleading. Likewise, the same may be true when using modern techniques to quantify AM colonisation, such as qPCR (Voříšková et al., 2017).

Multi-trophic plant biotic interactions with above and below ground organisms invariably take place within dynamic environments (Frew & Price, 2019). Increasing atmospheric [CO_2] may alleviate plant C limitation caused by aphids and restore plant C delivery to AM fungi. That said, the C drain induced by aphids could be greater at eCO_2, as discussed in Chapter 1 section 1.5. Briefly, the abundance of wheat-feeding aphids may increase at eCO_2 (e.g. Sun et al., 2009a; Wang et al., 2018), perhaps owing to the enhanced water status of target plants (Sun et al., 2015) or rising leaf temperatures (O'neill et al., 2011) driven by reduced stomatal conductance. Secondly, eCO_2 may dampen key phytohormonal signalling pathways in plants, like JA and ET, which may otherwise confer aphid resistance (Sun et al., 2018). Lastly, higher C:N ratios in plant sap at eCO_2 may induce compensatory feeding by aphids (Sun et al., 2009b), as determined by greater honeydew production at high [CO_2] (Sun et al., 2009b; Kremer et al., 2018). However, this is not always the case (Boullis et al., 2018), and has not before been studied in wheat-aphid systems.

Given the context dependent nature of resource exchange in plant-AM symbioses (Walder & van der Heijden, 2015), studies investigating the dual effects of biotic and abiotic factors on C-for-nutrient exchange between crops and AM fungi are lacking. I investigated how aphids and elevated [CO_2], in-line with climate estimates for the end of the century (IPCC, 2014), impact resource exchange between wheat and an AM fungus. Given wheat is often targeted by the bird cherry-oat aphids (*Rhopalosiphum padi*) (Blackman & Eastop, 2000) and at the same time colonised by AM fungi like the generalist species *R. irregularis* (van der Heijen et al., 2015), this experimental system represents a biologically-relevant tri-trophic interaction with broad societal relevance.

4.2 Key questions and hypotheses

- Do aphids reduce recently-fixed plant C transfer to the AM fungus?
  - Hypothesis 1: Exposure to the *R. padi* aphids is hypothesised to reduce plant C supply to *Rhizophagus irregularis*. This is owing to sink competition between aphids and the AM fungus for plant C resources (Larson & Whitham, 1997), with aphids limiting their availability (Gehring & Whitham, 1994; 2002).
- Does elevated [CO_2] increase recently-fixed plant C transfer to the AM fungus?
  - Hypothesis 2: eCO_2 will increase the C source strength of wheat owing to greater rates of photosynthetic C fixation (Ainsworth & Rogers, 2007). Plant C allocation to the AM fungus is hypothesised to be greater at eCO_2, as recorded previously in wild plant-AM fungal symbioses (Drigo et al., 2010; 2013; Field et al., 2012). That said, recent work in wheat found that plant C transfer to AM fungi was unresponsive to eCO_2 (Thirkell et al., 2019).
- Does eCO_2 mitigate aphid-induced plant C losses and restore plant C supply to an AM fungus?
  - Hypothesis 3: eCO_2 is hypothesised to compensate for the plant C drain imposed on wheat by aphids, and reinstate plant C supply to the AM fungus. However, if eCO_2 increases the abundance of aphids on wheat (e.g. Ryan et al., 2014) or their assimilation of plant phloem owing to compensatory feeding (Kremer et al., 2018) then this may not be the case.
- Does fungal-acquired nutrient uptake by wheat change with plant C supply to the AM fungus?
  - Hypothesis 4: If plant nutrient assimilation by AM fungi is tightly coupled with plant C allocation to the fungus (Kiers et al., 2011), mycorrhizal-mediated P and N uptake by wheat will be reduced in plants exposed to aphids. In contrast, eCO_2 is predicted to alleviate the effect of the external biotic C sink, and re-establish fungal-acquired nutrient uptake by wheat. In order to determine whether plant C supply predicts mycorrhizal-acquired P/N uptake and vice versa, correlation coefficients will investigate the relationship between multiple metrics of plant C transfer to the AM fungus and shoot ^{33}P and ^{15}N.

4.3 Materials and Methods

4.3.1 Plant material

Seeds of wheat cv. Skyfall were supplied by RAGT Seeds Ltd. (Saffron Walden, UK). cv. Skyfall was chosen as the host plant for this experiment as in Chapter 3, exposing cv. Skyfall to aphids perhaps determined the extent to which AM colonisation improved plant nutrient uptake. However, separating the effect of aphids on plant nutrient uptake via the mycorrhizal and direct pathway was not possible. As such, cv. Skyfall was considered the best candidate for studying the effect of plant C sinks and sources - aphid herbivory in combination with atmospheric [CO_2] - on plant-AM function.

Seeds were sterilised using chlorine gas and germinated as outlined in Chapter 2 section 2.3.1. To ensure the successful establishment of a healthy plant in each pot, 2 seedlings were planted in 4.5" pots in substrate consisting of a sand and perlite mix (3:1) that had been sterilised at 121˚C for 45 minutes. Seedlings were subsequently thinned down to one plant per pot after 2 weeks growth (48 plants, n=12).

4.3.2 Fungal material

All plants were inoculated with the AM fungus *Rhizophagus irregularis* (Schenck & Smith, 2009), a broadly distributed fungal isolate (Savary et al., 2018) previously shown to partake in tightly coupled carbon-for-nutrient exchange (Kiers et al., 2011; Fellbaum et al., 2012; Fellbaum et al., 2014). Root organ cultures of the fungus were cultured as described in Chapter 2 section 2.3.2. The AM fungal inoculum was made by blending six plates of *R. irregularis* with 200 mL sterile dH_2O in a counter-top food processor. Spore counts were carried out in triplicate and 15 mL of inoculum containing roughly 12,900 spores was mixed uniformly throughout the substrate of all plants of cv. Skyfall.

4.3.3 Growth conditions

48 plants were grown inside 4 insect rearing cages (47.5 cm^3) in two controlled environment growth chambers at the University of Leeds. Growth conditions were maintained as described in Chapter 2, section 2.3.3. Atmospheric CO_2 concentrations ([CO_2]) were kept at 440 ppm ('aCO_2') or 800 ppm ('eCO_2'), and light intensities averaged 210 µmol m^{-2} s^{-1} inside insect rearing tents. Plants were watered when needed and fed once weekly with 30 mL low-P (40%) Long Ashton Solution of the

nitrate formulation (Smith et al., 1983). Feeding frequencies were upped to twice a week between weeks 4 and 6, after which P strengths were halved (i.e. down to 20% of the original P content).

When plants were potted up, three windowed PVC cores were inserted into the substrate of each pot (Figure 4.1 & Figure 4.2). Core windows and bottoms were lined with 35 µm nylon mesh secured using Tensol® 12 plastic adhesive. Mesh excluded roots of wheat but enabled access of fungal hyphae (Johnson et al., 2001). Two of the cores contained bulk substrate (99.25% core volume) with crushed tertiary basalt added (0.75% core volume) to act as bait/promote the in-growth of fungal hyphae (Quirk et al., 2012). A silicone capillary tube was secured centrally to both substrate-filled cores, through which isotope tracers of ^{33}P and ^{15}N were subsequently added (see section 4.3.6). The third core was packed loosely with glass wool and an air-tight Suba-Seal® rubber septum was fitted on top. This core enabled sampling of below-ground respiration and flux of ^{14}C by the AM fungal network during the 16-hour $^{14}CO_2$ labelling period (see section 4.3.7).

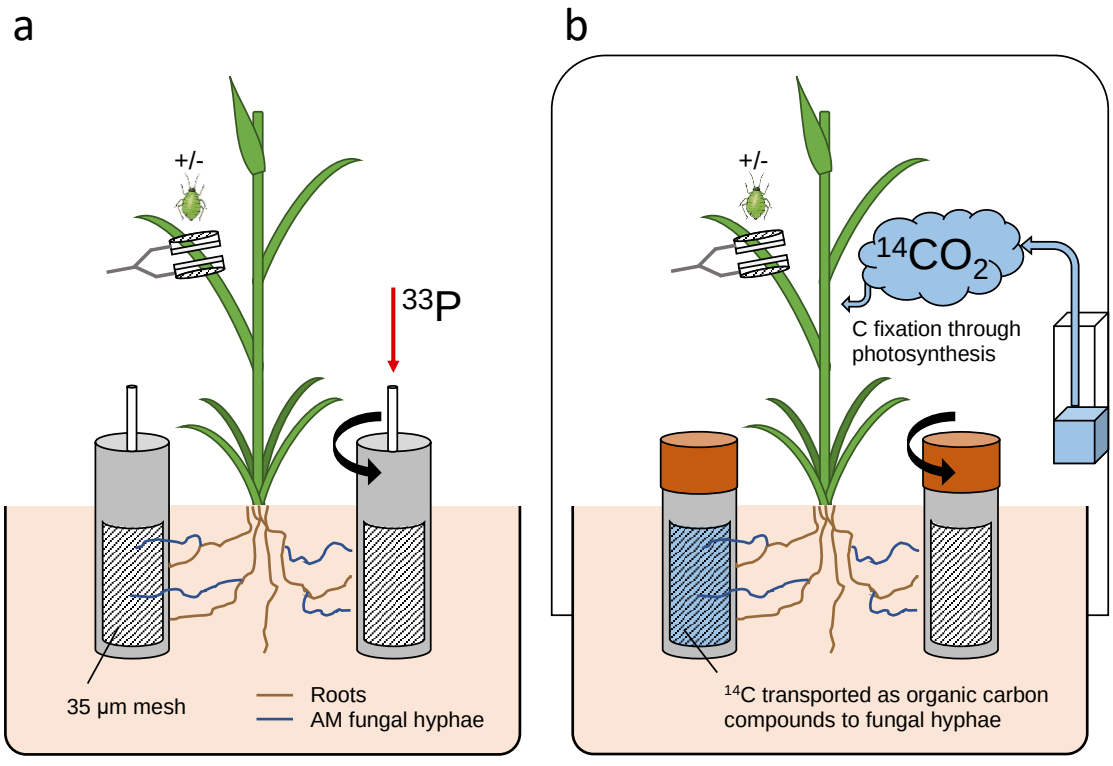

Figure 4.1: Experimental approach for quantifying C-for-nutrient exchange between wheat and AM fungi in the presence and absence of aphids. cv. Skyfall was grown at aCO_2 (440 ppm) and eCO_2 (800 ppm) and exposed or not to *R. padi* aphids during the dual-isotope labelling period. (a) ^{33}P-orthophosphate and ^{15}N-ammonium chloride were added to

mesh-lined cores accessible only to hyphae of the fungus. Fungal-mediated P and N uptake was determined by subtracting quantities of tracer recovered in wheat shoots in the 'rotated' treatment (shown) from those in the 'static' treatment. (b) Pots were enclosed in polythene bags and $^{14}CO_2$ released from ^{14}C-sodium bicarbonate. $^{14}CO_2$ was fixed by wheat and transferred to the hyphal network of the fungus or assimilated by aphids inside clip cages.

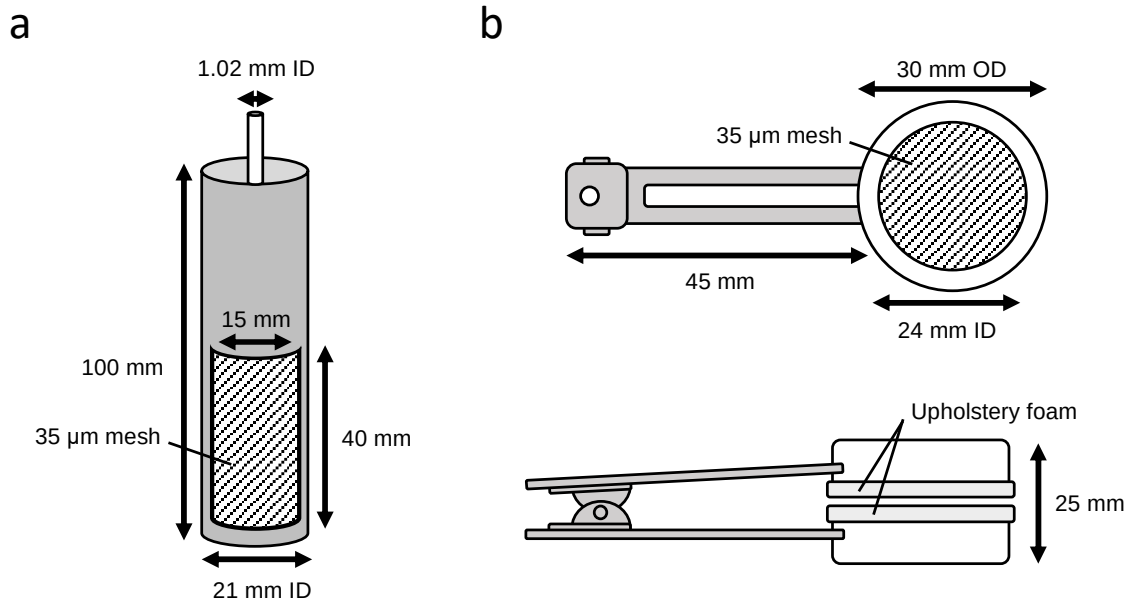

Figure 4.2: Schematic diagrams showing dimensions of the constructed mesh-walled cores and insect clip cages. 'ID' and 'OD' stands for inner and outer diameter. (a) PVC cores had two windows cut into opposing sides which were lined with 35 µm mesh. This excluded the roots of plants but allowed for the in-growth of fungal hyphae. A perforated capillary tube was affixed centrally, via which ^{33}P and ^{15}N were introduced to one of the two substrate-filled cores in each pot. (b) Aphid cages were assembled using clear acrylic tubing and double prong hair clips. 35 µm mesh was secured to the top and bottom of the cage in order to keep aphids confined. Modelling foam was affixed to the inside to prevent damage to the leaf. Cages were positioned on the third leaf of the primary tiller of plants and kept raised above the pot.

4.3.4 Aphid culture

Bird cherry-oat aphids (*Rhopalosiphum padi*) were provided by Dr. Tom Pope at Harper Adams University. Aphid cultures were established in semi-controlled glasshouse conditions in the Centre for Plant Sciences at the University of Leeds. *R. padi* aphids were maintained on wheat plants grown in composted soil inside nylon mesh-lined insect rearing cages. Growth conditions were kept at 20˚C during a 16:8 day-night cycle, supplemented with high pressure sodium lamps and blinds. Light

intensities averaged 150 µmol m^{-2} s^{-1}, and plants were watered twice weekly. Aphids later introduced to plants grown at eCO_2 (see section 4.3.5) were not acclimated to elevated [CO_2] prior to exposure to experimental plants.

4.3.5 Aphid exposure

8 weeks after germination, one insect clip cage was attached to the third leaf on the main tiller of each plant (Figure 4.1 & Figure 4.2b). Cages were suspended above the substrate surface using a skewer, so as not to separate the leaf from the plant. Half of all plants at aCO_2 and eCO_2 (24 plants, n=12) were exposed to five wingless *R. padi* aphids (hereafter termed the '+ aphids' treatment). Aphids were transferred from culture plants using a paint brush and placed on the adaxial surface of the leaf, with clip cages positioned on top. Clip cages on the remaining 50% of plants (24 plants, n=12) were kept empty (hereafter termed the '- aphids' treatment). Plants exposed and not exposed to aphids were temporally separated in order to make sure VOCs induced by aphids did not impact C-for-nutrient exchange in the '- aphids' treatment.

Given aphid abundance on wheat can respond positively to eCO_2 (Ryan et al., 2014), aphid counts were conducted at 9 time points throughout the 12-day isotope labelling period. The last abundance count was carried out on day 11 before pots were enclosed in airtight chambers for ^{14}C labelling (see section 4.3.7). Aphid abundance was plotted against time for each replicate and linear trend lines were fitted (Figure 4.3). Aphid growth rates were derived from the equation of the line for each plant (Equation 1).

$$A = rt + A_0$$

Equation 1: Where A = aphid abundance, r = growth rate, t = time (in days), and A_0 = starting aphid abundance (in this case 5).

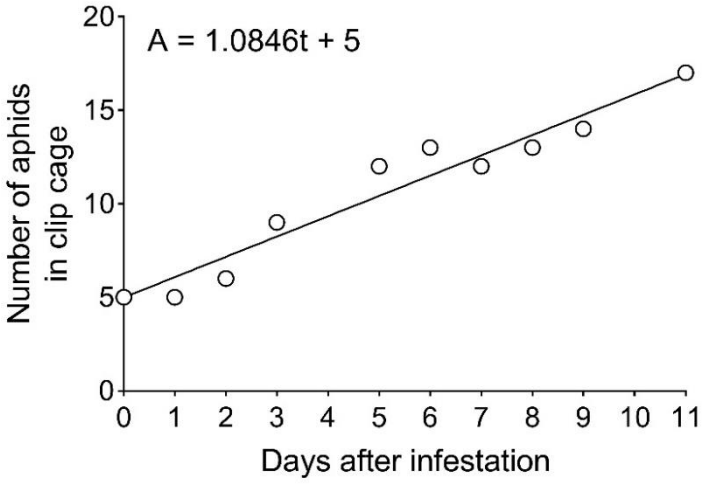

Figure 4.3: Abundance of bird cherry-oat aphids on a mycorrhizal wheat plant grown at elevated [CO_2]. Aphid counts were conducted roughly every 24-48 hours and plotted over time, and linear trend lines were fitted.

4.3.6 ^{33}P-orthophosphate and ^{15}N-ammonium chloride label

24 hours after insect clip cages were secured to leaves, an aqueous solution (100 µl) containing 1MBq ^{33}P-orthophosphate (specific activity: - aphids 5.76 TBq mg^{-1} [0.17 ng]; + aphids 3.12 TBq mg^{-1} [0.32 ng]) and 41.32 µg ^{15}N-ammonium chloride (>98 atom %) were introduced to one PVC mesh-walled core in each pot in, accessible only to fungal hyphae. Isotope solutions were pipetted into the core substrate via the capillary tube fitted centrally. Tubes had been perforated using a mounted needle every 5 mm below the soil surface, enabling an even distribution of isotope solution throughout the core substrate. In half of all pots, cores to which isotope tracers were added were gently rotated 90^{o} (hereafter termed the 'rotated' treatment) to break fungal hyphae connecting wheat and the core substrate (Figure 4.1a). Core rotation was carried out every 48 hours during the subsequent 12-day labelling period. The second substrate-filled PVC core in these pots remained static, maintaining hyphal connectivity between the plant and core. In remaining pots labelled cores were not rotated (hereafter termed the 'static' treatment) meaning hyphal connections between wheat and the mesh-walled core were preserved. Cores that were not radio-labelled within these pots were rotated, controlling for hyphal disturbance and effects on mass flow. By subtracting wheat-assimilated ^{33}P and ^{15}N in the 'rotated' treatment from the 'static' treatment, the diffusion of isotopes (or movement via microbial nutrient cycling processes) out of the cores and ultimately into the plant was controlled for.

Radioactivity of shoot material was recorded every 48 hours using a Geiger counter to monitor fungal-mediated tracer uptake in 'rotated' and 'static' treatments under both CO_2 atmospheres and in the absence and presence of aphids (Figure A1.1 in the appendix). Shoot radioactivity was lower in the 'rotated' treatment, confirming that the rotation of radio-labelled cores had severed fungal hyphal connections and prevented uptake of ^{33}P by plants.

4.3.7 ^{14}C-sodium bicarbonate label

12 days after labelling with ^{33}P and ^{15}N, the tops of both substrate-filled cores were sealed using scintillation vial caps and anhydrous lanolin, and pots enclosed in airtight zip-locked polyethylene bags using electrical tape (Figure 4.1b). At the beginning of the 16-hour photoperiod, a 1.036-MBq pulse of $^{14}CO_2$ was liberated into the headspace of plants by injecting 2 mL 10% lactic acid into a cuvette containing

$Na^{14}CO_3$ (specific activity: '- aphids' 1621 MBq $mmol^{-1}$; '+ aphids' 1850 MBq $mmol^{-1}$). Cuvettes were positioned near the base of each plant attached to plant labels inserted into the substrate. A 1 mL sample of above-ground gas was taken from the headspace of each plant using a hypodermic syringe immediately after $^{14}CO_2$ was released. 1 mL headspace gas samples were also taken 1.5 and 4.5 hours later to monitor drawdown of ^{14}C by wheat. 1 mL below-ground gas samples were taken from the glass-wool core immediately after $^{14}CO_2$ liberation and subsequently every 90 minutes. These samples were used as a measure of respiration and flux of ^{14}C through the hyphal network of the AM fungus. Above- and below-ground gas samples were injected into separate gas-evacuated 20 mL scintillation vials containing 10 mL of the liquid scintillant Carbo-Sorb® mixed with 10 mL of the liquid scintillant Permafluor®. ^{14}C radioactivity was calculated using a Tri-Carb® 3100TR liquid scintillation counter.

Plants were maintained under controlled conditions until the end of the 16-hour photoperiod during which time no peak in below-ground ^{14}C was recorded (Figure A1.2 in the appendix). 4 mL 2M KOH was injected into scintillation vial caps placed on top of the PVC cores inside each chamber, to trap remaining $^{14}CO_2$ gas at the end of the photoperiod. A final 1 mL headspace gas sample was taken the following morning to ensure ^{14}C had been trapped, before chambers were opened and plants harvested.

4.3.8 Harvest procedure and AM colonisation

Insect clip cages were removed from all plants and aphids stored in 15 mL Falcon tubes at -20°C until subsequent analysis of ^{14}C. PVC cores and plants were gently extracted from pots. Bulk substrate was loosened from wheat roots, and a sub-sample (approximately 10-15 g) was taken from each pot and stored in zip-lock bags at 5°C for quantification of extraradical hyphal lengths (see Chapter 2 section 2.3.6). Shoot and root material were parted using a scalpel. Roots were washed of any excess substrate with tap water, dried using paper towels, and fresh weights recorded using a 3-digit digital scale. Roots were then divided in two, with half being stored in 50 % EtOH (v/v) at 5°C and used to determine AM colonisation (see Chapter 2 section 2.3.5). Remaining roots were re-weighed. Bulk substrate, rotated core substrate, static core substrate, shoots, and remaining roots were stored at -20°C for 24 hours and freeze-dried with aphid material for 3 days. Dry weight measurements of each component were taken using a 5-digit digital scale before being analysed for P, ^{33}P,

N, ^{15}N, and ^{14}C. Aphid samples were analysed for ^{14}C only. Total root dry weights and root: shoot ratios were determined as outlined in Chapter 2 section 2.3.4.

4.3.9 Plant P and ^{33}P determination

Freeze-dried shoot and root material were homogenised using a mill, and 30-40 mg of bulk substrate, rotated core substrate, static core substrate, shoot material, and root material were weighed in triplicate into test tubes that had been acid washed with 1% HCl. Samples were digested as described in Chapter 2 section 2.3.7, and shoot and root P (i.e. plant- and fungal-acquired) was determined using spectrophotometric assays (Murphy & Riley, 1962; John, 1970) against a standard curve of known [P].

^{33}P-radioactivity in digest samples was quantified by liquid scintillation, using 2 mL of digest solution added to 10 mL Emulsify-safe scintillant. Fungal-mediated assimilation of ^{33}P by wheat was calculated for shoot and root samples using Equation 2 from Cameron et al., (2007), while correcting for the radioactive decay of the ^{33}P isotope which had a half-life of 25.34 days.

$$\mathrm{M}^{33}\mathrm{P} = \left\{\left[\frac{\frac{\mathrm{cDPM}}{60}}{\mathrm{SAct}}\right]\mathrm{Mwt}\right\}\mathrm{Df}$$

Equation 2: Where $M^{33}P$ = mass of ^{33}P (mg); cDPM = counts as disintegrations per minute; SAct = specific activity of the sourse (Bq mmol $^{-1}$); Df = dilution factor; and Mwt = molecular mass of P.

4.3.10 Shoot N and ^{15}N determination

2-4 mg of freeze-dried and homogenised shoot material was weighed into tin casings and analysed for ^{15}N by continuous-flow isotope ratio mass spectroscopy. Data was collected in the form of atom %, ^{15}N and % N utilising non-radiolabelled shoot material as control samples for background detection. Mycorrhizal-mediated uptake of ^{15}N by wheat was calculated using Equation 3 from Cameron et al., (2006).

$$M_{Ex} = \left(\frac{[At_{lab} - At_{cont}]}{100}\right)\left(M\left[\frac{\%\,N}{100}\right]\right)$$

Equation 3: Where M_{Ex} = mass excess of the isotope (g); At_{lab} = atom percentage of the isotope in labelled microcosm; At_{cont} = atom percentage of the isotope in paired control microcosm; M = biomass of sample (g); and % N = percentage of the nitrogen.

4.3.11 ^{14}C determination

20-30 mg of freeze-dried homogenised shoot and root material was weighed in triplicate into separate Combusto-cones (Perkin Elmer, Beaconsfield, UK), as was 30-40 mg of bulk substrate, rotated core substrate, and static core substrate from each pot. All aphids removed from each plant were likewise weighed into separate Combusto-cones. ^{14}C in plant, substrate, and aphid samples was released by sample oxidation and $^{14}CO_2$ gas trapped in a 20 mL cocktail of the liquid scintillants CarbonTrap and CarbonCount in equal volumes. Radioactivity within each component was then determined through liquid scintillation counting. Total carbon fixed by plants during the labelling period (i.e. $^{12}CO_2$ and $^{14}CO_2$) and allocated to the AM fungus or siphoned by aphids was quantified by calculating the CO_2 volume and content mass of the polyethylene bag and the proportion $^{14}CO_2$ assimilated by plants, using the Equations 4 and 5 from Cameron et al., (2008). By subtracting values of ^{14}C in the rotated core substrate from that in the static core, determination of plant C transfer from wheat to the hyphal network of the AM fungus was possible while controlling for ^{14}C detection through diffusion and/or alternative microbial C cycling processes (Thirkell et al., 2019). This value was scaled to the bulk substrate in each pot to calculate total plant C transfer to the hyphal network of the AM fungus (i.e. Fungal C).

$$T_{pf} \text{ or } T_{pa} = \left(\left(\frac{A}{A_{sp}} \right) m_a \right) + (P_r \times m_c)$$

Equation 4: Where T_{pf} or T_{pa} = total C transferred from plant to fungus or siphoned by aphids in any given pool (g); A = radioactivity of the tissue sample (Bq); A_{sp} = specific activity of the source (Bq Mol^{-1}); m_a = atomic mass of ^{14}C; P_r = proportion of the total ^{14}C label supplied present in the tissue; and m_c = mass of C (g) in the CO_2 present in the labelling chamber, from the ideal gas law (Equation 5):

$$m_{cd} = M_{cd} \left(\frac{PV_{cd}}{RT} \right) \therefore m_c = m_{cd} \times 0.27292$$

Equation 5: Where m_{cd} = mass of CO_2 (g); M_{cd} = molecular mass of CO2 (44.01 g mol^{-1}); P = pressure (kPa); V_{cd} = volume of CO_2 in the chamber (0.003 m^3); m_c = mass of unlabelled C in the labelling chamber (g); M = Molar mass (12.011 g); R = universal gas constant (J K^{-1} mol^{-1}); T = absolute temperature (K); m_c = mass of C (g) in the CO_2 present in the labelling chamber, where 0.27292 is the proportion of C in CO_2 on a mass fraction basis.

4.3.12 Statistical analyses

All data analyses were carried out using R Studio v1.1.453. Data were visually assessed using standard probability and residuals vs fitted plots to confirm that test assumptions of normality and homogeneity of variances were passed. Kruskall-Wallis tests, skewness tests, and kurtosis tests were carried out where appropriate (see figure legends and tables in Results). The effect of [CO_2] on aphid growth rate, final aphid abundance, aphid C, and the % of plant-fixed C assimilated by aphids were determined using Student's t tests. Given final aphid abundance at the time of the ^{14}C label did not differ between [CO_2] treatment and plant C assimilated by aphids was equivalent at eCO_2 and eCO_2 (see section 4.4.1), a categorical explanatory variable was used in the statistical model (i.e. aphid presence or absence). Shoot biomass, shoot N, shoot [N], shoot ^{33}P, shoot [^{33}P], and shoot C:N were analysed using a generalised linear model (GLM) with post hoc Tukey HSD tests, testing the effect of aphids, [CO_2], and their interaction. Shoot P, shoot [P], root P, shoot ^{15}N, shoot [^{15}N], root biomass, root [C], hyphal lengths, aphid growth rates, final aphid abundance, aphid C, % plant-fixed C allocated to the static core, and % plant-fixed C assimilated by aphids were Log_{10} transformed and analysed using a GLM. Shoot [C], root ^{33}P, and root [^{33}P] were square root transformed, and % root length colonisation, % arbuscule, and % vesicles were arcsine square root transformed and analysed using a GLM. Fungal C could not be transformed to pass GLM test assumptions, and so was analysed using multiple non-parametric Mann-Whitney U tests. Associations between fungal-acquired P and N uptake and plant C allocation - expressed either as % root length colonisation or fungal C - were determined using Spearman's rank correlation coefficients, in a test of hypothesis four. All figures were created using GraphPad Prism v8.2.0.

4.4 Results

4.4.1 Aphids

Population growth rates of aphids were marginally greater on plants of cv. Skyfall grown at eCO_2 than at aCO_2 (Figure 4.4a; Table 4.1). However, given the short-term exposure period, final aphid abundance in insect clip cages did not differ between [CO_2] treatments (aCO_2: 9.91 ± 1.18; eCO_2: 13.25 ± 1.48) (Figure 4.4b; Table 4.1). The amount of recently-fixed plant C assimilated by aphids was also unaffected by [CO_2] (Figure 4.4c; Table 4.1), averaging 456 ± 115 ng, and when expressed as a percentage of plant-fixed C (Figure 4.4d; Table 4.1). This suggested external C sink strengths were equivalent at eCO_2 and aCO_2 at the time of labelling.

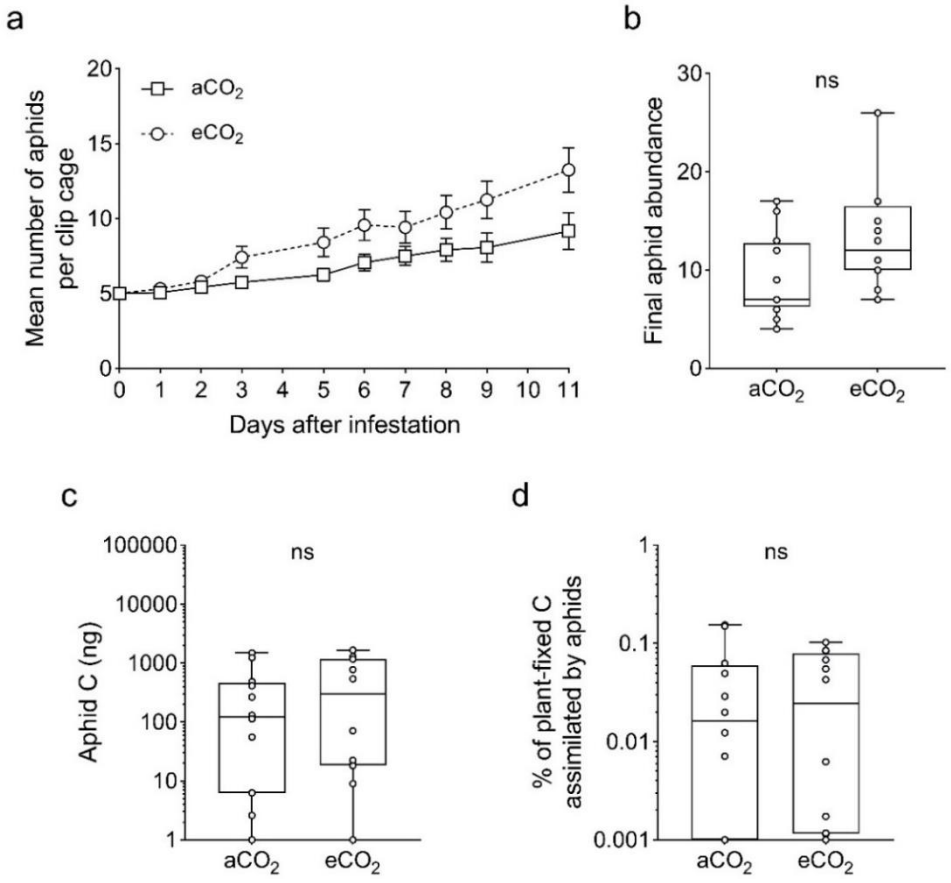

Figure 4.4: Aphid abundance and C assimilation on wheat grown at ambient and elevated [CO_2]. (a) Number of aphids per clip cage; (b) Final aphid abundance; (c) Aphid C (log scale); (d) % of recently-fixed plant C assimilated by aphids (log scale). cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to bird cherry-oat aphids (*R. padi*) in clip cages for 12 days, and labelled with $^{14}CO_2$. For panel (a), each marker represents the mean (± SE) of 12 replicates. For panels (b-d), boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and

whiskers range from minimum to maximum data points (open markers, n=12). 'ns' indicates no significant differences between means (where p > 0.05).

Table 4.1: Summary of Student t-test results investigating the effect of [CO_2] on aphid abundance and plant C assimilation on wheat. Significant p-values are in bold (n=12).

Variable	cv. Skyfall		
	t	*df*	p
Aphid growth rate	-2.02	19.11	**0.032**
Final aphid abundance	-1.75	20.94	0.094
Aphid C	-0.48	21.97	0.636
% of plant-fixed C assimilated by aphids	0.05	21.94	0.961

4.4.2 Plant growth

Shoot biomass of wheat was significantly affected by [CO_2] (Figure 4.5a; Table 4.2), being greater in plants grown at eCO_2 than at aCO_2 whether wheat was exposed to aphids or not (- aphids: +28%; + aphids: +30%). Aphids also affected shoot biomass, reducing it by 14% and 11% at aCO_2 and eCO_2, respectively. As such, greatest shoot biomass was achieved by plants in the '- aphids' treatment at eCO_2 (2.01 ± 0.07 g), while the lowest was recorded at aCO_2 in the presence of aphids (1.38 ± 0.06 g).

There was no effect of [CO_2] on root biomass of cv. Skyfall (Figure 4.5b; Table 4.2), which averaged 3.87 ± 0.21 g in the '- aphids' treatment. In contrast, aphids significantly reduced root biomass regardless of [CO_2] treatment (aCO_2: -54%; eCO_2: -57%) (Table 4.2). Mean root biomass in the '+ aphids' treatment was 1.71 ± 0.06 g.

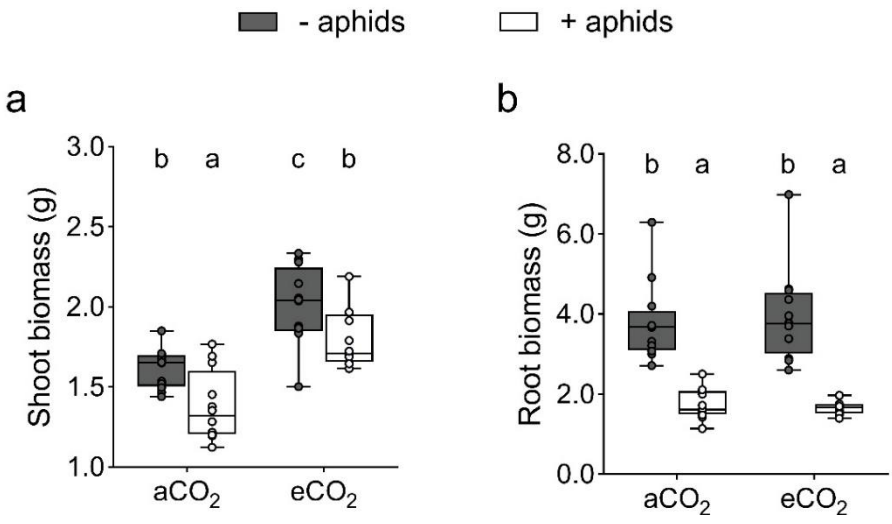

Figure 4.5: Biomass of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Shoot biomass; (b) Root biomass. cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for 12 days. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).

Table 4.2: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on plant biomass of wheat. Significant p-values are in bold (n=12).

Variable	**Factor**	**cv. Skyfall**		
		F	*df*	p
Shoot biomass	Aphids	16.01	1,44	**<0.001**
	[CO_2]	52.19	1,44	**<0.001**
	Aphids*[CO_2]	0.02	1,44	0.886
Root biomass	Aphids	172.48	1,44	**<0.001**
	[CO_2]	0.01	1,44	0.931
	Aphids*[CO_2]	0.23	1,44	0.636

4.4.3 Plant C source strength

eCO_2 increased shoot C concentrations ([C]) (Figure 4.6a; Table 4.3), indicating that plants at eCO_2 were a greater C source strength than those at aCO_2, and more so when plants were not exposed to aphids (- aphids: +76%; + aphids: +36%). In contrast, exposure to aphids reduced shoot [C], but only at eCO_2 (aCO_2: +8%; eCO_2: -17%) resulting in a significant interaction (Table 4.3).

Root C concentrations ([C]) were also affected by [CO_2] and aphids (Figure 4.6b; Table 4.3). Root [C] increased in aphid exposed plants by 69% and 170% at aCO_2 and eCO_2, respectively, likely driven in part by the reduction in root biomass. eCO_2 increased root [C], but to a larger degree in aphid exposed plants (- aphids: +30%; + aphids: +107%).

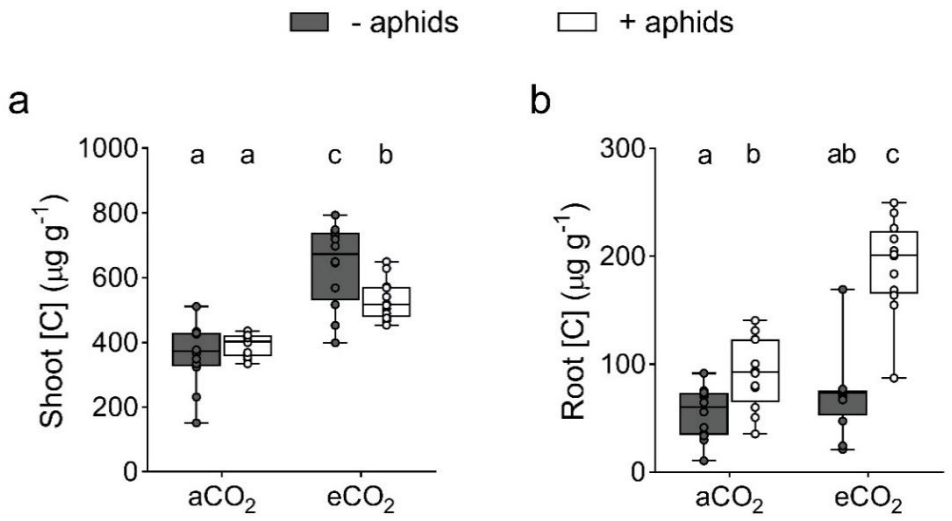

Figure 4.6: Shoot and root [C] of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. Shoot [C]; (b) Root [C]. cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for 12 days. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).

Table 4.3: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on plant C concentrations of wheat. Significant p-values are in bold (n=12).

Variable	Factor	cv. Skyfall		
		F	*df*	p
Shoot [C]	Aphids	1.03	1,44	0.315
	[CO_2]	63.03	1,44	**<0.001**
	Aphids*[CO_2]	6.33	1,44	**0.016**
Root [C]	Aphids	36.61	1,44	**<0.001**
	[CO_2]	13.92	1,44	**0.001**
	Aphids*[CO_2]	3.37	1,44	0.073

4.4.4 AM colonisation

All roots of cv. Skyfall were colonised by the AM fungus *Rhizophagus irregularis*. Atmospheric [CO_2] significantly affected % root length colonisation, being lower at eCO_2 than aCO_2 regardless of aphid treatment (- aphids: -44 %; + aphids: -29%)

(Figure 4.7a; Table 4.4). In contrast, AM colonisation was significantly greater in roots of plants exposed to aphids at aCO_2 (+41 %) and eCO_2 (+79%). Similar trends were recorded for % arbuscules (Figure 4.7b; Table 4.4), which were less frequent in roots of plants grown at eCO_2 than aCO_2, and more abundant when plants were exposed to aphids. No effect of aphid herbivory was recorded on % vesicles in roots of wheat (Figure 4.7c; Table 4.4). However, [CO_2] significantly reduced vesicle frequencies, which averaged 2.11 ± 0.56 % at aCO_2 and 0.83 ± 0.19 % eCO_2, respectively. Hyphal lengths of *R. irregularis* in bulk soils of cv. Skyfall were unaffected by aphids or atmospheric [CO_2] (Figure 4.7d; Table 4.4), averaging 1.49 ± 0.08 m g^{-1}.

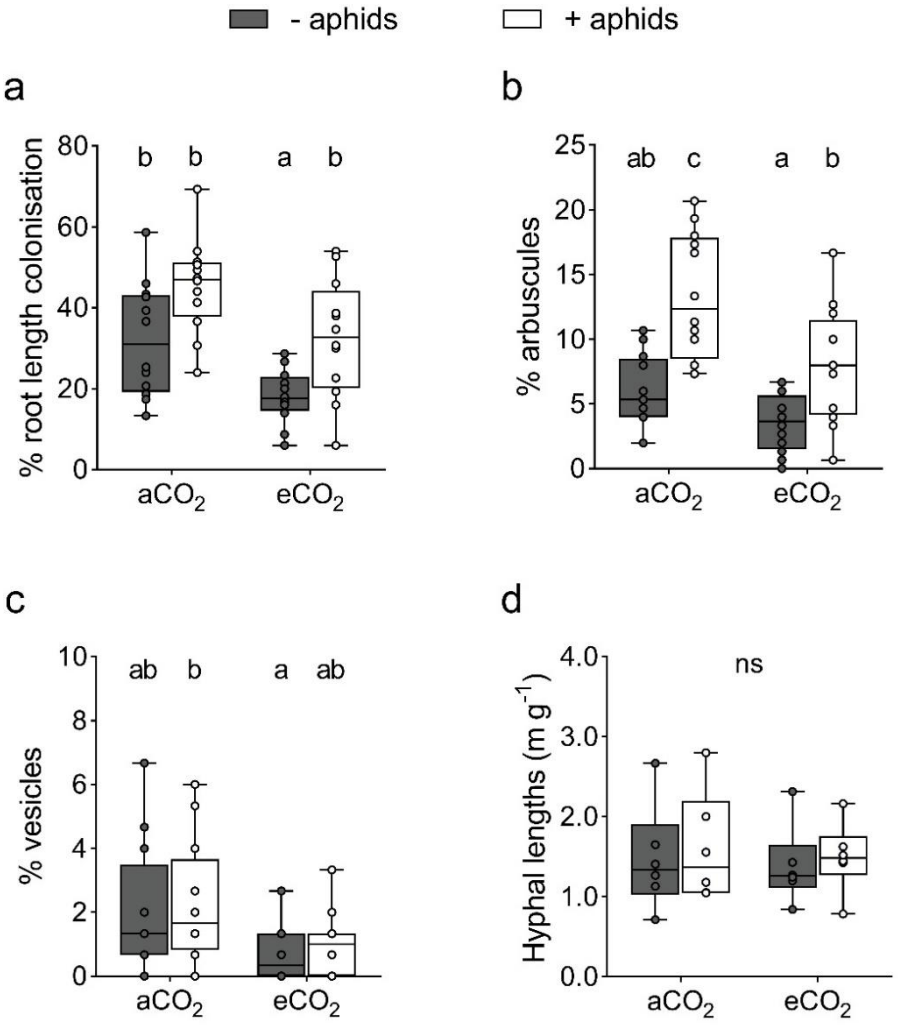

Figure 4.7: AM fungal abundance in roots and soils of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) % root length colonisation; (b) % arbuscules; (c) % vesicles; (d) Extra-radical hyphal lengths. cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for

12 days. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests). 'ns' indicates no differences.

Table 4.4: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on the AM colonisation of wheat. Significant p-values are in bold (n=12).

Variable	Factor	cv. Skyfall		
		F	*df*	p
% root length colonisation	Aphids	14.73	1,44	**<0.001**
	[CO_2]	14.94	1,44	**<0.001**
	Aphids*[CO_2]	0.05	1,44	0.823
% arbuscules	Aphids	27.03	1,44	**<0.001**
	[CO_2]	13.64	1,44	**0.001**
	Aphids*[CO_2]	0.26	1,44	0.610
% vesicles	Aphids	1.10	1,44	0.300
	[CO_2]	8.91	1,44	**0.005**
	Aphids*[CO_2]	0.01	1,44	0.915
Hyphal lengths	Aphids	0.34	1,44	0.565
	[CO_2]	0.06	1,44	0.810
	Aphids*[CO_2]	0.01	1,44	0.962

4.4.5 Plant C allocation to the AM fungus

Transfer of recently-fixed plant C to the extraradical mycelium of the AM fungus was recorded across all treatments during the ^{14}C labelling period. However, supply of newly-assimilated plant C to *Rhizophagus irregularis* was dramatically reduced in wheat exposed to aphids (Figure 4.8a; Table 4.5), declining by 97% and 73% at aCO_2 and eCO_2, respectively. Plant C transfer ceased entirely in 10 of 12 replicates exposed to aphids at aCO_2, and in 8 of 12 plants at eCO_2. In contrast, no effect of [CO_2] was recorded on plant C supply to the fungus regardless of aphid treatment (Table 4.5).

Similarly, recently-fixed plant C allocation was reduced in aphid exposed plants by 78% (aCO_2) and 63% (eCO_2) (Figure 4.8b; Table 4.6). Likewise, [CO_2] had no effect on the percentage of plant C allocated to the AM fungus by wheat, irrespective of aphid treatment (Table 4.6).

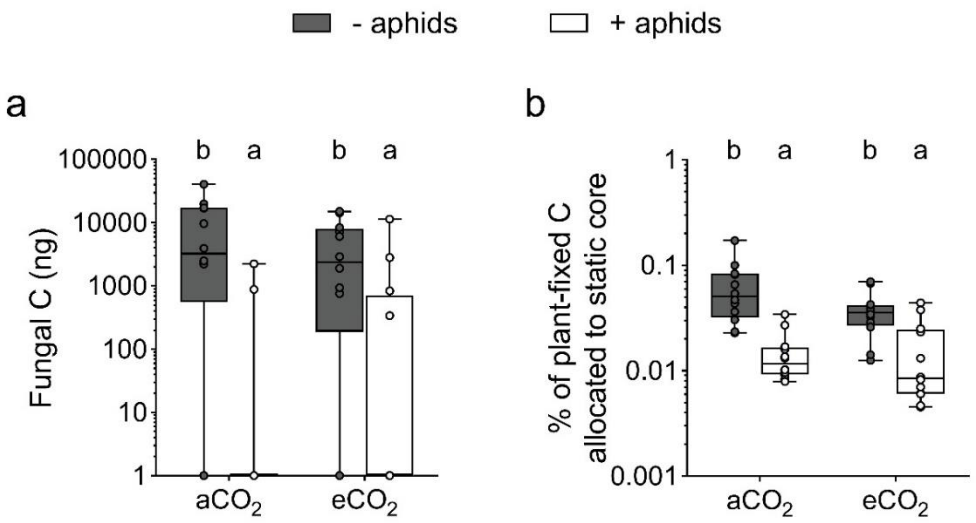

Figure 4.8: Plant C transfer and allocation to an AM fungus by wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Total plant C transfer the AM fungus (log scale); (b) % of recently-fixed plant C allocated to the static core (log scale). cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for 12 days, and labelled with $^{14}CO_2$. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12). Different letters denote significant differences (where $p < 0.05$, Mann-Whitney U tests [a] and Tukey HSD tests [b]).

Table 4.5: Summary of Mann-Whitney U-test results investigating the effect of aphids, [CO_2], and their interaction on recently-fixed plant C transfer from wheat to an AM fungus. Significant p-values are in bold (n=12).

Variable	Factor	U_{min}	U_{max}	$n_1 = n_2$	p
Fungal C	(i) aCO_2 - aphids * aCO_2 + aphids	22	122	12	**0.002**
	(ii) aCO_2 - aphids * eCO_2 - aphids	56.5	87.5	12	0.367
	(iii) aCO_2 - aphids * eCO_2 + aphids	32	112	12	**0.015**
	(iv) eCO_2 - aphids * eCO_2 + aphids	35	109	12	**0.025**
	(v) aCO_2 + aphids * eCO_2 + aphids	60	84	12	0.362
	(vi) aCO_2 + aphids * eCO_2 - aphids	25	119	12	**0.003**

Table 4.6: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on the allocation of recently-fixed plant C from wheat to an AM fungus. Significant p-values are in bold (n=12).

Variable	Factor	cv. Skyfall		
		F	***df***	**p**
% of plant-fixed C allocated to static core	Aphids	47.90	1,44	**<0.001**
	[CO_2]	2.96	1,44	0.092
	Aphids*[CO_2]	0.86	1,44	0.359

4.4.6 Plant- and mycorrhizal-acquired P

Total shoot P, this being the sum of plant- and fungal-mediated P uptake, was unaffected by [CO_2] (Figure 4.9a; Table 4.7). Instead, aphid treatment significantly impacted shoot P, being reduced in '+ aphids' plants by 34% and 38% at aCO_2 and eCO_2, respectively. When controlling for shoot biomass, plant- and fungal-acquired shoot P concentration ([P]) was significantly affected by aphids and [CO_2] (Figure 4.9b; Table 4.7). Again, aphid herbivory negatively impacted shoot [P] under both CO_2 concentrations (aCO_2: -21%; eCO_2: -31%). Shoot [P] also declined at eCO_2, being reduced by 9% and 20% in '- aphids' and '+ aphids' plants, respectively, most likely owing to increased shoot biomass at eCO_2. As such, lowest shoot [P] was achieved by wheat plants exposed to aphids at eCO_2, which averaged 1.39 ± 0.03 mg g^{-1}.

Mycorrhizal-acquired ^{33}P in the shoot was unaffected by aphid herbivory, but varied significantly according to [CO_2] (Figure 4.9c; Table 4.7). Fungal-acquired ^{33}P in the shoot was 42% greater at eCO_2 in plants not exposed to aphids, and 178% greater at eCO_2 in those that were. When controlling for shoot biomass, aphid and [CO_2] had a marginally significant effect on shoot ^{33}P concentration ([^{33}P]) (Figure 4.9d; Table 4.7). As per total shoot ^{33}P, concentrations of mycorrhizal-acquired ^{33}P in the shoot were higher in plants exposed to aphids, but to a larger extent at eCO_2 (+110%) than when plants were grown at aCO_2 (+13%).

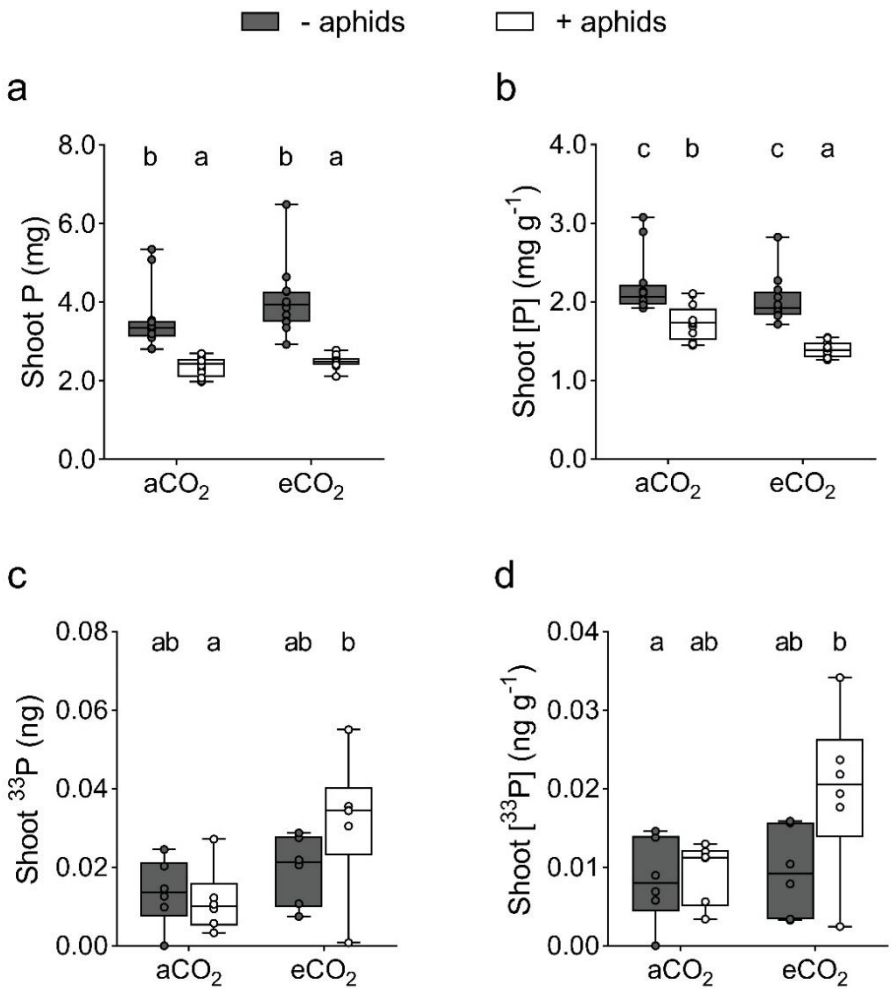

Figure 4.9: Plant- and fungal-acquired P in shoots of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Shoot P; (b) Shoot [P]; (c) Fungal-acquired shoot ^{33}P; (d) Fungal-acquired shoot [^{33}P]. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for 12 days, and ^{33}P was added to a region of substrate accessible only to the fungus. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12 except for panels [c] and [d] where n=6). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).

Table 4.7: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on plant- and fungal-acquired P in shoots of wheat. Significant p-vales are in bold (n=12;12;6;6).

Variable	**Factor**	**cv. Skyfall**		
		F	*df*	p
Shoot P	Aphids	92.78	1,44	**<0.001**
	[CO_2]	4.03	1,44	0.051
	Aphids*[CO_2]	0.68	1,44	0.412
Shoot [P]	Aphids	63.98	1,44	**<0.001**
	[CO_2]	16.77	1,44	**<0.001**
	Aphids*[CO_2]	2.93	1,44	0.094
Shoot ^{33}P	Aphids	1.16	1,20	0.293
	[CO_2]	7.87	1,20	**0.011**
	Aphids*[CO_2]	2.41	1,20	0.136
Shoot [^{33}P]	Aphids	4.36	1,20	**0.050**
	[CO_2]	4.36	1,20	**0.050**
	Aphids*[CO_2]	2.89	1,20	0.105

Over the course of the 12-day labelling period, between 3-11% of the ^{33}P-labelled tracer added to the mesh-walled cores was recovered in shoot material of wheat. ^{33}P was also detected in the roots of wheat plants, with root ^{33}P (Figure 4.10c; Table 4.8) and [^{33}P] (Figure 4.10d; Table 4.8) data broadly reflecting trends recorded in shoot material. However, these values invariably include tracer contained within root-internal AM fungal structures, and as such do not enable the drawing of conclusions as to the effect of aphids or [CO_2] on plant uptake of mycorrhizal-acquired P. The same is true of root P and [P], this being the combined total of plant- and fungal-acquired P in the root. Root P content was reduced by aphids at aCO_2 and eCO_2 (Figure 4.10a; Table 4.8), reflecting the effect of aphids on root biomass. In contrast, root [P] was greater in plants within the '+ aphids' treatment (Figure 4.10b; Table 4.8). No effect of [CO_2] was recorded on root P or [P].

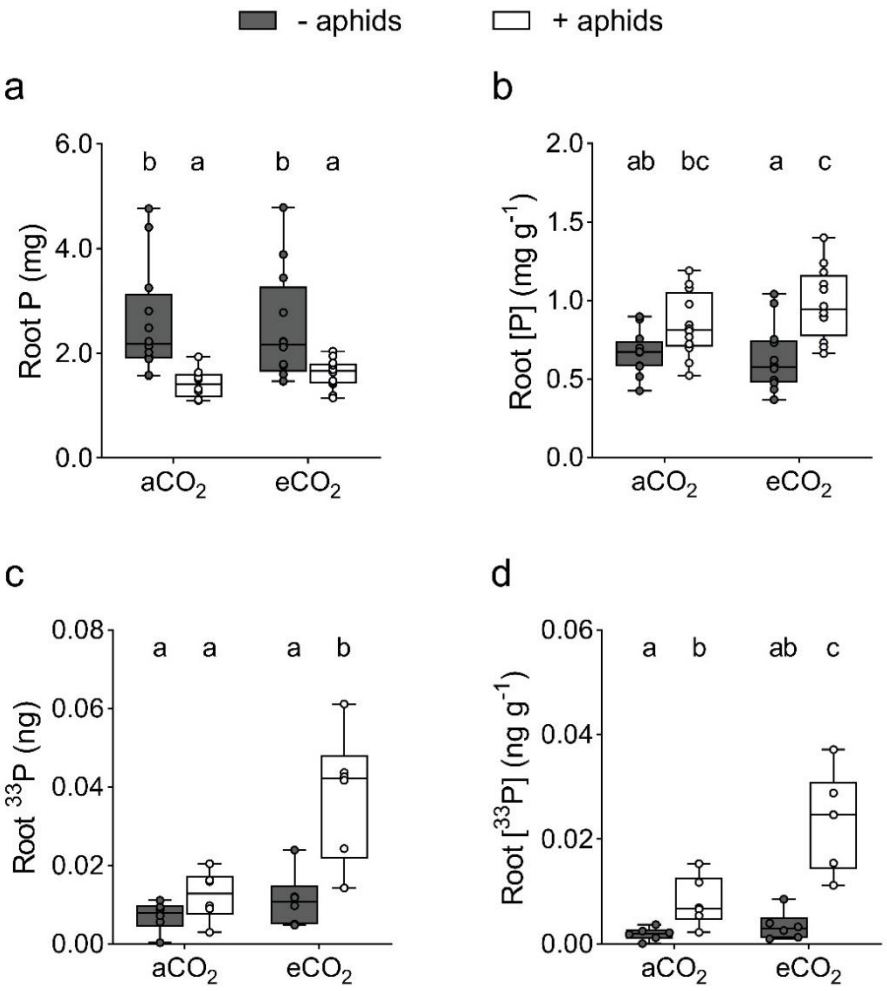

Figure 4.10: P in roots of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Root P; (b) Root [P]; (c) Root ^{33}P; (d) Root [^{33}P]. cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were then exposed to aphids (*R. padi*) (+ aphids, white boxes) or not exposed (- aphids, grey boxes) inside clip cages for 12 days, and ^{33}P was added to a region of substrate accessible only to the fungus. However, as AM fungi are present in the root cortex, all values reflect the sum of P and ^{33}P held in plant and AM fungal material (i.e. plant and fungal P in the root is indistinguishable). Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12 except for panels [c] and [d] where n=6). Different letters denote significant differences between means (where $p < 0.05$, Tukey HSD tests).

Table 4.8: Summary of two-way ANOVA results investigating the effect of aphids, [CO2], and their interaction on plant- and fungal-acquired P in roots of wheat. Significant p-vales are in bold (n=12;12;6;6).

Variable	Factor	cv. Skyfall		
		F	*df*	p
Root P	Aphids	28.72	1,44	**<0.001**
	$[CO_2]$	0.16	1,44	0.687
	Aphids*$[CO_2]$	1.21	1,44	0.278
Root [P]	Aphids	21.14	1,44	**<0.001**
	$[CO_2]$	0.67	1,44	0.416
	Aphids*$[CO_2]$	2.43	1,44	0.126
Root ^{33}P	Aphids	16.28	1,20	**0.001**
	$[CO_2]$	13.38	1,20	**0.002**
	Aphids*$[CO_2]$	4.29	1,20	0.051
Root [^{33}P]	Aphids	47.88	1,20	**<0.001**
	$[CO_2]$	15.02	1,20	**0.001**
	Aphids*$[CO_2]$	6.04	1,20	**0.023**

4.4.7 Plant- and fungal-acquired N

Total shoot nitrogen (N) content acquired by the plant and AM fungus differed significantly according to $[CO_2]$ (Figure 4.11a; Table 4.9), being greater at eCO_2 than aCO_2 regardless of aphid treatment (- aphids: +27%; + aphids: +21%). Aphids did not significantly affect shoot N content of wheat (Table 9), but there was a trend towards reduced total shoot N in plants exposed to aphids under both CO_2 regimes (aCO_2: -5%; eCO_2: -9%). As such, patterns in total shoot nitrogen reflected those of shoot biomass. When controlling for shoot biomass, there was no effect of aphids or $[CO_2]$ on shoot N concentration ([N]) (Figure 4.11b; Table 4.9), which averaged 1.39 ± 0.03 mg g^{-1} across all treatments.

All plants within the 'rotated' core treatment acquired N via the mycorrhizal pathway. Total shoot ^{15}N content of wheat was unaffected by aphids, $[CO_2]$, or the interaction between the two (Figure 4.11c; Table 4.9). Shoot ^{15}N averaged 6.54 ± 0.83 µg across all treatments, although there was a trend for values to be more variable at eCO_2. Likewise, when expressed as a concentration, no effect of aphid herbivory or atmospheric $[CO_2]$ was recorded on shoot [^{15}N] (Figure 4.11d; Table 4.9). Values averaged 3.99 ± 0.48 µg g^{-1} across all replicates, but broadly speaking followed

patterns of ^{33}P uptake with fungal-mediated N uptake being greatest in wheat grown at eCO_2 in the presence of aphids.

3-6% of the ^{15}N isotope administered into the mesh-lined cores was detected in wheat shoot material. Root ^{15}N content and concentration were not determined due to the confounding effect of ^{15}N being held within AM fungal structures inside the root.

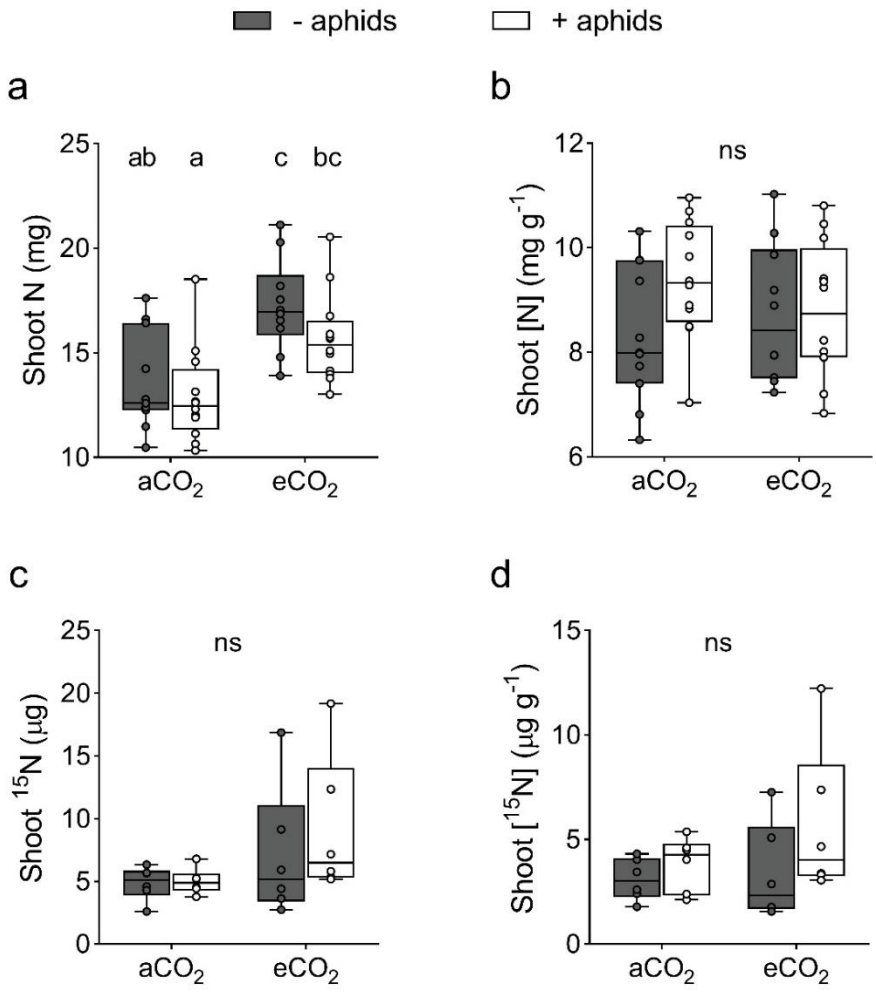

Figure 4.11: Plant- and fungal-acquired N uptake by wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Shoot N; (b) Shoot [N]; (c) Fungal-acquired shoot ^{15}N; (d) Fungal-acquired shoot [^{15}N]. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside clip cages for 12 days, and ^{15}N was added to a region of substrate accessible only to the fungus. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=11 except for panels [c] and [d] where n=6). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).

Table 4.9: Summary of two-way ANOVA results investigating the effect of aphids, [CO_2], and their interaction on plant- and fungal-acquired N in shoots of wheat. Significant p-vales are in bold (n=11,11,6,6).

Variable	Factor	cv. Skyfall		
		F	*df*	p
Shoot N	Aphids	2.68	1,41	0.109
	[CO_2]	22.54	1,41	**<0.001**
	Aphids*[CO_2]	0.46	1,41	0.500
Shoot [N]	Aphids	2.25	1,41	0.142
	[CO_2]	0.09	1,41	0.760
	Aphids*[CO_2]	1.70	1,41	0.199
Shoot ^{15}N	Aphids	0.95	1,20	0.342
	[CO_2]	3.57	1,20	0.073
	Aphids*[CO_2]	0.51	1,20	0.485
Shoot [^{15}N]	Aphids	0.95	1,20	0.073
	[CO_2]	3.57	1,20	0.526
	Aphids*[CO_2]	0.51	1,20	0.388

4.4.8 Correlations

In order to determine if mycorrhizal-mediated P and N uptake by wheat was a function of % root length colonisation, Spearman's rank correlation coefficients were performed between AM fungal abundance in the root and total shoot ^{33}P and ^{15}N. No association was recorded between AM colonisation and the supply of P or N by *Rhizophagus irregularis*, regardless of aphid or [CO_2] treatment (Figure 4.12). Then, in order to evaluate whether the exchange of carbon-for-nutrients between wheat and the AM fungus was tightly coordinated, Spearman's rank correlations were conducted between wheat C outlay and fungal-acquired P and N. No relationship was recorded between plant C transfer to the fungus and mycorrhizal-mediated P or N uptake by plants, in '- aphids' and '+ aphids' hosts under both CO_2 atmospheres (Figure 4.13).

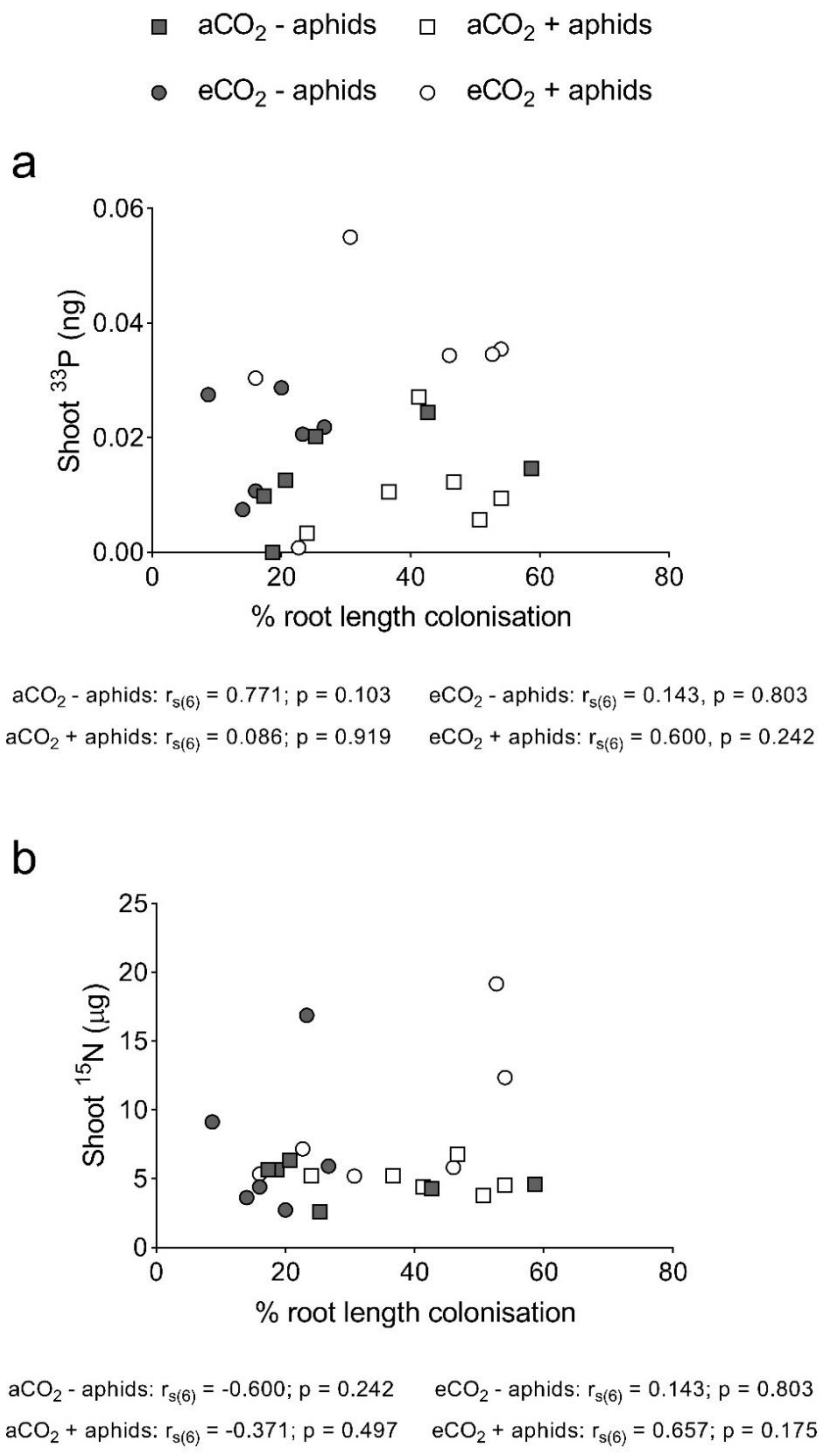

Figure 4.12: Correlations between mycorrhizal-mediated tracer uptake and fungal abundance in roots of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Shoot ^{33}P vs % root length colonisation; (b) Shoot ^{15}N vs % root length colonisation. Correlations were tested using Spearman's rank correlation coefficients

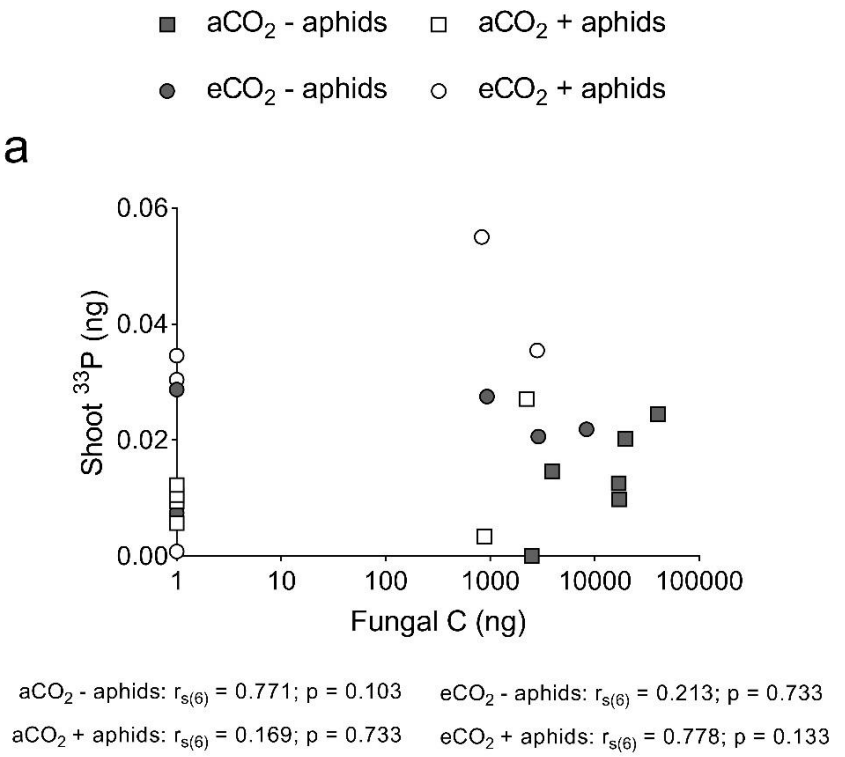

Figure 4.13: Correlations between mycorrhizal-mediated tracer uptake and plant C outlay to the AM fungus by wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Shoot ^{33}P vs fungal C; (b) Shoot ^{15}N vs fungal C. Correlations were tested using Spearman's rank correlation coefficients

4.5 Discussion

The bi-directional exchange of resources between plants and AM fungi is believed to be typical of mycorrhizal symbioses. Plants in all ecosystems interact with multiple organisms simultaneously within a changeable environment (Frew & Price, 2019). Despite this, how biotic and abiotic factors together impact C-for-nutrient exchange between plants and AM fungi has, until now, not been examined. Using a biologically-relevant multi-trophic system, this study explored how altering the availability of plant C resources through aphid herbivory and eCO_2 affects plant C allocation to an AM fungus, and the subsequent supply of P and N from fungus to plant.

Recently-fixed plant C transfer to the AM fungus *R. irregularis* all but ceased in wheat exposed to aphids at aCO_2 and eCO_2, declining by 97% and 73%, respectively. This finding was in-line with the first hypothesis, which predicted an external biotic sink for plant C would limit the availability of photosynthate for AM fungi (Gehring & Whitham, 1994; 2002; Barto & Rillig, 2010). Elevated [CO_2] did not increase plant C delivery to the AM fungus, as recorded in undomesticated plants (Drigo et al., 2010), and failed to restore mycorrhizal-allocated C in plants exposed to aphids. In spite of this powerful biotic effect, uptake of fungal-acquired ^{33}P and ^{15}N by plants was maintained in the presence of aphids, even increasing at eCO_2. This is the first time that above-ground biotic interactions have been shown to influence resource exchange between crops and an AM fungus, and further stresses the context dependent nature of C-for-nutrient exchange between symbionts.

4.5.1 Plant C dynamics: Biotic and abiotic factors

4.5.1.1 An aphid-induced C sink reduces plant C supply to an AM fungus

Exposure to *R. padi* aphids reduced shoot biomass of wheat (Figure 4.5a), in line with previous studies (Reidell et al., 2003). Shoot C concentrations also declined (Figure 4.6a), suggesting that plant C resource availability was lower in the presence of an external biotic C sink. In turn, transfer of recently-fixed plant C to the AM fungus was greatly reduced when plants were exposed to aphids (Figure 4.8a). This discovery supports the first hypothesis, that predicted aphid herbivory would limit the availability, and subsequent delivery, of plant C to AM fungi. Aphids assimilated plant C by feeding on phloem of wheat (Figure 4.4c), and may have manipulated normal plant C transport (Larson & Whitham, 1997) in order to divert C resources to aphid exposed leaves, as

seen previously (Girousse et al., 2003). Moreover, aphids may have further reduced plant C availability by triggering defence-signalling pathways in wheat. The build-up of SA is typical of plants challenged by aphids (Mohase & van der Westhuizen, 2002; Donovan et al., 2012), which when induced may also impact other phytohormones (Ali & Agrawal, 2012). The accumulation of secondary metabolites like benzoxazinoids has also been seen in cereals infested with aphids (Ahmad et al., 2011). These compounds are produced across a range of wheat cultivars (Kowalska & Kowalczyk, 2019), and slow the development of root-feeding pests (Dematheis et al., 2013) and aphids (Meihls et al., 2013). AM colonisation may too induce the production of these anti-herbivore compounds (Walker et al., 2012), which could have further limited plant C resource availability for the fungus.

AMs are obligated to form symbioses with plants in order to acquire organic C compounds required for their continued growth and function (Smith & Read, 2010). Root-internal fungal structures are produced and degraded continuously during the lifespan of the association (Kobae & Hata, 2010; Luginbuehl & Oldroyd, 2017), with turnover of root-external AM fungal hyphae also considered to be constant and rapid (Staddon et al., 2003). Consequently, the degree to which plant roots are colonised by AM fungi, as well as the extent of fugnal hyphal networks in soils, may reflect plant C allocation over longer intervals than the transitory measurements made using radio-isotope techniques (Müller et al., 2017). Nonetheless, when considered in isolation, % root length colonisation by AM fungi may not accurately predict physiological function in plant-mycorrhizal symbioses (Smith et al., 2004; Nagy et al., 2009). While considering this limitation, when plant C availability declines - for instance following aphid herbivory - AM colonisation might be predicted to be reduced (Gehring & Whitham, 1994; 2002; Barto & Rillig, 2010). However, in this study, % root length colonisation by *R. irregularis* increased in roots of plants exposed to aphids at aCO_2 and eCO_2 (Figure 4.7a), as did the frequency of arbuscules (Figure 4.7b), suggesting perhaps increased long-term plant C supply to the AM fungus in the presence of aphids. Variable effects of aphid herbivory have been recorded on % root length colonisation of target plants using the same microscopical methods, being increased, unaffected, or reduced (see Chapter 1 section 1.4.1; Table 1.1). These differences are thought to be driven in part by host-plant genotype, as contrasting outcomes have been recorded even between plants within the same genus (Meier & Hunter, 2018).

Increases in % root length colonisation detected here may have been driven by changes in root biomass, which declined considerably in '+ aphids' plants (Figure 4.5b). While striking, reduced below-ground biomass following aphid exposure corresponds with findings reported previously in spring wheat where infestation (and that of two other Aphididae species) halved root lengths of wheat, which only recovered 4 weeks after herbivore removal (Riedell & Kieckhefer, 1995). Similarly, aphids reduced root biomass of the perennial grass species *Phleum pratense* (Hempel et al., 2009), and mycorrhizal plants of cv. Skyfall in Chapter 3 (see section 3.4.3). Negative effects of aphid herbivory on root growth have been recorded in other plant-aphid pairings (Hosted et al., 2018), highlighting that % root length colonisation may poorly reflect plant-mycorrhizal function within tri-trophic contexts. The use of alternative methods, such as qPCR-based approaches, in quantifying fungal abundance could be of benefit when considering above-ground below-ground interactions (Thonar et al., 2012). That said, there is limited positive correlation between estimates of % root length colonization and qPCR signal strengths, even in simplified systems under controlled conditions (Voříšková et al., 2017). As such, future studies investigating the effect of insect herbivory on plant-mycorrhizal symbioses should - when possible - employ multiple approaches, using isotope tracing techniques in conjunction with cytological and molecular methods rather than relying on these metrics alone to infer changes in plant C allocation.

4.5.1.2 Elevated [CO_2] does not impact fungal C provisioning by wheat

Wheat grown at eCO_2 achieved greater shoot biomass (Figure 4.5a) and shoot C concentrations (Figure 4.6a) than plants grown at aCO_2. As such, more plant C was available at eCO_2 for allocation to *R. irregularis*, which could have compensated for the loss of plant C to the phloem-feeding herbivore. Despite this, recently-fixed plant C transfer to the AM fungus was equivalent across [CO_2] treatments (Figure 4.8a), contrasting hypothesis two which had anticipated greater plant C provisioning at eCO_2 based on previous findings in wild plant-AM fungal symbioses (Drigo et al., 2010; Field et al., 2012). In fact, the proportion of plant-fixed C allocated to *R. irregularis* by wheat was lower than that described previously (Khaschuk et al., 2009; Tomè et al., 2015). Reasons for the modest allocation of recently-fixed plant C to AM fungi could be two-fold. Firstly, this finding may reflect the low mycorrhizal receptivity of modern wheat cultivars compared to both their ancestral relatives (Hetrick et al., 1992; Lehmann et

al., 2012) and undomesticated plants. Strong selection pressures imposed by crop breeders for aerial, yield-related traits, such a plant pathogen resistance and sensitivity to high fertiliser inputs may have - unknowingly - selected against below-ground wheat characteristics through linkage drag (Voss-Fels et al., 2017). As a result, modern varieties may exhibit reduced root biomass (Den Herder et al., 2010), root: shoot ratios (Siddique et al., 1990), and root length densities (White et al., 2015) than older cultivars, and also allocate fewer plant C resources to AM fungi than wild plants even when plant C resources are abundant (Thirkell et al., 2019; Elliott et al., 2020). The second explanation for the small allocation of fungal C could be the duration of the ^{14}C labelling window. Outlay of plant C to the AM fungus was determined after a 16-hour photoperiod, from ^{14}C recovered in the extra-radical hyphal network of *R. irregularis*. Previous studies to have quantified plant C flux to root mutualists over longer intervals have recorded increases in plant C allocation over time (Bever et al., 2009), perhaps accounting for any delay in the drawdown and translocation of plant C from the root to AM fungal network. As such, it is possible that stored plant C resources, which were non-radiolabelled and therefore undetectable, were also initially transferred to the AM fungus during the tracing period, perhaps explaining differences in allocations between studies. That said, large variation exists in the % of recently-fixed plant C supplied to AM fungi when monitored at the same time point (i.e. after 24 hours) (Johnson et al., 2002; Tomè et al., 2015; Thirkell et al., 2019), driven perhaps by the above-mentioned effect of host genotype or differing C demands of AM fungi (Lendenmann et al., 2011).

AM colonisation was reduced in roots of plants grown at eCO_2 compared to plants grown at aCO_2 (Figure 4.7a), perhaps suggesting that longer-term plant C allocation to *R. irregularis* over the duration of plant growth was lower at eCO_2. This contrasts with findings from some natural (Rillig et al., 2000) and experimental systems (Zhu et al., 2016; Jakobsen et al., 2016), with meta-analyses concluding increases of 22% in root-internal and root-external abundances of AM fungi at eCO_2 (Alberton et al., 2005; Compant et al., 2010; Dong et al., 2018). Combined with the ^{14}C tracing data, these results underline that greater plant C resource availability does not necessarily mean increased plant C supply to AM fungi either in the short- or long-term, and may not compensate for an aphid-induced plant C “drain”. Although beyond the scope of this study, investigating whether insect herbivory or [CO_2] affects the allocation of plant C to mycorrhizal fungi across a range of host plants that differ in AM receptivity and

responsiveness is now required. Plants of different functional groups (i.e. grasses, forbs, crops etc.) seemingly vary in the extent to which herbivory limits plant C availability for AM fungi (Barto & Rillig, 2010), so could also differ in the degree to which [CO_2] compensates for this external biotic C sink.

4.5.2 AM fungal-mediated P and N uptake was not linked to plant C allocation

Typically, plants acquire mineral nutrients across the root epidermis as well as via mycorrhizal symbionts. Consequently, AM fungi are usually not the sole means by which plants assimilate soil nutrients, with total shoot P and N content instead equalling the sum of nutrient uptake via the direct and mycorrhizal pathways (see Chapter 1 section 1.2.1). By introducing ^{33}P and ^{15}N to a region of substrate permissible only to extra-radical hyphae of *Rhizophagus irregularis*, wheat P (Figure 4.9c & 4.9d) and N (Figure 4.11c & 4.11d) assimilation via the AM fungus was found to be unchanged by aphid feeding at aCO_2, and increased in the presence of herbivores at eCO_2. Considering exposure to aphids drastically reduced recently-fixed plant C allocation to the AM fungus, these findings together imply that aphids drove asymmetry in carbon-for-nutrient exchange between wheat and *R. irregularis* during the dual-isotope labelling period. Reduced total shoot P (Figure 4.9a) and N content (Figure 4.11a) were rather the result of smaller root biomass of wheat in aphid exposed plants, which limited root assimilation of mineral soil nutrients via the direct pathway.

Mycorrhizal-mediated P and N uptake by wheat was not determined by the extent to which plant roots were occupied by the AM fungus (Figure 4.12), as recorded previously in maize (Sawers et al., 2017). This result adds to the perception that % root length colonisation does not necessarily equate to physiological function (Smith et al., 2004; Nagy et al., 2009). Similarly, mycorrhizal-acquired nutrient uptake was not governed by plant C provisioning of the fungus (Figure 4.13). The breakdown in carbon-for-nutrient exchange between wheat and an AM fungus when exposed to aphid herbivores may infer that resource exchange is not tightly coordinated in complex multi-trophic systems, as proposed in highly-simplified studies (Kiers et al., 2011; Fellbaum et al., 2012). Such a conclusion would lead to the rejection of the fourth hypothesis, which predicted plant C allocation to the AM fungus would determine *R. irregularis*-mediated supply of plant P and N, in line with the reciprocal rewards model (Kiers et al., 2011). On the other hand, the asymmetry in resource

exchange could be driven by the AM fungus having no alternative host to associate with, and thus no secondary source of organic C compounds. In such circumstances, the continued provisioning of mineral nutrients may represent the only viable option for the fungus, as to do otherwise may lessen plant tolerance to herbivory (Maschinski and Whitham, 1989) and compromise the subsequent transfer of plant C. In more biologically-relevant scenarios in which AM fungi form common mycorrhizal networks (CMN) that link roots of multiple hosts simultaneously, preferential nutrient supply by the fungus may be expected based on the source strength of plant C (Merrild et al., 2013; Fellbaum et al., 2014; Weremijewicz et al., 2016). Future work should therefore seek determine the effects of biotic and abiotic factors on carbon-for-nutrient exchanges between neighbouring plants (i.e. alternative C sources) and a CMN, in what would represent more complex but ecologically-realistic systems.

The percentage of ^{33}P and ^{15}N isotopes recovered in shoot tissues of wheat was low compared to 30% of tracer acquired via *Glomus intraradicies* (formerly *Rhizophagus irregularis*) by *Medicago truncatula* (Lendenmann et al., 2011), likely driven by differences in the duration of the labelling period. Isotope tracing experiments provide only a brief insight into carbon-for-nutrient exchanges between plants and AM fungi, with lifetime fitness benefits (Field et al., 2017) and non-nutritional benefits of AM symbioses (Evelin et al., 2009; Chitarra et al., 2016) also likely governing resource exchange between mutualists. Consequently, my findings may not reflect longer-term dynamics in bi-directional resource exchange between mutualists, nor the broader impact of insect herbivory on plant-mycorrhizal functioning across the lifecycle of wheat. That said, this study was conducted during shoot elongation of wheat (Zadoks et al., 1974), allowing for the determination of how aphids and [CO_2] - independently and in combination - impact nutrient trade during an important period of considerable plant-nutrient demand (Weih et al., 2016). Going forward, future investigations should study the effect of sink-source strength dynamics on carbon-for-nutrient exchanges at multiple stages of plant growth, as mycorrhizal functionality could shift along the parasitic-mutualistic continuum (see Chapter 1 section 1.2.5) (Johnson et al.,1997), for example when nutrients are reapportioned to the grain during flowering and ripening (Shewry, 2009).

4.5.3 Aphid impacts

The striking decline in plant C transfer to an AM fungus was recorded under both atmospheric CO_2 concentrations (Figure 4.8a). Recently-fixed plant C assimilation by aphids was the same at aCO_2 and eCO_2 (Figure 4.4c & 4.4d), suggesting aphid feeding behaviour during the ^{14}C labelling period was unchanged. This equivalence in sink strength was recorded despite a 40% increase in the C:N ratio of shoot material at eCO_2 (Figure A1.3 in the appendix), and declining shoot P concentrations (Figure 4.9b). This finding contrasts those of some previous studies which recorded enhanced siphoning of phloem per aphid at eCO_2, in order to compensate for the reduced nutritional quality of target plants (Sun et al., 2009b; Kremer et al., 2018). Three potential reasons exist for the discrepancy in these findings. Firstly, aphids could have manipulated phloem amino acid concentration at eCO_2, as shown previously in other plant-aphid systems (Guo et al., 2013), and therefore negated the need to imbibe more sap at eCO_2. Alternatively, aphid C may not have reflected the external C sink strength of aphids, in that honeydew secretions during the ^{14}C pulse-chase were not collected. Lastly, increased phloem siphoning by the pea aphid (*Acyrthosiphon pisum*) was not recorded on broad bean (*V. faba* L.) at eCO_2 (Boullis et al., 2018), suggesting plant-aphid combination may drive variation in this response. The compensatory feeding response of cereal aphids on wheat at eCO_2 have not before been studied, meaning this finding may be significant for future pest management practices as it suggests that aphid siphoning of plant C resources may be the same at elevated [CO_2].

Given the localised and transient nature of aphid exposure, reduced plant C transfer to the AM fungus reinforces the notion that aphids can have strong and systemic effects on how target plants partition their C resources (Girousse et al., 2003; 2005). Although plant C was seemingly withheld from root mutualists, root C concentrations were greater in plants exposed to aphids, particularly at elevated [CO_2] (Figure 4.6b), suggesting plants were sequestering C resources below-ground in response to herbivory. Specialist pests, such as bird cherry-oat aphids, can resist (Schwachtje et al., 2006) or assimilate defence compounds (Erb & Robert, 2016), meaning plants may opt to tolerate herbivory by redistributing carbohydrates away from the site of defoliation (Holland et al., 1996; Babst et al., 2008). This response is thought to be driven by the down-regulation of specific protein kinases (Schwachtje et al., 2006).

Accordingly, when plants are challenged by root-feeding pests, plant C may be reapportioned above-ground (Newingham et al., 2007; Anten & Pierik, 2010).

eCO_2 had no effect on ^{33}P and ^{15}N uptake via the AM-pathway in the absence of aphids, in accordance with previous work using a range of host plants (Gavito et al., 2003; Jakobsen et al., 2016), including wheat colonised by a mixed AM fungal community (Thirkell et al., 2019). Intriguingly, eCO_2 did increase ^{33}P uptake - and ^{15}N to a lesser extent - in the presence of aphids, emphasising the importance of considering abiotic and biotic factors in combination when investigating plant-AM functioning. This finding was perhaps a result of differing aphid growth rates under contrasting [CO_2] (Figure 4.4a). Phloem-feeders herbivores assimilate not only sucrose but also organic N in the form of amino acids and macroelements such as P from plant (Dinant et al., 2010). Aphid siphoning of P and N has been shown through the use of isotope tracers. For instance, by cultivating barley (*Hordeum vulgare* L.) with a ^{15}N-labelled nutrient solution, Kuhlmann et al., (2013) showed that ^{15}N ingestion by *R. padi* aphids correlated positively with the duration of feeding phases in the phloem, with similar findings recorded previously using ^{32}P (Tjallingii, 1978). As such, the drain on these resources at eCO_2 when aphid growth rates were marginally faster may have necessitated greater nutrient uptake via the AM pathway, particularly as root biomass declined. Recently, it has been shown that cereal-feeding aphids can assimilate mineral nutrients acquired by plants via their mycorrhizal symbionts, as ^{15}N was recovered in English grain aphids (*S. avenae*) feeding on *H. vulgare* L. from a radio-labelled organic patch permissible only to fungal hyphae (Wilkinson et al., 2019). Although not the focus of this study, whether mycorrhizal-acquired P and N is also ultimately siphoned by aphids from wheat phloem may be of interest for future studies.

4.5.4 Conclusions

This study investigated the effect of manipulating plant C sink-source strength dynamics on resource exchange between wheat and an AM fungus. Increasing external C sink strengths by exposing plants to *Rhopalosiphum padi* aphids dramatically reduced recently-fixed plant C allocation to *Rhizophagus irregularis*. Increasing plant C source strength at eCO_2 did not affect plant C transfer to the AM fungus, nor compensate for the aphid-induced decline in plant C outlay. Despite this, mycorrhizal-acquired P and N uptake by wheat was maintained in the presence of aphids at aCO_2, and increased following herbivory at eCO_2. That resource exchange

was found not to be tightly coordinated suggests that symmetrically regulated carbon-for-nutrient exchange in plant-AM symbioses may not be universal, particularly within multi-trophic contexts and when using poorly mycorrhizal-responsive host plants (Walder & van der Heijden, 2015). Results from this experiment provide an insight into how biotic (i.e. aphid herbivory) and abiotic (i.e. atmospheric [CO_2]) drivers can impact carbon-for-nutrient exchange between wheat and an AM fungus, and how multi-trophic interactions may be affected by increases in [CO_2] (Frew & Price, 2019). Future work must now consider the effect of aphids and [CO_2] on carbon-for-nutrient exchange at multiple time-points of plant growth, using alternative host plants that vary in their receptivity to AM fungi, and within more complex social settings.

Chapter 5 Native AM fungi reduce wheat yields regardless of aphid exposure and atmospheric [CO_2]

5.1 Introduction

To date, approximately 250 species of AM fungi have been morphologically described (Öpik et al., 2010; Lee et al., 2013), with recent molecular approaches putatively identifying between 300 (Öpik et al., 2013) and 1,600 taxa (Koljalg et al., 2013). The majority of these AM fungal species are broadly distributed; over 90% of isolates recorded by Davison et al., (2015) were present in soils on multiple continents, with 35% occurring on all continents except Antarctica. As such, AM fungal diversity can be high on a local scale, with plants sometimes interacting with up to 75 species at the same time (van der Heijden et al., 2015). However, as outlined in Chapter 1 section 1.2.4, modern farming practices may reduce AM fungal species richness in farm soils (Helgason et al., 1998; Oehl et al., 2003). Management approaches like high-intensity tillage, long fallow periods, fertilization, and the application of fungicides may select for a “poorly mutualistic” sub-set of AM fungi, which do not participate in resource exchange (Verbruggen & Kiers, 2010). This could be because disruption to extra-radical hyphae results in long periods during which mycorrhizal fungi have reduced access to plant C, which may advantage species that favour reproduction over those that prioritise resource investment in nutrient uptake.

Despite this, AM fungal communities native to farm soils are often more species rich than those used in many laboratory experiments. As per Chapters 2-4 of this thesis, plants grown in the laboratory are typically inoculated with single-strain inoculants of easily cultured AM fungi (García de León et al., 2020), which may readily provide host plants with mineral nutrients. In contrast, farm soils can harbour over 20 species of mycorrhizal fungi (Oehl et al., 2017) which likely differ in their colonisation and nutrient foraging/acquisition strategies (Hart & Reader, 2002; Jansa et al., 2008). How AM fungal diversity impacts plant growth responses following colonisation can vary, with species either competing with or complementing one another depending on composition (Thonar et al., 2014). Growth responses of plants to mixtures of AM fungi may react additively with each extra strain (van de Heijden et al., 1998), or reflect the average symbiotic function of all species in the community, or be driven by the presence of dominant isolates (Bennett & Bever, 2007). Plant growth responses may be more positive when inoculated with complex AM fungal communities than simplified

ones (Hoeksema et al., 2010), as colonisation by several mycorrhizal species can improve resource exchange efficiency (Argüello et al., 2016). On the other hand, less positive growth responses have been recorded in crops colonised by multiple fungal isolates than when inoculated with one (van Geel et al., 2016), as diverse assemblages may enable less mutualistic symbionts to persist in roots (Hart et al., 2013). Lastly, Bennett & Bever (2007) found that dominant fungal taxa, such as *Glomus* species, may determine growth responses of plants inoculated with mixed AM fungal communities. Dominant species may be those that colonise the roots of plants first, thus restricting the niche space available to other isolates that later arrive at the root (Werner & Kiers, 2015). Alternatively, dominant taxa could be more capable of acquiring plant C from their hosts, either through more optimal growth strategies or as a result of higher affinity sucrose transporters (Cotton et al., 2015).

Soils used in laboratory and field experiments also differ in terms of the wider microbial communities they host. In order to create comparable AMF and non-AMF treatments in the laboratory, plants are typically grown using inert substrates (e.g. sand: perlite mixes, as per Chapters 2-4) or soils that have been sterilised, both of which are devoid of all other soil microorganisms. This is noteworthy because certain components of native soil microbial communities may suppress or facilitate plant-AM function. For instance, bacterial and fungal taxa have been shown to impede mycorrhizal-mediated ^{33}P uptake by *Medicago truncatula* in cultivated (Svenningsen et al., 2018) and natural soils (Cruz-Paredes et al., 2019). However, *Streptomyces* species can facilitate AM fungal-acquired ^{33}P uptake in maize (Battini et al., 2017), with synergies between AM fungi and free-living microorganisms also shown to drive non-additive increases in N uptake in other grasses (Hestrin et al., 2019).

A recent meta-analysis by Zhang et al., (2018) suggested that the positive effects of AM colonisation on wheat yields may be less pronounced in field experiments than those in the laboratory. This finding may be due to the aforementioned presence of less nutritionally beneficial AM fungi in farm soils, or competition between mycorrhizal species, or AM fungal-suppressive components of the wider soil microbiome (Ryan & Graham, 2018). However, evidence from field studies is lacking, due to limited access to existing long-term trails (Lekberg & Helgason, 2018) and difficulties associated with creating control treatments in the field (Gryndler et al., 2018). As such, more studies are now required which investigate crop-mycorrhizal interactions using farm soils

containing native AM fungi and their wider microbial communities (García de León et al., 2020; Frew, 2020).

The same is true of experiments involving AM fungal-plant-aphid interactions. The non-nutritional benefits afforded to plants by AM fungi also perhaps depend on mycorrhizal identity and diversity (Powell & Rillig, 2018). AM fungi differ in their ability to alter plant tolerance of drought stress (Grümberg et al., 2015), pathogen infection (Sikes et al., 2009; Wehner et al., 2010), and herbivory (Bennett & Bever, 2007). Fungal identity may too determine how colonisation impacts aphid performance (see Chapter 1 section 1.4.2; Table 1.2); *Gigaspora margarita* increased the abundance of bird cherry-oat aphids on wheat (cv. Pioneer '26R22') relative to control plants, but no effect was recorded when inoculated with *Glomus intraradices* (formerly *R. irregularis*) (Abdelkarim et al., 2011). To date, how colonisation of wheat by AM fungi native to farm soils impacts wheat tolerance of aphids (this being the capacity of plants to maintain or increase yields despite herbivory [Girvin et al., 2017]) and aphid abundance remains unstudied. Considered from the 'top-down' perspective, how aphids affect AM colonisation may also depend on the identity or diversity of AM fungi occupying roots of plants (see Chapter 1 section 1.4.1; Table 1.1). For example, aphid siphoning of plant C reduced AM infection of broad bean (*V. faba* L.) when colonised by mixed AM fungi (Babikova et al., 2014a), but no effect was recorded when inoculated with *R. irregularis* alone (Cabral et al., 2018). This finding could mean certain mycorrhizal fungi are more tolerant of insect feeding than others (Gehring & Bennett, 2009). However, this aspect of mycorrhizal ecology is poorly understood; evidence from other AM fungal-plant-insect systems suggests the destabilising effect of herbivory on AM colonisation is less pronounced in plants associated with multiple AM fungi than single taxa (Bennett & Bever, 2009). Recently, no effect of grain aphids was recorded on AM colonisation of barley grown with wild AM fungi (Wilkinson et al., 2019). Crucially, no such study has been conducted in wheat, despite mycorrhizal fungi and aphids like *R. padi* being universal in food production systems.

Abiotic conditions, such as elevated [CO_2] (eCO_2), may alleviate plant C limitation cause by aphids by increasing C fixation and ultimately the supply photosynthate to AM fungi (Drigo et al., 2010; 2013; Field et al., 2012). However, eCO_2 has been shown to differentially benefit AM fungal species, favouring taxa capable of exploiting greater plant C supply (e.g. Glomeraceae species) at the expense of isolates which cannot

(e.g. Gigasporaceae species) (Cotton et al., 2015). As such, the effect of [CO_2] on plant growth responses to AM infection may also depend on fungal identity/diversity.

The individual effects of [CO_2] (a plant C source) and aphids (a plant C sink) on the growth and nutritional response of wheat to colonisation by the AM fungus *R. irregularis* were investigated in Chapters 2 and 3, using an inert and otherwise sterilised substrate. The effect of [CO_2] and aphids together on wheat-AM function was investigated using similar experimental conditions (Chapter 4). Here, the added complexity of a mixed AM fungal community native to farm soils is introduced to this tri-trophic interaction, alongside an intact microbial assemblage. This experiment investigated how field-collected AM fungi impact wheat yield when grown in the presence and absence of a cereal-feeding aphid (*R. padi*) at ambient and elevated [CO_2], in line with future climate change projections for 2100 (IPCC, 2014).

5.2 Key questions and hypotheses

- Do field-collected AM fungi improve wheat grain yield and nutrition?
  - Hypothesis 1: Wheat growth responses to wild AM fungi are hypothesised to be less positive than those recorded in Chapters 2 and 3, in which wheat was inoculated with a laboratory-cultured fungal species without a wider microbiome. This is because complex mycorrhizal communities native to farm soils may harbour less mutualistic taxa (Verbruggen & Kiers, 2010; Hart et al., 2013) and/or bacteria or fungi which suppress symbiotic function (Svenningsen et al., 2018). Alternatively, wheat growth responses may be more positive if AM fungal diversity increases resource exchange efficiency (Argüello et al., 2016) and/or the soil microbiome facilitates plant-AM function (Battini et al., 2017).
- Do aphids effect AM colonisation of wheat inoculated with a wild AM fungal community, and is this effect mitigated at eCO_2?
  - Hypothesis 2: Exposure to aphids is hypothesised to reduce AM colonisation of wheat. This is because evidence from other AM fungal-plant-aphid systems suggests that aphids may disrupt % root length colonisation to a greater extent when plants are colonised by a mixture of AM fungi (Babikova et al., 2014) than when grown with single strains (Cabral et al., 2018). This may be due to certain species being competitively excluded by others once plant C becomes limited. eCO_2 is not hypothesised to mitigate the effect of aphids on AM colonisation, as

plant C transfer to an AM fungus did not increase at eCO_2 in Chapter 4, nor in a recent study growing wheat with field-collected AM fungi (Thirkell et al., 2019).

- Do aphids and [CO_2] effect wheat yield responses to native AM fungi?
- Do AM fungi native to farm soils impact wheat tolerance of aphids and their abundance, at ambient and elevated [CO_2]?
    - Hypothesis 4: AM colonisation of cv. Skyfall is hypothesised to improve wheat tolerance against *R. padi* aphids, but not impact aphid abundance as seen in Chapter 3. However, plant tolerance and aphid fitness may respond differently to colonisation by resident AM fungi than a single-strain inoculum, as AM fungal genotype can determine these 'bottom-up' impacts (Bennet & Bever, 2007; Abdelkarim et al., 2011). Aphid growth rates and abundance may be greater on AM plants if colonisation increases plant nutrient status (Hartley & Gange, 2009). *R. padi* abundance is hypothesised to be greater on plants grown at eCO_2, as growth rates of *R. padi* aphids were found to be faster at elevated [CO_2] in Chapter 4 and in previous studies (Sun et al., 2009a).

5.3 Materials and Methods

5.3.1 Plant material

Wheat seeds of cv. Skyfall were supplied by RAGT Seeds Ltd.. cv. Skyfall was selected as the host plant for this experiment because aphid herbivory was shown in Chapter 4 to alter carbon-for-nutrient exchange between this variety and an AM fungus at ambient and elevated [CO_2] (Charters et al., 2020). Seeds were sterilised and germinated as described in Chapter 3 section 3.3.1. 48 seedlings (48 plants, n=6) were potted up in 4.5” pots in substrate consisting of farm soil (see section 5.3.2), sterilised sand, and perlite in a 4:3:1 ratio.

5.3.2 Soil collection, sterilisation, and AM fungal material

Farm soil was collected from Leeds University Farm (53°52'30.5"N 1°19'16.5”W) on August 7th 2018. Soil physical, chemical, and biological properties, as well as management practices on site (i.e. crop rotations, tilling, fertilisation, and fungicide applications) as described by Holden et al., (2019).

Soil was dried at room temperature and passed through a 3.35 mm sieve. Half of the farm soil, containing its native AM fungal community and wider microbial assemblage, was used to create plants in the ‘+ AMF’ treatment (24, n=6). An AM fungal inoculum of *Rhizophagus irregularis* (Schenck & Smith, 2009) was added to the substrate to ensure successful colonisation of plant roots (Köhl et al., 2016). This supplementary inoculum was cultured as described in Chapter 2 section 2.3.2. Eight 20 cm Petri dishes of *R. irregularis* were blended with 160 mL dH_2O, and 15 mL of inoculum containing 16,200 spores was mixed into the substrate of each mycorrhizal plant.

Remaining farm soil used for plants in the ‘- AMF’ treatment (24, n=6) was sterilised using the Cobalt-60 gamma irradiator (Model 812 Research Irradiator, FTS Inc., USA) at the University of Manchester’s Dalton Nuclear Institute. Soil was irradiated on a turntable receiving an evenly distributed absorbed dose of 50-74 kGy, shown previously to eliminate even the most radio-resistant soil microorganisms (McNamara et al., 2003; Buchan et al., 2012). In order to reinstate the wider microbial community removed during sterilisation (i.e. bacteria and non-mycorrhizal fungal species), a microbial wash was performed. 10 g of unsterilised sieved farm soil was stirred for 5 minutes in 1 L of phosphate-buffered saline (PBS) adjusted to 7.4 pH, which contained

8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 dissolved in dH_2O. Sediment in solution was allowed to settle for 7 minutes before the PBS was filtered through a Whatman No. 1 filter paper. The 11 um pore size blocked/retained spores of AM fungi which range in diameter from 30-120 µm (Marleau et al., 2011), but not bacteria or propagules of other fungal species which can be far smaller (Christensen et al., 1999; Aguilar-Trigueros et al., 2019). 30 mL of the resulting wash was watered into the substrate of plants in the '- AMF' treatment in order to reintroduce the broader microbial assemblage, while plants in the '+ AMF' treatment were watered with equal volumes of dH_2O. In order to control for the effect of adding the supplementary *R. irregularis* inoculum, 15 mL carrot root-only inoculum was mixed evenly throughout the irradiated substrate of plants in the '- AMF' treatment.

The rationale behind this method of sterilisation was that a preliminary experiment in which all farm soil was autoclaved at 121˚C and then half re-inoculated with an AM fungus had been unsuccessful, in that wheat grown in this '+ AMF' treatment failed to establish AM symbioses. Changes to the substrate's structure (Lees et al., 2018), dissolved organic C content (Berns et al., 2008), and heavy metal concentrations (Williams-Linera & Ewel, 1984) following heating are hypothesised to have impeded the development of the association. In contrast, gamma irradiation is thought to be less disruptive to the physical and chemical properties of soil than autoclaving or fumigation (Lees et al., 2018), and thus was considered preferable.

5.3.3 Plant growth conditions and aphid exposure

Plants were grown inside 4 insect rearing cages within two separate controlled environmental growth chambers at the University of Leeds, maintained at 440 ppm ('aCO_2'; 24, n=6) or 800 ppm ('eCO_2'; 24, n=6) atmospheric [CO_2]. Other environmental parameters (i.e. temperature, relative humidity, and light intensity) were identical to those described in Chapter 4 section 4.3.3. Plants were watered with tap water when required, and fed with 40% nitrate-type Long Ashton Solution between weeks 7 and 12 having shown mild symptoms of nutrient depletion.

After 8 weeks of growth, one insect clip cage (see Chapter 4, Figure 4.2b) was affixed to leaves of all plants, as described in Chapter 4 section 4.3.5. 50% of plants, hereafter termed the '+ aphids' treatment (24, n=6), were exposed to five winged bird cherry-oat aphids (*Rhopalosiphum padi*). Aphids were moved with care from culture plants (which

had been maintained as described in Chapter 3 section 3.3.4) onto the third leaf on the primary tiller of each '+ aphids' replicate, and insect clip cages were placed on top. Remaining plants, hereafter termed the '- aphids' treatment (24, n=6), did not have aphids added to their insect clip cages. Cages were kept raised above the soil surface in order to prevent damage to the plant. Aphid exposed and aphid unexposed plants were grown between different dates in order to ensure volatile organic compounds (VOCs) induced by *R. padi* feeding did not impact plant-fungal interactions within the '- aphids' treatment.

As aphid growth rates on wheat can be impacted by AM fungi (Abdelkarim et al., 2011) and atmospheric [CO_2] (e.g. Sun et al., 2009a), abundance counts were performed at five time points during the 14-day exposure period. The final count was conducted on day 14 before insect clip cages and aphids were removed from all plants. Aphid abundance was plotted against time for each replicate and exponential trend lines were fitted (see Figure 5.1 for an example growth curve). Growth rates were derived from the equation of the trend line for each plant (Equation 1).

In order to investigate the effect of aphid feeding on AM colonisation, roots were sampled following the removal of clip cages (i.e. 10 weeks after planting) by inserting a 10 mm core borer into the substrate. Subsequent clearing and staining (see section 5.3.4) showed that AM fungi were present in roots of plants in the '+ AMF' treatment and absent in those of '- AMF' replicates. Extracted substrate was replaced with autoclaved sand, and plants were grown to yield. As per Chapter 2 section 2.3.3, watering frequencies were slowly reduced from week 15 until yield to avoid waterlogging of the substrate and a build-up of soil pathogens.

$$A = A_0 e^{(rt)}$$

Equation 1: Where A = aphid abundance, A_0 = starting aphid abundance (in this case 5), *e* = Euler's number (2.71828), r = growth rate, and t = time (in days).

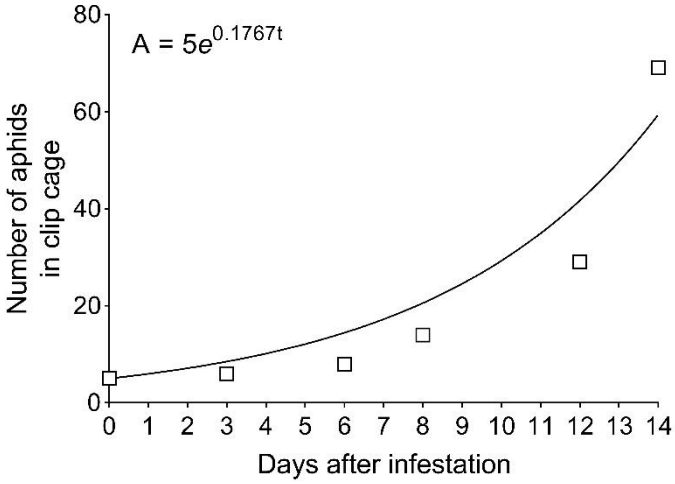

Figure 5.1: An example growth curve of bird cherry-oat aphids on one mycorrhizal wheat plant grown at ambient [CO_2]. Abundance counts were conducted roughly every 72 hours and plotted over time, and exponential trend lines were fitted.

The rationale behind this mode of aphid exposure was three-fold. Firstly, although localised, such an exposure was found previously to alter carbon-for-nutrient exchange between wheat and an arbuscular mycorrhizal fungus, as presented in Chapter 4. This finding suggests that a profound and possibly systemic response occurs in wheat fed upon by aphids, removing the need for aphid infestation on a larger scale. Secondly, a more wholesale exposure to aphids akin to that conducted in Chapter 3 may have risked the failure of plants before they achieved yield. To this end, plants were checked every 48 hours after the removal of aphids to ensure that no insects remained. Finally, the controlled environment chambers used to maintain aCO_2 and eCO_2 were communal. Consequently, two protective measures were used at all times, these being insect clip cages *and* insect rearing cages, in order to avoid an outbreak of aphids.

5.3.4 Harvest procedure and grain P and N analysis

All plants were destructively harvested at yield, approximately 18 weeks after germinated seedlings were sown and 8 weeks after insect clip cages had been removed. Above-ground material was separated into stems, ears, and grain. Fresh weight measurements were taken of each aerial component using a 3-digit digital scale, and plant material was stored at -20˚C overnight and freeze dried for at least 72

hours. Dry weights were recorded using a 5-digit digital scale, and grain material was homogenised using a mill. Grain P and N status was determined using colourimetry of acid digest samples, as described in Chapter 2 (see sections 5.3.9 and 5.3.10). AM colonisation of roots collected when insect clip cages were removed (i.e. after 10 weeks growth) was quantified following root clearing and staining using the gridline intersection methodology described in Chapter 2, section 2.3.5.

5.3.5 Statistical analyses

All data analyses were conducted using R Studio v1.1.453. The normality and uniformity of variances within each data set were assessed using conventional Q-Q and residuals vs fitted plots. Shapiro-Wilk tests, skewness tests, and kurtosis tests were used when visual assessments were equivocal, and data was transformed before analysis if results indicated that test assumptions were not met. The effects of AMF, [CO_2], and their interaction on aphid growth rate and aphid abundance on days 6, 8, and 12 were tested using a two-way ANOVA (GLM) with additional post hoc Tukey HSD tests. Aphid abundance on days 3 and 14 were Log10 transformed and analysed using a GLM. Three-way ANOVAs (GLM) were performed investigating the effect of AMF, aphids, [CO_2], and their interactions on grain P and grain [P]. Given that exponential growth rates appeared to diverge half way though the exposure period resulting in significantly different final aphid abundances across treatments (see section 5.4.1), a continuous explanatory variable was used in the statistical model for the *Aphid* factor. Exponential aphid growth rates on each plant replaced the categorical (i.e. two level) approach favoured in Chapters 3 and 4, thereby controlling for any potential confounding effect of differing herbivore pressure on mycorrhizal plants or at eCO_2. Grain number, grain biomass, grain N, and grain [N] were Log_{10} transformed before analysis using GLM. The effect of aphids, [CO_2], and their interaction was tested on total root length colonisation, arbuscular colonisation, and vesicular colonisation. In order to study the effect of AM colonisation by field-collected AM fungi on grain nutrient status, and in turn grain nutrient status on aphid fitness, Spearman's rank correlation coefficients were performed between % root length colonisation or % arbuscules and grain P/N, and grain P/N and aphid performance, in a partial test of hypotheses one and four, repsectively. All figures were produced using GraphPad Prism v8.2.0.

5.4 Results

5.4.1 Aphids

Aphid growth rates did not differ significantly when feeding upon mycorrhizal and non-mycorrhizal wheat plants grown at ambient or elevated atmopsheric [CO_2] (Table 5.1). However, growth rates diverged after day 8 (Figure 5.2a), with aphid numbers on '- AMF' plants at eCO_2 being lower than those of the other three treatments. Final aphid abundance on day 14 was significantly affected by AMF and [CO_2] individually (Figure 5.2b; Table 5.1). Lowest aphid numbers of 18 ± 1.5 per clip cage were recorded on non-mycorrhizal plants of cv. Skyfall at eCO_2, while aphid abundance was highest (38 ± 7.3) on '+ AMF' plants at aCO_2.

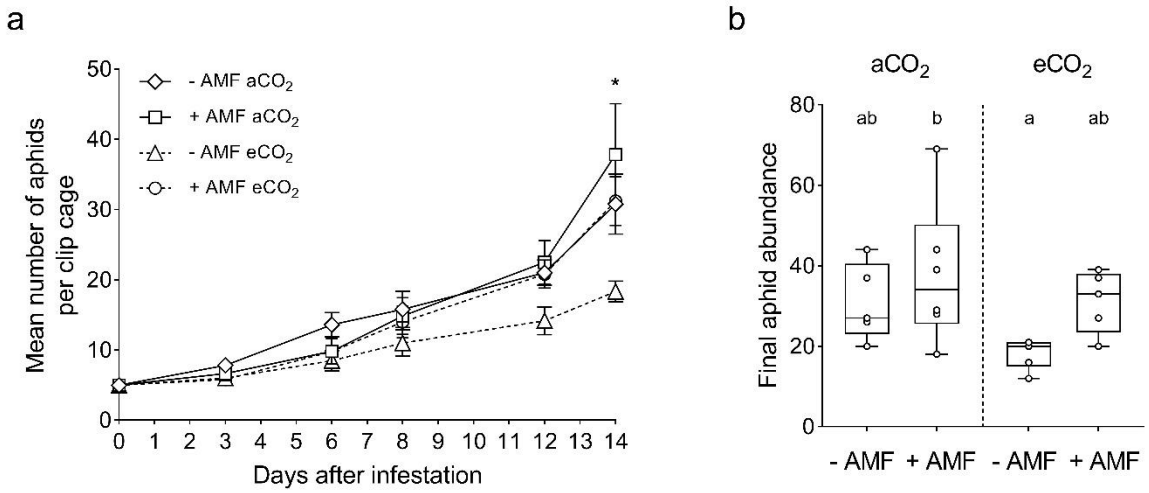

Figure 5.2: Aphid abundance on mycorrhizal and non-mycorrhizal wheat grown at ambient and elevated [CO_2]. (a) Number of aphids per clip cage; (b) Final aphid abundance. cv. Skyfall was grown in farm soil with native AM fungi and an added inoculum of *R. irregularis* (+ AMF), or a sterilise version of the substrate with the wider microbiome reintroduced (- AMF). Plants were grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks, and exposed to bird cherry-oat aphids (*R. padi*) in clip cages for 14 days. For panel (a), each marker represents the mean (± SE) of 6 replicates. For panel (b), boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers encompass all data points (open markers, n=6). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).

Table 5.1: Summary of two-way ANOVA results investigating the effect of AMF, [CO_2], and their interaction on aphid performance on wheat. Significant p-values are in bold (n=6).

	Aphid growth rate			Final aphid		
	F	*df*	p	F	*df*	p
AMF	4.09	1,18	0.058	6.78	1,18	**0.018**
[CO_2]	3.90	1,18	0.064	4.95	1,18	**0.039**
AMF*[CO_2]	1.68	1,18	0.211	1.66	1,18	0.213

5.4.2 AM colonisation

No effect of atmospheric [CO_2] was recorded on % root length colonisation of wheat grown with a resident AM fungal community (Figure 5.3a; Table 5.2). Instead, roots of plants exposed to aphids were significantly less colonised by mycorrhizal fungi than plants which were not infested with aphids (Table 5.2). AM colonisation averaged 68.7% in '- aphids' plants, but fell to 31.8% in '+ aphids' replicates. In contrast, the effect of [CO_2] on % arbuscules differed in the presence and absence of aphids, resulting in a significant interaction between the two factors (Figure 5.3b; Table 5.2). Within the '- aphids' treatment, arbuscule frequencies were greater at eCO_2 (36.1%) than at aCO_2 (22.2%). However, no stimulatory effect of [CO_2] was recorded on arbuscular colonisation in plants exposed to aphids, which averaged 17.8%. As with total root length colonisation, % vesicles were significantly reduced in plants infested with aphids compared to those not subjected to herbivory (Figure 5.3c; Table 5.2). Vesicle frequencies in roots of wheat were unaffected by [CO_2] (Table 5.2).

Table 5.2: Summary of two-way ANOVA results investigating the effect of [CO_2], aphids, and their interaction on AM colonisation wheat. Significant p-values are in bold (n=6).

	% root length			% arbuscules			% vesicles		
	F	*df*	p	F	*df*	p	F	*df*	p
[CO_2]	0.01	1,18	0.934	4.84	1,18	**0.041**	1.20	1,18	0.288
Aphids	54.47	1,18	**<0.001**	13.25	1,18	**0.002**	13.36	1,18	**0.002**
[CO_2]*Aphids	2.30	1,18	0.147	9.17	1,18	**0.007**	0.01	1,18	0.923

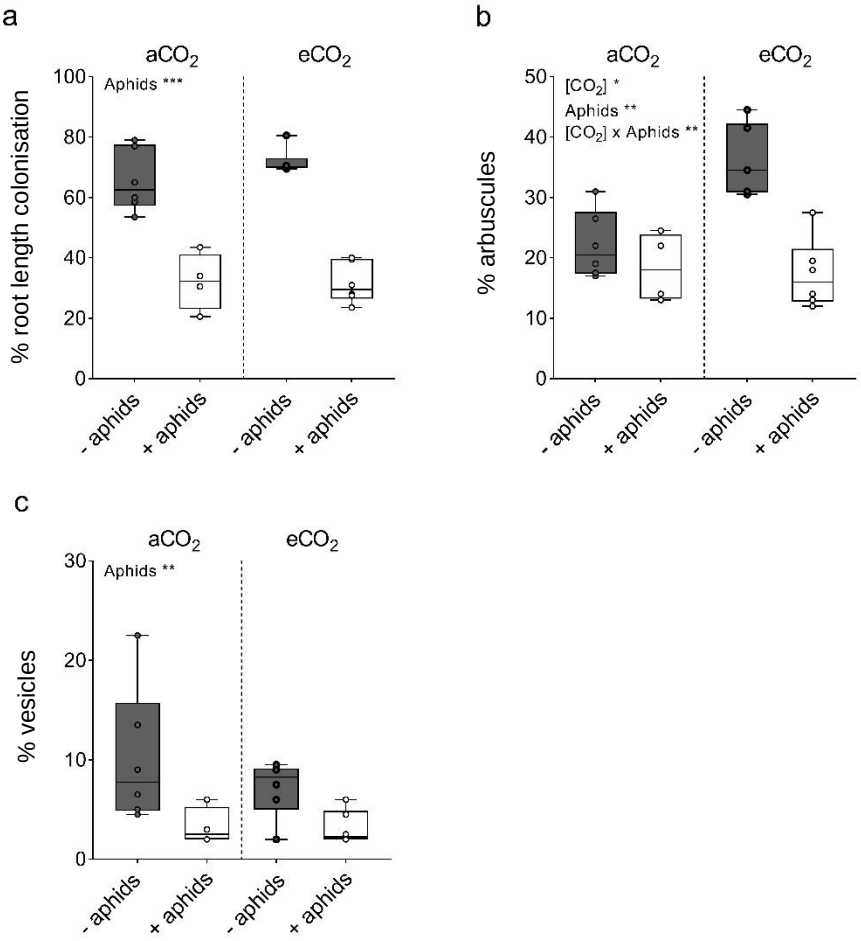

Figure 5.3: AM fungal abundance in roots of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) % root length colonisation; (b) % arbuscules; (c) % vesicles. cv. Skyfall was grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) in farm soil with native AM fungi and an added inoculum of *R. irregularis*. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) between weeks 8 and 10, and roots sampled using a core borer following the removal of aphids. Boxes cover the interquartile range. Median values are denoted by middle lines, and whiskers encompass all data points (closed or open markers, n=6). Asterisks indicate significant factors in the GLM, where p = 0.05 (*), <0.01 (**), <0.001 (***).

5.4.3 Plant growth

Wheat grown in the '+ AMF' treatment produced significantly fewer grains than plants not inoculated with native AM fungi (Figure 5.4a; Table 5.3). This was true regardless of whether cv. Skyfall was infested with aphids or grown at aCO_2 or eCO_2. No effect of [CO_2] or aphids was recorded individually on grain number, but a significant

interactive effect was recorded between [CO_2] and aphid on grain number (Table 5.3). In general, the number of grain produced by wheat was greater in plants exposed to aphids than those which were not, although this effect was more pronounced at eCO_2 (- AMF: +44.8%; + AMF: +50%) than it was at aCO_2 (- AMF: +21%; - AMF: -21.4%).

Grain biomass of plants of cv. Skyfall was also significantly affected by AMF, being reduced in plants colonised by a resident AM fungal community (Figure 5.4b; Table 5.3). A significant interaction between [CO_2] and aphids was also recorded for grain biomass (Table 5.3). At aCO_2, aphids reduced grain biomass whether plants were mycorrhizal or not (- AMF: -30.6%; + AMF: -37.2%). In contrast, at eCO_2, aphids had little effect on grain biomass of '- AMF' plants (-4.2%) but increased dry grain weight of replicates within the '+ AMF' treatment (+24.4%). eCO_2 increased biomass of wheat grains, but to a greater extent in '+ aphids' plants than those not exposed to aphids.

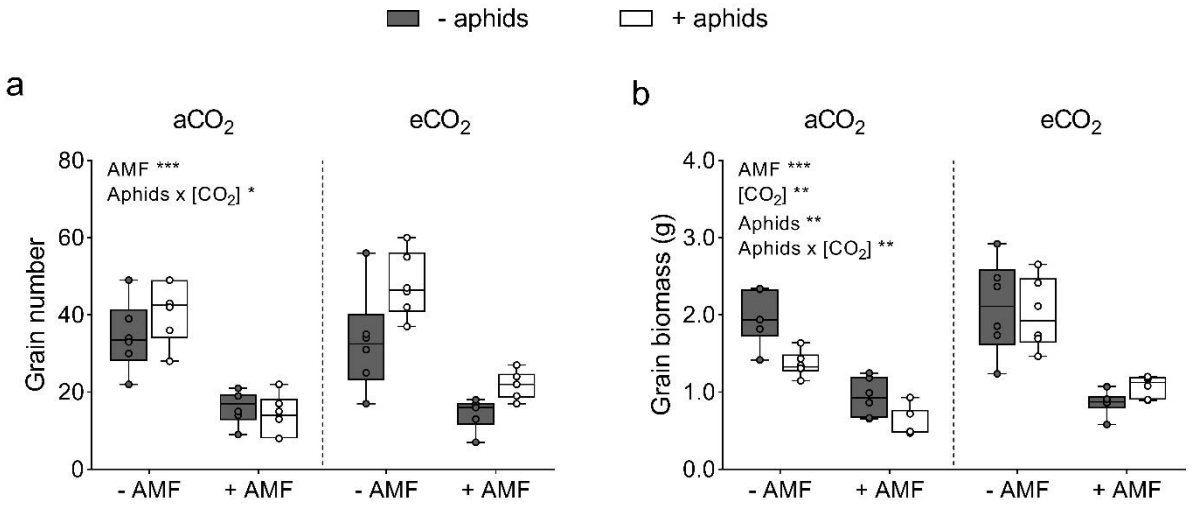

Figure 5.4: Yield of non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Grain number; (B) Grain biomass. cv. Skyfall was grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) in farm soil with native AM fungi and *R. irregularis* (+ AMF) or in sterilised soil with the wider microbiome reintroduced (- AMF). Plants were exposed to aphids (*R. padi*) (+ aphids, white bars) or not (- aphids, grey bars) between weeks 8-10. Boxes cover the interquartile range. Median values are represented by middle lines, and whiskers encompass all data points (closed or open markers, n=6). Asterisks indicate significant factors in the GLM, where p = 0.05 (*), <0.01 (**), <0.001 (***).

Table 5.3: Summary of three-way ANOVA results testing the effect of AMF, [CO_2], aphids, and their interactions on grain yield of wheat. Significant P-values are in bold (n=6).

	Grain number			Grain biomass		
	F	*df*	p	F	*df*	p
AMF	91.00	1,40	**<0.001**	128.40	1,40	**<0.001**
[CO_2]	1.66	1,40	0.204	12.46	1,40	**0.001**
Aphids	3.08	1,40	0.087	7.37	1,40	**0.010**
AMF*[CO_2]	0.78	1,40	0.384	0.24	1,40	0.629
AMF*Aphids	0.50	1,40	0.485	0.69	1,40	0.412
[CO_2]*Aphids	5.03	1,40	**0.031**	8.33	1,40	**0.006**
AMF*[CO_2]*Aphids	0.51	1,40	0.478	1.05	1,40	0.312

5.4.4 Grain P

Grain P was lower in plants of cv. Skyfall inoculated with field-collected AM fungi than in plants which were not (Figure 5.5a). Grain P was also lower in plants exposed to aphids than in those which were not, although this was not true of '+ AMF' plants grown at eCO_2, resulting in a significant interaction between AMF and aphid treatments (Table 5.4). A significant effect of [CO_2] was also recorded individually, as grain P was increased at eCO_2 particularly within '+ aphids' plants (- AMF: +15.6%; + AMF: +56.4%), reflecting patterns in grain biomass.

When controlling for biomass, grain P concentration ([P]) was unaffected by AMF treatment (Figure 5.5b; Table 5.4). A significant interaction was recorded between [CO_2] and aphids, however (Table 5.4). No effect of eCO_2 was recorded on grain [P] of plants grown in the absence of aphids, regardless of AMF treatment. The same was not true cv. Skyfall plants exposed to aphids, as eCO_2 reduced grain [P] by 20.6% and 13.7% in '- AMF' and '+ AMF' plants, respectively.

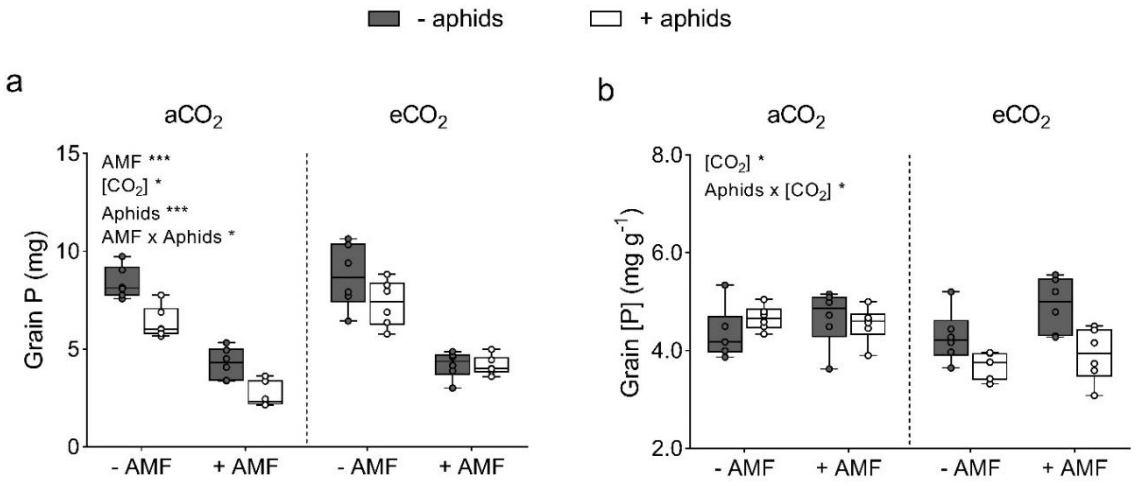

Figure 5.5: Grain P status of non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Grain P; (B) Grain [P]. Skyfall was grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) in farm soil with native AM fungi and *R. irregularis* (+ AMF) or in sterilised soil with the wider microbiome reintroduced (- AMF). Plants were exposed to aphids (*R. padi*) (+ aphids, white bars) or not (- aphids, grey bars) between weeks 8-10. Boxes cover the interquartile range. Median values are represented by middle lines, and whiskers encompass all data points (closed or open markers, n=6). Asterisks indicate significant factors in the GLM, where p = 0.05 (*), <0.01 (**), <0.001 (***).

Table 5.4: Summary of three-way ANOVA results testing the effect of AMF, [CO_2], aphids, and their interactions on grain P status of wheat. Significant P-values are in bold (n=6).

	Grain P			Grain [P]		
	F	*df*	p	F	*df*	p
AMF	200.75	1,40	**<0.001**	3.37	1,40	0.074
[CO_2]	6.33	1,40	**0.016**	5.68	1,40	**0.022**
Aphids	22.34	1,40	**<0.001**	2.71	1,40	0.108
AMF*[CO_2]	0.01	1,40	0.941	1.23	1,40	0.274
AMF*Aphids	4.40	1,40	**0.042**	1.48	1,40	0.231
[CO_2]*Aphids	1.31	1,40	0.260	7.30	1,40	**0.010**
AMF*[CO_2]*Aphids	0.96	1,40	0.333	0.02	1,40	0.888

5.4.5 Grain N

As per grain P, a significant interaction was recorded between AMF and aphid treatment for grain N (Figure 5.6a; Table 5.5). Grain N was lower in plants inoculated with an AM fungal community native to farm soils than in plants not colonised by mycorrhizas. Likewise, in general, grain N was reduced in wheat plans exposed to

aphids that in plants which were not, although the degree to which aphids reduced grain N varied between '- AMF' plants (aCO_2: -35.3%; eCO_2: -14.8%) and '+ AMF' plants (aCO_2: -20.7%; eCO_2: -3.2%). [CO_2] significantly impacted grain N individually (Table 5.5), increasing grain N content in '+ aphids' replicates (- AMF: +24.4%; + AMF: +22.8%) reflecting patterns in grain biomass.

A significant effect of AMF treatment was recorded on grain N concentration ([N]), as plants inoculated with the wild mycorrhizal inoculum achieved higher grain [N] (Figure 5.6b; Table 5.5). As per grain [P], grain N concentration was significantly affected by the interaction between [CO_2] and aphid treatment (Table 5.5). Grain [N] of plants not exposed to aphids was largely similar at aCO_2 and eCO_2, irrespective of AMF treatment. In contrast, eCO_2 reduced grain [N] of cv. Skyfall plants exposed to aphids (- AMF: -14.9%; + AMF: -34.1%).

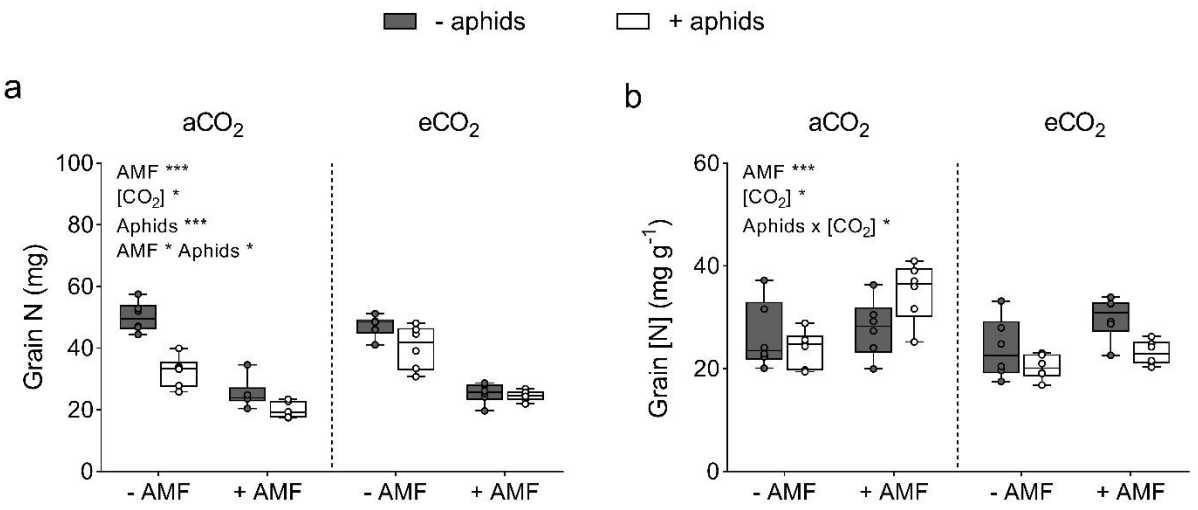

Figure 5.6: Grain N status of non-mycorrhizal and mycorrhizal wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. (a) Grain N; (B) Grain [N]. Skyfall was grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) in farm soil with native AM fungi and *R. irregularis* (+ AMF) or in sterilised soil with the wider microbiome reintroduced (- AMF). Plants were exposed to aphids (*R. padi*) (+ aphids, white bars) or not (- aphids, grey bars) between weeks 8-10. Boxes cover the interquartile range. Median values are represented by middle lines, and whiskers encompass all data points (closed or open markers, n=6). Asterisks indicate significant factors in the GLM, where p = 0.05 (*), <0.01 (**), <0.001 (***).

Table 5.5: Summary of three-way ANOVA results testing the effect of AMF, [CO_2], aphids, and their interactions on grain N status of wheat. Significant P-values are in bold (n=6).

	Grain N			Grain [N]		
	F	*df*	p	F	*df*	p
AMF	188.19	1,40	**<0.001**	13.45	1,40	**0.001**
[CO_2]	5.25	1,40	**0.027**	6.96	1,40	**0.012**
Aphids	24.24	1,40	**0.001**	0.13	1,40	0.722
AMF*[CO_2]	0.13	1,40	0.718	0.11	1,40	0.745
AMF*Aphids	6.17	1,40	**0.017**	0.72	1,40	0.401
[CO_2]*Aphids	3.23	1,40	0.080	4.90	1,40	**0.033**
AMF*[CO_2]*Aphids	0.00	1,40	0.986	1.57	1,40	0.217

5.4.6 Correlations

In order to examine whether aphid performance was determined by plant nutrient status, Spearman's rank correlation coefficients were carried out between grain [P] and [N] and final aphid abundance and aphid growth rates. There was a positive correlation between grain [P] and aphid abundance at the end of the 14-day exposure period (Figure 5.7a). The same was true of grain [N] (Figure 5.7b). Grain [P] and grain [N] were also positively associated with aphid growth rates, a metric which accounted for insect abundance across all five time points during the exposure period.

In order to study whether AM colonisation determined grain nutrient concentrations, correlations were performed between % root length colonisation and % arbuscules and grain [P] and [N]. % root length colonisation after 10 weeks was positively correlated wheat grain [P] (Figure 5.8a), as were arbuscule frequencies (Figure 5.8c). The same was not true of grain [N], which was not correlated with either % root length colonisation (Figure 5.8b) or % arbuscules (Figure 5.8d).

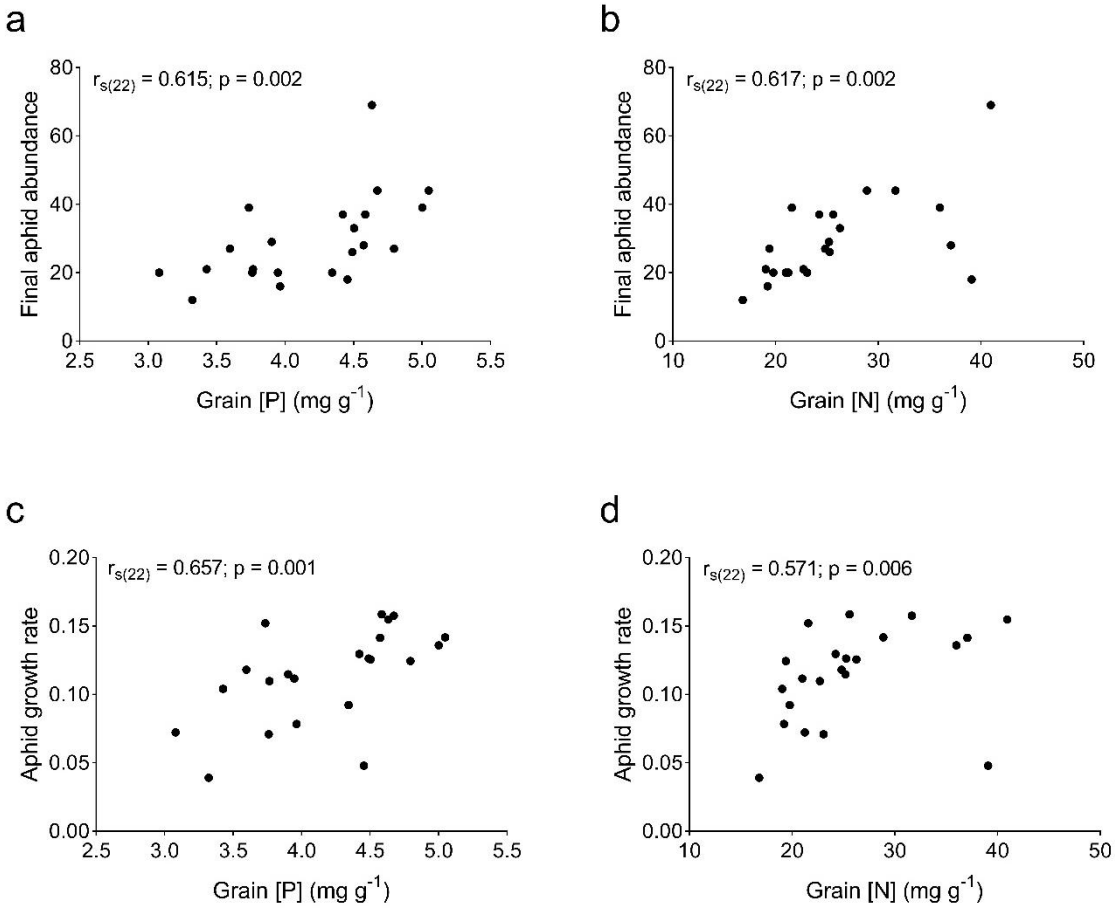

Figure 5.7: Correlations between grain nutrient concentrations and aphid performance on wheat. (a) Grain [P] vs final aphid abundance; (b) Grain [N] vs final aphid abundance; (c) Grain [P] vs aphid growth rate; (d) Grain [N] vs aphid growth rate. 8-week plants were exposed to *R. padi* aphids in clip cages for 14 days, after which plants were grown to yield. All data pooled across AMF (- AMF and + AMF) and [CO_2] treatments (aCO_2 and eCO_2). Correlations were tested using Spearman's rank correlation coefficients.

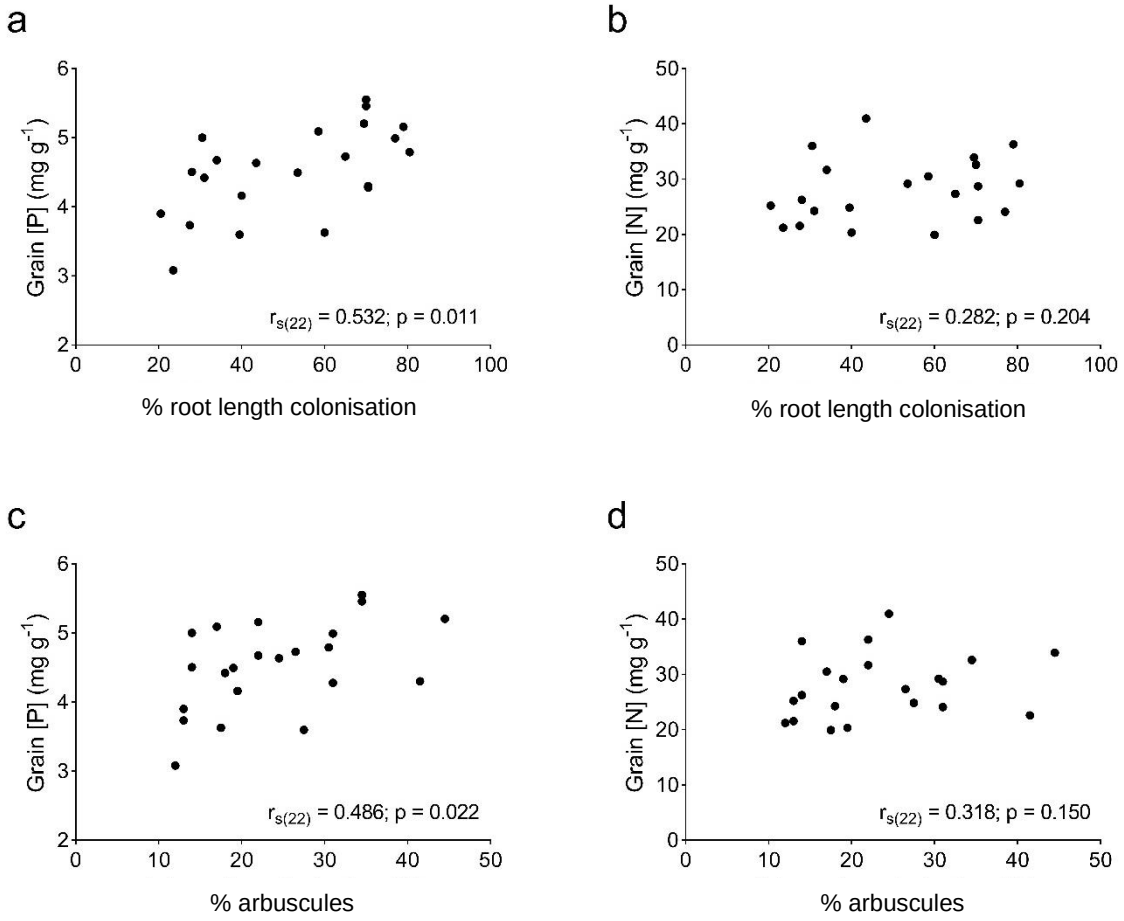

Figure 5.8: Correlations between grain nutrient concentrations and AM fungal abundance in roots of wheat. (a) Grain [P] vs % root length colonisation; (b) Grain [N] vs % root length colonisation; (c) Grain [P] vs % arbuscules; (d) Grain [N] vs % arbuscules. All data was pooled across aphid treatments ('- aphids' and '+ aphids') and [CO_2] treatments ('aCO_2' and 'eCO_2'). Correlations were tested using Spearman's rank correlation coefficients.

5.5 Discussion

Most studies investigating plant-AM interactions use single-species inoculants (García de León et al., 2020). These experiments do not account for functional variation in mixed AM fungal communities, nor antagonistic or synergistic interactions between mycorrhizal taxa (Argüello et al., 2016) and/or soil microbes (Svenningsen et al., 2018). The same is true of experiments into AM fungal-plant-insect interactions, and how these manifest at eCO_2 (Frew & Price, 2019). In this study, the below-ground above-ground interactions between AM fungi native to farm soils, wheat, and a cereal-feeding aphid (*R. padi*) were studied at ambient [CO_2] and at levels forecast for 2100 (IPCC, 2014). Wheat not inoculated with field-collected AM fungi achieved higher grain yield than plants which were, regardless of [CO_2] or aphids. Likewise, grain P and N were greater in non-AM hosts, although concentrations were unchanged. These findings reinforce the notion that wheat-AM interactions may be parasitic in certain systems, including those involving wild AM fungi (Zhang et al., 2018). AM colonisation was reduced by aphids, contrasting Chapters 3 and 4. Aphid abundance was increased by AM infection, but only at eCO_2.

5.5.1 Wheat yield responses to field-collected AM fungi

Colonisation of wheat by native AM fungi reduced grain yield of cv. Skyfall relative to the non-mycorrhizal treatment. This was true whether plants were exposed to aphids or not, or grown at aCO_2 or eCO_2 (Figure 5.4). This finding confirms the first hypothesis of this study, which predicted plant growth responses of wheat to colonisation by field-collected AM fungi may be parasitic, and therefore less positive than when using a single, laboratory-cultured generalist mycorrhizal species (see Chapters 2 & 3). There are a number of potential reasons for this. AM fungi are wholly reliant on plants for organic C which sustains their growth and function (Smith & Read, 2010). Because of this, the extent to which roots are colonised by AM fungi may be used as an indication of plant C supply to fungal partners (although see Chapter 4). % root length colonisation of AM plants not exposed to aphids averaged 69% at aCO_2 and eCO_2 10 weeks after planting (Figure 5.3a). The use of resident AM fungi, and thus perhaps a mixed mycorrhizal community, may have driven these relatively high colonisation levels, as different taxa in species-rich AM fungal assemblages may occupy different regions (or niches) of host-plant roots. Alternatively, the presence of one AM fungus

can enable root colonisation by others (Thonar et al., 2014). The extent of AM colonisation, and therefore the size of the below-ground plant C sink, may have reduced wheat yields. However, reduced grain yield was also recorded in mycorrhizal wheat plants exposed to aphids, in which AM colonisation averaged just 32% (Figure 5.3a). This finding suggests that wheat growth depressions were not solely driven by the outlay of plant C to AM fungi (Grace et al., 2009; Smith et al., 2009).

A second explanation for the negative effect of mycorrhization on grain yield could be the presence of “poorly mutualistic” wild AM fungi in wheat roots, these being taxa that do not readily engage in resource exchange. Selection pressures imposed on AM fungi in farms soils by intensive practices may favour isolates that produce abundant spores over those that invest in plant nutrient uptake (Verbruggen & Kiers, 2010). Plants may be adept at recognising and sanctioning symbionts that provide few nutritional benefits if colonised by these AM fungi alone (Kiers et al., 2011), although how exploiters are identified is unknown. When associated with mixed AM fungal communities, however, evidence suggests that less nutritionally beneficial taxa may persist within host-plant roots (Hart et al., 2013). To confirm this, further experiments are needed to determine the identity of AM fungi in the roots of wheat when grown in this soil, ideally through microbial profiling techniques like 18S rRNA gene sequencing (Nakatsu et al., 2019). By using an older approach termed terminal-restriction fragment length polymorphism (T-RFLP), Elliott et al., (2020) detected 18 T-RFs within roots of wheat grown in farm soil collected from the same site. Although genotypes of all mycorrhizal species were not determined, one likely matched *Funneliformis mosseae* or *F. caledonium* (previously of the genus *Glomus*). The presence of the former species may be of significance; Hetrick et al., (1992) recorded that colonisation of wheat cvs. Newton and Kanzler by *F. mosseae* reduced plant growth by 51 % and 60 %, respectively, despite only occupying 5 % and 42 % of the root system. However, *F. mosseae* has been shown to increase yield parameters of other wheat varieties (Kumar et al., 2011), and mitigate against yield losses caused by abiotic stress, such as hypersalinity (Yi et al., 2017). Cultivar-specific responses to AM colonisation may be a consequence of the year of cultivar release, as mentioned in Chapters 2-4 (Zhu et al., 2001; Martín-Robles et al., 2018; Zhang et al., 2018). Future work ought to investigate the effect of native AM fungi on wheat yields across a range of wheat lines that differ in release date.

Aside from *F. mosseae* and *F. caledonium,* it is also likely that the roots of wheat plants were colonised by *Rhizophagus irregularis*, owing not only to its ubiquitous distribution (Savary et al., 2018), but also the addition of a supplementary *R. irregularis* inoculum. The introduction of this inoculum was designed to ensure wheat was colonised in the mycorrhizal treatment, as it has been shown that adding inocula to soils can increase AM colonisation of plants beyond that achieved by the resident AM fungal community alone (Köhl et al., 2016). The potential exists, however, for inoculants to reduce the abundance and/or diversity of native AM fungi in plant roots (Jin et al., 2013; Janoušková et al., 2017). This may be because once plants become associated with one fungal species, secondary taxa achieve lower abundance in the root cortex (Werner & Kiers, 2015). Mycorrhizal community profiling using the approaches outlined above could reveal whether inoculating wheat with *R. irregularis* affected AM fungal community composition in roots of wheat grown in soil with native mycorrhizas.

A third explanation for the negative effect of AM colonisation on wheat yields could be the nutritional status of the substrate. Soils were collected from a conventionally managed arable site which received annual applications of fertilisers and manure (see Holden et al., 2019). However, farm soil was diluted with sand and perlite (see section 3.3.1), with plants being fed between weeks 7 and 12 having exhibited symptoms of nutrient deficiency (see section 5.3.3). N:P ratios in the grain were < 9 at harvest (data not shown), these being below the threshold of 14 which is indicative of N limitation (Koerselman & Meuleman, 1996). While plants may exhibit greater dependency on AM fungi when grown in P-limited soils, parasitism may be expected in N-deficient substrates (Johnson et al., 2015). This is because plants and AM fungi may compete for N (Hodge & Fitter, 2010), and low-N may slow rates of photosynthesis (Jin et al., 2005) thereby reducing carbon-for-nutrient exchange (Johnson, 2010). It is feasible that the N status of the substrate resulted in poorly mutualistic associations between cv. Skyfall and native AM fungi, driving lower yields in the '+ AMF' treatment. % root length colonisation and % arbuscules were not correlated with grain [N] at yield (Figure 5.9b & 5.9d), perhaps suggesting fungal-mediated N uptake did not occur. However, the use of isotopic tracers would be needed to confirm this. Similarly, though AM colonisation was positively associated with total grain [P] (Figure 5.9a & 5.9c), separating P uptake via AM fungi and directly across the root was not possible. Roots biomass was not measured at yield, as rates of root growth differ during the foundation

and construction phases of crop development and decline at maturity (AHDB, 2018). As such, root size at yield may not have been indicative of earlier stages when nutrient uptake peaks, like during stem elongation (GS30-GS40). That said, it remains possible that smaller roots of AM plants (as seen in Chapters 2 and 3) could also be responsible for reduced grain yield, grain P, and grain N in the '+ AMF' treatment.

Lastly, differences in yield characteristics between mycorrhizal and non-mycorrhizal plants may be a consequence of how each treatment was created. After gamma irradiating soil for use in the non-mycorrhizal group, bacteria and non-AM fungi native to farm soils were reintroduced using a microbial wash. This was performed in order to ensure any treatment effects were driven solely by the presence/absence of AM fungi, and not also due to the lack of a wider soil microbial community in the non-AMF treatment. 30 mL of filtrate was added to each pot, in line with previous studies (García de León et al., 2020). However, microbes were extracted from just 10 g of soil (see section 5.3.2), meaning bacteria and non-AM fungi in soils of the non-mycorrhizal treatment were likely less abundant than they were in the '+ AMF' treatment. This difference could have reduced yields of AM plants if disease-causing bacteria or pathogenic fungi were more present in unsterilised soils. This potential confounding effect emphasises the difficulty of creating suitable non-mycorrhizal treatments in the laboratory when using natural soils (Gryndler et al., 2018). Moreover, even if the wider microbiome was reinstated, interactions that occur between mycorrhizal fungi and root-associated bacteria could not be replicated in the non-AM treatment. AM fungi can increase or suppress the growth of native soil microbes, meaning the addition of AM fungal inoculants to farm soils can change microbial community structure (Akyol et al., 2018).

5.5.2 Impact of aphids on AM colonisation

Aphid exposure reduced AM colonisation of wheat by 53% (Figure 5.3a). This finding was in line with hypothesis two, which predicted that aphids would decrease fungal biomass by limiting plant C availability and thus supply (see Chapter 4) (Gehring & Whitham, 2002; Drigo & Rillig, 2010). The effect of aphids on AM colonisation was consistent at aCO_2 and eCO_2, however, countering the second part of hypothesis two, which anticipated eCO_2 would mitigate against the presence of an external biotic C sink. Reduced % root length colonisation by a mixture of AM fungal species following aphid exposure has been seen in broad bean (Babikova et al., 2014a) and *Asclepias*

species (Meier & Hunter, 2018), but this is first time it has been documented in wheat inoculated with wild AM fungi. The effect size recorded here was striking, being greater than the 20-36% reduction in the above-mentioned studies. However, changes of this magnitude have been recorded in the opposite direction, as *Aphis nerii* aphids increased colonisation of *A. incarnata* by 56% (Meier & Hunter, 2018). Together, these finding confirm that phloem feeders can significantly impact AM fungal abundance, and plant-AM function by extension (Charters et al., 2020).

Although one must be cautious when comparing across studies, the finding that *R. padi* aphids reduced AM colonisation of cv. Skyfall when inoculated with a field-collected AM fungal community but not when colonised by *R. irregularis* alone in Chapters 3 and 4 is intriguing. This observation may mirror differences recorded between experiments using *V. faba* L., in which aphids reduced colonisation by multiple AM fungi (Babikova et al., 2014a) but not *R. irregularis* individually (Cabral et al., 2018). One reason for this could be that mycorrhizal species differ in their plant C requirements, and thus susceptibility to an external biotic C sink. AM fungal taxa with high C demands, such as *Gigaspora margarita* (Lendenmann et al., 2011) or *F. mosseae* (Argüello et al., 2016), may be more sensitive to plant C limitation caused by aphids. Similarly, fungal species less capable of quickly acquiring photosynthate from plants, either because of sub-optimal growth strategies (i.e. fewer root-internal AM fungal structures) or lower affinity hexose transporters (Cotton et al., 2012), may fail to compete with more dominant mycorrhizal species for plant C once it's availability declines. Both factors could result in certain AM fungal taxa becoming less abundant in roots and soils of plants exposed to aphids, or even excluded, leading to reduced colonisation and a restructuring of AM fungal communities. Very little is known about how different mycorrhizal taxa respond to herbivory (Gehring & Bennett, 2009), and the knock-on effect this has on AM fungal diversity and community structure. To date, only one study has investigated the effect of aphids on mycorrhizal composition; despite reporting no effect of aphid feeding on % root length colonisation of barley, Wilkinson et al., (2019) observed (via DNA sequencing) an increase in the relative abundance of AM fungal species of the family Gigasporaceae, these being slow-growing k-strategists (Boddington & Dodd, 1999). This finding was counter to their expectations, as an external biotic C sink was predicted to favour fast-growing r-strategist AM fungi (e.g. Glomeraceae species), which may be more adept at

competing for plant C once supply to AM fungi declined. However, plant C dynamics were not tested. Future research on AM fungal-crop-aphid interactions should combine the use of molecular techniques and ^{14}C tracers to identify which mycorrhizal taxa are least and most resilient to changes in plant C supply caused by aphids. This knowledge could inform growers as to which inoculants to avoid or select if their crops are frequently challenged by phloem feeders. Moreover, by exposing plants to aphids at different densities, it may be possible to identify aphid thresholds beyond which certain AM fungal isolates are excluded from roots. Plants in this experiment were exposed to aphids inside insect clip cages for the reasons outlined in section 3.3.3, meaning the scale of the infestation was modest. Field experiments are now required in which wheat is systemically exposed to *R. padi* aphids and the impact on AM colonisation assessed.

Alternatively, differences in % root length colonisation between aphid treatments seen here could relate to how soils within each treatment were handled. Plants exposed to aphids were grown between different dates to those which were not. This was to ensure that aphid-induced plant VOCs did not impact wheat-AM interactions in the '- aphids' treatment (see Chapter 3 section 3.5.1.1). However, this meant that farm soils used in the '+ aphids' treatment were stored while wheat plants not exposed to aphids were grown to yield. It is possible that the community composition and/or abundance of AM fungi may have changed in soils during this intervening period; Rubin et al., (2013) observed that both the temperature at which soils were stored and duration of the storage impacted microbial diversity and community structure. Both factors could ultimately have impacted AM colonisation of wheat in the '+ aphid' treatment. However, using soil collected on the same day across both aphid groups was considered preferable to making a second collection. This is because seasonality can significantly alter AM fungal community composition, with abiotic factors like temperature, sunlight hours, and soil pH driving the formation of distinct AM fungal assemblages between summer and winter months (Dumbrell et al., 2010; Dumbrell et al., 2011). The use of molecular tools similar to those described above would have helped determine whether the storage of soils impacted AM fungal abundance and/or species richness.

5.5.3 Impact of AM colonisation and [CO_2] on aphids

No effect of [CO_2] or AM colonisation was recorded on aphid growth rates (Figure 5.2a). However, final aphid abundance differed significantly according to [CO_2] and

AM fungal treatment, being lowest in non-mycorrhizal plants at eCO_2 and highest in mycorrhizal ones at aCO_2 (Figure 5.2b). The reason behind this may lie in the nutritional status of plants in each treatment. Aphid performance was positively associated with grain [P] and grain [N] (Figure 5.7). Aphids assimilate trace quantities of both P and N when siphoning plant phloem, the latter in the form of amino acids. Lowest shoot [P] was recorded in the same treatment in which aphids were least abundance after 14 days experiment. Future studies ought to look into how AM fungi and eCO_2 impact more nuanced aspects of aphid performance, such as relative growth rates, development times, and feeding behaviour, as these traits may impact aphid performance over longer exposure periods more typical of field settings.

5.5.4 Impacts of aphids and [CO_2] on wheat yield

Aphids reduced grain biomass of wheat by 30-37% at ambient CO_2 (Figure 5.2b). This finding was in line with previously reported direct feeding effects of aphids on wheat yields (Zeb et al., 2016), with the loss of plant C and other trace elements through phloem siphoning slowing their translocation to sink tissues, like the grain, in different parts of the plant (Aqueel & Leather, 2011). Interestingly, the negative effect of aphids on grain biomass was mitigated at eCO_2, suggesting perhaps that an abiotic plant C source counterbalanced the effect the biotic plant C sink. This increase in tolerance against *R. padi* aphids at eCO_2 may suggest that the negative effects of aphids on yields could be mitigated somewhat under future climate change scenarios. This contrasts the effect of temperature on wheat yield losses to pests, which are predicted to grow by 10-25% with every 1˚C of warming (Deutsch et al., 2018). However, losses to aphids may be greater in the field due to the transmission of plant viruses by phloem feeders, like BYDV (Fereres & Moreno, 2009). AM fungi may prime plants against viruses, which warrants study in this AM fungal-plant-aphid system (Miozzi et al., 2019).

5.5.5 Summary

This study investigated the effect of aphids and atmospheric [CO_2] on the symbioses formed between the wheat cv. Skyfall and a wild mycorrhizal community native to farm soils. Colonisation by field-collected AM fungi reduced yield parameters of wheat markedly, perhaps owing to the presence of poorly symbiotic fungi in soils or the nutritional status of the substrate itself. In contrast with previous findings in this thesis, aphids reduced AM colonisation of the field-collected mycorrhizal assemblage, hinting

perhaps at the presence of certain fungal species in roots that were sensitive to an aphid induced plant C drain. Increasing plant C source strength at eCO_2 did not impact plant growth responses to AM fungi, nor did the presence of an external plant C sink (i.e. *R. padi* aphids). [CO_2] appeared to mitigate the effect of aphids on yield loss.

Chapter 6 General discussion

Demand for food production is predicted to double by 2050 (Godfray et al., 2010), but annual yield increases for crops like wheat are below that required to meet this increasing global need (Ray et al., 2013). Compounding this food security threat is the over-dependence of agriculture on finite and environmentally-damaging fertilisers and pesticides. Rock phosphate reserves - from which most P fertilisers originate - are diminishing (Cordell et al., 2009), and emissions from N fertiliser production must be curbed in order to avoid rising CO_2 and irreversible climate change (IPCC, 2018). Similarly, evidence suggests insect pests, like aphids, have developed resistance to insecticides (Foster et al., 2014), with some compounds now banned because of their impact on non-target species (VanDoorn & de Vos, 2013). Thus, agriculture faces the challenge of increasing yields but reducing its reliance on on-farm chemicals.

Most crops, including wheat, form symbioses with AM fungi (Smith & Read, 2010), which can increase grain nutrient concentrations and ultimately yield (Pellegrino et al., 2015; Lehmann & Rillig, 2015; Zhang et al., 2019). AM colonisation may also enhance plant resistance against insect herbivores (Koricheva et al., 2009), although their effect on aphids is variable (see Table 1.1). Consequently, the potential may exist to use AM fungi in agriculture to help increase productivity and reduce fertiliser and pesticide usage (Thirkell et al., 2017). However, growth responses of plants to AM fungi can be influenced by environmental factors (Johnson et al., 2015), and vary between cultivars (Hetrick et al., 1992). Therefore, an improved comprehension of the abiotic, biotic, and genotypic factors driving wheat growth responses to mycorrhizal fungi is essential if they are to be functionally important in future agro-ecosystems (Smith & Smith, 2011b).

The overarching aim of this thesis was to study how environmental drivers that affect wheat sink-source strength dynamics for plant C impact crop growth and nutritional responses to colonisation by AM fungi. How atmospheric [CO_2] and aphids impact resource exchange dynamics between plants and AM fungi was also studied. At the same time, plant-mediated interactions between mycorrhizal fungi and wheat-feeding aphids were explored, this being an under-studied system in multi-trophic ecology.

Research presented in Chapter 2 investigated whether [CO_2] levels projected for 2100 (IPCC, 2014) accentuate the functional variability observed in wheat-AM symbioses, and if so, whether certain cultivars are more responsive to AM fungi under future [CO_2]

than others. eCO_2 increased plant C source strength of cvs. Avalon, Cadenza, and Skyfall, which - despite being shown to impact wild plant-AM symbioses (Johnson et al., 2005) - had little effect on AM colonisation by *R. irregularis*, or the lifetime fitness benefits afforded to wheat by the AM fungus. Partial evidence hinted at a functional mycorrhizal uptake pathway, although isotope tracers were needed to validate this.

How biotic interactions, such as those between crops and aphid herbivores, impact wheat-AM symbioses were then addressed in Chapter 3. Aphids may reduce the source strength of wheat for plant C by siphoning phloem, with aphids shown in other tri-trophic systems to negatively impact plant-AM symbioses (Babikova et al., 2014a; 2014b; Meier & Hunter, 2018). Using the same cultivars, this study found no effect of aphids on AM colonisation of wheat, with growth and nutritional responses of plants to mycorrhization being largely unchanged. For cv. Skyfall, however, the effect of AM colonisation on shoot [P] and [N] seemingly differed in the presence and absence of aphids, perhaps suggesting a change in plant-AM functioning. Mycorrhization improved wheat tolerance of aphids in this cultivar, but aphid abundance was universally unaffected by AM colonisation.

Using multiple isotopic tracers (^{33}P, ^{15}N, and ^{14}C), the impact of atmospheric [CO_2] and aphids on carbon-for-nutrient exchange between cv. Skyfall and *R. irregularis* was studied in Chapter 4. From a theoretical viewpoint, this research aimed to reveal whether resource exchange in complex plant-AM interactions is reciprocally regulated as evidenced in simplified systems (Kiers et al., 2011; Fellbaum et al., 2012), which is a matter of debate (Walder & van der Heijden, 2015; Kiers et al., 2016), or instead if source-sink dynamics govern C-for-nutrient exchange. As hypothesised, aphids reduced plant C transfer to the fungus, but mycorrhizal-acquired nutrient uptake by wheat was unchanged. eCO_2 had no effect on plant C supply to AM fungi, as seen previously (Thirkell et al., 2019; Elliott et al., 2020), and thus did not mitigate the decline in plant C flux to the AM fungus caused by aphids. Broad patterns in AM colonisation did not match those of plant C transfer to the fungal symbiont, underlining that caution must be taken when using these metrics interchangeably.

Finally, how AM fungi native to arable soils impact wheat yields when grown with and without aphids at aCO_2 and eCO_2 was studied in Chapter 5. A strong negative effect of field-collected AM fungi was recorded on wheat yields, which were unchanged by insect herbivory or [CO_2].

6.1.1 Carbon-for-nutrient exchange is impacted by aphids but not [CO_2]

Atmospheric [CO_2] was found to have little impact on wheat-AM symbioses. AM colonisation of wheat was largely equivalent at aCO_2 and eCO_2 when inoculated with *R. irregularis* alone (Chapter 2) or native AM fungi to farm soil (Chapter 5), as was the supply of plant C to an AM fungus as determined using ^{14}C-labelled CO_2 (Chapter 4). In contrast, although no effect of aphids was recorded on AM colonisation in Chapter 3, aphid feeding reduced plant C transfer to *R. irregularis* (Chapter 4), and decreased AM colonisation of wheat colonised by a resident AM fungal community (Chapter 5).

Given the increase in plant C source strength at eCO_2 was likely greater than the external biotic C sink strength represented by aphids, the impact of aphids on crop-AM symbioses and not atmospheric [CO_2] is of interest. There are a number of possible explanations for this. Firstly, the acclimation (or lack thereof) of wheat plants to their respective treatments might have driven these contrasting outcomes. Wheat plants grown at 800 ppm were subject to eCO_2 from the beginning of each experiment. Therefore, at the time point at which plant C transfer to the AM fungus was assessed (either by using AM colonisation measurements or directly through the use of isotopic tracers), plants were already physiologically acclimatised to high [CO_2], and had invested additional plant C resources above-ground as determined by shoot or grain biomass measurements. In contrast, wheat plants were exposed to aphids between weeks 8 and 10 in Chapters 3-5, and not acclimated to the presence of insect herbivores prior to their introduction. Aphid recognition by target plants may elicit SA-dependent defence responses which, via cross-talk between pathways, can also impact JA biosynthesis (Ali & Agrawal, 2012). It is possible, therefore, that a transient increase in endogenous SA in response to herbivory resulted in a short-term trade-off between plant C allocation to AM fungi and plant defence. In order to test this hypothesis, wheat plants grown at 800 ppm should only be subject to eCO_2 for the same duration as plants are exposed to *R. padi* aphids.

A second explanation could be that biotic factors are more powerful regulators of crop-mycorrhizal function than abiotic factors. Other studies into the effect of [CO_2] on wheat-AM resource exchange recorded no effect of eCO_2 on plant C outlay to AM fungi, in both cases across a range of modern UK cultivars (Thirkell et al., 2019; Elliott et al., 2020). These findings contrast those of Walder et al., (2012) who, in growing *Sorghum bicolor* and flax (*Linum usitatissimum*) with a CMN, recorded unequal

carbon-for-nutrient exchange between cultivated crops and AM fungi based solely on plant identity (i.e. a biotic factor).

Lastly, the divergent effects of [CO_2] and aphids on plant C provisioning of AM fungi may be a consequence of sink competition impacting resource exchange to a greater extent than plant C source strength. In order to unpick this, experiments must now test the effect of abiotic factors that reduce plant C availability, such as sub-ambient [CO_2] (Williams et al., 2013) or shading treatments (Merild et al., 2013; Fellbaum et al., 2014), and biotic factors that increase plant C availability. Fewer options exist for this, but using plants that differ in age and/or development stage was the approach favoured by Merrild et al., (2013) when investigating source-sink strength dynamics on mycorrhizal-mediated P uptake in cucumber. Of course, in the case of wheat, using plants of different developmental stages may confound results, as nutrient demands change throughout the life cycle of wheat (AHDB, 2018).

The finding that aphids reduced plant C allocation to an AM fungus opens a number of potential avenues for future work. Primarily, new research is required into how insect herbivores of different feeding approaches effect carbon-for-nutrient exchange between wheat and AM fungi. Although bird cherry-oat aphids are major pests of wheat (Blackman & Eastop, 2017), cereals are also subject to herbivory from chewing insects, such as caterpillars of the northern armyworm (*Mythimna separate*) and fall armyworm (*Spodoptera frugiperda*), and leaf mining insects, like the cereal leaf miner (*Syringopais temperatella*). The consumption of foliar biomass by chewing insects invariably reduces the surface area of plants for photosynthesis, but may also impair gas exchange in remaining (i.e. undamaged) tissues (Zangerl et al., 2002). Multiple mechanisms have been proposed for this, including disruption to plant vasculature and the induction of JA-dependent responses (Nabity et al., 2008; Nabity et al., 2013). Chewing insects may represent greater external sinks for plant C than phloem feeders, therefore, and drive C limitation in wheat to a greater extent than that recorded by aphids. What impact this may have on the function of crop-AM symbioses is unclear, and therefore warrants experimentation.

6.1.2 Contrasting multi-trophic outcomes in AM fungal-wheat-aphid interactions

Research into interactions between AM fungi and aphids have reported variable outcomes for both above- and below-ground organisms. The 'top down' impact of aphids on AM colonisation varies (Chapter 1 section 1.4.4; Table 1.1). What drives this is unknown, but different AM fungal-plant-aphid combinations elicit contrasting outcomes. Until now, studies addressing how cereal aphids impact AM colonisation of wheat were entirely lacking, which represented a crucial knowledge gap.

Here, variable effects of aphid feeding were recorded on % root length colonisation of wheat, even when using the same genotype of fungus, host, and aphid. In Chapter 3, no impact of aphid feeding was recorded on colonisation by *R. irregularis* of three wheat cultivars. In Chapter 4, however, aphids increased AM colonisation of cv. Skyfall when inoculated with the same fungal species. This finding suggests that not only does organism identity determine outcome of AM fungal-plant-aphid interactions, but differences in abiotic conditions between studies may too impact results. Wheat plants in Chapter 3 were grown under semi-controlled greenhouse conditions, in which temperature and relative humidity were not tightly regulated, and light intensities were determined in part by the weather outside. In contrast, plants in Chapter 4 were grown in controlled environment chambers, and therefore subject to smaller diurnal fluctuations in abiotic conditions at canopy level (Poorter et al., 2016). Plants of cv. Skyfall achieved greater shoot biomass when grown in the greenhouse (Chapter 3) than when grown at aCO_2 in growth chambers (Chapter 4), so it is possible that these abiotic factors could have impacted the source strength of wheat for plant C. This may, in turn, have determined the extent to which plant C became limited when wheat was exposed to aphids, and therefore governed how aphids impacted % root length colonisation.

Environmental conditions could also have impacted aphid and AM fungal ecology. The development and fecundity of *R. padi* aphids is known to be influenced by temperature (Park et al., 2017) and humidity (Leather, 1985), with temperature also directly impacting AM fungi; hyphal growth may be faster under warmer soil conditions (Gavito et al., 2005) owing to greater plant C supply (Hawkes et al., 2008). While experiments involving [CO_2] are crucial to understanding our future world (Becklin et al., 2017), climate change is expected impact a suite of other abiotic factors at the same time,

including air temperature which are already 0.8-1.2 °C higher than before the industrial revolution, and continue to rise 0.2 °C every decade (IPCC, 2018). Together, these factors may have additive or interactive effects on plant physiology; the decline in wheat stomatal conductance at eCO_2 may be greater at high temperatures (Bunce, 2001), perhaps impacting rates of photosynthesis and thus plant C availability for aphids and AM fungi. As such, there is an increasing understanding that in order to better understand future below-ground ecology, and by extension how these processes impact those above ground, multiple drivers of environmental change must investigated concurrently (Rillig et al., 2019b).

In contrast to the variable 'top down' effects of aphids on AM colonisation, the 'bottom up' impacts of AM fungi on aphid fitness were consistent across Chapters. In Chapters 3 and 5, I found that AM colonisation of wheat had little effect on the abundance of *R. padi* aphids. This was true when three cultivars were colonised by *R. irregularis* alone (Chapters 3), and when cv. Skyfall was inoculated with an AM fungal community native to farm soils (Chapter 5). This finding alleviates some concerns that AM fungi may increase aphid pest pressures (Koricheva et al., 2009), either through improving host-plant quality (Hartley & Gange, 2009) or by altering the internal leaf anatomy of AM plants (Simon et al., 2017). However, it may also raises doubts as to the efficacy of promoting AM fungi as sustainable alternatives to pesticides. Future studies into the effects of AM fungi on wheat-feeding aphids should study more subtle aspects of aphid performance on modern cultivars, such as development rates and feeding behaviours. Changes in these traits could impact aphid performance and pest pressure over longer exposure periods, such as those experienced by wheat in summer months.

Rather than being determined by the AM status of host plants, aphid abundance was instead impacted by shoot [P] (Chapter 3 and 5) and [N] (Chapter 5). This finding is in-line with previous work on wheat-feeding aphids in which insect growth and survival was higher on wheat supplemented with fertilisers (Aqueel & Leather, 2011). This finding also underlines that AM colonisation could increase aphid pest pressure in more AM responsive crops, and that the potential exists for breeding efforts that aim to reinstate AM responsiveness into modern wheat to inadvertently make cultivars more palatable to aphids. The variable aphid numbers recorded on wheat varieties in Chapter 3 should be the focus of future research into developing aphid-resistant lines. Ultimately, this could help reduce not only direct feeding damage caused by aphids

but also limit population sizes and therefore the transmission of plant viruses like BYDV, the symptoms of which may increase with rising [CO_2] (Trębicki et al., 2017). Multiple studies have putatively reported resistance to *R. padi* aphids in wheat cultivars, for instance in China (Zhang et al. 2016) and Brazil (de Jesus Correa et al. 2020). The need for plant resistance is likely to grow if aphid numbers rise at elevated [CO_2] and insect pests become increasingly immune to the effects of insecticides.

6.1.3 The wider ecological picture

The final chapter of this thesis introduced a greater degree of complexity to this AM fungal-plant-aphid interaction by utilising mycorrhizal fungi native to arable soils. Future studies ought to also consider the plant-mediated effects of AM fungi on organisms occupying higher trophic levels than herbivores, such as natural enemies of aphids. As alluded to in Chapter 1 section 1.4.1, AM colonisation may change the composition of aerial chemicals released by plants, in some instances increasing the production of VOCs that attract parasitoids of aphids (Guerrieri et al., 2004; Volpe et al., 2018). This could explain increased rates of aphid parasitism on some AM plants, including grasses (*Phleum pratense*) targeted by *R. padi* aphids (Hempel et al., 2009). However, as per plant-aphid interactions, how mycorrhizal fungi impact plant-aphid-parasitoid interactions varies, with AM fungi found to both increase and decrease parasitoid success (Bennett et al., 2016). To the best of my knowledge, no study to date has investigated how AM colonisation of wheat indirectly impacts % parasitism of aphids feeding on these hosts, which represents a large knowledge gap. Natural enemies of aphids may impact aphid performance to a greater extent than host-plant quality (Vidal & Murphy, 2018). As such, the effect of AM fungi on parasitoids may determine the performance of wheat-feeding aphids to a larger degree than their impact on plant nutrient status.

The study of this multi-trophic interaction is now needed using more complex social settings. CMNs may be capable of transferring signals between connected plants that warn unexposed hosts of the presence of insect pests (Johnson & Gilbert, 2015). When fungal hyphae connect roots of aphid infested plants (termed “donors”) with hosts that have not yet been the subject of herbivory (termed “receivers”), the latter may produce VOCs with similar aphid-repellent and parasitoid-attractive properties as the former (Babikova et al., 2013a). Although the signal is unclear, this communication occurs rapidly (Babikova et al., 2013b) and has the potential to mitigate crop losses to

pests like aphids that multiply rapidly and have aggregated distributions. The potential also exists for these signals to alter the function of plant-AM symbioses in uninfested plants, which warrants close study. Which plants receive these AM fungal-mediated signals is also of interest; mycorrhizal fungi may preferentially warn hosts that deliver the most plant C to the CMN so as to maximise future C supply (Bücking et al., 2016).

Lastly, moving these experiments into the field also seems a crucial step. Free-air carbon dioxide enrichment (FACE) systems involve the continuous release of CO_2 into the canopy of plants grown in the field, with concentrations being maintained by altering rates of CO_2 injection with respect to wind speed and direction (Högy et al., 2009). How this multi-trophic interaction manifests in the field may reflect the results recorded in Chapter 5, owing to the use of native AM fungi and farm soil. However, despite using the same CO_2 concentrations, the effect of eCO_2 may be less pronounced in FACE experiments than those conducted in controlled-environment chambers (Long et al., 2006). This is perhaps due to abiotic conditions in the field that laboratory experiments inevitably fail to replicate, such as plant density, potting volume, and variation in light intensity or temperature (Poorter et al., 2016). Thus, field studies must be conducted.

Conclusions

Inconsistent growth responses in crops to colonisation by arbuscular mycorrhizal fungi represents one of the largest hurdles preventing the wide-spread use of AM fungi in agriculture. Despite the ancient origins of AM fungi and their potential nutritional and non-nutritional benefits, how ecologically-relevant environmental factors impact plant-AM function remains poorly characterised. This thesis advances our awareness of the context-dependent nature of crop-mycorrhizal symbioses. The impact of abiotic and biotic drivers, these being atmospheric [CO_2] and aphids, on wheat growth responses to AM colonisation and on carbon-for-nutrients exchange between symbionts were revealed. It remains to be determined whether other climate change drivers and insect pests of different feeding approaches impact resource exchange similarly.

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Appendix

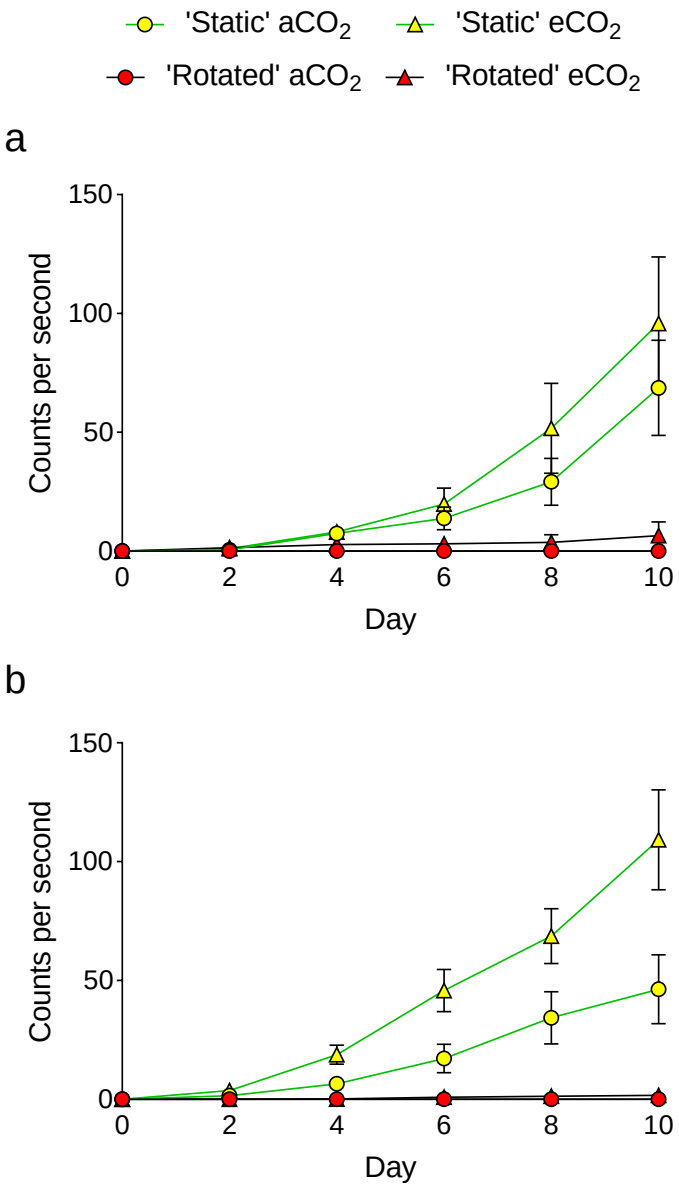

Figure A1. Shoot radioactivity of wheat plants grown in the presence and absence of aphids at ambient and elevated [CO_2] during the 12-day isotope labelling period. (a) '- aphids' plants; (b) '+ aphids' plants. Radioactivity, in counts per second, was recorded using a Geiger counter at 5 time points at 48-hour intervals (n=6, mean ± SE). Pots in which hyphal connections between the plant and the labelled core were severed (i.e. the 'rotated' treatment) are denoted by red markers and black lines (aCO_2: circles; eCO_2: triangles). Pots in which hyphal connections were maintained (i.e. the 'static' treatment) are denoted by yellow markers (aCO_2: circles; eCO_2: triangles) and green lines.

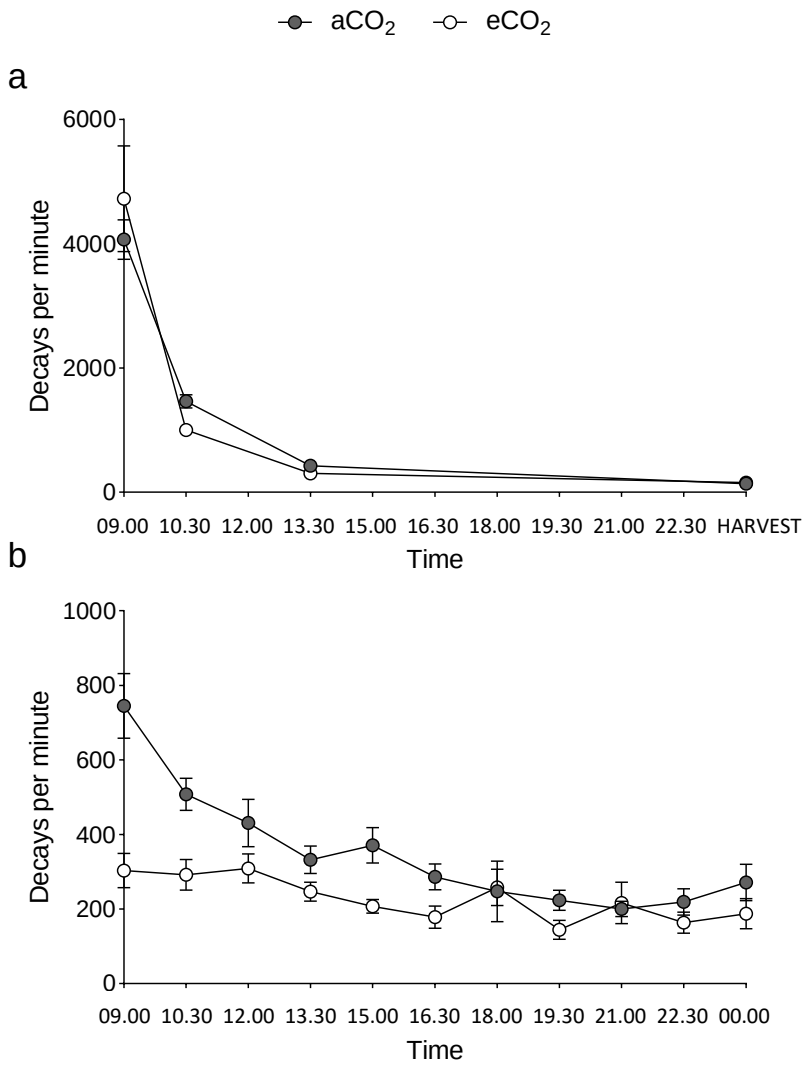

Figure A2. Radioactivity of above- and below-ground gas samples taken throughout the ^{14}C-labelling photoperiod from wheat plants grown in the absence of aphids at ambient and elevated [CO_2]. Radioactivity, in decays per minute, was quantified through liquid scintillation counting (n=12, mean ± SE). ^{14}C was liberated at 09.00 and KOH traps administered after the final gas sample was taken at 00.00. Above-ground samples recorded the drawdown of ^{14}C by wheat plants. Below-ground samples recorded the flux of ^{14}C by the AM fungal network, but no discernible peak was recorded. Data shown are for plants not exposed to aphids. Equivalent trends were recorded in the '+ aphids' treatment.

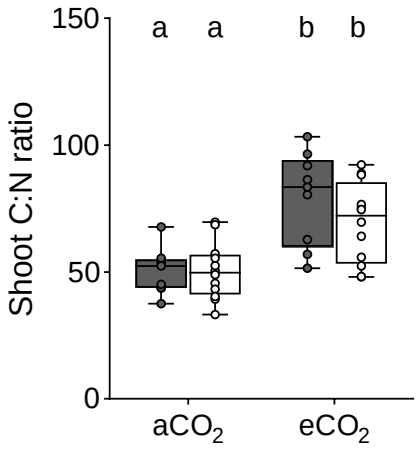

Figure A3. Shoot carbon-to-nitrogen ratio (C:N) of wheat grown in the presence and absence of aphids at ambient and elevated [CO_2]. cv. Skyfall was inoculated with *R. irregularis* and grown at aCO_2 (440 ppm) or eCO_2 (800 ppm) for 8 weeks. Plants were exposed to aphids (*R. padi*) (+ aphids, white boxes) or not (- aphids, grey boxes) inside insect clip cages for 12 days. Boxes extend from Q_1 to Q_3. Median values are represented by middle lines, and whiskers range from minimum to maximum data points (closed or open markers, n=12). Different letters denote significant differences (where $p < 0.05$, Tukey HSD tests).